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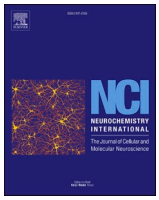

Alterations in hippocampal cholinergic dynamics following CRF infusions into the medial septum of male and female rats

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ABSTRACT

Corticoptropin releasing factor (CRF) is implicated in stress-related physiological and behavioral changes. The septohippocampal pathway regulates hippocampal-dependent mnemonic processes, which are affected in stress-related disorders, and given the abundance of CRF receptors in the medial septum (MS), this pathway is influenced by CRF. Moreover, there are sex differences in the MS sensitivity to CRF and its impact on hippocampal function. However, the mechanisms underlying these associations remain elusive. In the present study, we utilized an *in vivo* biosensor-based electrochemistry approach to examine the impact of MS CRF infusions on hippocampal cholinergic signaling dynamics in male and female rats. Our results show increased amplitudes of depolarization-evoked phasic cholinergic signals in the hippocampus following MS infusion of CRF at the 3 ng dose as compared to the infusion involving artificial cerebrospinal fluid (aCSF). Moreover, a trend for a sex × infusion interaction indicated larger cholinergic transients in females. On the contrary, intraseptal infusion of a physiologically high dose (100 ng) of CRF produced a subsequent reduction in phasic cholinergic transients in both males and females. The assessment of tonic cholinergic activity over 30 min post-infusion revealed no changes at the 3 ng CRF dose in either sex, but a significant infusion × sex interaction indicated a reduction in females at the 100 ng dose of CRF as compared to the aCSF. Taken together, our results show differential, dose-dependent modulatory effects of MS CRF on the dynamics of phasic and tonic modes of cholinergic signaling in the hippocampus of male and female rats. These cholinergic signaling modes are critical for memory encoding and maintaining arousal states, and may underlie sex differences in cognitive vulnerability to stress and stress-related psychiatric disorders.

1. Introduction

Stress is a physiological response to negative life experiences or environmental changes that could take a toll on mental health and increase the risk of developing psychiatric disorders. Cognitive impairments are a core feature of a multitude of psychiatric disorders linked to stress. Specifically, deficits in hippocampal-dependent memory are commonly observed in stress-related psychopathologies (Kim and Kim, 2023; Luine et al., 1994).

The prevalence of stress-related psychiatric disorders differs between men and women. Clinically, women are at an elevated risk of suffering from anxiety-related disorders, post-traumatic stress disorder (PTSD), and major depressive disorder, while men are more likely to suffer from substance use disorders and an earlier onset for schizophrenia (Bangasser and Valentino, 2014). This sex difference in the clinical population may be due to biological differences in how the brain responds to stress and stress hormones.

The stress response is triggered by a neuropeptide, corticotropin-releasing factor (CRF), that is released from the hypothalamus-pituitary-adrenal (HPA) axis, causing physiological, behavioral, and cognitive changes via activation of CRF receptors at the level of the pituitary and centrally in brain regions the mediate cognition (Hupalo et al., 2019). CRF-overexpressing transgenic mice exhibited impairments in spatial memory (Heinrichs et al., 1996). Moreover, studies from our group and others found attentional impairments in rats that received acute central infusions of CRF (Cole et al., 2016; Van't Veer et al., 2012).

CRF exerts neuromodulatory effects of stress on cognition via interactions with various neurotransmitter systems. The septohippocampal pathway has long been implicated in learning and memory and

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its involvement in mnemonic function and more recently has been a target for stress research. The medial septum (MS) is rich in CRF_1 receptors and is therefore well positioned to have a direct impact of CRF release (Van Pett et al., 2000). Previously, we found that micro-infusions of a high dose of CRF into the MS impaired performance of both male and female rats in the Object-Location Memory task (Wiersielis et al., 2019). However, male rats were more sensitive than females to the memory-impairing effects of a low dose of intraseptal CRF, and the female resistance to these detriments was not due to circulating ovarian hormones. The mechanisms underlying the association between CRF-mediated modulation of MS neurons and sex-dependent changes in hippocampal-dependent memory are unclear.

The septohippocampal projections from the MS cholinergic neurons to the hippocampus are known to modulate the activity in hippocampal networks. In the present study, we utilized an *in vivo* biosensor-based electrochemistry approach to examine the impact of MS infusions of a low and a high dose CRF on hippocampal cholinergic signaling in male and female rats. We hypothesized that intrseptal CRF infusions would produce dose- and sex-dependent alterations in the dynamics of acetylcholine (ACh) release in the dorsal hippocampus.

2. Materials and methods

2.1. Animals

Adult (3–4 months old) male (N = 10) and female (N = 12) Long Evans rats (Charles River, Wilmington, MA) were used. Animal were housed as same sex pairs in a temperature (23 °C) controlled environment with 12hr light/dark cycle (lights ON at 09:00 a.m.). Food and water were available *ad libitum*. A total of 26 animals were included in the study. Data from 4 animals were excluded due to either loss of biosensor sensitivity or misplacement of infusion cannula. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Temple University and were conducted in accordance with the National Institute of Healthy guidelines.

2.2. Reagents

Lyophilized ovian CRF (American Peptides, Vista, CA, USA) was reconstituted in DI water, aliquoted, and kept frozen at -20^{o} C. On day of use, CRF was diluted with sterile artificial cerebrospinal fluid (aCSF) to infuse a total dose of either 3 ng or 100 ng into the MS. Choline oxidase (ChOx) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Meta-phenylene diamine (m-PD) was obtained from Fluka Biochemika (Buchs, Switzerland).

2.3. Biosensor-based electrochemical recordings of cholinergic transmission

2.3.1. Choline biosensor microprobes

Preparation and calibration of ceramic-based choline biosensor microprobes for amperometric measurement of presynaptic ACh release is detailed in (Duggan et al., 2021). The characteristics for choline-sensitive platinum channels (n = 37) subsequently used for *in vivo* recordings were: slope/sensitivity for choline (10.15 ± 1.21 pA/μM), choline/AA selectivity (280.19 ± 103.08), linearity (R^2) for choline (0.9956 ± 0.0008), and limit of detection (0.50 ± 0.08 μM).

2.3.2. In vivo recordings and experimental design

Animals were anesthetized with urethane (1.25–1.5 g/kg; i.p.) and placed in a stereotaxic frame. ChOx-coated microprobes were lowered into the right dorsal hippocampus (from bregma; A/P: 3.84 mm, M/L: 3.0 mm, D/V: −2.5 mm) of rats using a microdrive (MO-10, Narishige International, East Meadow, NY, USA). A reference electrode (Ag/AgCl) was implanted into the rostral cortical region of the contralateral hemisphere. An infusion cannula (30ga, Braintree Scientific, Braintree, MA, USA) connected to a 10 μL Hamilton Syringe was positioned into the MS, directed towards the left ventricle at a 10-degree angle (AP: 0.4 mm; ML: 1 mm, DV: 4.9 mm from bregma). Amperometric recordings were conducted at 2 Hz by applying a fixed potential of +0.7V, and data were digitized using a FAST-16 potentiostat (Quanteon).

Illustrations depicting the experimental design summarizing the sequence of drug manipulations, and simultaneous placements of infusion cannula with the microelectrode in the target brain regions, are shown in Fig. 1A and B. Experiments began after the stabilization of baseline current (60–90 min) following which aCSF was infused into the MS at a rate of 0.1 μL/min for 5 min to assess resting (minute-based) changes in extracellular choline concentration reflective of tonic ACh release. After recording resting choline changes for 30 min post-infusion, the depolarization-evoked ACh release depicting phasic cholinergic signaling was measured. For these recordings, local pulses of sterile K+ solution (70 mM; 200 nL) were applied using a picospritzer into the dorsal hippocampus using a glass capillary that was attached to the microprobe and pulled to an internal tip diameter of 15–20 μm. The ejection volumes were monitored through a stereoscope equipped with a reticule. Next, the aCSF cannula was removed and replaced with CRF infusion cannula (loaded with either 3 ng dose or 100 ng dose) into the same location within the MS. The current was once again allowed to stabilize and the recording process was repeated with the CRF infusion. Separate cohorts of animals were used for the two doses of CRF [3 ng: N = 11 (6F,5M); 100 ng: N = 11 (6F, 5M)], and for each experimental condition, separate control (aCSF) recordings were conducted. Data from the two experimental groups were extracted from ChOx-coated channels [3 ng: N = 20 channels (10 from 6F to 10 from 5M); 100 ng: N = 21 channels (11 from 6F to 10 from 5M)] for final analysis.

2.3.3. Data processing

Current recordings in ChOx channels were self-referenced to eliminate any artifacts due to background noise levels and/or local K^+ application by subtracting currents from sentinel channels. Depolarization-evoked cholinergic transients were analyzed with respect to peak signal amplitudes and signal decay rate or clearance (T80; time required for signal to decline 80% of peak amplitude). For these signals, the amperometric recording data were binned at 0.5 s. Data are represented as the average of two signals per condition. Basal choline signal data were box-car filtered by a moving average of 20 data points. These data were binned at 2-min time intervals, starting 6 min before infusion, to examine changes occurring over the 30 min aCSF/CRF post-infusion time interval, as well as comparing to pre-infusion levels. All data collected was analyzed using infusion as a within-subjects variable due to individual variability in animal and electrode calibration.

2.4. Histology

Brains collected via rapid decapitation after each recording, were post-fixed overnight in 4% paraformaldehyde and cryoprotected in 30% sucrose. Brains were sliced on a freezing microtome to obtain coronal sections which were later Nissl stained to verify the placements of infusion cannula in the MS and microelectrode in the dorsal hippocampus. An illustration depicting coronal sections with the location and tracks of cannulae and electrodes from experimental animals are shown in Fig. 1C.

2.5. Statistical analysis

Statistical analyses were performed using SPSS/PC + version 29.0 (IBM-SPSS, Armonk, NY, USA). All data were analyzed using a mixed model analysis of variance (ANOVA) with sex as a between-subjects factor and infusion as a within-subjects factor. The two doses of CRF were analyzed separately to investigate the physiological response to each dose but not across doses. For the analysis of basal choline signal

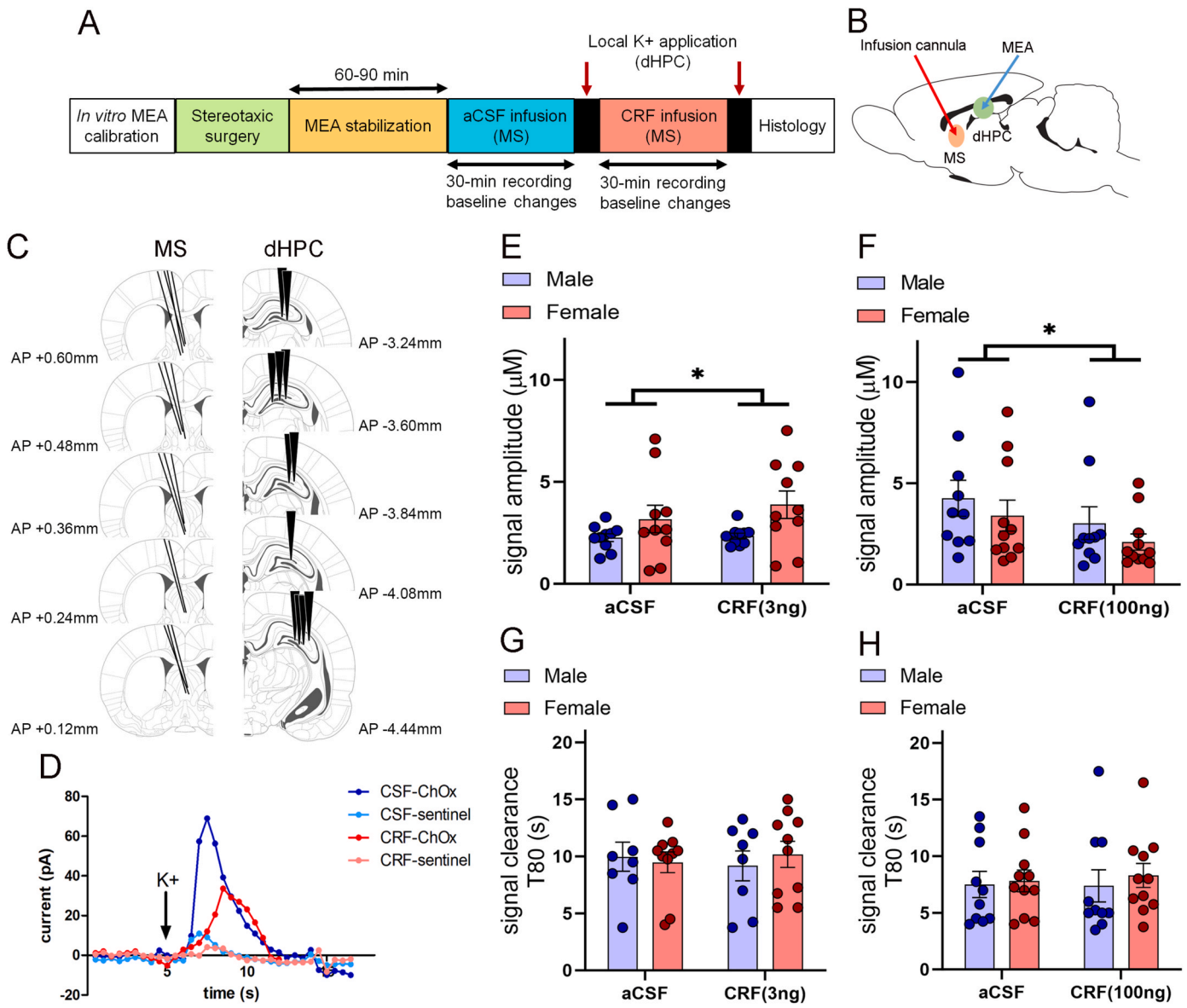

Fig. 1. Effects of intraseptal CRF infusions on phasic cholinergic signaling dynamics in the hippocampus. (A) Experimental design and timeline. (B) Sagittal brain cartoon illustrating the targeting of medial septum (MS) for aCSF/CRF infusions and simultaneous recordings from dorsal hippocampus (dHPC). (C) Schematic depicting cannula tracts in the MS and microprobe placements in the dHPC across a series of coronal sections on the rostral-caudal axis based on histological analysis. MS Plates = AP +0.60 mm to +0.12 mm; dHPC plates: AP -3.24 mm to −4.44 mm. D) Representative traces depicting choline oxidation currents from ChOx-sensitive and sentinel channels following K^+ depolarization recorded 30-min post-infusion of either aCSF or CRF (100 ng) into the MS. Increases in current reflecting phasic cholinergic transients were observed on ChOx-coated sites while sentinel channels showed only negligible changes. (E) Depolarization-evoked cholinergic signal amplitudes significantly increased after low-dose CRF (3 ng) CRF infusion as compared to the aCSF infusion. (F) On the contrary, high dose CRF (100 ng) suppressed the amplitudes of phasic cholinergic transients. (G,H) None of the CRF infusion doses affected the choline signal clearance time (T80). Data are represented as Mean ± SEM. *$p < 0.05$; main effect of infusion.

data, time was also used as a within-subjects factor. Significant main effects were further investigated with Bonferroni post-hoc pairwise comparisons. Effects were considered significant at the $p \leq .05$ level.

3. Results

3.1. Intraseptal CRF infusions altered the amplitudes but not the clearance of depolarization-evoked phasic cholinergic signals in the hippocampus

Representative traces of the oxidation current from ChOx-coated and sentinel channels following brief depolarizing pulses of K^+ in the hippocampus from a rat injected with aCSF and CRF (100 ng) into the MS are shown in Fig. 1D. An increase in currents on ChOx-coated channels reflects the oxidation of choline generated from the hydrolysis of newly released ACh from presynaptic cholinergic terminals (Duggan et al., 2021). In the low-dose study, mixed ANOVA revealed a significant increase in amplitudes of depolarization-evoked cholinergic transients in the hippocampus following CRF (3 ng) into the MS as compared to the aCSF infusion (main effect: $F(1,18) = 6.22$, $p = 0.02$; Fig. 1E). Moreover, a trending interaction of sex × infusion was also observed ($F(1,18) = 4.04$, $p = 0.06$). The posthoc tests would have been invalid here, and therefore, were not conducted. However, visual inspection of amplitudes indicates this interaction was presumably driven by higher signal amplitudes in females (3.88 ± 0.67 μM) than males (2.34 ± 0.14 μM) post-CRF (3 ng) infusion indicating that the interaction effects depicting sex differences could have been more prominent with higher sample

sizes.

Mixed model ANOVA in the high-dose study show reduced amplitudes of K^+-elicited cholinergic signals in the hippocampus recorded 30-min after CRF (100 ng) MS infusions vs. aCSF infusions (main effect: $F(1,19) = 22.442$, $p < 0.001$; Fig. 1F). A lack of sex × infusion interaction ($F(1,19) = 0.02$, $p = 0.88$) illustrates that the 100 ng CRF dose suppressed phasic ACh release comparably in both males and females. The amplitudes of cholinergic signals generated with local terminal depolarizations following aCSF infusions remained comparable between the two experimental conditions ($F(1,39) = 1.60$, $p = 0.12$). These data suggest that dose-related differences in the CRF-mediated effects on septohippocampal cholinergic transmission were not confounded by variability in ACh release under baseline conditions.

The low dose CRF (3 ng) infusion neither affected the clearance of depolarization-evoked choline (main effect of infusion: $F(1,16) = 0.01$, $p = 0.94$; Fig. 1G) nor there was a sex × infusion interaction ($F(1,16) = 1.13$, $p = 0.30$). Likewise, repeated measures ANOVA show T80 values remained comparable between high dose CRF (100 ng) and aCSF infusions (main effect $F(1,19) = 0.24$,$p = 0.63$; Fig. 1H). Moreover, the infusion × sex interaction in the high CRF dose study remained insignificant ($F(1,19) = 0.70$, $p = 0.41$). The clearance time (T80) is a functional measure of choline uptake primarily mediated by the high-affinity choline transporters CHTs that is critical to sustaining cholinergic transmission (Parikh et al., 2013). The lack of difference in the T80 measure indicates that CRF-mediated alternations in phasic ACh release are presumably not driven by CHT-mediated transport mechanisms.

3.2. *Intraseptal CRF infusions and tonic changes in hippocampal cholinergic transmission*

To assess the effects of acute MS CRF infusion on slow (minute-based) tonic cholinergic changes in the hippocampus, changes in basal extracellular choline signals were compared following aCSF and CRF infusions with respect to pre-infusion signals. Data was analyzed using a mixed design repeated measures ANOVA with time and infusion as within-subjects factors and sex as between-subjects factors. At the 3 ng dose, an infusion by time × sex interaction was observed ($F(1,17) = 4.05$, $p < 0.001$). In order to investigate the source of these interactions, male and female data were analyzed separately and pairwise comparisons at each time point were conducted between aCSF and CRF infusion. However, these comparisons did not reveal significant differences at any time point in either male or female animals (Fig. 2A and B). It is possible that a high variability in data between recordings and trending drift in basal choline levels due to reduced microelectrode biosensing efficacy over time might have contributed to these results. For the 100 ng CRF

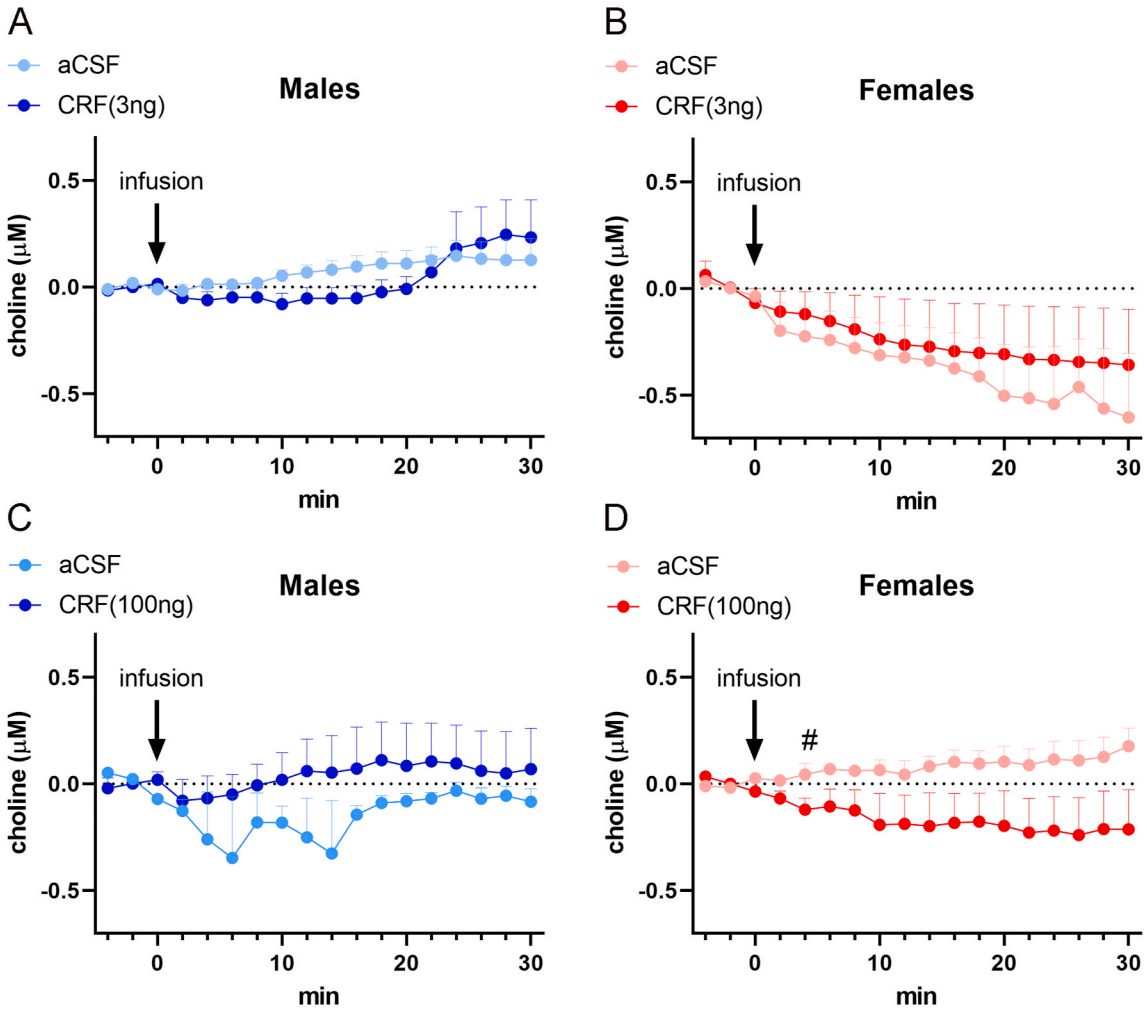

Fig. 2. Effects of intraseptal CRF infusions on tonic cholinergic signaling in the hippocampus. Currents expressed as changes in extracellular choline levels (μM) in the dorsal hippocampus from pre-infusion baseline with infusions into the medial septum starting at time 0. Basal (tonic) choline signal alterations in male rats (A) and female rats (B) with intraseptal infusions of aCSF and low-dose CRF (3 ng) over 30 min. Tonic cholinergic signals remained unaffected with high-dose CRF (100 ng) in males (C) but declined in females with a significant reduction noted at the 4-min time point post-CRF infusion (D). Data are represented as Mean ± SEM. #$p < 0.05$; pairwise comparison.

infusion animals, a significant infusion × sex ($F(1,8) = 5.20$, $p = 0.05$), and a significant time × infusion × sex ($F(1,17) = 1.80$, $p = 0.03$) interaction, was observed. To determine whether these interactions reflect CRF-induced changes in the temporal characteristics of tonic cholinergic activity, posthoc tests were conducted separately on data from extracellular choline levels in males and females. Pairwise comparisons did not show significant differences in males at any timepoint (Fig. 2C). However, at the 4-min time point, female animals exhibited significantly lower extracellular choline signal levels following the CRF infusion as compared to the aCSF infusion ($p = 0.04$; Fig. 2D). Although these data suggest that 100 ng CRF may produce time-dependent changes in tonic ACh release only in the females, it is still plausible that this difference is just an anomaly. The sex-specific neuromodulatory effects of the high dose of CRF may be more reflective of cumulative changes in tonic ACh release over the 30-min time period.

4. Discussion

Here, we investigated the neuromodulatory influence of the stress-related peptide CRF on septohippocampal cholinergic circuits. To target these circuits, we directly infused CRF into the MS and concurrently recorded cholinergic signaling dynamics from the dorsal hippocampus using choline biosensor microprobes. While infusion of a physiologically high dose of CRF led to a subsequent reduction in depolarization-evoked phasic cholinergic transients in the hippocampus of both males and females, higher signal amplitudes were observed with the lower CRF dose and this effect was more pronounced in the females. In contrast, tonic cholinergic activity remain unaffected with the lower dose of CRF, but transiently reduced in the females at the higher CRF dose. Given the evidence that fluctuations in ACh within the hippocampal networks on both phasic and tonic timescales orchestrate different cognitive operations including memory encoding and transition between vigilance states (Teles-Grilo Ruivo et al., 2017), our findings have functional implications that may explain sex differences in cognitive vulnerability to stress.

CRF receptors are present in the MS (Van Pett et al., 2000; Wiersielis et al., 2019). Given the sparse population of local CRF cell bodies within the MS (Hupalo et al., 2019), the sources of CRF projections to the MS, such as the paraventricular nucleus of the hypothalamus (Jiang et al., 2019), may be the primary drivers of changes in septohippocampal function following stress. Within the MS, CRF_1 receptors are predominantly localized on cholinergic neurons (Sauvage and Steckler, 2001) which may explain the stimulatory effects of low-dose CRF on phasic cholinergic signals in the hippocampus. It should be noted that K^+ depolarization only stimulate presynaptic cholinergic terminals. Because the low-dose CRF did not impact CHT-mediated clearance mechanisms, increases in the amplitudes of cholinergic transients may plausibly be driven by augmented releasable vesicular pools of ACh due to sustained activation of MS cholinergic neurons. The suppression of cholinergic transients at the higher dose of CRF remains an intriguing observation and could plausibly be linked to GABA-mediated inhibition. Indeed, the presence of CRF_1 receptors have also been observed at the non-cholinergic, presumably GABAergic neurons in the MS that also project to the hippocampus (Sauvage and Steckler, 2001). Likewise, any changes in CRF-mediated changes in tonic cholinergic activity likely remained overridden by the activation of MS GABAergic neurons. Thus, CRF modulation of MS neurons and its downstream effects on hippocampal cholinergic signaling may likely involve an interplay between cholinergic and GABAergic neurons.

An interesting observation of our study was the neuromodulatory effects of MS CRF on phasic and tonic cholinergic signaling in the hippocampus differ between males and females. Although, these results are preliminary given the low sample sizes and further studies are warranted to confirm sex-specific effects, these are important observations. Previously, we examined CRF_1 receptor mRNA levels in the MS and found no sex differences (Wiersielis et al., 2019). However, our attempts to detect CRF_1 protein levels in the MS of rat brain either using immunohistochemistry or Western blotting with the commercially available antibodies were not successful due to issues with sensitivity and/or specificity to rat CRF_1 receptors. Thus, it remains to be determined whether protein levels are different. Howerton et al. (2014) reported a blunted neural excitability response to CRF_1 antagonist in the dorsal raphae of the female but not male mice despite the absence of sex differences in the expression of these receptors (Howerton et al., 2014). This effect could be linked to sex differences in CRF_1 signaling, because in the locus coeruleus, there are sex differences in CRF_1 signaling that drive sex differences in neuronal excitability (Bangasser et al., 2010). Beyond changes in receptor sensitivity, we previously found elevated levels of CRF-binding protein (CRF-BP), a protein that is known to limit the bioavailability of CRF, in the MS of female rats as compared to the males (Wiersielis et al., 2019). Therefore, the low-dose CRF sex difference trend in phasic cholinergic transients may plausibly be associated with females' resistance to CRF effects on MS GABAergic neurons either due to higher CRF-BP or blunted CRF_1 receptor signaling, keeping the cholinergic activation unopposed. On the other hand, diminished hippocampal cholinergic transients with the higher CRF dose in both males and females may be a consequence of a more balanced modulation of MS cholinergic and GABAergic neurons. These interpretations align with a previous study that reported deficits in spatial reference memory only in the males but not females with the intraseptal infusion of low-dose CRF, while the high-dose CRF impaired performance in both sexes.

While the mechanistic basis underlying sex differences in tonic cholinergic transmission following high-dose of CRF remains unclear, altered distribution and responsivity of CRF_1 receptors in the MS of females as a plausible cause of these differences could not be dismissed. Because tonic cholinergic signaling has shown to be associated with arousal (Teles-Grilo Ruivo et al., 2017), our findings may have functional relevance to understand the differential impact of stress-related arousal on memory encoding in males and females. Further research is warranted to tease apart the contributions of MS cholinergic and GABAergic neurons to CRF-mediated alterations in septohippocampal function and whether sex differences in these alterations involve translational/post-translation changes in the CRF_1 receptors or their downstream signaling pathways.

CRediT authorship contribution statement

Alyssa Kniffin: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Miranda Targum:** Investigation, Data curation. **Aryan Patel:** Investigation, Data curation. **Debra A. Bangasser:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Vinay Parikh:** Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that the study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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