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## The Role Of Dentate Gyrus Pituitary Adenylate Cyclase Activating Polypeptide (pacap) In Contextual Fear Discrimination

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THE ROLE OF DENTATE GYRUS PITUITARY ADENYLATE CYCLASE  
ACTIVATING POLYPEPTIDE (PACAP) IN CONTEXTUAL FEAR  
DISCRIMINATION

A Thesis Presented

by

Samantha Moriarty

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## ABSTRACT

When dysregulated, neural systems important for fear behaviors can contribute to mental health disorders such as anxiety, phobias, and post-traumatic stress disorder (PTSD). In PTSD, a myriad of symptoms is possible, but a hallmark feature of the disorder is generalizing fear. This occurs when fear is experienced inappropriately in relation to the environment or circumstances. To study this behavior in rodent models, contextual fear conditioning is used. Contextual fear conditioning is a learning theory preparation where rodents are conditioned with an aversive stimulus such as foot-shock in one distinct context (A), while concurrently being exposed to a safe context (B). This training results in low fear behavior, usually measured in terms of freezing, in the safe context B, and high fear behavior in context A. This paradigm allows investigation of contextual discrimination, and also gives the experimenter the ability to observe context generalization, where the fear from the aversive context A extends to the safe context B. In this thesis, three experiments were performed. The purpose of the first experiment was to pilot a novel contextual fear conditioning paradigm so that it trained male rats to discriminate fearful and safe contexts. The second experiment served to extend this experimental design to include female rats, and therefore compare performance of males and females in contextual discrimination. Finally, the third experiment sought to expand on prior work demonstrating that pituitary adenylate cyclase activating polypeptide (PACAP), a peptide with strong associations to stress responses, significantly excites cells of the dentate gyrus. The dentate gyrus is a component of the hippocampus that is commonly thought to be involved in pattern separation, a process that may be integral to context discrimination. In addition, PACAP has been associated with aspects of PTSD, including symptom severity, particularly in women. With the connection of PACAP and PTSD as well as the dentate gyrus, experiment 3 aimed to test the result of a dentate gyrus PACAP infusion during a test session in either context A or B following the contextual fear conditioning design used in experiment 2. Experiment 1 showed that male rats could discriminate between contexts A and B in our experimental design. Experiment 2 built off of these results and extended the findings to females, showing that female rats also discriminated using this paradigm. Additionally, experiment 2 showed a sex difference between male and females, whereas male rats fear conditioned stronger than females, and females discriminated context better than males. Experiment 3 replicated these results in the first stage of conditioning. The results of the test day in experiment 3 showed that PACAP infused into the dentate gyrus had no effect on context discrimination in either male or female rats. Taken together, the experiments in this thesis show an experimental design that is capable of producing contextual discrimination, highlights possible sex differences in context fear conditioning, and suggests that if PACAP has an effect on generalization as our design measures it, it is likely not stemming from actions on the dentate gyrus.

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## **CHAPTER 1: Literature Review**

### **1.1. Fear and Related Pathologies**

Fear can be an outcome of the brain detecting a threat. Fear not only warns of danger, it readies the body to evade or fight off a threat through activation of the sympathetic nervous system and other stress pathways (Milad & Quirk, 2002);(Davis, 1992; Fanselow, 1994). Fear is associated with many physiological responses, including rapid heartbeat, sweating, and increased vigilance. Emotionally, we might feel “on edge” or have a foreboding feeling of danger. Sometimes, in the case of horror movies and amusement park rides, people use the feeling of fear for enjoyment. The fleeting adrenaline rush turns fear into an exciting thrill. However, in situations where fear is not produced intentionally, the physical and emotional symptoms are unpleasant and a cue for potentially life-threatening danger, thus organisms avoid fearful situations when possible. One way to do this is to rely on fear memories. In addition to fear systems activating to coordinate a response to threats that are immediate, memories of fear allow organisms to remember what environments and stimuli have been threatening in the past in order to facilitate avoidance or other adaptive behavior towards them in the future. Evolutionarily, fear is a vital and beneficial system for survival, but it may also become dysregulated to produce states of persistent fear, or fear that is inappropriate to the situation.

Dysregulation of fear systems may be associated with a number of psychological disorders. For example, if fear to a specific environment or stimulus is persistent or exaggerated, this might be classified as a phobia (Garcia, 2017). Alternatively, if the persistent fear is not specific and is instead a generalized fear of what might happen in the

future, the diagnosis might be generalized anxiety disorder (Showraki, Showraki, & Brown, 2020). In some cases, a person may experience acute trauma that triggers symptoms that persist well past the event, leading to post-traumatic stress disorder, or PTSD (Ressler et al., 2022).

PTSD is complex, where the cause and symptoms of the disorder may widely differ among those diagnosed. The complexity of PTSD makes it difficult to pinpoint what mechanisms are at work behind the pathology, which in turn causes a difficulty in treatment and inconsistent outcomes. Due to the wide range in events precipitating PTSD and the variability in symptoms, the symptoms and criteria for diagnosis have evolved over several iterations of the Diagnostic and Statistical Manual of Mental Disorders (DSM). Currently, there are 8 criteria that must be met to reach a PTSD diagnosis, including exposure to actual or threatened death, injury, or sexual violence through either direct exposure, indirect exposure, witnessing the trauma, or learning that someone close to the person had been exposed to the trauma. In addition, criterion B requires that the traumatic event is persistently re-experienced, either by upsetting memories, nightmares, flashbacks, or emotional or physical distress after reminders of the trauma. Criterion C is an avoidance of trauma related stimuli after the trauma, and criterion D is that negative thoughts or feelings began or worsened after the trauma. Similarly, criterion E states that trauma-related arousal and reactivity began or worsened after the trauma. Criteria F, G and H, confirm that the symptoms discussed in the prior criterion have lasted longer than one month, create distress or functional impairment for the person, and that no symptom is due to medication, substance use, or illness. While previously PTSD had been included

amongst a wide range of anxiety disorders, it has been reclassified as a trauma and stressor related disorder in the most recent diagnostic statistical manual (Messent, 2013).

Similar to many anxiety disorders, prevalence of PTSD is higher in women. Not only is the lifetime prevalence for PTSD two times higher in women, the symptoms are often more severe and chronic than in men (Ressler et al., 2022). This disparity is robust, even when taking into account differences in rates of reporting, type of trauma, and lifetime likelihood of experiencing a trauma for men and women. These data suggest that it is important to consider the possible mechanistic differences in physiology between males and females that may contribute to a sex difference in pathology.

## **1.2. Relevant Physiology**

### *HPA and HPG Axes*

The hypothalamic-pituitary-adrenal (HPA) axis plays a central role in stress-related responses (DeMorrow, 2018). This system responds to circadian rhythm and environmental cues with a chain of activity. Neuroendocrine neurons in the hypothalamus secrete corticotropin-releasing hormone (CRH), which then acts upon the anterior pituitary gland to cause the production and release of adrenocorticotrophic hormone (ACTH). This circulating ACTH then acts on the adrenal gland, which synthesizes and releases corticosteroids such as cortisol/corticosterone. Circulating corticosteroids regulate many physiological processes, including stress responding, and also regulate the glucocorticoid receptor activity that is responsible for inhibiting the HPA axis and keeping the system in homeostasis. HPA axis function can impact reproduction, sleep, disease, and pathologies such as anxiety and depression (Toufexis, Rivarola, Lara, & Viau, 2014). The HPA axis is also known to interact with the hypothalamic-pituitary-

gonadal (HPG) axis, which leads many to believe there is a sex difference in stress responses (Viau, 2002), (Bangasser & Wicks, 2017). Some evidence for this is that females are shown to have faster HPA axis reactivity, and higher release of stress hormones (Goel, Workman, Lee, Innala, & Viau, 2014).

Gonadal hormones such as testosterone, progesterone, and estrogen are known to interact with the stress response in different ways. Estrogen receptor (ER) activity has been shown to have the ability to either enhance (ER alpha) or inhibit (ER beta) HPA axis reactivity to stress based on subtype. ER alpha agonists have been shown to limit the negative feedback on the glucocorticoid receptors that are responsible for inhibiting the HPA axis response (Weiser & Handa, 2009). Conversely, ER beta mediates anxiolytic effects of estrogen. Administration of the ER beta agonist propyl-pyrazole-triol (PPT) causes decreased anxiety-like behavior in rodents in the light-dark box paradigm, the elevated plus maze, and the open field test (Weiser & Handa, 2009). Testosterone, a sex hormone that is found in higher concentrations in males, may have anxiolytic actions, due to interacting with the HPA axis by decreasing the glucocorticoid and ACTH response to stress in male rats (Viau & Meaney, 1996).

Just as the HPG axis influences the HPA axis, the reverse is also true. Activation of the HPA axis inhibits secretion of gonadal hormones (Viau, 2002). In female humans, the interaction of hormones is more complex due to menstrual cycling, where levels of estrogen, progesterone, and its metabolites rise and fall. Studies have shown that where females are in their cycle has an impact on the stress response (Nillni, Rasmusson, Paul, & Pineles, 2021). In female rats, who cycle every 4-5 days, ACTH levels are higher following acute stress when in proestrus (high levels of ovarian hormone) (Viau &

Meaney, 1991). Moreover, in women with PTSD, the menstrual cycle may regulate fear related symptoms, worsening certain symptoms at different phases in the cycle. For example, during the mid-luteal phase, intrusive sensory symptoms such as flashbacks are exacerbated (Bryant et al., 2011). One possible explanation for the influence of hormone cycling on anxiety and fear related pathologies is the actions of the metabolites of progesterone: allopregnanolone and pregnanolone (ALLO) (Rasmusson et al., 2006), (Pineles et al., 2018). They are believed to positively modulate GABA(A) receptors, resulting in an anxiolytic effect. Because these levels fluctuate throughout the menstrual cycle, one hypothesis is that when the amount of ALLO circulating drops off, as in the premenstrual and menstrual phases, this anxiolytic effect also decreases (Rasmusson et al., 2017). This is supported by women reporting a worsening of anxiety and PTSD symptoms in the premenstrual and menstrual phases (McLeod, Hoehn-Saric, Foster, & Hipsley, 1993) (Bryant et al., 2011).

With its wide-ranging symptoms and complex pathology, PTSD is extremely difficult to treat. Moreover, the neural mechanisms that produce PTSD are unknown, and it is also unknown whether PTSD associated with acute versus chronic trauma have different underlying pathologies. A prolonged stress response may not in of itself be maladaptive, however, when stress-related symptoms do not abate there is need for intervention. In order to best treat PTSD, it is important to understand how its symptoms arise, including the underlying physiological changes and brain regions involved.

### *Fear Circuitry*

Attributing behaviors to a single brain structure is not probable considering the extent of neural interconnectivity; however, several brain regions have been highly

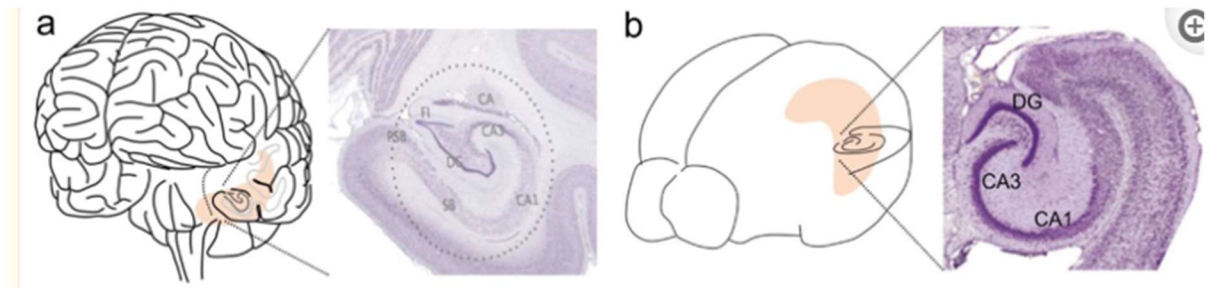
implicated in fear responding and together likely form fear circuits that have been reviewed in detail (Tovote, Fadok, & Lüthi, 2015), (Davis, 1992), (Perusini & Fanselow, 2015). The main fear circuit in rodents is complex and associated with several brain regions and circuits, and can involve input from the medial prefrontal cortex (mPFC) travelling to the basolateral amygdala. From there, the central nucleus of the amygdala is activated which is responsible for enhanced fear expression. The infralimbic cortex (IL) of the mPFC projects to amygdala and activates inhibitory intercalated cells and inhibits fear output. Conversely, input from the mPFC prelimbic cortex (PL) enhances fear expression. In humans, the dorsal anterior cingulate cortex (dACC) is homologous to the rodent PL, and the ventromedial PFC is the homologous to the rodent IL (Fenster, Lebois, Ressler, & Suh, 2018). Additional brain regions play roles in the generation and expression of fear such as the hippocampus. The hippocampus can suppress or induce fear memory expression depending on the contextual cues. In rodents for example, in a context where a previously threatening cue has been extinguished, the hippocampus will activate the IL to suppress fear expression. In fact, the hippocampus has been widely studied for its role in learning and memory, particularly episodic memory, spatial learning, and contextual fear {Fanselow, 2010 #35}. Additionally, as a limbic structure, the hippocampus is involved in emotion and cognition. It is also heavily impacted by HPA axis activity, where it is not only a regulator of the stress response, but also becomes dysfunctional upon elevations in stress hormones {Herman, 2005 #84}.

### *Hippocampus*

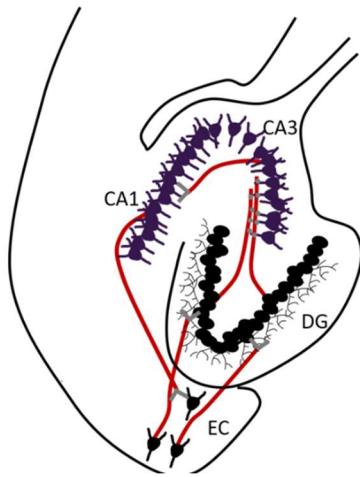
The hippocampus is a layered brain structure that is commonly functionally split into dorsal and ventral regions in rodents (Moser & Moser, 1998). Although it is likely

not strictly divided dorsally and ventrally, lesion studies have shown that each region is responsible for different functions. The dorsal hippocampus (DH) is thought to be involved in spatial representation and short-term memory, and is equivalent to the human anterior hippocampus (Moser, Moser, Forrest, Andersen, & Morris, 1995). When the DH is lesioned in rats, there is impaired performance in the Morris water maze. This behavior is not impacted by ventral hippocampus (VH) lesions (Moser et al., 1995). The VH is involved in stress and emotional valence, and is most closely related to the human posterior hippocampus. When the VH is lesioned in rodents, stress responses and emotional behavior is impaired (Henke, 1990). In addition to dorsal and ventral divisions, the layers of the hippocampus are also distinct (Fanselow & Dong, 2010). They are regions CA1, CA2, CA3, and the dentate gyrus. Each layer has unique cytology, with CA1 consisting of small pyramidal neurons, large pyramidal neurons with mossy fibers making up CA3, and CA2 containing large pyramidal neurons without mossy fibers. The principal cell type of the dentate gyrus is the glutamatergic granule cell. The connections between the layers are also unique (Amaral & Witter, 1989). Within the hippocampus, there is a tri-synaptic loop where input from the entorhinal cortex (EC) synapses on dendrites in the molecular layer of the dentate, creating the perforant path. From the dentate, mossy fibers synapse with pyramidal cells in CA3, which then project to Schaffer collaterals in CA1. These then project back to the EC, completing the loop. CA1 also projects to the retrosplenial and anterior cingulate cortices, two areas known for visuospatial and memory processing. Considering the diversity of the hippocampus, we will focus on one region for this thesis- the dentate gyrus.

**Figure 1.** Adapted from Hainmuller & Bartos (2020). Hippocampus Morphology. A) Human brain with Nissl-stained hippocampus B) Rodent brain with Nissl-stained hippocampus



**Figure 2.** Adapted from Johnson (2019). Hippocampal trisynaptic loop

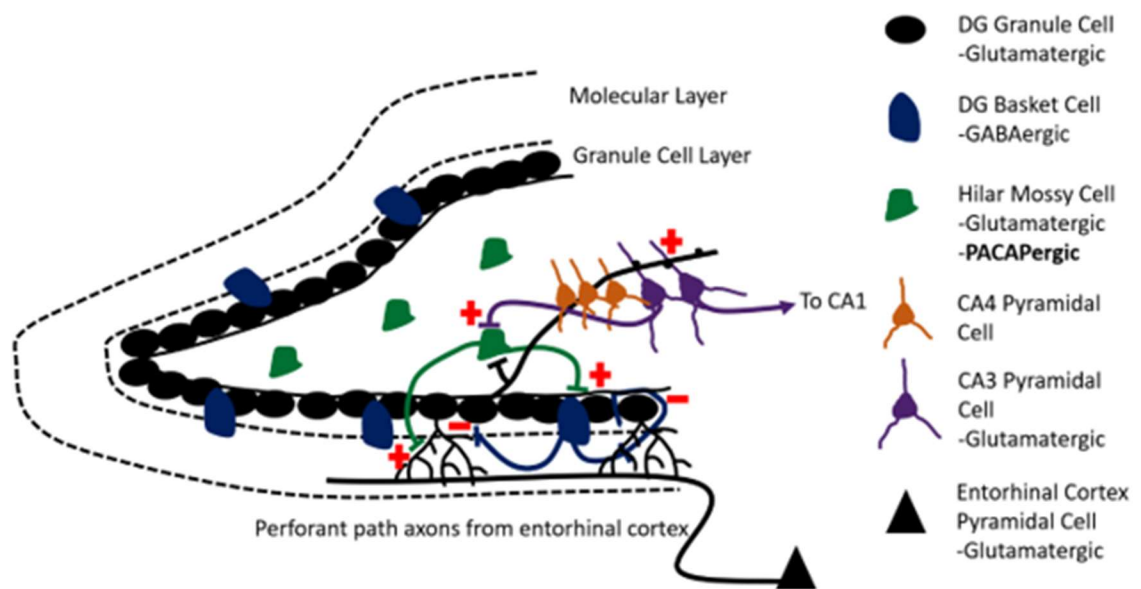


The dentate gyrus is the most ventral aspect of the hippocampus, directly lateral to the third ventricle. Like the hippocampus itself, the dentate is also layered, consisting of an upper blade and a lower blade, and three distinct regions: the molecular layer, the granule layer, and the hilar region. As mentioned before, granule cells are the principal

cell type, although only a small portion of these cells are active under basal conditions, as they are tonically inhibited by GABAergic inputs (Krueppel, Remy, & Beck, 2011).

Besides granule cells, which are the most abundant, there are also mossy cells and basket cells in the dentate. Mossy cells are glutamatergic and the only excitatory cell type that has connections to granule cells, although their overall effect on cells in the dentate is not known. Basket cells are inhibitory and reside mostly on the borders of the upper and lower blade (Amaral, Scharfman, & Lavenex, 2007).

**Figure 3.** Adapted from Johnson (2019). Dentate gyrus circuitry.



Input to the dentate gyrus from outside the hippocampus is limited. Besides the entorhinal cortex, there is input from the raphe, which provides serotonergic input, the locus coeruleus which provides noradrenergic input, and the medial septum which provides cholinergic input (Amaral et al., 2007). Additionally, the supramammillary nucleus, which contains many neuropeptides, most notably pituitary adenylate cyclase activating polypeptide (PACAP), also has afferents to the dentate. These

extrahippocampal inputs innervate the molecular layer of the dentate. PACAP mRNA has also been found in mossy cells, suggesting there may be local PACAP in the dentate as well (Lein et al., 2007).

### **1.3. PACAP**

#### *PACAP Overview*

PACAP itself has been highly implicated in stress responding and PTSD (Vaudry et al., 2009), (Boucher, May, Braas, & Hammack, 2021) (King, Toufexis, & Hammack, 2017) (Hammack & May, 2015) , and it is well conserved and widely distributed in central and peripheral tissues. PACAP functions not only as a neurotransmitter but also as an endocrine regulator, taking part in homeostatic regulation of endocrine hormone production and secretion, cardiovascular responses, glucose metabolism, intestinal motility, micturition, and germ cell maturation. In the nervous system, PACAP has a variety of roles in feeding and satiety, nociceptive sensitivity, learning and memory, and behaviors related to chronic stress.

There are two forms of PACAP: PACAP27 and PACAP38, with PACAP38 being vastly more abundant, especially in the brain. Both forms activate the cognate receptor, PAC1, as well as VPAC1 and VPAC2, which vasoactive intestinal polypeptide (VIP) also binds to (Harmar et al., 2012). PAC1 receptors have two splice variants, Hip and Hop, that cause differential second messenger potency, efficacy, or duration. Once activated, PAC1 receptors can also be internalized for beta arrestin mediated endosomal ERK signaling.

PACAP is expressed in the hypothalamus, bed nucleus of the stria terminalis, the amygdala, prefrontal cortex, hilar mossy cells in the dentate gyrus as mentioned before, and the parabrachial nucleus (Hashimoto et al., 1996). It is believed that PACAP's ability to influence behaviors related to stress is due in part to its expression in many limbic structures. This PACAP effect is shown in experiments designed to test anxiety-like behavior. For example, PACAP infusion increases stress-like responses (Agarwal, Halvorson, & Legradi, 2005), anxiety-like behavior (Roman et al., 2014), and startle responses in rats (Hammack et al., 2009). Additionally, activation of PACAP afferents to the BNST increased anxiety-like behavior in mice (Boucher, Aktar, Braas, May, & Hammack, 2022). For this reason, PACAP has been a peptide of interest in studying pathology of stress disorders.

#### *PACAP and PTSD*

There has been substantial evidence associating PACAP dysregulation with PTSD in humans. Ressler and colleagues found in 2011 that in a traumatized population, high PACAP38 blood levels in females but not males predicted PTSD symptoms and diagnosis (Ressler et al., 2011). Additionally, high vs low PACAP levels in women also predicted number of PTSD symptoms experienced, with high PACAP women experiencing significantly more symptoms. Women, but not men, also displayed an increased fear response in an acoustic startle task, suggesting their response to conditioned fear had been heightened. Together, these results suggest there is a sex specific component that influences PACAP expression and its effects. Upon investigation, Ressler and others found that within the gene for the PAC1 receptor, there is a single nucleotide polymorphism (SNP) in an estrogen response element that is associated with

PTSD in females (Ressler et al., 2011). This work suggests that PACAP and the PAC1 receptor plays an important role in traumatic stress, and that males and females are differently affected.

### *PACAP in the Dentate Gyrus*

As mentioned previously, PACAP is thought to have actions in the hippocampus (Sauvage, Brabet, Holsboer, Bockaert, & Steckler, 2000), (Otto et al., 2001). In the CA1 region of the hippocampus, PACAP infusions enhance consolidation of contextual fear conditioning, while infusion of PACAP(6-38), a PACAP antagonist, impaired consolidation as well as extinction. Importantly, there are PACAP expressing cells and PAC1 receptor transcripts in the hilus of the dentate gyrus. Behaviorally, there is decreased functioning in hippocampal dependent tasks in PAC1 receptor knock out mice, suggesting that PACAP may serve an important role in the hippocampus, particularly the dentate gyrus.

A hallmark behavioral function of the dentate gyrus is context discrimination. It is thought to be a “pattern separator” meaning it distinguishes between environments despite similarities between them. This has been shown in rodents in radial arm mazes where lesions of the DG have impaired spatial memory (Gilbert, Kesner, & Lee, 2001), and where removal of dentate granule cells impaired pattern separation of similar contexts (Nakashiba et al., 2012). Additionally, optogenetic activation of a memory engram in the DG is sufficient to produce behavior appropriate to that memory (Liu et al., 2012).

## *PACAP and Fear Conditioning*

When studying behavior in the dentate gyrus, a common paradigm used in rodents is contextual fear conditioning (Sanders, Wiltgen, & Fanselow, 2003). Contextual fear conditioning is a form of Pavlovian fear conditioning, where a neutral conditioned stimulus (CS) is paired with an aversive unconditioned stimulus (US) to produce a conditioned response (CR). In contextual fear conditioning, the US is a fearful stimulus, such as a mild foot shock, and is paired with a distinct context as the CS. The context can consist of everything in the environment, from the physical features to the auditory and olfactory components of the space. When the organism connects the context to the imminent presence of the aversive stimulus, they display a fear response to the context alone, and have been contextually fear conditioned. The level of fear they exhibit is operationalized as freezing behavior, or the absence of movement besides what is required for breathing. This is an evolutionarily adaptive behavior and is shown in wild, naturally behaving rodents. It is a robust measure that is used widely among researchers (Trott, Hoffman, Zhuravka, & Fanselow, 2022). There is some debate as to whether this behavior is sex biased, meaning that female rodents are thought to not show freezing as a sign of fear as often as males. Some alternative behaviors proposed for females are rearing, grooming, and in cued fear conditioning, darting. However, there is much debate surrounding these claims (Trott et al., 2022), (Colom-Lapetina, Li, Pelegrina-Perez, & Shansky, 2019).

Due to the work implicating the role of PACAP in the hippocampus, Johnson and colleagues used whole-cell patch clamp electrophysiological methods *ex vivo* to apply PACAP38 to neurons in rat brain slices of the dentate gyrus, observing an increase in

spiking of 95% of the active cells (Johnson, Parsons, May, & Hammack, 2020).

Following this characterization, these investigators utilized contextual fear conditioning to determine if infusion of PACAP38 had an impact on fear memories. In the first iteration of the experiment, the rats were given an infusion of either PACAP38 or vehicle, and conditioned in a chamber to four mild foot shocks. The following day, they were returned to the same chamber in the absence of shock, and percent time freezing was recorded as a measure of fear. It was found that PACAP infusion had no impact on fear when infused prior to conditioning, in other words, PACAP had no effect on fear consolidation. The next iteration of the experiment therefore tested PACAP on fear retrieval. To do this, rats were conditioned and then received the infusion of either PACAP or vehicle directly prior to the test session. In this experiment, a small main effect of PACAP on freezing was discovered, where PACAP infusion caused a slight increase in freezing as compared to the vehicle group. This led to the hypothesis that PACAP was interfering with the retrieval of fear memories when in the context where the fear was learned.

Based on this work, we argue that PACAP related effects in the DG may represent a mechanism behind the generalization symptoms of PTSD. As discussed before, there is already an existing literature connecting PACAP and PTSD, so there is reason to believe PACAP has a role in generating symptoms related to PTSD pathology. However, the experiments in Johnson (2019) are not designed to test generalization. To do that, two distinct contexts are needed, one that was the conditioning context and is therefore fearful to the animal (context A), and one that is a safe context (context B), where no fear experiences were had. With this design, it is possible to observe any behavior change in

both the fearful and safe environments. Additionally, considering the role of sex in both PACAP and PTSD, the Hammack lab was interested in how both male and female subjects would behave in contextual fear experiments with the influence of PACAP. In this thesis, we will be presenting work that establishes a context discrimination paradigm, compares contextual fear discrimination between males and females, and tests the effect of PACAP infusion on discrimination of contexts in both males and females.

## **CHAPTER 2: Experiment 1: Contextual fear discrimination in male rats**

The purpose of Experiment 1 was to demonstrate contextual fear discrimination learning in rats. While prior studies have previously demonstrated contextual discriminations, the majority of these studies typically involve a single session of conditioning in Context A, followed by a test session in either Context A or a novel Context B {Tronson, 2009 #76}.

However, this procedure has the potential to either over- or underestimate fear to Context B. For instance, if the novel Context B elicits unconditional fear behavior, this could result in an overestimation of fear. In contrast, if Context B elicits exploratory behavior, this could interfere with fear-related behaviors and therefore underestimate fear in B. To remedy this, the current experiment ensured equal familiarity to Context A and Context B by including repeated exposures to Context A with shock and Context B in the absence of shock. Thus, on each conditioning day, all rats are exposed to both Context A and Context B.

Bucci et al., (2002) examined contextual fear discrimination using a similar method. In their experiments, rats were repeatedly exposed to Context A with foot-shock and Context B in the absence of shock. Unsurprisingly, they reported stronger freezing in

Context A than Context B (Bucci, Saddoris, & Burwell, 2002). However, one potential confound to this study was that all rats always received the Context A session in the morning. Therefore, it is not possible to rule out “time-of-day” as contributing to the discrimination. Another potential issue with the experiment by Bucci et al., (2002) was that the contexts consistently predicted each other, so if the animal had just received exposure to Context A, they could expect Context B to follow and vice versa. Both confounds were controlled for in Experiment 1.

### *Subjects*

The subjects were 16 male experimentally naïve Sprague- Dawley rats. Rats were allowed one week to acclimate to the vivarium while paired housed in 12 x 7.5 x 7.5 plastic caging where they remained for the duration of the experiment. Food and water were available ad libitum (LabDiet 5P00 Prolab RMH 3000, LabDiet, St. Louis, MO) in a climate-controlled colony room on a 12:12 light-dark cycle. Throughout the experiment, rats were monitored and cared for in compliance with the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and the University of Vermont Institutional Animal Care and Use Committee.

### *Behavioral Apparatus*

Behavioral procedures occurred in 8 conditioning chambers (Med Associates, Inc., St. Albans, VT, ENV-007; 24 cm W × 30.5 cm L × 29 cm H), which were modified to create 2 sets of distinct “contexts”. All chambers had the following common features. Each chamber was housed in a sound-attenuating cabinet (Med Associates, ENV-017M; 66 cm W × 56 cm L × 56 cm H) outfitted with an exhaust fan to provide airflow and background noise (68 dB). All 8 chambers were outfitted with a food cup, recessed in the

center of the front wall, a retracted lever (Med Associates, ENV-112CM), located on the right of the front wall, and an inactive nose-poke aperture (2 cm in diameter) located 3 cm above the food cup. All chambers also had a panel light (Med Associates, ENV-221M) on the right front wall (16 cm above the grid floor), a house light (Med Associates, ENV-215M) centered on the back wall 24 cm above the grid floor, and a speaker (Med Associates, ENV-224AM) located 20 cm above and to the right of the food cup. The grid floor was used to deliver a 1.0-mA, 1.0- sec shock. Security cameras were mounted to the wall outside each sound-attenuating cabinet, and an 8-cm hole in the chamber wall allowed for video recording from the wall opposite the door.

The 8 conditioning chambers were divided into 2 distinct contexts, with 4 chambers per context. The two distinct contexts were counterbalanced as “Context A” and “Context B”. The first set, the “Anise” context, had a grid floor with thick and thin alternating bars and the tray beneath was painted matte gray. The side walls were stainless steel, and the front and back walls were plastic with a horizontal black bar taped to the center. There was a geometric insert attached at an angle to the left between the front and side wall. Two key lights were on during the sessions and the house lights were off. A fan within the cabinet provided background noise and a dish of 20% anise extract diluted in water was placed within the cabinet to scent the chambers.

The second set of chambers, the “Coconut” context, had a grid floor with bars arranged in an arch above a stainless-steel tray. The ceiling and front wall of each box featured a blue polka dot pattern. The two key lights were turned off, and the house light was turned on. The exhaust fans were turned off in this context, and the chambers were scented with a dish of 10% coconut extract diluted in water placed within the cabinet.

### *Behavioral methods*

*Pre-exposure.* Day one of the experiment consisted of 12-minute preexposure sessions in both contexts, where each subject experienced one context in the morning and the other in the afternoon with the contexts counterbalanced for time of day.

*Conditioning.* Day two of the experiment marked the beginning of conditioning. All sessions were 8 minutes long and the subjects experienced both contexts, one in the morning and one in the afternoon. In Context A, 1.0-mA 1.0-sec shocks occurred at 4.5 minutes, 5.5 minutes, and 6.5 minutes after placement in the chamber. No shocks occurred in Context B. Exposure to contexts A and B were counterbalanced for physical context and time of day. This ensured that exposure to each context was not predictable based on time of day or what context had been experienced in the previous session. This procedure was carried out for 7 additional days, for a total of 8 days of conditioning.

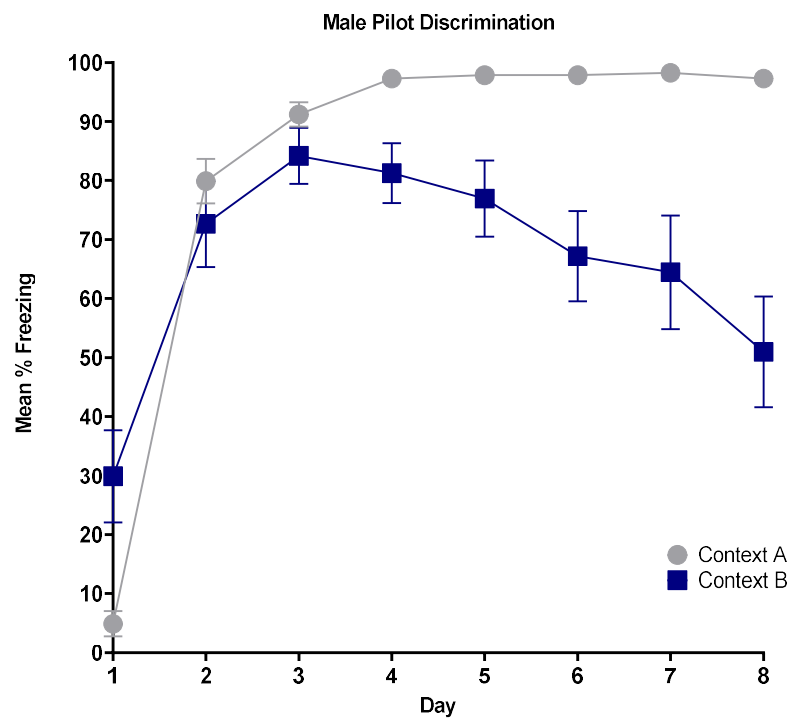
### *Behavioral Observations and Data Analysis*

The ability to discriminate between contexts was determined by analyzing freezing as the dependent variable, which is a known behavioral marker of fear. Freezing is defined as total motor immobility except for breathing (Blanchard & Blanchard, 1969). For the conditioning sessions, behavior was assessed (freezing or not) for each rat every 8 seconds during the first 4.5 min of the session, which is the time before the first shock occurs in Context A. The frequency of freezing indices was converted to a percentage of time spent freezing. A low percentage of freezing would indicate the subject was not fearful in the context, while a high percentage of freezing would indicate the subject was fearful.

## Results and Discussion

Freezing in Contexts A and B is presented in Figure 1. Freezing was analyzed with a 2 (Context: A vs B)  $\times$  8 (day) ANOVA, which revealed a main effect of day [ $F(7, 105) = 63.56 p < 0.001$ ]. There was also a main effect of context, [ $F(1, 15) = 12.41 p < 0.01$ ], indicating more freezing in Context A than B. Additionally, there was a significant interaction of day and context [ $F(7, 105) = 14.40 p < 0.001$ ], indicating that the strength of the discrimination increased over the course of training.

**Figure 4.** Mean percent freezing in Context A and Context B over 8 days of conditioning.



### **CHAPTER 3: Experiment 2: Contextual fear discrimination in male and female rats**

Experiment 1 demonstrated contextual discrimination in male rats as a result of our contextual fear conditioning protocol. However, we know from past literature that males and females might differ in how they express fear behaviors, with many reporting that male rats show stronger conditioning. For example, Russo and Parsons found that male rats exhibit higher levels of freezing behavior than females (Russo & Parsons, 2021). Nevertheless, the literature is not always consistent. Pryce and colleagues found that sex differences may depend on the strain of rat (Pryce, Lehmann, & Feldon, 1999), and in mice, some studies have found that sex differences depend on parameters, with male mice show stronger conditioning to context only when there is no pre-exposure (Wiltgen, Sanders, Behne, & Fanselow, 2001). Additionally, reports of how the sexes discriminate contexts differ as well. A multitude of studies suggest that female mice show more generalization than males (Asok et al., 2019); (Keiser et al., 2017). However, this finding may not be applicable to other species and parameters. When generalization is tested soon after conditioning in rats, this sex difference does not appear (J. Lynch, 3rd, Cullen, Jasnow, & Riccio, 2013), and (Colon, Odynocki, Santarelli, & Poulos, 2018) found that while generalization of fear changes in male rats as they develop, this is not true for female rats.

Taking into consideration the breadth of research supporting potential sex differences in both fear conditioning and generalization, the purpose of experiment 2 was to test our contextual fear conditioning paradigm in female rats, and compare the two sexes. Prior to the start of experiment 2, we also made a few changes to the experimental

parameters from experiment 1 to increase the efficacy of the paradigm. An additional preexposure day was included as it has been shown that preexposure sessions significantly assist in learning about contexts (Rudy & O'Reilly, 1999). Additionally, the original three shock exposure protocol was reduced to one in order to avoid a “ceiling effect” where fear in context A would be too high to observe behavior accurately. Finally, because the shock exposures were reduced, the total session length was also reduced, where instead of 8 minutes total, the session was 6 minutes total.

### *Subjects*

The subjects were 16 male and 16 female experimentally naïve Sprague- Dawley rats. Rats were allowed one week to acclimate to the vivarium while housed in pairs in 12 x 7.5 x 7.5 plastic caging where they remained for the duration of the experiment. Food and water were available ad libitum (LabDiet 5P00 Prolab RMH 3000, LabDiet, St. Louis, MO) in a climate-controlled colony room on a 12:12 light-dark cycle. Throughout the experiment, rats were monitored and cared for in compliance with the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and the University of Vermont Institutional Animal Care and Use Committee.

### *Behavioral Apparatus*

The behavioral apparatus used in Experiment 2 was identical to Experiment 1.

### *Behavioral Methods*

*Pre-exposure.* Days one and two of the experiment consisted of 10-minute preexposure sessions in both contexts, where each subject experienced one context in the morning and the other in the afternoon with the contexts counterbalanced for time of day.

*Conditioning.* Day three of the experiment marked the beginning of conditioning. All sessions were 6 minutes long and the subjects experienced both contexts, one in the morning and one in the afternoon. A single 1.0-mA 1.0-sec shock occurred at 4.5 minutes into the session, while in context B no shocks occurred. Exposure to Contexts A and B were counterbalanced for physical context and time of day. This procedure was carried out for 7 additional days, for a total of 8 days of conditioning.

#### *Behavioral Observations and Data Analysis*

As in experiment 1, discrimination was quantified using fear behavior. Freezing is defined as total motor immobility except for breathing (Blanchard & Blanchard, 1969). For the conditioning sessions, behavior was assessed (freezing or not) for each rat every 8 seconds during the first 4.5 min of the session, which is the time before the first shock occurs in Context A. The frequency of freezing indices was converted to a percentage of time spent freezing. A low percentage of freezing would indicate the subject was not fearful in the context, while a high percentage of freezing would indicate the subject was fearful.

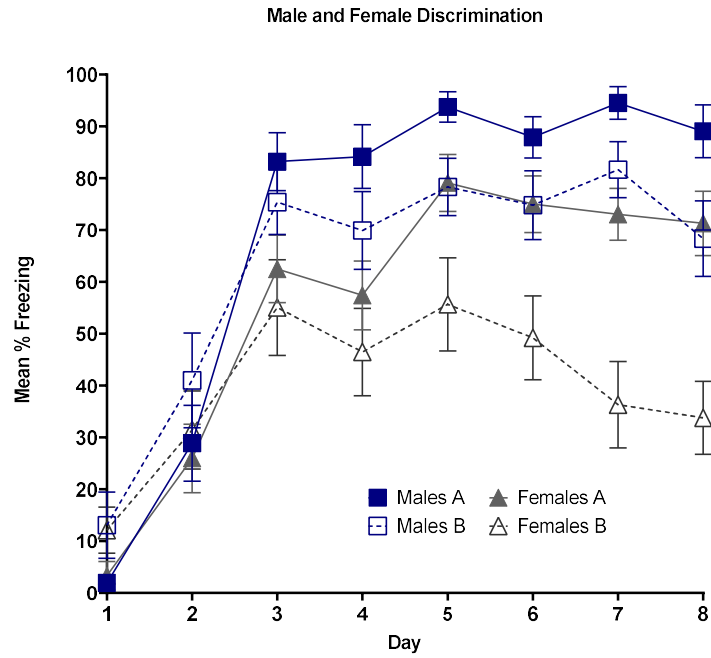
#### *Results and Discussion*

Mean percent freezing is presented in Figure 2. A 2 (context: A vs. B)  $\times$  2 (sex: M vs. F)  $\times$  8 (Session) ANOVA revealed a significant main effect of day [ $F(7, 210) = 74.52$   $p < 0.001$ ]. There was also a main effect of context [ $F(1, 30) = 21.33$   $p < 0.001$ ], with more freezing in A than B. Additionally, there was a main effect of sex [ $F(1, 30) = 10.21$   $p < 0.01$ ], with males freezing more than females. Finally, there were interactions of day and sex [ $F(7, 210) = 3.71$   $p < 0.001$ ], as well as day and context [ $F(7, 210) = 13.24$   $p < 0.001$ ].

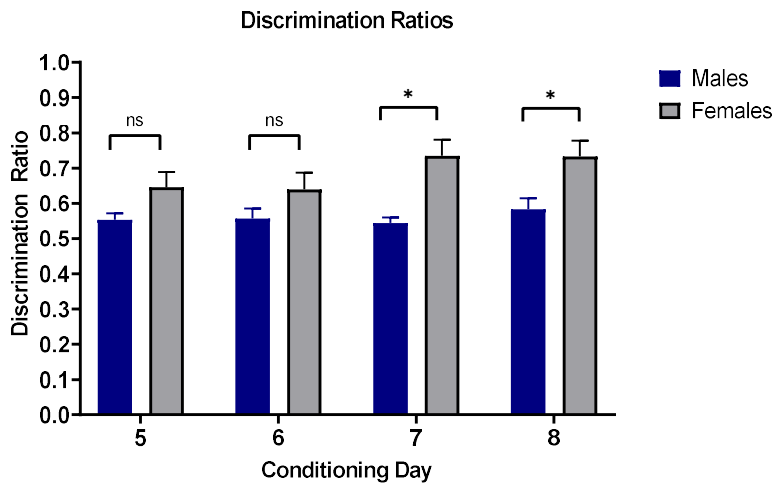
Although the above analysis did not detect differences in the strength of the *discrimination* as a function of sex, inspection of Figure 2 suggests that near the end of training, females may have shown a greater difference between A and B than males. To further examine this possibility, we completed a supplementary analysis of discrimination ratios (freezing in A/ (freezing in A + freezing in B)) for conditioning days 5 through 8 (Figure 3). An independent samples t-test revealed a significant difference between males and females on conditioning days 7 ( $t(30) = -3.94, p < 0.001$ ) and 8 ( $t(30) = -2.73, p = 0.01$ ). The discrimination ratios for female rats were significantly higher than male rats.

Experiment 2 served to investigate sex as a variable in our contextual fear discrimination paradigm. We found that both male and female rats were able to discriminate between the contexts, where both groups froze more in context A than B. Interestingly, male rats froze more overall than female rats, replicating prior findings that suggest males fear condition stronger than females (Maren, De Oca, & Fanselow, 1994), (Gresack, Schafe, Orr, & Frick, 2009). Additionally, while overall there was no main effect of sex or an interaction with context, the means suggested that a sex effect emerged later in conditioning, specifically day 7 and 8. This was supported by the calculated discrimination ratios and statistical tests. The most important conclusion to take from this experiment is that sex as a variable must be considered in future experiments, since the level of freezing is differential between sexes.

**Figure 5.** Mean percent freezing in Context A and B of male and female rats



**Figure 6.** Comparison of discrimination ratios of male and female rats



## **CHAPTER 4: Experiment 3: DG PACAP and contextual fear discrimination**

Experiment 1 demonstrated contextual discrimination in rats, and experiment 2 showed that while both sexes are able to discriminate, males show overall higher freezing and females show a stronger discrimination. The purpose of experiment 3 was to investigate the potential role of dentate gyrus PACAP in contextual discriminations. As previously discussed, Johnson (2019) had examined dentate gyrus PACAP's impact on contextual fear conditioning. His finding, that PACAP in the dentate effects retrieval of context fear and not encoding, suggests that PACAP is influential in fear memories, and gives reason to investigate other facets of fear learning.

Given that the dentate gyrus is known to contribute to discriminating contexts (Biedenkapp & Rudy, 2007), (Wiltgen et al., 2010), the present experiment sought to look at the potential effect of PACAP in the dentate gyrus on contextual fear discrimination. Building off Johnson's findings, experiment 3 looked at PACAP's influence on the retrieval of fear not only in the conditioning context, but also in a safe context. This was done by training both male and female rats to discriminate between a fearful context (A), and a safe context (B), as in Experiment 2. At the test session, subjects were infused with either vehicle or PACAP38 into the dentate gyrus and males and females were both split into 4 groups: PACAP infusion and tested in context A, PACAP infusion and tested in context B, vehicle infusion and tested in context A, and vehicle infusion tested in context B. This design allowed for the comparison of not only vehicle vs PACAP, but also PACAP in A vs B, and male vs female in all conditions.

### *Subjects*

The subjects were 32 male and 32 female experimentally naïve Sprague- Dawley rats. Rats were allowed one week to acclimate to the vivarium while housed in pairs in 12 x 7.5 x 7.5 plastic caging where they remained for the duration of the experiment. Food and water were available ad libitum (LabDiet 5P00 Prolab RMH 3000, LabDiet, St. Louis, MO) in a climate-controlled colony room on a 12:12 light-dark cycle. Throughout the experiment, rats were monitored and cared for in compliance with the Association for the Assessment and Accreditation of Laboratory Animal Care guidelines and the University of Vermont Institutional Animal Care and Use Committee.

### *Behavioral Apparatus*

The behavioral apparatus used in Experiment 3 was identical to Experiments 1 and 2.

### *Behavioral Methods*

*Pre-exposure.* Days one and two of the experiment consisted of preexposure sessions identical to experiment 2.

*Conditioning.* Day three of the experiment marked the beginning of conditioning which lasted 8 total days and was identical to experiment 2.

*Surgical Cannulation.* Following the initial conditioning phase, all rats underwent bilateral implantation of cannula to the dentate gyrus. Rats were anesthetized with 1.5-3% isoflurane and placed in a stereotaxic frame for cannula placement. The guide cannulae were lowered to the molecular layer of the dentate gyrus, using coordinates relative to bregma in mm used in Johnson (2019) (10-degree angle, AP=-3.5; ML  $\pm$ 1.9; and DV=-3.5 to the dural surface). They were secured using a head cap of dental cement, with

screws placed in the skull for extra strength. Dummy cannulae were screwed into the guide cannula to ensure debris did not clog them during the recovery process. Rats were allowed to recover for a week minimum.

*Post-surgery conditioning.* After recovery, rats were given five additional days of conditioning to refresh their ability to discriminate contexts. The conditioning was identical to the pre-surgery conditioning protocol.

*Infusion and Test.* The next day following post-surgery conditioning, all rats underwent an infusion and test session. Prior to the test, the rats were allocated to their test groups. Male and female rats were split into four groups each; vehicle infusion and test session in Context A, vehicle infusion and test session in Context B, PACAP infusion and test session in Context A, or PACAP infusion and test session in Context B. This made for 8 groups total. Each group was balanced to have a similar mean percentage of freezing in their test context on the last day of conditioning. This was done so any behavioral differences between the groups at test could more accurately be attributed to the infusion manipulation. On the test day, rats were intracranially infused based on their assigned group with either 1 $\mu$ g/ $\mu$ l PACAP38 or 1 $\mu$ l of PBS. After an approximately 30-minute lead time, they were placed in their respective conditioning chambers and experienced a 20-minute shock free test session.

*Histology.* Rats were perfused with 4% paraformaldehyde and brain tissue was postfixed for 24 hours, then cryoprotected in 30% sucrose for an additional 24 hours. Brain tissue was then sliced on a cryostat at 60 microns for cannula placement confirmation. Cannula placements in rats allocated to a PACAP condition that did not terminate in the dentate gyrus were excluded from the results and data analysis.

### *Behavioral Observations and Data Analysis*

As in experiment 1 and 2, fear behavior was quantified as mean percent time spent freezing. For this experiment, an automated method of scoring freezing was used as opposed to hand scoring. Automated scoring of freezing was conducted using the following method: video streams were acquired in near-infrared (720P resolution, 29.97 frames per second) by Anpviz IPCameras (model IPC-B850W) mounted in each chamber. Streams were delivered over a dedicated ethernet network, and captured by a computer running ffmpeg. Recordings were subsequently scored by first computing the absolute difference in pixel intensity at every pixel on each pair of subsequent frames. A per-frame activity measure was produced by averaging this difference over all pixels. Inspection of the distribution of (log10-transformed) activity scores revealed a clear bimodal distribution of activity, with the mode of the lowest scores reflecting video noise and mode of the higher scores reflecting rat movement. These distributions varied almost solely by chamber/camera. Presumptive freezing was therefore defined as occurring, on a per-chamber basis, when the activity score fell below the value visually marking the beginning of the rat-movement related portion of the distribution. Activity scores were then averaged in 1 s bins, and only 1 s bins that fall below the threshold were defined to represent freezing (approximating procedures used by the Fanselow laboratory, (Fanselow, Hoffman, & Zhuravka, 2019)). Algorithmically scored freezing correlated well with freezing scored by trained human observers, with all R-values exceeding 0.80.

### *Results and Discussion*

All analyses included all vehicle rats and only PACAP rats with acceptable cannula placements (Males: Vehicle in Context A n= 9, PACAP in Context A n= 4,

Vehicle in Context B n= 6, PACAP in Context B n= 5. Females: Vehicle in Context A n= 7, PACAP in Context A n= 5, Vehicle in Context B n= 7, PACAP in Context B n= 5). Pre-surgery conditioning mean percent freezing is presented in Figure 7. A 2 (context: A vs. B) × 2 (sex: M vs. F) × 8 (Session) ANOVA revealed a significant main effect of session [ $F(7,1) = 85.67 p < 0.001$ ], a main effect of context [ $F(1,0) = 9.55 p < 0.004$ ], and a main effect of sex [ $F(1,0) = 6.48 p < 0.015$ ]. There was also an interaction of session and context [ $F(7,1) = 8.43 p < 0.001$ ]. The results from the pre-surgery conditioning phase of experiment 3 replicates the sex difference found in experiment 2, where females discriminate contexts better than males, and also that males fear conditioned stronger than females.

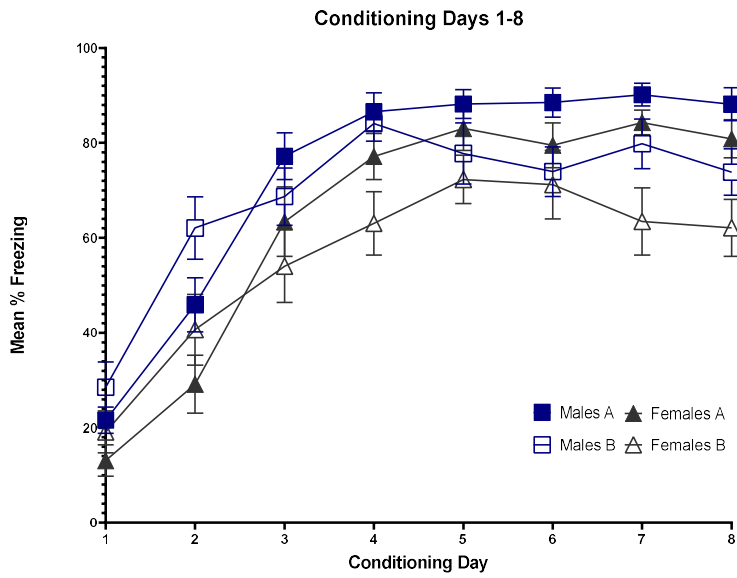
Post-surgery conditioning freezing is presented in Figure 8. A 2 (context: A vs. B) × 2 (sex: M vs. F) × 5 (Session) ANOVA showed a main effect of context [ $F(1,0) = 46.22 p < 0.001$ ] and an interaction of session and context [ $F(4,1) = 9.01 p < 0.001$ ]. This indicated that freezing significantly differed between contexts with higher freezing in context A, and that as conditioning progressed, discrimination of the contexts improved.

Test data is presented in Figures 9 and 10. Although groups were matched prior to testing, unfortunately the exclusion of rats for missed placements resulted in mean differences during the last session of conditioning. Thus, in order to assess if PACAP produced changes in behavior, we analyzed the last day of conditioning and the test day with a 2 (context: A vs B) x 2 (sex: male vs female) x 2 (time point: last day of conditioning vs test day) x 2 (infusion: vehicle vs PACAP) ANOVA. This would prevent any preexisting differences in freezing between groups from being misrepresented as an effect of infusion. There was a main effect of session [ $F(1,0) = 7.18 p < 0.011$ ], a main

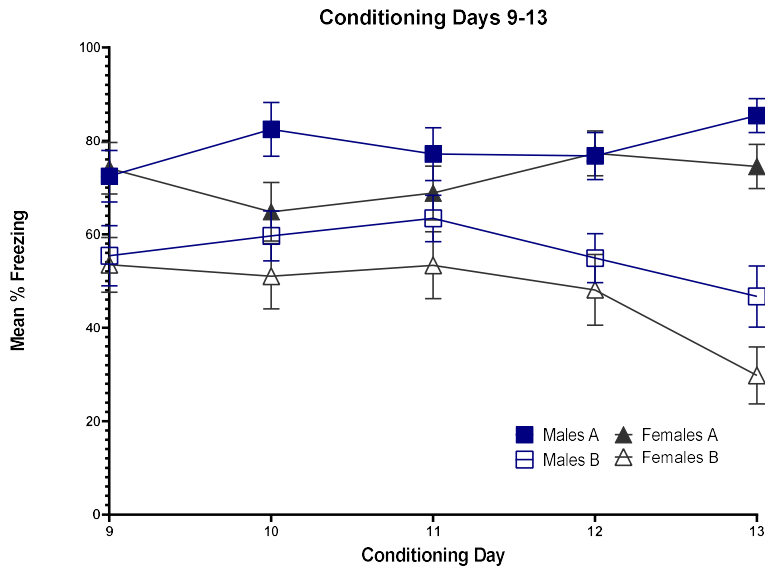
effect of sex [  $F(1,0) = 4.24$   $p < 0.046$ ] and an interaction of session and context [  $F(1,1) = 6.76$   $p < 0.013$ ]. This meant that freezing was significantly different between the last conditioning session and the test session, that freezing was significantly different between males and females, and that the amount of freezing in each context was influenced by the session.

The purpose of experiment 3 was to test the effect of a PACAP infusion on contextual fear discrimination in male and female rats. The results indicate that there was no effect of PACAP infusion on contextual fear discrimination. The lack of a statistical main effect of infusion indicates that there was no significant difference between the vehicle and PACAP groups in terms of behavior during the test session. While there was a difference in amount of freezing between the conditioning and test sessions, this is more likely an outcome of extinguishing fear as time goes on, and not due to an effect of our PACAP manipulation. The interaction between time and context supports this, since it shows that the freezing level in each context depends on time, and becomes lower as time goes on, as would be expected in extinction. Interestingly, the effect of sex that we have seen in our previous experiments has persisted in this experiment. Once again, females are overall freezing less than males in a contextual fear conditioning task, suggesting that males show stronger fear conditioning. The results of experiment 3 also suggests that female rats discriminate contexts better than males.

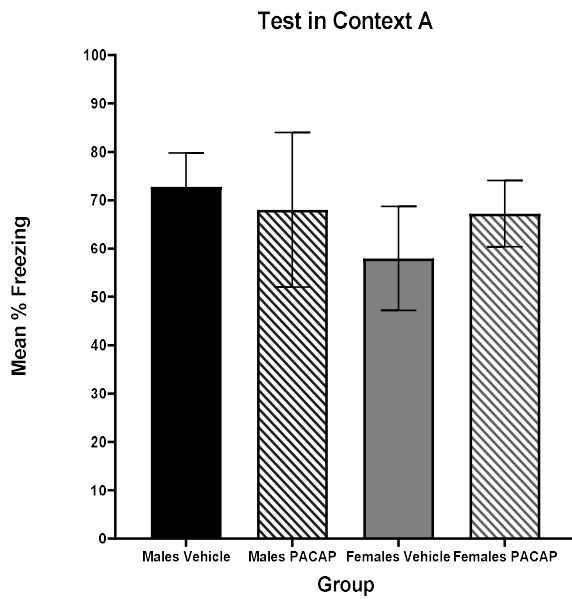
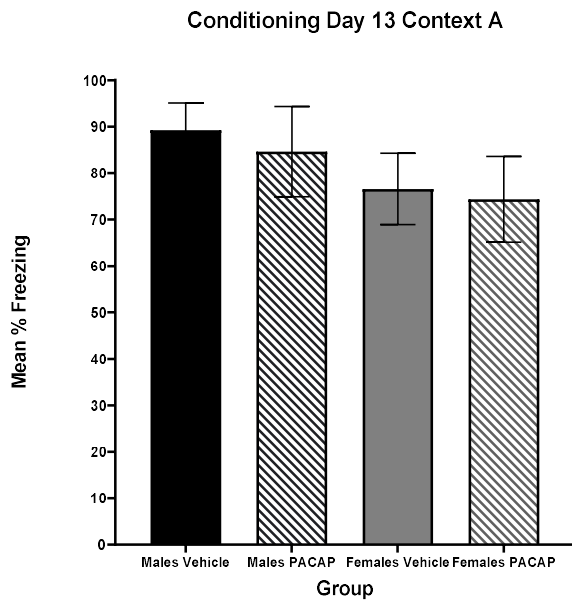
**Figure 7.** Pre-surgery conditioning for males and females in Contexts A and B



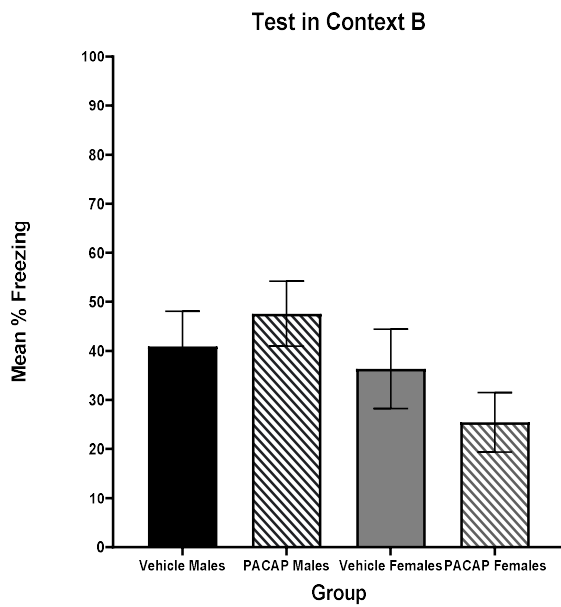
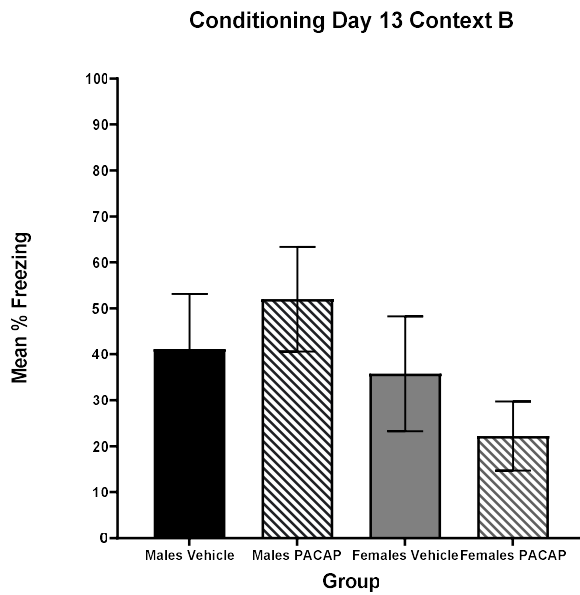
**Figure 8.** Post-surgery conditioning for males and females in contexts A and B



**Figure 9.** Mean percent freezing of male and female PACAP and vehicle rats in context A on the last day of conditioning (left) compared to the test session (right).



**Figure 10.** Mean percent freezing of male and female PACAP and vehicle rats in context B on the last day of conditioning (left) compared to the test session (right).



## CHAPTER 5: General Discussion

### 5.1. Summary of Results

The experiments in this thesis examined contextual fear discrimination learning in male and female rats, and the impact of dentate gyrus PACAP in fear responding in aversive and safe contexts. In Experiment 1, male Sprague-Dawley rats demonstrated a robust discrimination between the aversive and safe context. This finding extends prior work (Bucci et al., 2002) that demonstrated that male rats can discriminate between a context where they received foot shock, and a safe context. However, as noted, these authors always conditioned in the morning, and did not counterbalance sessions. Therefore, counterbalancing for time of day, session, and context, allowed for more control over potential confounding variables, particularly the ability to predict context exposure based on the time of day or what the animal had experienced last. This is an important feature of this experiment that had not been controlled for in previous studies.

This finding replicates past contextual fear conditioning studies that have been able to achieve a discrimination (Tronson et al., 2009), (Keiser et al., 2017), (Bucci et al., 2002). In the literature, contextual fear conditioning protocols commonly only have one conditioning day followed by a test day (Tronson et al., 2009), (Keiser et al., 2017). A strength of extending the conditioning period in experiment 1 was that the discrimination of the contexts was observable over the course of training, and that we were able to measure a strengthening of the discrimination over time.

A limitation of experiment 1 is that although it establishes working parameters for contextual fear conditioning in males, it is not applicable to female rats. There are many studies that show that male and female rats do not fear condition similarly (Gresack

et al., 2009), (Maren et al., 1994), (Keiser et al., 2017), therefore it was not clear if we would observe similar results from experiment 1 in female rats. Experiment 2 was performed to address this limitation and explore any differences between male and females in contextual fear conditioning. In addition, in Experiment 2 the experimental protocol was adjusted to a single 1-mA 1-sec shock from the original three shocks. This was done because in Experiment 1, the mean percentage of freezing in context A was consistently near or at 100%. While this indicates strong fear conditioning, it also limits the ability to observe increases in fear that future manipulations may cause.

Experiment 2 demonstrated that male and female rats both statistically significantly discriminated between context A and B. However, female rats had a lower mean percent freezing in both contexts as compared to males. The higher level of freezing in males observed in experiment 2 is congruent with other studies reporting that males show stronger fear conditioning than females (Maren et al., 1994), (Gresack et al., 2009). In addition, although conditioning was not as strong as the males, females were significantly fear conditioned and discriminated contexts significantly better than the males on days 7 and 8 of conditioning. This is in contrast to many studies that suggest that females show more generalization between contexts than males (Yagi, Chow, Lieblich, & Galea, 2016). Based on discrimination ratios, males and females were not different in discrimination of contexts on all days except for the last two days of conditioning (day 7 and day 8). On these days, females discriminated significantly better than males, which is in contradiction to the previous literature. One reason for this might be the length of conditioning. In the experiments that reported a stronger discrimination in males, conditioning took place for only a day (Wiltgen et al., 2001). Conversely, our

experiment took place over the course of 8 conditioning days. The fact that the sex difference in discrimination appeared on the last two days of conditioning might indicate that the strength of the discrimination between males and females may be time or experience dependent. These nuances in performance are not observable in shorter protocols, which is a strength of this design. However, a caveat of this latent effect is that it may not be as robust and therefore may not appear in all experiments.

Experiment 3 used the conditioning protocol from experiment 2 to test the effect of a PACAP38 infusion into the dentate gyrus in both male and female rats on context discrimination. To do this, all rats were first contextually fear conditioned for 8 days, just as in experiments 1 and 2. After this initial conditioning phase, rats underwent surgical cannulation to the dentate gyrus and were allowed at least one week to recover. Following recovery, rats were conditioned for an additional 5 days before the test day, where they received infusions of either PACAP or vehicle into the dentate gyrus and experienced a shock-free session in either context A or B while mean percent freezing was measured.

In the test phase of experiment 3, it was found that PACAP38 infusion did not significantly alter context discrimination as compared to performance in conditioning. This suggests that PACAP in the dentate gyrus is not altering retrieval of fearful and safe contextual representations in our experimental preparation.

Johnson (2019) had found in their contextual fear conditioning study that an infusion of PACAP prior to retrieval of a fear memory led to increased freezing as compared to a vehicle infusion. Based on this finding, we were expecting to see similar results in experiment 3, and hypothesized that an infusion of PACAP might increase fear in our paradigm as well, producing generalization. Considering the extensive literature

on PACAP and its involvement with stress and PTSD, we theorized that contributing to generalization of fear memories may be a process that PACAP is involved in. Therefore, the null finding in experiment 3 was surprising, however, there are possible explanations as to why this occurred.

In the electrophysiology studies that Johnson (2019) conducted, application of PACAP onto dentate gyrus slices resulted in increased spiking in cells that were already active. While initially this had led us to the hypothesis that PACAP might affect the recall of the context representations as it may increase the spiking of cells involved in the engram for the contexts, there are other possibilities. Although it has been shown to be associated with stress mechanisms (Stroth, Holighaus, Ait-Ali, & Eiden, 2011), PACAP also has other functions. It is a highly conserved peptide, and has neuroprotective functions. It could be that the actions on the cells in the dentate are more connected with survivability, and not stress or fear related. Alternatively, since fear conditioning heavily involves the amygdala, and PACAP has been shown to have stress effects in the extended amygdala, particularly the bed nucleus of the stria terminalis (BNST), that might be a region where PACAP is having the effects we expected it to have in the dentate gyrus.

Another possibility is that the dosage of PACAP used in experiment 3 was not the optimal amount. There is a lack of previous studies involving PACAP infusions into the hippocampus, so this possibility would have to be studied in future experiments. However, the amount of PACAP used and the lead time for it to take effect in experiment 3 was based on Johnson (2019), as well as past experiments done by colleagues targeting the BNST. Additionally, experiments using the startle paradigm have observed persisting

PACAP effects weeks following infusion, indicating that the time-course of PACAP was not a concern for this experiment.

Another possible factor influencing our results is that after multiple conditioning sessions, the dentate gyrus may no longer be necessary to retrieve context representations (Kitamura et al., 2017). If this is the case, the infusion directly into the dentate might not be affecting how the context representations are being retrieved. If the dentate gyrus is not necessary after several conditioning sessions, any PACAP effect that might be occurring may not be visible. This could explain why Johnson (2019) would see an effect with a one trial conditioning paradigm, while we do not.

Since the extended conditioning may have impacted the necessity of the dentate gyrus in contextual fear retrieval, the timeline of experiment 3 may be considered a limitation. However, to look at contextual discrimination, it was important to ensure that the animals were appropriately recognizing both contexts before test, making multiple conditioning days necessary. In the future, it may be interesting to look at a one conditioning day preparation, much like Johnson (2019) performed, to investigate if the result he saw was robust before going forward. It may also be interesting to look at markers of cellular activity in the dentate gyrus, such as cFos, to get a better idea of how active the dentate is in retrieval of contexts after many sessions of conditioning. Additionally, as a follow up to experiment 3, it may be informative to infuse PACAP into the amygdala rather than the dentate gyrus at test to see if the results differ since it is a crucial brain region in fear conditioning. Finally, it is interesting to consider if there is a different learning phenomenon that may be better suited to testing PACAP's effects on context discrimination. For example, the literature emphasizes extinction as a crucial

phase to test when investigating symptoms related to PTSD (Alexandra Kredlow, Fenster, Laurent, Ressler, & Phelps, 2022). Related to this, renewal of contextual fear may be a future direction worth pursuing, as it also features similarities to some hallmark symptoms of PTSD.

## **5.2 General Conclusion**

In addition to the results from the infusion test day of experiment 3, the conditioning data proved to be informative as well. Pre-cannulation conditioning was identical to Experiment 2, therefore the first phase of experiment 3 was a direct replication of experiment 2. This provided the opportunity to see if the sex effect in strength of conditioning and discrimination observed in experiment 2 (stronger fear conditioning in males, females discriminating better on days 7 and 8) was also present in the initial conditioning of experiment 3.

In experiment 3, male rats showed higher mean percent freezing in both context A and B in the first 8 days of conditioning as compared to the females, suggesting stronger fear conditioning. This replicates the same finding from experiment 2. The sex difference in discrimination that was observed in experiment 2, where females showed stronger contextual discrimination than males, was also replicated in experiment 3. There was a main effect of sex in experiment 3, where females discriminated better than males overall. This result is in agreement with the significant difference in discrimination we observed in conditioning days 7 and 8 in experiment 2. This finding is seemingly in contradiction to reports of females having more rapid loss of context specificity with time (J. Lynch, 3rd et al., 2013) and being less adept at pattern separation (Yagi et al., 2016).

A possible explanation for the sex difference in the strength of contextual discrimination and fear conditioning we observed is that males and females are relying on different pathways to produce the behavior we see in the context fear discrimination experiment. It has been suggested that estradiol in the hippocampus causes a decrease in retrieval of hippocampal dependent memories (J. F. Lynch, 3rd et al., 2014), while testosterone in males have been shown to decrease activations of the amygdala (Chen et al., 2014). This hormone influence may alter what mechanisms are used by each sex to retrieve and respond to contextual fear memories. A sex bias towards different pathways to inform responses to a context may result in slightly different behavioral outputs, such as the ones we have observed here. However, it is important to note that although there were sex differences, both sexes fear conditioned and were able to discriminate contexts, meaning that although male and female behavior is not identical, the overall outcome is similar.

Altogether, the collection of experiments in this thesis demonstrated contextual fear conditioning in male and female rats, observing a sex difference in the strength of fear conditioning as well as the strength of context discrimination. The sex difference found was in partial agreement with prior literature, but also offered new evidence that sex differences in contextual fear discrimination are yet to be understood. Additionally, experiment 3 followed up on results from past work and explored the role of dentate gyrus PACAP in contextual fear discrimination. The current results indicate that with these experimental parameters, PACAP in the dentate gyrus does not affect contextual discrimination in male or female rats. This finding opens the door to different avenues of investigation and highlights the complexity of fear and stress related pathologies.

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