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*Mol Psychiatry*. 2010 September ; 15(9): 877–904. doi:10.1038/mp.2010.66.**Sex Differences in Corticotropin-Releasing Factor Receptor Signaling and Trafficking: Potential Role in Female Vulnerability to Stress-Related Psychopathology****Debra A. Bangasser, PhD<sup>1</sup>, Andre Curtis, PhD<sup>1</sup>, Beverly A.S. Reyes, PhD<sup>3</sup>, Thelma T. Bethea<sup>1</sup>, Ioannis Parastatidis, MD<sup>2</sup>, Harry Ischiropoulos, PhD<sup>2</sup>, Elisabeth J. Van Bockstaele, PhD<sup>3</sup>, and Rita J. Valentino, PhD<sup>1</sup>**<sup>1</sup>Department of Anesthesiology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104<sup>2</sup> Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA 19104<sup>3</sup>Department of Neurosurgery, Thomas Jefferson University, Farber Institute for Neurosciences, Philadelphia, PA 19107**Abstract**

Although the higher incidence of stress-related psychiatric disorders in females is well documented, its basis is unknown. Here we demonstrate that the receptor for corticotropin-releasing factor (CRF), the neuropeptide that orchestrates the stress response, signals and is trafficked differently in female rats in a manner that could result in a greater response and decreased adaptation to stressors. Most cellular responses to CRF in the brain are mediated by CRF receptor (CRFr) association with the GTP-binding protein, G<sub>s</sub>. Receptor immunoprecipitation studies revealed enhanced CRFr-G<sub>s</sub> coupling in cortical tissue of unstressed female rats. Previous stressor exposure abolished this sex difference by increasing CRFr-G<sub>s</sub> coupling selectively in males. These molecular results mirrored the effects of sex and stress on sensitivity of locus ceruleus (LC)-norepinephrine neurons to CRF. Differences in CRFr trafficking were also identified that could compromise stress adaptation in females. Specifically, stress-induced CRFr association with  $\beta$ -arrestin2, an integral step in receptor internalization, occurred only in male rats. Immunoelectron microscopy confirmed that stress elicited CRFr internalization in LC neurons of male rats exclusively, consistent with reported electrophysiological evidence for stress-induced desensitization to CRF in males. Together, these studies identified two aspects of CRFr function, increased cellular signaling and compromised internalization, which render CRF-receptive neurons of females more sensitive to low levels of CRF and less adaptable to high levels of CRF. CRFr dysfunction in females may underlie their increased vulnerability to develop stress-related pathology, particularly that related to increased activity of the LC-norepinephrine system, such as depression or post-traumatic stress disorder.

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**CONFLICT OF INTEREST:**

We have no competing financial interests in relation to the work described.

## Keywords

stress; locus ceruleus; norepinephrine; corticotropin-releasing hormone; depression; post-traumatic stress disorder

Stress-related psychiatric disorders (e.g., depression, post-traumatic stress disorder) are twice as prevalent in women compared to men (1-3). Although the neurobiological basis for this is unknown, differences in stress reactivity have been implicated in this disparity (4-7). Because corticotropin-releasing factor (CRF), a primary mediator of the stress response, is dysregulated in stress-related psychiatric disorders, it is a likely substrate for sex differences in stress vulnerability (8-11). Indeed, evidence for direct estrogenic regulation of CRF gene expression provides a compelling mechanism for sexual dimorphism of stress reactivity and prevalence of stress-related psychopathology in women (12, 13).

CRF acts as a neurohormone to initiate the hypothalamic-pituitary-adrenal response to stress and as a neurotransmitter to initiate autonomic, behavioral, and cognitive components of the stress response (14-16). One target of CRF neurotransmission is the locus ceruleus (LC), the source of the major brain norepinephrine system that regulates emotional arousal (17-20). CRF activates LC neurons during stress and this is associated with heightened arousal (19, 21, 22). Although these effects are adaptive in response to an acute stressor, persistent or inappropriate LC-norepinephrine activation has pathological consequences. Indeed, excessive activity of CRF and LC-norepinephrine systems is thought to underlie the core feature of hyperarousal in melancholic depression (8, 10, 23). Similarly, CRF hypersecretion and increased LC sensitivity have been implicated in post-traumatic stress disorder (24, 25). Thus, sex differences in these systems or their interaction could contribute to female vulnerability to these stress-related illnesses.

Our previous electrophysiological studies demonstrated sex differences in LC sensitivity to CRF and its regulation by prior stress that could be expressed as excessive activation of the LC-norepinephrine system in females (26). LC neurons of unstressed female rats were more sensitive to CRF compared to males, as indicated by a leftward shift in the CRF dose-response curve. Additionally, prior swim stress sensitized LC neurons of male rats only to low doses of CRF and desensitized them to high doses, such that the CRF dose-response curve shifted to the left to match that seen in unstressed females but plateaued at a lower level. Together, the findings suggested that the CRF receptor (CRFr), which mediates LC activation by CRF, signals and/or is trafficked differently in males and females.

This study was designed to identify the molecular basis for sex differences in neuronal sensitivity to CRF. Because CRFr signaling occurs primarily through its coupling to the GTP binding protein,  $G_s$ , receptor immunoprecipitation was used to determine whether CRFr- $G_s$  coupling differed in male and female rats (27). To examine potential sex differences in CRFr trafficking, CRFr phosphorylation and association with  $\beta$ -arrestin2 were compared, as these are important steps in the CRFr internalization process (28, 29). To confirm that the molecular events had cellular consequences, immunoelectron microscopy was used to visualize cellular compartmentalization of the receptor and stress-induced internalization. The results converged to reveal sexual dimorphism in CRFr function at

molecular and cellular levels that could contribute to the higher incidence of certain stress-related psychiatric disorders in females.

## METHODS AND MATERIALS

### Subjects

The subjects were male and female Sprague-Dawley rats (Charles River, Wilmington, MA). Females were intact or ovariectomized by the vendor at 42 days. Rats were approximately 47 days of age when shipped and used approximately 2 weeks after arrival. Shipments of male and female rats were age matched. See Supplementary Information (SI) Methods for details on subjects, housing conditions, and tracking the estrous cycle. Care and use of animals was approved by the Children's Hospital of Philadelphia Institutional Animal Care and Use Committee and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### Electrophysiology

Extracellular single unit LC activity was recorded in the halothane-anesthetized state 24 h after a 15 min swim stress or brief handling as described (26). The 24 h timepoint was chosen to correspond to the time that sex differences in stress regulation of LC sensitivity to CRF were observed (26). Surgery and procedures for LC recording coupled with drug microinfusion were as previously described with modifications detailed in SI Methods (26). Artificial cerebrospinal fluid (ACSF) or the cyclic adenosine monophosphate (cAMP)/protein kinase A antagonist, Rp-cAMP-S (adenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer, triethylammonium salt), was microinfused into the LC (600 ng in 120 nl ACSF) 20-40 min before CRF (1-30 ng in 30 nl ACSF). LC activity was recorded at least 6 min before and 10 min after CRF. Only one dose of CRF was tested on a single cell and only one cell tested in an individual rat. Recording sites were histologically identified as previously described (26).

### CRFr immunoprecipitation

Tissue was usually collected 24 h after stress or handling, to match electrophysiological timepoints. For receptor phosphorylation and  $\beta$ -arrestin2 studies tissue also was collected immediately after and one hour after stressor exposure. Unanesthetized rats were placed in a flexible plastic restrainer (Decapicone) and rapidly decapitated. Frontal cortex (anterior to the +3.20 AP coordinate, relative to Bregma) was dissected and frozen ( $-80^{\circ}\text{C}$ ). Initial studies revealed no sex- or stress-induced differences in cortical CRFr expression indicating that cortical samples prepared for the immunoprecipitation procedure contained comparable amounts of CRFr protein in each group (see SI Results). CRFr was immunoprecipitated from 3 pooled samples for a single determination as detailed in SI Methods.

### Western blotting

Immunoprecipitated samples (5  $\mu\text{g}$  per condition) were subjected to SDS-PAGE gel electrophoresis and proteins transferred to polyvinylidene fluoride membranes (Immobilon-FL) as described (26). Membranes were probed for specific proteins as previously described with modifications detailed in SI Methods (30). Odyssey Infrared Imaging software

quantified the integrated intensity of each band and determined molecular weights based on Biorad Precision Plus Protein Standards. The ratio of target protein ( $G_s$ ,  $G_o$ ,  $G_{q/11}$ , phosphothreonine, or  $\beta$ -arrestin2) to CRFr was calculated and the mean ratios were compared between groups using ANOVAs. For the Figures, each individual fluorescent channel of the image was adjusted for brightness and contrast using the Odyssey Infrared Imaging Software.

### Immunoelectron microscopy

Tissue preparation, immunolabeling, and quantification for the immunoelectron microscopy studies were as previously described (31, 32). Immunogold-silver and immunoperoxidase labeling were used to detect CRFr- and tyrosine hydroxylase (TH)-immunoreactivity, respectively. Further details on immunoelectron microscopy methods and quantification are in SI (methods and Fig. S3).

### Antibody controls

Evidence that the antibodies used are detecting only CRF<sub>1</sub> in cortical and LC tissue in these studies is described in detail in SI Methods. Thus, CRFr refers to CRF<sub>1</sub> in cortical and LC tissue in this study.

## RESULTS

### CRFr signaling is increased in females and differentially regulated by stress compared to males

To determine whether sex differences in LC sensitivity to CRF were related to differences in cAMP-mediated cellular signaling, initial studies assessed the cAMP-dependent component of LC responses to CRF (Fig. 1). LC activation by a relatively low CRF dose (3 ng for males and the equieffective dose of 1 ng for females) or a near maximally effective dose of CRF for both groups (30 ng) was recorded in the presence of the cAMP and protein kinase A antagonist, Rp-cAMP-S, or vehicle. As previously reported (26), in the unstressed state, LC neurons of female rats were activated by a dose of CRF that was ineffective in male rats (Fig. 1A,B). This response was completely cAMP-dependent because it was abolished by Rp-cAMP-S (Fig. 1B). For both sexes, LC activation by the higher dose (30 ng) was mediated by both cAMP-dependent and independent processes (Fig. 1A,B). Following swim stress, LC neurons of male rats were activated by a CRF dose (3 ng) that was ineffective in unstressed male rats, confirming previous findings (26) (Fig. 1C). This sensitized response was completely cAMP-dependent, whereas the neuronal response to the higher dose of CRF (on the plateau) was cAMP-independent (Fig. 1C). In contrast to what was seen in males, the cAMP-dependent profile in females was unchanged by prior stress history (Fig. 1D). Finally, there were no significant effects of stress or sex in cAMP-independent signaling.

To determine whether sex differences in neuronal responses to CRF reflected differential CRFr- $G_s$  coupling, the amount of  $G_s$  pulled down with immunoprecipitated CRFr was quantified. The quantity of protein required for receptor immunoprecipitation necessitated the use of cortical tissue for these studies. Notably, the CRF<sub>1</sub> receptor that is thought to mediate LC activation is in high density in the cortex and linked to the cAMP signaling

pathway (33-36). Receptor binding and *in situ* hybridization studies suggest that this is the sole CRF receptor subtype in cortex and a lack of staining with a CRF<sub>2</sub> receptor antibody supported this (33, 37) (See Figs. S2, S3 in SI). Figure 2 shows representative blots of immunoprecipitated CRFr and the associated G<sub>s</sub> protein pulled down in the different experimental groups. In the unstressed condition, the amount of G<sub>s</sub> protein immunoprecipitated with CRFr was significantly greater for females (either ovariectomized or intact) compared to males, indicating greater CRFr-G<sub>s</sub> coupling in females (Fig. 2A,D). This mirrors the electrophysiological findings of an increased neuronal response of female rats to low doses of CRF (26)(Fig.1A,B).

Swim stress increased CRFr-G<sub>s</sub> coupling in males to a level comparable to that of unstressed females (Fig. 2A,D) and this occurred at the same time that LC neuronal sensitivity to low doses of CRF was increased in male rats (26) (Fig. 1C). Swim stress did not significantly alter CRFr-G<sub>s</sub> coupling in females (Fig. 2A,D). CRFr-G<sub>s</sub> association was comparable in ovariectomized and intact females in both stressed and unstressed conditions, suggesting no contribution of circulating ovarian hormones to this effect. These biochemical results in the cortex match the electrophysiological findings in the LC and suggest that enhanced neuronal responses to CRF in females and stressed males result from increased CRFr-G<sub>s</sub> coupling.

There were no sex or stress-related differences in CRFr association with either G<sub>o</sub> or G<sub>q/11</sub> (Fig. 2 B,C,E,F). This is consistent with a lack of sex or stress differences in the non-cAMP mediated component of the electrophysiological response to CRF and underscores the contribution of CRFr-G<sub>s</sub> coupling to sex differences in CRFr function (Fig. 1).

### **Stress-elicited association of CRFr to $\beta$ -arrestin2 is not observed in females**

The process of receptor internalization regulates cell sensitivity to ligands and agonists of G-protein coupled receptors (38, 39). CRFr internalization is initiated by phosphorylation of a threonine residue on the carboxy terminus and subsequent binding of  $\beta$ -arrestin2 in cultured cells and primary cortical neurons (28, 29, 40). Sex differences in CRFr phosphorylation were assessed by probing immunoprecipitated CRFr with an antibody directed against phosphothreonine. Merging the channels used to visualize phosphothreonine- and CRFr-immunoreactivity revealed an identical band, consistent with detection of the phosphorylated receptor (Fig. 3A). There were no sex- or stress-related differences in phosphothreonine labeling of CRFr at any timepoint after swim stress (Fig. 3B1-B3).

Detection of  $\beta$ -arrestin2 in immunoprecipitated samples revealed an effect of both sex and stress on CRFr- $\beta$ -arrestin2 association (Fig. 3C, D1-D3). In the unstressed condition, CRFr- $\beta$ -arrestin2 association was relatively low and similar in males and females. Stress increased CRFr- $\beta$ -arrestin2 association solely in males at 1 h and 24 h after swim stress, consistent with the internalization process (Fig. 3D1-D3) (32). In contrast, stress failed to increase CRFr- $\beta$ -arrestin2 association in females at any timepoint, suggesting that the important adaptive process of CRFr internalization may be compromised in females (Fig. 3 D1-D3). CRFr- $\beta$ -arrestin2 association was similar in ovariectomized and intact females in either unstressed or stressed conditions.

### **Stress-elicited CRFr internalization is not observed in females**

Immunoelectron microscopy was used to compare the cellular localization of CRFr between groups. Immunogold-silver labeled CRFr was identified in LC dendrites in both male and female rats (Fig. 4A-C). CRFr was found within TH-labeled dendrites of female rats as has been demonstrated for LC neurons of male rats (32) (Fig. 4C1-C2). Consistent with previous reports (32), in unstressed male rats CRFr was more prevalent on the plasma membrane. In contrast, in unstressed female rats CRFr was predominantly cytoplasmic (Fig. 4A1, B1, D).

Swim stress induced CRFr internalization in male rats as indicated by a greater ratio of cytoplasmic-to-total silver grains 24 h after stress (Fig. 4A2,D). In contrast, a decreased ratio of cytoplasmic-to-total silver grains was apparent in females after swim stress, suggestive of CRFr recruitment to the plasma membrane (Fig. 4B2, D). Together, the immunoelectron microscopy data support the immunoprecipitation and electrophysiological studies suggesting that stress causes CRFr internalization in male rats only.

## **DISCUSSION**

The present study provides convergent evidence for sexual dimorphism in CRFr signaling and trafficking. Receptor immunoprecipitation revealed greater CRFr-G<sub>s</sub> coupling in females compared to males in unstressed conditions, consistent with a greater sensitivity to CRF determined electrophysiologically. A history of stress increased CRFr-G<sub>s</sub> coupling only in males to a magnitude comparable to that seen in females, mirroring stress-induced changes in neuronal sensitivity to CRF. Sex differences in CRFr- $\beta$ -arrestin2 association corresponded to sex differences in CRFr trafficking determined by electron microscopy. These results can account for the earlier plateau in the CRF dose response curve determined in male rats in electrophysiological studies (26). Together, the findings identify molecular and cellular mechanisms that could result in enhanced sensitivity of females to CRF and a decreased ability to adapt to excessive CRF. Because hyperactivity of CRF and LC systems are features of certain stress-related disorders that are more prevalent in females (e.g., depression, post-traumatic stress disorder), these mechanisms may underlie the well recognized vulnerability of females to these conditions.

### **Sex differences in the CRF system and the role of gonadal hormones**

Sexual dimorphism of the CRF system has been demonstrated at multiple levels (for review see, (13, 41). Hypothalamic CRF expression is greater in female humans and rodents, and certain stressors increase hypothalamic CRF exclusively in females (42-45). Sex differences in CRF expression are established by organizational and activational effects of gonadal hormones. The perinatal testosterone surge organizes sex differences in adult CRF gene expression and mRNA (46, 47). In adulthood, circulating estrogen positively regulates CRF and CRF binding protein (CRF-BP) mRNA expression through estrogen response elements on their genes (12, 48-50).

The present study provides the first evidence for sexual dimorphism at the level of CRFr. Unlike the case for CRF or CRF-BP, circulating gonadal hormones are not involved in sex differences in CRFr function, as indicated by molecular findings of the present study or our



previous electrophysiological findings (26). Thus, sex differences in CRFr likely result from organizational effects of testosterone at critical developmental periods.

### Sex differences in CRFr signaling

LC neurons of male and female rats have comparable spontaneous discharge rates and responses to sensory stimuli (26). However in the unstressed state, LC neurons of females are more sensitive to CRF, as indicated by a shift to the left of the CRF dose-response curve for LC activation compared to that determined in male rats (26). This is physiologically relevant because it translates to enhanced activation of the LC-norepinephrine system by stress (26). Consistent with *in vitro* studies, a substantial component of LC activation by CRF *in vivo* is mediated by a cAMP-dependent pathway (51). LC activation in unstressed females by a CRF dose that was ineffective in males was completely cAMP-dependent. Thus, the greater CRFr-G<sub>s</sub> coupling in unstressed females revealed by CRFr immunoprecipitation studies accounts for the ability of a low CRF dose to activate LC neurons by a cAMP-dependent mechanism in females and not males.

Although it would be ideal to use LC tissue for CRFr immunoprecipitation studies, this was not feasible given the amount of protein required. Because CRF effects on LC neurons are mediated by CRF<sub>1</sub>, the cortex was an appropriate brain region to use to assess sex differences in LC sensitivity to CRF. CRF<sub>1</sub> is abundant in cortex and coupled to G<sub>s</sub> and cAMP formation (52, 53). In contrast to hippocampus and amygdala, CRF<sub>1</sub> in cortex is not linked to p-ERK1/2 activation (54). The parallel results in these anatomically distinct regions suggest that sex differences in CRFr signaling may be a more widespread.

In male rats, previous stress changes the CRF dose-response relationship for LC activation in a complex manner: causing a shift to the left in the low dose range and decreasing the maximum response. This has been documented for repeated shock, repeated intraperitoneal saline injections, and swim stress (55-57). The stress-induced sensitization of male LC neurons to low doses of CRF makes the response comparable to that seen in unstressed females and, as in females, this response is completely cAMP dependent. Sex and stress differences in CRFr-G<sub>s</sub> coupling mirrored the electrophysiological differences, supporting this as an underlying mechanism for stress-induced neuronal sensitization in males.

### Sex differences in CRFr internalization

Internalization of G-protein coupled receptors regulates cell sensitivity to agonists (38, 39). Both agonist- and swim stress-induced CRFr internalization in LC neurons of male rats have been previously documented (31, 32). As time increases from 1 to 24 h after swim stress, CRFr incorporation into multivesicular bodies increases, consistent with receptor downregulation (32). The functional consequence of this is an earlier plateau of the CRF dose-response curve for LC activation in male rats exposed to swim stress (26). The cellular processes involved in CRFr internalization in cultured cells indicate a requirement for phosphorylation of a threonine residue Thr399 on the carboxy tail and recruitment of  $\beta$ -arrestin2 (28). The finding that stress did not alter CRFr-threonine phosphorylation may be an indication that stress-induced CRFr internalization in brain requires phosphorylation at other sites on the receptor. Alternatively, the technique used in this study may not be

sufficiently sensitive to detect differences in phosphorylation sites that are required for internalization.

Stress increased CRFr- $\beta$ -arrestin2 association in males at 1 and 24 h after stress, times at which CRFr internalization was apparent in LC neurons (32). The inability of swim stress to alter CRFr- $\beta$ -arrestin2 association in females predicted that the internalization process would be impaired and this was confirmed in electron microscopy studies. The finding that CRFr was prominent in the cytoplasm in unstressed females and on the plasma membrane in stressed females was unexpected. This could reflect stress-induced recruitment of CRFr to the plasma membrane. Alternatively, it is possible that unstressed females produce more receptors that remain cytoplasmic and this is attenuated by stress, effectively increasing the proportion of receptors on the plasma membrane by decreasing the amount in the cytoplasm. Evidence for simultaneous receptor internalization and increased coupling in previously stressed male rats completely accounts for the complex shift in the CRF dose-response curve in male rats with a history of stress (i.e., a shift to the left with a decreased maximum response). This suggests that CRFr remaining on plasma membrane after stress is more highly coupled.

### Relevance of enhanced CRFr signaling in females

Sex differences in CRFr in rat studies should translate to increased sensitivity in rodent models of stress-related psychopathology. However, most rodent models of these disorders use males exclusively and studies using both sexes are equivocal with regard to whether females are more sensitive (for review see, (58-60)). An important issue is that many of these models (e.g. the forced swim test, open field) use an inhibition of motor activity as an endpoint indicative of depressive- or anxiety-like behavior. This is problematic because there are baseline sex differences in activity in many rodent species (60). Moreover, it is unclear that a decrease in activity appropriately models the hyperarousal that characterizes melancholic depression and PTSD, and which is thought to involve LC hyperactivity and/or sensitivity.

CRF and stressors shift the mode of LC discharge towards a high tonic-low phasic state that is associated with heightened arousal and a shift from a focused to labile attention that facilitates scanning the environment (61-63). This is an adaptive behavioral response to acute stress. However, if this response is engaged inappropriately or if it persists, this is expressed as pathology similar to that described in depression or PTSD (e.g., hyperarousal, sleep disturbance, inability to concentrate, anxiogenic behaviors). As a result of increased CRFr- $G_s$  coupling, the LC system of females will be activated by stressors (i.e., stimuli that release CRF) that are subthreshold for activating the system in males. The lack of CRFr internalization in LC neurons of females would be translated to an inability to adapt to high levels of CRF as might be produced with chronic stress or in depression, conditions in which CRF hypersecretion is hypothesized (64). The vulnerability of females to depression or PTSD may in part involve this lower threshold for stress-induced activation of the LC-norepinephrine system and the potential for a more persistent activation.



## Summary

Complementary approaches identified sex differences in two aspects of CRFr function that can contribute to increased CRF sensitivity in females. Increased CRFr signaling and compromised internalization would be expressed as increased sensitivity to low levels of CRF and compromised adaptation to high levels of CRF in females. This enhanced postsynaptic CRF function may be an important molecular mechanism underlying the vulnerability of women to stress-related psychiatric disorders. Finally, these findings underscore the importance of considering sexual dimorphism in CRFr function in developing CRFr antagonists for the treatment of psychiatric disorders that are more prevalent in females.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

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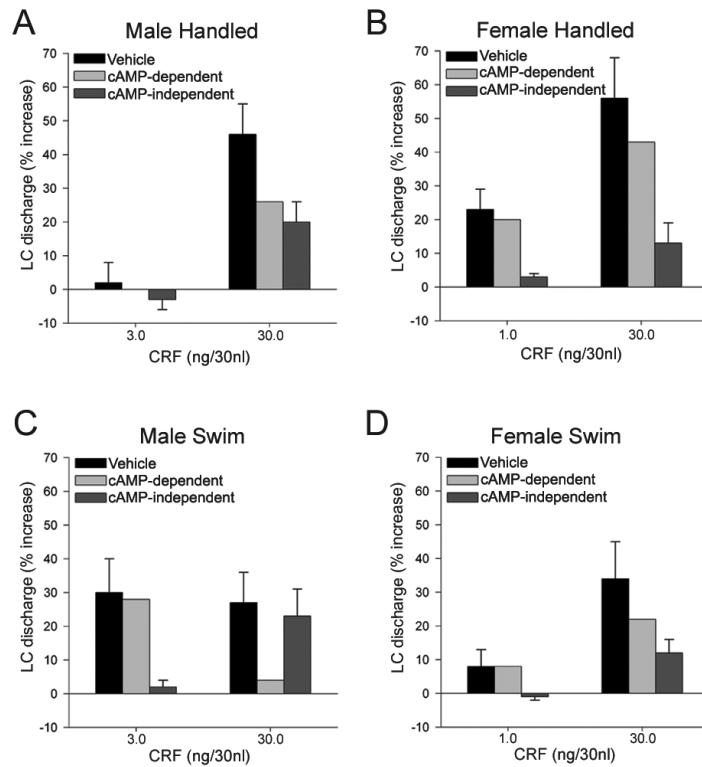
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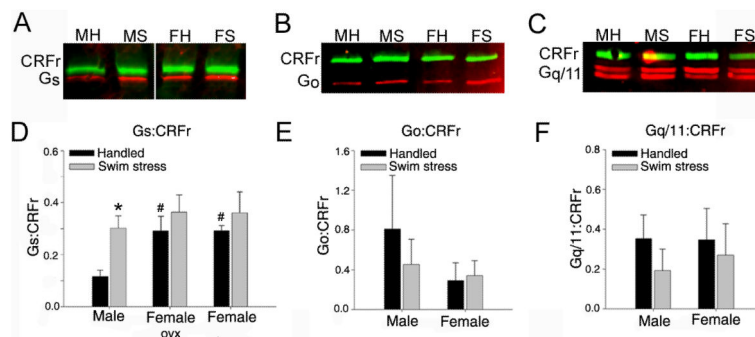
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**Figure 1.**

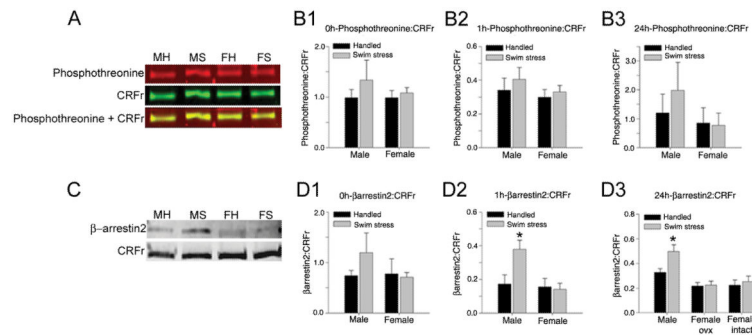
The role of cAMP signaling in the sex- and stress-related differences in LC neuronal activation by CRF. (A-D) LC activation by local CRF after pretreatment with Rp-cAMP-S (600 ng in 120 nl, intra-LC) or ACSF (120 nl) is shown for unstressed male (n=5-8) and female rats (n=4-6) and male (n=6-8) and female rats 24 h after swim stress (n=4-6). Bars depict the average response to CRF following ACSF (black) or Rp-cAMP-S (dark gray). Light gray bars represent the cAMP-mediated component (calculated by taking the difference between the vehicle and Rp-cAMP-S treated groups). In the unstressed state, a relatively low CRF dose activated LC neurons in females only [ $t(6)=3.56$ ,  $p<0.05$ ], and this response was completely cAMP-dependent. For both males and females, neuronal responses to the higher dose (30 ng) were mediated by cAMP-dependent and independent processes [ $F(1,26)=12.88$ ,  $p<0.05$ ]. Swim stress changed the cAMP signaling profile in males {stress $\times$ dose $\times$ drug interaction [ $F(1,46)=4.45$ ,  $p<0.05$ ]}, but not in females [ $F(1,39)=1.87$ ,  $p>0.05$ ].



**Figure 2.**

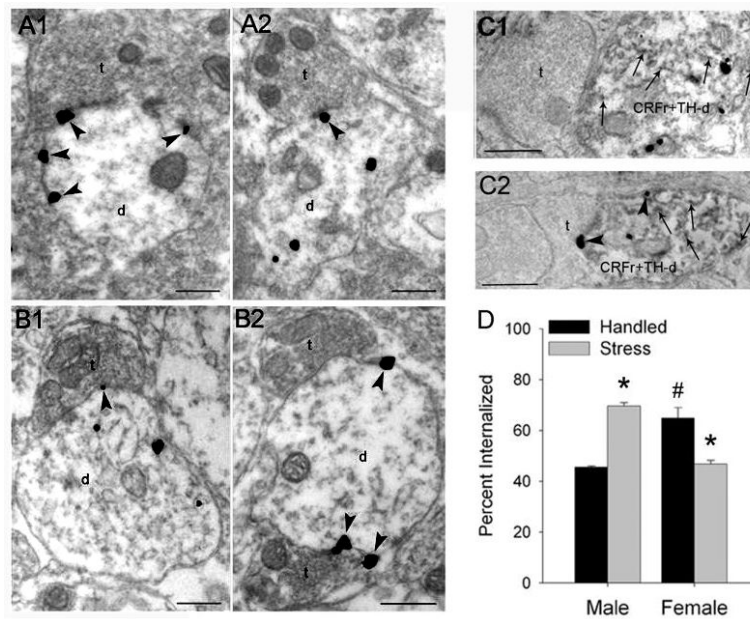
Sex- and stress-related differences in CRFr association with different G proteins. (**A-C**) Representative blots of immunoprecipitated CRFr (green, MW=52kD) from different groups and (**A**) the  $G_s$  protein (red, MW=48kDa), (**B**) the  $G_o$  protein (red, MW=40kDa) and (**C**) the  $G_{q/11}$  protein (red, MW=40kDa). So that the presentation in A, matched that in B and C, a lane containing the molecular weight marker that was between female and male samples on the same gel was deleted and the image of male samples that were to the right of this lane were moved to the left of female samples. (**D-F**) Graphs show the mean ratio of the integrated intensity of each band of G proteins to the corresponding band of CRFr from the same samples ( $n=4-6$  determinations, pooled 3 rats per determination). CRFr- $G_s$  coupling was greater in unstressed ovariectomized and intact females compared to unstressed males [ $F(5,26)=2.56$ ,  $p<0.05$ , post-hocs  $p<0.05$ ]. Stress increased coupling in males ( $p<0.05$ ) to a level comparable to that of unstressed females ( $p>0.05$ ), but had no further effect on females (regardless of hormonal status;  $p>0.05$ ). There were no significant differences in coupling of the CRFr to  $G_o$  [ $F(3,20)=0.55$ ,  $p>0.05$ ] or  $G_{q/11}$  [ $F(3,12)=0.55$ ,  $p>0.05$ ] (top band quantified). Data are represented as the mean ( $\pm$ SEM). Number sign indicates sex difference under basal (unstressed) conditions (i.e., greater coupling in unstressed females vs. unstressed males;  $p<0.05$ ). Asterisks indicate a significant stress-induced increase compared to the unstressed same sex control ( $p<0.05$ ).





**Figure 3.**

Sex differences in proteins involved in CRFr internalization processes. **(A)** Blots represent the phosphothreonine band (red), CRFr band (green) and the merged image (yellow) indicating that both label the same protein (i.e., phosphorylated CRFr, MW=52 kDa). Protein for this blot was collected 24 h after stressor exposure. **(B1-3)** Bar graphs show the mean ratio of phosphothreonine:CRFr for each condition from tissue collected immediately [ $F(3,12)=0.39$ ,  $p>0.05$ ], 1 h [ $F(3,20)=0.58$ ,  $p>0.05$ ] or 24 h [ $F(3,20)=0.66$ ,  $p>0.05$ ] post-stress ( $n=4-6$  determinations, pooled 3 rats per determination). **(C)** The Western blot shows the  $\beta$ -arrestin2 band (MW = 54 kDa) and the CRFr band 24 h after stressor exposure or handling. **(D1-3)** Graphs illustrate the ratio of  $\beta$ -arrestin2:CRFr for rats sacrificed immediately [ $F(3,12)=0.52$ ,  $p>0.05$ ], 1 h [ $F(3,16)=4.74$ ,  $p<0.05$ ] or 24 h post-stress [ $F(5,30)=5.77$ ,  $p<0.05$ ] ( $n=4-6$  determinations, pooled 3 rats per determination). At both 1 and 24 h after stress,  $\beta$ -arrestin2 association with the CRFr was significantly increased in males ( $p<0.05$ ) but not in females ( $p>0.05$ ). Cycling females were included for an additional comparison at the 24 h timepoint, and there was no statistically significant difference in CRFr- $\beta$ -arrestin2 association between ovariectomized and cycling females in either the unstressed or stressed condition ( $p>0.05$ ). Data are represented as the mean ( $\pm$ SEM). Asterisks indicate a significant effect of stress compared to unstressed same sex control ( $p<0.05$ ).



**Figure 4.**

Electron microscopic visualization of CRFr compartmentalization and stress-induced trafficking in LC dendrites. A-C are electron photomicrographs of sections through the LC. **(A1)** LC dendritic profile (d) in an unstressed male rat with immunogold-silver labeling for the CRFr along the plasma membrane (arrowheads). The dendrite receives synaptic contacts from axon terminals (t). **(A2)** Dendrite from a male rat 24 h following swim stress. CRFr labeling shifts from the plasma membrane to the cytoplasm. **(B1)** Dendrite from an unstressed female rat shows that CRFr is prominent in the cytoplasm. **(B2)** Dendrite from a female rat 24 h following swim stress shows that CRFr labeling shifts from the cytoplasm to the plasma membrane. **(C1-2)** TH-immunoperoxidase-labeled dendrites containing immunogold-silver labeling for CRFr (CRFr+TH) in a female control **(C1)** and a stressed rat **(C2)**. Arrowheads point to CRFr on the plasma membrane in C2. Arrows point to immunoperoxidase reaction product. **(D)** Bar graph indicating the percentage of internalized receptors for each condition (n=3, mean per rat generated from at least 125 dendritic profiles). Unstressed females had a significantly greater percentage of cytoplasmic receptors than unstressed males [ $F(1,8)=45.3$ ,  $p<0.05$ , post-hoc,  $p<0.05$ ]. Swim stress increased the percentage of CRFr in cytoplasm in males rats ( $p<0.05$ ). In contrast, swim stress decreased the percentage of cytoplasmic CRFr in females ( $p<0.05$ ). Data are represented as the mean ( $\pm$  SEM). Number sign indicates sex difference under unstressed conditions ( $p<0.05$ ). Asterisks indicate a significant effect of stress compared to the unstressed same sex control ( $p<0.05$ ). Scale bars=500 nm (A-C).