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Supplementary Materials for

Hyperexcitation of ovBNST CRF neurons during stress contributes to female-biased expression of anxiety-like avoidance behaviors

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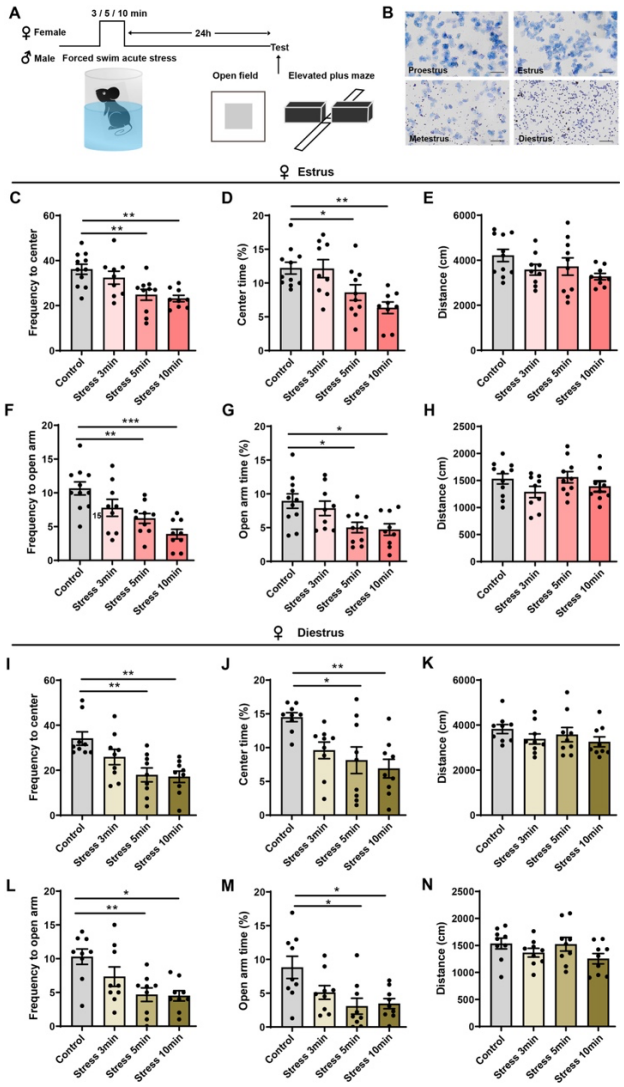

Fig. S1. The estrus cycle had nothing to do with the response to acute strong stress in female mice. (A) Experimental timeline for measuring anxiety-like behaviors following 3 or 5 or 10-min treatment of forced swimming. **(B)** Representative vaginal smear images. **(C-E)** Quantification of frequency to center zone **(C)**, percentage time spent in the center zone **(D)** and locomotion distance **(E)** of OFT in estrus female mice (*P<0.05, **P<0.01, one-way ANOVA and post hoc test). **(F-H)** Quantification of frequency to open arms **(F)**, percentage time spent in the open arms **(G)** and locomotion distance **(H)** of EPM in estrus female mice (*P<0.05, **P<0.01, ***P<0.001, one-way ANOVA and post hoc test). **(I-K)** Quantification of frequency to center zone **(I)**, percentage time spent in the center zone **(J)** and locomotion distance **(K)** of OFT in diestrus female mice (*P<0.05, **P<0.01, one-way ANOVA and post hoc test). **(L-N)** Quantification of frequency to open arms (L), percentage time spent in the open arms **(M)** and locomotion distance **(N)** of EPM in diestrus female mice (*P<0.05, **P<0.01, one-way ANOVA and post hoc test).

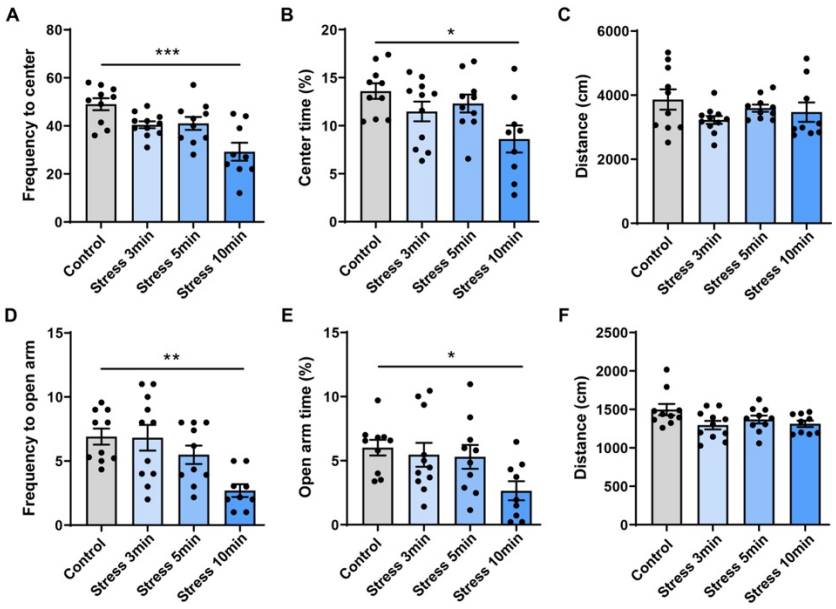

Fig. S2. Male mice display less susceptibility to acute stress induced-anxiety than female mice. (A-C) Quantification of frequency to center zone **(A)**, percentage time spent in the center zone **(B)** and locomotion distance **(C)** of OFT in male mice (*P<0.05, ***P<0.001, one-way ANOVA and post hoc test). **(D-F)** Quantification of frequency to open arms **(D)**, percentage time spent in the open arms **(E)** and locomotion distance **(F)** of EPM in male mice (*P<0.05, **P<0.01, one-way ANOVA and post hoc test).

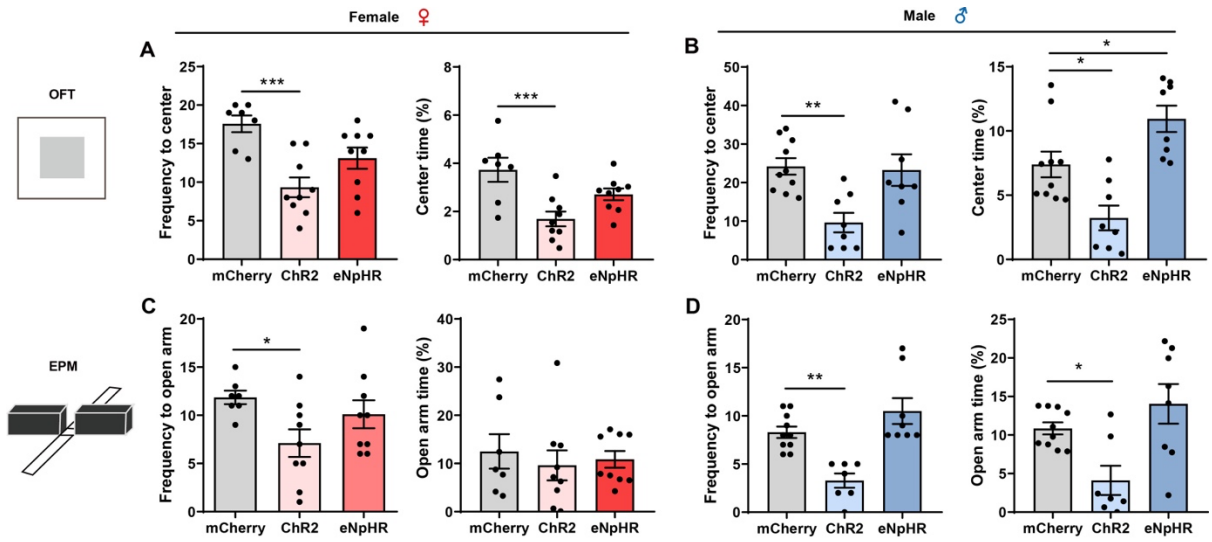

Fig. S3. Optogenetic activation or inhibition of CeA CRF projections in ovBNST modulate anxiety-like behaviors in both sexes. (A-B) Quantification of frequency to center zone and percentage time spent in the center zone of OFT in female **(A)** and male **(B)** mice (*P<0.05, ***P<0.001, one-way ANOVA and post hoc test). **(C-D)** Quantification of frequency to open arms and percentage time spent in the open arms of EPM in female **(C)** and male **(D)** mice (*P<0.05, **P<0.01, one-way ANOVA and post hoc test).

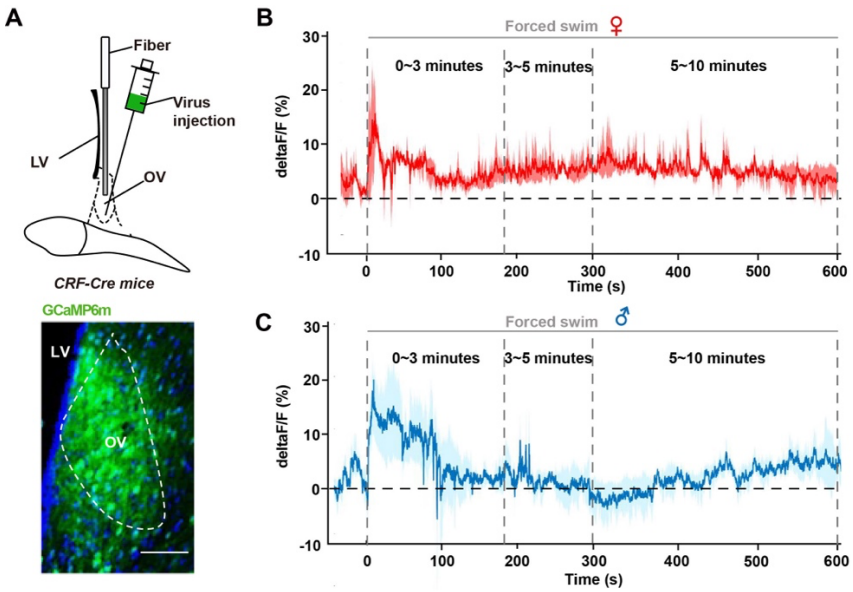

Fig. S4. The activation pattern of ovBNST CRF neurons during 10-min forced swimming stress shows sexual difference. (A) Top, virus injection and fiber configuration. Bottom, representative image showing expression of GCaMP6m in ovBNST. Scale bar: 200μm. **(B-C)** Example trace of ovBNST CRF neuronal calcium signals during 10-min forced swimming stress in female **(B)** and male **(C)** mice.

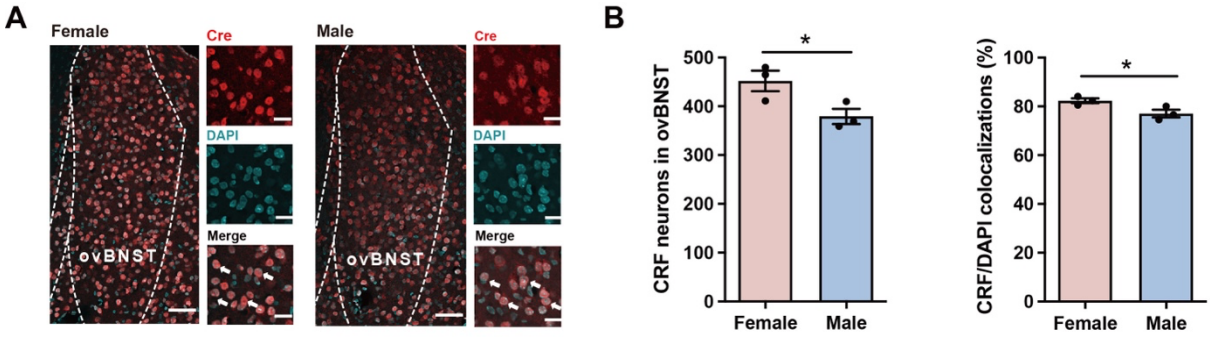

Fig. S5. The number of ovBNST CRF neurons shows sex differences. (A) Immunofluorescence staining of Cre (red) and DAPI (blue) in the ovBNST of female (left) and male (right) *CRF-Cre* mice. Scale bars=50 μm. **(B)** Left, quantification of CRF neurons number between the ovBNST of female and male mice. Right, quantification of CRF/DAPI colocalization (%) between the ovBNST of female and male mice (*P<0.05, unpaired t-test).

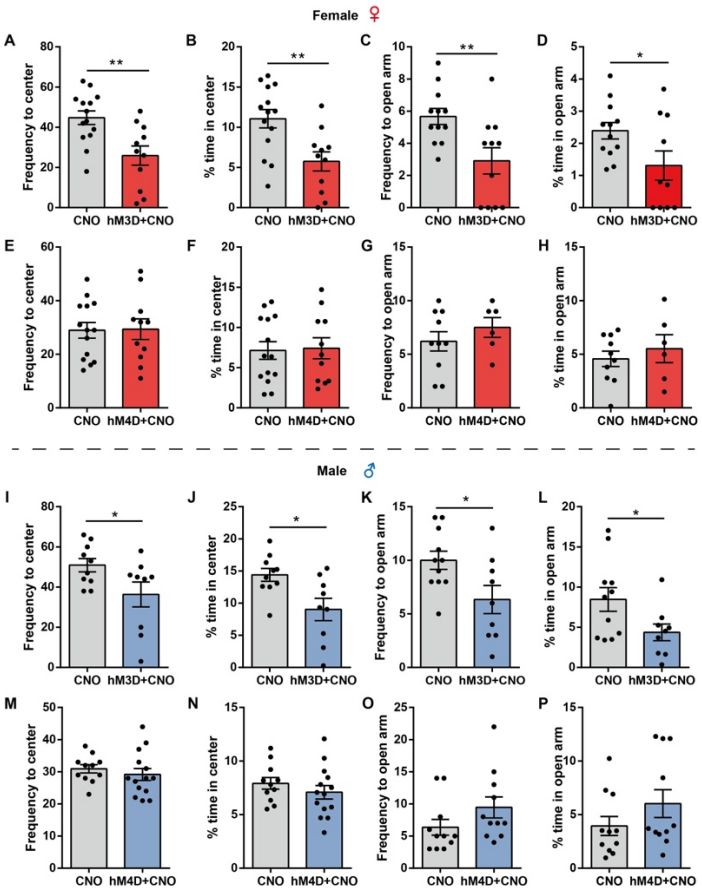

Fig. S6. Chemogenetic activation of ovBNST CRF neurons increases anxiety-like behaviors and inhibition of ovBNST CRF neurons has no effect in female and male mice. (A-D) Quantification of frequency to center zone **(A)** and percentage time spent in the center zone **(B)** of OFT, as well as frequency to open arms **(C)** and percentage time spent in the open arms **(D)** of EPM after specific activation of CRF neurons in female mice (*P<0.05, **P<0.01, unpaired t-test). **(E-H)** Quantification of frequency to center zone **(E)** and percentage time spent in the center zone **(F)** of OFT, as well as frequency to open arms **(G)** and percentage time spent in the open arms **(H)** of EPM after specific inhibition of CRF neurons in female mice. **(I-L)** Quantification of frequency to center zone **(I)** and percentage time spent in the center zone **(J)** of OFT, as well as frequency to open arms **(K)** and percentage time spent in the open arms **(L)** of EPM after specific activation of CRF neurons in male mice (*P<0.05, unpaired t-test). **(M-P)** Quantification of frequency to center zone **(M)** and percentage time spent in the center zone **(N)** of OFT Quantification of frequency to open arms **(O)** and percentage time spent in the open arms **(P)** of EPM after specific inhibition of CRF neurons in male mice.

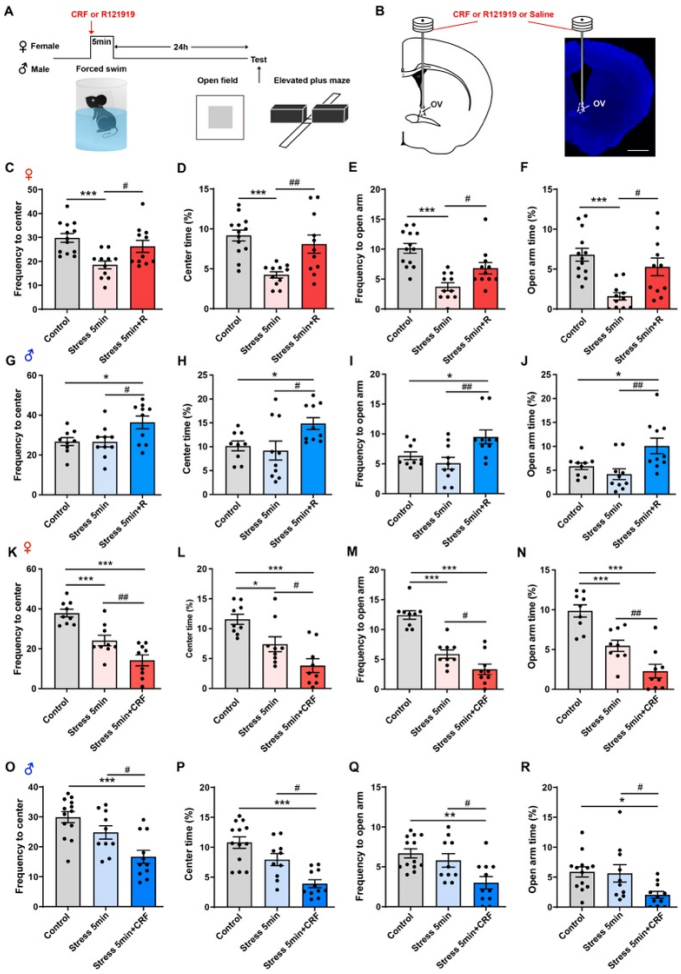

Fig. S7. The CRFR1 in the ovBNST is required for the generation of sex-specific susceptibility to acute stress-induced anxiety. (A) Experimental timeline. **(B)** Left, drug injection configuration. Right, representative image showing tract of cannula. Scale bar: 1mm. **(C-F)** Quantification of frequency to center zone **(C)**, percentage time spent in the center zone **(D)** of OFT, as well as frequency to open arm **(E)**, percentage time spent in the open arm **(F)** of EPM in female mice with intra-ovBNST administration of R121919 (***P<0.001, $^{\#}$P<0.05, $^{\#\#}$P<0.01, one-way ANOVA and post hoc test). **(G-J)** Quantification of frequency to center zone **(G)**, percentage time spent in the center zone **(H)** of OFT, as well as frequency to open arm **(I)**, percentage time spent in the open arm **(J)** of EPM in male mice with intra-ovBNST administration of R121919 (*P<0.05, $^{\#}$P<0.05, $^{\#\#}$P<0.01, one-way ANOVA and post hoc test). **(K-N)** Quantification of frequency to center zone **(K)**, percentage time spent in the center zone **(L)** of OFT, as well as frequency to open arm **(M)**, percentage time spent in the open arm **(N)** of EPM in female mice with intra-ovBNST administration of CRF (*P<0.05, ***P<0.001, $^{\#}$P<0.05, $^{\#\#}$P<0.01, one-way ANOVA and post hoc test). **(O-R)** Quantification of frequency to center zone **(O)**, percentage time spent in the center zone **(P)** of OFT, as well as frequency to open arm **(Q)**, percentage time spent in the open arm **(R)** of EPM in male mice with intra-ovBNST administration of CRF (*P<0.05, **P<0.01, ***P<0.001, $^{\#}$P<0.05, one-way ANOVA and post hoc test).

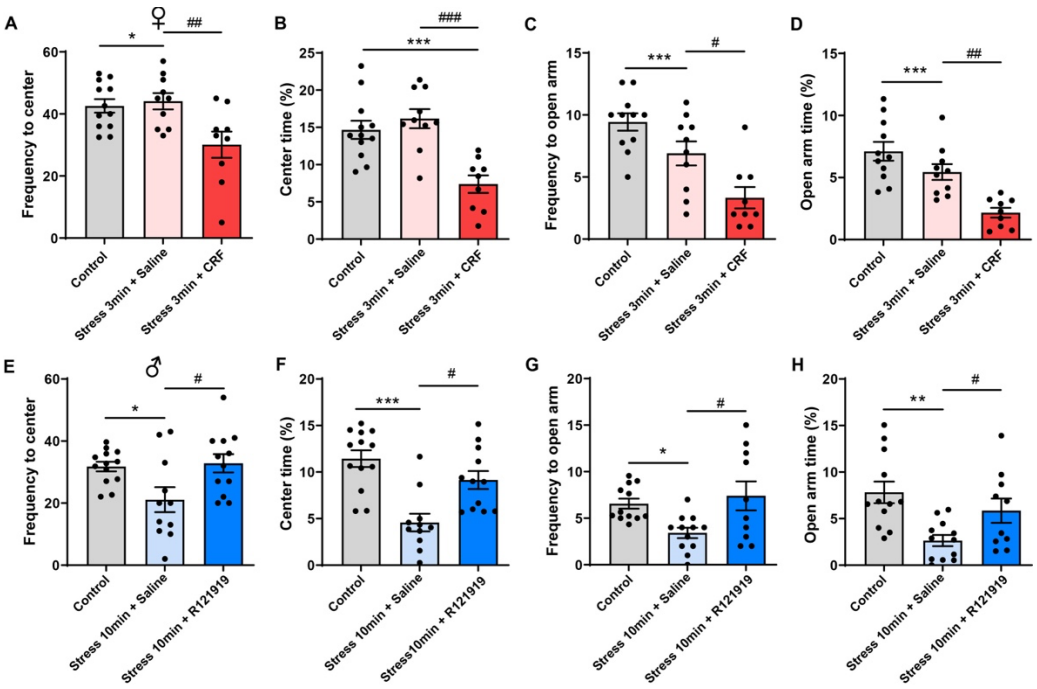

Fig. S8. The CRF-CRFR1 system in the ovBNST regulates the susceptibility to anxiety in both male and female mice. (A-B) Quantification of frequency to center zone **(A)**, percentage time spent in the center zone **(B)** of OFT in female mice (*P<0.05, ***P<0.001, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001, one-way ANOVA and post hoc test). **(C-D)** Quantification of frequency to open arm **(C)**, percentage time spent in the open arm **(D)** of EPM in female mice (***P<0.001, $^{\#}$P<0.05, $^{\#\#}$P<0.01, one-way ANOVA and post hoc test). **(E-F)** Quantification of frequency to center zone **(E)**, percentage time spent in the center zone **(F)** of OFT in male mice (*P<0.05, ***P<0.001, $^{\#}$P<0.05, one-way ANOVA and post hoc test). **(G-H)** Quantification of frequency to open arm **(G)**, percentage time spent in the open arm **(H)** of EPM in male mice (*P<0.05, **P<0.01, $^{\#}$P<0.05, one-way ANOVA and post hoc test).

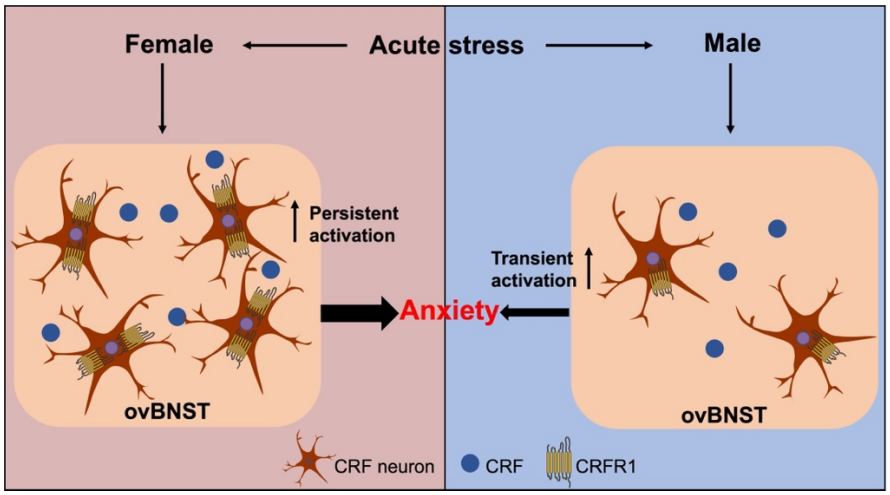

Fig. S9. Graphical abstract to summarize the potential mechanism underlying the female-biased susceptibility to stress-induced anxiety. First, CRF release in ovBNST is higher in female than male mice. Second, the activity of ovBNST CRF neurons show persistent activation during stress in female, but transient activation in male mice. Third, the expression of ovBNST CRFR1 in female mice outnumbered that in male mice, and CRFR1 could mediate the CRF-induced excitation of ovBNST CRF neurons. In total, the above mechanisms all contribute to the female-biased susceptibility to anxiety.