Corticotropin Releasing Factor in the Bed Nucleus of the Stria Terminalis modulates the behavioral consequences of unpredictable threat

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ABSTRACT

Fear is a protective response to perceived danger that allows an organism to identify and respond to threats to avoid harm. Though fear is critical for survival, excessive fear can impede normal biological processes; thus, accurate risk assessment is key for well-being. Here we investigate the neural underpinnings of two distinct behavioral states: phasic and sustained fear. Phasic fear is considered an adaptive response and is characterized by response to a clear and discrete cue that dissipates rapidly once the threat is no longer present. Conversely, sustained fear or anxiety is a heightened state of arousal and apprehension that is not clearly associated with specific cues and lasts for longer periods of time. Here, we directly examine the contribution of BNST CRF signaling to phasic and sustained fear in male and female mice using a partially reinforced fear paradigm to test the overarching hypothesis that plasticity in BNST CRF neurons drive distinct behavioral responses to unpredictable threat in males and females.

INTRODUCTION

Fear is a protective response to perceived danger that allows an organism to identify and respond to threats to avoid harm. Though fear is critical for survival, excessive fear can impede essential biological processes[1–5]; thus, accurate risk assessment is key for well-being. Here we investigate the neural underpinnings of two distinct behavioral states: phasic and sustained fear. Phasic fear is considered an adaptive response and is characterized by response to a clear and discrete cue that dissipates rapidly once the threat is no longer present. Conversely, sustained fear or anxiety is a heightened state of arousal and apprehension that is not clearly associated with specific cues and lasts for longer periods of time[6,7]. In preclinical

studies, paradigms for sustained fear better capture the symptoms of human anxiety; thus, understanding the neural correlates that distinguish phasic and sustained fear is critical for improving treatments for anxiety disorders[7]. Though often excluded from canonical fear circuitry, the bed nucleus of the stria terminalis (BNST) is recruited when there is uncertainty regarding when or if harm will occur and is thought to drive sustained fear[7–12].

One system in the brain that has been implicated in sustained fear is the corticotropin releasing factor (CRF) system. CRF is highly expressed in the BNST and potently shapes the function of this region,[13–16] yet the role of BNST CRF in phasic and sustained fear remains unclear. CRF overexpression in the BNST prior to conditioning weakens sustained fear[17]; however, CRF receptor antagonism in the BNST also blocks sustained fear[7,18]. Though both studies suggest that CRF signaling in the BNST is particularly important for sustained fear, the mechanism by which CRF shapes fear response is unknown.

Critically, though mood disorders are more prevalent in women and non-binary individuals, preclinical studies have classically only included males[19,20]. Sex differences in defensive behavioral strategies, BNST structure and function, and CRF signaling have been reported and thus, investigation into how these features shape negative emotional states is prudent[21–28]. Here, we directly examine the contribution of BNST CRF signaling to phasic and sustained fear in male and female mice using a partially reinforced fear paradigm to test the overarching hypothesis that plasticity in BNST CRF neurons ($BNST^{CRF}$) drive distinct behavioral responses to unpredictable threat in males and females.

METHODS

Mice: All experiments were conducted in accordance with the University of North Carolina at Chapel Hill's Institutional Animal Care Use Committee's guidelines. All mice were group housed and maintained on a 12h/12h light-dark cycle with lights on at 7 AM, and had access to standard rodent chow and water ad libitum. Male and Female C57BL/6J mice >8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). CRF-cre, $CRF^{fl/fl}$, and $CRFR1^{fl/fl}$, mice were bred in house and maintained on a C57BL/6J background.

Surgery: Mice were anesthetized using isoflurane (4% induction, 1-2% maintenance). Mice were administered buprenorphine (.1 mg/kg, s.c.) during surgery, and were also given acetaminophen (80 mg/200mL in drinking water) one day before and for at least 3 days following surgery. AAV constructs were delivered to target regions using Hamilton microsyringes at the following coordinates; dBNST: AP +.3mm, ML +/- .9mm, DV -4.35. vBNST: AP +.3mm, ML +/- .9mm, DV -4.6. For fiber photometry experiments, fiber optic cannulae (FOC) were cut to length and implanted at the same coordinates and cemented to the skull with dental cement (C&B Metabond, Parkell). For miniature microscope experiments, GRIN lenses (.6 x 7.3mm) with integrated baseplates (Inscopix, Mountain View, CA) were implanted .2mm above the target coordinates and lowered at a rate of .2mm/min. Mice recovered for >3 weeks prior to the start of experiments.

Viruses: For CRF deletion experiments, AAV5-CaMKiia-cre-GFP and AAV5-CaMKiia-GFP were obtained from the UNC Vector Core (Chapel Hill, NC). Viruses for fiber photometry and 1-photon calcium imaging detailed below.

Behavior

Fear conditioning: Fear conditioning was conducted using a three-day protocol. On the first day (habituation), mice were placed into a fear conditioning chamber (context A, Med Associates, St. Albans, VT) equipped with a shock grid floor and clear plexiglass walls, and allowed to forage for five minutes. Context A was cleaned with a 20% ethanol + 1% vanilla extract solution to provide a scent cue. On day 2 (fear learning), mice were returned to context A. Following a 3-minute baseline, mice were presented with tone-shock pairings (tone: 30 s, 80 dB, 3 KHz, shock: 0.6 mA, 2 s), or unpaired tones depending on the experimental group. Mice subjected to fully-reinforced fear (Full) received 3 tone-shock pairings separated by inter-trial intervals of 60 and 90s. Mice undergoing partially reinforced fear (Part) received 3 tone-shock pairings as well as 3 unpaired tones interspersed such that tones 1, 2, and 4 ended in shock, and 3, 5, and 6 did not. Inter-trial intervals were as follows: 60-20-40-20-20. Control mice received 6 tones at the same timing as the Part group, except the mice did not receive foot shocks. Following tone-shock presentations, mice remained in context A for a 2-minute consolidation period. On the third day, mice were placed into a novel context with white plastic flooring, curved walls, and cleaned with 0.5% acetic acid. Following a 3-minute baseline, 6 tones (identical to fear learning) were presented separated by a random ITI of 20-60 s. Behavior hardware was controlled by Ethovision XT software (Noldus Inc.).

Novelty-Induced suppression of feeding (NSF): Mice were food deprived overnight prior to the start of NSF. On test day, a single pellet of standard chow was placed into the center of a brightly lit (1350 lux) white Plexiglas open field chamber. The floor was covered with normal cage bedding. Mice were placed into the corner of the chamber. The trial was ended when the

mouse ate for a period of 3 seconds or more. If mice did not eat, the trial was ended after 10 minutes and mice were assigned a maximum latency of 600 seconds. After the test, mice were returned to a dimly lit clean cage and allowed to eat for 10 minutes, and amount consumed was recorded.

Acoustic startle: Mice were semi-restrained in Plexiglas tubes mounted to a platform with an accelerometer attached and placed inside a sound attenuating chamber (all hardware and software from San Diego Instruments, San Diego, CA). This context was separate from fear conditioning chambers. Following a 5-minute acclimation period, white noise bursts of varying intensity were played, and startle responses were measured. Four different stimulus intensities were tested: 90 dB, 105 dB, 120dB, and 0 dB (control) separated by a random ITI of 30-60 seconds. Each session consisted of 10 blocks of 4 trials, one of each intensity, such that there were 10 total trials at each stimulus intensity. Startle response peaks were detected automatically using Startle Response Lab software.

Fiber Photometry

GCaMP6s (AAV5-hsyn-GCaMP6s, UPenn Vector Core, Philadelphia, PA) was injected into the BNST of C57BL/6J mice and fibers were implanted unilaterally in the BNST. Fiber optic cannulae (200μm, .37nA, Neurophotometrics, San Diego, CA) were cut to length and tested for light transmission prior to implantation. Prior to behavioral testing, mice were habituated to patch cord tethering daily for 3 days. Using a Neurophotometrics FP3001 system (Neurophotometrics, San Diego, CA), alternating pulses of 470 nm and 415 nm LED light (~50 μW, 40 Hz) were bandpass filtered and focused onto a branching patch cord (Doric, Quebec City, Quebec) by a 20x objective lens. A custom-built Arduino was used to time lock recordings to behavior.

Custom MATLAB scripts were used for all analysis. Background fluorescence was subtracted from 415 and 470 traces, then traces were lowpass filtered at 5 Hz and fit to a biexponential curve. Curve fit was subtracted from each trace to correct for baseline drift. dF/F for 415 and 470 traces were calculated (raw signal-fitted signal)/(fitted signal), and traces were z-scored. The 415 signal was fit to the 470 signal using non-negative robust linear regression to correct for motion artifacts [30]. Spike analysis: Spikes were identified from processed traces using a custom MATLAB script, and were defined as peaks greater than 2* the mean absolute deviation of baseline (first minute of each recording). Peak timing was then aligned with freezing data to determine whether each spike occurred during freezing or mobility.

1-Photon Calcium Imaging

Prior to the start of behavioral testing, mice were habituated to miniature head-mounted microendoscope attachment daily for 3 days. For all experiments, 470 LED light was delivered at .9 - 1.1mW, and data were recorded at 20 Hz using an Inscopix nVoke imaging system (Inscopix, Mountain View, CA). Recordings were time-locked to behavior hardware via TTL. Data were analyzed using Inscopix Data Processing Software. Briefly, data were spatially downsampled by a factor of 4, and temporally downsampled by a factor of 2 to reduce computational load. Videos were then spatial bandpass filtered, motion corrected, and normalized with dF/F to prepare the video for automated cell detection using PCA-ICA. Detected regions of interest were then manually accepted or rejected based on signal quality, and overlapping regions were discarded. Spikes were then identified as events with a minimum peak of 3* the median absolute deviation and minimum decay time of .2 s. Traces were then further processed using a custom MATLAB script. Briefly, traces were z-scored, and peri-event

plots were generated. Responses to tone and shock were determined for each trial for each cell using a Wilcoxon rank-sum test comparing the 2-second window before and after stimulus. For GCaMP recordings in $BNST^{CRF}$, GCaMP8m AAV1-syn-flex-GCaMP8m, Addgene) was diluted 1:1 in sterile PBS and injected unilaterally into the BNST.

Automated Behavior Tracking

Behavior videos were tracked using DeepLabCut software that uses deep neural networks to estimate mouse position [31]. Behavior was then scored by analyzing DLC tracking output using SimBA [32], a machine learning platform that can be trained to identify behaviors. A classifier for freezing behavior was created in house. A custom MATLAB script (Mathworks, Natick, Massachusetts) was used to determine freezing bout start and end times, and to calculate % freezing during various behavior epochs.

Histology

To verify virus, fiber, and GRIN lens placement, mice were transcardially perfused with 30mls each of phosphate buffered saline and 4% paraformaldehyde. Brains were post-fixed in 4% PFA for 24h after extraction, then sectioned on a vibratome. GCaMP fluorescence was amplified using immunohistochemistry. Mice with off-target virus expression, fiber, or lens placement were excluded from analysis.

RESULTS

Partially reinforced fear drives a hyperarousal phenotype in males

To characterize the behavioral profiles and underlying neural processing that distinguish phasic and sustained fear, we subjected male and female C57BL/6J mice to either partially reinforced fear (Part), fully reinforced fear (Full) or tone only exposure (Ctrl). In fully reinforced fear, mice are presented with 3 tone-shock pairings such that 100% of tones are paired with a shock. In partially reinforced fear, mice are presented with 6 tones, 50% of which are paired with a shock in a pseudo-random order[11]. Control mice were presented with 6 tones, all unpaired with a shock. Twenty-four hours after fear learning, all mice were subjected to a fear recall protocol consisting of 6 tone presentations (Fig 1A). Freezing was scored as a measure of defensive behavior. We found that during conditioning, Full and Part mice in both sexes froze more than no shock controls, and Part mice froze more than Full on the last tone (Fig 1B-C, F-G). During recall, Full and Part mice again froze more than controls, but there were no significant differences between Full and Part groups in either sex (Fig 1D-E, H-I).

Studies in rodents and humans have shown that paradigms in which aversive stimuli are uncertain or temporally unpredictable drive increased arousal and avoidance behaviors[9–11,33,34]. To test whether Part fear alters anxiety-like behaviors, we subjected mice to conditioning as described above. However, instead of fear recall, mice underwent a novelty-induced suppression of feeding (NSF) test twenty four hours after conditioning to assess avoidance behavior (Fig 1J). We did not find differences in latency to feed or amount consumed during the refeed portion of the test between groups in either sex (Fig 1L-M, P-Q), suggesting that Part and Full fear do not alter approach-avoidance behavior. Next, we assessed arousal using an acoustic startle reflex test, where mice are presented with white noise burst stimuli and startle reflex

size is measured. This test is valuable as a measure of psychomotor arousal, and increased startle responses are observed in humans with anxiety disorders[35,36]. We found that Part fear potentiated acoustic startle response in males but not females, measured as increased startle amplitude (Fig 1N, R). Our results indicate that Part but not Full fear conditioning drives hypervigilance in males.

BNST dynamics differ during Part and Full fear

Given that the BNST is recruited in unpredictable fear paradigms[7,9–11], we hypothesized that BNST activity differs in Part and Full fear conditioning and recall. To characterize BNST dynamics, we injected an adeno-associated virus (AAV) construct containing the genetically encoded calcium sensor GCaMP6s into the BNST of C57BL/6J mice and implanted fiber optic cannulae (FOC). We recorded calcium activity in BNST using fiber photometry (Fig 2A-C). We found that during fear learning, BNST was responsive to foot shock in all groups (Fig 2E, H, J, M), measured as a significant increase in GCaMP signal from baseline. Shock response increased in the BNST over the course of the session in Full fear males, but not Part fear or either group in females (Fig 2I, N). Additionally, BNST was responsive to tone onset in Full and Part fear in males (Fig 2F). In Full males only, BNST tone response increased significantly between first and last trials (Fig 2G), suggesting that BNST becomes responsive to threat-predicting cues when harm is expected, but not when the outcome is uncertain. In females, tone responses were less pronounced, and only the BNST response in Part fear was significantly higher than baseline (Fig 2K), indicating sex differences in BNST response to threat-predicting stimuli. Overall, BNST

dynamics were highly similar across groups and sexes during learning, and thus the subtle differences in signaling may communicate the nuanced distinctions between Full and Part fear.

In contrast to conditioning, sex differences in BNST response emerged during fear recall 24 hours later. In males, BNST activity dipped below baseline during tone presentations, and this suppression was significantly greater in Part fear (Fig 2O, Q). Additionally, the BNST tone onset peak was significantly above baseline in Part but not Full fear (Fig 2P), suggesting that a greater suppression in BNST activity is not due to a smaller signal overall. These results indicate that Part and Full fear give rise to dissociable effects on BNST activity in males. In females, the BNST responded to tone onset with an initial spike that decayed slowly over the course of the tone, and this peak was significantly above baseline in both Full and Part fear (Fig 2R-T). Unlike in males, differences between fear groups were not observed. Overall, our findings suggest that BNST activity differs between Part and Full fear in males but not females. This mirrors the acoustic startle behavior in Figure 1, where Part conditioning leads to increased vigilance in males but not females. In this way, distinct alterations in BNST activity after fear learning may mediate the behavioral consequences of Part fear in males.

CRF knockdown in the BNST potentiates partially reinforced fear in females

CRF signaling in the BNST is critical for sustained fear[6,7], but classic studies do not distinguish local CRF signaling from CRF inputs to the BNST. Interestingly, chemogenetic inhibition of $BNST^{CRF}$ neurons during phasic (cued) fear conditioning has no effect on fear[37,38], however, CRF infusion into the BNST after conditioning enhances recall of emotional memory[39]. Taken together, these findings suggest that the role of CRF in the BNST on fear is highly nuanced.

To determine the role of CRF in BNST neurons in Part fear, we used a viral approach to genetically knockdown CRF in the BNST[40]. To do this, we injected an AAV encoding cre recombinase or GFP bilaterally into the BNST of $CRF^{lox/lox}$ mice[40] three weeks prior to behavior testing (Figure 3A-C). Importantly, we found that CRF knockdown did not alter freezing during conditioning in any groups (Fig 3D-G), indicating that this approach does not impair defensive responses to proximal threat. In males, there was no effect of CRF knockdown on fear recall in either group (Figure 3H-I). However, in females, CRF knockdown selectively increased freezing during both baseline and tones in Part fear, with no effect on Full fear (Figure 3J-K). These results indicate that CRF knockdown in the BNST drives fear generalization after partially reinforced fear in females, and that CRF differentially shapes passive coping in males and females.

CRF knockdown in the BNST modulates anxiety-like behavior after fear

Intra-BNST infusion of CRF potentiates acoustic startle response[41], and locally-projecting BNST CRF neurons drive anxiety by inhibiting anxiolytic outputs from BNST[37,42]. Thus, we hypothesized that knockdown of CRF in the BNST would block the increased acoustic startle we observed in males after Part fear (Fig 1N). Using the same approach described above, we knocked down CRF in the BNST, and examined changes in anxiety-like behavior (Figure 4A-B). In males, we found CRF knockdown did not alter latency to feed in the NSF assay in control or Full fear groups. However, knockdown significantly decreased latency to feed in Part fear, indicating that BNST CRF mediates differences in avoidance behavior after Part fear (Fig 4C). In females, there was a main effect of fear on latency to feed, but no effect of CRF knockdown in any group

(Fig 4E). Importantly, there was no effect of CRF knockdown on the amount of food consumed during the refeed portion of the test in any group, indicating that this approach does not alter consummatory behavior (Fig 4D, F). In the acoustic startle test, CRF knockdown did not alter startle amplitude in control mice in either sex, indicating that knockdown has no effect on startle under basal conditions (Fig 4G,I). However, in males, there was a significant main effect of CRF knockdown, and knockdown x fear interaction after fear conditioning (Fig 4H). At 105dB, GFP controls showed the same trend as in Figure 1 where startle response was increased in Part fear relative to Full, though this effect was not significant. Surprisingly, however, CRF knockdown did not alter startle in Part males, but increased startle amplitude in Full males (Fig 4K). CRF knockdown had no effect on startle behavior in females (Fig 4J,L). These findings suggest that CRF in the BNST does not shape avoidance behavior or arousal under basal conditions in either sex, but that plasticity occurs after fear conditioning such that CRF signaling becomes engaged in males.

Activity of BNST CRF neurons during fear

We previously found that $BNST^{CRF}$ neurons contribute to fear encoding[37] but only when serotonin levels are increased in the BNST. This suggests that in some situations, $BNST^{CRF}$ can directly regulate fear learning. Given the effects of CRF deletion on fear and anxiety-like behavior, we hypothesized that differential engagement of $BNST^{CRF}$ during fear learning and recall drives the behavioral consequences of Part fear. To record $BNST^{CRF}$ activity, we injected an AAV encoding a cre-dependent GCaMP8m into the BNST of CRF-cre mice and implanted GRIN lenses for 1-photon calcium imaging (Fig 5A-D). During fear conditioning, we observed

that roughly a third of cells were excited by shock in both sexes, and the rest were inhibited (Fig 5E-F, K). In all groups, the spike frequency in BNST^{CRF} declined gradually over the course of the trial, and changes in activity between baseline and trial end (last 2 minutes of trial) did not differ between fear groups (Fig 5G-H, L-M). Following the same trend, spike frequency significantly decreased between first and last tone presentations in all groups except Full males (Fig 5I-J, N-O). Overall, no major differences in BNST^{CRF} activity between groups and sexes were noted during conditioning

In contrast, we noted sex differences in Part fear encoding. In Part fear males, we observed a difference in activity after shock and omission trials. After shock trials, BNST^{CRF} were largely silent for a period of 10 seconds, whereas spike frequency remained significantly higher after omission trials (Fig S2B-C). In females, BNST^{CRF} were similarly inhibited after both shock and omission trials (Fig S2D). Though spike frequency was significantly higher after shock compared to omission trials (Fig S2E), this effect is likely driven by the initial spike in response to shock. These findings indicate sex differences in how BNST^{CRF} encode negative prediction error during Part fear conditioning.

Similar to conditioning, BNST^{CRF} activity decreased significantly between baseline and session end during recall (Fig 5P-Q,U). Interestingly, the magnitude of decrease was greater after Full fear in both sexes (Fig 5P,R,V), suggesting that CRF neuron activity remains elevated after Part fear. Further, despite an overall decrease in activity, spike frequency during tones increased between the first block (first 2 tones) and last block (last 2 tones) in part fear only (Fig 5S-T, W-X). Overall, these findings suggest two distinct phenomena. First, BNST^{CRF} neurons selectively escalate firing to ambiguous cues. Second, Part fear results in higher activity in CRF

neurons during fear expression, which may drive the behavioral consequences observed after fear conditioning.

DISCUSSION

Partially reinforced fear conditioning drives sustained fear in males

We show that Part but not Full fear conditioning gives rise to hypervigilance in males indicated by increased acoustic startle response. Importantly, this effect was observed three days after fear learning, suggesting sustained increases in arousal. This finding is of translational interest because hypervigilance and increased threat responsivity are core symptom of post-traumatic stress disorder (PTSD) in humans[36,43,44]. Additionally, this effect was observed in the absence of differences in freezing and avoidance behavior, which suggests that this paradigm does not confer an overall change in fear memory strength or anxiety-like behavior, but rather, has a selective effect on vigilance. Further, Part fear did not result in any behavioral differences in females. This finding is particularly interesting because it suggests that the conditions that induce sustained fear in males do not do so in females. To date, very little work has studied sustained fear in females; thus, more studies are needed to understand the factors that drive anxiety-like behavior in females after an emotionally salient event.

BNST dynamics during fear

Using fiber photometry, we showed that the BNST is responsive to shock in both sexes, and that dynamics were largely the same between groups during fear learning. However, one notable difference we observed was that both tone and shock responses increased over time in

Full but not Part males. This may represent that the BNST encodes the expected likelihood of a negative outcome. This is consistent with *in vivo* electrophysiology recordings suggesting that roughly 40% of BNST neurons encode attention or uncertainty of aversive contingency[45]. Further, human fMRI studies have linked BNST activity to anticipation of threat, particularly those that are ambiguous .

Sex and fear differences in BNST dynamics emerged during fear recall, where we showed that BNST activity differed between Part and Full fear in males but not females. In males, BNST activity was suppressed during tones, and to a larger degree in Part fear. This is consistent with findings that an inhibitory input to the BNST from the medial prefrontal cortex is engaged during Part fear recall[11]. Notably, differentiation between Full and Part fear in males but not females parallel the behavioral findings in Figure 1. This suggests that post-fear alterations in BNST signaling may drive the behavioral consequences of unpredictable fear conditioning in males. Additionally, during both conditioning and recall, activity in the BNST negatively correlated with freezing (Fig S1), suggesting that this is an innate property of the BNST and is not altered by fear conditioning. These results are consistent with findings that the BNST mediates the expression of proactive avoidance behaviors to prevent harm[47].

CRF knockdown alters post-fear behaviors in a sex and fear-specific manner.

Sustained fear is a diffuse state of apprehension that is driven by distant or diffuse cues and persists for longer periods of time. In contrast, phasic fear is defined as defensive responses to clear and imminent threats that dissipate quickly once a threat is no longer present[7]. Both the BNST and CRF are hypothesized to play a critical role in sustained fear in

rodents and humans[7,18,25,28,48]. However, the mechanism by which CRF in the BNST contributes to sustained fear is unknown. In males, we showed that knockdown of CRF in the BNST had no impact on fear conditioning or recall, but differentially altered avoidance behavior and arousal in a fear paradigm-dependent manner. Further, CRF knockdown had no effect on behavior in control mice. These findings suggest that there is an interaction between fear and CRF signaling such that CRF is recruited after fear conditioning and mediates behavioral disruptions post-conditioning. Additionally, this effect is dependent on the conditioning paradigm used.

Specifically, we showed that in the NSF test, CRF knockdown normalized latency to feed after Part fear such that Part/cre mice resembled Full fear mice. This would suggest that Part fear engenders changes in approach-avoidance behavior that are mediated by CRF. Interestingly, this pattern did not generalize to acoustic startle behavior. CRF knockdown had no effect on startle in controls or after Part fear conditioning, but robustly increased startle reflex after Full fear. This finding was unexpected given that intra-BNST infusion of CRF potentiates acoustic startle under basal conditions[41]. However, we previously showed that $BNST^{CRF}$ neurons project both locally, and out of the BNST[37,42]. Taken together, this may indicate that CRF signaling within the BNST versus in target regions of $BNST^{CRF}$ differentially alters acoustic startle reflex, and that reduced CRF in downstream regions may drive increased startle after Full fear. Alternatively, this may reflect that Full, but not Part fear leads to recruitment of CRF signaling that enhances recovery from a traumatic stressor. Others have shown that following traumatic stress, CRF_{R2} signaling in the posterior BNST speeds return to baseline behavior[49,50]. The lack of an effect of CRF knockdown on acoustic startle after Part fear suggests a potential CRF-independent role of $BNST^{CRF}$ neurons instead, as this population also

releases GABA[51]. This is especially interesting, as we recently found that knockdown of GABA vs CRF $BNST^{CRF}$ neurons led to differential regulation of operant ethanol self-administration[52].

Interestingly, our findings in females do not follow this same pattern. First, we showed that CRF KD selectively increased Part, but not Full fear recall. The lack of effect in Full fear is not surprising given that the BNST is not required for cued fear[9–11,53]. However, an explanation for increased freezing in Part fear recall is less clear. Interestingly, we noted both an increase in freezing to tones and during baseline after CRF KD in Part fear, suggestive of increased fear generalization. In this way, CRF may dampen fear generalization to ambiguous cues in females, akin to the traumatic stress-induced engagement of CRF_{R2}. Additionally, CRF KD had no effect on avoidance behavior or arousal under basal conditions or after fear. Overall, these findings are consistent with reports that CRF plays divergent roles in anxiety-like and stress behaviors in males and females[25,28,29].

BNST CRF neurons are persistently active during Part fear expression

We identified two distinct characteristics of CRF neuron activity that may contribute to Part fear conditioning. First, CRF neurons selectively increase firing to unpredictable cues during fear expression, and second, $BNST^{CRF}$ firing frequency is elevated after Part fear recall in both sexes. Persistent $BNST^{CRF}$ activity is particularly interesting, as elevated central CRF levels and CRF signaling hyperactivity have been documented in people with stress-related disorders[54]. Accordingly, elevated BNST CRF neuron activity may drive the behavioral alterations observed following Part fear.

Interestingly, we did not see any differences between CRF neuron activity in Part and Full fear conditioning. Consistent with our previous findings that chemogenetic inhibition of CRF neurons does not alter Full fear[37], this suggests that CRF neuron activity during conditioning itself is not important for later behavioral distinctions between phasic and sustained fear.

Notably, we did not observe sex differences in $BNST^{CRF}$ activity despite robust behavioral differences after CRF knockdown. There are a few possible explanations for this. First, Ca^{2+} activity in CRF cell bodies may not necessarily reflect release of CRF itself. Others have shown that regulation of dopamine terminals leads to changes in dopamine release that do not reflect cell body activity[55]; thus, it is possible that there are sex differences in CRF release that we cannot capture using this method. Second, CRF signaling in the BNST drives distinct behavioral and cellular responses to stress in male and female rats[26–28]. Thus sex differences in behavior may emerge downstream of BNST CRF neuron activity.

We previously showed that there are 2 dissociable populations of CRF neurons in the BNST that play opposing roles in fear: CRF interneurons that project to other targets in the BNST drive fear and anxiety by inhibiting anxiolytic and fear-dampening outputs to the ventral tegmental area and lateral hypothalamus. A subset of these outputs also express CRF[37,42]. Therefore, recording all BNST CRF neurons together may mask changes occurring within $BNST^{CRF}$ subpopulations. Further studies characterizing the role of these two populations in partially reinforced fear would be informative to understanding the contribution of $BNST^{CRF}$ microcircuits to sustained fear.

Overall, these findings identify that high CRF neuron tone during fear expression may contribute to the behavioral distinctions between Full and Part fear, and reveal novel sex

differences in BNST CRF function. Further, our CRF knockdown studies suggest that heightened arousal following Part fear is not driven by CRF in BNST CRF neurons. We propose that fear gives rise to a shift in the internal state of the mouse and that the internal state then influences the impact of BNST CRF neuronal signaling on defensive behaviors. Taken together, these findings highlight that understanding the distinct mechanisms that drive hyperarousal after Part fear may provide insight into the circumstances that mediate susceptibility and resilience to trauma.

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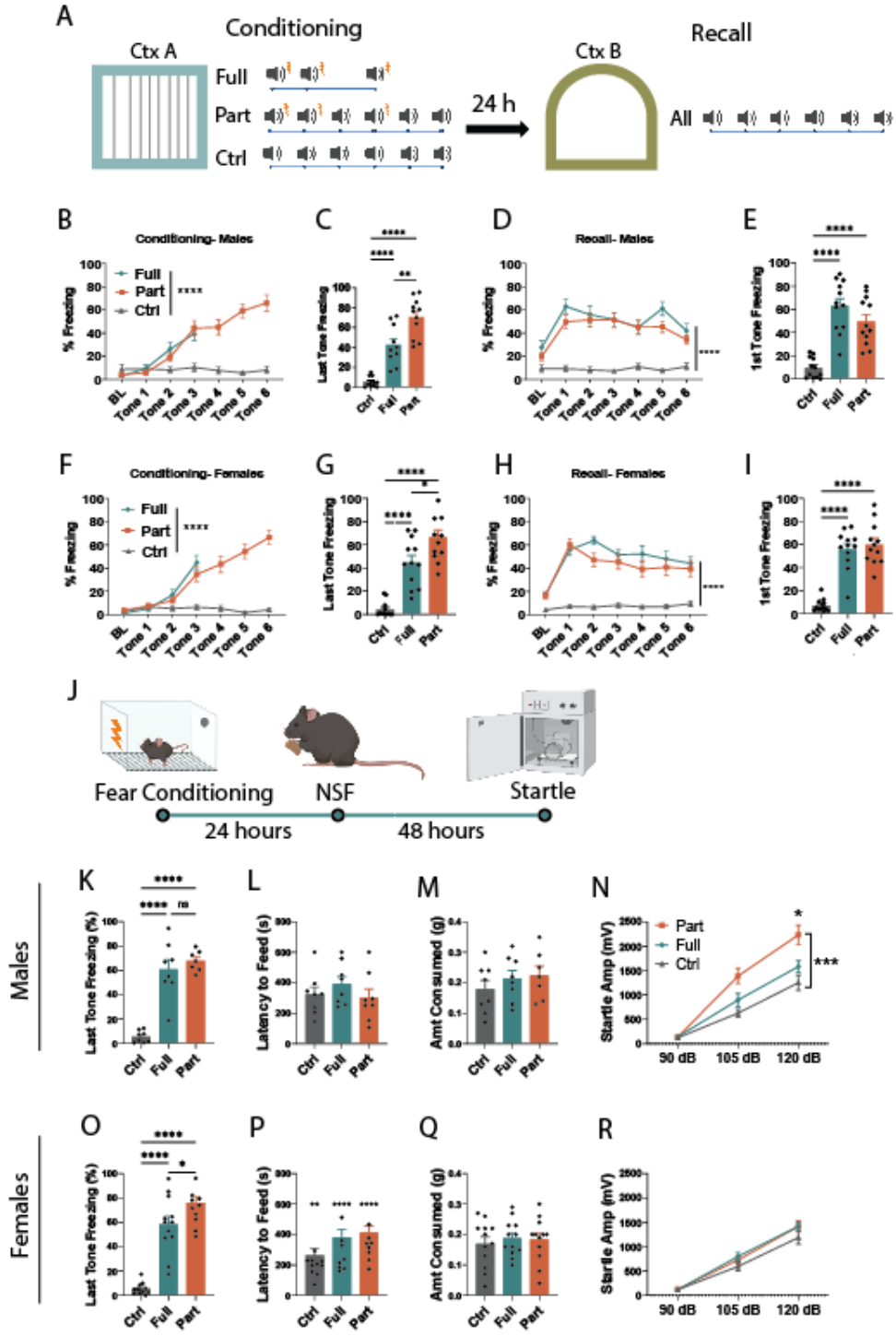

Figure 1: Partially reinforced fear drives a hyperarousal phenotype in males. A, Schematic of behavior chambers and tone/shock presentations for fully- and partially reinforced fear conditioning and recall. **B,** Freezing during fear conditioning in males (Mixed-effects model, effect of tone F(2.482, 71.99)=16.76 p<.0001, effect of fear F(2,33)=27.24 p<.0001). **C**, last tone freezing during conditioning in males (one-way ANOVA F(2,29)=44.53 p<.0001, Tukey's multiple comparisons test Full v. Part p=.0014, Full v. Ctrl p<.0001, Part v. Ctrl p<.0001). **D,** Freezing during fear recall in males (two-way RM ANOVA, main effect of tone: F(4.445, 146.7)=8.836 p<.0001, fear: F(2,33)=57.04 p<.0001, tone x fear: F(12,198)=3.121 p=.0004). **E,** Freezing during first tone of recall in males (one way ANOVA F(2,33)=28.49 p<.0001, Tukey's multiple comparisons test Full v. Ctrl p<.0001, Part v. Ctrl p<.0001). **F,** Freezing during fear conditioning in females

(Mixed-effects model, effect of tone $F(2.024, 58.70)=20.85$ $p<.0001$, effect of fear $F(2,33)=27.28$ $p<.0001$). **G**, last tone freezing during conditioning in females (one-way ANOVA $F(2,33)=39.74$ $p<.0001$, Tukey's post-hoc Full v. Part $p=.0122$, Full v. Ctrl $p<.0001$, Part v. Ctrl $p<.0001$). **H,** Freezing during fear recall in females (two-way RM ANOVA, main effect of tone: $F(4.499, 148.5)=18.16$ $p<.0001$, fear: $F(2,33)=49.20$ $p<.0001$, tone x fear: $F(12,198)=4.640$ $p<.0001$). **I,** Freezing during first tone of recall in females (one way ANOVA $F(2,33)=49.52$ $p<.0001$, Tukey's post-hoc Full v. Ctrl $p<.0001$, Part v. Ctrl $p<.0001$). **J,** behavioral timeline for anxiety-like behavior tests after fear. **K,** last tone freezing during fear conditioning in males (one-way ANOVA $F(2,20)=42.71$ $p<.0001$, Tukey's multiple comparisons test: Ctrl v. Full $p<.0001$, Ctrl v. Part $p<.0001$). **L,** Latency to feed in the novelty-induced suppression of feeding task in males. **M**, food consumed during NSF refeed test in males. **N,** acoustic startle response in males (two-way RM ANOVA, main effect of fear ($F(2,20)=11.61$ $p=.0005$, main effect of stim intensity: $F(1.808, 36.15)=166.9$ $p<.0001$, interaction: $F(4,40)=6.096$ $p=.0006$, Tukey post-hoc 120 dB Full v. Part $p=.0395$). **O**, last tone freezing during fear conditioning in females (one way ANOVA, $F(2, 33)=57.11$, Tukey's multiple comparisons test Full v. Ctrl $p<.0001$, Part v. Ctrl $p<.0001$, Full v. Part $p=.0383$). **P**, Latency to feed in the novelty-induced suppression of feeding task in females. **Q**, food consumed during NSF refeed test in females. **R**, acoustic startle response in females (two-way RM ANOVA, main effect of stim intensity $F(2,66)=223.1$ $p<.0001$). n=8-12/group.

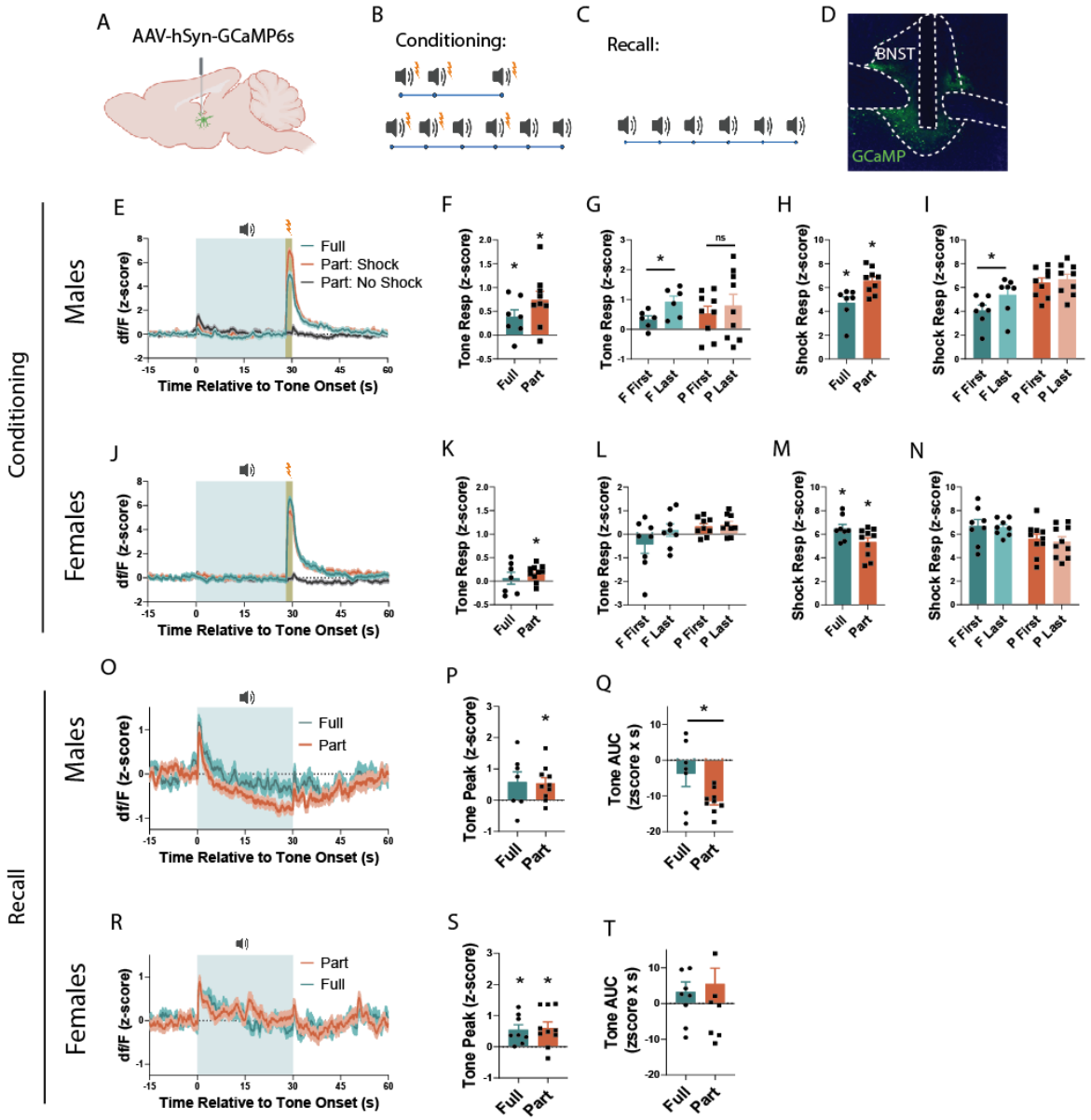

Figure 2: BNST is dynamically engaged during fear conditioning and recall. A, surgical schematic for fiber photometry experiments. **B,C** schematic of tone and shock presentations during fear conditioning and recall. **D,** BNST representative images showing fiber placement. **E,** Fear Conditioning traces in males. **F,** tone response in males averaged from t=0-2 across all trials (paired two-tailed t-tests, Full v. BL t(6)=2.504 p=.0463, Part v. BL t(8)=3.935 p=.0043). **G,** comparison of first and last tone response in males (paired two-tailed t-test: F First v. F Last t(5)=3723 p=.0137). **H,** shock response in males averaged from t=28-30 across all trials (paired two-tailed t-tests, Full v. BL t(6)=9.423 p<.0001, Part v. BL t(8)=18.36 p<.0001). **I,** comparison of first and last shock response in males (paired two-tailed t-tests, F BNST v. BL. t(6)=2.587 p=.0414). **J,** Fear Conditioning traces in females. **K,** tone response in females averaged from t=0-2 across all trials (paired two-tailed t-tests, Part v. BL t(9)=3.218 p=.0105). **L,** comparison of first and last tone response in females. **M,** shock response in females averaged from t=28-30 across all trials (paired two-tailed t-tests, Full v. BL t(7)=18.04 p<.0001, Part v. BL t(9)=13.97 p<.0001). **N,** comparison of first and last shock response in females. **O**, Fear recall traces in males. **P,** tone onset response during fear recall in males (paired two-tailed t-tests, Part v. BL t(9)=3.111 p=.0125. **Q,** area under the curve during tone presentations in males from t=5-30 (two-way RM ANOVA, Part v. Full: main effect of fear F(1,14)=5.69

p=.0409). **R,** Fear recall traces in females. **A,** tone onset response during fear recall in females (paired two-tailed t-test, Full v. BL t(7)=3.361 p=.0121, Part v. BL t(9)=3.241 p=.0101). **T,** area under the curve during tone presentations in females from t=0-30. n=7-10/group.

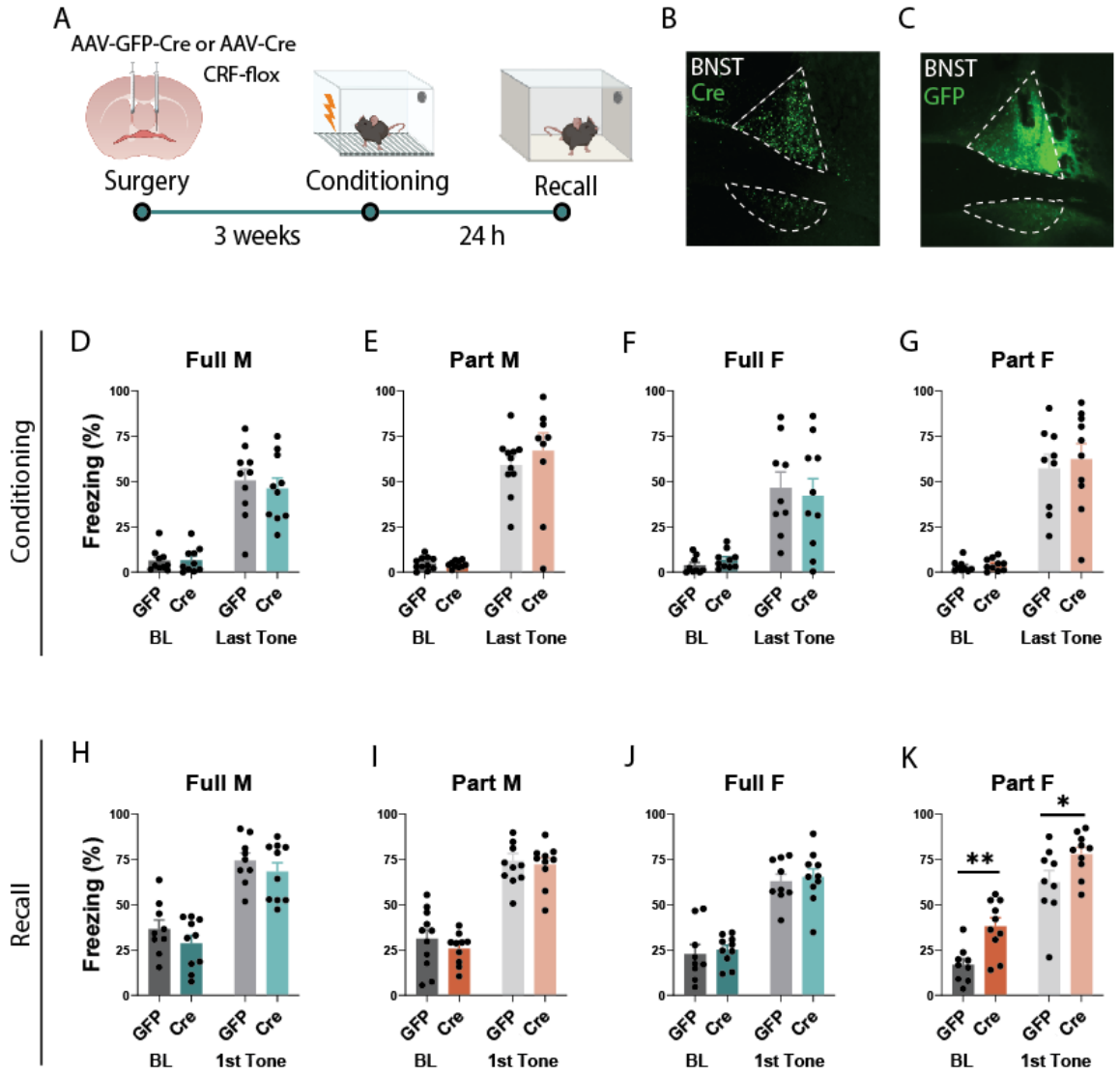

Figure 3: CRF knockdown in the BNST potentiates partial but not fully reinforced fear in females. A, Surgical schematic and timeline CRF knockdown experiments. **B,C,** representative image of cre and GFP virus expression in BNST. **D,E,** freezing during full and partial fear conditioning in males. **F,G,** freezing during full and partial fear conditioning in females. **H,I** freezing during recall for full and partial fear groups in males. **J,** Full recall in females. **K,** Part recall in females (unpaired two-tailed t-test: baseline GFP v Cre $t(17)=3.660$ $p=.0019$, first tone GFP v. Cre $t(17)=2.213$ $p=.0487$).

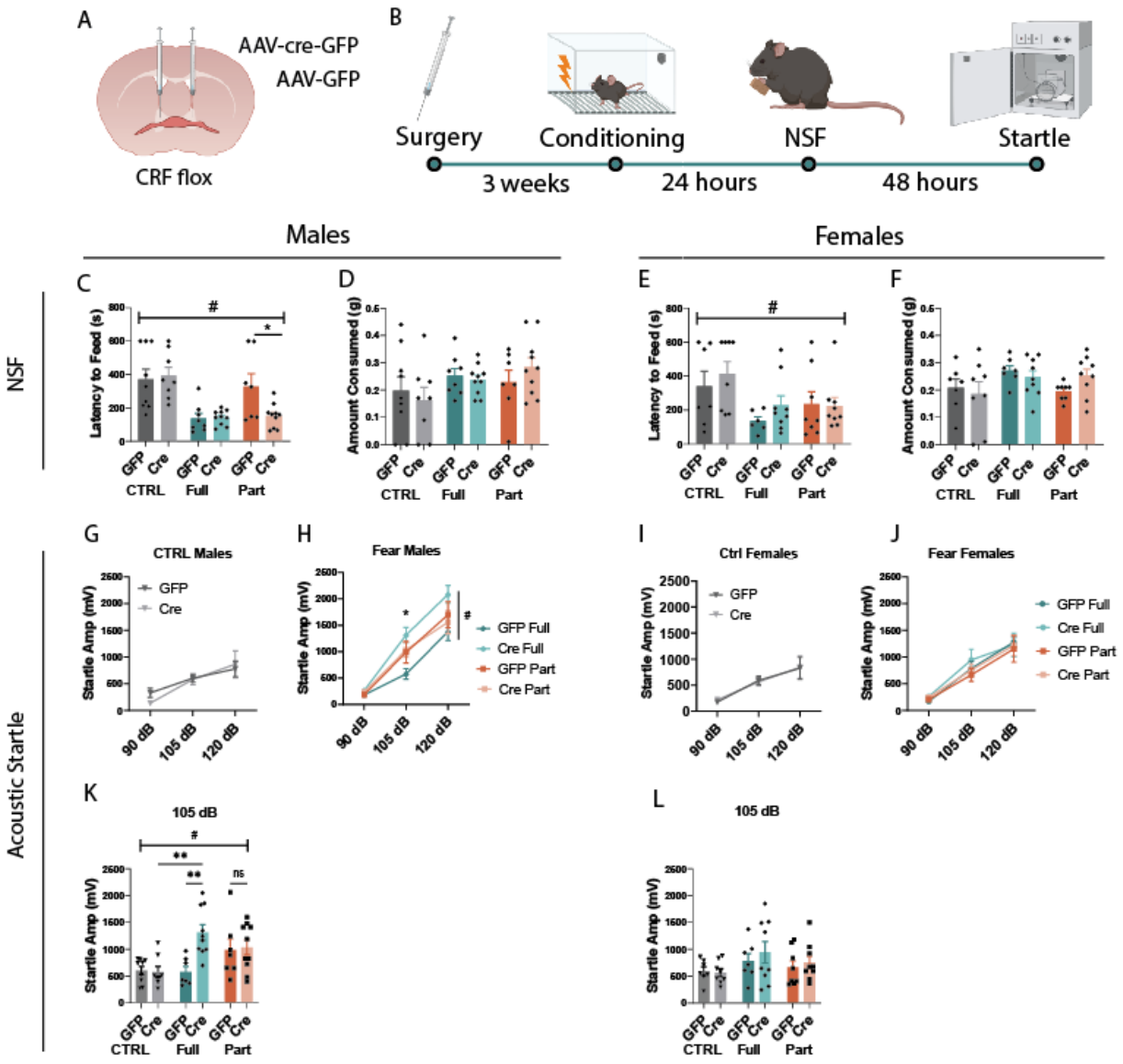

Figure 4: CRF knockdown in the BNST modulates anxiety-like behavior after fear. A, Surgical schematic. **B,** behavioral timeline. **C,** Latency to feed in the novelty-induced suppression of feeding test in males (two-way ANOVA, main effect of fear: F(2,46)=15.24 p<.0001, Fear x virus interaction F(2,46)=2.972 p<.0611, Bonferroni post-hoc GFP part v. Cre part p=.0266). **D**, amount consumed during refeed test in males. **E**, Latency to feed in the novelty-induced suppression of feeding test in females (two-way ANOVA, main effect of fear F(2,41)=4.627 p<.0154). **F,** amount consumed during refeed test in females. **G**, acoustic startle response in control males. **H**, acoustic startle response in fear males (three way anova, main effect of virus: F(1,30)=5.101 p=.0313, fear x virus interaction: F(1,30)=5.700 p=.0235,Tukey post-hoc: 105 dB Full Cre v. 105 dB Full GFP p=.0357). **I**, acoustic startle response in control females. **J**, acoustic startle response in fear females. **K,** acoustic startle response at 105dB in males (two-way ANOVA, main effect of virus: F(1,45)=5.335 p=.0256, main effect of fear: F(2,45)= 5.759 p=.0059. interaction: F(2,45)=.0119, Tukey post-hoc: Full GFP v. Full cre p=.0045, Ctrl Cre v. full cre p=.0029). **L,** acoustic startle response at 105dB in females. N=7-10/group. All data are shown as mean ± SEM. # denotes significant main effect or interaction, * denotes significant post-hoc comparison.

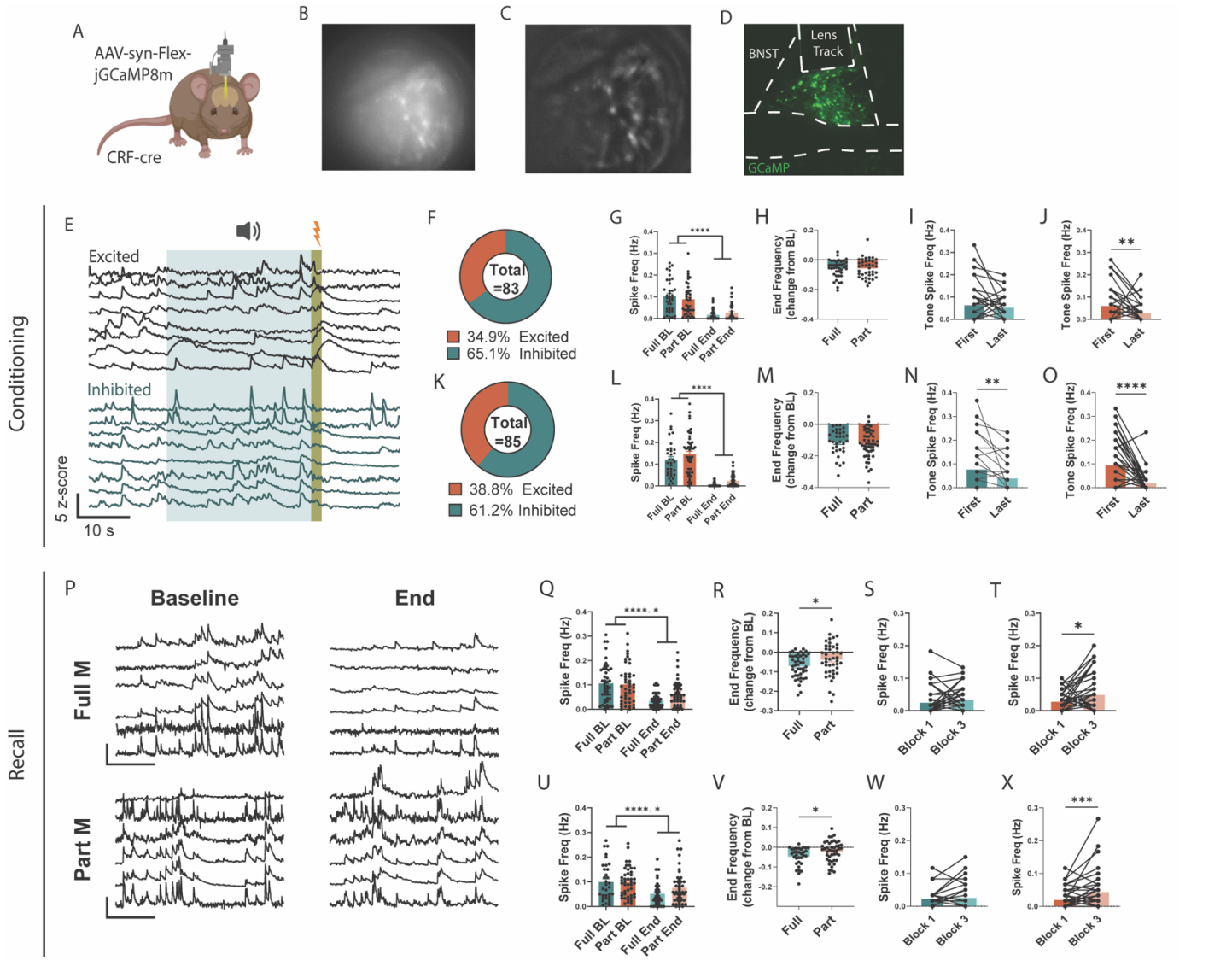

Figure 5: CRF neurons differentially encode partial fear in males and females. A, Viral strategy schematic. **B**, representative raw FOV. **C,** representative processed FOV. **D,** representative image showing GCaMP expression and GRIN lens placement. **E,** example traces of different responses to footshock. **F,** Pie charts showing the proportion of cells excited and inhibited in response to the first footshock in males, fear groups combined. **G**, Spike frequency during conditioning baseline and trial end (last 2 min of session) in males (two-way RM ANOVA main effect of epoch $F(1,76)=99.61$ $p<.0001$). Full: n=39 cells from 6 mice. Part: n=39 cells from 5 mice. **H**, Comparison of trial end spike frequency as a change from BL between full and part fear in males. **I,** spike frequency during first and last tones for Full fear in males. **J,** spike frequency during first and last tones for Part fear in males (paired two-tailed t-test $t(40)=2.270$ $p=.0096$. **K,** Pie charts showing the proportion of cells excited and inhibited in response to the first footshock in females. **L**, Spike frequency during conditioning baseline and trial end (last 2 min of session) in females (two way RM ANOVA main effect of epoch $F(1,82)=163.1$ $p<.0001$). Full: n= 33 cells from 5 mice. Part: n=51 cells from 5 mice. **M**, Comparison of trial end spike frequency as a change from BL frequency between full and part fear in females. **N,** spike frequency during first and last tones for Full fear in females (paired two-tailed t-test $t(32)=2.832$ $p=.0079$). **O,** spike frequency during first and last tones for Part fear in females (paired two-tailed t-test $t(51)=6.385$ $p=.0001$). **P**, example traces during baseline and

trial end in fear recall. **Q,** Spike frequency during baseline and trial end during recall in males (two way RM ANOVA: main effect of epoch $F(1,87)=47.88$ $p<.0001$, epoch x fear interaction $F(1,87)=4.677$ $p=.0333$). Full: n= 45 cells from 6 mice. Part: n=44 cells from 5 mice. **R,** Comparison of spike frequency during the last 2 minutes of the recall trial as a change from BL between full and part fear in males (unpaired t-test: $t(86)=2.048$ $p=.0436$). **S,** Spike frequency during first and last blocks of tone presentations during Full fear recall in males. **T**, Spike frequency during first and last blocks of tone presentations during Part fear recall in males (paired two-tailed t-test $t(44)=3.729$ $p=.0005$). **U,** Spike frequency during baseline and trial end during recall in females (two way RM ANOVA: main effect of epoch $F(1,76)=35.69$ $p<.0001$, epoch x fear interaction $F(1,76)=4.908$ $p=.0297$). Full: n=33 cells from 5 mice. Part: n=45 cells from 5 mice. **V,** Comparison of trial end spike frequency as a change from BL during recall between full and part fear in females (unpaired t-test: $t(76)=2.215$ $p=.0297$). **W,** Spike frequency during first and last blocks of tone presentations during Full fear recall in females. **X**, Spike frequency during first and last blocks of tone presentations during Part fear recall in females (paired two-tailed t-test $t(44)=3.729$ $p=.0005$).

Supplement

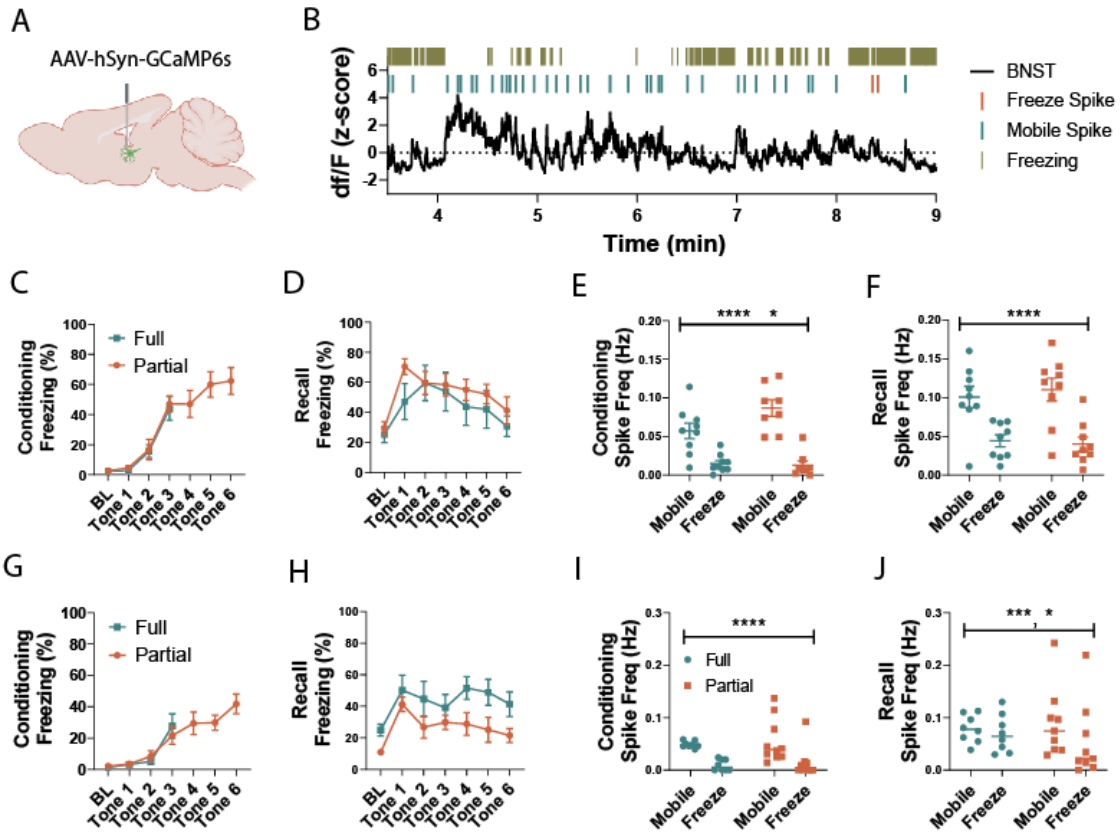

Figure S1: BNST activity negatively correlate with freezing. A schematic**. B.** spike freeze. **C-D,** freezing males. **E.** spikes freeze main effect of mobility F(1,15)=72.38 p<.0001, mobility x fear interaction F(1,15)=5.358 p<.0352. **F** spike freeze recall. Main effect mobility F(1,16)=53.70 p<.0001. **G-H.** female freezing. **I.** spikes freeze main effect of mobility F(1,16)=54.49 p<.0001. **J.** F spike freeze recall. Main effect mobility F(1,15)=17.79 p<.0007, mobility x fear F(1,15)=6.095 p=.0261

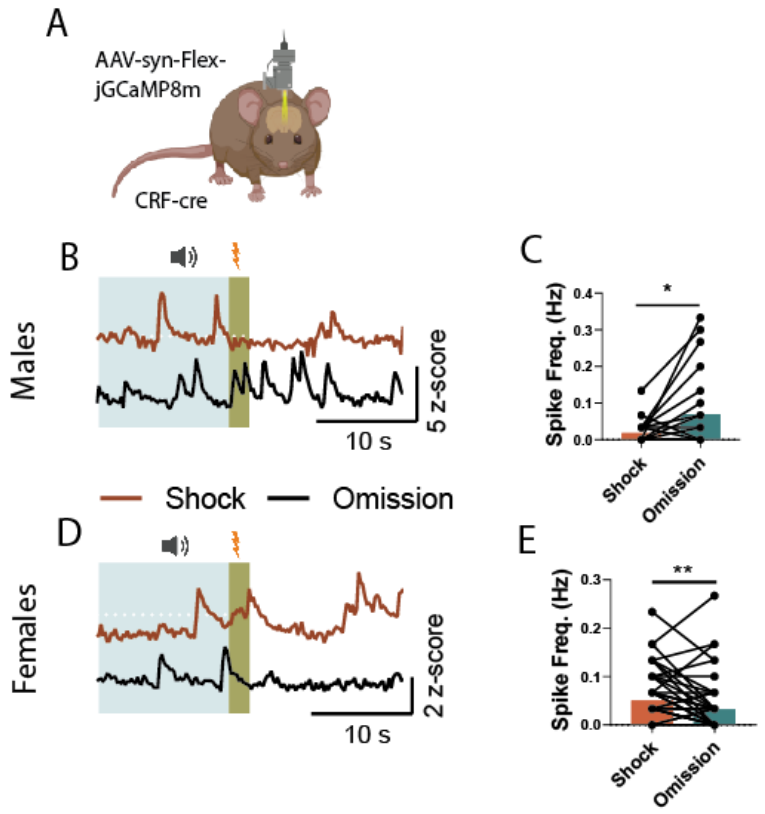

Figure S2: Sex differences in BNST^{CRF} response to shock omission. A, experimental design schematic for 1-photon recordings. **B,D,** representative traces showing a response to shock and shock omission trials for partial fear only in males and females. **C,** Spike frequency during the 10 s window post-shock or post-omission during partial fear in males (two-way RM ANOVA: main effect of shock $F(1,40)=5.790$ $p=.0208$). **E,** Spike frequency during the 10 s window post-shock or post-omission during partial fear in females (two-way RM ANOVA: main effect of shock $F(1,51)=8.022$ $p=.0066$, main effect of trial $F(2,102)=5.950$ $p=.0036$.