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Sex Biased Stress Signaling: the Corticotropin-releasing Factor Receptor as a Model

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Abstract

Sex differences in the prevalence or severity of many diseases as well as in the response to pharmacological agents are well recognized. Elucidating the biological bases of these differences can advance our understanding of the pathophysiology of disease and facilitate the development of treatments. Despite the importance to medicine, this has been an area of limited research. Here we review physiological, cellular and molecular findings supporting the idea that there are sex differences in receptor signaling and trafficking that can be determinants of pathology. The focus is on the receptor for corticotropin-releasing factor (CRF), the orchestrator of the stress response, which has been implicated in diverse stress-related diseases that show a female prevalence. Data are reviewed showing sex differences in the association of the CRF receptor (CRF1) with the Gs protein and β-arrestin 2 that would render females more responsive to acute stress and less able to adapt to chronic stress as a result of compromised CRF1 internalization. Because β-arrestin 2 serves to link CRF1 to Gs-independent signaling pathways, this sex biased signaling is proposed to result in distinct cellular responses to stress that are translated to different physiological and behavioral coping mechanisms and that can have different pathological consequences. Because stress has been implicated in diverse medical and psychiatric diseases, these sex differences in CRF1 signaling could explain sex differences in a multitude of disorders. The possibility that analogous sex differences may occur with other G-protein coupled receptors underscores the impact of this effect and is discussed.

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Introduction

Many diseases exhibit sex differences such that their prevalence and/or severity are greater in one sex. Identifying the biological bases for sex differences in specific diseases can advance our understanding of their etiology and lead to the development of more effective individualized therapies. Sex differences are particularly prominent in neuropsychiatric diseases (Table 1). For example, autism, substance abuse and attention deficit disorder are more prevalent in males (Table 1) (Gaub and Carlson, 1997; Ramtekkar et al., 2010). In contrast, affective disorders and many anxiety disorders are nearly twice as prevalent in females compared to males (Table 1) (Kessler, 2003; Kessler et al., 1994). Similarly, the incidence of posttraumatic stress disorder (PTSD) is greater in females despite males being exposed to more traumatic events (Breslau, 2001; Breslau, 2002). These mood and anxiety disorders are debilitating mental illnesses and are a leading cause of disability in the United States for younger adults (Alonso et al., 2011; Bromet et al., 2011). A common underlying feature of the psychiatric disorders that are more prevalent in females is an association with stress. Stressor exposure is associated with the onset and severity of depression and several anxiety disorders (Kendler et al., 1995). For PTSD, stress is the precipitating event. Importantly, the sex bias in PTSD remains after adjusting for the type of trauma, preexisting psychiatric disorders, and sex differences in reporting (Breslau, 2009; Breslau et al., 1999; Tolin and Foa, 2006). Together these data support the notion that the increased incidence of stress-related psychiatric diseases in females is biologically determined.

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In addition to an association with stress, anxiety disorders, PTSD and depression all share a core symptom of hyperarousal, which is a defining feature of the diseases and is characterized by sleep disturbances, inability to concentrate, restlessness and increased vigilance (Gold and Chrousos, 2002; Southwick et al., 1999). To elucidate the biological bases for the female prevalence of these diseases we examined a major point of intersection between stress and arousal systems. This was the interaction between the orchestrator of the stress response, corticotropin-releasing factor (CRF) and a major arousal system, the locus coeruleus (LC)-norepinephrine system (Valentino and Van Bockstaele, 2008). Below we describe the role of these systems in stress and how their interaction is an important cognitive limb of the stress response but can become dysfunctional and result in stress-related pathology. We then describe sex differences in functional endpoints of CRF-LC interactions and parallel differences in CRF receptor signaling and trafficking as identified in cortical tissue. The global implications of the discovery that sex can determine the direction of receptor signaling are discussed with respect to clinical and therapeutic applications.

Corticotropin-releasing factor and stress-related psychiatric disorders

The hypothalamic neuropeptide that orchestrates the stress response is CRF, which functions as a neurohormone that is released from paraventricular hypothalamic neurons into the hypophysial portal system to elicit secretion of adrenocorticotropin (ACTH) from anterior pituitary corticotrophs (Vale et al., 1981). This is the initial step in a cascade that results in adrenal corticosteroid release that is considered to be a hallmark of stress. Synaptically released CRF in extrahypophyseal neural circuits that

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innervate the limbic forebrain, midbrain and pontine monoamine nuclei and autonomic-related brainstem nuclei facilitates the coordination of autonomic, behavioral and cognitive responses to stress with the endocrine limb (de Kloet et al., 2005). Whereas coordinated CRF release in these circuits would be well designed for coping in a dynamic environment with potential life-threatening challenges, the initiation of these responses would be maladaptive in the absence of stressors or if the responses persisted after stress termination. In this regard, inappropriate or excessive CRF is thought to be a pathophysiological factor in the stress-related psychiatric disorders that are more prevalent in females including PTSD and depression. For example, CRF levels are elevated in the cerebrospinal fluid of patients with PTSD or depression (Arborelius et al., 1999; Baker et al., 1999; Bremner et al., 1997; Nemeroff et al., 1984; Sautter et al., 2003). CRF protein and mRNA are elevated in numerous brain regions of depressed suicide victims (Austin et al., 2003; Bissette et al., 2003; Leake et al., 1990). Finally, many of the symptoms in a subset of individuals with severe depression resemble overactivity of the stress axis including increased basal cortisol secretion, blunted rhythm and resistance to dexamethasone suppression (Gold and Chrousos, 2002; Gold et al., 1988a; Gold et al., 1988b).

CRF effects are mediated through two CRF receptor subtypes, CRF1 and CRF2, whose genes have been cloned (Chen et al., 2005; Lovenberg et al., 1995; Perrin et al., 1993). These receptors have been well characterized with respect to their distribution and pharmacological specificity (see for review (Bale and Vale, 2004; Chalmers et al., 1996; Hauger et al., 2003; Hauger et al., 2006). Additionally, many details related to signaling and trafficking of the receptors have been identified (Hauger et al., 2006;

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Hauger et al., 2009; Hillhouse and Grammatopoulos, 2006). The present review focuses on CRF1, as this is the subtype through which CRF acts to elicit ACTH release and to initiate many of the central effects associated with the stress response, including arousal and anxiogenic effects. As such, novel therapeutics for stress-related disorders are being developed to target CRF1 (Grammatopoulos and Chrousos, 2002; Holsboer, 1999). Additionally, this is the CRF receptor subtype for which sex differences in signaling have been described (Bangasser et al., 2010). In contrast, the role of CRF2 in stress is less well defined, with some studies suggesting stress-protective effects and others providing evidence for pro-depressive effects mediated by this receptor (Bale et al., 2000; Hammack et al., 2003; Valentino and Commons, 2005). Reviews of CRF2 structure, signaling and function can be found in (Hauger et al., 2006; Hillhouse and Grammatopoulos, 2006).

Corticotropin-releasing factor and the locus coeruleus-norepinephrine system

The norepinephrine nucleus, locus coeruleus (LC), in the pons is a primary target of CRF during stress (Valentino and Van Bockstaele, 2008). The LC is the major source of norepinephrine in forebrain and has long been implicated in arousal and directing the mode of attention (Aston-Jones et al., 1995; Berridge and Waterhouse, 2003; Waterhouse et al., 1998). CRF axon terminals synapse with LC dendrites and also are apposed to afferent axon terminals in the LC, suggesting that CRF has both direct and indirect actions on LC neurons (Van Bockstaele et al., 1996). Consistent with this, CRF increases the spontaneous discharge rates of LC neurons *in vivo* when directly applied onto LC neurons and *in vitro* in the presence of tetrodotoxin (Curtis et

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al., 1997; Jedema and Grace, 2004). This action is involves cyclic AMP mediated inhibition of potassium conductance (Jedema and Grace, 2004). Additionally, CRF decreases the signal-to-noise ratio of LC responses to sensory stimuli (Valentino and Foote, 1987; Valentino and Foote, 1988). Related to this it is noteworthy that at least a third of CRF axon terminals in the LC co-localize glutamate, the neurotransmitter that mediates LC activation by many stimuli (Valentino et al., 2001).

The net effect of CRF on LC neurons is to shift discharge towards a high tonic state that favors increased tonic and decreased phasic, stimulus-evoked activity (Valentino and Foote, 1987; Valentino and Foote, 1988). This mode of discharge has been associated with a shift from focused attention to a scanning mode that would promote increased arousal and behavioral flexibility (Aston-Jones and Cohen, 2005; Aston-Jones et al., 1999). Supporting this, CRF microinfusion into the LC desynchronizes cortical electroencephalographic activity, a sign of elevated arousal, and promotes behavioral flexibility in an attentional set shifting task (Curtis et al., 1997; Snyder et al., 2012). Stressors mimic the electrophysiological effects on LC neurons and this can be prevented by microinfusion of CRF antagonists into the LC providing evidence for CRF neurotransmission in the LC during stress (Curtis et al., 2001; Curtis et al., 2012; Valentino et al., 1991).

In addition to its electrophysiological effects, CRF has enduring effects on LC dendritic morphology as seen in cultured slices from neonatal rats or in CATH.a cells, immortalized norepinephrine cells that resemble LC neurons (Cibelli et al., 2001; Swinny and Valentino, 2006). In the cultured explants CRF increases dendritic growth and in CATH.a cells it promotes neurite outgrowth and these effects are CRF1

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mediated. Both actions require cyclic AMP dependent protein kinase and mitogen-activated protein kinase (MAPK) pathways. The morphological effects of CRF on LC dendrites in cultured slices, were additionally demonstrated to require activation of the small GTPase, Rac, which promotes and stablizes dendritic branches (Swinny and Valentino, 2006). This was the first evidence linking CRF receptor actions to actin cytoskeletal regulation through Rho GTPases and suggested molecular mechanisms for stress-induced remodeling of the actin cytoskeleton.

The LC system is designed in a way that would allow CRF effects on LC dendritic growth to have a profound impact on how the LC-norepinephrine system is regulated. LC dendrites extend for hundreds of microns outside of the LC core (Shipley et al., 1996) and afferents to these peri-coerulear (peri-LC) regions are functionally distinct from afferents to the nuclear core. The majority of CRF afferents to the nuclear core of the LC are related to autonomic function. These include Barrington's nucleus, which regulates the parasympathetic neurons that control pelvic visceral function and the nucleus paragigantocellularis, which regulates sympathetic preganglionics involved in cardiovascular function (Valentino et al., 1992). In contrast, CRF afferents terminating outside of the nuclear core, in peri-LC regions into which LC dendrites extend, derive from limbic regions such as the central amygdalar nucleus (Van Bockstaele et al., 2001; Van Bockstaele et al., 1998; Van Bockstaele et al., 1999). These afferents convey emotion-related information and their links to the LC provide an anatomical substrate for emotional arousal. The further LC dendrites extend into the peri-LC space, the higher the probability that they will contact limbic afferents relaying emotion-related information to the LC arousal system, and the more likely the system will be regulated by emotion-

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related stimuli. By promoting LC dendritic growth, CRF shapes a structural basis for emotional arousal. One speculation from these data is that early life stressors create a foundation for higher emotional arousal through the process of CRF release in the LC. Potential sex differences in this effect are discussed below.

In addition, to these morphological effects, CRF acting at CRF1 potentiates the ability of tropomyosin receptor kinase B (TrkB) agonists, brain derived neurotrophic factor (BDNF) and neurotrophin-4, to induce the norepinephrine phenotype of LC neurons in culture (Traver et al., 2006). In this preparation, BDNF increases the number of tyrosine hydroxylase expressing neurons and although CRF has no effect on its own, it enhances this effect of BDNF. These CRF1 actions have been associated with activation of adenylyl cyclase, induction of cyclic AMP and activation of Epac (see below).

Despite the electrophysiological and immunohistochemical evidence for CRF1 in LC neurons, many in situ hybridization studies have failed to detect CRF1 mRNA in LC neurons of rodents although LC neurons of humans and non-human primates express CRF1 mRNA (Hiroi et al., 2001; Sanchez et al., 1999; Van Pett et al., 2000). Although difficult to reconcile, this could be attributed to distribution of the mRNA in the extensive LC dendritic system, which could dilute the signal, making it difficult to detect. Notably, a relatively recent in situ hybridization study provided evidence for CRF1 mRNA in rat LC (Zeng et al., 2003). Additionally CRF1 mRNA (but not CRF2 mRNA) was detected in LC neurons cultured from embryonic rats (Day 14) in the study described above (Traver et al., 2006).

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Sex differences in corticotropin-releasing factor regulation of the locus coeruleus

The first evidence suggesting sex differences in CRF receptor-mediated effects came from electrophysiological comparisons of LC neuronal activity in male and female rats (Curtis et al., 2006). The electrophysiological characteristics of LC neurons are mostly comparable between sexes. For example, LC firing rate and the magnitude of sensory-evoked responses that are mediated by glutamatergic inputs are similar in male and female rats. However, the magnitude of LC activation by hypotensive challenge, which activates LC neurons through CRF release in the LC, was greater in female compared to male rats. Notably, this effect was unrelated to adult hormonal status of either males or females. These sex differences in the magnitude of LC activation by hypotensive stress could be attributed to differences in postsynaptic sensitivity to CRF. Thus, the CRF dose-response curve for LC activation was shifted to the left in females compared to males and doses of CRF that were below threshold for increasing LC discharge rate in male rats were effective in females. In addition to sex differences in the acute effect of CRF, sex differences were apparent in the manner in which a prior history of stress regulated LC sensitivity to CRF (Curtis et al., 2006). For example, in male rats, a history of shock or swim stress shifts the CRF dose-response curve for LC activation to the left with a decrease in the maximal response such that neurons are more sensitive to low doses of CRF and less sensitive to higher doses. In contrast, in female rats the CRF dose-response curve for LC activation is not altered by a history of stress.

With respect to the morphological effects of CRF on LC neurons, it is noteworthy that LC dendrites of female rats are longer and more complex, having more branch

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points and extend further into the peri-LC than LC dendrites of male rats (Bangasser et al., 2011). This is reminiscent of the effects of CRF on LC dendrites and would result in a greater magnitude of arousal in response to emotion-related stimuli by favoring more contacts with limbic terminals that convey emotion-related information (Fig. 1). Nonetheless, the role of CRF in these sex differences has yet to be established.

CRF1 signaling and trafficking

The sex differences described above suggest distinctions in CRF1 signaling. CRF1 is of the Class B family of seven transmembrane G protein-coupled receptors (GPCR). In brain, the primary mode of signaling is through Gsα, which binds to the third intracellular loop (Hauger et al., 2006; Hauger et al., 2009). This results in activation of adenylyl cyclase with consequent formation of cyclic AMP and phosphorylation of protein kinase A (PKA). Activated PKA phosphorylates diverse cellular proteins including potassium ion channels and inactivation of potassium channels through phosphorylation has been implicated in the ability of CRF to increase LC neuronal discharge rates (Jedema and Grace, 2004). Phosphorylation of cyclic AMP response element binding protein (CREB) by PKA provides a potential mechanism whereby stress can affect cell transcription via CRF. Evidence suggests that brain derived neurotrophic factor BDNF is upregulated through this pathway (Bayatti et al., 2005). PKA signaling through CRF1 has also been linked to activation of the serine threonine kinase, SGK-1 that is associated with synaptic plasticity and cell survival (Sheng et al., 2008). CRF1 signaling through cyclic AMP that is independent of PKA results in activation of the guanine nucleotide exchange factor, Epac (Traver et al.,

2006). This proceeds to activate extracellular signal-regulated kinase-mitogen activated protein kinase (ERK-MAPK) pathways that regulate BDNF signaling. This cascade is involved in the ability of CRF to potentiate the phenotypic determination of LC neurons by BDNF (Traver et al., 2006). CRF1 has been linked to other signaling pathways particularly in other tissues or cells in culture and these are reviewed in (Hillhouse and Grammatopoulos, 2006).

Like other G protein-coupled receptors, CRF1 is internalized after agonist binding. Both agonist and stress-induced CRF1 internalization have been described *in vivo* in rat LC neurons (Reyes et al., 2006; Reyes et al., 2008). In male rats, CRF microinfusion into the LC causes CRF1 internalization into early endosomes in LC dendrites that is apparent by 5 min after injection and is more pronounced by 30 min after injection (Reyes et al., 2006). Evidence for CRF1 internalization into male rat LC dendrites is also apparent 1 h and 24 h after a single 15 min swim stress and this is completely prevented by pretreatment with a selective CRF1 antagonist (Reyes et al., 2008). At both timepoints, CRF1 is associated with early endosomes in the cytoplasm. However, by 24 h after swim stress significantly more CRF1 is associated with multivesicular bodies compared to 1 h after stress, indicative of degradation and downregulation of the receptor. This effect would be consistent with the decreased maximum response of the CRF dose-response curve for LC neuronal activation seen in male rats 24 h following swim stress.

The molecular mechanisms guiding agonist-induced CRF1 internalization have been well characterized in cell preparations including primary cortical neurons and HEK293 cells (Holmes et al., 2006; Oakley et al., 2007). Following agonist binding,

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CRF1 undergoes sequential phosphorylation steps by G protein receptor kinases on both the carboxyl tail and the third intracellular loop. This promotes the recruitment and binding of β-arrestin 2 and internalization by a dynamin-dependent process.

Sex differences in corticotropin-releasing factor signaling

The sex differences observed in LC neuronal responses to CRF could be attributed to differences in CRF1 signaling. Consistent with this, in females LC activation by CRF was almost completely prevented by the PKA antagonist, Rp-cAMP-S, whereas only a fraction (approximately 50%) of the CRF-elicited LC activation was prevented by the PKA antagonist in males (Bangasser et al., 2010). To examine the possibility of sex differences in CRF1 signaling, CRF1 was immunoprecipitated from rat cortex (Bangasser et al., 2010), a tissue of high CRF1 expression and lacking CRF2 (Van Pett et al., 2000). Although the cortex exhibits one of the highest densities of CRF1 expression in brain, relatively few studies have examined the effects of activating these receptors on behavior presumably because its greater area makes it less amenable to microinjection experiments compared to more discrete regions such as the amygdala. Nonetheless, several animal studies provide evidence for a role of cortical CRF1 in stress-related behaviors and pathology (Bijlsma et al., 2011; Jaferi and Bhatnagar, 2007; Magalhaes et al., 2010). Additionally, chronic stress increases in CRF1 mRNA in cortex in rodents and CRF1 mRNA is decreased in the cortex of depressed suicide victims, possibly in response to increased CRF peptide (Anisman et al., 2007; Merali et al., 2004).

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Immunoprecipitated CRF1 from the cortex of unstressed female rats was associated with approximately three times more Gs compared to unstressed male rats (Bangasser et al., 2010). In contrast, there were no sex differences in Go or Gq/11 association with CRF1. Similar to the sex differences in LC responses to CRF, adult hormonal status was not a determinant of the sex differences in CRF1-Gs coupling because similar results were obtained with intact and ovariectomized females. Importantly, in rats with a history of swim stress CRF_1-Gs association increased in males to a magnitude that matched that of unstressed females but the same stress history had no effect in females. Although it is not feasible to perform similar receptor immunoprecipitation studies with LC tissue, these sex differences in CRF_1-Gs coupling determined in cortical tissue mirrored sex differences in LC sensitivity to CRF and provide potential molecular mechanisms for these physiological differences. It is noteworthy that these were the first data to link sex differences in physiology to sex differences in the coupling of G proteins to receptors.

Sex differences in CRF_1 receptor trafficking

In contrast to its ability to induce CRF1 internalization in male LC neurons, swim stress does not result in CRF1 internalization in LC neurons of female rats (Bangasser et al., 2010). Indeed the cellular localization of CRF1 is opposite in female and male LC neurons. In the unstressed male rats approximately 50% CRF1 immunogold labeling is on the plasma membrane of LC neurons and 50% is cytoplasmic. Swim stress shifts the distribution of CRF1 immunolabeling such that approximately 70% is cytoplasmic, indicative of internalization. This is consistent with the decreased maximal activation of

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LC discharge produced by CRF. In contrast, in female LC neurons in the unstressed condition, CRF1 is predominantly cytoplasmic and swim stress shifts the distribution to the plasma membrane. In this case it is not clear whether swim stress actually recruits CRF1 to the plasma membrane or whether there is a decrease in the synthesis of CRF1 that would render relatively less in the cytoplasm compared to the plasma membrane. The inability of CRF1 to be internalized in female LC neurons by 24 h after swim stress is consistent with the lack of change in the maximum effect of CRF.

The compromised ability to internalize CRF1 in female LC neurons is likely the result of an inability of β-arrestin 2 to associate with CRF1, a critical molecular step in the process of receptor internalization. In the unstressed condition CRF1 immunoprecipitated from cortical tissue of male and female rats pulled down an equivalent amount of β-arrestin 2 (Bangasser et al., 2010). Following swim stress the association of β-arrestin 2 with CRF1 greatly increased in males, consistent with stress-induced CRF1 internalization. However, in females stress did not alter CRF1- β-arrestin 2 association. The deficit in β-arrestin 2 association with CRF1 in females relative to males can account for the compromised ability to internalize CRF1 following stress and this molecular mechanism may account for the inability of stress to decrease the maximal CRF response of female LC neurons. As for CRF_1-Gs coupling, the differences in β-arrestin 2 association were unrelated to adult hormonal status. Notably, the decreased ability of CRF_1 receptors in females to associate with β-arrestin 2 could also account for an enhanced association of CRF_1 with Gs, as β-arrestin 2 sterically hinders binding of Gs to GPCRs (Kohout and Lefkowitz, 2003).

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Sex differences in receptor association with G proteins and β-arrestins are unique and suggest structural differences in CRF1, perhaps as a result of post-translational modifications. CRF1 is glycosylated at many points, however differences in glycosylation would be predicted to alter molecular weight and there was no indication of sex differences in the molecular weight of the receptor (Grigoriadis and De Souza, 1989). Sex differences in GRK phosphorylation at one or more of several sites at which CRF1 is phosphorylated following agonist binding could account for differences in Gs or β-arrestin 2 association.

Sex differences in the consequences of CRF overexpression

The sex differences in CRF1 signaling described above would render female neurons more sensitive to CRF and less able to adapt to excessive CRF. However, sex differences in CRF1 signaling and trafficking have little consequence in the absence of CRF and so are unlikely to be manifest in the absence of stress. Rather, the presentation of a stressor that releases CRF is necessary to reveal the consequences of these molecular differences. Sex differences in CRF signaling and trafficking would have the greatest impact in conditions of excessive CRF as have been proposed to occur in the same stress-related psychiatric disorders that are more prevalent in females, PTSD and depression. Different strains of CRF overexpressing mice (CRF-OE) have been used to model the pathological condition of excessive CRF (Dirks et al., 2002; Groenink et al., 2003; Lu et al., 2008; Stenzel-Poore et al., 1994). The best characterized of these is a transgenic line in which CRF expression is under control of the metallothionein promoter (Stenzel-Poore et al., 1994). These mice have elevated

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CRF expression in brain neurons in most regions that normally express CRF. They exhibit evidence of hypothalamic-pituitary-adrenal axis overactivity, including adrenal hypertrophy and elevated plasma adrenocorticotropin and corticosterone. Additionally, these mice also show anxiogenic effects in many animal models. Conditional CRF-OE mice have been generated in which CRF can be overexpressed throughout the entire brain (Lu et al., 2008). A disadvantage of the conditional model is that CRF is expressed ubiquitously in both neurons and glia in brain and unlike the CRF transgenic mice, which exhibit CRF overexpression primarily in brain regions where CRF is typically expressed, the pattern of CRF expression in the conditional line is substantially different with the highest expression being in the olfactory bulb, cortex and hippocampus.

Sex differences were apparent in CRF-OE transgenic mice (metallothionein promoter) with respect to LC activity recorded from slice preparations *in vitro* and CRF1 cellular localization (Bangasser et al., 2012). The characteristics of LC neuronal activity were comparable in male and female wild type mice. Despite a greater CRF innervation of LC neurons of male CRF-OE mice compared to wild type mice, LC discharge rates were similar. In contrast, LC neurons of female CRF-OE mice had discharge rates that were nearly three times greater than those of wild type mice or male CRF-OE mice, suggesting that neurons of male mice have mechanisms to adapt to excessive CRF that are not present in females. CRF1 trafficking could account for the sex differences in LC activity of CRF-OE mice. The cellular distribution of CRF1 in LC neurons of male wild type and CRF-OE mice were analogous to CRF1 distribution in unstressed and stressed male rats, respectively. In wild type mice CRF1 was equally distributed within the cytoplasm and on the plasma membrane and in CRF-OE mice 80% of CRF receptor

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labeling was within the cytoplasm, consistent with internalization and accounting for the lack of effect of excess CRF on male LC neurons. The situation was opposite for female wild type and CRF-OE mice, such that CRF1 was predominantly cytoplasmic in wild type mice and on the plasma membrane in CRF-OE mice where it would be available to be activated by excessive levels of CRF. The inability of neurons in female CRF-OE mice to internalize CRF1, an effect that may be in part attributed to diminished CRF1-β-arrestin 2 association, results in an overactivated LC-norepinephrine system.

As overactivation of the LC-NE system translates to the hyperarousal symptoms that define stress-related psychiatric disorders, it is not surprising that these disorders are more prevalent in females. Although the molecular sex differences described here may bias towards stress-related pathology, they may also be programmed for evolutionary advantages in species in which the female must remain vigilant and exhibit behavioral flexibility in performing multiple tasks including foraging for food while protecting from predators.

Although sex differences in the behavioral phenotype of CRF-OE mice have not been systematically explored, some differences have been noted. Female CRF-OE mice gain more weight and have larger adrenals compared to their wild type counterparts whereas these differences are not apparent in males (Bangasser et al., 2012). Female CRF-OE mice also have enhanced pelvic visceral responses to stressors and show more behavioral inhibition in novel environments compared to males (Million et al., 2007). Finally, female CRF-OE mice exhibit impaired social interaction with males, whereas male CRF-OE mice do not show this reaction (Heinrichs et al., 1997).

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Sex Biased CRF Signaling

Although sex differences in CRF1 trafficking and signaling have important implications for stress-related disorders, new perspectives about β-arrestin function suggest even broader implications of the findings. In addition to its role in receptor internalization, β-arrestin2 can engage G-protein-independent signaling cascades by scaffolding receptors to signaling molecules, including mitogen-activated protein kinase (e.g., ERK2, JNK3, and p38), tyrosine kinases (e.g., c-SRC), as well as AKT, PI3 kinase and RhoA (for review see, (Lefkowitz and Shenoy, 2005; Violin and Lefkowitz, 2007). A compromised ability of female CRF_1 receptors to associate with β-arrestin2 would shunt CRF1 signaling down Gs-related pathways. In contrast, in male neurons β-arrestin 2, G-protein independent pathways would be favored, at least relative to CRF1 signaling in females. By engaging sex specific signaling pathways, CRF released during stress could have sex-specific cellular consequences that translate to distinct physiological and/or behavioral responses and distinct pathology (Fig. 2).

The consequences of CRF1 sex biased signaling would be magnified when CRF is in excess as has been proposed to occur in stress-related psychiatric disorders that are more prevalent in females. In these conditions, sex differences in signaling cascades engaged by CRF could underlie sex differences in the pathological presentation of stress. Because Gs-protein and β-arrestin 2 signaling regulate phosphorylation dynamics in cells, the excessive CRF would be predicted to result in sex specific phosphoprotein profiles. Keys to understanding sex differences in stress-related psychiatric disorders may lie in the differences between these profiles. This is

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currently being examined by performing a deep phosphoproteomic analysis of cortex of male and female CRF overexpressing (CRF-OE) mice using stable isotope labeling of whole mouse (SILAM) and high resolution mass spectrometry (Valentino et al., in press). The initial results confirm the model of sex biased signaling in that approximately 15% of the phosphopeptides that could be quantified in both groups were significantly enriched in either the female (10%) or male (5%) based on a false discovery rate of 1% which translated to a 1.52 fold difference. Ingenuity pathway analysis supported the concept of sex biased CRF1 signaling and indicated that kinases (including PKA) were prominent in the top five canonical pathways in which phosphopeptides that were enriched in the female CRF-OE phosphoproteome were overrepresented. In contrast phosphopeptides that were enriched in male CRF-OE cortex were overrepresented in pathways related to Rho signaling, which has been linked to β-arrestin. Interestingly, phosphopeptides enriched in the female CRF-OE were overrepresented in an amyloid processing pathway and included enzymes that process amyloid precursor protein to neuropathological amyloid β and kinases that phosphorylate tau, which leads to the formation of fibrillary tangles. Given that Alzheimer's disease is more prevalent in females and has been linked to stress (Csernansky et al., 2006; Figueira and Ouakinin, 2010; Ruitenberg et al., 2001; Wilson et al., 2006), these findings suggest a mechanism by which sex biased CRF1 signaling favors pathways that increase vulnerability to this disease in females.

Sex biased CRF_1 receptor signaling has important therapeutic implications for novel compounds that can shift the bias of CRF_1 receptor signaling. By shifting CRF signaling towards a β-arrestin2 pathway, such compounds could potentially make

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females more resilient to the pathophysiological consequences of stress, particularly hyperarousal. “Biased agonists” are currently being designed to direct receptor-mediated cellular events towards specific pathways in an effort to promote efficacy for desired effects while diminishing adverse effects (Bohn and McDonald, 2010; Rajagopal et al., 2011; Whalen et al., 2011). One example of this is the angiotensin II receptor, which may be targeted to take advantage of the anti-apototic effects mediated through β-arrestin, while at the same time avoiding G-protein mediated hypertensive effects (Rajagopal et al., 2011). Pharmaceuticals aimed at shifting CRF1 signaling from G-protein mediated to β-arrestin-mediated may reduce the hyperarousal symptoms associated with stress-related psychiatric diseases.

Overview and implications

This review integrates convergent findings supporting the novel concept of sex differences in receptor signaling and trafficking, using CRF1 as a model. A caveat is that much of the evidence for sex biased CRF1 signaling derives from studies using cortical tissue and there may be region specific effects of CRF1 signaling. Even post-translational modifications, such as receptor glycosylation, can be region specific (Grigoriadis and De Souza, 1989). Nonetheless, the finding that sex differences in CRF1 signaling identified in cortex were consistent with sex differences in CRF1 trafficking and physiological sensitivity in LC neurons supports the notion that these differences generalize to other regions expressing CRF1. Sex differences in Gs coupling would confer differences in agonist sensitivity and in the case of CRF, differences in acute responses to stressors. Notably, small molecule non-peptide CRF1

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antagonists bind to transmembrane domains of the receptor that are in proximity to the Gs-protein coupling site in the third intracellular loop. Potential sex specific phosphorylation of sites in this loop could confer sex differences in the ability of these antagonists to bind (Hauger et al., 2006; Hoare et al., 2004). Differences in receptor association with β-arrestin influence receptor trafficking and the ability to adapt to the excessive CRF that is predicted to be present in diseases related to severe or chronic stress. For females this would translate to an enhanced sensitivity to acute stress and decreased ability to adapt to chronic or repeated stress. The broader implication of this model, given evidence for β-arrestin 2/Gs-protein independent signaling, is that sex can be a determinant of the coping responses and pathology elicited by stress (Fig. 2). Identifying the consequences of sex biased CRF1 signaling will help us to understand why females are more vulnerable to certain stress-related diseases.

Sex differences in CRF function have important implications for drug development. Currently CRF antagonists are being designed to treat mood and anxiety disorders. Because the data reviewed here suggest that CRF hypersecretion has a greater impact in females compared to males, CRF antagonists may be more effective in women than men. Alternatively, sex differences in the CRF_1 receptor could affect the ability of these drugs to prevent CRF1 signaling. Pharmacotherapies designed with sex differences in mind may prove necessary in treating psychiatric consequences of stress.

Although this review focused on CRF1 signaling, given many of the shared characteristics of different GPCRs, it is unlikely that sex biased signaling is limited to CRF1. Recently, sex differences were reported in phosphorylation of cannabinoid receptors that could lead to differences in trafficking (Xing et al., 2011). Sex differences

have also been reported in ERK and AKT pathways and their regulation in a model of schizophrenia, the neonatal ventral hippocampal lesion model, suggesting mechanisms by which altered dopamine signaling may underlie sex differences in vulnerability to schizophrenia (Bychkov et al., 2011). This unexplored area of sex differences in receptor signaling has the potential to greatly impact our knowledge of pathophysiology of diverse diseases and to transform their related therapeutics.

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Author Contribution

Wrote or contributed to the writing of the manuscript: R.J. Valentino, D. Bangasser, E.J. Van Bockstaele

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Footnotes

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Figure Legends

Figure 1. Schematic depiction of how sex differences in LC dendritic morphology could affect emotional arousal. LC neurons of female rats have longer and more complex dendrites than neurons of males. Thus, the probability that LC dendrites will contact limbic afferents that convey emotion-related information and terminate in the peri-LC rather than the core is greater in females compared to males. This would be predicted to result in a greater magnitude of arousal in response to emotion-related stimuli. Abbreviations: BNST, bed nucleus of the stria terminalis; CNA, central nucleus of the amygdala; PGi, paragigantocellularis; PVN, paraventricular nucleus of the hypothalamus.

Reproduced from Bangasser et al., 2011.

Figure 2. Schematic depicting the predicted model of sex biased signaling. In females, the decreased ability of CRF_1 to associate with β-arrestin2 biases signaling through Gs-related pathways. In contrast, in males CRF_1 receptor can associate with β-arrestin2, which results in a relative bias towards β-arrestin2-related pathways. As a result of differential signaling, stress-induced release of CRF can produce sex specific cellular responses that can translate to different physiological and behavioral responses. Because CRF1-Gs signaling is linked to tonic LC activation and increased LC dendritic length, this is predicted to be greater in females and to result in hyperarousal in response to stress. At a clinical pathological level this would predispose to stress-related psychiatric disorders such as depression and PTSD where hyperarousal is a

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significant component. Consequences of CRF1-β-arrestin2 signaling are less well known but these may lead to more adaptive responses and perhaps pathology that is more prevalent in males as indicated in Table 1. The question marks underscore the speculation here.

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Table 1. Lifetime Prevalence of Psychiatric Disorders by Sex (%>9,000 subjects)[1]

Disorder	Female	Males	Female:Male Ratio
Panic	6.2	3.1	2.0
Generalized Anxiety	7.1	4.2	1.7
Any Anxiety Disorder	36.4	25.4	1.4
PTSD	9.7	3.6	2.7
Major Depression	20.2	13.2	1.5
Any Affective Disorder	24.4	17.5	1.4
Bipolar	4.5	4.3	1.0
Conduct Disorder	7.1	12.0	0.6
ADHD	6.4	9.8	0.7
Alcohol Abuse	7.5	19.6	0.4
Drug Abuse	4.8	11.6	0.4

[1]NCS-R http://www.hcp.med.harvard.edu/ncs/

Figure 1

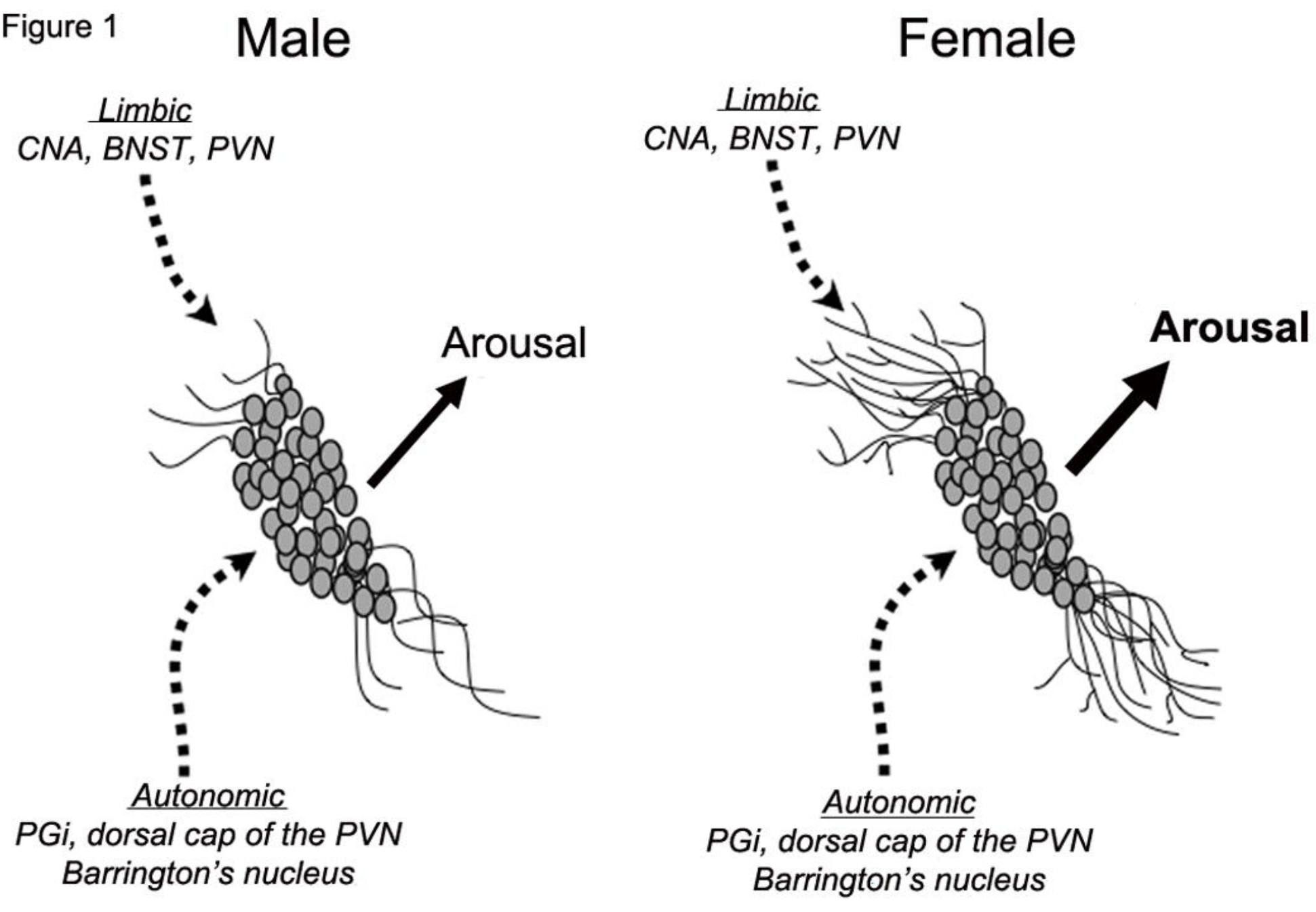
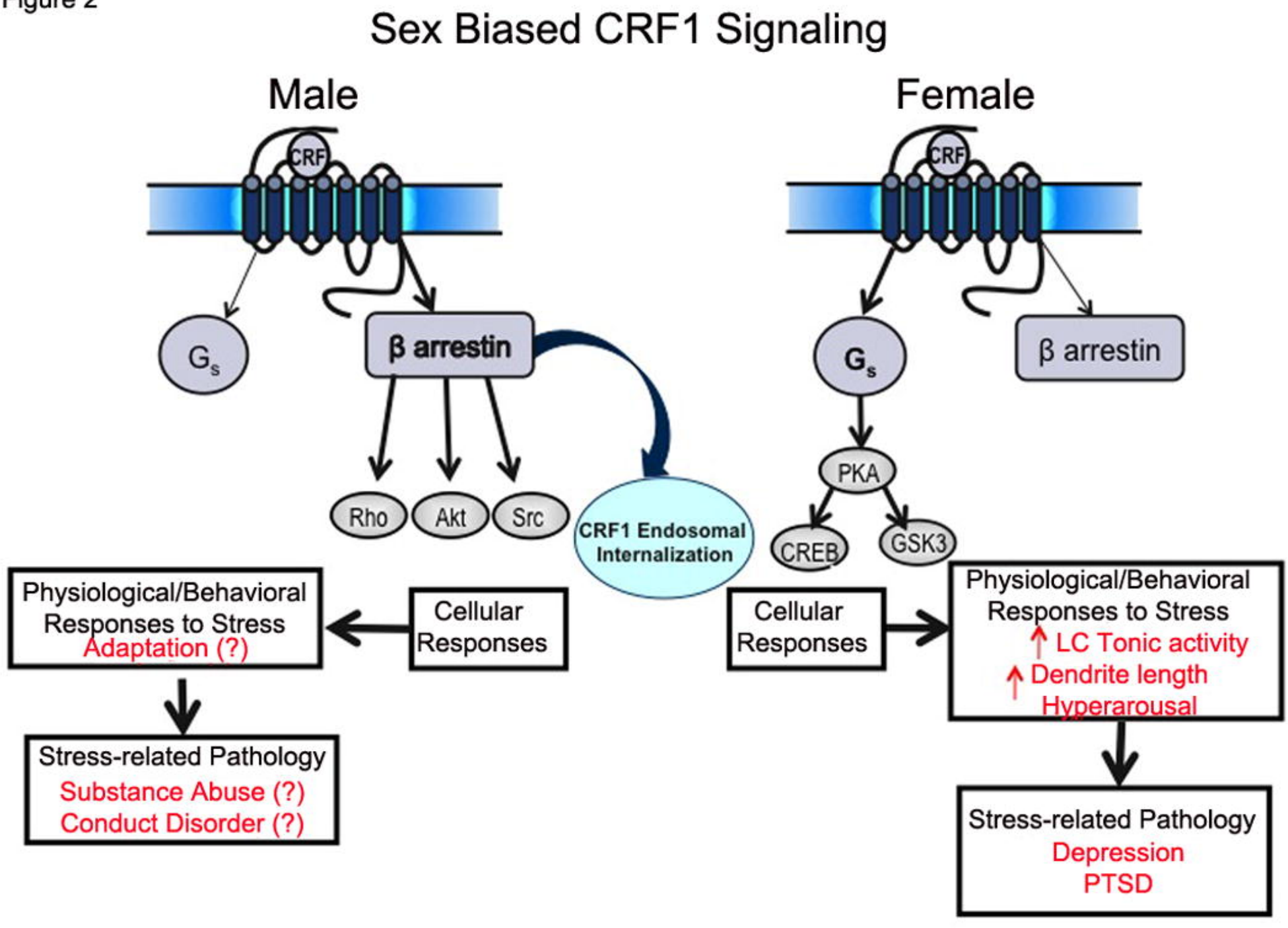

Male
Limbic
CNA, BNST, PVN
Arousal
Autonomic
PGi, dorsal cap of the PVN
Barrington's nucleus
Female
Limbic
CNA, BNST, PVN
Arousal
Autonomic
PGi, dorsal cap of the PVN
Barrington's nucleus

Figure 2


Sex Biased CRF1 Signaling
Male
Female
CRF
CRF
G_s
β arrestin
G_s
β arrestin
PKA
Rho
Akt
Src
CREB
GSK3
CRF1 Endosomal Internalization
Physiological/Behavioral Responses to Stress
Adaptation (?)
Cellular Responses
Cellular Responses
Physiological/Behavioral Responses to Stress
↑ LC Tonic activity
↑ Dendrite length
Hyperarousal
Stress-related Pathology
Substance Abuse (?)
Conduct Disorder (?)
Stress-related Pathology
Depression
PTSD