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**Interactions between BNST PACAP stress system and Estrous Cycling in Non-
overiectomized Female Rats**

UVM Undergraduate Thesis

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Abstract:

The objective of this investigation is to examine possible interactions between the BNST PACAP stress system and hormone levels during the estrous cycle of naturally cycling female rats. This is significant as behavioral consequences, analogous to anxiety disorders in humans, including increased startle, anxious behavior during open arm tests, and decreased feeding are associated with increased transcripts of PACAP and PAC1 within the BNST of animals undergoing a chronic variant stress paradigm (reviewed by Hammack & May, 2014). As females are at a greater risk to suffer from symptoms of PTSD than men (Veteran Affairs, 2017), studying females is of great importance to stress researchers. To study the interaction between estrous cycle and the BNST PACAP system, this research examines PACAP mRNA transcript levels as well as its associated PAC1 receptor mRNA transcript levels in non-overectomized female rats. PACAP and PAC1 transcript levels were determined at each of the four stages of the estrous cycle using qPCR technique. In rat models, estradiol treatment increased BNST PACAP transcription in females suggesting that PACAP and estradiol may have an interaction that explains the sexual dimorphism seen in human PTSD pathologies (Lezak et al., 2014; Ressler et al., 2011). Further investigation into natural hormone cycling in females may lead to clearer answers regarding the regulation of PACAP in the presence of estrogen hormones and why this previously mentioned sexual dimorphism occurs in stress-related disorders like PTSD.

Introduction

Post-traumatic stress disorder (PTSD) is a debilitating psychological illness characterized by detrimental behavioral and mood changes following the occurrence of one or more traumatic events. According to The Department of Veteran Affairs, more than 7 percent of the United States population will experience PTSD in their lifetime. Within that group, females are twice as likely to experience PTSD as their male counterparts (Veteran Affairs, 2017). Though females are less likely to experience trauma overall in comparison to males, they are at a higher risk of experiencing sexual assault (PTSD, 2007). This is significant as sexual assault is more likely to result in PTSD than other traumatic events, according to the National Center for PTSD. Both environmental and biological factors characterize any disorder's etiology. The high prevalence of PTSD in the female population leads to questions as to the possible biological causes underlying this sexually dependent difference. This investigation will attempt to add to current research regarding sexual dimorphism in anxiety pathologies by investigating PACAP and PAC1 transcription levels during the estrous cycle in naturally cycling female rats.

The response of the central nervous system (CNS) and incorporated nuclei when exposed to stressors is essential to understanding human psychopathologies such as PTSD. In general, when an organism responds to an environmental or biological stressor, multiple regions in the brain and endocrine system are activated in a compensatory manner gauged to return homeostasis to the system. This initiation of a compensatory stress response, is the fundamental function of the hypothalamic-pituitary-adrenal (HPA) axis. Proceeding exposure to a stressor, the hypothalamus becomes activated in response to excitatory inputs from areas such as the prefrontal cortex, amygdala and hippocampus. This excitation signals the periventricular nucleus of the hypothalamus to secrete the hormone corticotropin-releasing hormone or CRH, which

projects to the anterior pituitary gland. The pituitary gland releases adrenocorticotrophic hormone (ACTH) into the blood stream transporting it to the adrenal gland. Glucocorticoids, such as cortisol, are then released by the adrenals and flood the system. Through this process, the sympathetic branch of the autonomic nervous system is activated. Sympathetic activation causes pupillary dilation, an increase in heart rate, and blood pressure and is an adaptive response to stressor exposure. Once the stressor is gone, negative feedback to multiple points in the HPA axis circuitry leads to reactivation of the parasympathetic nervous system and a return in homeostasis.

However, with sustained stressor activation, analogous to human anxiety psychopathologies, the HPA axis becomes chronically activated leading to deregulation of its negative feedback loop. There is evidence that specific nuclei in the limbic system, including the bed nucleus of the stria terminalis (BNST) undergo functional and morphological changes as a result of this chronic anxiety state (reviewed by Hammack & May, 2014; Schulkin et al., 1998; Vyas et al., 2003). In fact, with sustained activation of the HPA axis in response to chronic stress, the BNST increases in volume, dendritic branching, and synaptic efficacy (Pego et al., 2008; Dumont, Rycroft, Maiz, & Williams, 2008). These findings implicate the BNST as a structure of importance in the HPA responding to chronic stress. Further anatomical evidence suggests that the BNST has connections to regions crucial to the HPA circuitry. These include the central amygdala, the primary source of GABAergic inputs to the BNST (Dong et al., 2001), the basolateral amygdala, and prefrontal cortex, both exerting glutamatergic inputs on the BNST (as reviewed by Vranjkovic, Pina, Kash & Winder, 2017). Moreover, certain chronic stress disorders seen in humans, such as PTSD, may be producing similarly maladaptive neuroplastic

changes within the BNST nuclei (reviewed by Hammack & May, 2014), leading to significant changes in the brain of rodent models.

Along with specific CNS nuclei, a number of neuropeptides are also implicated in mediating the effects of stress. In particular, pituitary adenylate cyclase-activating peptide (PACAP) is thought to be a regulator of the stress response, as evident by transcription changes following stressful experiences (reviewed by Hammack et al., 2014; Hashimoto et al., 2011). PACAP as well as its receptor, PAC1, are widely expressed in stress-associated brain regions, including the BNST (Hammack et al., 2009). Consequently research has shown that chronic stress paradigms consisting of stressors such as forced swim, foot shock, and restraint can increase expression of PACAP messenger RNA within the BNST (Hammack et al., 2009). This leads to the conclusion that significant change is occurring in both the PACAP system as well as the BNST limbic region after chronic stress.

As mentioned, the BNST is an area implicated in chronic stress response (reviewed by Hammack & May, 2014; Schulkin et al., 1998; Vyas et al., 2003). Expression of PACAP within this limbic nucleus stimulates the production of stress hormones, such as CRH, creating a dynamic system. In fact, the BNST PACAP system is argued to be a critical component contributing to the behavioral consequences of repeated stress exposure. In support of this, the Hammack lab has shown that intra-BNST PACAP infusions mimic many of the behavioral consequences seen in chronic stress, even in the absence of any environmental stressor; while, intra-BNST PACAP receptor antagonists prevent the normal behavioral responses to a chronic stressor (Hammack et al., 2009). This gives credibility to the BNST PACAP system being a mediator for stress related disorders.

PACAP expression within the BNST is shown to be a dynamic system that contributes to

the behavioral consequences of stress exposure in both male and female rat models. PTSD, as well as other anxiety disorders, is sexually dimorphic in the human (Veteran Affairs, 2017) and rodent populations. In fact, elevated blood levels for the PACAP precursor PACAP38 were significantly correlated with PTSD in females but not in males (Ressler et al., 2011). Evidence suggests that PTSD in humans is connected to a single nucleotide polymorphism (SNP) in the PAC1 receptor gene that resides within an estrogen response element (Ressler et al., 2011). Further evidence found that exogenous estradiol treatment increased BNST PACAP transcription in female rats (Lezak et al., 2014). All this suggests that the interaction of PACAP and estradiol may explain the sexual dimorphism seen in PTSD pathologies. The current investigation was conducted in an attempt to shed light on natural hormone cycling in females and how PACAP and its receptor PAC1 are regulated in the presence of estrogen hormones. Little research has been completed on the sexual dimorphism within the BNST PACAP system. Taking a closer look at these interactions will broaden the scope of BNST PACAP investigations and bring about a better understanding of the functional differences in male and female stress responses.

Methods

Subjects.

Adult cycling female (n=28) Sprague-Dawley rats from Charles River Laboratories weighing between 225-275 g were acquired for this experiment. Rats were housed two to a compartment, with a 12h light/dark cycle maintained throughout the span of the experimental procedures. They had food and water readily available throughout their stay. Rats were given one week of habituation in their home cages prior to estrous tracking. At this time point they were 4 weeks of age. A total of two cohorts with n=16 and n=12 were used in this experimental design.

Tracking Estrous.

Vaginal Swabs were taken between 1:00pm and 3:00pm daily for 4 weeks using cotton tipped applicators that were soaked in a sterile water solution. Cells from the applicator were carefully rolled onto a glass slide and were air dried for 30 seconds.

Phase Identification.

Once vaginal swabs were collected, the correct estrous phase was identified through vaginal cytology. Slides were viewed under a microscope at 200x magnification under bright illumination. Stages of the estrous cycle were tracked based on presence or absence of leukocytes, nucleated epithelial cells, and cornified cell types. Proestrous, metestrous, diestrous, and estrous were all tracked and accounted for through qualitative microscopic examination of the specimens.

Euthanasia/BNST punches.

Animals were sacrificed on days corresponding to the phase of interest resulting in even groups of animals in each of the four estrous phases. Animals were euthanized through quick decapitation technique after anesthetization with isoflurine solution. The brains were removed and roughly cut into coronal sections for full visually identification of the BNST nuclei. Using a tissue punch tool, a punch of both the dorsal and lateral aspects of the anterior BNST regions were removed and set aside for qPCR.

Qualitative Polymerase Chain Reaction (qPCR).

Tissue was homogenized in Stat-60 (Tel-Test "B") and total RNA was isolated. 2.5 µg RNA were used to synthesize cDNA using SuperScript II reverse transcriptase and random hexamer primers. cDNA samples were treated with Rnase H, and real-time Taqman qPCR amplification using oligonucleotide primers was completed. Cycle threshold data were

normalized to the ribosomal protein (18s) reference gene and fold change relative to control animals was determined.

Statistics.

Statistical Analyses including one-way ANOVA for Figures 2&3 and a *t*-test for Figure 4 were completed using GraphPad Prism Version 7 Software (GraphPad Software, San Diego, CA). Results were expressed as mean \pm SEM. All phases of estrous were normalized to estrous control.

Results

Vaginal Cytology and Estrous Cycling

Previous literature suggests that there are specific cell types present at differing stages during the estrous cycle in female rats (Goldman, Murr, & Cooper, 2007). Overall, the female rats consistently cycled over the course of four days. This cycling was consistent throughout the 4 weeks for the majority of the animals. A small minority experienced disturbances in their estrous cycling; including extended cycling for animals that had stressful power struggles with the other member of the cage. Cells types present after vaginal swabbing are indicative of the animals phase. Proestrous phase of the estrous cycle is predominantly identified through the presence of nucleated epithelial cells (**Figure 1, A**). Following proestrous, estrous was identified primarily through cornified epithelial cells (**Figure 1, B**). Cornified epithelial cell types continue into metestrous with the addition of leukocytes to a far smaller proportion (**Figure 1, C**). Diestrous followed with leukocytes as the prominent cell type at this stage in the cycle (**Figure 1, D**). Accurate identification of specific phase in the estrous cycle was critical to determining what time points animals should be sacrificed for transcript level analysis.

PACAP Transcript Levels Across Phase

In the dorsal BNST, PACAP transcript levels were compared to estrous animals as control. PACAP transcript levels were analyzed as a fold control. This was completed across all four estrus cycles with 7 samples in each phase. Outliers were removed from the data based on Grubb's test in Prism, three outliers were removed from proestrous, metestrous, and diestrous groups. No significance was found when a one-way ANOVA was completed, $F(3, 20)=0.9674$, $p > 0.05$. Post hoc comparisons also found no significant difference overall (**Figure 2, A&B.**).

In the ventral BNST, PACAP transcript levels were compared to estrous animals as control. PACAP transcript levels were analyzed as a fold control. This was completed across all four estrus cycles with 7 samples in each phase. A total of 2 outliers were removed from the data, from estrous and diestrous phases. No significance was found when a one-way ANOVA was completed, $F(3,20)=1.944$, $p > 0.05$. The Ventral BNST also showed no statistical significance between phases of estrous and there were no difference when post hoc was completed (**Figure 2, C&D.**).

PAC1 Transcript Levels Across Phase

PAC1 transcript was compared to estrous as a control. This was completed across all four estrus cycles with 7 samples in each phase. One outlier was removed from the data. No significance was found between PAC1 transcript and estrous cycling for the dorsal BNST region. No significance was found when a one-way ANOVA was completed, $F(3, 81)=0.87$, $p > 0.05$. Post hoc comparisons also found no significant difference overall. However, qualitatively proestrous showed a higher trend (**Figure 3, A&B.**).

Static versus Fluctuating Estrous

As there seemed to be a qualitative split between the fluctuating estrous phase, proestrous and metestrous, and the static estrous phases, diestrous and estrous, in (**Figure 3, A&B.**) a

statistical analysis of these collapsed phases was completed. The proestrous phase has the highest estrogen levels overall. PAC1 transcript was assessed in static versus fluctuating estrous phases for the dorsal BNST region. No significant interaction was found between fluctuating versus static phases of the estrous cycle, $t(24)=0.1898$, $p > 0.05$. A total of 2 outliers were excluded from the data.

Discussion

This project's aim was to study the interaction between PACAP, the BNST, and estrous cycle in naturally cycling females. As previously mentioned, there is a considerable sexually dependent difference when looking at the incidence of PTSD in the human population. In fact, females are at a greater risk to suffer from symptoms of PTSD than males (Veteran Affairs, 2017), suggesting that there might be some underlying biological mechanism connecting stress related circuitry in anxiety disorders like PTSD to estradiol present during the estrous cycle.

The BNST PACAP system is highly implicated in chronic stress paradigms (Hammack et al., 2009; Schulkin et al., 1998; Vyas et al., 2003) and this system can be influenced by estradiol. Evidence suggests that PTSD in humans is connected to a SNP in the PAC1 receptor gene that resides within an estrogen response element (Ressler et al., 2011). This finding, along with the fact that increased PACAP transcript was seen in female rats that were given exogenous estradiol (Lezak et al., 2014; Ressler et al., 2011) suggests that PACAP and estradiol may create a dynamic system that adds weight to a biological basis for the sexual dimorphism seen in anxiety pathologies such as PTSD.

Further investigation into natural hormone cycling in our experiments has led to some new findings about the estrous cycle and its implications on chronic stress. Fortuitous behavioral evidence found that when animals were stressed due to power struggles between cage mates,

there were irregularities in the estrous cycling of these females. Females who were bullied exhibited prolonged phasic cycling as opposed to their non-stressed counterparts. This is indicative of a connection between stress and the natural hormone levels seen during the estrous cycle. Further data collected from the dorsal BNST found that there was a trend toward higher PAC1 transcript levels during high estrous phases, specifically proestrous (**Figure 3, A & B**). Though not statistically significant, $p > 0.5$, a trend is clearly evident suggesting that there are more PAC1 receptors present in the BNST during high estrogen. Though predominantly speculative, this could mean that higher estradiol levels are sensitizing the system to increased stress output when presented with chronic stress.

In order to give credibility to this thinking, a number of follow up experiments should be conducted both to add validity to the current data presented and to support the mechanism of action. Foremost, transcription levels for VPAC1 and VPAC2 should be taken during the four phases of estrous. As PACAP has three receptors, PAC1, VPAC1, & VPAC2, it is critical that these other two receptors are accounted for because a change in PACAP transcript cannot be directly attributed to the PAC1 receptor but must be considered in conjunction to the other receptors as well. Following this investigation, western blots should be conducted to look at the resultant protein levels associated with the mRNA transcripts. This is important because increases in RNA do not necessarily correlate to an up regulation in protein production. To test the validity of phase identification from vaginal cytology, blood serum estradiol levels should be analyzed at the time of tissue collection. This is particularly important in this study as visualization of cell types between metestrous and diestrous proved difficult and most likely resulted in overlap in data collected between these cycles. Further research into how estradiol levels specifically influence PACAP and PAC1 transcript would be worthwhile research

direction as well. Looking at ovariectomized females and then giving exogenous estradiol every four days would be useful, as it would rule out other sex hormones that might be influencing PACAP/PAC1 transcript levels. This additional research might lend credence to the thinking that estradiol levels are sensitizing the system to increased stress output when presented with chronic stress.

An original paper published by Mercer et al., 2016, found that the presence of estrogen receptor ($ER\alpha$) was sufficient to induce expression of the PAC1 receptor and that disruption of this binding site due to a PTSD risk allele decreased the expression of PAC1 overall. This suggested that estrogen could contribute to higher levels of PAC1 that might suggest down regulation of PACAP release and lead to a compensatory response when chronic stress is present, according to Mercer et al., 2016. Functionally, this suggests that increased PAC1 levels would be indicative of decrease in stressor effects. Limitations to this finding include their testing of PAC1 levels in whole blood and not directly from the BNST region. This could be why this conclusion does not fit with the data found during this investigation. Evidence from this research found that with the increase of estradiol in the high estrogen phases resulted in increased PACAP and PAC1 transcripts suggestive of a deleterious impact of estradiol on the stress system. However, further work must be conducted to solidify that assumption.

Evidence as to the effect of mood regulation due to estrogen fluctuation is highly varied. Data suggests that girls going through puberty, a time which estrogen levels dramatically increase, are more vulnerable to stress as opposed to girls before or after puberty (Caspi & Moffitt, 1991). Along this line, the proportion of depression in females directly after puberty increases two fold in prevalence as compared to males (Kessler & Walters, 1998). However, during menopause, a time when estrogen levels drop sharply, mood symptoms include

irritability, depressed mood, and anxiety are prominent (Prior, 1998). Because hormonal changes occur in all women throughout different stages in life, it is impossible to conclude that anxiety disorders are not solely reliant on the fluctuation of female hormones. That said, the possibility that differing hormone levels might increase the susceptibility to certain disorders warrants further investigation. If the conclusion of this current investigation holds true, it could mean that females are more susceptible to anxiety phenotypes during times of high estrous. This would provide a therapeutic target for females who are seeking treatment after a highly traumatic experience with presents likelihood for PTSD development.

Our investigations offer the potential for a clearer understanding of why there are sexually dependent differences in male versus female stress response. Current research suggests that high estrogen phases are sensitizing the system to respond with increased stress responding when chronic stress. As anxiety disorders such as PTSD remain a continued concern for the United States female population, studying the PACAP BNST circuitry in connection to the estrous cycle and estrogen could lead to the improved understanding of the maladaptive limbic circuitry implicated in females with chronic stress. In fact, through this area of study, viable therapeutic techniques could be implemented in an attempt to decrease the incidence of anxiety disorders in the female population.

Acknowledgments

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Author declares no conflict of interest

References

- Caspi, A. & Moffitt, T.E. (1991). Individual differences are accentuated during periods of social change: the sample case of girls at puberty. *J Pers Soc Psychol*, 61: 57-68.
- Dong, H.W., Petrovich, G.D., Watts, A.G., Swanson, L.W., 2001. Basic organization of projections from the oval and fusiform nuclei of the bed nuclei of the stria terminalis in adult rat brain. *J. Comp. Neurol.* 436 (4), 430e455.
- Dumont, E.C., Rycroft, B.K., Maiz, J., & Williams, J.T. (2008). Morphine produces circuit-specific neuroplasticity in the bed nucleus of the stria terminalis. *Neuroscience*. Retrieved from <http://www.sciencedirect.com/science/article/pii/S030645220800167X>
- Goldman, J. M., Murr, A. S., & Cooper, R. L. (2007). The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth defects research. Part B, Developmental and reproductive toxicology*, 80(2), 84–97.
- Hammack, S. & May, V. (2014). Pituitary Adenylate Cyclase Activating Polypeptide in Stress-Related Disorders: Data Convergence from Animal and Human Studies. *Biological Psychiatry* doi:10.1016/j.biopsych.2014.12.003.
- Hammack, S.E., Roman, C.W., Lezak, K.R., Kocho-Shellenberg, M., Grimmig, B., Falls, W.A., et al. (2010). Roles for pituitary adenylate cyclase-activating peptide (PACAP) expression and signaling in the bed nucleus of the stria terminalis (BNST) in mediating the behavioral consequences of chronic stress. *J Mol Neurosci*, 42, 327–40.
- Hammack, S.E., Cheung, J., Rhodes, K.M., Schutz, K.C., Falls, W.A., Braas, K.M., May, V. (2009) Chronic stress increases pituitary adenylate cyclase-activating peptide (PACAP) and brain-derived neurotrophic factor (BDNF) mRNA expression in the bed nucleus of

- the stria terminalis (BNST): roles for PACAP in anxiety-like behavior. *Psychoneuroendocrinology*, 34(6), 833-43.
- Hashimoto, H., Shitani, N., Tanida, M., Hayata, A., Hashimoto, R., & Baba, A. (2011). PACAP is implicated in the stress axes. *Curr Pharm Des* 17: 985-989.
- Kessler, K.C. & Walters, E.E. (1998). Epidemiology of DSM-III-R major depression and minor depression among adolescents and young adults in the National Comorbidity Survey. *Depress Anxiety*, 7: 3-14.
- King, S. B., Lezak, K. R., O'Reilly, M., Toufexis, D. J., Falls, W. A., Braas, K., May, V., et al. (2017). The Effects of Prior Stress on Anxiety-Like Responding to Intra-BNST Pituitary Adenylate Cyclase Activating Polypeptide (PACAP) in Male and Female Rats. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*
- Kocho-Schellenberg et al. (2014). PACAP in the BNST produces anorexia and weight loss in male and female rats. *Neuropsychopharmacology*, 39,1614-1623.
- Lezak, K.R., Roelke, E., Harris, O.M., Choi, I., Edwards, S., Gick, N., Cocchiaro, G., Missig, G., Roman, C.W., Braas, K.M., Toufexis, D.J., May, V., & Hammack, S.E. (2014). Pituitary adenylate cyclase- activating polypeptide (PACAP) in the bed nucleus of the stria terminalis (BNST) increases corticosterone in male and female rats. *Psychoneuroendocrinology* 45: 11-20.
- Pego, J.M., Morgado, P., Pinto, L.G., Cerqueira, J.J., Almeida, O., & Sousa, N. (2008). Dissociation of the morphological correlates of stress induced anxiety and fear. *European Journal of Neuroscience*, 27(6), 1503–1516.

Prior, J.C. (1998). Perimenopause: The complex endocrinology of the menopausal transition.

Endocr Rev, 19: 397-428

Mercer, K. B., Dias, B., Shafer, D., Maddox, S. A., Mulle, J. G., Hu, P., Walton, J., et al. (2016).

Functional evaluation of a PTSD-associated genetic variant: estradiol regulation and

ADCYAP1R1. *Translational psychiatry*, 6(12), e978

PTSD: National Center for PTSD. (2007). From [https://www.ptsd.va.gov/public/PTSD-](https://www.ptsd.va.gov/public/PTSD-overview/women/women-trauma-and-ptsd.asp)

[overview/women/women-trauma-and-ptsd.asp](https://www.ptsd.va.gov/public/PTSD-overview/women/women-trauma-and-ptsd.asp)

Ressler , K.J., Mercer , K.B., Bradley , B., Jovanovic, T ., Mahan, A., Kerley , K., Norrholm,

S.D., Kilaru, V ., Smith, A.K., Myers, A.J., Ramirez, M., Engel, A., Hammack, S.E.,

Toufexis, D., Braas, K.M., Binder , E.B., May , V ., 2011. Post-traumatic stress disorder

is associated with PACAP and the PAC1 receptor. *Nature* 470, 492—497.

Schulkin, J., Gold, P.W., & McEwen, B.S. (1998). Induction of corticotropin-releasing hormone

gene expression by glucocorticoids: implication for understanding the states of fear and

anxiety and allostatic load. *Psychoneuroendocrinology*, 23(3), 219-43.

Veterans Affairs. (2017). Fact Sheet. PTSD: National center for PTSD. *U.S. Department of*

Veterans Affairs.

Vranjkovic, O., Pina, M., Kash, T. L., & Winder, D. G. (2017). The bed nucleus of the stria

terminalis in drug-associated behavior and affect: A circuit-based

perspective. *Neuropharmacology*.

Vyas, A., Bernal, S., & Chattarji, S. (2003). Effects of chronic stress on dendritic arborization in

the central and extended amygdala. *Brain research* 965: 290–4.

Figures

Figure 1. Vaginal cytology looking at cell types present at each of the four phases of the estrous cycle. **(A).** Cell types present in proestrous. Predominately present are nucleated epithelial cells (black arrow). **(B).** Cell types present in estrus. Cornified Epithelial cells are predominately present at this stage in the cycle (blue arrow). **(C).** Cell types in Metestrous. Cornified epithelial cells (black arrow) as well as leukocytes are predominate at this stage (black circle). **(D).** Cell types in Diestrous. Leukocytes are the prominent cell type at this stage in the cycle (black circle).

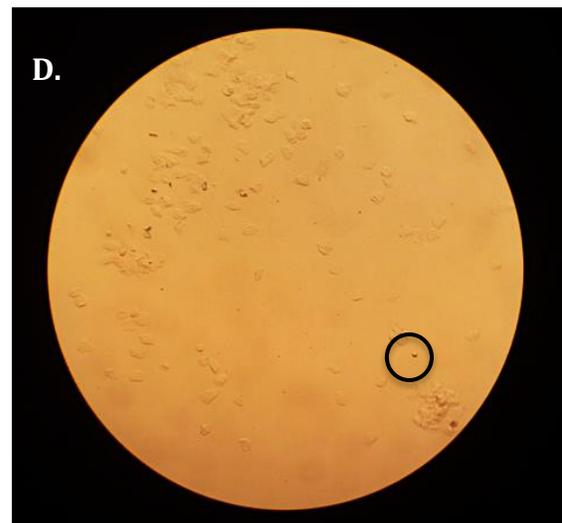
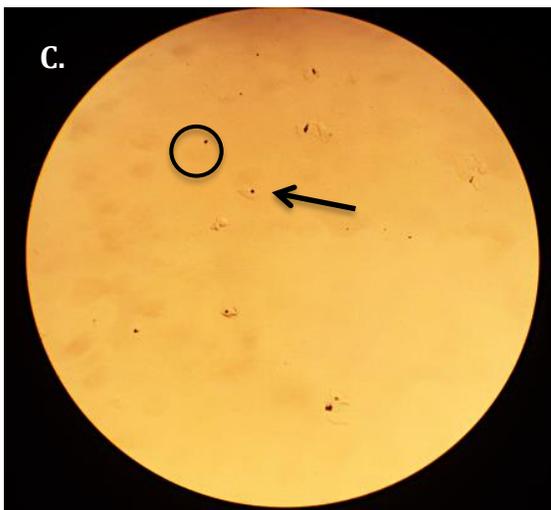
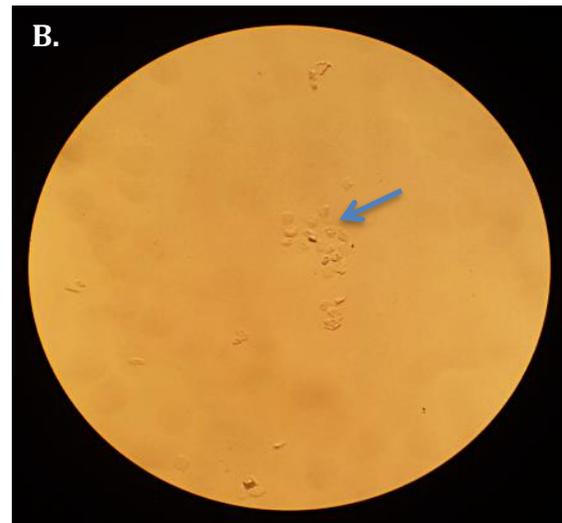
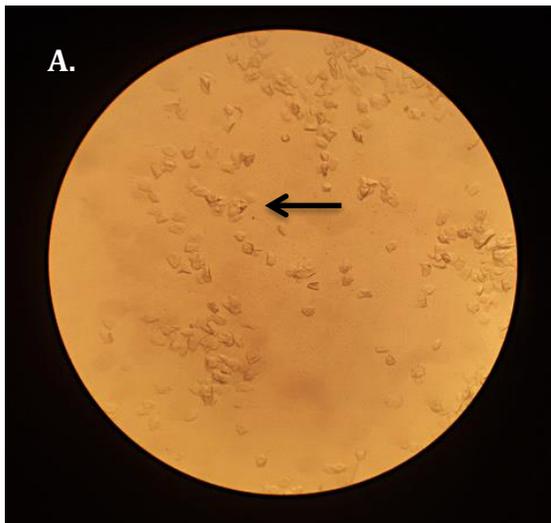


Figure 2. *PACAP* transcript Levels in the BNST. **(A&B).** *PACAP* transcript levels in the dorsal BNST for combined cohorts. No significance was found when a one-way ANOVA was completed, $F(3,18)=1.184, p > 0.05$ **(C&D).** *PACAP* transcript levels in the ventral BNST for combined cohorts. No significance was found when a one-way ANOVA was completed, $F(3,20)=1.944, p > 0.05$. Outliers were removed.

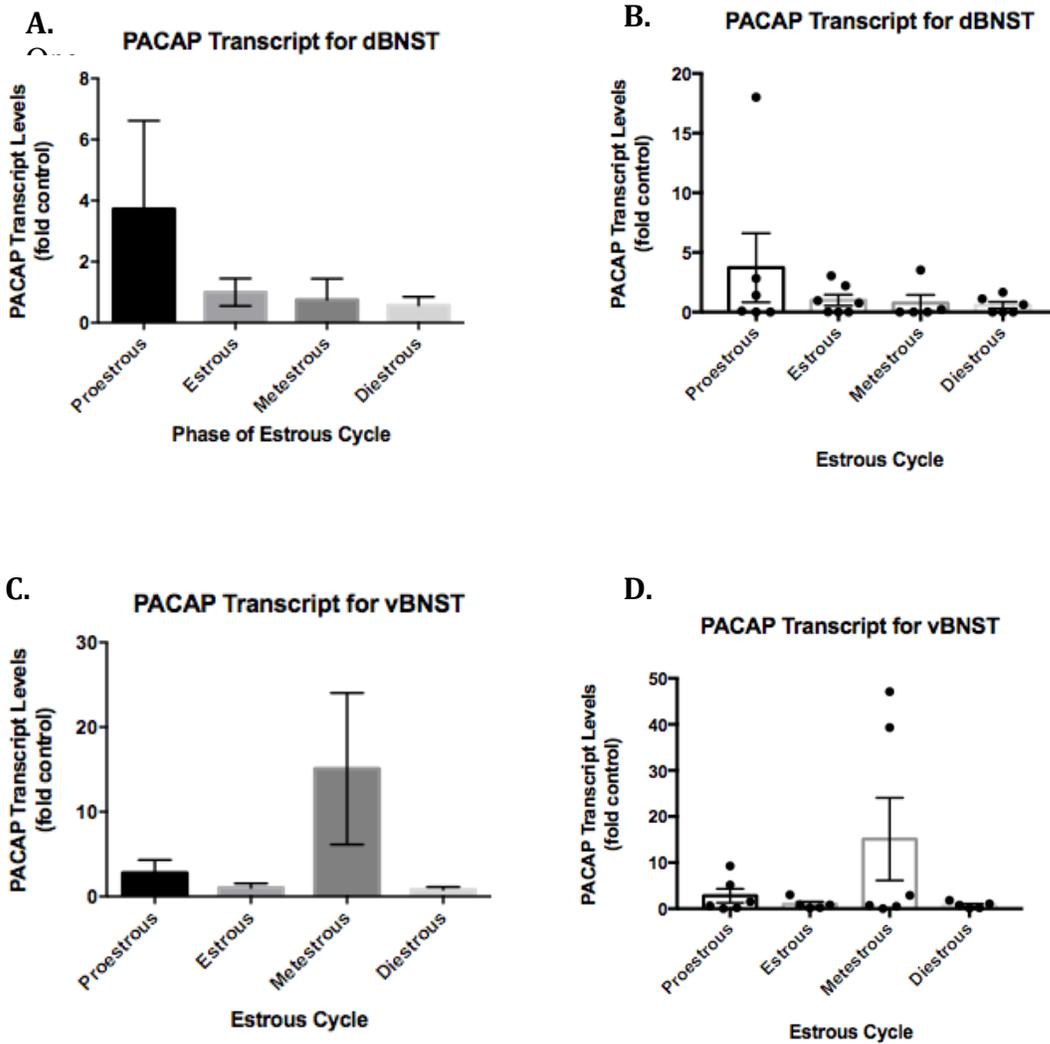


Figure 3. *PAC1* transcript Levels in the BNST. (A&B). *PAC1* transcript levels for the dorsal BNST of combined cohorts, n=28. No significance was found when a one-way ANOVA was completed, $F(3, 81)=0.87$, $p > 0.05$. Outliers were removed.

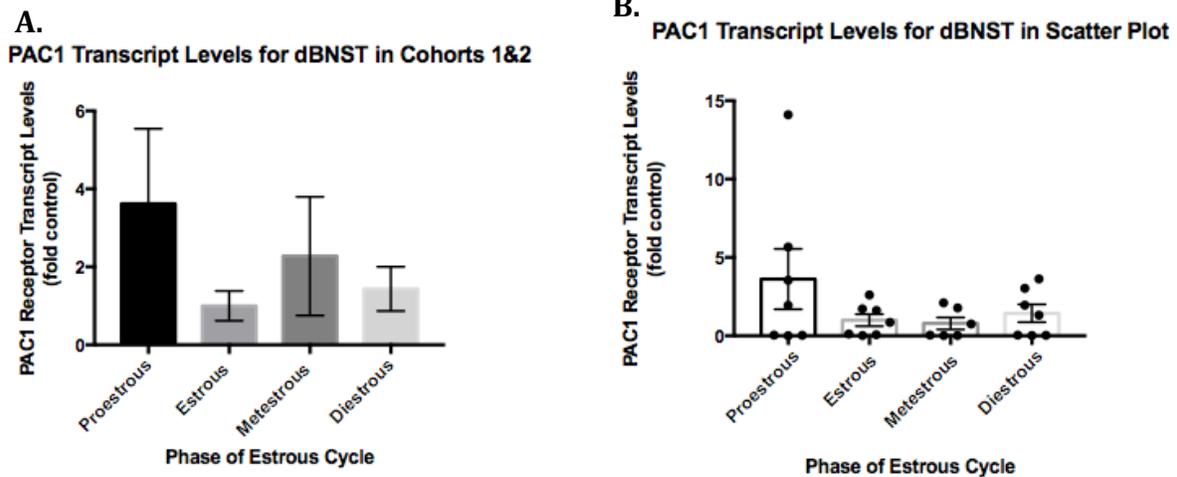


Figure 4. *PAC1* transcript during static versus fluctuation phases. (A&B).

Graph of fluctuating versus static estrous cycle transcript for cohorts 1 and 2 in the dBNST. No significant interaction was found between fluctuating versus static phases of the estrous cycle, $t(24)=0.1898$, $p > 0.05$. Outliers were excluded in the data.

