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Effects Of Chronic Stress On The Pituitary Adenylate Cyclase Activating Polypeptide (pacap) In The Bed Nucleus Of The Stria Terminalis (bnst)

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EFFECTS OF CHRONIC STRESS ON THE PITUITARY ADENYLATE CYCLASE ACTIVATING POLYPEPTIDE (PACAP) IN THE BED NUCLEUS OF THE STRIA TERMINALIS (BNST)

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Abstract

Exposure to chronic stressors can produce maladaptive behavioral and physiological consequences. Previous work has demonstrated that chronic variant stress exposure enhances anxiety-like behavior and increases pituitary adenylate cyclase activating polypeptide (PACAP) and PAC1 receptor transcripts in the anterolateral bed nucleus of the stria terminalis (BNST) in rats. The studies described here demonstrate that treatment with a chronic variant stress paradigm produced anxiety-like behavior in transgenic PACAP-Cre mice. Additionally, the stressed group did not gain weight during the 14 days of chronic stressors exposure compared to control mice. Furthermore, fewer PACAP-expressing neurons were observed in the posterior BNST and lateral hypothalamus following chronic variate stress. In aggregate, these data suggest that chronic stress has behavioral and physiological consequences in mice and that PACAP systems in the posterior BNST and the lateral hypothalamus may play a role in these behavioral changes.

Acknowledgement

I would like to acknowledge my advisor, Dr. Sayamwong Hammack, for his insight, enouncement and help from the beginning to end of this process. I would also like to thank my committee members - Donna Toufexis and Anthony Morielli for their feedback. Lastly, I would like to thank Department of Neuroscience and Department of Psychological Science for the support.

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Introduction

The stress response is important for survival, adaptation, and maintaining homeostasis. However, chronic stress can cause maladaptive changes leading to a variety of psychopathologies, including anxiety disorders (Gungor et al., 2016). According to the National Institute of Mental Health, anxiety disorders are the most common mental illness, affecting more than 40 million American adults (DuPont et al., 1996). Exposure to chronic stress is an important risk factor for these disorders. However, the underlying mechanisms that produce anxiety are still unknown. Clarifying the underlying mechanisms will help to produce better therapeutic strategies to manage anxiety.

Stress can be defined as the response to a homeostatic challenge (Selye, H.,1956). Organisms exhibit an array of defensive behaviors in response to perceived threat. Though stress is necessary for normal physiological homeostasis, chronic stress may become maladaptive and produce inappropriate responding. The types of defensive behaviors exhibited in response to threats are often species and stimulus specific (Blanchard et al., 2003). For example, rodents may demonstrate an increase in freezing, startle response, defensive withdrawal, and avoidance of threatening areas. These behaviors are often measured as indices of anxiety-like behavior (Kliethemes et al., 2005).

There are several brain regions shown to be involved in mediating behavioral response following stress, including the hypothalamus, the central nucleus of amygdala (CeA), and the bed nucleus of stria terminalis (BNST). The BNST plays an important role in producing both the physiological and behavioral responses to stress (Tran et al., 2014).

The BNST is a complex, heterogeneous limbic structure (Crestani et al., 2013) that is composed of a collection of subnuclei (Hashimoto et al., 2001). While most BNST

neurons are GABAergic, the BNST has a small proportion of glutamatergic cells, some of which are projection fibers (Hashimoto et al., 2001). Many BNST neurons co-express multiple peptides, including pituitary adenylate cyclase- activating polypeptide (PACAP) (Moga et al. 1989). The BNST has been argued to play important roles in coordinating behavioral responses following exposure to stressors (Walker et al., 2003). For example, multiple studies have suggested that the BNST mediates anxiety-like behavior, including the behavioral changes observed after uncontrollable stress (Hammack et al., 2004), freezing after the introduction of predator odor (Fendt et al., 2003), and increased startle response to bright light (Walker et al., 2002). According to Davis and colleagues (2003, 2009), the BNST responds to diffuse stimuli, which persists long after the termination of the threat, and this response was described as an anxiety response. On the contrary, the CeA responds to rapid phasic response to a specific immediate thread, which was described as a fear response. Moreover, BNST activity among non-humans are described as an anxiety response (Kalin et al., 2005). Additionally, humans who are more hypervigilant and anxious showed increased recruitment and activation of BNST (Avery et al., 2014, Somerville et al., 2010). Together these data suggest the BNST is a critical structure for anxiety-like behavior after stressor exposure.

The BNST can be activated by different types of stressors, and evidence suggests that repeated exposure to stressors can alter BNST plasticity. For example, the volume and dendritic length of the BNST is increased after exposure to a chronic unpredictable stress paradigm (Pego et al., 2008) and chronic immobilization increased dendritic arborization in BNST neurons (Vyas et al., 2003). Our lab has observed an increase of BNST dendrite length after one-week exposure to a variate stress paradigm (unpublished data). Chronic

stress may alter the BNST neurocircuits by altering neuroplasticity, which augments BNST function and produces maladaptive anxiety-like behavior.

In 1989, Akira Arimura and colleagues discovered a novel peptide from an ovine hypothalamus extract that stimulated adenylyl cyclase activity in the anterior pituitary cells, which they named pituitary adenylate cyclase activating polypeptide (PACAP) (Miyara et al., 1989). PACAP is an important polypeptide that produces pleiotropic effects on cell proliferation, survival, and acts as a neurotransmitter (For review, see Vaudry et al., 2009). The precursor for PACAP can be processed into bioactive alpha-amidated PACAP38 and PACAP27 (Miyara et al., 1989). The biologically active regions of PACAP have been preserved for 380 million years, indicating the importance of PACAP for basic biological functioning (Sherwood et al., 2000). PACAP38 is the predominant form of PACAP found in brain tissue (Arimura et al., 1991) and is a member of the vasoactive intestinal polypeptide (VIP)-secretine GHRH- glucagon superfamily.

PACAP cells are widely distributed in the central nervous system as well as throughout the peripheral nervous system. In the central nervous system, PACAP expressing neurons are prominent in the hypothalamic area, specifically in paraventricular (PVN), supra-optic (SO), and arcuate nuclei (ARC) (Köves et al., 1991, Hannibal et al., 1995). Among limbic structures, the BNST has a high number of PACAP expressing neurons and fibers (Kozicz et al., 1997).

PACAP serves several important physiological functions via different regions of the brain, particularly in the hypothalamus (Arimura, 1992). PACAP also plays important roles in different pathological disease responses, such as pain-related behavior, anxiety-like behavior, psychomotor behavior (Vaudry et al., 2009), and post-traumatic stress

disorder (PTSD). Ressler and colleagues (2010) showed polymorphism at the estrogen responsive element (ERE) of the PAC1 (encoded by ADCYAP1R1) receptor gene was associated with PTSD in women. Additionally, methylation of the PAC1 expressing gene has been also associated with PTSD symptoms in both men and women (Ressler et al. 2011).

There are three types of PACAP receptors with varying affinity for PACAP and VIP. The PAC1 receptor has higher affinity for PACAP (both PACAP38 and PACAP27) compared to VIP (Gottschall et al., 1990). VPAC1 and VPAC2 receptors have a similar affinity for PACAP and VIP (Gottschall et al., 1990). All of the PACAP receptors are G-protein couple receptors (GPCR) and have both rapid and prolonged cellular signaling events that are important for multiple physiological functions such as cell survival, cell proliferation, and neuroplasticity (Braas & May, 1999). Prolonged and sustained activation of receptors may also produce maladaptive neuroplasticity (May & Parsons, 2017).

In rats, PACAP activation in the BNST can produce anxiety-like behavior (Hammack et al., 2009). After 7 days of a chronic variant stress paradigm that produced anxiety-like behavior, PACAP transcripts were increased 10-fold in the dorsolateral BNST of adult male rats, while the PAC1 transcripts increased two-fold. The data suggested that PACAP signaling in the BNST may represent a potential mechanism by which chronic stress produces anxiety-like behavior. To determine if BNST PACAP could produce anxiety-like behavior, PACAP38 was infused bilaterally into the anterior BNST in adult rats. Infusion of PACAP38 into the BNST produced anxiogenic behavior in a dose-dependent manner. Even one week after the PACAP38 infusion, the rats showed increased startle response compared to baseline response (Roman et al., 2014). Above data indicate

that PACAP can produce long-lasting increases in anxiety. Blocking the BNST activation using the antagonist PACAP6-38 prevented the increased anxiety and decreased the weight loss normally observed following exposure to chronic stressors (Roman et al., 2014). In summary, the BNST PACAP neuronal circuit produces anxiety-like behavior following chronic stress.

The goal of the current study is to replicate the previous study using transgenic PACAP mice. Using immunohistochemistry, it is difficult to visualize PACAP expressing cells because of preferential expression in the fibers rather than cell bodies (Condro et al., 2016). Transgenic PACAP mice will allow for the clear quantification of PACAP-producing cells. Moreover, using these mice will allow us to endogenously manipulate PACAP population of a specific anatomical location with temporal and spacial precision using chemo-genetic and optogenetic technologies. Here, PACAP-Ires-Cre mice underwent 14 days chronic stress paradigm. These mice would allow direct observation of PACAP cells and any changes that happened due to chronic stress.

.

Methodology

Subjects: PACAP-Ires-Cre mice were obtained from Dr. Bradford Lowell's at Deaconess Hospital, Harvard University (Khodai et al., 2018, Krashes et al., 2014). They were bred at a University of Vermont animal facility and used for the experiments at the age of 2-3 months old. The mice were housed in groups and maintained on a 12 hours light/ dark cycle with ad libitum food and water. All of the experiments and procedures were approved by the Institutional Animal Care and Use Committee of University of Vermont.

Virus injections: In order to identify PACAP cells, Adeno-associated-virus-2-EF1a-DIO-mCherry ($\sim 1 \times 10_{12}$ viral genomes/ml) was obtained from the University of North Carolina at Chapel Hill Vector core and injected into the BNST.

Stereotactic injections: 2-3 month old PACAP-Ires-Cre mice were injected stereotaxically with adeno-associated-virus-2-EF1a-DIO-mCherry virus into the BNST to identify the PACAP-expressing cells. Mice were anesthetized using 1.5-2 percent isoflurane in an induction chamber. They were placed into the stereotaxic frame (David Kopf Instruments, Tujunga, CA), their heads were shaved, and they were secured in place with ear bars. The scalp was sterilized using povidon-iodine and an incision was made to expose the skull. Two burr holes were drilled into the exposed skull. After the two holes were drilled, 0.5 µl of the AAV2-EF1a-DIO-mCherry reporter vector was injected using Hamilton 7001 KH syringe, 1ul, needle size- 25s gauge and length -70 mm into the BNST using the following coordinates: AP = +0. 1 mm, ML= -1/+1 mm, DV = - 4.6 mm. The virus was injected using a pump over 5 minutes into each burr hole. The syringe was kept in place for another 5 minutes to prevent efflux of the virus. After 5 minutes, the syringe was withdrawn slowly over 3 minutes and the scalp was sutured using Vicryl. The mice

received an analgesic injection of ketoprofen (5mg/kg) to alleviate pain and were placed on a heating pad. After the mice woke, they were returned to their home cage. On next day, the mice were weighed and given another dose of analgesic. The animals were given 7 to 10 days to recover.

Chronic Variate Stress (CVS): After surgical recovery, each cage of mice was randomly assigned to one of two groups – control or stress. Both groups were weighed daily until they were euthanized. The control group was handled each day for 14 days. In contrast, the stress group was exposed to one of five different stressors – foot shock, forced swim, pedestal standing, oscillation, and restraint - for 14 days (see Table 1). The animals from the chronic stress group were exposed to the stressor for the same duration in the same order. On the first day, animals from the stress group experienced foot shock. The animals from the stress group were placed into a Plexiglas conditioning chamber (Med Associates, St. Albans, VT) 30cm x 25 cm x 35cm (L x W x H) and habituated for 5 minutes. After that, the animals were administered two 1.0 mA, 2 second foot shocks in 5-minute intervals, delivered through the grid floor. The animals were returned to their home cage 3 mins after the last foot shock. The second day of the chronic variate stress was the forced swim. The animals from the stress group were placed in a cylindrical container 29 cm x 37 cm (D X H), which was 2/3 filled with room temperature water and the animals were forced to swim for 5 minutes. The animals were then put into a fresh cage for 5 minutes to let them dry. After the animals were relatively dry, they were returned to their home cage. On the third day, stress group animals were placed on a pedestal for 30 minutes and returned back to their home cage. The next day, the cages from stress group were placed and secured on a clinical rotator (Fisher Science, Morris Plains, NJ) for 30 minutes and oscillated at a

medium speed. On fifth day, mice of the stress group were placed into a 200 ml glass beaker and restrained for one hour. The stressors were repeated for fourteen days following the same sequence. 24 hours following the last stressor or control handling mice were tested for anxiety-like behavior on an elevated-plus maze.

Table 1:

Days	Stressor	Duration
1	Foot shock	2s (X 2)
2	Swim	5 mins
3	Oscillation	30 mins
4	Pedestal	30 mins
5	Restraint	60 mins
6	Foot shock	2s (X 2)
7	Swim	5 mins
8	Oscillation	30 mins
9	Pedestal	30 mins
10	Restraint	60 mins
11	Foot shock	2s (X 2)
12	Swim	5 mins
13	Oscillation	30 mins
14	Pedestal	30 mins

Elevated plus maze: The elevated plus maze consists of two sets of arms respectively: open- 25 cm x 5 cm x 0.5 cm (L x W x H) - and closed – 25 cm x 5 cm x 16 cm (L x W x H) arms, which are perpendicular to each other. The maze is 50 cm above the floor and made of plastic. There is a central square area - 5 cm x 5 cm x 0.5 cm (L x W x H). The closed arms have black walls on its both sides. The room is illuminated with a red bulb 6 Lux (lx) in the center and open arms. Each animal was placed individually in the center of the elevated plus maze facing towards the closed arm and the door of room was closed behind to prevent any noise entering in the room. Animals were allowed to explore the maze for 5 minutes. The session was captured by overhead webcam and recorded in a Mac

laptop using the software Quicktime player. The time spent in the open arm, the closed arm, and how many times the mice crossed from closed arm to open arm were scored by an observer blind to treatment condition. The animals that were stressed spent more time in the closed arm rather in the open arm and crossed less from the closed arm to the open arm.

Slide preparation: Immediately after the elevated plus maze, mice were anesthetized and transcardially perfused using PBS and then 4% paraformaldehyde (PFA). After the perfusion, the brains were removed from the skull and post fixed in 4% PFA for one day. Next, the brains were transferred to 30% sucrose for cryoprotection. After cryoprotection, brains were frozen and sectioned into 40 µm thick sections using a cryostat. The sections were then mounted onto Superfrost Plus (Fisher Scientific) slides and covered using Citifluor antifadent solution.

Microscope: Images of the mounted slices were obtained on an epifluorescence microscope. Fluorescent neurons (presumptive PACAP-expressing neurons) in the BNST cells were counted using Stereo Investigator.

Statistics and software: Student t-test and correlation tests were done for statistical analysis for the experiments using Graph Pad Prism 7 software.

Results

Chronic variate stress induces anxiety like behavior in PACAP-ires-Cre mice:

Mice undergoing 14 days of chronic variant stress paradigm exhibited anxiety-like behavior compare to the control group, entering the open arms of the elevated-plus maze less (Fig. 1, t (14) = 1.930; p <0.05) and spending less time in the open arms (Fig.*2*, t (14) = 2.269; $p < 0.05$) consistent with prior reports in rats (Hammack et al. 2009 and Roman et al. 2014). Although there was increased anxiety-like behavior among the stressed group, there was no significant difference in total open and closed arms crossing (Fig. 3, t (14) = 1.415, $p = 0.0894$) among the stressed and control group, which suggests there was no locomotor effect of chronic stress amongst the groups. Additionally, the ratio of open arm entries and total arm entries was consistent with an anxiogenic response to chronic stress (Fig. 4, t (14) = 2.384, p <0.05). The number of PACAP BNST cells and open arm entries (Fig. 7, r = 0.5315, p <0.05) had a significant positive correlation.

After the 14 days chronic variant stress paradigm, the stressed group lost significant weight compared to the control group (Fig. 5, t (14) = 2.546, p <0.05), which is in line with our previous finding (Roman et al. 2014).

The number of PACAP cells were counted. There were variabilities regarding injection sites in the brains. Additionally, one animal from the control group and three animas from stress group had viral injections that missed the BNST. Those samples were excluded from the total sample size. There was no significant difference in the number of total BNST (anterior and posterior) PACAP cells among the stressed and control groups (Fig. 6, t (10) = 0.6971, $p = 0.250$).

When the regional distribution of PACAP cells were observe, there were significantly less PACAP cells in the stress group than the control group in posterior BNST (Fig. 8, $t(3) = 2.758$, $p < 0.05$).

The PACAP-positive cells extended up to the lateral hypothalamus (LH) and there were significantly fewer PACAP cells in the stress group than the control group (Fig. 9, $t(7) = 2.715$, $p < 0.05$) in this region.

Discussion

Chronic stress produced maladaptive anxiety-like behavior among adult male mice, which is consistent with previous reports where adult male rats underwent chronic stress showed anxiety-like behavior (Hammack et al, 2009). The mice that exhibited anxiety like behavior also did not gain as much weight as non-stressed mice. While it was intended that viral infusions would target the anterior BNST, instead placements were much more posterior, with many in the region of the. We used Adeno-associated-virus containing the promoter EF1-a, which is an ubiquitous promoter that can label cells, including neuron and glia; due to the morphology of the labeled cells, we believe most staining was likely neuronal, but counterstaining with neuronal and glial markers would be required to determine cell type. Interestingly, we observed a regional distribution of PACAP cells in the posterior BNST and in the LH. The posterior BNST and LH had fewer PACAP-expressing cells in the stress group as compared to the control group. Given the high concentration of viral particles injected (.5ul), it may be surprising that we were able to observe changes in fluorescence, which could represent either changes in the amount of florescence proteins expressed per cell, and/or a loss of PACAP-expressing cells. Further study is required to delineate the source of this change.

Previously published data demonstrate that chronic immobilization stress reduced open arm entries in the elevated plus maze (Vyas et al., 2002). Additionally, chronic variant stress increased light enhanced startle responding in rats, suggesting chronic stress produces anxiety-like behavior (Hammack et al., 2009). Moreover, chronic unpredictable stress can produce anxiety-like behavior (reduction in open arm entries on an elevated plus maze and increased acoustic startle responding) without affecting the fear response and

also produced neuronal hypertrophy in the anterolateral BNST and increased dendritic arborization (Pego et al. 2008, Vygas et al., 2002), suggesting anxiety-related behavioral changes due to chronic stress may be due to the neuroplasticity in the anterolateral BNST. Our data are consistent with above reports showing that a chronic variate stress paradigm produces anxiety-like behavior in mice.

There are different stress paradigms often used in the literature, including chronic variate stress, the social defeat model, restraint, forced swim and others. Additionally, stressor characteristics can be manipulated using different variables including, the predictability, the controllability, the change in temporal duration and homotypic/heterotypic stressor. Temporal components of stressor exposure are an important variable, as Lezak et al. (2014) demonstrated that chronic stressors exposure is crucial for increased PACAP and PAC1 transcripts in the anterolateral BNST, since a single stressor exposure was inadequate. Hence, chronic stressors exposure is necessary to increase PACAP activation and the BNST gradually recruit its PACAP cell population in the latter time of CVS exposure to produce anxiety like behavior (Roman et al, 2012).

Previous reports have showed that upon exposure to repeated homotypic stressor, HPA axis hormones were attenuated progressively via a habituation process. After initial exposure of the stressor, there is an increased level of adrenocorticotropic hormone (ACTH) and corticosterone, which decreased eventually due to habituation of exposure to homotypic stressor (Babb et al., 2014). Conversely, the exposure to variate stressors exhibited increase in baseline hypersecretion of corticosterone, ACTH and adrenal hypertrophy (Herman et al.,1995). CVS paradigm showed to induce physiological, behavioral changes and change in HPA axis observed in psychological disorders (Borrow

et al., 2019). While we currently do not know whether stressor exposure in our own paradigm habituates, and whether a variate stressor paradigm is necessary, we have used the CVS paradigm based on the argument that changing the stressor type prevents or slows habituation. In our previous reports, we showed that CVS for 7 days was able to produce anxiety-like behavior in rats (Hammack et al., 2010). Hare et al. (2018) showed that following 14 days of CVS, mice exhibited anxiety like behavior and as we are using PACAP-Ires-Cre mice, we used the same duration of CVS for our experiments.

The BNST has been divided mainly into two parts - the anterior and posterior divisions assessed by the proximity to the stria terminalis main fiber bundle. The anterior BNST can be further divided in relation to the anterior commissure fibers into dorsal, lateral and ventral areas (Swanson, 1998), of which anterolateral BNST has been shown to be important for autonomic, energy homeostasis and feeding behavior (Dong & Swanson, 2004). The anterolateral BNST is also associated with anxiety-like behavior (Walker et al., 2003). Chronic stress has been shown to increase corticotropin releasing factor (CRF) in the anterolateral BNST (Stout et al., 2000). CRF infused into this region also enhanced acoustic startle response in rats (Lee & Davis, 1997). The BNST contains a dense collection of CRF cells which appear to be innervated by PACAP expressing fibers (Kozicz et al., 1997). Previous reports have shown that PACAP and its cognate receptor PAC1 mRNA and peptide were substantially increased following chronic stress in the anterolateral aspect of the BNST and infusion of PACAP in this same region produced stress- and anxiety-like behaviors in rats (Hammack et al. 2009), which suggested that PACAP- PAC1 signaling in the anterolateral BNST mediates the maladaptive anxiety like behavior, maybe by

producing neuroplasticity by dendritic remodeling of the BNST neurons (Vyas et al., 2002).

Kocho-schellenberg et al. (2014) showed that only posterior BNTS infusions of PACAP, and not anterior BNST PACAP infusions reduced weight in rats. Additionally, the posterior BNST has been argued to have an inhibitory role in controlling the HPA axis (Choi et al., 2008). Choi et al. (2008) also showed through lesion studies that posterior BNST inhibit PVN and HPA responses through GABAergic projection. Additionally, posterior BNST projection showed to be important for inhibiting HPA axis response to emotional stress to the PVN (Bingham et al., 2011). The posterior BNST also have one of the highest concentrations of sex steroid hormone receptors that projects to PVN of hypothalamus, which is important for regulation of HPA axis (Shughrue et al., 1997). Collectively, these data indicate that posterior BNST is important for metabolic and behavioral influence of HPA axis. In our data, we observe a reduction of PACAP expressing cells in the posterior BNST among the stressed animal. Because of the injection variability, the sample size was small and more detailed investigation is needed.

The LH is also a heterogeneous area with multiple nuclei expressing different neurotransmitters and neuropeptides important for cognitive, autonomic, motor and endocrine functions, with rostral connections to the preoptic area and caudal projections to the ventral tegmental area (For more detailed review, see Berthound & Munzberg, 2011). The LH has also been implicated in feeding, drinking, and reward motivation (Teitelbaum et al.,1962, Urstadt & Berridge, 2020), and electrolytic ablation of LH led to the suppression of feeding and drinking behavior (Stuber & Wise, 2016). Among the neuropeptides expressed in the LH, PACAP has been found in high concentration (Arimura

et al., 1991, Masuo et al., 1993). Chance et al. showed that PACAP injection to perifornical LH reduced food and water intake. Furthermore, bilateral PACAP38 injection into the perifornical LH could modulate the drinking behavior in rats. These data together suggested that PACAP cells in LH are important for feeding and drinking behavior.

We observed that the number of PACAP expressing cells in the LH was significantly less in the stress group than the control group, which may suggest that PACAP in LH can alter the feeding behavior in mice. As the sample size of this experiment is small, we need to increase the power in order to be more confident. Additionally, how LH PACAP systems alter behavior including the feeding behavior is unknown. We need to further investigate this aspect of the PACAP system.

We used Adeno-associated-virus-2-EF1a-DIO-mCherry to activate the Cre recombinase enzyme of the PACAP-Ires-Cre mice. The promoter – EF1a was derived from human elongation factor-1 aphha. It is 1.2 kb in size and is not specific to neurons. There was a possibility that PACAP could have expressed in population of progenitor cells (Condro et al., 2016) and other cells, which were not neurons in the BNST. But the morphology of the neurons was clear to identify because of the distribution of the florescent protein. The m-cherry protein was expressed in the cell body as well as in the processes including axons, dendrites and axon hillocks, which made it possible to count the PACAP expressing neurons in an accurate manner.

PACAP produces its actions by binding to one of several G-protein coupled receptors - PAC1, VPAC1 and VPAC2. Among those, PAC1 is the specific receptor for PACAP (Harman et al., 2012). PACAP binds to PAC1 receptors and activates downstream ERK signaling and may produce behavioral and endocrine changes (Hammack & May,

2015). Previous reports have shown PAC1 receptor antagonism blocked behavioral and physiological changes - like stress induced anxiety like behavior and weight gain (Roman et al., 2014). Together these data indicate that PAC1-receptor signaling system in BNST is critically involved in the behavioral and physiological consequences of chronic stress.

Repeated exposure to various stressors can mediate different behavioral changes including change in weight gain and anorexia (Marti et al.,1994). These effects have been linked to several anatomical locations that include the paraventricular nucleus of the hypothalamus (PVN), central nucleus of the amygdala (CeA), and BNST, where the concentration of CRH is high (Roman et al., 2012). CRH intraventricular injection has been demonstrated to produce anorexia and weight loss in the rats (Buwalde et al., 1998). Additionally, the anorexia produced by activation of BNST CRH receptors can be reversed by BNST nociceptin/orphanin FQ system, a system that often produces effects that are functionally opposite of CRF action (Ciccocioppo et al., 2003). Moreover, when the BNST is excitotoxicity lesioned, the effect of repeated stressors exposure on weight gain is reduced (Roman et al., 2012), which suggests that BNST activity plays an important role in the anorexia and weight reductions observed following chronic stress. In the BNST, CRH neurons are closely related to PACAP neuros (Kozicz et al., 1997). Additionally, by chronically blocking PAC1 receptors in the BNST, the weight change following chronic stress was prevented (Roman et al., 2014). Our data demonstrated that after chronic stress, there was a reduction in weight gain among the stress group, which may be due to activation of BNST PACAP.

Stress related psychological disorders have significant sex- difference. For example, women are affected by anxiety disorders in a higher rate than men (Dalla &

Shores, 2009). Ressler et al. (2011) reported an association between an ERE in PAC1 gene SNP with PTSD in female, but not in men. Additionally, they showed a strong association between increased level of PACAP38 and PTSD symptoms in female. These data suggested that the PACAP-PAC1 receptor signaling is sex specific in a traumatic stress. Moreover, estrogen (E) has been demonstrated to increase BNST PACAP and PAC1 transcripts in rodents (Ressler et al., 2011) Previous reports have demonstrated that chronic stress differentially alters the behavioral and endocrine consequences in male and female rats (For detailed review, see King et al., 2017). King et al., 2017 shown that prior stress did not increase the acoustic startle response in intact, cycling female rats, which was opposite in male rats in the same experiment after subthreshold intra-BNST PACAP infusion. The result observed in female rats in above experiment can be due to several factors, importantly – cyclical nature of gonadal hormones (i.e – E and progesterone) and interaction between stress and different gonadal hormones (King et al., 2017). Frye and Walf (2002) observed females in proestrus where the level of E and progesterone are high, which showed less anxiety- like behavior compared to diestrus females and males. King et al. (2017) also showed that proestrus females who were exposed to stressor showed reduced startle amplitude following PACAP infusion, but the proestrus females who were in not exposed to stressor showed increased startle response, similar to male rats. On the other hand, and irrespective of prior stress, females in other phases showed basal level of startle amplitude following PACAP infusion, which suggested that the phase of estrus cycle is important for mediating the response of PACAP effect in females (King et al. 2017). We also found that following chronic stressors exposure, there is a reduction of PACAP cells in the posterior BNST. The BNST is a sexually dimorphic region (Allen et al., 1990),

specifically the posterior BNST (Kocho-shellenberg et al., 2014). The posterior BNST in male rats has been observed to be higher in volume than female rats, an effect that is controlled by the gonadal steroids (Del Abril et al., 1987, Hines et al., 1992). Additionally, the posterior BNST expressed higher number of sex steroid hormone receptors (Shughrue et al., 1997). In aggregate, we acknowledge the importance of repeating the same experiments using intact cycling female mice and evaluate the sexual dimorphic characteristic of posterior BNST following chronic stressors. Future studies will address this issue.

Here we showed that exposure to chronic stressors gave rise to anxiety-like behavior among adult male mice. Moreover, mice that manifested anxiety-like behavior did not gain weight as non-stressed mice. Additionally, we observed that posterior BNST and LH had fewer PACAP-expressing cells in the stress group compared to the control group. We suggested that chronic stressors mediated the anxiety-like behavior by modulating neuronal plasticity in the BNST PACAP cells and the weight change maybe due to change in the number of PACAP cells in the posterior BNST and LH. The stress induced neuroplasticity may be the mechanism of human anxiety disorders and can be a potential therapeutic target.

Figures:

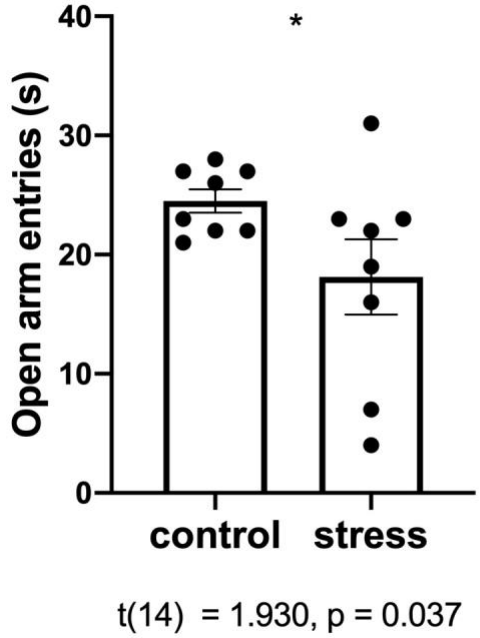


Figure 1:

Chronic stressed PACAP- ires Cre male mice exhibit anxiety-like behavior. After 14 days of chronic variant stress, the stressed group entered the open arm of elevated plus maze significantly less than control group. Data represent mean ± SEM. * significantly different from control group $p < 0.05$.

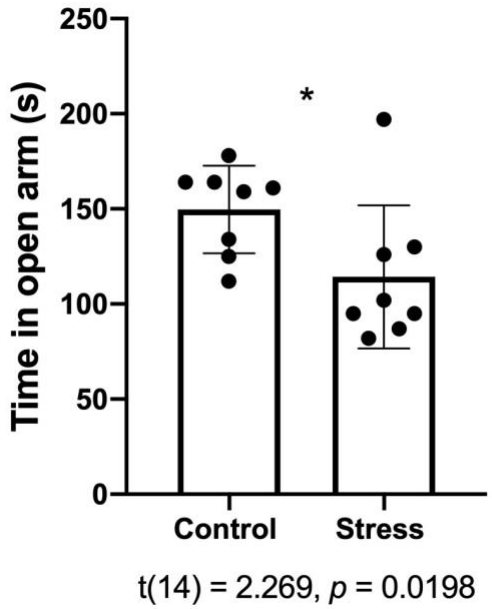


Figure 2:

Chronic stressed PACAP- ires Cre male mice exhibit anxiety-like behavior. After 14 days of chronic variant stress, the stressed group spend significantly less time in the open arm of elevated plus maze than control group. Data represent mean ± SEM. * significantly different from control group $p < 0.05$.

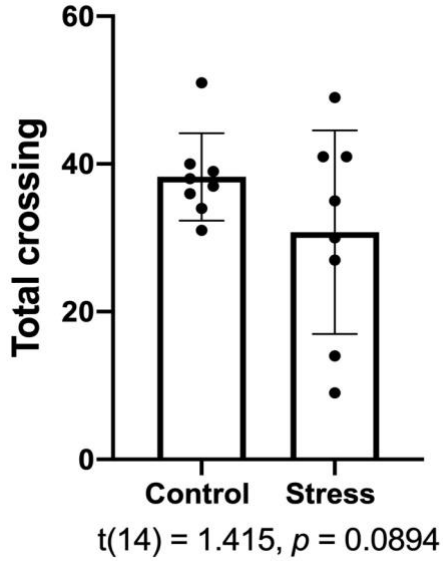


Figure 3:

Chronic stress has no significant effect on overall locomotor activity. After 14 days of chronic variant stress paradigm, both the stressed and the control groups exhibited the same number of open/closed arm entries, indicative of no difference in overall locomotor activity. Data represent mean ± SEM.

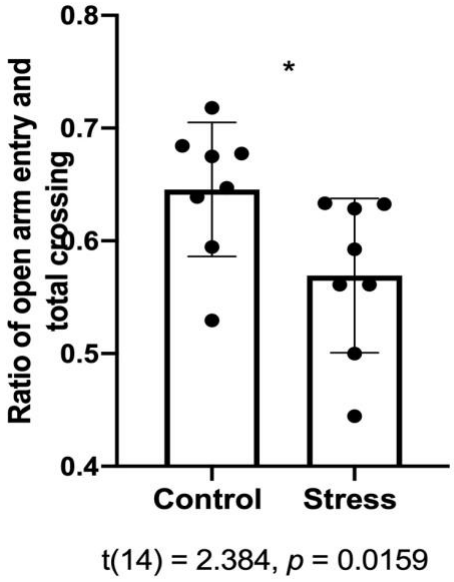


Figure 4:

Further demonstrating that the effects of chronic stress were not dependent on changes in locomotor activity, an anxiogenic response was still observed when assessing the ratio of open arm entry over total entries. Data represent mean ± SEM. * significantly different from control group $p < 0.05$

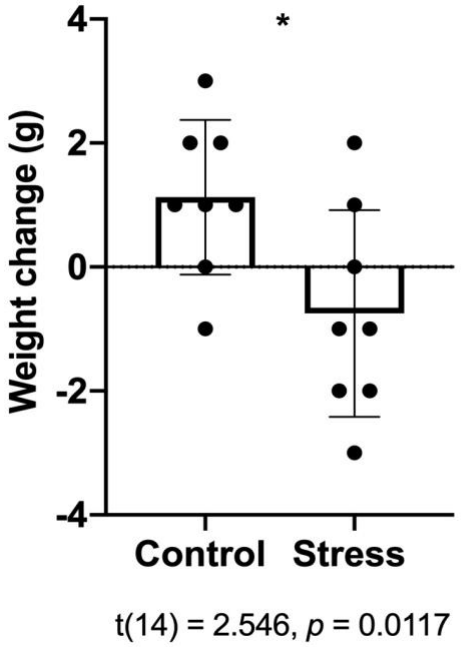


Figure 5:

Chronic stress reduced weight gain in PACAP- ires Cre male mice. After 14 days of chronic variant stress paradigm, the stressed group lost significant weight compare to control group. Data represent mean ± SEM. * significantly different from control group $p < 0.05$

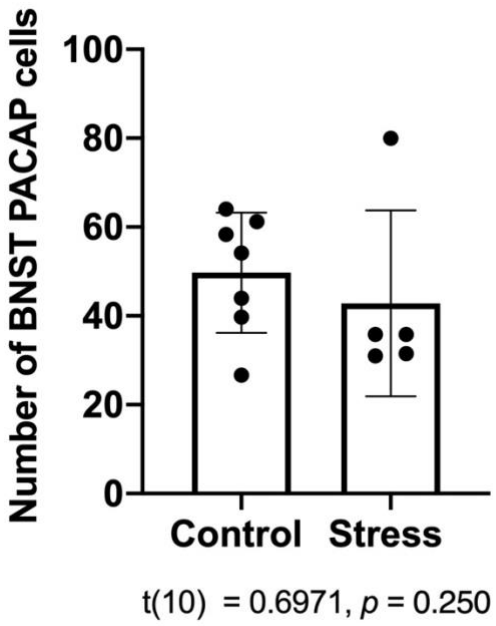


Figure 6:

Chronic stress did not alter the number of BNST PACAP neurons. Data represent mean ± SEM. * significantly different from control group $p < 0.05$

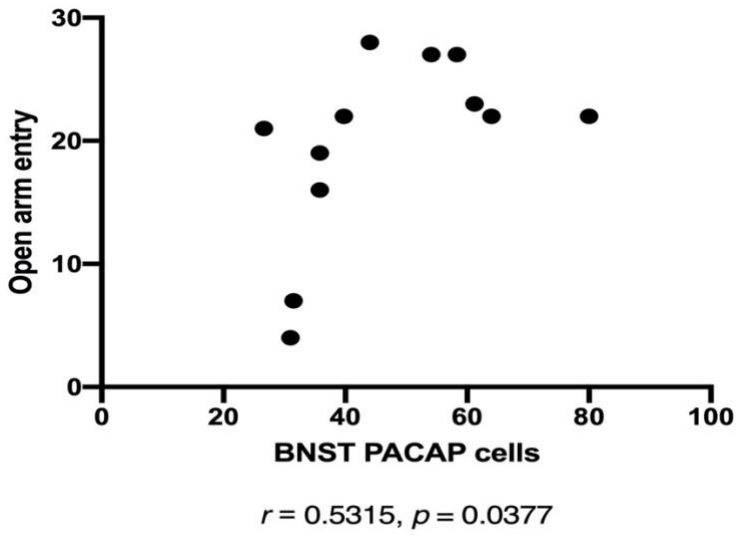


Figure 7:

There was a significant positive correlation between number of PACAP BNST cells and open arm entries.

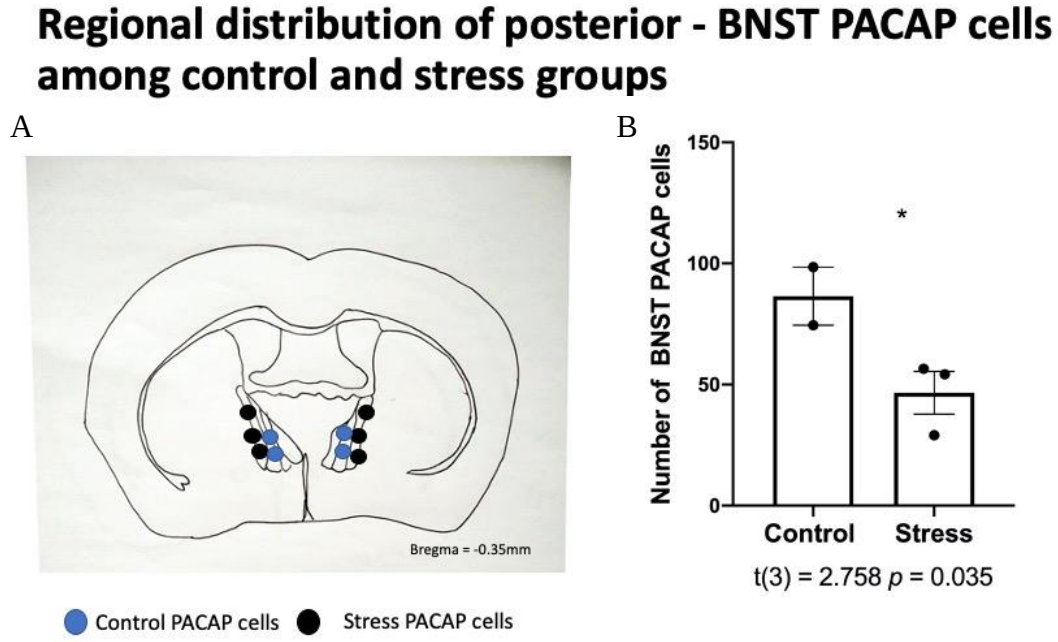
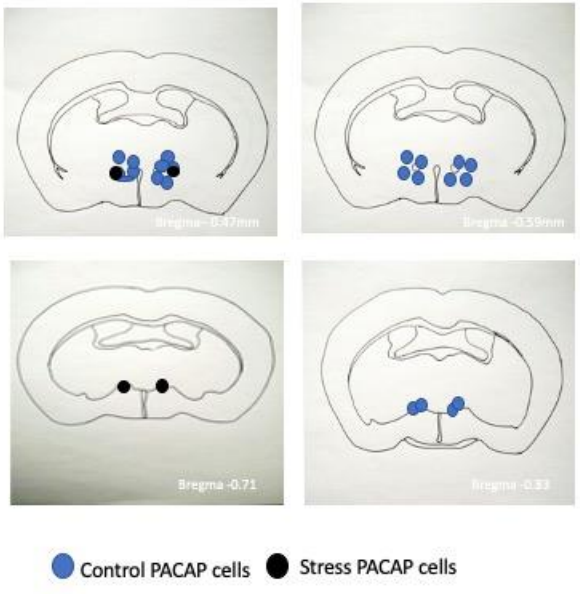


Figure 8:

Chronic stress alters the number of PACAP neurons in posterior BNST. A. The schematic showing the anatomical location of PACAP cells in the posterior BNST. B. Bar graph showing significant difference in the number of BNST cells in posterior BNST between control group and stress group. Data represent mean ± SEM. * significantly different from control group $p < 0.05$

A



B

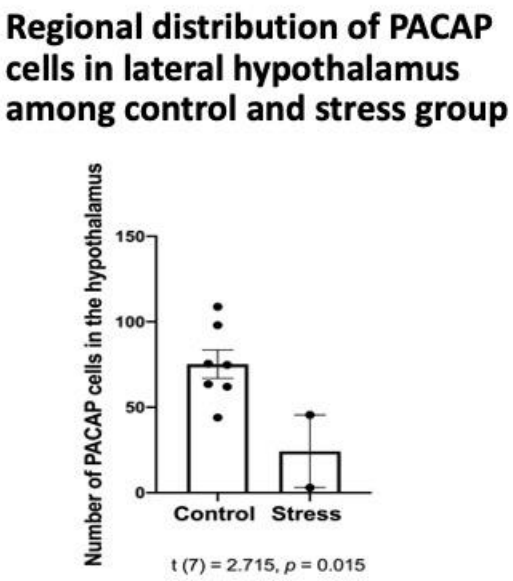


Figure 9:

Chronic stress alters the number of PACAP neurons in the lateral hypothalamus. A. The schematic showing the anatomical location of PACAP cells in the lateral hypothalamus. B. Bar graph showing significant difference in the number of BNST cells in lateral hypothalamus between control group and stress group. Data represent mean ± SEM. * significantly different from control group $p < 0.05$

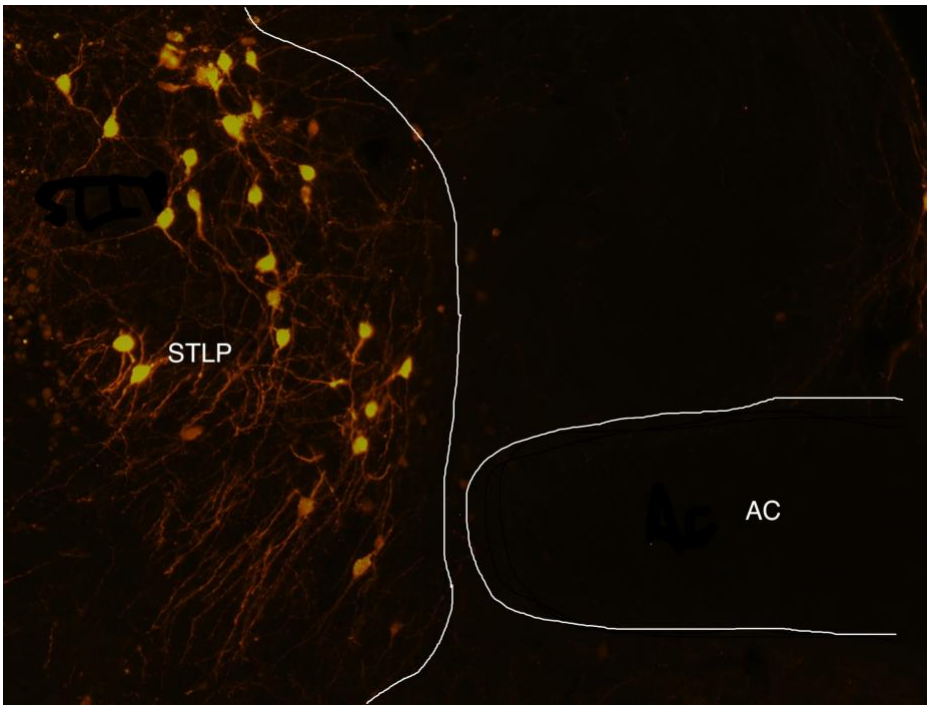


Figure 10:

A representative image of BNST PACAP cells from control group

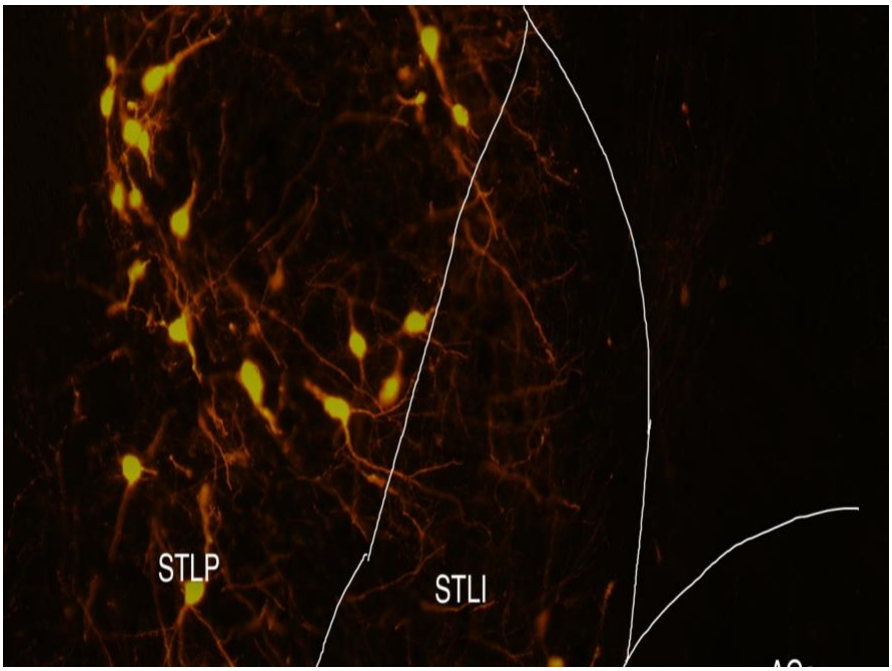


Figure 11:

A representative image of BNST PACAP cells from stress group

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