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Claire Deckers

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Anxiety Responses Across the Estrous Cycle in Relation to BNST-PACAP Interactions

UVM Undergraduate Thesis

Claire Deckers

Advisor: Sayamwong Hammack

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Abstract

Previous studies have provided strong evidence that the bed nucleus of the stria terminalis (BNST) is involved in anxiety behavior expression following chronic exposure to stressful stimuli. Pituitary adenylate cyclase-activating polypeptide (PACAP) mediated signaling has been linked to the regulation of anxiety related behavior within this region. PACAP functioning appears to be linked to sex, with PACAP blood levels being strongly correlated with post-traumatic stress disorder (PTSD) diagnosis and symptoms in female humans (Ressler et al., 2011). The goal of this study was to further explore this sex specific association by examining anxiety behaviors across the estrous cycle in relation to the presence or absence of PACAP in the BNST. This line of inquiry was examined using free cycling non-ovariectomized female rats at various stages of the estrous cycle that were intracranially injected with either PACAP or a vehicle. Anxiety behaviors were operationalized by categorizing responses in an elevated plus maze. Significantly higher instances of anxiety-like behaviors were observed in female rats injected with PCAP during proestrus and estrus, phases in which ovarian hormones are fluctuating. Thus, the findings of this experiment lend credence to the idea that sex hormones interact with the BNST in a manner that can lead to amplified anxiety-like behaviors. Further investigation of this relationship is warranted in order to further understand apparent sexual dimorphism in the display of anxiety-related disorders, such as PTSD.

Introduction

Post-traumatic stress disorder (PTSD) is a psychological disorder in which patients suffer from marked changes in behavior and mental/emotional wellness following the experience of a traumatic event as defined by the Diagnostic and Statistical Manual of Mental Disorders (5th ed.)
to include instance in which an individual is exposed to threatened death, serious injury, or sexual violence. This exposure can occur via direct experience, witnessing a traumatic event, learning of close friend or family member’s traumatic experience, or undergoing repeated exposure to traumatic events. Exposure to traumatic events is not unusual—around 60% of men and 50% of women will experience some form of trauma within their lifetime (Veteran’s Affairs, 2017). While most of these individuals will not go on to develop PTSD, a significant number of people do. The lifetime prevalence of PTSD within the United States is estimated to be around 7 – 8% (VA, 2017). It is notable that upon examination of PTSD diagnoses in relation to biological sex, lifetime prevalence of PTSD in men is estimated to be around 5%, whereas prevalence in women is around 10.4% (VA, 2017). Therefore, even though men are experiencing traumatic events at a higher rate than women, women are actually more than two times as likely to develop PTSD at some point in their lives.

To place some of these demographic incidence rates within the context of current world events, around 30.2% of individuals who have experienced severe COVID-19 infection have gone on to develop PTSD (Janiri et al., 2021). 55.7% of these PTSD patients were women, and biological sex was identified as an important risk factor for development of PTSD following acute critical infection (Janiri et al., 2021).

It is hard to fully estimate the economic burden of PTSD—as to our knowledge, studies examining health care costs due to PTSD are rather limited. However, it is known that within the United States the annual cost of anxiety disorders (including PTSD) is estimated to be between $56.97 billion and $62.76 billion (adjusted for inflation as of 2021) including both direct (medical) and indirect (productivity/mortality) costs (DeVane et al., 2005). In 2012, the Department of Defense spent around $294.1 million (around $336.91 million when adjusted for
inflation) on PTSD-related care for service members (National Academies of Sciences, 2014). Importantly, this number only includes direct medical costs for service members alone, and thus does not fully demonstrate the total economic impact of the disorder. One study examining direct medical costs places the economic burden of PTSD at around $12,815-21,927 (adjusted for inflation) per patient per year (Ivanova et al., 2011).

PTSD is marked by distinctive “intrusion symptoms”, including recurrent memories and dreams of the traumatic event(s) (American Psychiatric Association, 2013). Additionally, PTSD patients often experience dissociation (i.e. flashbacks), in which patients feel as if the event is re-occurring (APA, 2013). Patients will also display psychological distress and physiological stimulation when exposed to cues that remind them of the traumatic event(s) in some way (APA, 2013). In addition to these intrusion symptoms, patients with PTSD will also display systematic avoidance of stimuli associated with the traumatic event(s), as well as a pattern of negative cognitions and mood (APA, 2013). Importantly, individuals with PTSD will also often display alterations in arousal and reactivity (APA, 2013). This includes, but is not limited to, display of hypervigilant behaviors and exaggerated startle response, both of which are reflective of a state of heightened anxiety.

The disruptive nature of the disorder presents a strong incentive and motivation to better understand the underlying biological mechanisms behind PTSD. By doing so, treatment solutions can be better optimized to help patients with PTSD.

The Genetics of PTSD.

Previous studies have investigated genetic links with PTSD, in part to identify risk factors for development of the disorder. It has previously been demonstrated that adult children of
Holocaust survivors with PTSD are more likely to also develop the disorder compared to adult children of survivors without PTSD (Yehuda et al., 2001). This observation is consistent with the idea of a genetic basis for PTSD. Twin studies have also demonstrated that genetic influences are able to explain a large proportion of PTSD vulnerability, with most variance being accounted for by non-shared environmental factors (True et al., 1993; Stein et al., 2002).

Although these studies do suggest genetic influences in the development of PTSD, they do not demonstrate exactly which genes play a role in the etiology of the disorder. More recent research has focused on identifying genetic targets through genome wide association studies. Stein et al., 2021 found that several genes are implicated within the expression of the PTSD phenotype (Table 1). Most notably, \textit{MAD1L1}, a gene thought to play a role in cell cycle control, was repeatedly shown to be associated with PTSD across racial and ethnic groups (Stein et al., 2021). Mutations in this gene have also been shown to be associated with both schizophrenia (Ripke et al., 2014) and bipolar disorder (Stahl et al. 2019), suggesting that the gene might represent a general risk factor for the development of psychological disorders.

\textit{CRHR1}, a gene encoding a receptor for corticotropin releasing hormone (a hormone highly implicated in stress responding), has been found to be associated specifically with intrusive symptoms related to PTSD (Gelernter et al., 2019). In individuals diagnosed with PTSD, the gene has been found to be highly expressed within the amygdala, hippocampus, frontal cortex, and anterior cingulate – all brain structures thought to be involved in PTSD circuitry (Stein et al., 2021). Despite this, trials using a CRHR1 antagonist in women were not found to significantly reduce PTSD symptoms (Dunlop et al., 2017).
TSNARE1 (involved in intracellular protein transport) and EXD3 (involved in nucleic acid binding) were also associated with PTSD (Stein et al., 2021). Importantly, TSNARE1 has been shown to be associated with increased risk-taking behaviors, which might increase the likelihood that one will experience a traumatic event (Linner et al., 2019). Conversely, EXD3 was actually negatively correlated with PTSD diagnosis, although this relationship was found to be mediated by socioeconomic status (Polimanti et al., 2019).

Additionally, PARK2 and PACRG were both found to be positively associated with PTSD diagnosis (Stein et al. 2021). Both of these genes have previously been connected with susceptibility to leprosy and Parkinson’s Disease, but it is currently unclear what role they may play within PTSD etiology (Meschede et al., 2020).

Compelling results have also been reported in the case of mutations within ADCYAP1R1, the gene encoding PAC1, a receptor for pituitary adenylate cyclase activating polypeptide (PACAP) (Ressler et al., 2011). The association between ADCYAP1R1 and PTSD has been found in women alone. Because of this sex difference, it is possible that PACAP dysregulation could play a role in the unequal prevalence between men and women. PACAP is a polypeptide that has wide ranging effects throughout the body and brain (Shioda, 2000). PACAP has been referred to as a “master regulator” of stress systems, with its functioning being a point of focus for the Hammack Laboratory. Accordingly, it is reasonable to examine PACAPergic systems in relation to stress and anxiety-related disorders such as PTSD.

PACAP.

PACAP exists in two isoforms—PACAP38 and PACAP27 (named for the number of amino acids assembling the peptide), with the PACAP38 form being predominant within the
nervous system. It binds to multiple receptor subtypes, including the PAC₁, VPAC₁, and VPAC₂ receptors. The most common of these is the PAC₁ receptor. This receptor is a G-protein coupled receptor that is a member of the vasoactive intestinal peptide family. Although different subtypes are coupled with different signaling pathways, they all ultimately converge on the MEK/ERK pathway (May et al., 2014). PACAP-mediated ERK phosphorylation then functions to stimulate cAMP. In this manner, PACAP functions as a neurotransmitter, neuromodulator, and neurotrophic factor within the central nervous system (Arimura and Shioda, 1995).

PACAP has previously been implicated in the stress response and the display of anxiety-like behavior (Hammack et al., 2009; Hashimoto et al., 2011; Roman et al., 2014; Kocho-Schellenberg et al., 2014; Hammack and May, 2015). PACAP receptors are densely expressed in anxiety-related regions, and their expression can be upregulated in some of these regions following exposure to chronic stress. Because of this, PACAP is thought to be involved in stress-related neurocircuitry within these brain regions, and is implicated in control of physiological and psychological responding to stress (Ressler et al., 2011; Lehmann et al., 2013).

In this case, variations within ADCYAP1R1 are especially interesting, as the association with PTSD was located within a single nucleotide polymorphism (SNP) “risk allele” that is found in conjunction with an estrogen response element (ERE) involved in PAC₁ gene regulation. Additionally, this SNP was associated with PTSD diagnosis in women, but not men.

Social and cultural factors likely play a role in PTSD development and may contribute to differences in PTSD prevalence among demographic groups. However, there may also be differences in underlying biological mechanisms. Note that none of the previously mentioned genes seem to address a potential biological basis for sex differences observed in PTSD.
diagnosis rates. Thus, _ADcyAP1R1_ presents itself as a potential candidate for further illumination of sex-related differences in demographic incidence of PTSD.

In support, in humans that have been diagnosed with PTSD, there is an association between PACAP and PAC₁ blood levels and sex, with higher levels and expression in females (Ressler et al., 2011; Lezak et al., 2014; Ramikie and Ressler, 2016; Mercer et al., 2016).

As previously mentioned, PACAP and its receptors display dense distribution within stress and anxiety related brain areas, including the medial prefrontal cortex, hippocampus, amygdala, and bed nucleus of the stria terminalis (BNST) (Hashimoto et al., 2011). As to our knowledge, only one study has examined sex differences in PACAP expression and receptor distribution without inducing stress, finding that male rats express higher levels of PACAP within the adrenal gland than females (Mosca et al., 2015). Male rats also displayed significantly lower levels of PACAP within the superior cervical ganglia, as well as a significantly lower number of VPAC2 receptors within the hypothalamus as compared to female rats (Mosca et al., 2015). The scope of this study was limited, focusing only on a few structures, so it is hard to draw complete conclusions about PACAP-related expression in males and females.

The relationship between PACAP and the BNST has been a strong point of interest within the Hammack Laboratory. A number of previous studies within the laboratory suggest that PACAP in the BNST is critical for stress-induced anxiety (Hammack et al., 2009; Hammack et al., 2010; Lezak et al., 2014; Roman et al., 2014; Hammack and May, 2015; Goode and Maren, 2017; King et al., 2017). Consequently, the lab is interested in studying PACAP functioning within the BNST in the context of anxiety-related disorders like PTSD.

**The BNST.**
The BNST is a sexually dimorphic structure. General morphology of the BNST differs between males and females. In male animals, the volume of the medial posterior region of the BNST is larger than that in females (del Abril et al., 1987; Shah et al., 2004). Conversely, the medial anterior region of the BNST shows a greater volume in females than in males (del Abril et al., 1987). Outside of structural differences, it seems that functional connectivity and circuitry within the BNST also display sex differences. It has been demonstrated that the BNST binds high levels of sex hormones early in life, which is the period when functional differentiation occurs (Hines et al., 1985). Because of this, it is likely that this hormone-binding contributes to both morphological and functional sex differences. Males appear to display more robust projections to other brain regions, as well as displaying a higher density of arginine vasopressin and androgen receptors (Hutton et al., 1998; Gu et al., 2003; Han and de Vries, 2003; Shah et al., 2004). Importantly, females display a higher density of CRH neurons than males, which is relevant when examining fear and anxiety related behaviors (Uchida et al., 2019).

The BNST is considered to be a part of the extended amygdala, and has been implicated in the stress response and human psychiatric disorders (Lebow and Chen, 2016). The BNST is thought to play a role in the mediation of the hypothalamic-pituitary-adrenal axis (HPA), the sympathetic nervous system, and other central nervous system (CNS) circuits that are engaged following exposure to a stressor (Walker et al., 2003; Herman et al., 2005; Walker and Davis, 2008; Walker et al., 2009; Radley and Sawchenko, 2011). Importantly, the BNST plays a major role in the regulation of the paraventricular nucleus (PVN) of the hypothalamus, which then serves to regulate the HPA axis and endocrine responses to stress (Radley and Sawchenko, 2011). Because of this, it is important to further explore dysregulation of the BNST and how it contributes to the regulation of other stress-related neurocircuitry.
The BNST has also been shown to play a critical role in regulating response to long-duration stressors. Accordingly, the BNST is argued to function to mediate anxiety-related behaviors (Walker et al., 2003; Waddell, et al., 2006; Walker and Davis, 2008; Walker, et al., 2009; Radley and Sawchenko, 2011; Hammack et al., 2015). Based upon these findings, it then follows that altered or maladaptive BNST functioning is likely involved in the development of anxiety disorders. It is thought that lengthy exposure to stressors may increase BNST functioning, leading to anxiety. To evidence this, chronic exposure to stressors has been associated with enhanced BNST neuroplasticity (Stout et al., 2000; Vyas et al., 2003; Choi et al., 2006; Pego et al., 2008; Hammack et al., 2009).

Past examinations of the neural basis of PTSD symptomatology have highlighted the role of the amygdala in the experiencing of flashbacks (Liberzon & Sripada, 2007; Hayes et al., 2011; Bourne et al., 2013; Storm et al., 2013; Andrewes & Jenkins, 2019). However, anxiety-associated symptoms like hypervigilance also are distinctive markers of PTSD. Because the BNST has been shown to be heavily involved in anxiety processes, the BNST may contribute to hypervigilance and enhanced startle observed in PTSD.

To support this, it has previously been demonstrated that veterans diagnosed with PTSD display amplified dark-enhanced startle behaviors (Grillon et al., 1998). Dark-enhanced startle is a form of anxiety responding that has been shown to be dependent upon the BNST (Walker et al., 2009). Kamkwala et al., 2012 performed a similar study in civilian populations, but found that dark-enhanced startle was only increased in females diagnosed with PTSD. The ADCYAP1R1 SNP has also been studied relation to dark-enhanced startle, and has been found to be associated with increased startle responding in both male and female children (Jovanovic et
al., 2013). However, when studied in adult populations, dark-enhanced startle is only shown to be significantly increased in females with the *ADCYAP1R1* SNP (Ressler et al., 2011).

**Sex Differences in BNST-PACAP Interactions.**

Previous work has also demonstrated that sex hormones may differentially affect PACAP’s functioning within the BNST. It has been found that increased PACAP and PAC₁ transcript levels were found in ovariectomized female rats that were given estradiol (Ressler et al., 2011). It has also been noted that when PACAP is injected into the posterior BNST, anorexia and weight loss can be observed in both male and female rats (Kocho-Schellenberg, et al., 2014). Anorexic behaviors were also found to be stronger in rats that were treated with estradiol, further suggesting an interaction of estradiol with PACAPergic systems.

Additionally, it has been found that startle amplitude and corticosterone levels increased following intra-BNST injection of PACAP in male rats that has previously been exposed to CVS (King et al., 2017). However, CVS was not found to increase startle response in female rats. However, BNST PAC₁ receptor transcripts were shown to be upregulated in both males and females. These results drew researchers to the conclusion that exposure to stressors alters neural circuitry in males and females in different ways and intra-BNST PACAP effects are related to sex, which remains to be clarified.

Based upon this previous literature, it is likely that sex hormones differentially interact with central PACAP function, thus creating dissimilarities in stress-related neural circuitry. Interactions between estradiol and PACAP within the BNST are of particular interest, due to the *ADCYAP1R1* SNP being located within an ERE. This suggests that the PAC₁ receptor may be regulated by estradiol, and could imply that estradiol may regulate PACAP-dependent behaviors.
such as anxiety. **Figure 1** illustrates the hypothesized model of estradiol’s interaction with the *ADCYAP1R1* SNP, and how dysregulation within this system may lead to increased incidence of PTSD symptomatology.

Estradiol, like other sex hormones, fluctuates throughout the estrous cycle in females. In rats the estrous cycle lasts for four days and consists of four distinct stages—proestrus, estrus, diestrus 1, and diestrus 2. Estradiol levels rapidly climb and peak during proestrus (Marcondes et al., 2002). Ovulation occurs at the start of estrus, after which estradiol levels plummet and return to baseline by the end of estrus (Marcondes et al., 2002; Paccola et al., 2013). Other sex hormones such as prolactin, luteinizing hormone (LH), and follicle stimulating hormone (FSH) increase during proestrus and begin to return to baseline levels after estrus (Marcondes et al., 2002). Progesterone levels have two peaks—one during diestrus 1 and one towards the end of proestrus, with fluctuating levels in between (Marcondes et al., 2002). See **Figure 2** for an illustration of relative hormone levels over the course of the estrous cycle.

Based upon this background of research, we theorized that increased presence of estradiol during certain phases of the estrus cycle might interact with PACAPergic circuitry in the BNST, regulating the behavioral output following BNST PACAP infusion in the form of amplified anxiety-like responding. We examined this theory in our experiment by looking at the effects of intra-BNST PACAP on anxiety-like behaviors across the course of the estrous cycle in rats. Contrary to our initial hypothesis, significantly higher instances of anxiety-like behaviors were observed in female rats in proestrus and estrus that had been injected with PACAP. Therefore, we concluded that intra-BSNT PACAP increases anxiety when ovarian hormones are in flux.

**Methods**
Subjects.

Adult female Sprague-Dawley rats (225 – 275 g) were purchased from Charles River Laboratories (Wilmington, MA). After being delivered to the Dewey Hall research facilities, rats were allowed to habituate for at least five days prior to any experimental procedures. Subjects were placed in grouped housing prior to surgery, and then were moved to single housing after cannulation surgeries to ensure optimal recovery conditions. In both housing environments, rats were maintained on a 12-hour light/dark cycle, with lights turning on at 7:00. Rats had access to food and water ad libitum both before and after surgery.

The experimental groups consisted of rats in each of the four stages of the estrous cycle (proestrus, estrus, diestrus 1, and diestrus 2), with the rats in each group having either PACAP or a vehicle infused. There were eight rats in each experimental group, for a total of sixty-four subjects. All of the procedures and use of animal subjects were approved by the University of Vermont’s Animal Care and Use Committee.

Surgical Procedures.

Rats were deeply anesthetized with isoflurane vapor prior to undergoing surgery. The rats’ heads were completely shaved of fur. Rats were then placed on the surgical table, with their noses inserted into a nose cone in order to provide and constant and steady supply of isoflurane and oxygen. Throughout the surgical process, sedation was monitored by using a gentle toe pinch and checking for physiological response. Thermoregulation was provided with a heating pad. Rats where then mounted onto the stereotaxic axis by inserting ear bars into the ear canal, and then tightening into place and ensuring that the head would not move and would maintain its placement. Lubricant was placed on the rat’s eyes in order to protect them from drying during
the surgical process. The shaved skin was then cleaned thoroughly with alcohol and iodine. An incision (about 2 cm long) was then made, and bulldog clamps were used to pull the skin back and expose the skull. Soft tissue was lightly scrubbed from the skull. Using a scalpel, a scoring pattern was made upon the skull. The area was then cleaned with hydrogen peroxide to ensure that the skull was completely dry. Bregma was then marked, and then four holes were drilled into the surrounding plates, with care being taken so the drill bit did not penetrate into the brain itself. Four screws were then inserted into each of these holes to provide stability for the headcap. Additional holes were then drilled at the following coordinates relative to bregma in order to target the BNST region: 0.4 on the anterior/posterior plane, +3.8 on the medial/lateral plane, and -5.3 on the dorsal/ventral plane. Using a stereotaxic device, cannulas (26 gauge, Plastics One, Roanoke, VA) were inserted at a 20-degree angle. Dental cement and dental acrylic liquid were applied to the skull in order to make a headcap to hold the cannula in place. Following complete drying of the headcap, Lactated Ringers and carprofen (0.5 mg/kg) were injected subcutaneously.

**Recovery and Estrous Cycle Tracking.**

The rats were given at least one week of recovery time following surgery. During this time, rats were weighed daily and monitored for eating behaviors due to concerns about possible anorexic behaviors or slow recovery from surgery. Rats were given one additional injection of carprofen (0.5 mg/kg) on the day following surgery and then on an as-needed basis if the animal appeared to be in pain.

After this post-op period, vaginal swabs of each rat were taken, and examined for their cytology in order to determine which phase of the estrous cycle the subject was in (**Figure 3**).
Swabs were taken as close to noon as possible. Swabbing continued until an established pattern was formed and the subsequent phase was able to be accurately predicted. The minimum time required to accurately determine estrous cycle was four to five days—equating to the observation of one full estrous cycle.

**PACAP Infusion and Elevated Plus Maze Testing.**

Behavioral testing for all animals took place in the afternoon (12:00 h – 16:00 h). Rats were removed from their home cages and gently restrained in a towel. They were then infused bilaterally via the surgically placed cannulas over a one-minute period with either PACAP (American Peptide Co., Sunnyvale, CA; 0.1–1 μg in 0.5 μl per side) or a vehicle (0.05% BSA in saline) using an 11 mm internal cannulae attached to a 10 μL 700 series Hamilton syringe. Rats were then returned to their home cages and left to sit for fifteen minutes to allow PACAP to take effect.

Behavioral testing then took place, using an elevated plus maze. The arms of the maze that measured 60 cm long and 10 cm wide and extended from a 9 cm x 9 cm central platform. The two enclosed arms had opaque walls that were around 30 cm high. The room in which behavioral testing took place was dimly lit by bulb emitting red light. Rats were placed in the maze in the center square facing a closed arm away from the door. Their behavior was then recorded via an external camera. A five-minute segment of the video was then analyzed by two observers, both blind to the treatment condition. Analysis of videos began at the time when the researcher had left the room and the door was completely shut. Those analyzing the videos recorded time spent in open arms, time spent in closed arms, number of arm entries (defined by all four paws crossing a border into an open or closed arm), and number of open arm entries.
Cannula Verification.

Within the days following the completion of behavioral testing procedures, rats were transcardially perfused with saline followed by 4% paraformaldehyde. Brains were then removed and fixed with 4% paraformaldehyde for an additional 48 hr before transferring to a 30% sucrose solution for cryoprotection.

For sectioning within a cryostat, brains were placed within a mold and frozen in Tissue-Tek. 60 micrometer slices were taken and cannula placement was verified visually. If cannula placement fell outside of the BNST region, subjects were not included in analysis.

Statistics.

Results were first analyzed using Levene’s test for equality of variance. Data was then examined via factorial ANOVA and pairwise comparisons using SPSS version 27 (IBM Software, Armonk, NY). Additionally, post hoc Tukey’s comparisons were performed utilizing the SPSS software.

Results

Cannula Verification.

A visual examination showed that most cannulae were correctly aimed at the region of the BNST. A total of 5 animals were removed from analysis due to improper cannula placement. Three of these were treated with PACAP—one within estrus, one within diestrus 1, and one in diestrus 2. Additionally, two animals in the vehicle condition were removed from the diestrus 2 group.
**Time in Open Arms.**

There was an overall effect of PACAP on open arm time, indicative of an anxiogenic effect, $F(1,58) = 0.001, p < 0.05$. Moreover, there was a significant interaction between estrous phase and PACAP treatment, $F(3,58) = 0.042, p < 0.05$, suggesting that the increase in the anxiogenic effects of PACAP depended on phase of estrous. A visual inspection of the data suggests that the anxiogenic effects of PACAP may be stronger during phases when hormones are fluctuating significantly (proestrus and estrus), although post-hoc were not able to confirm these observations. Interestingly, there was no main effect of estrous phase on open arm time, $F(3,58) = 0.407, p > 0.05$ ([Figure 4](#)).

**Arm Entries.**

In order to determine whether PACAP infusion altered locomotor activity more generally, the total number of arm entries into open and closed arms were analyzed. The animals were scored on the number of times that all four paws crossed a border entering an open or closed arm.

There was no effect of PACAP on total arm entries, $F(1,58) = 0.134, p > 0.05$, indicating that PACAP does not have an effect on overall locomotion. Additionally, there was no effect of phase on total arm entries $F(3, 58) = 0.199, p > 0.05$, nor was there a significant interaction between estrous phase and PACAP treatment $F(3,58) = 0.289, p > 0.05$. Interestingly, while the interaction was not significant, visual inspection of the data shows that PACAP trended towards a decrease in total arm entries during proestrus and estrus, consistent with open arm time ([Figure 5](#)).
**Open Arm Entries.**

In order to determine whether PACAP infusion altered entrances in to open arms alone, the animals were then scored on the number of times that all four paws crossed a border entering an open arm.

There was an overall effect of PACAP on open arm entries, indicative of an anxiogenic effect, F(1,58) 0.010, p < 0.05. However, there was not an effect of phase F(3,58) 0.264 > 0.05 or a significant interaction between estrous phase and PACAP treatment, F(3,58) 0.063, p < 0.05, suggesting that the increase in the anxiogenic effects of PACAP do not depend on phase of estrous. A visual inspection of the data suggests that the anxiogenic effects of PACAP may be stronger during phases when hormones are fluctuating significantly (proestrus and estrus) ([Figure 6](#)).

**Open Arm Entries Ratio.**

Open arm entries were then examined as a function of total arm entries in order to control for overall entries (i.e. locomotion).

There was an overall effect of PACAP on the ratio of open arm entries, indicative of an anxiogenic effect, F(1,58) 0.002, p < 0.05. Despite this, there was not an effect of phase F(3,58) 0.326, p > 0.05 or a significant interaction between estrous phase and treatment F(3,58) 0.187, p > 0.05. Similar to the above observations, there is a trend towards an increased anxiogenic effect when treated with PACAP, especially during proestrus and estrus ([Figure 7](#)).

**Discussion**
Our data suggested that PACAP increased anxiety-like behaviors during both proestrus and estrus, but PACAP did not increase anxiety-like behavior during the other estrous phases. Proestrus and estrus are phases in which ovarian hormones, particularly estradiol and progesterone, are fluctuating rapidly and dramatically. Because there are multiple hormones changing during this time period, we cannot localize the effects to one hormone alone. Instead, we can conclude that intra-BNST PACAP infusion results in increased anxiety likely during estrous cycle phases in which ovarian hormones are in flux.

Previous studies have demonstrated that hormonal flux appears to increase the likelihood of experiencing mood disturbances and the development of affective disorders (Ahokas et al., 2001, Parker & Brotchie, 2004, Douma et al., 2005, Solomon and Herman, 2009, McHenry, et al., 2013). Within our experiment, we also observed that anxiety was not increased during estrous phases with more stable hormone levels. This provides a potential role for hormone stability as having a protective effect on anxiety-like behaviors.

While this study did not evaluate the effects of particular hormones, prior work has highly implicated estradiol’s interactions with the PACAP system, due to the ADCYAPIR1 SNP’s location within an ERE. In order to analyze the effects of estradiol alone, future studies could be conducted with ovariectomized rats given exogenous estradiol once every four days in order to simulate fluctuating levels of estradiol without the influence of other female sex hormones. It would also be recommended that blood serum levels of estradiol should be collected at the time of behavioral testing and examined in order to confirm hormone levels in a more precise manner. Doing this will allow for the development of a more precise model of estradiol-PACAP interactions, and provide more direction for future lines of inquiry.
Estradiol has two receptors-- ERα and ERβ (Gross & Yee, 2002). These receptors are differentially distributed within males and females. ERα is primarily expressed within female sex organs, whereas ERβ is expressed in a much wider variety of cells, but to a higher degree in females than in males (Chen et al., 2019). ERα and ERβ are two distinct receptors, with non-redundant roles. Both receptors have been found to be associated with different aspects of synaptogenesis and synaptic plasticity (Bondesson et al., 2014). Additionally, ERα is associated with behaviors related to mood regulation and is thought to have an anxiogenic effect, whereas ERβ is implicated in cognitive processes and is associated with anxiolytic properties (Lee et al., 2012; Borrow & Handa, 2017).

Although the roles of these two receptors are discrete, they both have similar mechanisms of action. The first of these is the “classical” mechanism of action, which acts to ultimately affect genomic transcription (Deroo & Korach, 2006). In this mechanism, estrogens diffuse into the cell and bind to the estrogen receptor (ER) in the nucleus. This estrogen-ER complex will then bind to EREs. This can occur either directly or indirectly via interactions with proteins within the promotor region of genes that respond to estrogen. This binding will then function to recruit coregulatory proteins to the promotor regions, thus facilitating the recruitment of RNA polymerase II transcriptional activity (Heldring et al., 2007). The exact mechanisms surrounding this binding are not well understood in terms of ligand specificity, ER subtype specificity, and interaction domains. Ultimately, this process results in the increase or decrease of mRNA and changes in associated protein production, which can contribute to alterations in physiological response. To contextualize this process within PACAPergic systems, Given the ADCYAP1R1 SNP’s location within an ERE, it follows that the presence of estrogen is able to induce changes in PAC1 expression, potentially contributing to alterations in anxiety-like behavior. This
regulation will be discussed further below. Importantly, the ER’s classical mechanism of action is a longer-term mechanism, occurring over the course of multiple hours (Deroo & Korach, 2006).

Conversely, the non-genomic mechanisms of ERs are much faster-acting, and can initiate an effect on the order of minutes or even seconds. In this case, estrogens interact with ERs or non-ER estrogen binding proteins located on the plasma membrane (Deroo & Korach, 2006; Heldring et al., 2007). In this case, binding causes the activation of second messenger cascades that ultimately function to increase intracellular levels of Ca\(^{2+}\) or NO, as well as the activation of kinases. **Figure 8** provides an illustration of both the classical and non-genomic pathways associated with estrogen-binding to ERs.

This division of genomic and non-genomic mechanisms might contribute to the effects seen within this experiment. The immediate effects of estradiol binding and activating the non-genomic pathway could contribute to significantly increased anxiety seen during proestrus. However, the classical pathway takes hours to reach its full effect, which means that the elevated level of estradiol leading up until ovulation in estrus could generate prolonged effects that generate physiological and behavioral changes even after estradiol levels have begun to decline and return to baseline (**Figure 9**). This model could be tested by utilizing a transcription factor decoy against the promoter region of the ERE (as in Wang et al., 2003 and Lambertini et al., 2005), thus preventing the estradiol-ER complex binding to this region and effecting downstream classical effects. By doing this, the non-genomic mechanisms can be isolated and examined with a greater degree of specificity.
Because ERα and ERβ have distinct pathways and roles, it is possible that these two pathways could be selectively stimulated/inhibited and analyzed in relation to a functional role in anxiety-like pathologies. Potentially, these pathways could be therapeutic targets if they are found to play specific roles in anxiety-related circuitry. Importantly, half of the neurons in the principal nucleus of the BNST express ERα, and most of these neurons co-express ERβ (Shughrue et al., 1998; Leite et al., 2014). The presence of ERα is modulated by the relative presence of female sex hormones, with higher hormone levels at proestrus correlated with a lower number of ERα positive neurons within the BNST (Leite et al., 2014). This downregulation most likely is directly tied to the increased presence of estradiol within the system, as progesterone has been demonstrated to have no effect on the total number of ERα positive neurons within the principal nucleus of the BNST (BNSTpr) (Leite et al., 2014). Additional evidence suggests that ERα and ERβ can be selectively activated, and that activation of ERβ can result in the modulation of ERα gene transcription (Frasor et al., 2003, Hall and McDonnell, 1999, Lindberg et al., 2003, Sá et al., 2009, Sá et al., 2013). Thus, it is thought that estradiol decreases total number of ERα-positive BNSTpr neurons by activating ERβ, which contributes to a decrease in behavioral and physiological responses associated with ERα alone.

Previous work has demonstrated that the combination of fear conditioning and estradiol treatment able to induce the expression of PAC1 receptors through ERα binding and signaling via the classical mechanism of action (Mercer et al., 2016). In individuals with the ADCYAPIR1 SNP, normal signaling within this pathway is most likely disrupted, causing increased vulnerability to anxiety-related disorders such as PTSD. Additional work demonstrates that DNA methylation is reduced by ERα binding, which suggests that epigenetic mechanisms may be involved in the upregulation of ADCYAPIR1 (Ung et al., 2014).
In addition to estradiol, progesterone levels show large fluctuations during proestrus and estrus. Future studies could explore the effects of progesterone alone by utilizing ovariectomized rats given exogenous progesterone or its metabolite allopregnanolone once every four days to simulate fluctuating levels of progesterone without the influence of other female sex hormones. Studies examining the anxiety-related effects of progesterone have produced somewhat conflicting results. Progesterone is traditionally thought to have anxiolytic properties, mediating the activity of anxiety-related circuitry through its role as a positive allosteric modulator of GABA_A activity (Picazo & Fernandez-Guasti, 1995; Le Melledo & Baker, 2004; Reddy et al., 2005; Flores et al., 2020). However, some evidence in humans suggests that progesterone can have anxiogenic effects by selectively increasing amygdala reactivity (van Wingen et al., 2007; Reynolds et al., 2018), which may be due to changes in GABA_A expression over the course of the estrous cycle, but this effect remains to be completely elucidated (Maguire et al., 2005). Interestingly, several studies have reported that rapid decreases in progesterone levels may increase anxiety-like behavior (Perna et al., 1995; Smith et al., 1998; Gulinello et al., 2002; Smith et al., 2006; Nillni et al., 2011), which may be relevant for the increased anxiety-like behavior seen during estrus within our experiment.

Research examining LH and FSH is somewhat limited, but there is some evidence to demonstrate that both may have anxiogenic effects (Warnock & Bundren, 1997). Additional studies similar to the proposed estradiol/progesterone examinations would allow for further untangling of complex interactions between the individual ovarian hormones and stress-related neurocircuirty.

Although results of this experiment did seem to point to heightened anxiety behaviors in PACAP-treated animals during phases in which ovarian hormones are in flux, we did not
perform post-hoc analyses in order to confirm this observation. Because of this, it is possible that this effect was actually restricted to one estrous cycle phase. If this is the case, it seems likely that the effect is localized within estrous as opposed to proestrus based upon a visual observation of the data. This would mean that PACAP increases anxiety-like behaviors in phases with especially low ovarian hormone levels. Several studies have reported that low ovarian hormone levels are associated with an increased display of anxiety-related behaviors (Zimmerberg & Farley, 1993; deChaves et al., 2009; Lebron-Milad & Milad, 2012; American Physiological Society, 2018), but the interaction between low female sex hormone levels and PACAPergic circuitry has not been explored. To examine this question, future studies could be conducted with ovariectomized rats given no hormone replacements in order to simulate especially low hormone levels.

An additional avenue to explore is potential sex differences in PACAPergic signaling. As mentioned earlier, there are multiple pathways that can be stimulated by PACAP binding to its receptors. Generally, PAC$_1$ is thought to be positively coupled to adenylyl cyclase and phospholipase C. However, it has previously been shown that different variants of the PACAP receptor result in differential signal transduction (Spongier et al., 1993; Zhou et al., 2002). Depending on which PAC$_1$ isoform is present, PACAP binding can differentially act through Gs, Gq, or β-arrestin in order to activate multiple canonical signaling pathways, including AC/cAMP/PKA, PLC/DAG/IP$_3$/PKC, cAMP/NCS-RapGef2/Rep1/MEK, and MEK/ERK (King et al., 2017; Johnson et al., 2019). Through these pathways, PAC$_1$ receptor signaling is able to mediate a wide variety of cellular functions, and can produce diverse downstream consequences. This can include enhanced neurotransmitter and neuropeptide synthesis and release, and changes in structural plasticity within stress and anxiety-related neurocircuitry (King et al., 2017). Both
forms of VPAC receptors are thought to couple Gs, activating adenylyl cyclase, and increasing intracellular cAMP levels (Laburthe & Couvineau, 2002; Laburthe et al., 2007; Johnson et al., 2019). There have been other studies that have provided evidence that VPAC receptors might also trigger biological responses through other signaling pathways, although findings from these studies have been slightly contradictory (Laburthe & Convineau, 2002). Further clarity is needed in order to illuminate the exact functioning and downstream effects of these receptors.

Sex differences in coupling and functioning should be examined within these signaling cascades. Sex differences have been found within other stress and anxiety-related receptor activity, particularly within CRH systems. The CRH1 receptor is normally coupled to either β-arrestin and Gs pathways. However, following exposure to stress, CRH1 receptors in females demonstrates decreased linkage to β-arrestin, and enhanced coupling to Gs (Bangasser et al., 2010). This results in an amplified response to acute stress in females, as well as a weakened ability to develop compensatory mechanisms in response to chronic stress. These findings are especially important in light of the similarities between the PAC1 receptors and CRH1—both belong to the Class B family of seven transmembrane G-protein coupled receptors, and activate similar downstream signaling pathways following ligand binding (Valentino et al., 2013). Thus, it is possible that analogous sex differences may occur within PACAPergic systems, with estrous cycle phase biasing the type of PACAP signaling pathway utilized.

Another route of exploration could be to examine if physiological changes in PACAPergic systems correlate hormonal fluctuations across the course of the estrous cycle. Transcript levels for all of the PACAP receptors (PAC1, VPAC1, and VPAC2) should be examined during each of the four phases of the estrous cycle in order to see if the expression pattern of receptors is changing throughout the estrous cycle. It is necessary to analyze mRNA
transcript levels for all three of PACAP’s receptors in order to fully account for any changes or fluctuations that might be taking place. In conjunction with this, western blots should be conducted to analyze any changes in actual protein levels associated with mRNA transcripts, as changes in transcript level do not necessarily equate to changes in protein production/presence.

These physiological changes within the context of PACPergic-BNST symptoms are valuable to explore, as they may contribute to increased incidence of BNST-mediated anxiety-related symptomatology (as discussed within the introduction). As females with PTSD report higher incidence of hypervigilance and hyperarousal (i.e., anxiety-related symptoms) than men, changes in PTSD-related PACAPergic circuitry may contribute to increased susceptibility to anxiety phenotypes during certain phases of the estrous cycle (Birkeland et al., 2017).

Studies have demonstrated that rats within the proestrus and estrus stages have enhanced contextual fear acquisition and extinction (Chang et al., 2009). This finding was replicated in human females, using analogous menstrual cycle phases, noting that low estradiol levels were associated with continued expression of fear responses during the extinction phase (Lebron-Milad & Milad, 2012). Furthermore, women with low estradiol levels during extinction reported a higher incidence of intrusive thoughts within the following days, which may suggest both a phasic vulnerability and protective effect based upon whether fear extinction or acquisition is taking place. Importantly, this may provide a potential pathway for optimized therapeutic treatments for PTSD patients—perhaps administration of exogenous estrogen could help to increase the efficacy of cognitive behavioral therapy.

The findings of this experiment contribute to current knowledge surrounding sex-dependent differences in male and female stress responses. These results suggest that perhaps
hormone fluctuations might sensitize anxiety-related neurocircuitry, causing increased incidence of stress responding and anxiety-like behaviors. Continuing to examine PACAPergic BNST neurocircuitry in relation to female sex hormones is warranted, as understanding of this system could lead to a clearer portrait of maladaptive functioning of anxiety-related neurocircuitry. Through this area of study, viable therapeutic targets and techniques could be developed and implemented in order to try and mitigate the presence and effects of anxiety-related disorders such as PTSD in women.
Acknowledgements

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**Figures and Tables**

**Table 1.** Genetic markers that have been associated with PTSD.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Functional Role</th>
<th>Other Relevant Information</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAD1L1</strong></td>
<td>Component of mitotic spindle assembly checkpoint; prevents onset of anaphase until chromosomes are aligned properly along metaphase plate</td>
<td>Associated with PTSD across racial and ethnic groups; also associated with schizophrenia and bipolar disorder</td>
<td>Stein et al., 2021; Ripke et al., 2014; Stahl et al., 2019</td>
</tr>
<tr>
<td><strong>CRHR1</strong></td>
<td>Encodes CRHR1, a corticotropin releasing hormone receptor</td>
<td>Associated with intrusive symptoms of PTSD; antagonism of CRHR1 has not been found to be an effective PTSD treatment</td>
<td>Stein et al., 2021; Gelernter et al., 2019; Dunlop et al., 2017</td>
</tr>
<tr>
<td><strong>TSNARE1</strong></td>
<td>Involved in intracellular protein transport, particularly in SNARE binding and SNAP receptor activity</td>
<td>Associated with risk taking behaviors, schizophrenia</td>
<td>Stein et al., 2021; Plooster et al., 2021; Linner et al., 2019</td>
</tr>
<tr>
<td><strong>EXD3</strong></td>
<td>Involved in nucleic acid binding and 3’ to 5’ exonuclease activity</td>
<td>Negatively correlates with PTSD diagnosis, with relationship mediated by socioeconomic status</td>
<td>Stein et al., 2021; Polimanti et al., 2019</td>
</tr>
<tr>
<td><strong>PARK2</strong></td>
<td>Precise function not known</td>
<td>Connected with susceptibility to leprosy, Parkinson’s Disease</td>
<td>Stein et al., 2021; Meschede et al., 2020</td>
</tr>
<tr>
<td><strong>PACRG</strong></td>
<td>Encodes Parkin Coregulated, a gene that can function to suppress cell death and may be involved in the formation of Lewy bodies</td>
<td>Connected with susceptibility to leprosy, Parkinson’s Disease</td>
<td>Stein et al., 2021; Meschede et al., 2021</td>
</tr>
<tr>
<td><strong>ADCYAP1R1</strong></td>
<td>Encodes PAC_y, a pituitary adenylate cyclase activating polypeptide receptor</td>
<td>Single nucleotide polymorphism within an estrogen response element is associated with PTSD in females</td>
<td>Ressler et al., 2011; Mercer et al., 2016; Smoller, 2016</td>
</tr>
</tbody>
</table>

* this is not an exhaustive list of all genes that have been associated with PTSD
**Figure 1.** A proposed model for estradiol-PAC₁ interactions in both normal conditions, and in the presence of the *ADCYAPIRI* SNP. From “Stress-related disorders, pituitary adenylate cyclase-activating peptide (PACAP)ergic system, and sex differences”. Ramikie, T.S. & Ressler, K.J. (2016). *Dialogues in Clinical Neuroscience, Vol 18*(4): 403 – 413. DOI: 10.31887/DCNS.2016.18.4/kressler.

Left: In normal responding, experience of a traumatic event activates stress systems, including PACAPergic circuitry. Any estradiol within the system is able to bind normally to the ERE. This causes normal fear and stress responding. Right: Experience of a traumatic event activates stress systems, including PACAPergic circuitry. However, because the variant ERE is present (indicated by the red dot), these PACAPergic systems do not activate normally, causing dysregulation within the stress response. This leads to increased display of fear and stress (i.e. PTSD-like symptoms).

Top Left: Estradiol (E2) levels over the course of the rat estrous cycle. Top Right: Follicle Stimulating Hormone (FSH) levels over the course of the rat estrous cycle. Bottom Left: Progesterone (Prog.) levels over the course of the rat estrous cycle. Bottom Right: Luteinizing Hormone (LH) levels over the course of the rat estrous cycle.

A: Proestrus vaginal smear, marked by the predominance of nucleated epithelial cells (E). B: Estrus vaginal smear, marked by the predominance of anucleated cornified cells (C). C: Diestrus 1 vaginal smear, marked by equal proportions of leukocytes (L), anucleated cornified cells (C), and nucleated epithelial cells (E). D: Diestrus 2 vaginal smear, marked by the predominance of leukocytes (L).
Phase/treatment interaction and main effects were analyzed via factorial ANOVA. Interaction between phase and treatment is significant, $p = 0.042 < 0.05$. Main effect for treatment is significant, $p = 0.001 < 0.05$. Main effect for phase is not significant, $p = 0.407 > 0.05$. Anxiogenic effects of PACAP appear to be increased during phases in which hormones are fluctuating significantly (i.e., proestrus and estrus).
**Figure 5.** *Number of Arm Entries vs. Phase/Treatment.*

Phase/treatment interaction and main effects were analyzed via factorial ANOVA. Interaction between phase and treatment is not significant, $p = 0.289 > 0.05$. Main effect for treatment is not significant, $p = 0.134 > 0.05$. Main effect for phase is not significant, $p = 0.199 > 0.05$. 

Error bars: 95% CI
Figure 6. Number of Open Arm Entries vs. Phase/Treatment.

Phase/treatment interaction and main effects were analyzed via factorial ANOVA. Interaction between phase and treatment is not significant, $p = 0.063 > 0.05$. Main effect for treatment is significant, $p = 0.010 < 0.05$. Main effect for phase is not significant, $p = 0.264 > 0.05$. Anxiogenic effects of PACAP appear to be increased during phases in which hormones are fluctuating significantly (i.e., proestrus and estrus).
Figure 7. Ratio of Open Arm Entries to Total Arm Entries vs. Phase/Treatment

Phase/treatment interaction and main effects were analyzed via factorial ANOVA. Interaction between phase and treatment is not significant, p = 0.187 > 0.05. Main effect for treatment is significant, p = 0.002 < 0.05. Main effect for phase is not significant, p = 0.187 > 0.05. Anxiogenic effects of PACAP appear to be increased during phases in which hormones are fluctuating significantly (i.e., proestrus and estrus).

The “classical” pathway, in which estrogen diffuses through the plasma membrane and binds to ERs within the cell nucleus. ii. Non-genomic pathway, in which estrogen binds to ERs on the plasma membrane and then leads to downstream effects through second messenger signaling. iii. Estrogen may also act in a non-genomic manner by binding to non-ER membrane associated estrogen binding proteins. After binding, effects will continue in a similar manner as (ii).
The division of genomic and non-genomic mechanisms may contribute to increased anxiety-like behavior during proestrus and estrus in rats that have been treated with intra-BNST PACAP. The immediate effects of estradiol binding to transmembrane estrogen receptors results in modulation of the activity of the PAC_1 receptor, ultimately leading to amplified anxiety-like behavior during proestrus. In the classical pathway, estradiol diffuses into the cell and binds to estrogen receptors (ER) within the nucleus. The estradiol-ER complex then alters genomic transcription, taking hours to fully take effect. Thus, the elevated level of estradiol leading up until ovulation in estrus could generate prolonged effects that cause physiological and behavioral changes even after estradiol levels have begun to decline and return to baseline.