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Corticotropin-releasing factor overexpression gives rise to sex differences in Alzheimer's disease-related signaling

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Abstract

Several neuropsychiatric and neurodegenerative disorders share stress as a risk factor and are more prevalent in women than men. Corticotropin releasing factor (CRF) orchestrates the stress response and excessive CRF is thought to contribute to the pathophysiology of these diseases. We previously found that the CRF₁ receptor (CRF₁) is sex-biased whereby coupling to its GTP-binding protein, Gs, is greater in females, while β -arrestin-2 coupling is greater in males. This study used a phosphoproteomic approach in CRF-overexpressing mice (CRF-OE) to test the proof of principle that when CRF is in excess, sex-biased CRF₁ coupling translates into divergent cell signaling that is expressed as different brain phosphoprotein profiles. Cortical phosphopeptides that distinguished female and male CRF-OE mice were overrepresented in unique pathways that were consistent with Gs-dependent signaling in females and β -arrestin-2 signaling in males. Notably, phosphopeptides that were more abundant in female CRF-OE mice were overrepresented in an Alzheimer's disease (AD) pathway. Phosphoproteomic results were validated by demonstrating that CRF overexpression in females was associated with increased tau phosphorylation and, in a mouse model of AD pathology, phosphorylation of β -secretase, the enzyme involved in formation of amyloid β . These females exhibited increased formation of amyloid β plaques and cognitive impairments relative to males. Collectively, the findings are

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consistent with a mechanism whereby the excess CRF that characterizes stress-related diseases initiates distinct cellular processes in male and female brains, as a result of sex biased CRF₁ signaling. Promotion of AD-related signaling pathways through this mechanism may contribute to female vulnerability to AD.

Women are nearly twice as likely as men to suffer from stress-related psychiatric disorders, such as depression and post-traumatic stress disorder^{1, 2}. Alzheimer's disease (AD) is also more common in women than men, and stress is a risk factor for this neurodegenerative disorder³⁻⁵. Hypersecretion of corticotropin-releasing factor (CRF), the neuropeptide that orchestrates the stress response⁶, has been implicated in the pathophysiology of these disorders⁷⁻⁹. Therefore, sex differences in CRF sensitivity may underlie the increased prevalence of stress-related neuropsychiatric diseases in females.

Our laboratory identified sex differences in CRF₁ receptor (CRF₁) coupling and trafficking that could be a molecular basis for the increased vulnerability of females to stress and stress-related pathology¹⁰. CRF₁ was more highly coupled to its GTP binding protein (Gs) in female compared to male rats¹⁰. Moreover, in male rats, acute stress promoted CRF₁ association with β -arrestin-2, an effect not observed in females. Sex differences in CRF₁ coupling translated to physiological differences¹⁰. Specifically, locus coeruleus (LC)-norepinephrine neurons of female rats were sensitized to CRF and this sensitization was mediated by cyclic adenosine monophosphate (cAMP) signaling, the signaling pathway activated by Gs^{10, 11}. Sex differences in CRF₁ association with Gs and β -arrestin-2 and neuronal sensitivity to CRF were unrelated to circulating hormones^{10, 11}. Finally, CRF₁ internalization, which requires CRF₁- β -arrestin-2 association, was apparent only in male LC neurons following stress¹⁰. Collectively, these molecular sex differences render neurons of female rats more sensitive to CRF and less able to adapt to excess CRF through the compensatory mechanism of internalization. This was particularly apparent in CRF-overexpressing mice (CRF-OE) that model the CRF hypersecretion of stress-related psychiatric disorders. In these mice, only female LC neurons were hyperactive and CRF₁ was not internalized in female CRF-OE mice as it was in male CRF-OE mice¹².

In addition to its role in internalization, β -arrestin-2 links receptor activation to signaling cascades that are distinct from G-protein-induced signaling^{13, 14}. Therefore, sex differences in CRF₁ coupling should translate to sex-biased CRF₁ signaling, favoring Gs-dependent signaling in females and β -arrestin-2 signaling in males¹⁵. Because β -arrestin-2 and Gs signaling pathways regulate protein phosphorylation dynamics, sex-biased CRF₁ signaling should give rise to sexually distinct profiles of protein phosphorylation when CRF₁ is activated. Through this mechanism, stressors could initiate sex-specific cellular effects that have different pathological consequences. Sex differences in CRF₁ signaling would be magnified when CRF is in excess, as modeled by CRF-OE mice. This study used a global phosphoproteomic approach with CRF-OE mice to test the hypothesis that the excessive CRF that occurs in stress-related disorders results in sexually distinct phosphoprotein profiles that can contribute to sex differences in the expression of pathology. Because sex differences in Alzheimer's disease (AD)-related signaling emerged from this analysis, sex differences in certain phosphorylated proteins within this pathway were validated using

Western blots. Additionally, sex differences in AD-related neuropathology were further investigated in an AD mouse model that also overexpresses CRF.

Materials and Methods

Animals

Animals were allocated to experimental groups based on genetic background and sex. Within a specific gene/sex group animals were randomized by selection from different litters. Phosphoproteomic studies and tau western blot studies used adult (>60 d) male and female CRF-OE mice and wild type (WT) littermates purchased from Jackson laboratories. CRF-OE mice were originally generated using a chimeric CRF transgene comprising the mMT-1 promoter driving the rat CRF gene (including introns) and backcrossed onto the C57BL/6 mouse strain¹⁶. Tissue for Western blot studies was taken from mice of different litters than those for phosphoproteomic studies. For analysis of amyloid- β (A β) plaques, cognitive function and β -site amyloid precursor protein (APP) cleaving enzyme (BACE-1), Tg2576 mice that express human APP were compared to triple transgenic (TT) mice that both express human APP and overexpress CRF selectively in forebrain. TT mice were generated by crossing Tg2576 mice with Tetop-CRF+ and CaMKII-tTA+ mice to obtain the APP+/CRF+/tTA+ (TT) mouse line¹⁷. In this line CRF gene expression can be suppressed by doxycycline administration, but doxycycline was not administered in this study so that CRF overexpression in TT mice was maintained throughout life. Care and use of animals was approved by the Children's Hospital of Philadelphia's and Northwestern University's Institutional Animal Care and Use Committee and was in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Tissue Preparation

Phosphoproteomic analysis used tissue (50-70 mg per animal) from the anterior (1.98 A/P relative to Bregma) and dorsal (-1.00 mm from the surface of the brain) portions of cerebral cortex. Samples from three mice from different litters were pooled to yield ~190 mg of starting material for each condition (male WT, female WT, male CRF-OE, and female CRF-OE). Pooled samples were homogenized, lysed, and proteins were precipitated as detailed in Supplemental Information (SI) Methods. A stable heavy Lysine 6 (13C6, 97%) isotope labeling of whole mouse (SILAM) reference sample (Cambridge Isotope Laboratories, Inc.) was generated by combining one whole SILAM female brain with one whole SILAM male brain. Equal amounts of SILAM reference brain protein were mixed with each cortical protein sample. Samples were digested with trypsin and tryptic peptides were desalted using Sep-Pak tC18 cartridges (Waters) (SI Methods). Hydrophilic interaction chromatography (HILIC) was used to separate tryptic peptides¹⁸ (SI Methods). Phosphopeptides were enriched from HILIC fractions using immobilized metal affinity chromatography (IMAC) in batch mode (SI Methods).

Mass Spectrometry Analysis

A hybrid LTQ Orbitrap-Elite mass spectrometer (ThermoFisher Scientific) coupled with a Nano2D Ultra LC system (Eksigent Technologies) and an autosampler analyzed tryptic digests. Raw MS files were processed using MaxQuant (version 3.1.0.5)¹⁹. MaxQuant

output files were searched against a mouse protein sequence database comprising reviewed canonical plus isoforms for taxonomy:10090 (Uniprot.org) using Andromeda search algorithm. The false-discovery rate for peptides and proteins was set at 1%. The data was analyzed by log (base 2) transforming SILAC ratios and fitting them to a three-component mixture model based on the approach outlined by Margolin *et al*²⁰. For further details on database searching and statistical analysis see the SI methods. Phosphopeptides that were significantly different between groups were then analyzed by Ingenuity Pathway Analysis (Ingenuity®Systems, www.ingenuity.com).

AD-related pathology

For Western blots, A β plaque quantification and behavior group sample size was estimated using the sample size and power test in the DOE category of JMP 9.0.0 (2010, SAS) assuming $\alpha=0.05$ and power=0.8. Cortical tissue (n=5 mice each group) was homogenized and subjected to Western Blotting^{17, 21}. Immunoblots from CRF-OE cortex were probed with a monoclonal antibody to assess paired helical filament (PHF-1, 1:1,000, V. Lee, Univ. Penn). This antibody recognizes tau phosphorylated at Ser³⁹⁶/Ser⁴⁰⁴. These phosphorylation sites are two of many on the tau protein associated with various stages of neurofibrillary tangle development in the tauopathy disease process. However, these particular sites are strongly related to late-stage extracellular neurofibrillary tangles, which contain substantial filamentous tau in both AD cases and mouse models of AD²². The same blots were probed with antibodies to assess total human tau1 (17025 antibody, 1:1000, V. Lee, Univ. Penn) and a GAPDH loading control (1:2,000, Sigma G9545) (SI Methods)²¹. Data was obtained from 2 blots each containing samples from all groups. Immunoblots from Tg2576 (n=6 for male and female) and TT (n=4 for male and female) mice were probed with antibodies directed against BACE-1(1:1000, R. Vassar, Northwestern Univ.), pSer⁴⁹⁸-BACE-1 (1:1000, Thermo Fisher Life Technologies, PA5-12549) and a β -actin loading control (1:1000, Santa Cruz, sc130300). Cleavage of APP by BACE-1 is the initial step in the formation of A β . A β plaques were evaluated in whole brain sections from TG2576 mice (n=6 male and 4 female) and TT mice (n=4 male and female) by two individuals blinded to experimental group using A β immunohistochemistry (rabbit anti-A β 1:1000, Thermo Scientific #44136; SI Methods)¹⁷.

Spontaneous alternation

Tg2576 (n=9 male and 22 female) and TT (n=7 male and 8 female) were placed in the center of a Y maze (5 × 21 × 15.5 cm) and allowed to explore the arms for 5 min as previously described¹⁷. Two trained observers blinded to genotypes recorded the sequence of arm entries. Successful alternation was defined as entry into all three arms in consecutive choices.

Results

Sex differences in CRF-OE phosphopeptide profiles

Our previous findings of sex differences in CRF₁ coupling to Gs and β -arrestin-2 predicted that agonist binding would result in different phosphoprotein profiles, indicative of Gs-related signaling in females and β -arrestin-2 signaling in males¹⁰. Of the phosphopeptides

that could be quantified by having a ratio of the SILAM standard heavy-labeled peptide/light peptide, 5,070 were present in both male and female CRF-OE mice (Table SI1). Based on the 1% false discovery rate (>1.5 fold difference) approximately 15% of these were sexually distinct, with approximately 10% being more abundant in females (FOE phosphoproteome) and 5% being more abundant in males (MOE phosphoproteome) (Fig. 1A). Phosphopeptide differences were unrelated to overall changes in protein levels, as indicated by an analysis of proteins in cortical samples of these groups that were not enriched for phosphopeptides (Fig. 1B; Table SI2). Of the 5,151 proteins quantified based on the identification and quantification of SILAM ratios for two or more lysine-containing peptides unique to each protein, 22 (<1%) were at least 1.5 times more abundant in female CRF-OE cortex and 25 (<1%) were more abundant in male CRF-OE cortex based on the same fold difference (Fig. 1B).

Consistent with sex-biased CRF₁ signaling, MOE phosphopeptides and FOE phosphopeptides were overrepresented in functionally distinct pathways as determined by Ingenuity Pathway Analysis (Fig. 1C). MOE phosphopeptides were overrepresented in twelve pathways, five being related to signaling by the small GTPases, Rho and Rac, consistent with a role for β -arrestin-2 in regulating Rho function (Fig. 1C, Table SI3)²³. Figure 2A shows roles of those individual phosphopeptides from the MOE phosphoproteome in the Rho signaling pathway. FOE phosphopeptides were less well represented in the Rho A signaling pathway (Fig. 1C, 2A).

As predicted by a female bias towards Gs-dependent signaling, protein kinase A (PKA) signaling was among the top canonical pathways in which FOE phosphopeptides were overrepresented (Fig. 1C, Table SI4). Other pathways in which FOE phosphopeptides, but not MOE phosphopeptides were overrepresented, and that would be predicted by enhanced CRF₁ receptor-Gs association in females, included cyclic AMP and GPCR signaling pathways (Fig. 1C; Table SI4). Many pathways in which FOE phosphopeptides were overrepresented, including PKA, contained an insufficient number of phosphopeptides from the MOE phosphoproteome to reach statistical significance for determining overrepresentation (Fig. 1C). Notably, FOE phosphopeptides were highly overrepresented in the GABA signaling pathway and MOE phosphopeptides were not present at all, suggesting that CRF overexpression affects cortical GABA signaling in females substantially more than males (Figs. 1C, 2B).

FOE but not MOE phosphopeptides were also overrepresented in an Alzheimer's Disease (AD)-related signaling pathway (Fig. 1C). FOE phosphopeptides converged on two neuropathological processes that contribute to AD, the processing of APP to A β that forms plaques and the phosphorylation of microtubule-associated protein tau (MAPT) that is involved in the formation of fibrillary tangles (Fig. 2C). A phosphorylated form of BACE-1 (pSer⁴⁹⁸), the enzyme that converts APP to the plaque-forming A β ²⁴ was 8 times more abundant in the FOE compared to the MOE phosphoproteome. Several kinases in the FOE phosphoproteome regulate tau phosphorylation, a critical step in the formation of fibrillary tangles²⁵. Additionally, some of the top canonical pathways in which FOE phosphopeptides are overrepresented are involved in tau phosphorylation including 14-3-3, P70S6K and ephrin signaling²⁶⁻³¹.

Comparison of male and female WT phosphopeptide profiles

A parallel comparison of phosphopeptides that distinguish male and female WT mice (MWT and FWT, respectively) revealed sex differences that were less extensive than those seen in CRF-OE mice. Of 3953 phosphopeptides that could be quantified in both males and females, approximately 9% were more abundant in the FWT and 4% were more abundant in the MWT. Importantly, the top canonical pathways in which phosphopeptides that were more abundant in either sex were overrepresented differed from those that distinguished the FOE and MOE phosphoproteomes (Fig. 1D; Tables SI5, SI6). MWT phosphopeptides were overrepresented in only five canonical pathways (Table SI5). With the exception of semaphorin signaling, these were not among the top canonical pathways in which MOE phosphopeptides were overrepresented. Notably, Rho signaling, which distinguished the MOE from the FOE phosphoproteomes, did not distinguish MWT and FWT phosphoproteomes. Although phosphopeptides that were more abundant in FWT compared to MWT were overrepresented in some of the same pathways as were phosphopeptides in the FOE phosphoproteome (e.g., 14-3-3, calcium), they were not overrepresented in many major pathways in which FOE phosphopeptides were overrepresented (e.g., ERK5, PKA, GABA, amyloid signaling), suggesting that the distinctions between FOE and MOE result from a sex-genotype interaction (Fig. 1D, Table SI6).

Sex differences in AD-related pathology

The finding that phosphopeptides that distinguished female from male CRF-OE mice were overrepresented in AD-related signaling pathways prompted follow-up studies to validate sex differences in AD-related pathology in mouse models. First, MAPT phosphorylation was assessed by Western blots of PHF1 in CRFOE and WT mice. Cortical tissue of female CRF-OE mice had higher PHF1 levels than female WT mice, but there were no differences in PHF1 levels between CRF-OE and WT for males (Fig. 3A,B). Importantly, there were no sex or gene differences in cortical levels of total tau (Tau1) (Fig. 3C). Similar results were obtained with hippocampal tissue (Fig. SI1).

Because aging in mouse models of AD-related tauopathy was reported to be associated with increased insoluble phosphorylated tau and decreased soluble phosphorylated tau, insoluble tau was also quantified³². Female CRF-OE mice had greater levels of PHF1 in the insoluble fraction compared to all other groups (Fig. 3D). Together, these results support the phosphoproteomic findings suggesting that CRF overexpression biases MAPT phosphorylation in females.

Sex differences in aspects of amyloid processing were then evaluated in mice that express human APP without (Tg2576) or with forebrain CRF overexpression (TT mice). In agreement with the phosphoproteomic results, Western blotting of cortical tissue revealed that pSer⁴⁹⁸BACE-1, but not BACE-1 levels, were increased in female TT relative to male TT mice (Fig. 4A-C, Fig. SI2A,B). No sex differences were found in Tg2576 mice (Fig. 4A-C).

Figure 4D shows representative A β plaques in male and female TT mice. As previously reported, A β plaque formation was greater in TT mice compared to Tg2576 mice¹⁷.

Notably, female TT mice had a greater number of plaques compared to all other groups (Fig. 4D,E, Fig. SI2,C).

Spontaneous alternation, which is based on the tendency of mice to enter novel arms of a Y-maze was used to assess short term working memory. Female TT rats were impaired compared to all other groups as indicated by a decreased number of corrected alternations (Fig. 4F).

Discussion

This study used the systems biology approach of phosphoproteomics to test a hypothesis generated from receptor immunoprecipitation studies that sex-biased CRF₁ coupling results in the engagement of sexually distinct cellular processes under conditions of excess CRF similar to those seen in stress-related psychiatric disorders. Supporting this hypothesis, a sexual divergence in the cortical phosphoproteome was identified in CRF-OE mice that was consistent with increased CRF₁-Gs coupling in females and CRF₁- β -arrestin-2 association in males. The divergence in the cortical phosphoproteome was due to differential phosphorylation rather than changes in protein expression. That similar sex differences were not apparent in WT mice underscores that they were a product of the interaction between sex and CRF-overexpression. The phosphoproteomic data set was further used as a discovery tool to probe the basis of sex differences in disease prevalence, with a focus on AD-related signaling. Increased MAPT and BACE-1 phosphorylation in female mice under conditions of CRF overexpression provided chemical validation that sex-biased CRF₁-Gs coupling can enhance signaling involved in the neuropathology of AD. That this may translate to functional sex differences was suggested by enhanced A β plaque formation and cognitive impairment seen in female TT mice. Together the results suggest that as a result of sex-biased CRF₁ signaling, excess CRF as seen in stress-related disorders can engage different cellular processes in male and female neurons. These different processes can translate to sex differences in the expression of stress-related neuropathology. AD is an example of one disease in which this mechanism may contribute to female vulnerability.

Sex differences in cell signaling emerging from sex-biased CRF₁ coupling

Initial evidence for sex differences in CRF signaling came from physiological studies showing that LC neurons of female rats were more sensitive to CRF and these CRF effects were more sensitive to PKA antagonists in female compared to male rats^{10, 11}. Receptor immunoprecipitation studies provided a molecular basis for these functional sex differences by demonstrating greater CRF₁-Gs association in unstressed females compared to males coupled with decreased stress-induced CRF₁- β -arrestin-2 association in females compared to males¹⁰. Because Gs and β -arrestin-2 engage distinct cellular signaling cascades that regulate protein phosphorylation in cells it was hypothesized that CRF₁ activation would initiate distinct cellular reactions that are expressed as different phosphoproteins profiles in male and female rats. Moreover, these sex differences would be most apparent when CRF is overexpressed, as has been hypothesized to occur in stress-related psychiatric disorders⁷⁻⁹. The current study using a systems biology approach of global phosphoproteomics in a model of CRF overexpression supported this hypothesis. Given that the interaction of CRF with

CRF₁ receptors during stress is considered to orchestrate many behavioral, autonomic and endocrine aspects of the stress response, the concept that this will potentially initiate different cellular reactions in males and females has broad implications for sex differences in stress responses and in stress-related pathology. Using the phosphoproteomic data set to guide discovery, potential disease-related consequences of sex-biased CRF₁ signaling can be examined. The overrepresentation of FOE phosphopeptides in the AD pathway guided our focus on signaling related to this disease.

Sex, CRF-OE and AD

Psychosocial and environmental stress have long been considered to be risk factors for AD and thought to accelerate AD progression in vulnerable individuals^{5, 33}. Evidence of hypothalamic-pituitary-adrenal dysfunction in AD implicated glucocorticoids as a causal link between stress and AD³⁴⁻³⁷. However, other studies argue against this and implicated CRF as a more likely causal factor. For example, stress promotion of tau phosphorylation is prevented in mice lacking CRF₁ and in WT mice administered CRF₁ antagonists^{21, 38}. Stress-induced enhancement of tau phosphorylation is clinically relevant because hyperphosphorylation of MAPT reduces its association with microtubules, resulting in tau aggregation and the formation of the neurofibrillary tangles that are a hallmark of AD^{39, 40}. The co-morbidity of AD with psychiatric disorders that are characterized by excessive CRF, such as depression is also consistent with a causal role for CRF^{37, 41}. Similar to these psychiatric disorders, AD is nearly twice as prevalent in females compared to males even when correcting for age⁴²⁻⁴⁴. Sex differences in CRF sensitivity as a result of biased CRF₁-Gs coupling could underlie sex differences in AD vulnerability. This was supported by the present phosphoproteomic analysis, which highlighted two AD-related pathways as distinguishing female and male CRF-OE mice. One pathway involved tau phosphorylation. This was further validated by Western blot analysis of cortical tissue that demonstrated that CRF overexpression increased tau phosphorylation on the Ser³⁹⁶/Ser⁴⁰⁴ residues in female, but not male, CRF-OE mice. The finding that insoluble phosphorylated tau was greater in female CRF-OE mice compared to other groups lends further support to the premise that CRF overexpression in females contributes to AD neuropathology, as increases in insoluble tau may be a better indicator of aging-related neuropathology³². Another hallmark of AD is the formation of A β plaques. A β is formed by the cleavage of APP to the fragment, C99, by BACE, followed by γ -secretase cleavage of C99 to release A β ⁴⁵. CRF mimics the ability of stress to increase A β levels and plaque formation in mouse models of AD, including the Tg2576 mouse used in the present study, and CRF₁ antagonists block the effects of stress in these models^{17, 46, 47}. CRF elicits a concentration-dependent secretion of A β from primary hippocampal neurons of Tg2576 mice that is mediated by CRF₁ and requires PKA signaling⁴⁷. The PKA dependence of CRF effects on A β secretion predicts that this pathway would be favored specifically in females that overexpress CRF. The present demonstration that female TT mice have a greater number of A β plaques compared to other groups supports this prediction. This may be attributed to sex differences in levels of pS⁴⁹⁸BACE, which was detected both by the phosphoproteomic analysis and by Western blot as being more abundant in the cortex of female mice that overexpress CRF. BACE phosphorylation favors its localization to more acidic intracellular compartments where it is found with higher levels of APP⁴⁸. This leads to the speculation that when CRF is overexpressed, the

female bias towards CRF₁-PKA-related signaling promotes BACE phosphorylation, which biases its cellular localization to compartments where A β formation may be accelerated. That this sex difference is functionally relevant was evidenced by both the increased number of plaques in female TT mice and greater cognitive impairment in spontaneous alternation. Although there were no sex differences in phosphorylation of casein kinase-1 (CK-1), an enzyme that phosphorylates Ser⁴⁹⁸-BACE-1⁴⁸, sex differences in activity or expression of CK1 could account for sex differences in Ser⁴⁹⁸-BACE-1 phosphorylation. Notably, CRF can induce A β formation in cells through CRF₁ association with γ -secretase and this is dependent on both β -arrestin-1 and β -arrestin-2, as well as CRF₁-independent mechanisms⁴⁹. The CRF₁ effect mediated by β -arrestins is consistent with other studies supporting a role for β -arrestin-2 in γ -secretase activity and A β formation⁵⁰. Sex differences in these effects have not been reported to our knowledge. However, taken with the present findings, this might predict different targets for treating Alzheimer's disease based on sex.

Together the findings suggest a model whereby, as a result of sex-biased CRF₁ coupling, conditions of elevated CRF in females engage signaling that underlies two AD-related neuropathological processes, tau phosphorylation and synthesis of A β (Fig. 5). This can account for an interaction between stress and sex that may contribute to female vulnerability to AD. Importantly, the findings suggest that manipulation of CRF₁-induced PKA signaling may be a potential therapeutic target for slowing the progression of AD, particularly in women.

Conclusions

The present findings demonstrate that conditions of excessive CRF, which are characteristic of stress-related psychiatric disorders, give rise to sexual differentiation of the cortical phosphoproteome as a result of sex biased signaling. Distinctions between male and female phosphoproteomes suggest a potential molecular basis for sex differences in stress-related psychiatric and neurodegenerative disorders, such as AD. Our further analysis in an AD model supported the phosphoproteomic results that revealed female vulnerability to CRF-induced AD pathology, and suggested that stress may be a risk factor for AD, specifically in women. Finally, the phosphoproteomic results can be used as a discovery tool, and this unique, rich data set can be mined at more specific levels in future studies to identify how stress and sex intersect to impact selective cellular functions. These explorations will lead to a better understanding of how stress contributes to disease and the determinants of individual vulnerability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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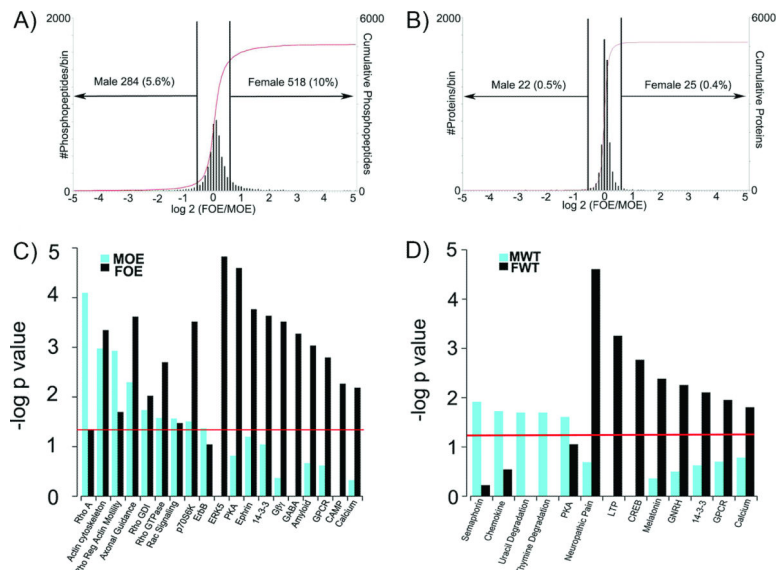


Figure 1.

CRF-overexpression results in sex differences in cortical phosphoproteins that are represented in distinct pathways. A) Histograms of the distribution of phosphopeptides that could be quantified in both CRF-OE males and CRF-OE females by having a ratio of the SILAM standard heavy-labeled peptide/light peptide. The abscissa (\log_2 FOE/MOE) indicates the relative abundance of phosphopeptides in female CRF-OE compared to the male CRF-OE. When they are equally present in both groups the $\log_2=0$. Phosphopeptides that are more prevalent in females or males would be to the right (positive bins) or left (negative bins) of 0, respectively. The number of phosphopeptides/bin is indicated on the left ordinate and the cumulative number of phosphopeptides is shown by the sigmoidal curve and indicated on the right ordinate. Vertical lines indicate the statistical cutoff (see Methods). Approximately 10% of the phosphoproteins were more prevalent in females and 5.6% were more prevalent in males. B) Histogram of the distribution of proteins identified from the HILIC fraction that was not enriched for phosphopeptides. Graphical description as in A. The majority of nonphosphorylated peptides were in equal abundance between the sexes. C) FOE and MOE phosphopeptides are overrepresented in distinct pathways that are different than those that distinguish FWT and MWT. Bars indicate some of the top canonical pathways in which phosphopeptides of the MOE (blue) or FOE (black) are overrepresented as determined by Ingenuity Pathway Analysis. The ordinate indicates $-\log$ of the p-values. The red line represents $p=0.05$. The first six pathways (RhoA to RhoGDI) are the top six canonical pathways in which MOE phosphopeptides were overrepresented. D) Bars indicate some of the top canonical pathways in which phosphopeptides of the MWT (blue) or FWT (black) are overrepresented as determined by Ingenuity Pathway Analysis. Graphical description as in C.

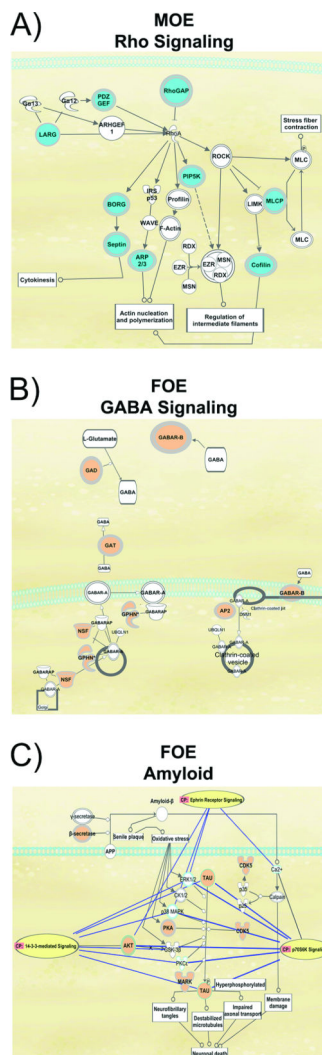


Figure 2. Phosphopeptides from the MOE and FOE phosphoproteomes are represented in some distinct signaling pathways, including Rho signaling for MOE (A), GABA signaling for FOE (B) and amyloid processing for FOE (C). Within specific pathways blue shading indicates MOE phosphopeptides and orange shading indicates FOE phosphopeptides. The schematic for the amyloid pathway also indicates how some of the major pathways represented by the FOE phosphoproteome (indicated in yellow) are also linked to the amyloid processing. The pathways were generated by Ingenuity Pathway Analysis (Ingenuity[®]Systems, www.ingenuity.com).

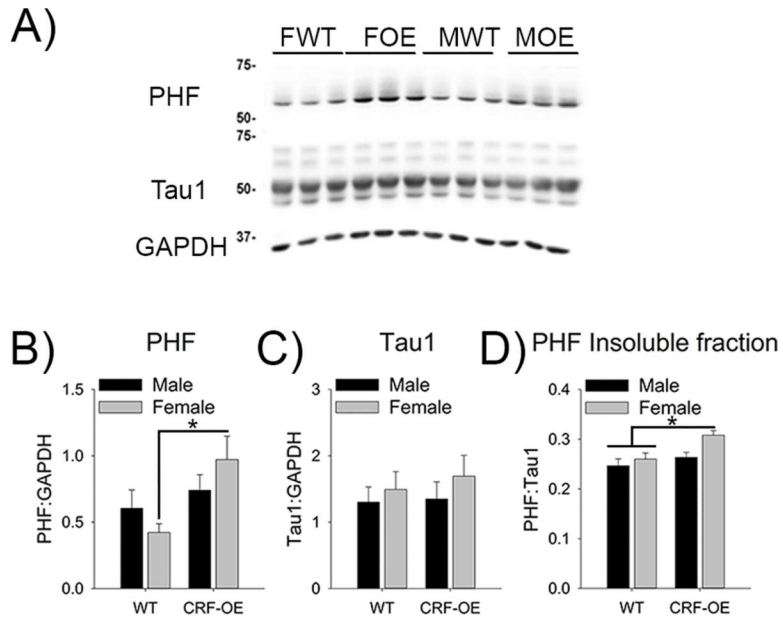


Figure 3. Sex differences in MAPT phosphorylation in CRF overexpressing mice. A-C) Quantification of pS^{396/404}tau (assessed by PHF-1) and tau1 in CRF-OE mice. A shows representative Western blots of PHF1, Tau1 and GAPDH from male and female CRF-OE and WT mice. Bars in B and C show the mean PHF:GAPDH or Tau1:GAPDH ratio (X 100) of male and female CRF-OE and WT mice, respectively (n=5 each group). PHF1, was increased in FOE compared to FWT mice ([$F(3,16) = 3.14, p = 0.054$]; Tukey HSD post-hoc, $p = 0.040$). There were no differences in PHF1 between males ($p > 0.05$) or in Tau1 [$F(3,16) = .42, p > 0.05$], the marker for total tau. D) Bars show mean PHF:Tau1 in the insoluble fraction (n=5 each group). PHF was increased in FOE compared to FWT and MWT ([$F(3,16)=5.3, p < 0.01$]; Tukey HSD post-hoc, $p < 0.05$). Tests of variance between groups revealed equality. Vertical lines indicate SEM. Asterisks indicate $p < 0.05$.

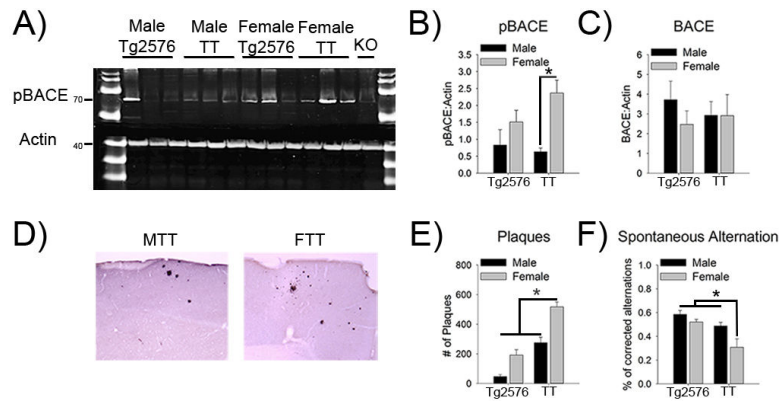


Figure 4.

Sex differences in A β signaling and related pathology. A) Representative Western blot of pS⁴⁹⁸BACE in male and female Tg2576 and TT mice and a BACE knockout mouse (KO). Bars in B and C show the mean pS⁴⁹⁸BACE:actin and BACE:actin ratio, respectively of male and female Tg2576 (n=6 each) and TT mice (n=4 each). Female TT mice had increased pBACE compared to their male counterparts ($[F(3,16) = 3.61, p = 0.037]$ Tukey HSD post hoc, $p < 0.05$). This sex difference was not observed in mice lacking CRF overexpression (Tg2576) ($p > 0.05$) and there were no group differences in BACE levels [$F(3,16) = .43, p > 0.05$]. Tests of variance between groups revealed equality. D,E) Quantification of A β plaques in male and female Tg2576 (n=4 female and 6 male) and TT (n=4 each) mice. Photomicrographs in D show representative examples of plaques in male and female TT mice. The bar graph in E shows the mean number of plaques in cortex of male and female Tg2576 and TT mice. Female TT mice had a greater mean number of A β plaques compared to all other groups ($[F(3,14) = 51.3, p > 0.001]$; Tukey HSD post-hoc, $p < 0.010$). Tests of variance between groups revealed equality. F) Quantification of performance on spontaneous alternation task. Bars show the mean percent correct alternation for male Tg2576 (n=9), female Tg2576 (n=22), male TT (n=7) and female TT (n=8) mice. FTT mice had a lower percent correct alternation compared to all other groups ($F(3,32)=7.6, p < 0.0005$; Tukeys HSD $p < 0.05$). Tests of variance between groups revealed equality. Vertical lines indicate SEM. Asterisks indicate $p < 0.05$.

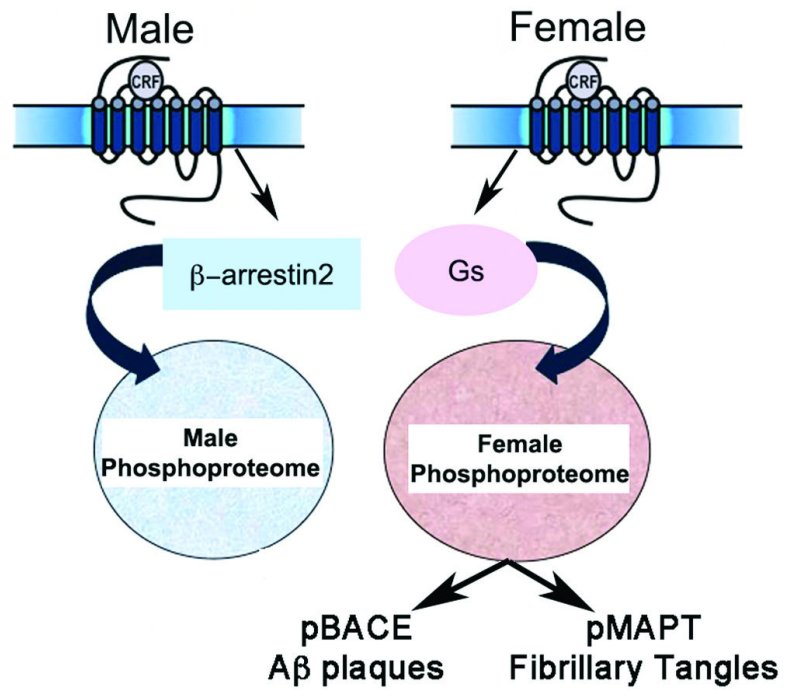


Figure 5. Schematic depicting how excessive CRF can promote AD-related pathology specifically in females because of sex-biased CRF₁ coupling.