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--Corticotropin releasing factor in the nucleus basalis of Meynert impairs attentional performance and reduces levels of glutamate and taurine in male and female rats

--Manuscript Draft--

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Abstract:	Psychiatric disorders that are characterized by impairments in sustained attention, including attention deficit hyperactivity disorder (ADHD), post-traumatic stress disorder (PTSD), and major depression are also sensitive to exacerbation by stress. Sustained attention relies on cholinergic and non-cholinergic projections from the nucleus basalis of Meynert (NBM) in the basal forebrain to the medial prefrontal cortex (mPFC). We have previously shown that central administration of the stress neuropeptide corticotropin releasing factor (CRF) impairs performance on the sustained attention task (SAT) in adult male and female rats. The present study investigated whether this effect was mediated by CRF's action in the NBM. Rats were administered CRF in the NBM and subsequent SAT performance was measured. A high dose of CRF (100ng) significantly impaired performance on non-signal trials across sex. Because non-signal trial performance is believed to depend on non-cholinergic (i.e., GABA and glutamate) signaling, high performance liquid chromatography was used to quantify amino acid levels in the NBM and mPFC. We found females have higher levels of glutamate, glutamine, GABA glycine, and alanine in the NBM than males. Importantly, CRF in the NBM led to a local decrease of taurine and several amino acids involved in glutamate synthesis in males and females, changes which may mediate the CRF-induced SAT performance deficit. Together these studies suggest that CRF regulation of amino acids in the NMB contributes to stress-induced attention deficits.

College of Liberal Arts
TEMPLE UNIVERSITY

DEPARTMENT OF PSYCHOLOGY AND NEUROSCIENCE PROGRAM

Dear Editors,

We are pleased to submit this Research Report entitled "Corticotropin releasing factor in the nucleus basalis of Meynert impairs attentional performance and reduces levels of glutamate and taurine in male and female rats" to *Neuropharmacology*.

Deficits in sustained attention characterize several psychiatric disorders, including attention deficit hyperactivity disorder and major depression. Stress can exacerbate attention deficits but the mechanisms by which this occurs are largely unknown. Here we demonstrate that the stress neuropeptide corticotropin releasing factor (CRF) in the nucleus basalis of Meynert (NBM), a region critical for sustained attention, disrupts aspects of performance in a sustained attention task in both male and female rats. The aspect of sustained attention performance affected is mediated by non-cholinergic (i.e., GABAergic and/or glutamatergic neurons) in the NBM. Thus, we evaluated how CRF in the NBM affected amino acid levels. CRF in the NBM decreased several amino acids involved in glutamate synthesis. This manipulation also reduced taurine, an amino acid associated with cognition and attention. Together these studies suggest that CRF regulation of amino acids in the NBM contributes to stress-induced attention deficits.

Thank you for considering this submission.

Sincerely,

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Dear Dr. Young,

The authors would like to thank the editors of the *Neuropharmacology* and the reviewers for taking the time to consider our manuscript for publication. We express our sincere thanks to the reviewers who were excited about our novel findings. We also thank them for identifying areas of our manuscript that needed clarification or modification. The revised manuscript has benefited from these insightful suggestions, and we have included additional analyses, which we believe strengthen the paper. Specific responses to the helpful comments are below. Changes in the manuscript are indicated with blue text.

Reviewer 1 comments and responses:

1. No Highlights are provided.
   - We have added highlights

2. Throughout the manuscript, the citation brackets are closely linked with the word preceding them. Please aim to correct this proofreading minor, but important, detail.
   - This has been fixed.

3. The Abstract is nicely written and clear. However, you may want to specific whether the rats used were male, female or both and whether there were differences in the performances and neurochemical effects between the two.
   - Thank you for the suggestion, these details have been added.

4. In the Introduction, it is unclear why the focus of this study was on the amino acid levels in the NBM and mPFC and not on cholinergic transients as well.
   - Our behavioral data indicated that CRF in the NBM was mediating performance measures mediated by amino acid neurotransmitters, not acetylcholine. We have clarified in the last paragraph of the introduction (p. 3) that this was the rationale for using HPLC to follow up.

5. The approval number for the procedures conducted on animals should be added in section 2.1.
   - This has been added.

6. Clarification should be given on why the infralimbic and prelimbic areas were combined for the HPLC-ED analysis study, although it was identified that they do play dissociable roles in response inhibition in rodents.

- We have rephrased the rationale (end of Section 2.2, p.5). For these studies we combined the infralimbic (IL) and prelimbic (PL) regions for technical reasons as very small tissue samples are challenging to analyze with HPLC-ED. Also, both substructures are known to be involved in inter-related supervisory attentional functions and to our knowledge, IL and PL distinctions have not been identified with SAT.

7. The font style and size in the Reference list should be corrected; currently it is not consistent in all references listed.

- This has been fixed.

8. The quality of Figures 1,3, 4 should be improved. It my just be a matter of increasing the size of each graph, but as they stand, it is somehow difficult to clearly follow their details.

- We redid the figures using vector graphics so they should be high quality (at any size). The journal does generate a pdf automatically which may affect the resolution but the clarity for the figures submitted is improved (at least in the format we submitted them).

Reviewer 2 comments and responses:

1. Some aspects of the experimental approach for the behavioral testing are unclear as written in the main text and many important details can only be found in Supplemental Methods. For example, how the vigilance index is calculated is missing in the main text and should be included as this is a major experimental readout of the study. In addition, as the SAT has many important components (signaled vs non-signaled, hits vs correct rejections vs false alarms, omissions, variable duration of signaled events) it might be useful to have a schematic or table summarizes the various conditions and potential outcomes.

- We initially had a lot of methodological details in the manuscript but had to move many to supplemental because of journal word limitations. We have put methods back because we agree that it helps with readability. Specifically, details about vigilance index and acquisition criteria are now in the main document. It was a great suggestion to have a schematic of the SAT outcomes and we have added a new Fig. 1 to illustrate the types of responses in SAT.

2. The data illustrated in Figure 1 and Figure 2 are also missing some information that could aid in the communication and interpretation of the findings. For example, it would be helpful to have the individual readouts for the vigilance index communicated. Hits is shown in Fig 2B for the different CRF doses, but not for the different signal durations. False alarms are not shown anywhere and none of the data in Figure 2 is shown for different signal durations. It is understandable that communicated every parameter would be cumbersome, but it would be worthwhile to illustrate additional data for some of the significant effects.

- Typically, people analyze and present response accuracies on signal and non-signal trials in SAT (i.e., hits and correct rejections). We stuck with convention and analyzed correct rejections, which is the inverse of false alarms. We hope that in response to above comment and through additional text, we have clarified outcome measures, including false alarms: text was added to the methods Section 2.3 (p. 3-4), discussion Section 4.1 (p.10), and we added Fig.1. In the initial submission we did make figures to display the significant results, but we agree with the reviewer that it is useful to show hits at the different signal durations, even though that analysis did not reveal any significant effects. We have added these results to the results to Section 3.1 (p. 6) and provided figures in Supplementary Materials (Figure 2).

3. The supplemental results pertaining to data depicted I Figure 2B should also be moved to main text as it is relevant to the main findings of the study.

- We moved the statistics for CRF effect on hits and omissions into the main text but had to leave some statistical results in supplemental due to space limitations.

4. Additional detail and consideration of the role of sex differences in amino acid levels in the changes observed with CRF should be included in the communication and discussion of the results. Some of the statements made in the communication of the results, while statistically accurate, do not accurately reflect the results overall. For example, the authors report that CRF significantly reduced glutamate, glutamine and taurine but the decreases are much larger in magnitude in the females, which is not discussed. The authors do report that they did not find any sex x CRF interactions, which, while accurately reflecting the statistical tests does obscure the clear observation that many of the effects of CRF appear to be primarily driven by effects in females. In addition, there may be a decrease in GABA levels in females that, were it analyzed separately from males might be significant.

- As noted, we did not have any significant interactions which means that post hoc analyses were not warranted. We also did not have any *a priori* predictions about sex differences in the magnitude of CRF's effect on amino acids, given the behavioral results were similar across sex, so planned comparisons were not justified. That said, we now acknowledge that with more power we may have been able to detect an interaction and larger effects for glutamate, glutamine, and taurine in females than males (noted at the end of results section 3.2, p.9). To address the reviewer's (and our own) curiosity, we did conduct a t-test for only females for GABA and it did not reach significance [$t(14) = 1.668$, $p = .118$].

5. The authors should also be consistent with their statistics: for the behavioral tests, the authors mention mixed factors ANOVA (implying a 2x3 with sex as between subjects and CRF dose as within subjects) but with HPLC studies the authors say "two by two ANOVA". This could be clarified by using staying consistent with terminology and clarifying the rationale for each test used.

- Thank you for pointing this out. We have clarified the type of ANOVA (mixed factors and 2-way) and detailed the levels of each factor and whether they are between- or within- subjects. Please see changes in the early paragraphs of Sections 3.1 and 3.2.

6. It is also unfortunate that the behavior and amino acid analysis was not performed in the same subjects, as that would strengthen the integration of the findings. It is understandable that the Latin-

square design precluded consistent tissue collection between groups but it is not clear why the cohort ran for amino acid analysis was not run through SAT testing at the single effective CRF dose? The authors acknowledge that testing could impact activity (and potentially amino acid levels) but they should add additional consideration of this and potentially include the food restriction used for behavioral testing which can also impact neuronal activity, notably in the PFC.

- The SAT task takes around 60 days to run and with the pandemic we were limited in what we could accomplish. We have added the point that food restriction used for behavioral testing could also impact amino acid levels in the first paragraph of Section 4.2 (p.12).

7. The authors present important effects of CRF on parallel measures of behavior and amino acid levels and provide a detailed discussion for how these effects may be occurring, however there is no discussion of the mechanism by which CRF could be having these effects. Specifically the mechanism by which CRF could interfere with glutamate synthesis or taurine levels is ignored. CRF-induced changes in signaling? Receptor interactions? More speculation on this in the discussion would strengthen the communication of the overall findings and their relevance in the broader context of CRF actions.

- This was a great suggestion and we're happy the reviewer pointed out this oversight. For glutamate, there is evidence that CRF can alter glutamatergic pathway synthetic enzymes in the inner hair cells of the cochlea (Graham et al., 2011) that we cite on Section 4.2, p. 12. We agree with the reviewer's suggestion re. signaling as a likely explanation for taurine (Section 4.3, p. 12-13)

Minor Comment
- There is an error in the Results section 3.2 where Fig 3D is incorrectly referred to as 2D. This should be corrected.

- We added figures so then numbers changed but have ensured that that now Fig. 4D is properly referenced.

Highlights

- CRF in the NBM impaired sustained attention
- CRF in the NBM decreased NBM levels of amino acids involved in glutamate synthesis
- CRF in the NBM decreased NBM taurine levels
- There are baseline sex differences in mPFC and NBM amino acid levels
- These data suggest CRF disrupts attention by suppressing glutamate and taurine

Corticotropin releasing factor in the nucleus basalis of Meynert impairs attentional performance and reduces levels of glutamate and taurine in male and female rats

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Abstract

Psychiatric disorders that are characterized by impairments in sustained attention, including attention deficit hyperactivity disorder (ADHD), post-traumatic stress disorder (PTSD), and major depression are also sensitive to exacerbation by stress. Sustained attention relies on cholinergic and non-cholinergic projections from the nucleus basalis of Meynert (NBM) in the basal forebrain to the medial prefrontal cortex (mPFC). We have previously shown that central administration of the stress neuropeptide corticotropin releasing factor (CRF) impairs performance on the sustained attention task (SAT) in adult male and female rats. The present study investigated whether this effect was mediated by CRF's action in the NBM. Rats were administered CRF in the NBM and subsequent SAT performance was measured. A high dose of CRF (100ng) significantly impaired performance on non-signal trials across sex. Because non-signal trial performance is believed to depend on non-cholinergic (i.e., GABA and glutamate) signaling, high performance liquid chromatography was used to quantify amino acid levels in the NBM and mPFC. We found females have higher levels of glutamate, glutamine, GABA glycine, and alanine in the NBM than males. Importantly, CRF in the NBM led to a local decrease of taurine and several amino acids involved in glutamate synthesis in males and females, changes which may mediate the CRF-induced SAT performance deficit. Together these studies suggest that CRF regulation of amino acids in the NMB contributes to stress-induced attention deficits.

1. Introduction

Stress can exacerbate psychiatric disorders with symptoms of attentional impairments, including attention-deficit/hyperactivity disorder (ADHD), major depression, and post-traumatic stress disorder (PTSD) (Paelecke-Habermann et al., 2005; Vasterling et al., 1998). One aspect of attention that can be disrupted by stress is sustained attention, the ability to continuously monitor a situation for intermittent or unpredictable events (Hancock, 1989). Sustained attention is a fundamental cognitive process that subserves other forms of attention including selective attention and divided attention, as well as general cognitive ability (Sarter et al., 2001). Thus, when sustained attention is impaired, many aspects of cognition are negatively impacted. Despite well-documented connections between stress and attention deficits, the mechanisms by which stress alters sustained attention are not well understood.

One translational task used to assess attentional capacities in both rodents and humans is the sustained attention task (SAT) (Demeter et al., 2008; McGaughy et al., 1996; Nuechterlein et al., 2009). In the rodent version, rats are trained to distinguish between signaled and non-signaled events by pressing distinct levers in an operant chamber (McGaughy et al., 1996). Accurate detection of signaled events depends on the release of brief cholinergic transients in the medial prefrontal cortex (mPFC) from neurons that originate in the nucleus basalis of Meynert (NBM) of the basal forebrain (Gritton et al., 2016; McGaughy et al., 1996; Parikh et al., 2007). The NBM also includes GABAergic and glutamatergic neurons (Fadel et al.; Zaborszky et al., 1999), which contribute to performance on non-signaled events (Burk and Sarter, 2001). Thus, the NBM is a crucial region for attention with different cell types contributing to different aspects of SAT performance.

One regulator of the stress response is corticotropin releasing factor (CRF). During a stressful event, the central release of CRF can modulate cognitive processes (Bangasser and Kawasumi, 2015; Hupalo et al., 2019). CRF has been documented to impair attention in a dose-dependent manner (Cole et al., 2016; Van't Veer et al., 2012). Previously, central administration of CRF caused a comparable decrease in performance in male and female rats on both signaled and non-signaled events (Cole et al., 2016). As noted, these aspects of SAT performance are mediated by NBM cholinergic and non-cholinergic neurons, respectively. CRF receptors are present on cholinergic and non-cholinergic neurons in the NBM (Sauvage and Steckler, 2001), suggesting this region may mediate the effects of central CRF administration on both aspects of SAT performance.

Here we tested the idea that CRF can impair sustained attention via direct regulation of the NBM. Our behavioral data indicated that CRF impaired performance measures in SAT that are mediated by amino acid neurotransmitters in the NBM. Thus, we next used high performance liquid chromatography (HPLC-ED) to determine if CRF in the NBM altered amino acid levels within the NBM and its target region, the mPFC.

2. **Methods and Materials**

2.1 Subjects

All procedures were conducted with approval from the Temple University Institutional Animal Care and Use Committee under protocol ACUP 4791 and were consistent with NIH guidelines. Adult (>70 days) male and female (sex determined by genitalia) Sprague Dawley rats (Charles River, Wilmington, MA) were used in all experiments. For more details on housing see Supplementary Materials (SM).

2.2 Surgery and microinfusions

Rats underwent aseptic stereotaxic surgery to implant guide cannula bilaterally in the NBM (as detailed in SM). Microinfusions were used to administer three different CRF doses—30ng, 100ng, or vehicle (aCSF) (see SM for more details). This experiment followed a repeated measures design with each animal receiving all 3 doses in a counterbalanced fashion with a minimum one-week washout period between doses as previously described(Cole et al., 2016). Animals began SAT 10min after infusion. Following completion of all 3 doses, animals were sacrificed via transcardial perfusion and brains were collected and sectioned to confirm accurate cannula placement.

Tissue from rats used in the SAT experiment was collected following one of the three infusion doses. However, because CRF doses for the SAT experiment were given using a counterbalanced within subjects design, we were underpowered to assess molecular changes in the NBM following behavior manipulations. Thus, we generated a separate set of animals for the HPLC-ED study. We compared vehicle-infused rats to the 100ng CRF dose because this dose had the most pronounced effect on SAT performance (see results). Each animal in the HPLC-ED study received one infusion of either 100ng CRF or vehicle 20 min prior to being sacrificed via rapid decapitation. The 20 min timepoint was chosen to be consistent with prior studies (Dalla et al., 2008; Kokras et al., 2020) and because the effect of CRF in the NBM on SAT performance analyzed across 3 blocks of trials (data not presented) was consistent throughout the entire 45 min session. NBM cannula placement was assessed during dissection and bilateral NBM and mPFC were collected, weighed, and immediately frozen on dry ice to await HPLC-ED testing.

The mPFC samples used for HPLC-ED analysis in the present study included infralimbic and prelimbic areas combined to ensure an optimal sample size for processing and because both substructures are involved in inter-related supervisory attentional functions (Dalley et al., 2004) and no functional distinction of these structures in SAT is known. Although in other tasks, the prelimbic and infralimbic areas of the mPFC play dissociable roles in response inhibition and behavioral flexibility in rodents (Oualian and Gisquet-Verrier, 2010; Seamans et al., 1995).

2.3 SAT

Rats (n=14 males, n=9 females) were trained in touchscreen SAT as described previously (Bangasser et al., 2017; Wicks et al., 2017). Rats were food restricted to 85% of their free feeding weight for the SAT task but given water *ad libitum*. After learning to nose poke for a food reward (45mg Precision Pellet, Bio-Serv), rats were trained to discriminate between signaled and non-signaled events. During signaled events, a light of varying durations (25ms, 50ms, 500ms) appeared at the top of the screen and rats nose poked on one side of the screen to earn a food pellet as reward. During non-signaled events, no light appeared, and rats responded to the absence of the light by nose poking on the opposite side of the screen to earn a food pellet. The locations of signaled versus non-signaled trial response areas were counterbalanced between rats. Correct responses on signaled events are called "hits", while incorrect responses are called "misses". Correct responses on non-signaled events are called "correct rejections", while incorrect responses are called "false alarms" (Fig. 1). Incorrect responses were not rewarded. We analyzed correct responses on SAT for data interpretation and presentation. We also calculated vigilance index, which considers hits (averaged over all signal durations) and false alarms and

reflects overall attentional performance [vigilance index=(hits–false alarms)/[2(hits+false alarms)−(hits+false alarms)^2] (McGaughy et al., 1996). In addition, vigilance index was also computed for each signal duration. Omissions were counted if rats did not nose poke in either response area during a trial. Finally, to ensure effects were not driven by a side bias in nose poking we calculated a side bias index: (hits+false alarms)/(hits+false alarms+misses+correct rejections). After reaching acquisition criteria (>70% hits on 500ms signal duration trials, >70% correct rejections, <20% omissions for 3 consecutive days), animals underwent NBM cannulation surgery as described above. Once recovered from surgery, rats were re-trained on SAT until reaching acquisition criteria again for 3 consecutive days before receiving any microinfusions.

2.4 Amino acid analysis

NBM (bilateral punches combined) and mPFC (n=6-9 per group included in analysis) were weighed and homogenized in chilled 0.1N HCLO4 through sonification, then centrifuged at 22000×*g* for 45min at 4^o C and the collected supernatants were used for neurochemical assays with HPLC-ED. Measurements were performed as previously described and detailed in Supplemental Methods (Kokras et al., 2018). Quantification of glutamate, serine, glycine, glutamine, alanine, taurine and γ-aminobutyric acid (GABA) was done using Clarity v.8.1 (DataApex, Czech Republic) by comparison of the area under the curve with that of reference external standards, as previously reported (Kokras et al., 2018). Ratios of Glutamine/Glutamate and GABA/Glutamate were also calculated, as previously described (Kokras et al., 2020)

3. Results

3.1 CRF in the NBM impairs SAT performance

Cannula placements in the NBM for animals that were included in SAT behavioral analysis are shown in SM Figure 1. To determine whether CRF in the NBM altered overall attentional performance, we first analyzed the vigilance index using a mixed factors ANOVA with sex (male, female) as the between-subjects factor and drug (aCSF, 30ng, 100ng) as the within-subjects factor. There was a trend for a main effect of CRF dose in the NBM to impair the vigilance index [$F(2,42)=2.788$, $p=.073$], but no effect of sex [$F(2,42)<1$] and no sex by dose interaction [$F(1,21)<1$] (Fig. 2A). The SAT task uses three different signal durations: 25ms, 50ms, and 100ms. In prior studies, we found that the 50ms signal duration is the most sensitive for detecting stress effects (Cole et al., 2016; Eck et al., 2020). Consistent with this, there was a significant main effect of CRF dose in the NBM on the vigilance index at the 50ms signal duration [$F(2,42)=3.951$, $p=.027$], such that 30ng ($p=.048$) and 100ng ($p=.009$) dose reduced performance relative to controls (LSD posthocs) (Fig. 2C). The vigilance index measures for the 500ms [$F(2,42)=1.496$, $p=.236$] and 25ms [$F(2,42)=1.115$, $p=.337$] signal durations were not altered by CRF in the NBM (Fig. 2B, D). There were no main effects of sex [$F(2,42)<1$] or sex by dose interactions [$F(1,21)<1$] for the vigilance index measures on any signal durations.

Next, we wanted to determine whether the change in the vigilance index was driven by effects of CRF in the NBM on measures of performance in non-signal events, signal events, or both. A mixed factors ANOVA (sex and dose as factors) revealed a significant effect of CRF dose in the NBM on correct rejections [$F(2,42)=5.131$, $p=.010$] and posthoc tests revealed that this was driven by the 100ng dose of CRF in the NBM which decreased correct rejections compared to vehicle ($p=.001$) (Fig. 3A). There was no significant effect of sex on correct rejections [$F(1,21)<1$] and no significant CRF by sex interaction [$F(1,42)<1$]. The CRF-induced

alteration in correct rejections was not due to rats developing a spatial bias towards a specific side of the lever, because there was no main effect of dose [F(2,42)=1.030, p=.366], sex [F(1,21)<1], or dose by sex interaction [F(1,42)<1] on the side bias index. In contrast to the impairing effects of the high dose of CRF in the NBM on correct rejections, CRF did not affect hits [F(2,42) <1] (Fig. 3B) or omissions [F(1.42, 29.77)<1] (Fig. 3C, additional statistics in SM). We also analyzed hits at the three different signal durations and did not find any significant effects (all p>0.5), underscoring that CRF-induced impairments in correct rejections drive the vigilance decrements (SM Fig. 2).

3.2 Some amino acids in the NBM were affected by local CRF administration and sex

For each amino acid quantified, we dropped outliers (greater than 2 standard deviations away from the mean for their sex and drug condition group). A maximum of 2 data points per amino acid were dropped for being outliers. Additionally, 2 samples were dropped because the total mass of NBM tissue collected was less than 1mg. This resulted in 6 to 9 subjects per group. For the analysis of the amino acids, we conducted 2-way ANOVAs with sex (male, female) and drug (aCSF, 100ng CRF) as between-subjects factors. Two by two ANOVAs found that intra-NBM infusions of 100ng CRF significantly decreased levels of glutamate [F(1,25)=6.713, p=.016] (Fig. 4A), glutamine [F(1,25)=4.991, p=.035] (Fig. 4B), taurine [F(1,25)=7.370, p=.012] (Fig. 4D), and alanine [F(1,26)=8.109, p=.008] (Fig. 4I),in the NBM compared to aCSF.

Additionally, sex differences in the levels of several amino acids in the NBM were identified. There was a significant main effect of sex such that females had higher levels of glutamate [F(1,25)=7.723, p=.010] (Fig.4A), glutamine [F(1,25)=4.462, p=.045] (Fig. 4B), GABA [F(1,25)=7.310, p=.012] (Fig. 4E), glycine [F(1,26)=7.607, p=.010] (Fig. 4H), and

alanine [F(1,26)=8.544, p=.007] (Fig. 4I), in the NBM compared to males. There was also a sex difference in the glutamine/glutamate ratio in the NBM with males having a higher ratio than females [F(1,25)=8.844, p=.006] (Fig. 4C). There was no effect of CRF on serine, glycine, or GABA in the NBM. There were no sex by CRF interactions on any NBM amino acid levels (all p>.05). However, the decreases in glutamate, glutamine, and taurine appear larger in female than male rats. Perhaps with more subjects the interaction for these analyses would reach significance.

3.3 Some amino acids were affected by sex in the mPFC

As in the NBM analysis, we dropped outliers (greater than 2 standard deviations away from the group mean), resulting in 7 to 10 rats per group. A maximum of 1 data point per amino acid was dropped for being an outlier. Intra-NBM infusions of CRF had no effect on amino acid levels in the mPFC and there were no sex by CRF interactions (all p>.05) (Fig. 5A-I). There was main effect of sex in glutamine levels in the mPFC such that females had higher levels than males [F(1,30)=14.290, p=.001] (Fig. 5B). Similarly, ANOVA revealed a significant sex difference in the glutamine/glutamate ratio in the mPFC, with females showing a higher ratio than males [F(1,30)=61.364, p<.001] (Fig. 5C). No other sex differences were detected (all p>.05).

4. Discussion

Our prior work found central administration of CRF impairs all aspects of SAT performance: the vigilance index, hits, and correct rejections (Cole et al., 2016). Here we investigated the NBM as a potential site for CRF to exert its impact on attention. Intra-NBM infusions of CRF impaired performance on the vigilance index, with a pronounced effect at the

50ms signal duration. The change in the vigilance index was driven by an effect of CRF in the NBM on correct rejections, not hits. To determine molecular changes induced by CRF in the NBM, we assessed whether CRF altered amino acids. We found CRF-induced decreases in glutamate, glutamine, alanine, and taurine in the NBM. These changes in amino acid levels may mediate the CRF-induced attention deficit by altering glutamate synthesis and inhibitory taurine action in the NBM. Novel sex differences in amino acid levels in the NBM were also observed.

4.1 CRF in the NBM impairs aspects of SAT performance

The NBM is critical for SAT and contains CRF_1 receptors (McGaughy et al., 1996; Sauvage and Steckler, 2001), so we tested whether CRF in the NBM impairs SAT. There was a trend for CRF in the NBM to reduce the vigilance index. The vigilance index was impaired by CRF for the 50ms, but not 500ms or 25ms durations. The vigilance index considers performance on signaled and non-signaled events. When these events were analyzed separately, we found no effect of intra-NBM CRF on hits. However, the high dose of CRF in the NBM reduced correct rejections (i.e., the inverse of false alarms) in both male and female rats. This effect was not attributable to a side bias, but instead suggests that rats in the CRF condition had a lower threshold for false alarms or reporting a signal when none was presented. It is therefore possible that CRF in the NBM causes a shift towards a riskier criterion for signal detection (Burk and Sarter, 2001; Sarter et al., 2001).

Hits rely on the release of cholinergic transients in the mPFC from NBM neurons, and CRF_1 are located on these cholinergic neurons (McGaughy et al., 1996; Sauvage and Steckler, 2001). Thus, it was surprising that local administration of CRF in the NBM did not impair hits. In the dorsal raphe, CRF_2 are cytoplasmic until after stressor exposure where they move to the membrane, a likely adaption for chronic stress (Waselus et al., 2009). The localization of CRF_1

within cholinergic neurons in the NBM has not been described. It is possible a similar effect occurs here and instead a deficit in hits would be observed if CRF was infused for a second time shortly after the first infusion (i.e., mimicking repeated stress). Future studies can test this idea. A lack of an effect of CRF in the NBM on hits also suggests that the impairing effect of central CRF on hits must be mediated by a different structure. Given the critical role of the mPFC in sustained attention and the high density of CRF_1 there (Gritton et al., 2016; Sarter et al., 2001; Van Pett et al., 2000), the mPFC is a likely candidate.

The NBM is heterogenous and, in addition to cholinergic neurons, contains GABAergic and glutamatergic neurons (Zaborszky et al., 2012). CRF_1 are also found on non-cholinergic neurons within this region (Sauvage and Steckler, 2001). Ibotenic acid lesions of the NBM, which largely spare cholinergic neurons, selectively impair correct rejections (Burk and Sarter, 2001). This effect has been attributed to GABAergic neurons because a negative GABA-modulator in the NBM also decreased correct rejections and GABAergic neurons are damaged by ibotenic acid lesions (Burk and Sarter, 2001; Holley et al., 1995). However, ibotenic acid lesions damage glutamate neurons as well (Heo et al., 2009), so these neurons could also play a role in correct rejections. Together, these findings indicate that CRF in the NBM impairs correct rejections through the modulation of non-cholinergic neurons.

4.2 CRF in the NBM reduces levels of amino acids involved in glutamate synthesis

The behavioral studies used a within-subjects design and counterbalanced dosing. Thus, tissue collected following the last intra-NBM CRF infusion on SAT varied by dose, limiting our ability to study molecular changes in behaviorally manipulated animals. We therefore generated new tissue to perform HPLC-ED to evaluate changes in amino acid levels in the NBM following

administration of the high dose of CRF or the vehicle control. It is possible training on the attention task itself or the food restriction used to motivate rats to complete the task affects amino acid levels, possibilities we hope to test with future studies. Thus, note that the results from the current analysis reflect the effect of CRF in the NBM in behaviorally naïve rats.

CRF in the NBM did not affect GABA or glycine. However, CRF in the NBM reduced amino acids involved in glutamate synthesis. Glutamate in the presynaptic terminal is synthesized from glutamine via glutaminase (Marcaggi and Attwell, 2004). CRF reduced both glutamine and glutamate in the NBM, effects which would likely decrease presynaptic glutamate release. After release, glutamate is taken up into astrocytes where it is recycled back into glutamine via glutamine synthetase. Another observation was that CRF reduced alanine in the NBM. This reduction could be a consequence of the reduced glutamate, because glia glutamate recycling can also occur via its transamination to alanine (Marcaggi and Attwell, 2004). It is unclear how CRF can affect these amino acid levels. However, there is evidence that CRF can regulate glutamine synthetase in inner hair cells of the cochlea (Graham et al., 2011). Thus, it is possible that in the NBM CRF alter enzymes the are crucial for the glutamate synthesis pathway.

The glutamate microcircuitry within the NBM is complex and several populations of glutamatergic neurons within this region could be impacted by CRF. There are glutamatergic projection neurons that originate in the NBM and terminate in the cortex (Hur and Zaborszky, 2005). However, our neurochemical results found no effect of CRF in the NBM on cortical glutamate or amino acids involved in cortical glutamate synthesis. Instead, our data suggests CRF-induced changes in glutamate synthesis within the NBM. Glutamate projections originating in the PFC terminate in the NBM and mediate top-down control of the basal forebrain corticopetal system (Sarter et al., 2001). The amygdala also sends glutamatergic

projections to the NBM (Carnes et al., 1990). Glutamatergic neurons in the basal forebrain also may act like local interneurons (Hajszan et al., 2004; Zaborszky et al., 2012), fine-tuning NBM function. Although typically postsynaptic, CRF receptors can be located on presynaptic neurons (Williams et al., 2014), although whether this occurs in the NBM is unknown. Future studies will be needed to determine if CRF administration in the NBM reduces glutamate release from projection neurons, interneurons, or both.

Although few studies have assessed how CRF affects glutamate synthesis, CRF alters physiological responses indicative of changes in presynaptic glutamate release. Specifically, CRF increases the frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) in the central nucleus of the amygdala, which could suggest increased presynaptic glutamate release (Liu et al., 2004; Silberman and Winder, 2013). In contrast, the CRF-related ligand, urocortin I, reduces presynaptic glutamate release as indicated by decreased mEPSC frequency accompanied by increased paired-pulse facilitation (Liu et al., 2004). In the VTA, CRF alters pair-pulse ratios: facilitating them via CRF_2 activation, while inhibiting them via CRF_1 activation (Williams et al., 2014). Although CRF could affect presynaptic glutamate release via several mechanisms, the present data provides evidence that altered glutamate synthesis may be a driving factor.

4.3 CRF in the NBM reduces taurine levels

Taurine is the second most abundant amino acid in the mammalian central nervous system (Jacobsen and Smith, 1968). Glia and neurons work together in the biosynthesis of taurine; astrocytes are the primary source (Vitvitsky et al., 2011). CRF receptors are present on both neurons and astrocytes in the brain (Kapcala and Dicke, 1992). CRF receptors are G-protein

coupled receptors that preferentially bind the GTP-binding protein Gs, which activates the cyclic AMP (cAMP)-protein kinase A (PKA) signaling pathway (Hauger et al., 2009). While we are not certain how CRF can alter taurine, however, in astrocytes, cAMP-PKA signaling can regulate taurine's rate limiting enzyme, cysteine sulfinate decarboxylase (Junyent et al., 2009).

Taurine can act as a neurotransmitter, causing a Cl- influx in neurons that is mediated by activation of the taurine-specific receptor as well as by taurine's actions as a $GABA_A$ and glycine receptor agonist (Bureau and Olsen, 1991; Okamoto et al., 1983). The taurine-specific receptor has not been extensively studied and it is unknown whether NBM cholinergic neurons contain this receptor. However, cholinergic neurons in the NBM are under inhibitory GABAergic control and there are postsynaptic $GABA_A$ receptors on rat NBM neurons (Smiley and Mesulam, 1999). Thus, while we found no decrease in NBM GABA levels following CRF infusion, the observed decrease in NBM taurine levels may disrupt inhibitory tone, decreasing agonistic activity of taurine at $GABA_A$ receptors.

Sufficient inhibitory tone on cholinergic neurons in the NBM is critical for accurate performance on non-signal events in SAT. Acetylcholine release from the NBM into the mPFC occurs prior to signal detection (Parikh et al., 2007) and optogenetic stimulation of NBM cholinergic neurons causes mice to increase false alarms (Gritton et al., 2016). A decrease in NBM inhibitory tone due to reduced taurine levels, could thus result in an improper activation of cholinergic neurons causing false alarms on non-signaled events. We found that CRF in the NBM reduces correct rejections (i.e., increases false alarms). Therefore, this behavioral effect could be linked to a disinhibition of cholinergic neurons resulting from a CRF reduction in taurine.

While taurine's role in the NBM has not been specifically studied, oral taurine administration is associated with enhancing cognition (Kim et al., 2014). It is a common ingredient in energy drinks and is being investigated as a potential therapeutic in the treatment of cognitive deficits associated with aging and neurological disease. Additionally, patients with Alzheimer's disease exhibit decreased CSF taurine levels (Basun et al., 1990; Vitvitsky et al., 2011). Much more research is needed to elucidate the role of taurine in the modulation of NBM-mediated attentional processes.

4.4 Sex differences in NBM and mPFC amino acids levels

The present study is the first to measure and identify sex differences in NBM amino acid levels. Female rats had more GABA and glycine in the NBM than male rats, suggesting an increase in inhibitory amino acids in females. However, in parallel, female rats had higher levels of amino acids involved in glutamate synthesis (glutamate, glutamine, and alanine) than males. One way to gauge inhibitory to excitatory tone is to assess the GABA/glutamate ratio. There were no sex differences in this ratio. Thus, the increase in inhibitory tone is offset by the increase in excitatory tone in the NBM of females.

The NBM sends cholinergic, GABAergic, and glutamatergic projections to this region (Zaborszky et al., 2012), so we hypothesized that CRF in the NBM might alter amino acid levels in the mPFC. However, we did not observe any effects of CRF in the NBM on mPFC amino acid levels. We did identify sex differences in mPFC amino acid levels. Glutamate turnover can be assessed by calculating the glutamine/glutamate ratio. Consistent with a prior report (Kokras et al., 2020), we found that female rats had greater glutamate turnover than males in the mPFC. This effect was driven by higher levels of glutamine in females. Therefore, females may have greater glutamate-to-glutamine cycle activity in this region than males. Sex differences in mPFC

taurine and alanine levels have previously been reported in Wistar rats, with females having higher levels of both amino acids (Kokras et al., 2018). We found no sex difference in taurine or alanine in the mPFC of Long-Evans rats, suggesting a strain difference in mPFC amino acid levels. Additionally, this is the first report of serine and glycine levels in the rat mPFC of both sexes.

We found several sex differences in amino acids in the NBM and mPFC. It might be surprising that there are no sex differences observed in SAT performance (Cole et al., 2016; Eck et al., 2020). However, many sex differences in the brain are compensatory, aimed at keeping behavior consistent between males and females (De Vries, 2004). One finding here is indicative of a compensatory sex difference: the female NBM has both increased GABA and glutamate compared to the male NBM, resulting in comparable excitatory/inhibitory tone in the NBM of females and males. Understanding compensatory sex differences in the basal forebrain attention system is important for drug development because therapeutics may need to be tailored differently for males and females to account for the sex differences in neurotransmitters and receptors in this circuit.

Conclusions

The present study demonstrated the effect of CRF in the NBM on attention and amino acid levels in basal forebrain attention circuit in both male and female rats. CRF in the NBM reduces aspects of SAT performance in both sexes. It also decreased amino acids involved in glutamate synthesis (including glutamate itself), while also reducing taurine in both males and females. Given the role of glutamate and taurine in attention, their perturbation by CRF may contribute to the deficit in SAT performance following CRF in the NBM. To confirm causality, we would need to determine whether increasing glutamate or taurine signaling rescues the

negative effect of CRF in the NBM on SAT performance. We plan to test this in future studies. If this is successful, these results would implicate glutamate and/or taurine as therapeutic targets to treat disorders with attention disruptions as a key feature and stress as a precipitating factor.

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Figure Captions

Figure 1. Outcome measures for the rat operant SAT. In this depiction of the touchscreen SAT, the left response area should be touched to indicate the rat perceived the signaled event while the right response area should be touched to indicate the rat perceived the non-signaled event. Correct responses on signaled events are scored as "hits", while incorrect responses are scored as "misses". Correct responses on non-signaled events are scored as "correct rejections", while incorrect responses are scored as "false alarms". Animals are rewarded with food pellets for correct responding. Incorrect responding is not rewarded.

Figure 2. The effect of CRF in the NBM on the vigilance index. There was a trend (p=.073) for CRF in the NBM to impair the vigilance index when all three signal durations were averaged (A). When the vigilance index was assessed for each signal duration, intra-NBM CRF infusions impaired the vigilance index at the 50ms duration (C), but not the 500ms (A) or 25ms (D) durations. *indicates $p<.05$

Figure 3. The effect of CRF in the NBM on correct rejection, hits, and omissions. The 100ng dose of CRF significantly impaired non-signal trial performance in SAT, reducing correct rejections in both males and females (A). Intra-NBM CRF infusions had no effect on hits (B) or omissions (C) in SAT. *indicates $p<.05$

Figure 4. The effect of intra-NBM CRF on NBM amino acid levels. Intra-NBM CRF infusions significantly decreased NBM levels of glutamate (A), glutamine (B), taurine (D), and alanine (I) compared to vehicle. Female rats showed significantly higher levels of glutamate (A),

glutamine (B), GABA (E), glycine (H), and alanine (I) compared to males. Males showed a significantly higher glutamine/glutamate ratio than females (C). * indicates $p<.05$

Figure 5. The effect of intra-NBM CRF on mPFC amino acid levels. There was no effect of intra-NBM CRF infusion on amino acid levels in the mPFC (A-I). Female rats had a significantly higher level of glutamine in the mPFC than males (B) and a higher glutamine/glutamate ratio than males (C). * indicates $p<.05$

Figure 1

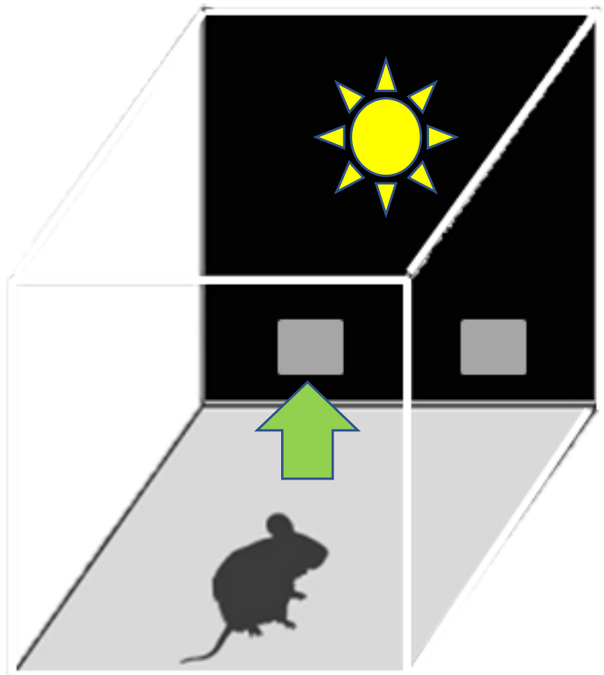
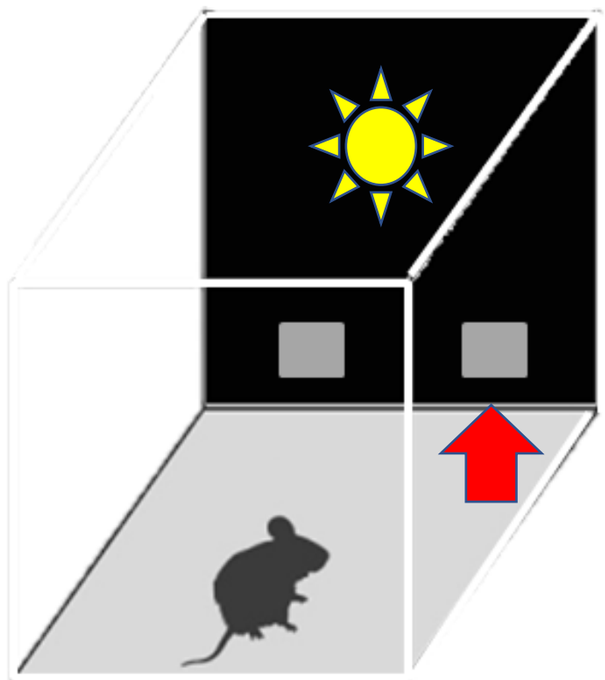
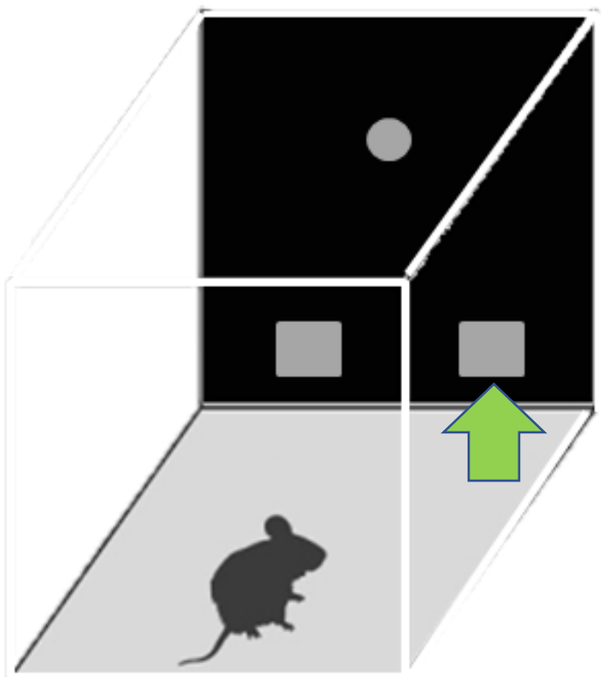
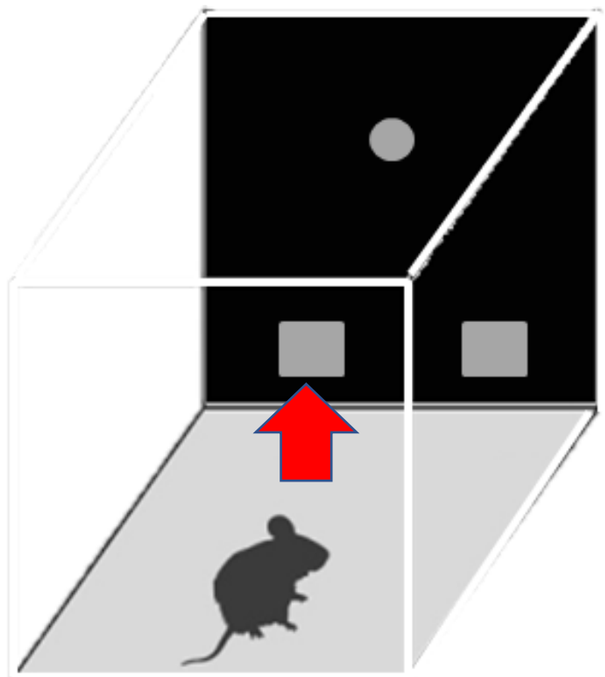
	Rewarded	Not Rewarded
Signaled Event	 Hit	 Miss
Non-Signaled Event	 Correct Rejection	 False Alarm

Figure 2

Click here to access/download;Figure(s);Fig. 2 VI resub copy.pdf

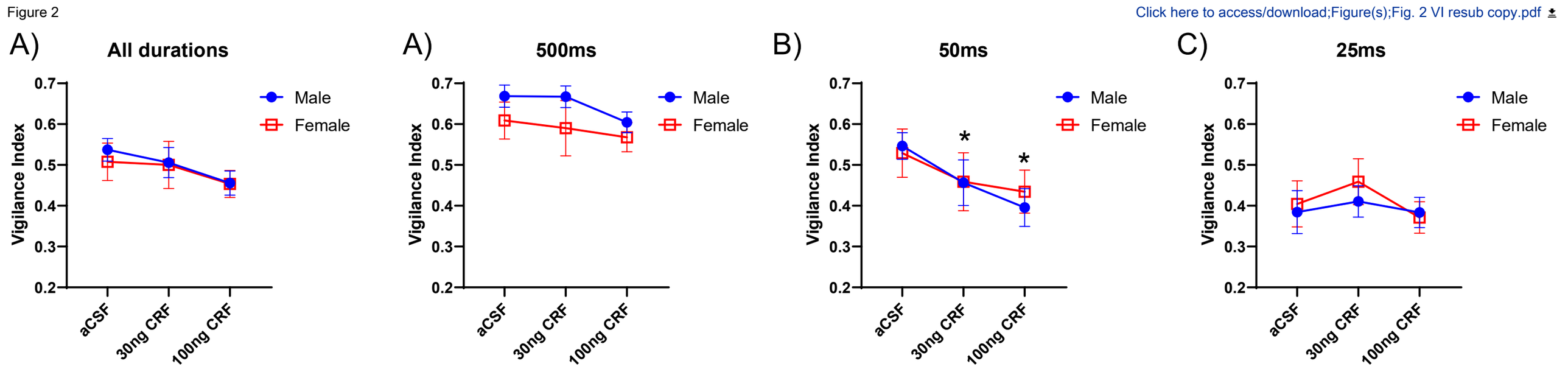


Figure 3

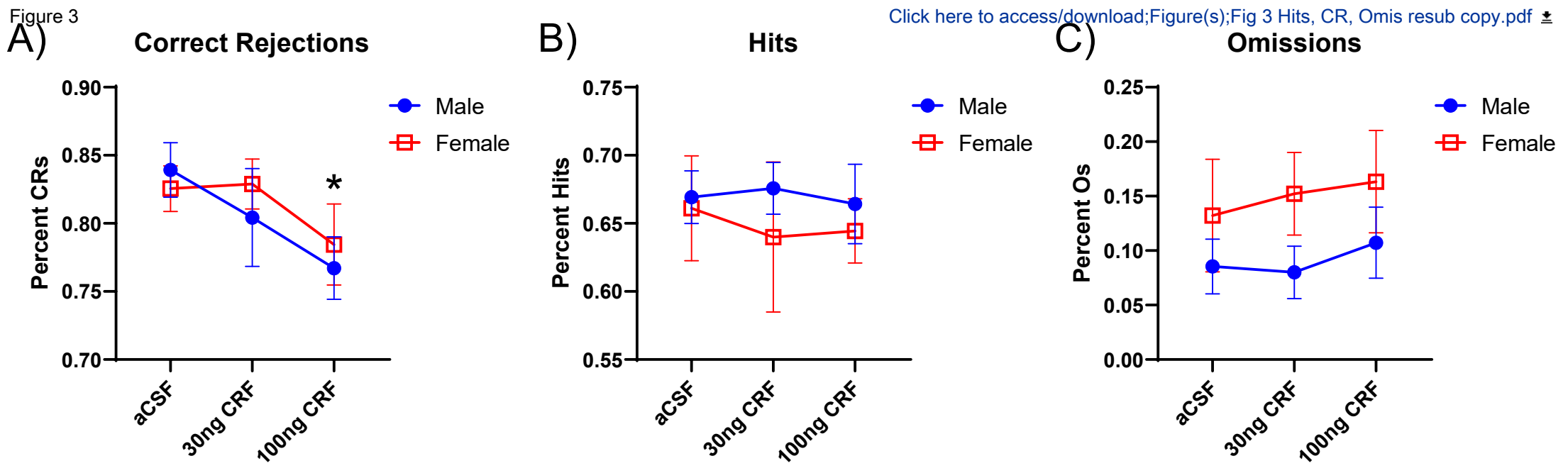


Figure 4

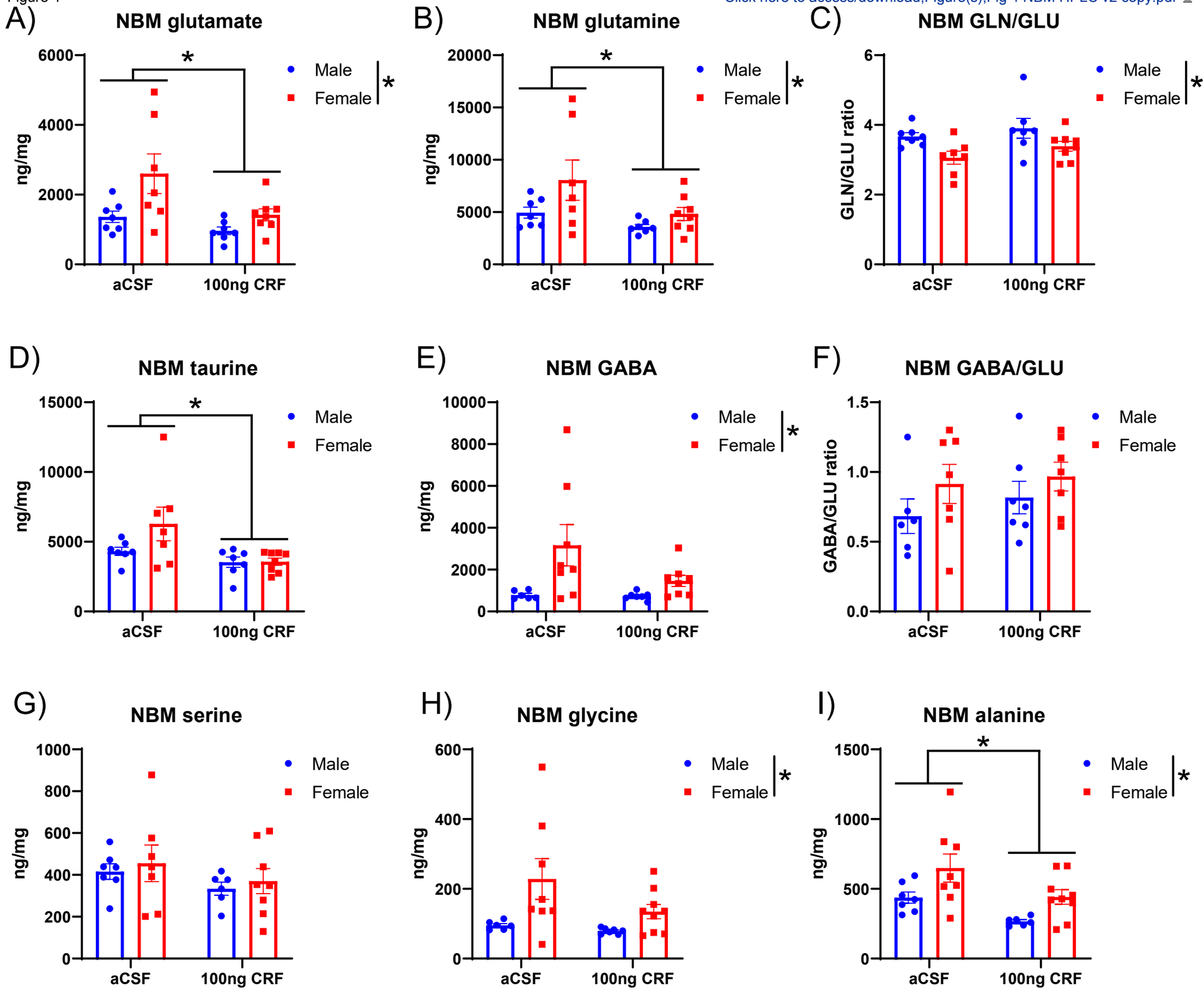
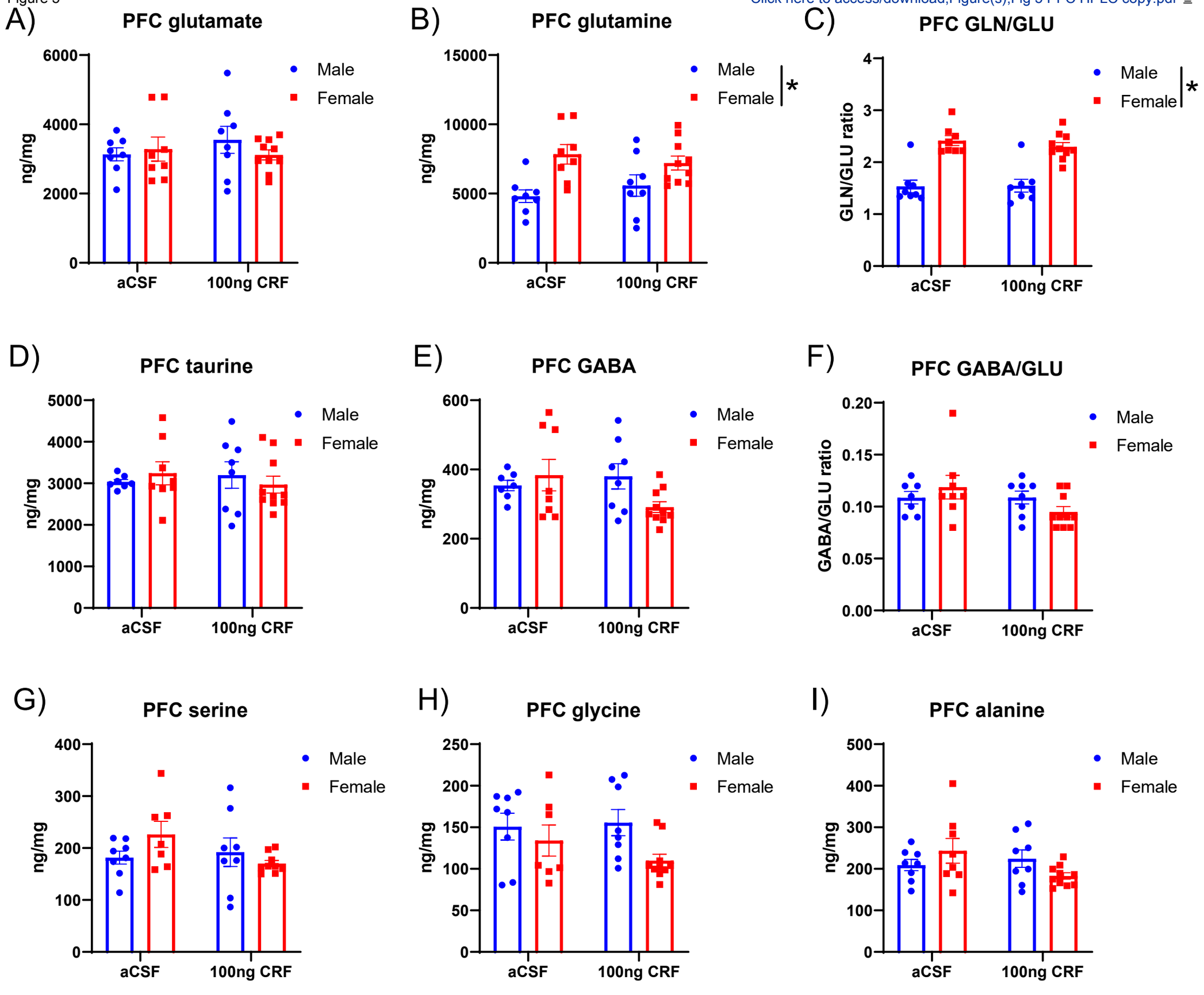


Figure 5



CRediT author statement

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