



Published in final edited form as:

*Mol Psychiatry*. 2013 February ; 18(2): 166–173. doi:10.1038/mp.2012.24.

## Increased vulnerability of the brain norepinephrine system of females to corticotropin-releasing factor overexpression

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### Abstract

Stress-related psychiatric disorders are more prevalent in women than men. Because hypersecretion of the stress neuromediator, corticotropin-releasing factor (CRF) has been implicated in these disorders, sex differences in CRF sensitivity could underlie this disparity. Hyperarousal is a core symptom that is shared by stress-related disorders and this has been attributed to CRF regulation of the locus coeruleus (LC)-norepinephrine arousal system. We recently identified sex differences in CRF<sub>1</sub> receptor (CRF<sub>1</sub>) signaling and trafficking that render LC neurons of female rats more sensitive to CRF and potentially less able to adapt to excess CRF compared to male rats. The present study used a genetic model of CRF overexpression to test the hypothesis that females would be more vulnerable to LC dysregulation by conditions of excess CRF. In both male and female CRF overexpressing (CRF-OE) mice, the LC was more densely innervated by CRF compared to wild type controls. Despite the equally dense CRF innervation of the LC in male and female CRF-OE mice, LC discharge rates recorded in slices *in vitro* were selectively elevated in female CRF-OE mice. Immunoelectron microscopy revealed that this sex difference resulted from differential CRF<sub>1</sub> trafficking. In male CRF-OE mice, CRF<sub>1</sub> immunolabeling was prominent in the cytoplasm of LC neurons, indicative of internalization, a process that would protect cells from excessive CRF. However, in female CRF-OE mice, CRF<sub>1</sub> labeling was more prominent on the plasma membrane, suggesting that the compensatory response of internalization was compromised. Together, the findings suggest that the LC-norepinephrine system of females will be particularly affected by conditions resulting in elevated CRF because of differences in receptor trafficking. As excessive LC activation has been implicated in the arousal components of stress-related psychiatric disorders, this may be a cellular mechanism that contributes to the increased incidence of these disorders in females.

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Stress-related psychiatric disorders, such as depression and post-traumatic stress disorder (PTSD), affect approximately 20% of the population.<sup>1,2</sup> Women are twice as likely to be affected as men.<sup>3–5</sup> This disparity may reflect sex differences in mediators underlying the

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stress response. For example estrogen positively regulates the gene for corticotropin-releasing factor (CRF), the primary mediator of the stress response and as a consequence, hypothalamic-pituitary adrenal (HPA) axis activity.<sup>6–8</sup> Recently, we identified sex differences in the postsynaptic response to CRF that could render females more sensitive to stress and less able to adapt to chronic stressors.<sup>9</sup>

CRF elicits adrenocorticotropin (ACTH) release in response to stress.<sup>10</sup> CRF also regulates biogenic amine systems during stress, including the locus ceruleus (LC)-norepinephrine system.<sup>11–13</sup> CRF activation of the LC initiates arousal and is thought to alter attention in response to stress.<sup>11,14–16</sup> Although this is part of an adaptive cognitive response to stress, dysregulation of the LC-norepinephrine system by excessive CRF has been proposed to occur in pathological conditions and to underlie the hyperarousal that characterizes stress-related psychopathology.<sup>17–20</sup>

LC neurons of female rats are more sensitive to stress and CRF.<sup>21</sup> We recently demonstrated that this results from enhanced coupling between the CRF<sub>1</sub> receptor (CRF<sub>1</sub>) and the Gs receptor binding protein through which it signals.<sup>9</sup> Additionally, stress-induced association of CRF<sub>1</sub> with  $\beta$ arrestin2 and subsequent internalization into LC neurons was compromised in female rats.<sup>9</sup> This could diminish the ability to adapt to high levels of CRF as might be present with chronic stress. Together, these sex differences in CRF<sub>1</sub> function could contribute to the higher incidence of stress-related disorders in females, particularly those characterized by hyperarousal.

Sex differences in the function of the LC system would be most prominent during conditions of excessive CRF, as has been proposed to occur in depression and PTSD.<sup>19,20,22–26</sup> This can be modeled with CRF-overexpressing (CRF-OE) mice.<sup>27–30</sup> The best characterized CRF-OE line is a transgenic in which CRF expression is under control of the metallothionein promoter (mMT-1).<sup>27</sup> In these mice, CRF is elevated in brain neurons in most regions that normally express CRF. This contrasts with conditional models in which CRF is expressed ubiquitously in brain or is limited to forebrain neurons where it is not typically expressed.<sup>29,30</sup> Given the relative restriction of CRF-overexpression to brain neurons that typically express CRF, this study used the transgenic CRF-OE line to best mimic CRF overexpression that would be expected to occur in stress-related psychiatric disorders. In these mice, LC neuronal discharge characteristics and morphology were compared between sexes and genotypes. Additionally, electron microscopy was used to compare CRF<sub>1</sub> cellular localization.

## Materials and Methods

### Subjects

Male and female CRF-OE mice and wild type (WT) littermates were purchased from Jackson laboratories. These mice were originally generated with the use of a chimeric CRF transgene comprising the mMT-1 promoter driving the rat CRF gene (including introns) and backcrossed onto the C57BL/6 mouse strain.<sup>27</sup> Mice representing each sex and strain arrived in the same shipment weekly so that ages were matched between groups for each experiment. Ages for each experiment are indicated in Table S1 in Supplemental

Information (SI). Care and use of animals was approved by the Children's Hospital of Philadelphia's Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

### **CRF Immunohistochemistry**

Mice (3–6/group, 8 months) were transcardially perfused with 4% paraformaldehyde. Coronal sections (30  $\mu\text{m}$ ) through the LC were processed to reveal CRF- and tyrosine hydroxylase (TH) immunoreactivity and this was quantified as previously described (see also SI).<sup>31</sup>

### **LC Slice Preparation and Whole Cell Recording**

Mice (2–5 months) were weighed, sacrificed, and adrenals, bladders, and hearts were weighed. Females were lavaged to assess estrous stage. All stages of the estrous cycle were represented in both genotypes. Most females (75%) were in estrus or diestrus, characterized by lower estrogen levels. Horizontal LC slices (200  $\mu\text{m}$ ) were prepared and visualized as previously described with minor modifications (SI Methods).<sup>32,33</sup> Biocytin-filled micropipettes were used to patch LC neurons.<sup>32–34</sup> Cell characteristics were recorded from a maximum of 2 cells per slice (SI methods). Data were analyzed with Clampfit software (Molecular Devices).

### **Morphological Analysis**

Slices were post-fixed in 4% paraformaldehyde and processed to visualize biocytin-filled cells and TH immunoreactivity (SI Methods). Cells were visualized using confocal microscopy. Only biocytin-filled cells in the LC core or double labeled with TH were included in the analysis. Dendritic morphology was assessed using Neurolucida (SI Methods).<sup>35,36</sup> Dendrite tracing and analysis was performed by an individual blind to genotype and sex.

### **Electron Microscopy**

Mice (5/group, 2 months old) were perfused for immunoelectron microscopic analysis of CRF<sub>1</sub> localization. Immunogold-silver was used to detect CRF<sub>1</sub>. The antibody (rabbit anti-CRF<sub>1</sub>, H-215 Santa Cruz) has been previously used for electron microscopic immunogold detection.<sup>37–39</sup> Tissue preparation, immunolabeling, specificity controls and quantification were as previously described and see SI Methods.<sup>9,37,38</sup>

### **Data analysis**

A 2 $\times$ 2 Analysis of Variance (ANOVA) compared genotype and sex. Mixed factors ANOVAs compared current-frequency relationships and the dendrite order: between subjects factors were genotype and sex, and the within subject factors were current depolarization or branch order, respectively. Newman-Keuls post-hoc tests were used. Values exceeding 2 SDs outside of the group mean were excluded. F-tests were not reported for main effects and interactions that failed to reach significance.

## Results

### CRF overexpression alters general physiological characteristics

CRF overexpression affected body and organ weights of male and female mice (Table S2). Female WT mice weighed less than male WT mice. CRF overexpression selectively increased female body weight to approximate that of males. Male mice had similar weights regardless of genotype. This agrees with previous reports of selective weight gain in CRF-OE females.<sup>40</sup>

For both sexes, CRF overexpression resulted in adrenal and bladder hypertrophy, as assessed by raw weight (Table S2). When organ to body weight ratios were calculated, female OE mice were no longer different than their WT counterparts because of their increased body weights. There were also sex effects in raw adrenal and bladder weight measures regardless of genotype, such that adrenals were heavier in females, while bladders were heavier in males. Heart weights were comparable between CRF-OE and WT mice (Table S2).

### Increased CRF innervation of the LC in CRF-OE mice

As previously described in rats, CRF-immunoreactive fibers were present in the mouse LC and were more prominent in the peri-LC region where LC dendrites extend (Fig. 1A).<sup>31</sup> CRF innervation of the core and peri-LC regions was denser in CRF-OE compared to WT mice (Fig. 1A). There were no sex differences in the relative density of CRF innervation of the LC or peri-LC in CRF-OE mice (Fig. 1B,C). Additionally, the area of TH expression was comparable for all groups (SI results).

### Sex specific effects of CRF overexpression on LC neuronal activity

LC neurons were spontaneously active in both WT and CRF-OE mice and firing rates in WT mice were comparable to those previously reported in mouse LC slices.<sup>41</sup> There was no sex difference in LC spontaneous discharge rate in WT mice (Fig. 2A). In spite of the increased CRF innervation of the LC, CRF overexpression did not affect LC discharge rates of male mice. In contrast, the discharge rate of LC neurons of female CRF-OE mice was nearly three times higher than all other groups (Fig. 2A).

To quantify cell excitability, the discharge frequency in response to depolarizing current steps was measured (Fig. 2B). There was no difference between female CRF-OE mice and female WT mice indicating that the elevated discharge rate that is selective to CRF-OE females is not due to a change in intrinsic excitability. Cells of male WT mice were less excitable than those of all other groups. Most of the general cell characteristics examined were comparable between groups (Table S3). The only characteristic that distinguished the female CRF-OE mice from all other groups was a significantly longer AP duration (Table S3).

### Sex specific effects of CRF overexpression on CRF<sub>1</sub> trafficking

Figure 3A shows representative electron photomicrographs of immunogold-silver labeled CRF<sub>1</sub> in LC dendrites of male and female WT and CRF-OE mice and demonstrates a

distinct cellular localization that is dependent on sex and genotype. Quantification of the CRF<sub>1</sub> cytoplasmic:total label ratio for individual dendritic profiles showed that CRF<sub>1</sub> was equally distributed between the cytoplasm and plasma membrane in WT males (Fig. 3B). By contrast, CRF<sub>1</sub> was more prominent in the cytoplasm in WT females (Fig. 3B). The pattern of cellular distribution of CRF<sub>1</sub> was reversed for both sexes in CRF-OE mice. In male CRF-OE mice CRF<sub>1</sub> was primarily cytoplasmic, indicative of internalization, whereas in female CRF-OE mice CRF<sub>1</sub> was more prominent on the plasma membrane (Fig. 3B).

### Effects of CRF overexpression on LC dendritic morphology

Figures 4A and B respectively show a representative biocytin-filled LC neuron and its NeuroLucida tracing used for quantification. The dendritic tree of WT males was less complex than that of CRF-OE males, because it had significantly fewer nodes and ends and tended to have shorter dendrites (Fig. 4C–E). In contrast, the length, nodes and ends of the dendritic tree of CRF-OE females were comparable to those of WT females, and tended to be greater than those of WT males. Finally, an assessment of the number of LC dendrites by branch order revealed that male WT mice had fewer branches than any other group (Fig. 4F).

Notably, LC neurons of male WT mice had less complex dendritic trees and also were less excitable than other groups. To evaluate a potential relationship, discharge rate in response to depolarizing current (80 pA) was correlated with dendrite node number. However, no significant correlation was found [ $r(33)=0.14$ ,  $p>0.05$ ].

## Discussion

This study used transgenic CRF-OE mice to model excessive CRF that is hypothesized to occur in stress-related psychiatric disorders and determine whether this would differentially impact females. Given previous evidence for increased sensitivity of female rat LC neurons to CRF and sex differences in stress-induced CRF<sub>1</sub> trafficking in LC neurons, the focus was on LC neurons.<sup>9,21</sup> The most striking finding was that CRF overexpression selectively increased LC firing rates of female mice up to three-fold. Sex differences in LC activity of CRF-OE mice could be attributed to differential trafficking of CRF<sub>1</sub>. LC neurons of male CRF-OE mice were protected from excessive CRF because of CRF<sub>1</sub> internalization. By contrast, the lack of CRF<sub>1</sub> internalization in female CRF-OE LC neurons rendered them vulnerable to CRF overexpression. This is reminiscent of the lack of stress-induced CRF<sub>1</sub> internalization in LC neurons of female rats, an effect attributed to decreased association of  $\beta$ arrestin2 with CRF<sub>1</sub>.<sup>9</sup> Together, these results suggest that conditions of CRF overexpression will result in excessive activation of the LC-norepinephrine system selectively in females. This finding is clinically relevant because overactivation of this system is implicated in the arousal symptoms that are core components of stress-related psychiatric disorders.<sup>17–20</sup> Importantly, compromised CRF<sub>1</sub> internalization in females may extend beyond the LC to other CRF<sub>1</sub>-expressing neurons resulting in a lack of adaptation to diverse CRF-elicited effects in addition to arousal. The diminished ability of neurons to adapt to excess CRF may account for female prevalence of stress-related psychiatric diseases.

## CRF-OE mice as models of excessive CRF in stress-related disorders

CRF overexpression or hypersecretion is associated with chronic stress and related disorders including PTSD and depression.<sup>18,22,24,25,42</sup> This has been modeled by chronic CRF administration,<sup>43,44</sup> viral vectors,<sup>45</sup> transgenic mice, and genetically altered mice that overexpress CRF.<sup>27–30,46</sup> In the transgenic mouse used in this study, CRF mRNA is increased throughout development in nearly all brain regions that normally express CRF, as well as in the dentate gyrus, which does not express CRF in WT mice.<sup>27</sup> These mice have evidence of an overactive HPA axis, including adrenal hypertrophy, elevated plasma ACTH and corticosterone, and a blunted corticosterone response to stress. They also exhibit a Cushing's-like phenotype.<sup>27</sup> Although CRF mRNA was detected in certain peripheral tissues using RT-PCR (testes, adrenal, heart), CRF is not present in the circulation. A CRF transgenic mouse line created using the murine Thy-1,2 promoter differs somewhat in having CRF overexpression confined to brain, spinal cord and adrenal medulla.<sup>28</sup> These mice display a similar endocrine profile with the exception of only a marginal increase in adrenocorticotropin and later development of a Cushing's phenotype.<sup>28,46</sup> A characteristic behavioral feature of both strains is a novelty dependent hypoactivity and decreased activity in approach-avoidance situations, an effect also seen with CRF administration.<sup>47–49</sup> Additionally, the CRF-OE mice used in this study show anxiogenic behavior in the light-dark box, decreased exploratory behavior in hole board and decreased immobility in response to swim stress.<sup>49</sup> Conditional CRF-OE mice have been generated in which CRF is overexpressed throughout the entire brain or is restricted to forebrain principal neurons or GABA forebrain neurons.<sup>29</sup> A comparison of these conditional lines revealed a hyperresponsive stress axis and increased active stress coping only in mice with CRF overexpression in the entire brain. An advantage of this conditional line CRF is the selective high CRF expression in brain. However, unlike the CRF transgenic mice used in the present study, 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. This atypical expression likely reflects the ubiquitous activity of the ROSA26 locus that is targeted by the CRF cDNA and the wide expression of nestin promoter, which drives Cre in both neurons and glia. Because excessive CRF that is thought to occur in stress-related psychiatric disorders is likely to result from overexpression within neurons that typically synthesize CRF rather than new expression in neurons that do not typically synthesize CRF, we chose the mMT1 transgenic model. One caveat of this model is that CRF overexpression is present throughout development and this can result in compensatory effects. Notably, it is unknown at what point during development the CRF dysregulation that leads to disease manifests in humans.

## Physiological features of CRF-OE mice

This study confirmed the general physiological phenotype of this CRF-OE line previously reported, including a selective weight gain in females.<sup>27,40</sup> Additionally, an increase in bladder weight in the CRF-OE mice was observed irrespective of sex. This is consistent with previous findings of stress-induced increases in bladder mass that have been attributed to CRF upregulation in Barrington's nucleus.<sup>50</sup>



## LC electrophysiological characteristics and CRF<sub>1</sub> trafficking

A major target of CRF neurotransmission is the LC-norepinephrine system.<sup>11</sup> CRF locally released into the LC during stress increases the firing rate of LC neurons and norepinephrine release in forebrain targets.<sup>15,16,51–54</sup> This is associated with cortical electroencephalographic indices of heightened arousal.<sup>15,16</sup> Additionally, LC activation by CRF is thought to bias attention towards a less focused, more labile state.<sup>11</sup> Although this may be adaptive in a life-threatening situation, if these responses persisted or occurred in the absence of a stressor, the consequences would be pathological. Indeed stress-related psychiatric disorders are characterized by a core symptom of hyperarousal that is expressed as sleep disturbances, inability to concentrate and obsessive thoughts and these have been attributed to overactivation of the LC-norepinephrine system.<sup>19,20,55,56</sup>

The potential for an impact of CRF overexpression on LC neurons was apparent in the substantially greater CRF innervation of the LC in CRF-OE compared to WT mice. Although this would be predicted to increase LC discharge rates of both male and female CRF-OE mice, only females were affected. LC discharge rates of male CRF-OE mice were remarkably similar to male and female WT mice. The lack of effect of CRF overexpression in males could be explained by the predominant cytoplasmic localization of CRF<sub>1</sub>, indicative of internalization. The shift in cellular distribution of CRF<sub>1</sub> from the plasma membrane in male WT mice to the cytoplasm in male CRF-OE mice suggests that the presence of excessive CRF induces CRF<sub>1</sub> internalization as a cellular adaptation to maintain LC discharge rates to normal levels. Agonist-induced CRF<sub>1</sub> internalization has been demonstrated in cells in culture and in LC neurons of male rats *in vivo*.<sup>37,57,58</sup> Swim stress-induced CRF<sub>1</sub> internalization in LC neurons of male rats is associated with a decreased maximum response to CRF, suggesting that this cellular mechanism plays a functional role in attenuating the response to excessive CRF.<sup>9,38</sup>

The cellular localization of CRF<sub>1</sub> in female WT and CRF-OE mice is analogous to its distribution in unstressed and stressed rats, respectively, and completely opposite the pattern of CRF<sub>1</sub> localization in male rodents.<sup>9</sup> Thus, in WT female mice and unstressed female rats CRF<sub>1</sub> is predominantly cytoplasmic, whereas in CRF-OE female mice and stressed female rats it becomes more prominent on the plasma membrane. In female rats, the lack of internalization following stress is functionally relevant because the plateau in the CRF effect seen following stress in male rats is not present.<sup>21</sup> In female CRF-OE mice, the lack of CRF<sub>1</sub> internalization results in higher tonic LC discharge rates. The molecular basis for the differential localization of CRF<sub>1</sub> in unstressed male and female rats and WT male and female mice is unknown. However, the lack of internalization in female CRF-OE mice may be explained by a decreased ability of CRF<sub>1</sub> to bind  $\beta$ arrestin2, a critical step in the CRF<sub>1</sub> internalization process.<sup>59,60</sup> This has been demonstrated in cortical tissue from female rats and explains the lack of CRF<sub>1</sub> internalization following stress in that species.<sup>9</sup> Together, the results suggest that this molecular mechanism may underlie a compromised ability to adapt to excessive CRF in females, resulting in an LC-norepinephrine system that has a higher tone.

Conditional CRF-OE mice that express CRF throughout the brain have behavioral and anatomical evidence of LC hyperactivity.<sup>29</sup> However, in contrast to the present study, no sex differences were seen for these effects. One explanation for differences between the studies is that anatomical endpoints of cellular activation (c-fos and zif268) may not translate to increased neuronal discharge. Alternatively, an overall higher level of CRF in the conditional model may be sufficient to activate the lower percentage of CRF<sub>1</sub> receptors that remain on the plasma membrane of LC neurons in male mice. Finally, the genetic manipulations used to generate the conditional CRF-OE may affect other molecular processes that interfere with the ability of LC cells in male CRF-OE mice to adapt.

The electrophysiological characteristics of LC neurons measured by whole cell recordings indicated that the increase in discharge rate seen in female CRF-OE mice was not due to an overall general excitability of LC neurons as indicated by the current-frequency curves. The finding that action potential duration was slightly, but significantly elevated in female CRF-OE mice is consistent with an increase in calcium conductance, an effect that could explain the elevated firing rate. The finding that the LC action potential threshold is significantly lower in females may also be a contributing factor. Although the consequence of a lower action potential threshold may not be relevant in the absence of an excitatory neuromodulator, this would have consequences for activity in a condition of CRF overexpression and activation.

### CRF overexpression and LC dendritic morphology

CRF can alter LC dendritic morphology. Specifically, CRF increases dendritic length in rat LC slice cultures and neurite outgrowth in CATHa cells, an LC-like cell line.<sup>61,62</sup> Additionally, LC dendrites are longer and more complex in female rats compared to males.<sup>35</sup> The present data in mice are generally consistent with increased LC dendritic complexity in females compared to males. The comparison of morphological characteristics between groups revealed a decreased dendritic complexity of male WT mice compared to all other groups. It is possible that CRF overexpression affects LC dendritogenesis at a time early in life, prior to the development of mechanisms for adapting to excessive CRF. This allows the dendrites of male CRF-OE mice to develop to the extent seen in females. The lack of further changes in female CRF-OE mice could indicate a ceiling effect.

A more complex LC dendritic tree is relevant to the sources of CRF that regulate the LC. Most limbic CRF-containing afferents terminate in the peri-LC where LC dendrites extend outside of the nuclear core.<sup>63–66</sup> The further LC dendrites extend and branch into the peri-LC region, the more likely they will contact limbic CRF afferents relaying emotion-related information. However, this cannot account for differences in LC discharge in male and female CRF-OE mice, which were not different in their dendritic characteristics.

### Functional implications

Stress-related psychiatric disorders are more prevalent in females. Two characteristic features of these disorders are excessive CRF and symptoms of hyperarousal. The core symptom of hyperarousal has been attributed to overactivity of the LC-norepinephrine system that is driven by high levels of CRF.<sup>19</sup> Using transgenic CRF-OE mice, the present



findings suggest that females will be particularly vulnerable to conditions of high CRF levels because cellular mechanisms important for adaptation to excess CRF are compromised. As symptoms related to hyperarousal characterize anxiety, depression and PTSD, this may explain the increased prevalence of these disorders in females. Studies that have systematically investigated sex differences in the CRF-OE mice used here provide evidence for increased autonomic and behavioral responses to acute mild stress in females only.<sup>67</sup> Similarly, the condition of CRF overexpression selectively alters sexual behavior of females, who exhibit active aggressive behaviors towards males.<sup>68</sup> Although this study focused on sex differences in LC activity in the condition of CRF overexpression, the impaired internalization likely occurs in other CRF<sub>1</sub>-expressing cells as well.<sup>9</sup> Thus, whereas CRF overexpression may bias females towards hyperarousal and associated symptoms as a result of enhanced LC activity, other effects may also be more prevalent in females as a result of CRF activation of other targets.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We would like to thank Kile McFadden and Dr. Susan Wood for their technical support. This work was supported by USPHS Grant MH40008 to RJV, MH084423 and MH092438 to DAB, and MH0754047 and MH089800 to SGB.

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and peri-LC in CRF-OE mice compared to WT mice as revealed by two main effects of genotype [ $F(1,12)= 15.5, p<0.01, F(1,12)= 25.9, p<0.001$ , respectively]. There were no significant effects of sex or interactions. Bars represent the mean ( $\pm$  SEM) of 3–5 mice/group.

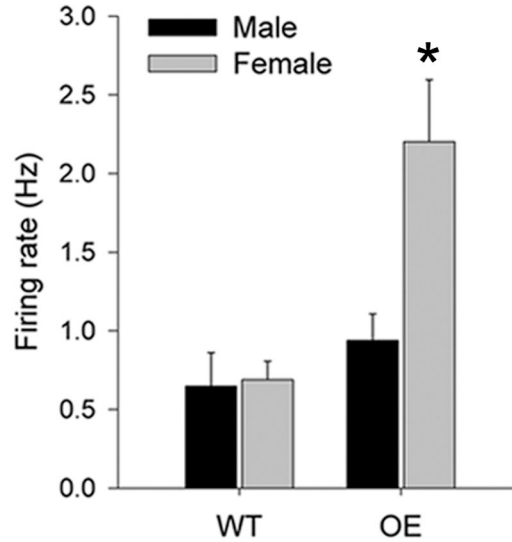
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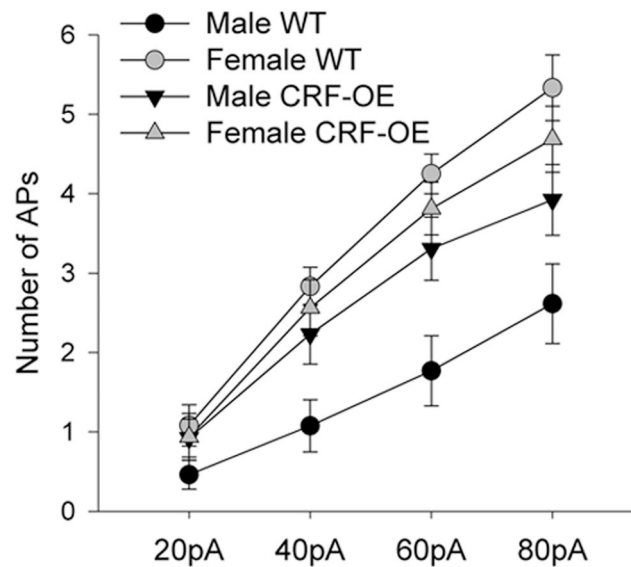
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### A) LC Neuronal Firing Rate



### B) Frequency-Current Plot



#### Figure 2.

Sex and genotype differences in electrophysiological properties of LC neurons. (A) Bars represent the mean firing rate (Hz) of LC neurons ( $n=12-16$  cells/group). There was a significant sex by genotype interaction for firing rate [ $F(1,50)=6.0$ ,  $p<0.05$ ]. Post-hocs revealed that neurons of female CRF-OE mice fired faster than all other groups ( $p<0.05$ ). (B) The plot displays the number of APs elicited by increasing depolarizing current injections ( $n=12-16$  cells/group). There was a significant sex by genotype by depolarizing current interaction [ $F(3,150)=2.7$ ,  $p<0.05$ ] and a significant sex by genotype interaction

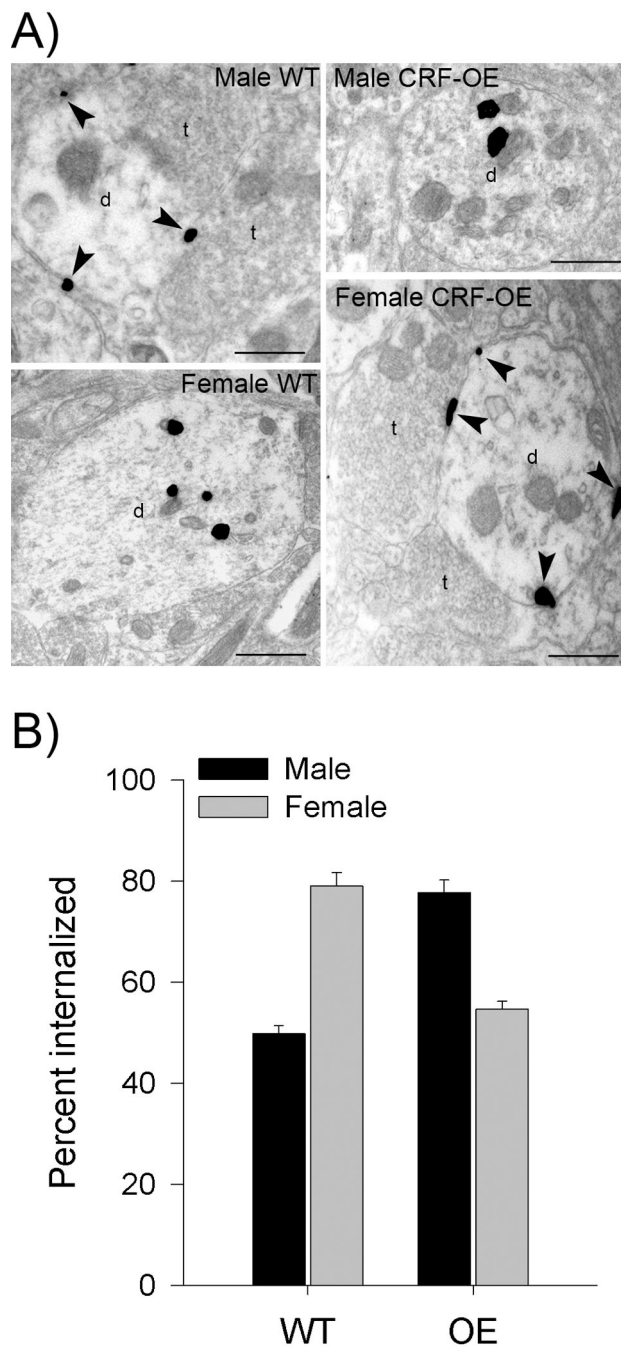
[ $F(1,50)=5.5$ ,  $p<0.05$ ]. Post-hocs revealed that male WT mice fired fewer APs than any other group ( $p<0.05$ ), indicating that their cells were less excitable. Data are represented as the mean ( $\pm$  SEM). Asterisks indicate a significant difference from all other groups ( $p<0.05$ ).

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**Figure 3.** Sex and genotype differences in CRF<sub>1</sub> compartmentalization. (A) Electron photomicrographs of LC dendrites (d) from a male WT, male CRF-OE, female WT and female CRF-OE showing CRF immunogold labeling. CRF-immunogold labeling is seen on the plasma membrane (arrowheads) in male WT mice and female CRF-OE mice whereas it is in the cytoplasm in male CRF-OE mice and female WT mice. t=axon terminal. Scale bars=500 nm. (B) Bar graph comparing the mean ratio of immunolabeled gold particles localized within cytoplasm to the total number (percent internalized). There was a

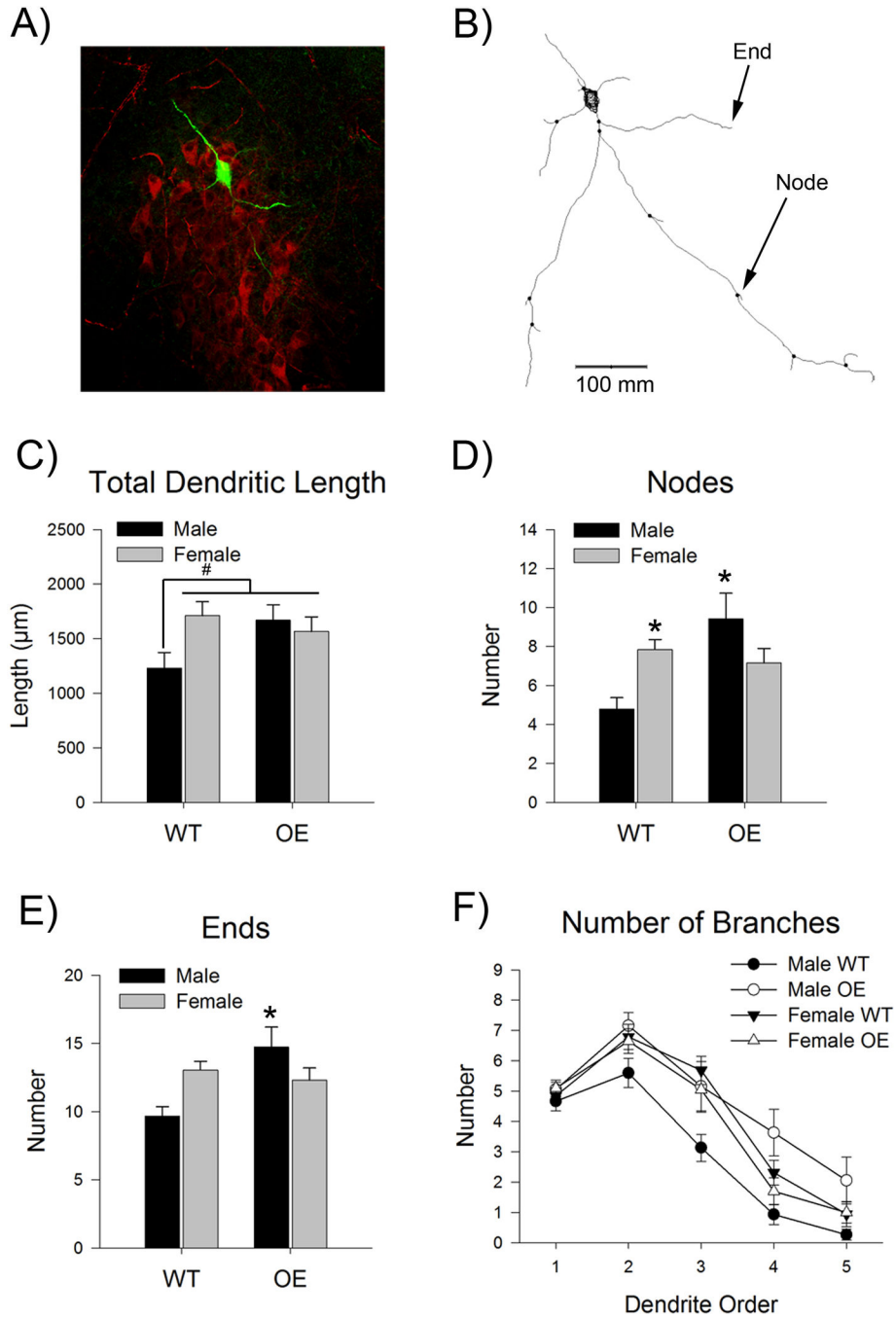
significant sex by genotype interaction [ $F(1,16)=144.9$ ,  $p<0.001$ ]. Post-hoc tests revealed that female WT mice had a significantly greater percentage of cytoplasmic CRF<sub>1</sub> labeling than male WT mice. However, the opposite effect was observed in CRF-OE mice where CRF<sub>1</sub> labeling is more prominent in the cytoplasm of male compared to female CRF-OE mice ( $p<0.001$ ). Data are represented as the mean ( $\pm$  SEM) of 5 mice/group.

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**Figure 4.** Sex and genotype differences in LC dendritic morphology. (A) Fluorescent photomicrograph of a biocytin labeled LC neuron (green) merged with TH labeling (red) from a male CRF-OE mouse. (B) Neurolucida tracing of the cell shown in A. (C) Bars represent the mean total dendritic length. There was a significant sex by genotype interaction for total dendrite length [ $F(1,69)=4.5, p<0.05$ ]. Post-hoc tests revealed that there was a trend for male WT mice to have shorter dendrites than all other groups ( $\#p<0.09$ ). (D) Bars depict the mean number of nodes (i.e., branchpoints). There was a significant sex by



genotype interaction for the number of nodes [ $F(1,69)=9.2$ ,  $p<0.01$ ]. Female WT and male OE mice had significantly more nodes than male WT mice ( $*p<0.05$ ). There was a trend for more nodes in female OE mice than male WT mice ( $p=0.06$ ). (E) Bars show the mean number of ends. There was a significant sex by genotype interaction for end number [ $F(1,69)=8.4$ ,  $p<0.01$ ]. Compared to WT males, CRF-OE males had more ends ( $*p<0.01$ ). There was a trend for female WT and OE mice to also have more ends than WT males ( $p=0.053$ ,  $p=0.07$ , respectively). (F) The graph represents the number of branches broken down by branch order (i.e., 1st, 2nd, 3rd, 4th, and 5th). Although the sex by genotype by branch order interaction failed to reach significance [ $F(4,276)=2.2$ ,  $p=0.07$ ], there was a significant sex by genotype interaction [ $F(1,69)=7.8$ ,  $p<0.01$ ]. Male WT mice had fewer branches than any other group [ $F(1,69)=7.8$ ,  $p<0.01$ , post hoc  $p<0.05$ ]. Data are represented as the mean ( $\pm$  SEM) of 15–20 cells/group.