

Regulation of behavioral responses to single prolonged stress in male and female rats: Role of PACAP

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ARTICLE INFO

Keywords:

Single prolonged stress
Post-traumatic stress disorder
PACAP
Prefrontal cortex
Sex

ABSTRACT

Post-Traumatic Stress Disorder (PTSD) is a debilitating condition in which a traumatic experience triggers symptoms related to re-experiencing, avoidance, arousal, and mood dysregulation. PTSD negatively impacts 6 % of people during their lifetime, with women being disproportionately affected and exhibiting different, more severe symptoms than men. Despite this widespread impact, the molecular mechanisms underlying PTSD and its sex differences remain poorly understood. Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is a neuropeptide which participates in fine-tuning circuitry throughout the brain and has been associated with PTSD in humans, especially in women. Here, we use Single Prolonged Stress (SPS), an animal model of PTSD, to explore the roles of PACAP and sex in PTSD-like behaviors. Specifically, a PACAP agonist or antagonist was infused into the infralimbic (IL) prefrontal cortex, a region key to regulating fear- and anxiety-related behaviors, prior to SPS in male and female rats. Rats were then tested in open field/novel object, elevated plus maze, and social interaction. Utilizing a behavioral indexing method, we were able to uncover SPS effects in PTSD-related behavioral domains that were differentially impacted by PACAP manipulations in males and females. While both sexes exhibited increased threat avoidance and decreased threat assessment following SPS, females increased sociability while males decreased sociability. Males also appeared to be protected by IL PACAP antagonism while female SPS phenotypes were exacerbated by IL PACAP agonism. Furthermore, RNAscope revealed that PACAP in the prefrontal cortex responds differently to SPS in males and females. Together, these findings suggest complex relationships between SPS, sex, and IL PACAP which may have important implications for treating PTSD in men and women.

1. Introduction

Post-Traumatic Stress Disorder (PTSD) is a detrimental psychiatric condition that impacts an estimated 6 % of people in their lifetime

(Schein et al., 2021; NIH, 2023). PTSD symptoms can emerge following a traumatic event and include intrusive re-experiencing, avoidance, altered arousal, and negative effects on mood and cognition (NIH, 2023; Lisieski et al., 2018). While PTSD has received widespread basic and clinical research attention, its underlying mechanisms remain poorly

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<https://doi.org/10.1016/j.ynstr.2025.100727>

Received 11 December 2024; Received in revised form 4 April 2025; Accepted 19 April 2025

Available online 26 April 2025

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Abbreviations

BLA	Basolateral Amygdala
BST	Bed Nucleus of the Stria Terminalis
CEA	Central Amygdala
CORT	Corticosterone
EPM	Elevated Plus Maze
HPA	Hypothalamic-Pituitary-Adrenal
IL	Infralimbic Prefrontal Cortex
NOR	Novel Object Recognition
OF	Open Field
PAC1	PACAP Receptor
PACAP	Pituitary Adenylate Cyclase-Activating Polypeptide
PFC	Prefrontal Cortex
PL	Prelimbic Prefrontal Cortex
PTSD	Post-Traumatic Stress Disorder
SI	Social Interaction
SPS	Single Prolonged Stress
vmPFC	Ventromedial Prefrontal Cortex

understood, and the field is still wanting for therapies that can effectively ameliorate PTSD symptoms.

One recently identified candidate for such a therapy is Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), an excitatory neuropeptide implicated in promoting hormonal and behavioral responses to stress (Hammack and May 2015; Mustafa, 2013). PACAP receptor (PAC1) polymorphisms are associated with risk for development of PTSD (Almli et al., 2013; Jovanovic et al., 2013; Ressler et al., 2011; Liao et al., 2019; Uddin et al., 2013), and circulating PACAP levels are positively associated with severity of PTSD symptoms (Ressler et al., 2011). PACAP and its receptors are present throughout many of the brain's key stress-responsive regions, including the prefrontal cortex (PFC), basolateral amygdala (BLA), central amygdala (CEA), bed nucleus of the stria terminalis (BST), paraventricular nucleus of the hypothalamus, and hippocampus (Hammack and May 2015; King et al., 2017; Martelle et al., 2021). PACAP is generally thought to fine-tune this stress circuitry to heighten an animal's response to environmental challenges (Hammack and May 2015; Mustafa, 2013; Boucher et al., 2021). For example, deletion of the PACAP gene in mice reduces hypothalamic pituitary adrenal (HPA) axis stress responses following various stressors (Lehmann et al., 2013; Morita et al., 2006). Behaviorally, PACAP knockout mice exhibit increased novelty seeking and reduced threat avoidance behaviors in the elevated plus maze, open field, and light-dark box (Hattori et al., 2012), consistent with reduced reactivity to novel stressor exposures in the absence of PACAP signaling.

One key circuit in which PACAP may act to modulate PTSD responsivity is the PFC to amygdala connection. PTSD pathology is linked to impaired activation of the ventromedial prefrontal cortex (vmPFC)-amygdala circuits promoting inhibition of fear (Cotella et al., 2023; Ramikie and Ressler, 2018). It is thought that PTSD reduces top-down control of the BLA by the vmPFC, reducing BLA-mediated inhibition of the central amygdaloid nucleus (CEA), resulting in enhanced activation of CEA neurons that drive fear responses (Marek et al., 2019). Studies in rodents reveal that central infusions of PACAP disrupt acquisition of fear conditioning, whereas infusions of a PAC1 antagonist disrupts cue learning in a trace conditioning paradigm (Kirry et al., 2018). A recent study from our group noted long-term enhancement of passive avoidance learning following injection of PACAP into the rodent vmPFC equivalent (infralimbic cortex (IL)), suggesting that the peptide is able to potentiate fear memories via direct modulation of this cortical circuitry (Martelle et al., 2021). PAC1 receptors in the IL are primarily located on inhibitory interneurons, suggesting that these behavioral effects may stem from PACAP driving inhibition within the

IL, impairing its ability to subsequently inhibit amygdala-driven fear responses (Martelle et al., 2021). In the BLA itself, infusion of PACAP *in vitro* activates neurons projecting to the CEA, thereby inhibiting consolidation of contextual fear conditioning (Cho et al., 2012). Together, these data suggest that PACAP can act within vmPFC-amygdala circuitry to exaggerate conditioned fear. The question which remains is how the actions of PACAP within this circuit directly participate in PTSD pathophysiology.

Many of the human PTSD-PACAP associations discussed above are either driven by or present exclusively in women (Almli et al., 2013; Jovanovic et al., 2013; Ressler et al., 2011; Liao et al., 2019; Uddin et al., 2013). These studies suggest that there are likely sex differences in the interplay between PTSD and PACAP that could have important consequences for the therapeutic potential of this neuropeptide. Despite these disproportionate effects on women, many animal studies of PTSD either focused on males alone or failed to elicit behavioral phenotypes in females. For example, single prolonged stress (SPS), a widely accepted animal model of PTSD, elicits predictable fear-, arousal-, and anxiety-related phenotypes in males (Lisieski et al., 2018; Souza et al., 2017). SPS in females is much less studied and has yielded more subtle, less consistent behavioral phenotypes (Pooley et al., 2018; Keller et al., 2015; Mancini et al., 2021). Recent studies in our lab have revealed female SPS effects on reinstatement of conditioned fear, but not on more classically studied fear acquisition and extinction-related endpoints that are impacted in males (Cotella et al., 2023). Despite these behavioral differences, females do exhibit unique molecular changes following SPS (Cotella et al., 2023). Sex differences in PACAP expression are noted rodent studies, with females showing higher PACAP tone in the BST and prefrontal cortex (PL) (Hammack and May 2015; Kirry et al., 2018).

Here, we utilized SPS in tandem with intra-IL pharmacological manipulations of PACAP to explore 1) the behavioral consequences of SPS in male and female rats and 2) the impact of PACAP agonism and antagonism within the IL on SPS behavioral phenotypes in both sexes. Furthermore, RNAscope was used to examine how SPS impacts PACAP in the PFC, providing clues for how PACAP manipulation could be causing differential effects in males and females. The present results offer insight into the roles of sex and vmPFC PACAP signaling in mediating an individual's response to a traumatic experience, deepening our understanding of the molecular mechanisms underlying PTSD and offering important considerations for the therapeutic treatment of this detrimental condition.

2. Materials and methods

2.1. Subjects

Eight-week-old male and female Sprague Dawley rats were purchased from Envigo. All animals were pair-housed in translucent polycarbonate shoebox cages with corncob bedding and left undisturbed for one week prior to any manipulations, with food and water available *ad libitum*. Animals receiving cannulation surgery (below) were single-housed post-operatively to limit damage to cannulae or cannula implant site. Animals who did not receive surgery were single housed immediately following SPS. The colony room was temperature and humidity-controlled and maintained on a 12-h light-dark cycle (07:00 h lights on, 19:00 h lights off). All experiments complied with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2. Experimental Design

Experiment 1 was designed to assess the interaction of SPS and IL PACAP manipulations. Male and female rats received intra-IL infusions of either vehicle (VEH), a PAC1 receptor agonist PACAP 1–38 (PACAP), or a PAC1 receptor antagonist PACAP 6–38 (ANTAG) 30 min prior to

SPS. Behavioral assessments began one week after SPS and were each separated by one week to prevent experience with one test from impacting subsequent testing (Fig. 1A). Experiment 2 examined the impact of SPS on PFC PACAP. Behaviorally naïve rats were sacrificed one week after SPS, and RNAscope was used to examine PACAP expression in the IL and prelimbic (PL) PFC (Fig. 1B). Males and females were run in separate cohorts to logistically accommodate the large n required by the present SPS x PACAP design (Cotella et al., 2020).

2.3. Surgical procedures

Male and female rats (n = 8–12 per group) were anesthetized with isoflurane and treated with a prophylactic dose (2 mg/kg) of meloxicam (Loxicom®; Norbrook, Overland Park, KS). A stereotaxic instrument (David Kopf Instruments, Tujunga, CA) was used to guide placement of double barreled 26-gauge guide cannulas (Plastics One Inc., Roanoke, VA) into the left and right IL cortices [from bregma: +3.0 mm anterior-posterior, ±0.6 mm mediolateral, -4.6 dorsoventral] under aseptic conditions. Guide cannulas were fixed with dental cement (Stoelting Company) to 3 anchoring screws on the skull. Each cannula was fitted with a dummy cannula that extended 0.2 mm beyond the tip of the guide. Rats were treated with meloxicam for 2 days following surgery and allowed to recover for 1 week prior to behavioral studies. The dummy cannula was manipulated daily during this period to maintain patency, but animals received no other handling during the recovery period. The locations of the cannula tracks were histologically verified at the end of experiments (Supplementary Fig. 1).

2.4. Pharmacological infusions

PACAP and ANTAG were obtained from BACHEM (Torrence, CA) and dissolved in saline vehicle (VEH) on the day of injection. Thirty minutes prior to SPS, rats received a bilateral intra-IL injection of VEH, PACAP (1µg/500 nl), or ANTAG (1µg/500 nl) at a rate of 160 nl/min for 3 min via a 33-gauge infusion cannula connected by polyethylene tubing to a 10 µl Hamilton micro-syringe (Hamilton Company). The injectors were left in place for 60 s after injection to allow for diffusion away from the cannula. These doses were chosen because they have been shown previously to be behaviorally active upon intraparenchymal infusion in rats (Lezak et al., 2014; Telegdy and Kokavszky, 2000). Rats were returned to their home cage until SPS commenced 30 min later.

2.5. Single prolonged stress

SPS was conducted in accordance with prior published procedures (Cotella et al., 2023). Rats were placed in a plastic restrainer for 2 h, followed immediately by a 20-min group swim (n = 6–8 animals per tub, 45 cm in diameter, water temperature 25 ± 2 °C). Following a 10-min rest, rats were placed in pairs into a glass desiccator containing ether until unconscious, removed to a clean cage until regaining consciousness, and then returned to the housing room. Animals were left to recover for 1 week before behavioral testing.

2.6. Behavioral testing

2.6.1. Open field and novel object recognition

One week after SPS, rats were subjected to open field (OF) and novel object recognition (NOR) testing. Testing occurred over 2 days and consisted of 4 phases: open field (day 1), familiarization 1 (day 1), familiarization 2 (day 2), and novel object testing (day 2). All phases were conducted in a black 75 × 75 × 40 cm plastic box and recorded from above. On the morning of day 1, rats were placed in the center of the empty box and allowed to freely explore for 10 min for the open field phase. Approximately 2 h later, two objects (500 mL bottles filled with blue dye) were placed in the middle of the box, and rats were allowed to freely explore the objects for the 10-min familiarization 1 phase. The next morning, rats were placed back in with the same 2 objects for the 10-min familiarization 2 phase. Four hours later, one of the objects was changed to a 12 × 12 × 7 cm tube holder, and rats were allowed to freely explore both objects for the 10-min novel object phase. Novel and familiar object locations were counterbalanced within each group. Ethovision 13 was used to assess center duration, border duration, and distance moved for the open field phase, as well as time spent exploring (nose within 2 cm) the novel and familiar objects during the novel object phase. A discrimination index was calculated using the formula: (novel duration – familiar duration)/(novel + familiar duration).

2.6.2. Social interaction

One week later, rats were subjected to social interaction (SI) testing. Rats were placed in a clean cage (40 × 20 cm) with a novel age- and sex-matched conspecific for 10 min. Videos of the interactions were scored by a blinded observer for total time conducting social (e.g., touching, sniffing), non-social (e.g., exploring the cage), aggressive (e.g., pinning), and submissive (e.g., presenting underside) behaviors.

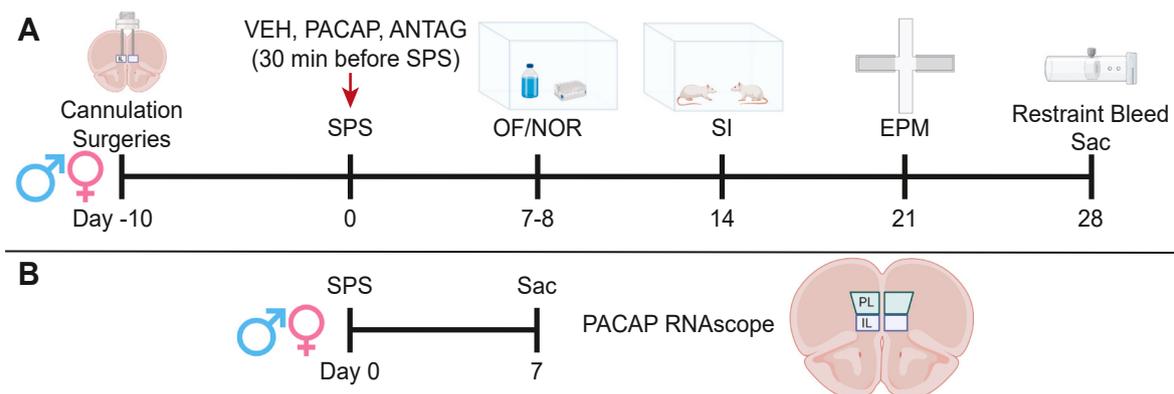


Fig. 1. Experimental Design. (A) Experiment 1 Timeline. Male and female rats were implanted with bilateral cannulae targeting the IL. Following recovery, rats were infused with either VEH, PACAP, or ANTAG 30 min prior to either SPS or CON. Behavioral testing commenced one week later and consisted of OF/NOR, SI, and EPM, each run a week apart. Rats were then sacrificed 90 min after a 30-min restraint, and brains were collected for verification of cannula placement. (B) Experiment 2 Timeline. Male and female rats were exposed to either SPS or CON, and sacrificed one week later. Brains were collected, frozen, and used for RNAscope analysis of PACAP mRNA expression in the IL and PL. Note that animals in this study were kept behaviorally naïve.

2.6.3. Elevated plus maze

One week later, rats were subjected to elevated plus maze (EPM) testing. The EPM apparatus consists of a 10 cm² center area, two open arms (50 × 10 cm), and two enclosed arms (50 × 10 × 30 cm). Rats were placed one of the open arms, facing the center, and allowed to freely explore the apparatus for 5 min. Ethovision 13 was used to assess time spent in the center, open arms, and closed arms, as well as the distance traveled. Additionally, a blinded observer scored the videos for nose exploration (poking the head into the open arms) and nose dips (dipping the head off of the open arms). It is important to note that, for the males, the EPM was run in white light, resulting in little exploration of the open arms. For that reason, the females were then run in dim light, resulting in greater time on the open arms. Differences between males and females on these endpoints thus represent procedural differences rather than differences by sex.

2.6.4. HPA axis assessment

One week later, rats were restrained in plastic restrainers for 30 min, and blood was sampled by tail bleed at 0, 15, 30, 60, and 120 min from the commencement of restraint for corticosterone (CORT) analysis.

2.7. Corticosterone radioimmunoassay

Blood samples were centrifuged (4 °C at 6000 rpm for 15 min), and plasma was collected and stored at -20 °C until assay preparation. Plasma CORT concentrations were determined using 125I radioimmunoassay kits (MP Biomedicals). All samples were run in duplicate.

2.8. Histological analysis

Following the final tail bleed, rats were administered an overdose of sodium pentobarbital and transcardially perfused with 0.1M phosphate buffer (PBS) followed by 4 % paraformaldehyde in 0.1M PBS, pH 7.4. Brains were post-fixed in 4 % paraformaldehyde at 4 °C for 24h, then transferred to 30 % sucrose in 0.1M PBS at 4 °C until the moment of tissue processing. Brains were sliced into serial 35 µm coronal sections using a freezing microtome (-20 °C). Sections (1/6) were collected into wells containing cryoprotectant solution (30 % Sucrose, 1 % Polyvinylpyrrolidone (PVP-40), and 30 % Ethylene glycol, in 0.1M PBS). Brains were stained with cresyl violet, and the PFC was imaged to assess cannula placement (Supplementary Fig. 1). The correct placement in the IL PFC was determined using the Paxinos & Watson Rat Brain Atlas (Paxinos, 2007).

2.9. Behavioral indexing

In order to gain further insights into the present data, we utilized a z-score method to analyze multiple behaviors together and generate indices in behavioral domains known to be negatively impacted in PTSD (Maren and Holmes, 2016). Z-scores were calculated with the following formula: $(x - \text{AVG})/\text{SD}$, where x = sample value, AVG = average of CON VEH group, and SD = standard deviation of CON VEH group. Z-scores from multiple behavioral endpoints in related domains were then averaged together to generate behavioral indices as follows (Fig. 5A). The Threat Avoidance Index included OF Border Duration and EPM closed arm duration. The Threat Appraisal Index included EPM nose exploration duration and EPM head dip duration. The Sociability Index included SI social interaction duration and the negative transform of non-social interaction duration. We additionally correlated these indices together in order to determine if more severe impairments in one domain were indicative of impairments in another.

2.10. RNAscope analysis of prefrontal PACAP

A new cohort of rats was generated (CON/SPS, $n = 6/\text{sex}/\text{group}$) and sacrificed by rapid decapitation one week after SPS (Fig. 1B). The goal of

this study was to determine SPS and sex effects on PFC PACAP expression. Brains were collected, flash frozen in cold isopentane, and stored at -80 °C. Brains were warmed to -20 °C for 1h and sectioned at 18 µm on a Microm cryostat. Sections containing IL and PL PFC were selected for Multiplex RNAscope (Advanced Chemical Diagnostics). The PL was selected as an additional analysis region because of prior studies implicating it in PTSD-related sex differences (Kirry et al., 2018). Tissue was labeled with a probe for PACAP RNA *adcyap1*. Tissue was fixed in 4 % PFA (room temp) for 1 h, dehydrated through an ethanol series, and stored in 100 % EtOH at -4 °C for between 24 h and a week before staining. Staining was done following the Advanced Chemical Diagnostics multiplex RNAscope protocol for fresh-frozen tissue. Images were obtained bilaterally for the IL and PL (AP +0.5 to +1.5) using a Stellaris confocal microscope and processed using the ACD Hi-plex software. Image settings were kept constant for all animals. Five to ten cells were analyzed per animal, each randomly selected from one section. PACAP puncta were quantified using Imaris by outlining points in the appropriate channel that matched the diameter of a selected punctum. These data are presented as average puncta per cell for each animal.

2.11. Statistics

It is important to note that we elected to analyze males and females independently in these studies. This decision was made because 1) males and females were assessed in different testing sessions and 2) large inherent male/female differences exist in many of the tested endpoints which could occlude the ability to examine individual endpoints within sex. Therefore, all results were analyzed within each sex and do not directly assess sex differences per se.

Normality and variance were assessed, and two-way (Stress x Infusion) ANOVAs (GraphPad Prism 9) were used to assess behavioral endpoints within sex. Outliers were determined by values that fall outside the mean ± 1.96 times the standard deviation. All post hoc testing utilized Sidak's multiple comparison tests, which were selected *a priori* to focus on stress effects within infusion groups. Pearson correlations were used where appropriate. CORT curves were analyzed using Two-way repeated measures ANOVAs within sex. RNAscope endpoints were analyzed using unpaired t-tests. Detailed statistical results are provided in Supplementary Table 1.

3. Results

3.1. Anatomical verification of cannula implantation

Only animals with both cannulae correctly hitting the IL were included in behavioral analyses. Maps of cannula placements are presented in Supplementary Fig. 1. Final n 's were as follows: Male CON VEH = 12, Male CON PACAP = 7, Male CON ANTAG = 8, Male SPS VEH = 8, Male SPS PACAP = 7, Male SPS ANTAG = 8, Female CON VEH = 10, Female CON PACAP = 10, Female CON ANTAG = 10, Female SPS VEH = 10, Female SPS PACAP = 9, Female SPS ANTAG = 10.

3.2. Impact of SPS and IL PACAP manipulations on behavior

SPS decreased OF center duration in males (main effect of stress: $F(1,41) = 4.197, p = 0.047$) and females (main effect of stress: $F(1,53) = 6.107, p = 0.017$) (Fig. 2A and B), suggesting increased anxiety-like behavior. While PACAP manipulations did not significantly impact this endpoint, it appears that ANTAG infusion was able to block this effect in males alone. Border duration was inversely affected, exhibiting increases in both sexes after SPS (male main effect of stress: $F(1,41) = 4.197, p = 0.047$; female main effect of stress $F(1,53) = 6.095, p = 0.017$); whereas distance moved was not altered in males but exhibited an interaction effect in females (interaction effect $F(2,52) = 4.382, p = 0.017$) (Supplementary Fig. 2A).

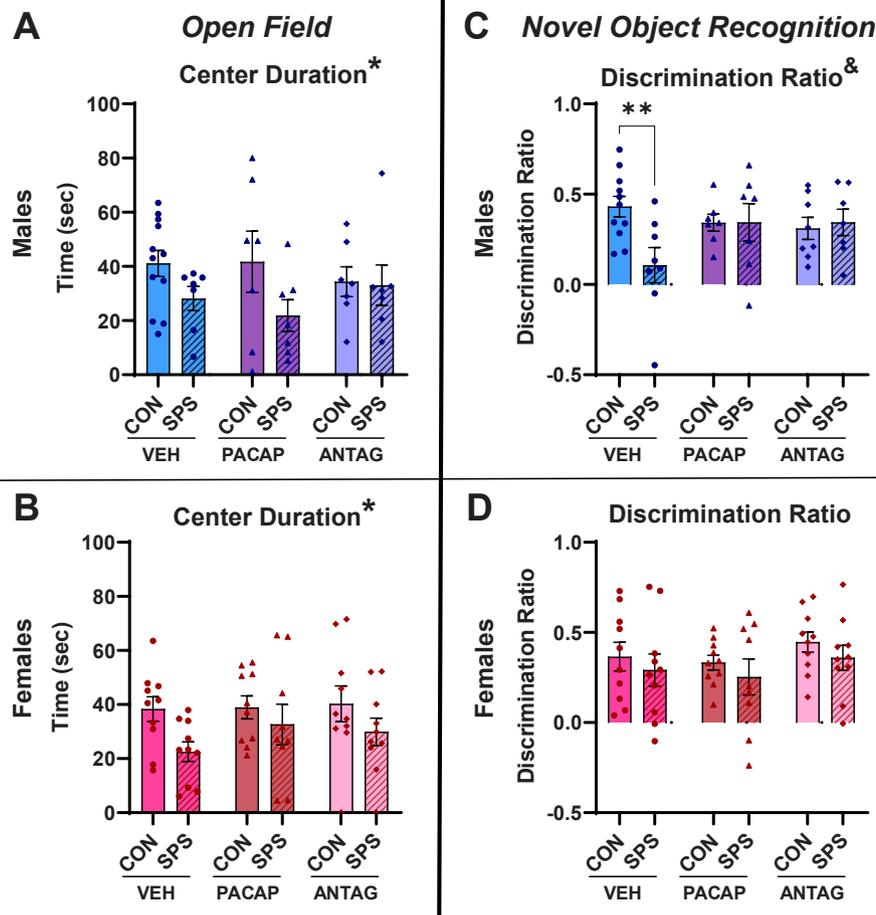


Fig. 2. SPS and PACAP Effects on Open Field and Novel Object Recognition Testing. (A) Male OF center duration. (B) Female OF center duration. (C) Male NOR discrimination ratio. (D) Female NOR discrimination ratio. $n = 7-12$. * = Main Effect of Stress. & = Interaction. * = post hoc $p < 0.05$.

NOR exhibited an interesting difference by sex, in that SPS disrupted discrimination in males (interaction effect: $F(2,41) = 3.733$, $p = 0.032$) but not in females (Fig. 2C and D). This effect was driven by enhanced exploration of the familiar object in males (interaction effect: $F(2,41) = 3.917$, $p = 0.028$). Females did explore the novel object less following SPS (main effect of stress: $F(1,51) = 4.195$, $p = 0.046$); however, this was not sufficient to impact the overall discrimination ratio (Supplementary Fig. 2B). PACAP and ANTAG did not significantly impact discrimination ratio in either sex.

In the SI test, males exhibited decreased social interaction (main effect of stress: $F(1,41) = 4.081$, $p = 0.049$) and increased non-social interaction (main effect of stress: $F(1,42) = 4.847$, $p = 0.033$) following SPS (Fig. 3A). These effects were strongest in the VEH animals and largely normalized in both PACAP and ANTAG conditions. Females did not show SPS or PACAP effects on these endpoints (Fig. 3B). Females did exhibit stress (main effect of stress: $F(1,48) = 6.898$, $p = 0.011$) and interaction (interaction effect: $F(2,48) = 4.125$, $p = 0.022$) effects on aggressive interactions; however, the low frequency of these events makes it difficult to draw conclusions from these data (Supplementary Fig. 2C).

Both sexes exhibited SPS effects on EPM endpoints. In males, closed arm duration was increased by SPS (main effect of stress: $F(1,44) = 4.634$, $p = 0.037$), and center duration was decreased (main effect of stress: $F(1,43) = 4.580$, $p = 0.038$) (Fig. 4A). Note that males largely did not go out onto the open arms due to brighter light conditions (Supplementary Fig. 1D). This effect was evident in both VEH and PACAP rats, but largely normalized in the ANTAG group. Males did not show any significant differences in head dip or nose exploration

durations. While females exhibited similar patterns, with SPS increasing closed arm duration (main effect of stress: $F(1,52) = 4.692$, $p = 0.035$; main effect of infusion: $F(2,52) = 5.069$, $p = 0.010$) and decreasing center duration (main effect of stress: $F(1,51) = 6.338$, $p = 0.015$), they appear to have been more affected than males by PACAP infusion (Fig. 4B; Supplementary Fig. 2D). SPS increased closed arm duration and decreased center and open arm durations the most in females that received PACAP. Similar effects were seen in the other groups, but to a smaller magnitude. Females also exhibited decreased head dip durations (main effect of infusion: $F(2,51) = 4.155$, $p = 0.021$) following SPS, suggesting decreased exploratory behaviors, again driven by the PACAP group.

The CORT response to restraint following SPS and PACAP manipulation was not impacted in either sex (Supplementary Fig. 3).

3.3. Behavioral indexing reveals SPS and PACAP effects on complex behaviors

While the standard behavioral endpoints presented in 3.2 revealed some interesting phenotypes, considering multiple endpoints at once has the potential to offer deeper insight into the interaction of SPS and IL PACAP. The Z-score method was used to generate such combined endpoints, each representing a behavioral domain known to be impacted by PTSD (Maren and Holmes, 2016) (Fig. 5A).

SPS enhanced threat avoidance and decreased threat appraisal in both males (avoidance main effect of stress: $F(1,41) = 6.509$, $p = 0.015$; appraisal interaction effect: $F(2,41) = 3.580$, $p = 0.037$) and females (avoidance main effect of stress: $F(1,49) = 19.29$, $p < 0.0001$; appraisal

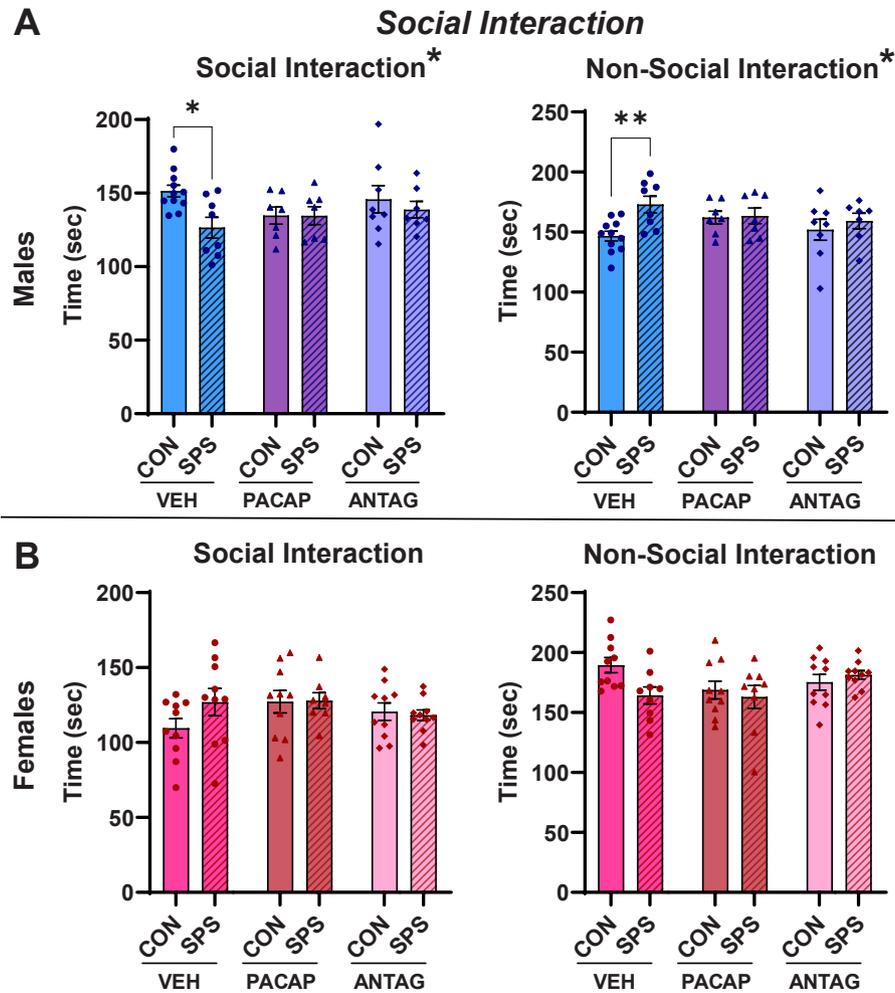


Fig. 3. SPS and PACAP Effects on Social Interaction Testing. (A) Male SI social and non-social interaction duration. (B) Female SI social and non-social interaction duration. $n = 7-12$. * = Main Effect of Stress. * = post hoc $p < 0.05$.

main effect of stress: $F(1,50) = 5.381$, $p = 0.025$ (Fig. 5B and C). In males, PACAP infusion did not alter these endpoints. However, ANTAG infusion blocked SPS effects on both indices. On the other hand, PACAP moderately enhanced these SPS phenotypes in females, while ANTAG had less of an impact. Thus, while SPS effects on threat-related indices were similar in males and females, the response to PACAP manipulation differed on the basis of sex.

The sociability index was also varied in males and females. While males exhibited decreased sociability following SPS (main effect of stress: $F(1,42) = 4.498$, $p = 0.040$), females exhibited enhanced sociability (interaction effect: $F(2,50) = 3.490$, $p = 0.038$). Opposing effects were seen in each sex with PACAP and ANTAG, although drug infusion normalized this difference in CON and SPS rats.

Correlating these behavioral indices offers further insight into individual differences in SPS coping strategies (Supplementary Fig. 4). In both males and females who experienced SPS, there was a strong negative correlation between threat avoidance and threat appraisal (male $R^2 = 0.603$, $p < 0.001$; female $R^2 = 0.382$, $p = 0.001$), suggesting that as rats became more avoidant, they also become less exploratory of their surroundings, indicating more severe SPS phenotypes. Females also exhibited a negative relationship between threat avoidance and sociability ($R^2 = 0.161$, $p = 0.042$) that was not present in males. This suggests that females who were more social were less avoidant, indicating less severe SPS phenotypes. PACAP manipulations did not significantly shift these relationships.

3.4. SPS differentially impacts PFC PACAP RNA in males and females

A new cohort of rats was generated to assess the impact of SPS on PACAP mRNA expression in the PFC. Brains were collected one week following SPS and RNAscope was used to measure PACAP expression (Fig. 6; Supplementary Fig. 5). In the IL, SPS decreased average PACAP puncta per cell in male rats ($t(6) = 2.699$, $p = 0.036$) (Fig. 6A). Females showed no effect of SPS in this region (Fig. 6B). Prior studies have implicated the PL in SPS phenotypes, especially in females, so we also assessed PACAP expression in this region. Here, SPS increased PACAP expression in females ($t(10) = 3.773$, $p = 0.004$), but not in males (Fig. 6C and D). While males did exhibit a similar pattern, this group was underpowered due to tissue loss, occluding our ability to observe significant differences in this analysis. This data suggests that the PL may play a role in SPS PACAP interactions, with particular importance in females.

4. Discussion

We utilized SPS and IL-targeted pharmacology to explore the role of PACAP and sex in PTSD-like phenotypes. These studies revealed differential effects within sexes following SPS, as well as a potential role for IL PACAP signaling in governing this divergence in stress resilience.

PTSD disproportionately impacts women, often yielding unique symptoms, making the study of sex differences key to understanding this disorder (Ramikie and Ressler, 2018; Eder-Moreau et al., 2022; Shansky,

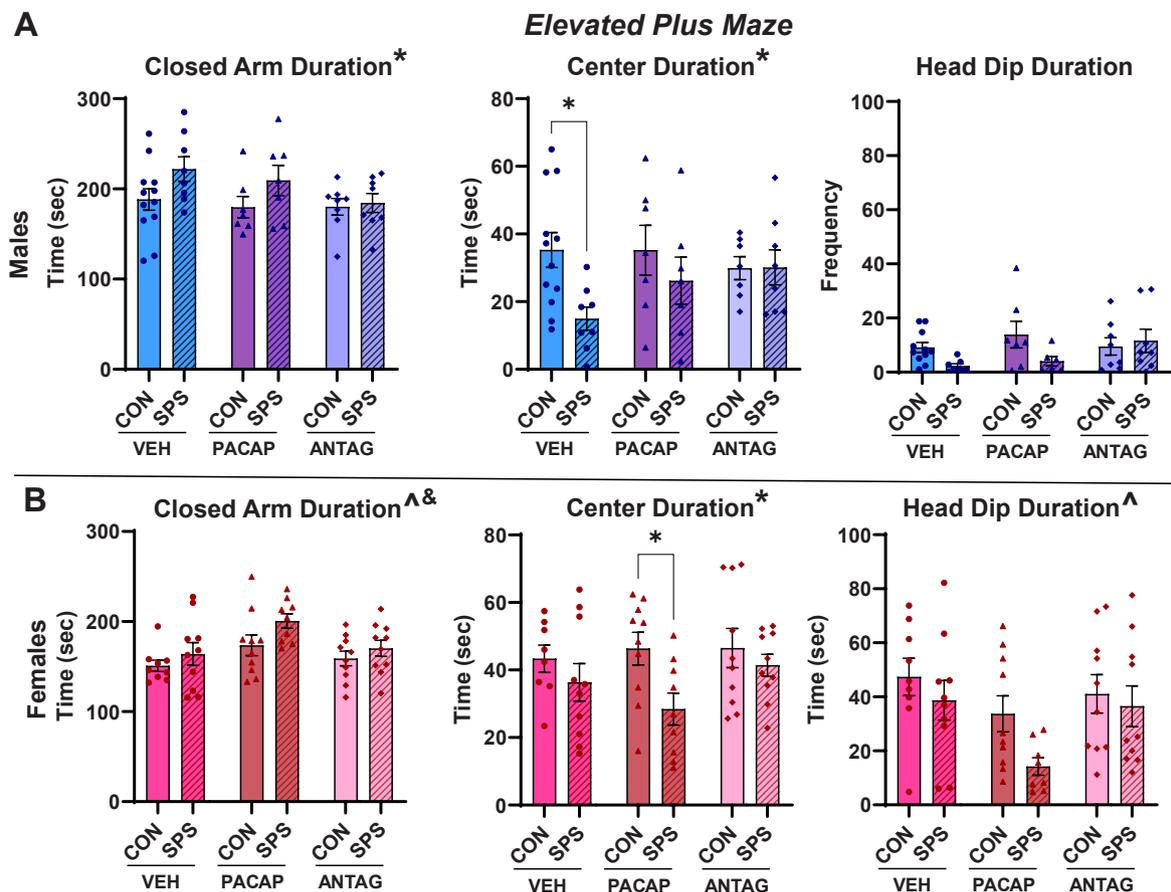


Fig. 4. SPS and PACAP Effects on Elevated Plus Maze Testing. (A) Male EPM closed arm duration, center duration, and head dip duration. (B) Female EPM closed arm duration, center duration, and head dip duration. $n = 7-12$. * = Main Effect of Stress. ^ = Main Effect of Infusion. & = Interaction. * = post hoc $p < 0.05$.

2015; Bangasser and Valentino, 2014; PTSD: National Center for PTSD, 2018). For example, most people experiencing PTSD will exhibit symptoms of re-experiencing and avoidance of cues related to the trauma, yet men tend to show more arousal-related symptoms and women tend to show more mood and cognition-related symptoms. Women also generally exhibit heightened symptom severity (Ramikie and Ressler, 2018; Pooley et al., 2018; Eder-Moreau et al., 2022; Hu et al., 2017). On the other hand, SPS in rats typically elicits stronger behavioral phenotypes in males, impacting domains associated with fear, anxiety, arousal, and sociability (Lisieski et al., 2018; Souza et al., 2017; Yamamoto et al., 2009; Eagle et al., 2013). While females do show some dysregulation in fear conditioning reinstatement and avoidance, these behaviors are often more subtle, drawing some concern about face validity (Cotella et al., 2023; Keller et al., 2015; Collins et al., 2023). This may be due to the fact that the SPS paradigm was developed in and validated with behavioral tests designed for males (Lisieski et al., 2018; Souza et al., 2017; Yamamoto et al., 2009). However, SPS does impact females on molecular level, often inducing changes in Fos and glucocorticoid receptor expression throughout the brain (Cotella et al., 2023; Pooley et al., 2018; Keller et al., 2015), suggesting that it is still a valuable model for understanding how females react to severely stressful experiences.

Here, we were able to uncover female SPS phenotypes in the PTSD-relevant domains of anxiety (i.e., decreased OF center time), threat (i.e., increased threat avoidance and decreased threat appraisal), and sociability (i.e., increased social interaction). Our ability to reveal these endpoints is primarily attributed to the approach of behavioral indexing, where instead of looking at individual behavioral endpoints, we examined broader behavioral domains by considering these individual

endpoints in tandem. While still not fully capturing the PTSD feature of stronger effects in females, the heightened threat avoidance and blunted threat appraisal phenotypes revealed with this method aligns nicely with PTSD symptomology in both men and women (NIH, 2023; Souza et al., 2017; Hu et al., 2017; Yamamoto et al., 2009). The negative correlation between threat avoidance and appraisal also suggests individual differences within SPS severity and coping strategies, another feature often seen in humans suffering from PTSD (NIH, 2023; Lisieski et al., 2018). An additional key finding here is the variance by sex in the sociability domain, with males having decreased sociability where females had increased sociability. This would suggest that where males may be more likely to withdraw from conspecifics, females may be more likely to seek out social peers. In humans, women are more likely to engage socially after trauma as a coping mechanism than men (Eder-Moreau et al., 2022; Hu et al., 2017; Olf, 2017). In fact, women who do not seek social support tend to have worse PTSD outcomes (Eder-Moreau et al., 2022; Hu et al., 2017), a notion that is supported here by a negative correlation between threat avoidance and sociability in female SPS rats. These findings not only show the value of behavioral indexing, but also have interesting implications for behavioral PTSD therapies in men vs. women.

We chose to focus on IL PACAP due to the established roles of the human vmPFC (analog to rodent IL) in governing PTSD symptoms (Martelle et al., 2021; Cotella et al., 2023; Bangasser and Valentino, 2014; Stark et al., 2015), along with known sex differences in PFC PACAP (Almli et al., 2013; Jovanovic et al., 2013; Ressler et al., 2011; Liao et al., 2019; Uddin et al., 2013). Using intra-IL cannulae, a PACAP agonist or antagonist was infused directly into the IL prior to SPS. While the resulting phenotypes were not overtly strong, there are some

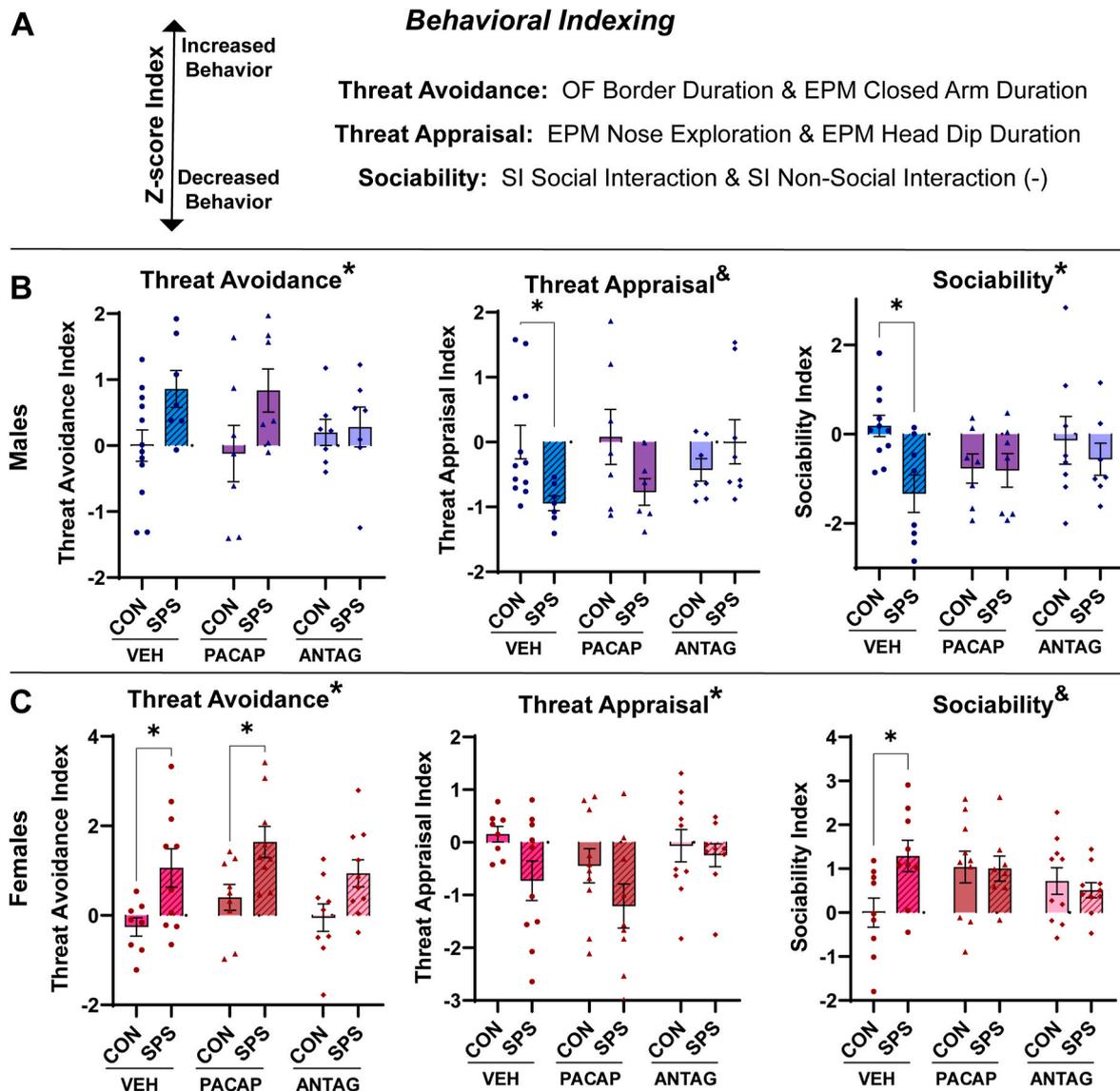


Fig. 5. SPS and PACAP Effects on Behavioral Indices. (A) Explanation of behavioral indexing. Z-scores were used to combine behavioral measures into PTSD-relevant indices of threat avoidance, threat appraisal, and sociability. A higher Z-score indicates an increase in that behavioral domain. (B) Male behavioral indices. (C) Female behavioral indices. $n = 7-12$. * = Main Effect of Stress. & = Interaction. * = post hoc $p < 0.05$.

patterns that are consistent with a role for IL PACAP in SPS, as well as variations by sex in PACAP signaling. Overall, it appears that IL ANTAG improved SPS phenotypes in males, while IL PACAP worsened SPS phenotypes in females. There is some important background information for interpreting these results in the present context. First, the IL-amygdala connection plays an important role in fear and anxiety-like behaviors (Martelle et al., 2021; Cotella et al., 2023; Ulrich-Lai and Herman, 2009). Typically, the IL inhibits the CEA via BLA connections, blunting expression of these phenotypes (Fig. 7A). Second, the main PACAP receptors (PAC1 receptors) are primarily expressed on inhibitory interneurons in the IL (Martelle et al., 2021). Therefore, increasing PACAP in this region would increase inhibitory drive, ultimately decreasing the excitatory output from the IL (Fig. 7B). Finally, this fear-related circuitry is differentially impacted by SPS in males and females (Cotella et al., 2023). In males, SPS decreases IL output, weakening the IL-BLA connection, permitting overall increased CEA output to drive SPS-related fear and anxiety-like phenotypes (Cotella et al., 2023; Marek et al., 2019; Nawreen et al., 2021) (Fig. 7C). However, different circuitry alterations seem to drive SPS phenotypes in females (Cotella et al., 2023). We have previously proposed that the IL-BLA activity

remains intact, while the CEA, this circuit's ultimate output node, instead exhibits direct hyperactivation (Cotella et al., 2023) (Fig. 7D). Thus, female SPS phenotypes emerge further downstream than male phenotypes. Considering these points together lends insight into the mechanism governing the present PACAP results. We propose that, in males, administering ANTAG to the IL would be expected to increase IL output via disinhibition of pyramidal neurons (Fig. 7B). This output increase counteracts the SPS-induced decrease in IL output (Nawreen et al., 2021), reducing overall amygdala output, helping to block SPS phenotypes (Fig. 7C). On the other hand, administering PACAP to females instead mimics decreased IL-BLA connectivity usually seen in males subjected to SPS, via increased interneuron activity in the IL (Fig. 7B). PACAP thus exacerbates SPS phenotypes in females by adding onto the preexisting hyperactivation of the CEA (Fig. 7D). Thus, underlying sex differences in PACAP physiology and SPS circuitry help to explain the divergent actions of IL PACAP manipulations by sex in the present studies.

A remaining question with this interpretation is why PACAP had minimal effects in males and ANTAG had minimal effects in females. It is important to remember that PACAP is a neuropeptide that acts to fine-

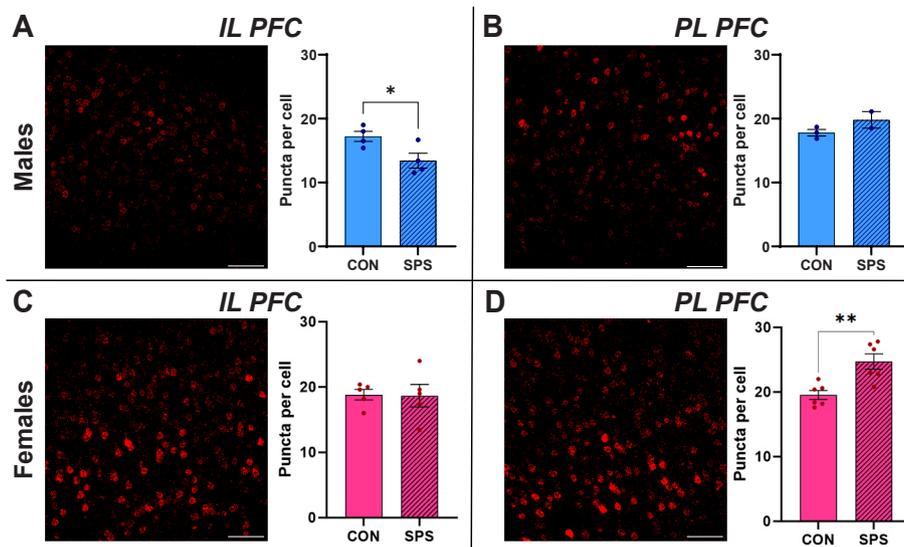


Fig. 6. SPS Effects on PFC PACAP RNA Expression. (A) Male IL PACAP Expression. (B) Male PL PACAP Expression. (C) Female IL PACAP Expression. (D) Female PL PACAP Expression. $n = 2-6$ rats, $N = 20-40$ cells. Scale bar = $100 \mu\text{m}$ * = $p < 0.05$.

tune circuitry (Hammack and May 2015; Mustafa, 2013; King et al., 2017; Bucher et al., 2021; Gilmartin and Ferrara, 2021). There is an inherent inverted-U to this type of molecule, with an optimal range of PACAP tone that helps to maintain normal function. We posit that inherent sex differences within this U-shaped curve contribute to the present results (Fig. 7E). The relationship between PACAP tone and SPS responsivity may be shifted in each sex, with females having higher sensitivity to increased PACAP (Fig. 7E, pink line) and males having higher sensitivity to decreased PACAP (Fig. 7E, blue line). Such a relationship would mean that females are less subject to stress-ameliorating effects of low PACAP but more vulnerable to stress-promoting effects of high PACAP. Therefore, in females, the PACAP agonist was sufficient to increase SPS severity (Fig. 7E, pink triangle), but ANTAG was unable to lower the levels enough to offer much benefit (Fig. 7E, pink square). On the other hand, males, with greater SPS responses to PACAP decreases than increases, were protected from harmful effects of too much PACAP (Fig. 7E, blue triangle) and benefited from ANTAG's effects (Fig. 7E, blue square). Thus, most of the animals in the present study fall within the optimal range of IL PACAP, including females receiving ANTAG and males receiving PACAP, and exhibit typical SPS effects. It is only in the PACAP females and ANTAG males that we move far enough along these differential curves to elicit heightened SPS effects in females and blunted SPS effects in males.

The present RNAscope results offer further insight into these relationships, namely that the IL may not be the most important PFC region for females with regard to SPS. Males exhibit decreased IL PACAP mRNA one week after SPS. This decrease in males may be compensatory, with the IL downshifting its production of PACAP in response to an initial increase acutely following SPS. While future studies examining acute responses to SPS are warranted, this supports the idea that males may benefit from IL ANTAG, as this may counteract an initial increase in PACAP following SPS. On the other hand, females show no change in IL PACAP mRNA after SPS. This could mean the female IL does not inherently respond as much to SPS as the male IL, a result which aligns with our lab's previous SPS FOS data (Cotella et al., 2023). Instead, the females show robust changes in the adjacent PL, with PACAP mRNA increasing one week after SPS. Where the IL inhibits amygdala output, the PL typically drives it, promoting defensive and stress-like behaviors (Ulrich-Lai and Herman, 2009; Knox et al., 2016; Giustino and Maren, 2015). An increase in PL PACAP following SPS, therefore, would be expected to serve a protective effect, inhibiting the PL via enhanced interneuron activation, thereby inhibiting the amygdala and its

stress-promoting effects. The PL has also been associated with PACAP sex differences, with females responding more to PL PACAP manipulations than males on fear conditioning endpoints (Kirry et al., 2018). Thus, the PL may be an intriguing future target for PACAP manipulations during SPS, especially in females.

There are a few important limitations to keep in mind for these interpretations. First, it is possible that the PACAP and ANTAG doses used here were not optimal, especially for females. These doses were chosen because they are behaviorally active upon intraparenchymal infusion in males. It is possible that drug sensitivity may differ by sex. A range of doses will be necessary to reveal the full nature of the inverted U curve proposed here for both sexes. Second, despite IL targeting (Supplementary Fig. 1) drugs could have diffused into the ventral PL. A study targeting the PL would be required help to untangle the differential effects of these two regions suggested by the present RNAscope data. Third, while we observe SPS and PACAP effects within each sex, future studies should be run in a way that is conducive to statistically testing for sex differences (i.e., running EPM with the same conditions for both sexes), thus enabling more definitive answers about the role of sex in an individual's response to SPS and PACAP manipulation (Garcia-Sifuentes and Maney, 2021).

There are also a few behavioral considerations that could strengthen these studies. One potential limitation here is that behaviors were run one week apart, putting the final test three weeks out from SPS. While we still observe SPS effects here, it is possible that we may have observed stronger effects if all behaviors were run closer to SPS. Another interesting future direction for these studies would be examining SPS effects on other PTSD-related behavioral domains in males and females. We have examined fear conditioning in the past and found that males are more susceptible to SPS-related extinction deficits, while females have stronger contextual and reinstatement phenotypes (Cotella et al., 2023). PTSD is also associated with impairments in cognition, motivation, and arousal (NIH, 2023; Bangasser et al., 2019; Donahue et al., 2016). SPS is known, at least in males, to impact similar behaviors, including spatial learning in the Morris water maze, attentional set shifting, sucrose preference, and acoustic startle (Mancini et al., 2021; Yamamoto et al., 2009). Given the divergent effects observed here, it would be interesting to examine SPS (and PACAP) effects on these behaviors in females, as well as on female exclusive behaviors such as maternal care (Ivy et al., 2008). Figuring out how to observe stronger SPS effects in females is a key question for the field to increase the face validity that we see with this model.

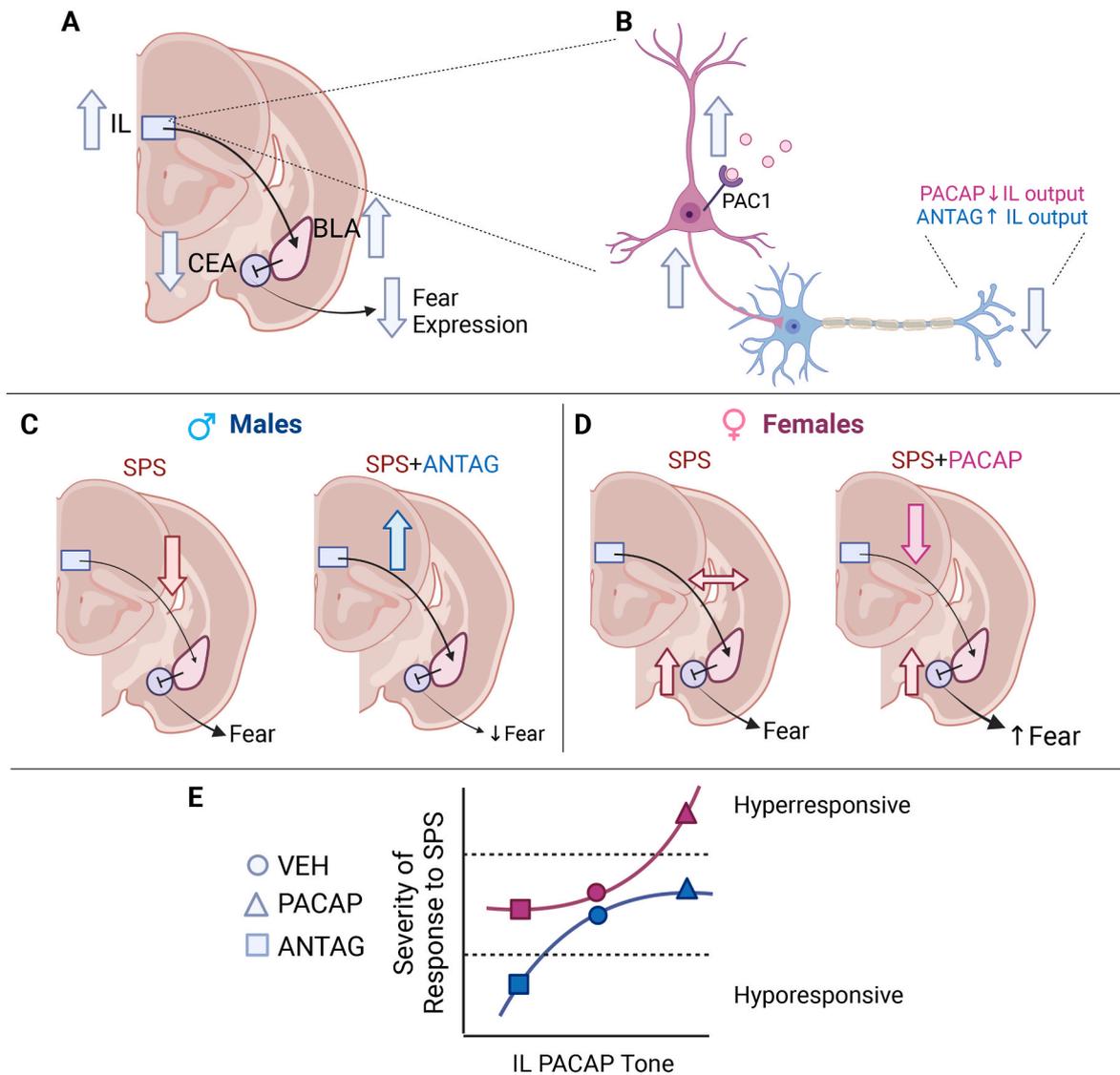


Fig. 7. Summary of the Interactions between SPS and PACAP in Males and Females. (A) The IL sends excitatory projections to the BLA, which inhibits the CEA via interneuron connections. This decreased CEA output suppresses fear expression. Thus, increased IL activity would typically decrease fear via this IL-amygdala circuit. (B) Within the IL, PAC1 receptors are primarily on inhibitory interneurons, suggesting that an increase in PACAP in this region would increase inhibitory activity, ultimately decreasing IL output. ANTAG would have the opposite effect, increasing IL output. (C) Previously our lab has demonstrated that, in males, SPS decreases IL excitation of the BLA, yielding fear expression in this model. In this case, infusing ANTAG into the IL restores this IL-BLA connection (via decreased interneuron activation), partly blocking the SPS effects observed here. (D) However, in females, SPS does not impact the IL-BLA connection, likely acting more downstream at the CEA level. In this case, increasing IL PACAP weakens the IL-BLA connection (via enhanced interneuron activation), enhancing SPS effects. (E) Note that the lack of effects in males receiving PACAP and females receiving ANTAG suggests a healthy range of IL PACAP tone, along with differences by sex within SPS \times PACAP interactions. It appears that the male response to SPS (blue) is not impacted by increasing PACAP but can be blunted in cases where PACAP tone is sufficiently decreased (ANTAG). On the other hand, the female response to SPS (pink) is not impacted by decreasing PACAP but can be exacerbated in cases where PACAP tone is sufficiently increased (PACAP). These unique male and female effects in pharmacology and circuitry have intriguing implications for not only how we understand the impact of neuropeptides on anxiety-like behaviors following traumatic stress, but also potential treatment options for PTSD in humans.

Further exploring the molecular components of these phenotypes would also be valuable. One element of this would be studying these relationships in other stress-regulating regions (Hammack and May 2015; Boucher et al., 2021; Ulrich-Lai and Herman, 2009). PACAP manipulation alone in the PL, BST, and BLA has been shown to impact fear- and anxiety-like behaviors (King et al., 2017; Kirry et al., 2018; Cho et al., 2012; Lezak et al., 2014). It would be interesting to see if SPS effects in these regions are similarly affected by PACAP. Another element would be a deeper examination of cell and receptor types. PACAP acts on multiple receptor subtypes, and while the target of these studies, the PAC1 receptor, has the highest affinity, PACAP also acts on VPAC1 and VPAC2 receptors which contribute to its function (Hammack and May 2015; King et al., 2017; Boucher et al., 2021; Hirabayashi et al.,

2018). These receptors are expressed on various cell types. While this is mostly interneurons in the PFC (Martelle et al., 2021), PACAP receptors are present in low levels on pyramidal neurons and glia which may also play a role in the present SPS phenotypes (King et al., 2017; Figiel and Engele, 2000; Kong et al., 2016; Taylor et al., 2014; Zhang et al., 2021). Furthermore, examining PACAP-related molecular changes at different times after SPS (such as acutely in the days immediately after drug administration and chronically towards the end of the behavioral timeline presented here) would yield valuable information about the temporal pattern of these effects and how they progress over time. Conducting studies on which elements in the PACAP system contribute to which SPS phenotypes is an important next step in understanding the complex relationships explored here.

One final future direction we want to propose here is testing the impact of PACAP manipulations after SPS. While these results suggest that PACAP plays a role in the molecular underpinnings of SPS, it is unclear if drug application after the fact could yield beneficial effects on SPS behavioral outcomes. Studies testing the therapeutic potential of PACAP manipulation will be an important step in applying this knowledge to the real world to help those suffering from PTSD.

5. Conclusions

These studies lend valuable insight into the interplay between SPS, sex, and PACAP which could have important implications for our understanding of PTSD. The unique male and female SPS behavioral phenotypes noted here are similar to PTSD symptomologies observed in men and women, namely congruent threat-related behaviors accompanied by divergent social characteristics. Male and female fear circuits exhibit differential responses to SPS, as well as IL PACAP, resulting in divergent effects of PACAP agonism and antagonism, with males showing improvement with ANTAG and females showing impairment with PACAP. Overall, the present results suggest that SPS can inform how PTSD impacts different behavioral domains in males and females, and that PACAP is an intriguing candidate contributing to these differences.

CRedit authorship contribution statement

Marissa A. Smail: Writing – review & editing, Writing – original draft, Visualization, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation. **Evelin M. Cotella:** Writing – review & editing, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Susan E. Martelle:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **James B. Chambers:** Methodology, Investigation, Data curation. **Ria K. Parikh:** Writing – review & editing, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Christine E. Moore:** Methodology, Investigation. **Ben A. Packard:** Project administration, Investigation. **Nawshaba Nawreen:** Writing – review & editing, Investigation, Data curation. **Rachel D. Moloney:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **James P. Herman:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Funding acquisition, Conceptualization.

Data availability

All data are available in the main text or supplementary materials.

Funding

This work was supported by I01BX003858, I01BX005923, MH049698, and MH119814 to JPH and MH125541 to MAS.

Declaration of competing interest

The authors declare no competing interests.

Acknowledgments

The authors would like to thank Rachel Morano, Parinaz Mahbod, and the Herman lab staff for their assistance with animal experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.yjnstr.2025.100727>.

Data availability

Data will be made available on request.

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