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# Lesioning of the Nucleus of the Hippocampal Commissure Followed by Food Deprivation Stress in Birds Demonstrates Simultaneous Involvement in both the Hypothalamo-Pituitary-Adrenal Axis and the Hypothalamo-Pituitary-Thyroid Axis

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of the requirements for the degree of
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by


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## Abstract

The hypothalamo-pituitary-adrenal (HPA) axis is the regulatory system for the neuroendocrine stress response within vertebrates. Within the HPA axis corticotropin releasing hormone (CRH) is a major regulator and driving hormone. A structure named the nucleus of the hippocampal commissure (NHpC) has been found to contain CRH neurons and also these neurons respond to early food deprivation stress significantly prior to the paraventricular nucleus (PVN), the major driving nucleus of the classic neuroendocrine HPA axis. The objective of this study was to perform a knock down of the NHpC via electrolytic lesioning, thus eliminating a significant portion of its population of CRH neurons. An experiment was designed to determine whether the elimination of CRH neurons within the NHpC would have a significant effect on HPA function following exposure of broiler chicks to food deprivation (FD). Male chicks (BW 300-350g, 10-14 d) were used in this experiment and split into 3 groups: 1) Sham surgical controls without FD (SHAM), 2) Sham surgical birds with 2h FD stress (SHAM+FD), and 3) Birds subjected to electrolytic lesioning and 2h FD (LES+FD). Blood, brain and anterior pituitary (APit) were sampled promptly from each bird at 2h of FD for the LES+FD and SHAM+FD groups and intermittently for SHAM CON birds. RT-PCR was performed for gene expression within the NHpC, PVN and anterior pituitary (APit) and a radioimmunoassay was performed to determine plasma corticosterone (CORT) concentrations. All RT-PCR data were analyzed with the Tukey Kramer HSD test and all CORT data were analyzed using one-way ANOVA. Electrolytic lesioning of the NHpC significantly reduced plasma CORT in the LES+FD group compared to intact levels in the SHAM+FD group. Decreased CORT occurred concurrently with decreased amounts of CRH mRNA within the NHpC of the LES + FD group. Supporting this, Proopiomelanocortin heteronuclear RNA (POMC hnRNA) within the APit was significantly downregulated. Interestingly, PVN CRH levels were found to be significantly decreased in the

LES+FD group of birds with no lesioning of the PVN itself.  Results suggest a possible neural connection from the NHpC to the PVN exists, resulting in down regulation of CRH expression in the PVN.  Corticotropin releasing hormone receptor 2 (CRHR2) was found to be significantly downregulated within the PVN and APit in the LES+FD group of birds.  Thyroid stimulating hormone beta (TSHβ) was also downregulated along with CRHR2 in the PVN and APit suggesting that CRHR2 expression within the PVN could be an important part of the hypothalamo-pituitary-thyroid (HPT) axis.  In conclusion, lesioning the NHpC had a significant effect on the HPA axis.  CRH neurons within the NHpC and/or PVN had a significant effect on the HPA and/or HPT axes.

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Lastly, I would like to thank my family for always supporting me in my academic career. My mother has always given me great advice and my father has always been there to assist me in my work as a researcher. Most importantly I would like to thank my wife Hadley who always given me great support and has sacrificed so much for my ability to complete my degree.

## Dedication

This thesis is dedicated to the scientific community.

## Table of Contents

# Introduction

Through the growth of the poultry industry many practices that have become common place due to their importance in the name of efficiency and growth management have come into question concerning their ethical implications. Two of these animal welfare practice issues such as broiler breeder feeding programs, for example, 4 days on feed 3 days off, and feed withdrawal at the end of broilers life span, are designed to manage body weight and to help in processing efficiency, respectively. Both practices directly alter natural feeding behaviors and can be considered feeding stressors. Thus, it is essential to investigate the neuroendocrine regulatory system of poultry food related stress, and its effects on the bird's health and well-being as food related stress is a common occurrence within the poultry industry. The hypothalamo-pituitary-adrenal (HPA) axis is the main neuroendocrine regulatory system in vertebrates and is responsible for the production of the major stress glucocorticoid, corticosterone (CORT), in birds or cortisol in mammals. The HPA axis is known to be involved in many different systems such as stress, feeding behavior and immune system function. One of the main driving neurohormones in this system is corticotropin releasing hormone (CRH), or also known as corticotropin releasing factor (CRF) in mammals, which is released during stress from the paraventricular nucleus (PVN), one of the major driving structures in the classical neuroendocrine HPA axis. The PVN is located within the hypothalamus which is located directly below the thalamus of the brain. The hypothalamus is the region contributing to many systems such as the autonomic nervous system (ANS) and the endocrine system due to its connections with the ANS and pituitary gland (Britannica Academic, 2019). Once CRH/CRF is released from the PVN it is transported to the anterior pituitary (APit) where it binds to its receptor corticotropin releasing hormone receptor 1 (CRHR1)/corticotropin releasing factor receptor 1 (CRFR1). This binding then triggers the release of adrenocorticotropic hormone

(ACTH) into the circulatory system where it is then transported to the adrenal cortex to stimulate the release of CORT into the blood (Smith and Vale, 2006). The purpose of this thesis is to address the function of CRH during a food deprivation stressor. Also, the 1mm wide structure known as the nucleus of the hippocampal commissure (NHpC) which is located dorsally from the PVN and in the septal region of the brain will be investigated as it has been found to contain CRH neurons. The reasoning for investigating the NHpC is that in a previous study it has been found to have a significant early upregulation of CRH mRNA within the NHpC as early as 2h of food deprivation stress (Nagarajan et al, 2017). The early significant upregulation of CRH within the NHpC could suggest influencing HPA function during food deprivation stress. The NHpC has also been found to have a greater percent activation of CRH neurons at 2h of FD stress than the PVN (Kadhim et al., 2019). The methodology of investigating this structure was done through electrolytic lesioning during stereotaxic surgery. Stereotaxic surgery is a methodology that was used as early as 1908 when Sir Victor Horsley and Robert Clarke invented the instrument, now coined the stereotaxic instrument, to perform electrolytic lesions, which destroys cells through the use of an electrical current, within a brain structure (Horsley and Clarke, 1908). Since then lesioning studies have been conducted on numerous areas of the brain to determine the functional roles of these areas. We hypothesize that surgical disruption of the NHpC will significantly decrease the neuroendocrine HPA axis stress response in birds.

**Chapter 1: Adrenocortical stress and lesion methodology: A literature review.**

## 1. Stress

Stress was termed a "general adaptation syndrome" in 1936 by Hans Selye (Selye, 1936). A stressor can be many things such as a physical condition such as atmosphere, temperature and air quality, or that causes a change in bodily homeostasis or it could be completely physiological due to a change in a sensory system such as noise and immobilization. A stress response is therefore the organism's response in correcting the situation in order to obtain homeostasis (Sapolsky, 2002). With understanding the conditions that cause stress, we can then define stress as a physiological change either induced by a physical or psychological action that causes a corrective action by an organism to maintain homeostasis.

## 2. Stressors

### 2.1 Physical Stressors

Physical stressors can often be referred to as environmental stressors. Some examples of these stressors are food derivation, thermal, atmospheric and air quality. All the previous examples have become challenges for the modern poultry industry today. For example, pulmonary arterial hypertension, or more commonly known as ascites syndrome, is greatly increased due to high altitude (Wideman et al, 2013). Heat stress is also extremely common as a large majority of the United States poultry production occurs in southern states where temperatures can rise over 100 °F in the summer. Models for heat stress have been created such as to induce gastrointestinal leakage in broilers due to its incidence in the poultry industry (Ruff et al, 2019).

## 2.2 Psychological Stressor

Psychological stressors can be more easily defined as social stressors or fear. Some examples of social stressors are mating behavior, isolation and pecking order. All the previous social stresses can activate the neuroendocrine stress response. Fear is also a very powerful psychological stress in examples such as an organism being preyed upon. Sudden environmental stressors can also by psychological stressors such as lighting changes or loud sounds. Furthermore, another psychological stressor that is used frequently in studies is immobilization stress where the animal has the limbs restrained and is unable to move about. An example of this is an acute restrain stress study that greatly increases CRH mRNA within the PVN but also within the central amygdala nucleus, which is thought to be involved in psychological stress (Hsu et al., 1998).

## 3. Adrenocortical Stress

The adrenocortical stress response occurs with three key anatomical structures of the organism: hypothalamus, anterior pituitary and the adrenals (Blas, 2014). These areas also make up what is known as the neuroendocrine stress axis or hypothalamo-pituitary-adrenal (HPA) axis in vertebrates. The hypothalamus, which is the primary neuronal site of the neuroendocrine stress response, contains a critical nucleus named the paraventricular nucleus (PVN). Within the PVN two very important neuropeptides are synthesized, arginine vasotocin (AVT) in birds and other vertebrates, arginine vasopressin (AVP) in mammals and corticotropin releasing hormone (CRH) in birds and mammals. These two neurohormones are released from the PVN and transported to the anterior pituitary (APit) where they interact with their respective receptors. There are three AVP receptors in mammalian species: V1a, V1b, and V2 and one oxytocin receptor. In birds, there are 4 protein coupled AVT receptors: VT1, VT2, VT3 and VT4

receptors (Cornett et al., 2013). The VT3 receptor also known as the mesotocin receptor, is comparable to the mammalian oxytocin receptor. Also, it has been proposed that the VT2 and VT4 receptors be changed to the mammalian terminology of V1bR for VT2R and VlaR for VT4R. With the release of AVT/AVP from the PVN magnocellular neurons, these neurohormonest with either target the V1aR or V1bR located on corticotropes in the APit resulting in the release of ACTH from. The release of AVT/AVP from the PVN to the V1bR/V1aR within the APit is also associated with chronic stress and has been positively upregulated with its receptors. Examples of this have been found in studies where food deprivation was used to illustrate a slow climbing stress response (Nagarajan et al, 2017; Kadhim et al., 2019). CRH has been shown to bind to two receptors, CRHR1 and CRHR2 (Potter et al, 1994). CRHR1, which is located on corticotropes within the APit, is the receptor responsible for CRF/CRH binding in the APit resulting in production of proopiomelanocortin (POMC) (Ramachandran et al., 1976). CRHR2 is located on thyrotropes within the APit and regulates the thyroid system (De Groef et al., 2003). POMC, the initial protein product is a common precursor for three peptides: adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (β-END) (Takei et al, 2016). ACTH is then transported into the general circulatory system where it is then brought to the adrenal cortex. This is where CORT is then produced and released into the blood. Once CORT is in the blood, regulatory systems are needed to control the level of CORT within the blood. One regulatory method is through CRH/CRF binding proteins (CRH-BP) located in the APit and another is glucocorticoid receptor (GR) negative feedback mechanisms. GRs are in various parts of the body and brain. These GRs regulate HPA components by modulating transcription factors needed to produce essential components of HPA (Smith and Vale, 2006). In the pituitary CRH-BP is found to be highly

expressed.  This high expression in the pituitary leads to the conclusion that CRH-BP regulates HPA feedback through the reduction of CRHR1 activity in the anterior pituitary as CRH/CRF secretions are transferred from brain into the anterior pituitary (Smith and Vale, 2006).

**4. Structures of the Neuroendocrine HPA and their Functional Genes in Stress**

**4.1 The Nucleus of the Hippocampal Commissure**

The Nucleus of the Hippocampal Commissure (NHpC) is a small nucleus located in the septal region of the brain just dorsal to the PVN.  The first link to the NHpC being involved in stress was its involvement in male bird social behavior (Xie et al., 2010).  This was followed by the discovery of a dense grouping of CRH neurons within the structure utilizing colchicine (Nagarajan et al., 2014).  This is significant as CRH neurons within the PVN are classically known for driving neuroendocrine stress responses.  The NHpC's involvement in stress responses was further solidified when the nucleus illustrated elevated expression of CRH mRNA during food deprivation stress at 2 h preceding a significant PVN CRH response at 4 h (Nagarajan et al., 2017).   Previous evidence reviewed is further supported by another food deprivation trial clearly illustrating that the NHpC has a greater percent increase s of CRH mRNA and earlier peak CRH mRNA compared to the PVN (Kadhim et al., 2019).  This study also illustrates that the NHpC's involvement in feeding behavior as CRH in excess can cause a decrease in feeding behavior.  CRHR1, the important receptor to CRH, is found within the NHpC as well.  CRHR1 appears to be inversely regulated.  When CRH mRNA response is elevated, CRHR1 mRNA is downregulated.

## 4.2 Paraventricular Nucleus

As previously stated in adrenocortical stress, the PVN is the classical major driving nucleus for neuroendocrine stress responses with its two essential peptides, CRH and AVT, during stress that stimulate the release of ACTH in vertebrates (Carsia et al., 1986; Salem et al., 1970). Although, both these neuropeptides are critical they are regulated differently. CRH in the PVN appears to be necessary for an initial response, although not before the NHpC, such as an acute stress. AVT however, acts as a long-term mediator of stress within the PVN as its levels may be elevated later and sustained if the animal appears to be headed into a chronic long-term stress response. This pattern has been found in both mammalian and avian systems (Aguilera, 1998; Nagarajan et al., 2017; Kadhim et al., 2019). Regarding CRH's receptors' CRHR1 and CRHR2 within the PVN, they are both positively regulated with CRH. For CRHR1, this is directly opposite of the NHpC as the nucleus illustrates both CRHR1 and CRHR2 show negative feedback during CRH upregulation. This is consistent with the theory that different groups of CRH neurons are regulated differently (Brunson et al., 2002).

## 4.3 Anterior Pituitary

In stress the APit's primary role is to produce and release ACTH into the blood stream to eventually stimulate production of CORT in the adrenals. This is done in corticotropes, of which CRHR1 resides to receive CRH from the PVN. In corticotropes POMC is then produced which is a precursor peptide for ACTH. Although CRHR1 resides on corticotropes CRHR2 is also present in the APit and is present on thyrotropes (De Groef et al., 2003). These thyrotropes function to release thyroid stimulating hormone (TSH) instead of ACTH but, bind the same neurohormone CRH as the corticotropes do. It has been demonstrated in birds that CRH in birds has the ability to activate the HPA axis and the hypothalamo-pituitary-thyroid (HPT) axis.

## 5. Stereotaxic Lesioning

The stereotaxic instrument and method stem back to 1908 where R. Clarke and V. Horsely investigated the cerebellum (Horsley and Clarke, 1908). The instrument allowed the selective location of brain structures if one possesses a map or atlas of the brain. Utilizing stereotaxic instruments allow for lesioning experiments. These experiments consist of using a stereotaxic instrument to guide an electrode into a specific area of the brain where an electrical current can be passed into it. This controlled electrical current ablates the tissue allowing for observations to be made in the absence of certain brain structures. An example of such a technique would be the lesioning of the nucleus accumbens in rats (Al'bertin et al., 2003).

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**Chapter 2: Lesioning of the nucleus of the hippocampal commissure followed by food deprivation stress in birds demonstrates simultaneous involvement of the hypothalamo-pituitary-adrenal axis and the hypothalamo-pituitary-thyroid axis.**

**Abstract**

The nucleus of the hippocampal commissure (NHpC) has been suggested to function within the hypothalamo-pituitary-adrenal (HPA) axis regarding the neuroendocrine regulation of stress in birds. The presence of corticotropin releasing hormone (CRH) neurons within the NHpC structure was one crucial finding suggesting its function within the HPA axis. The objective of this study was to perform a knock down of the NHpC via electrolytic lesioning, thus eliminating a significant portion of its population of CRH neurons. An experiment was designed to determine whether elimination of CRH neurons within the NHpC would have a significant effect on HPA axis function following exposure of broiler chicks to food deprivation (FD). Male chicks (BW 300-350g, 10-14 d of age) were used in this experiment and split into 3 groups: 1) Sham surgical control without FD (SHAM), 2) Sham surgical birds with 2h FD stress (SHAM+FD), and 3) Birds subjected to electrolytic lesioning and 2h FD (LES+FD). Blood, brain and anterior pituitary (APit) were sampled promptly from each bird at 2h of FD for the LES+FD and SHAM+FD groups and intermittently for SHAM CON birds. RT-PCR was performed for gene expression within the NHpC, paraventricular nucleus (PVN) and APit and a radioimmunoassay was performed to determine plasma corticosterone (CORT) concentrations. All RT-PCR data were analyzed with the Tukey Kramer HSD test and all CORT data were analyzed using one-way ANOVA. Electrolytic lesioning of the NHpC significantly reduced plasma CORT in the LES+FD group compared to intact levels in the SHAM+FD group. This directly correlates with a decreased amount of NHpC CRH mRNA detected within the nucleus due to the lesion. In addition. proopiomelanocortin heteronuclear RNA (POMC hnRNA) within the anterior pituitary (APit) was significantly downregulated. Interestingly, PVN CRH levels were found to be significantly decreased in the LES+FD group of birds even with no lesioning of

the PVN itself. Results suggest a possible neural connection from the NHpC to the PVN occurs resulting in down regulation of CRH expression in the PVN. Corticotropin releasing hormone receptor 2 (CRHR2) was found to be significantly downregulated within the PVN and APit in LES+FD group of birds. Thyroid stimulating hormone beta (TSHβ) was also shown to be downregulated in correlation with its receptor, CRHR2, in PVN and APit. In conclusion, lesioning of the NHpC has a significant effect on the normal function of the HPA axis. Additionally, CRH neurons within the PVN and possibly the NHpC appear to have a significant, positive effect on stimulating the hypothalamo-pituitary-thyroid axis during a stress response.

## 1. Introduction

Corticotropin releasing hormone (CRH/CRF), a 41-residue amino acid (Vale et al., 1981), is a major neurohormone in the regulation of stress within the hypothalamo-pituitary-adrenal (HPA) axis in vertebrates (Bale and Vale, 2004). CRH is classically released from parvocellular neurons within the paraventricular nucleus (PVN) during stress and is transported to the anterior pituitary (APit) where CRHR1 are located on corticotropes to produce adrenocorticotropic hormone (ACTH) (Smith and Vale, 2006). Once ACTH is released into the general circulatory system, the adrenal glands begin the production of corticosterone (CORT) in birds and many vertebrates, cortisol in mammals and fish, which is the major stress glucocorticoid (Sapolsky and Meaney, 1986). CRH is also supposedly involved in emotional stress responses (de Kloet et al., 2005).

The hypothalamo-pituitary-thyroid (HPT) axis has been shown to be involved in stress to varying degrees along with its relationship with the HPA axis. Several thyroid hormones have been found to be elevated during stressors in rats such as 3,5,3'-triodothyronine ($T_3$) and thyroxine $T_4$ during disturbance and immobilization stressors although their roles and

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relationship with the HPA axis is still not completely determined (Döhler et al., 1977;Langer et al., 1983). There are however studies that contradict this and demonstrate decreased T3 and T4 such as a study by (Servatius et al., 2000) that utilized tail-shock as an acute stressor. In birds, CRH has been linked directly to the HPT and HPA axes simultaneously through an in vitro study that injected ovine CRH which resulted in increased CORT and thyroid stimulating hormone (TSH) within the plasma as early as 1 hour (Meeuwis et al., 1989). The question remains how influential is CRH during stress on the HPT axis in live animals?

The nucleus of the hippocampal commissure (NHpC) has recently been discovered to contain a group of parvocellular CRH neurons with the use of colchicine injections (Nagarajan et al., 2014). Since then, significant CRH mRNA expression from within the NHpC has been observed during food deprivation (FD) stress as early as 2h, which occurs significantly earlier than the PVN (Nagarajan et al., 2017) or shows a greater percent activation of CRH neurons (Kadhim et al., 2019). Although, NHpC CRH mRNA expression is observed during stress, direct evidence is lacking of NHpC CRH neurons influencing APit ACTH output and blood CORT levels. With this past knowledge, we hypothesize that surgical disruption of the NHpC will significantly decrease the neuroendocrine HPA axis stress response in birds. Therefore, the objectives of this study are 1) knock down CRH neurons within the NHpC through the use of electrolytic lesioning, 2) observe HPA axis activity post lesioning during a 2h FD stress, 3) test the possibility that the NHpC CRH plays a critical role in the neuroendocrine HPA axis FD response in birds and 4) explore the possibility the NHpC CRH neurons play a role in the hypothalamo-pituitary-thyroid axis.

## 2. Materials and methods

### 2.1 Animals and rearing

Day old Cobb 500 male broilers were obtained from a commercial hatchery and reared in controlled heated battery cages with an environmental temperature of 32°C with 24h light for the first 3 days. Light was changed to a light (L), dark (D) cycle of 16L:8D with lights on at 5 AM starting on day 4 and temperature was decreased 2.5°C weekly. Birds were provided food and water *ad libitum* during their growing period and the diet was a standard broiler starter (22% protein and metabolizable energy of 3100 kcal/kg). At 7 days of age, birds were weighed and grouped with similar body weights to form the three treatment groups for stereotaxic surgery.

All methods and care of animals were approved by the University of Arkansas IACUC (protocol # is 19054).

### 2.2 Stereotaxic lesioning surgery and sample collection

Surgeries were performed on birds of BW 300-350g between 10 to 14 days of age. The underside of each bird's wing was cleaned with 70% ethanol and 1 ml per 300g BW of sodium pentobarbital (27mg kg$^{-1}$, i.v.) was injected into the brachial vein. Once the birds were fully anesthetized, they were placed within the stereotaxic instrument so that the centered ear bars aligned the head at a zero reference point. A typical set of coordinates used to position an electrode directly into the NHpC were the following: anterior/posterior (AP), A = 7.4, Depth =3.0, and LAT = 0.0 from the centered ear-bars (zero reference point). Once the bird's head was positioned and secured in place, the top head feathers of the bird were trimmed down to the skin, then betadine solution was applied to the surgical area of the head by a sterile swab. An anterior-posterior surgical cut was then performed through the dermis of the dorsal head region and the

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skin was separated and held apart by hemostats in order to expose the dorsal skull region. The electrode attached to the surgical arm of the stereotaxic instrument was then lowered just onto the top of the skull to mark the area in which a small hole could be made. A hole was then carefully drilled just enough through the skull to not puncture the dura mater via a sterile burr attached to a dental drill. Sterile swabs were used to control any bleeding that occurred from the drill site. The stereotaxic arm with the electrode was then lowered into the brain to a specific site, the NHpC, using predetermined coordinates. Once the stereotaxic arm was in place a positive wire from the electrical lesion maker (Grass Instruments, Inc.) was attached to the electrode and the negative wire attached to a small needle was dipped in physiological saline (0.9% sodium chloride) and inserted underneath the skin of the thigh muscle to complete the electrical circuit. An electrical current of 1 milliamp (1.0 mA) for 15 seconds was then applied to produce the lesion targeted for the NHpC. The electrode was then removed from the bird's brain and any bleeding from the surgical area was again controlled by sterile swabs. The skin on the bird's head was then sutured shut using surgical thread soaked in 70% ethanol. Finally, a small amount of a broad-spectrum antibiotic cream was applied to the surgical site. Each bird was placed in an isolated heated battery cage to recover.

Surgical birds were given 4 days to recover. On day 5 post surgery birds were subjected to 2h of FD stress starting at 8 AM, however water was made available. A blood sample was taken immediately from the brachial vein, then birds were cervically dislocated. Brain and anterior pituitary were dissected from the skull, brains were submerged in two-methyl butane at -30°C for 15s to freeze, while anterior pituitaries and brains were placed in dry ice, stored at -80°C to maintain their chemical and structural integrity for cryo-sectioning.

Plasma was separated from blood samples by centrifugation at 3,000 rpm for 20 min at 4°C, then stored at -20°C until a radioimmunoassay was performed for corticosterone. Cross sections of brain samples were cut at 40 µm via a cryostat (Leica CM3050 S, Leica Microsystems, Frisco, TX) until a lesion was seen present in the brain. The anterior commissure (AC) was used as a landmark structure to identify the NHpC as it is located at midline, just dorsal to the AC. Once a lesion was spotted in each brain, it was photographed by a camera attached to a dissecting scope (Leica MZ 125). The NHpC and PVN were then punched using a glass pipette with an internal diameter of 1.4 mm. The cryostat was kept at -15°C prior to sectioning. Sections 100 µm were then positioned on glass microscope slides and separate punches of the NHpC and PVN were stored in 1.5 ml sample tubes filled with trizol and stored at -20°C until RNA extraction and quantitative RT-PCR for each structure was determined.

**2.3 Colchicine injections and Immunocytochemistry**

Colchicine injection surgeries were performed on a separate group of male birds of BW 300-350g at 13 days of age. The underside of each bird's wing was cleaned with 70% ethanol and 1 ml per 300g BW of sodium pentobarbital (27mg $kg^{-1}$, i.v.) was injected into the brachial vein. Once the birds were fully anesthetized, they were placed within the stereotaxic instrument so that the centered ear bars aligned the head at a zero reference point. A typical set of coordinates used to position an electrode directly into the lambda skull marker were the following: anterior/posterior (AP), A = 7.4, Depth =3.0, and LAT = 0.8 from the centered ear-bars (zero reference point). Colchicine amounts of 30 µg/bird were injected 24 hours before the brain was harvested. Day 14 birds were anesthetized with sodium pentobarbital (27mg $kg^{-1}$, i.v.). Anesthetized birds were perfused via the left ventricle of the heart with 100 ml of cold heparinized 0.1 M phosphate buffer, containing 0.1% sodium nitrite at, pH 7.4 and lithium

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heparin (71 mg/L PB, Sigma).  150 ml of PB was then injected into the carotid arteries.

Thereafter, 250 ml PB containing freshly filtered Zamboni's fixative solution (4%

paraformaldehyde with 15% picric acid in 0.1 M phosphate buffer at 7.4 pH) were perfused.

Brains were blocked in a stereotaxic instrument and post fixed in Zamboni's fixative solution

overnight at 4 °C.  Blocked brains were then placed into a cryoprotectant solution (30% sucrose

in 0.1 M PB) at 4 °C until they sank.  Brains were then wrapped in parafilm and aluminum foil

and stored at -80 °C until sectioned.  For sectioning, brains were allowed to equilibrate at -20 °C

in a cryostat (LeicaCM3050S, LeicaMicrosystems, Austin, TX, USA) before being embedded in

Jung OTC medium (freezing media, Leica Microsystems, Wetzlar, Germany).  Cross-sections

were cut at 40μm using the cryostat.  NHpC sections were collected in a 24 well plate containing

2 ml of 0.02 M PBS (pH 7.4).  Immunostaining was performed as follows.  Sections were rinsed

in 0.02 M phosphate buffer saline (PBS, pH7.4) several times followed by treatment with 0.4%

Triton X-100 in PBS for 15 min. The sections were then incubated in 5% normal donkey serum

(0.1% sodium azide, 0.2% Triton X-100 in 0.02 M PBS) for 30 min.  After incubation, sections

were transferred to a primary antibody solution (1% normal donkey serum, 0.2% TritonX-100 in

0.02 M PBS) containing a cocktail of primary antibodies (CRH, host – guinea pig; T-5007,

Bachem; 1:2000) and incubated for at least 48hr at 4°C.  After incubation sections were washed

several times in 0.02 M PBS and incubated in a cocktail secondary antibody solution

(0.2%Triton X-100 in 0.02 M PBS) for 90 min. Secondary antibodies used were Alexa Flour®

594 AffiniPure Donkey anti-guinea pig IgG conjugated(AB_2340474). Sections were then

washed in 0.02 M PBS followed by a wash in distilled water and mounted on clean glass slides

and cover slipped using Vectashield.

## 2.4 RNA isolation and gene expression assay

Total RNA was extracted from micro dissected brain tissue and anterior pituitaries (n=10 birds/group) using Trizol-chloroform (Life Technologies) according to the protocol provided by the supplier. Total RNA was purified using an RNeasy mini kit (Qiagen), and RNA concentration was estimated using the Synergy HT multi-mode micro plate reader (Biotek). Firststrand cDNA was synthesized in 40 μl from total RNA (300 ng of NHpC, 600 ng of PVN and 800 ng of anterior pituitary) for each sample treated with DNase I (Ambion, Austin, TX, USA) using Superscript® III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. In brief, RNA was incubated with 2 μl Oligo (DT) and 2 μl dNTPs at 65 °C/ 5 min. Then, transferred to ice for 2–3 min. After that, the mixture was incubated with 20 μl cDNA synthesis mix (10X RT Buffer, 25mM MgCl2, 0.1M DTT, RNaseOUT, and superscript III RT) at 50 °C/5 min, and the reaction was terminated by 85 °C/5 min. Finally, the RNA was removed by adding 2 μl RNase H and incubated at 37 °C/20 min.. Primer sets used in the assays were (name, accession #, primer set, product size): CRH mRNA, NM 001123031, forward 5′GCCCACAGCAACAGGAAAC3′ and reverse 5′ GTGATGGCTCTGGTGCTGA C3′, 98 bp; CRH-R1 mRNA, NM_20432, forward 5′CCCTGCCCCGAGTATTTCTA3′ and reverse 5′CTTGCTCCTCTTCTCCTCACTG3′; CRH-R2 mRNA, XM_015281046, forward 5′GCAGTCTTTTCAGGGTTTCTTTG3′ and reverse 5′CGGTGCCATCTTTTCCTG; GR mRNA, XM_015294033, forward 5′GCCATCGTGAAAAGAGAAGG 3′ and reverse 5′TTTCAACCACATCGTGCAT3′, 94 POMC hnRNA, NM_001031098, forward 5′ATTTTACGCTTCCATTTCGC3′ and reverse 5′ATGGCTCATCACGTACTTGC3′ G3′, 87 bp, TSHβ NM205063, 128 bp, TSHβ-F: 5-CCACCATCTGCGCTGGAT-3; TSHβ-R: 5-GCCCGGAATCAGTGCTGTT-3. Power SYBR green PCR Master Mix was mixed with

sample products and primers and amplified using real-time quantitative PCR (Applied

Biosystems 7500Real-Time PCR system).  The assay was achieved in duplicate (30 µl) using the

following conditions: 1 cycle at 95 °C for 10m and amplified for 40 cycles at 95 °C for 30 s, 60

°C for 1 m, and 72 °C for 30 s.  The chicken glyceraldehyde 3-phosphate dehydrogenase

(GAPDH) and beta actin (βA) were used as internal controls to normalize the data.  We chose the

internal control that was most consistent in showing no differences between control and

treatment groups.  Relative gene expression levels of each specific gene were determined by the

$2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

## 2.5 Radioimmunoassay

A radioimmunoassay procedure was used to measure plasma corticosterone concentration

from each bird (Madison et al., 2008; Proudman and Opel, 1989).  A rabbit polyclonal primary

antibody against CORT, 100 µl rabbit anti-CORT #377, (Schmeling and Nockels, 1978; kindly

provided by Dr. Proudman) and 100 µl of $^{125}$I corticosterone tracer   and secondary antibody

(sheep anti-rabbit) were purchased from MP Biomedicals Inc. and used for the competitive

binding assay.  The samples were run in duplicate, then averaged to minimize the variation

among individual plasma samples.

## 2.6 Statistical analysis

Statistical analyses of both gene expression data and radioimmunoassay data were

performed using JMPR pro 13.0 (SAS Institute Inc., NC).  CORT plasma data were analyzed by

Tukey-Kramer HSD test to find significant differences among treatment groups.  A mean

separation test, Tukey's Kramer HSD procedure, was used for the NHpC, PVN and APit gene

expression data to find significant differences among treatment groups. A probability level of p < 0.05 was considered statistically significant.

## 3. Results

### 3.1 Anatomical lesions, immunocytochemistry and corticosterone

An unstained brain section (Fig. 1A) shows the result of a partial ablation of the nucleus of the hippocampal commissure (NHpC). A photograph was taken of the first sign of a lesion in all birds in the LES+FD group. This enabled an assessment of the degree of damage to the NHpC. The anterior commissure, a prominent structure located directly ventral to the beginning of the NHpC, was used as an anatomical marker. Fluorescence immunocytochemistry (Fig. 1B), illustrates the NHpC, a septal nucleus containing a dense population of CRH (red) neurons that is just dorsal to the third ventricle and anterior commissure.

Plasma corticosterone (CORT) data (Fig. 2) show that partial ablation of the NHpC resulted in reduced plasma CORT in the LES+FD group, compared to the SHAM+FD group ($p < 0.05$). A slight, but significant increase was observed in the LES+FD group in comparison with the SHAM CON group ($p < 0.05$) due to the partial knockdown of CRH neurons within the NHpC of the LES+FD birds.
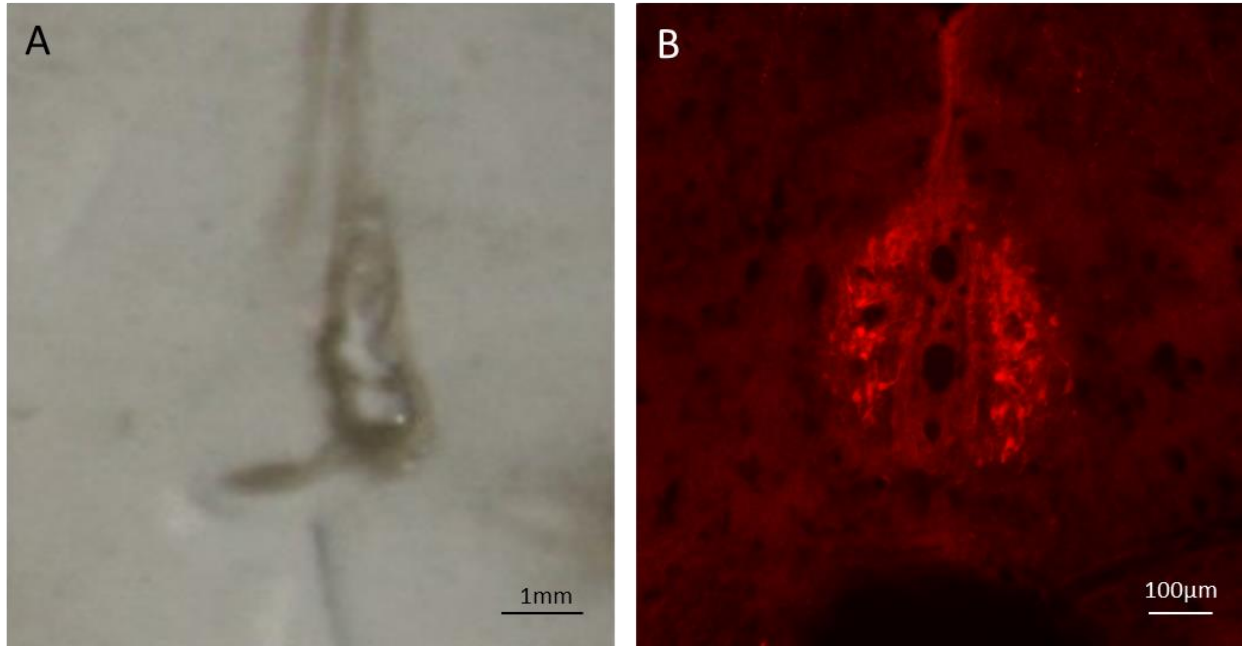
**Fig. 1.** A. Photomicrograph of an electrolytic lesion in the nucleus of the hippocampal commissure (NHpC). B. Photomicrograph of histofluorescence microscopy of the NHpC. corticotropin releasing hormone (CRH) neurons (red) are shown with greater number in the dorsal region of the structure. Scale bars = 1 mm in A; 100 µm in B.

Plasma CORT data (Fig. 2) shows that partial ablation of the NHpC resulted in reduced plasma CORT in the LES+FD group, compared to the SHAM+FD group ($p < 0.05$). A slight, but significant increase was observed in the LES+FD group in comparison with the SHAM CON group ($p < 0.05$) due to the partial knockdown of CRH neurons within the NHpC of the LES+FD birds.
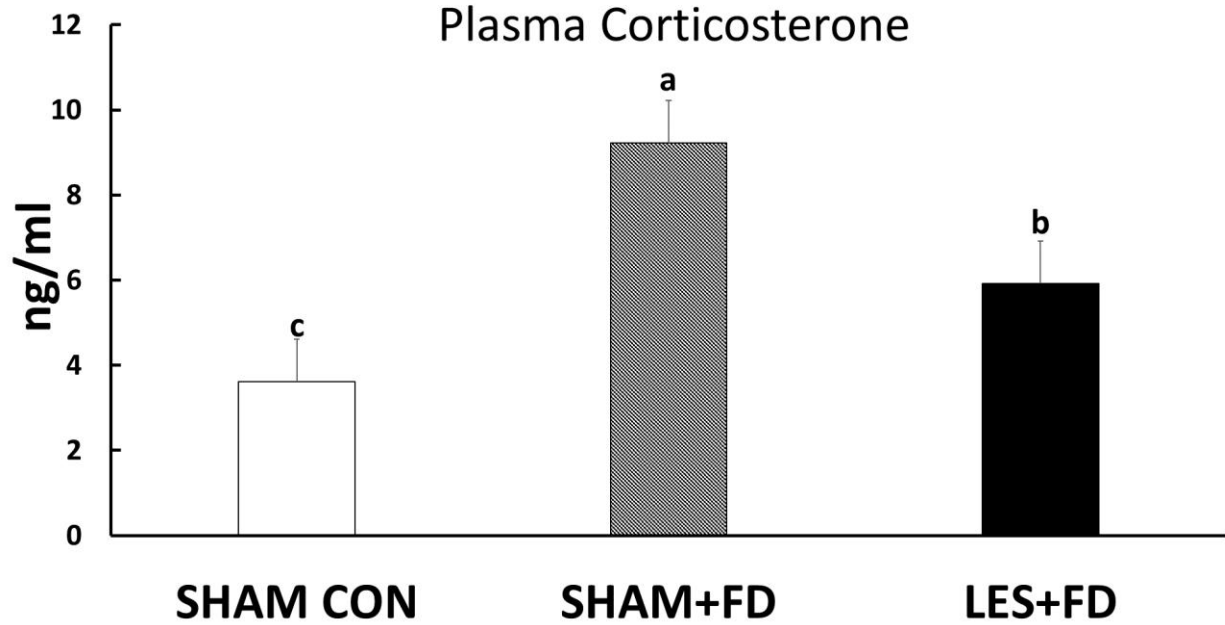
**Fig. 2.** Corticosterone (CORT) concentration in male broilers. SHAM CON (n=10) subjected to surgical procedures but no passage of electrical current. SHAM+FD (n=6) subjected to sham surgery along with 2h of food deprivation (FD) stress. LES+FD (n=10) subjected to lesioning of the NHpC and 2h of food deprivation stress. Data were analyzed by Tukey-Kramer HSD test. Different letters above columns indicate significant differences ($p < 0.05$). Error bars represent standard errors.
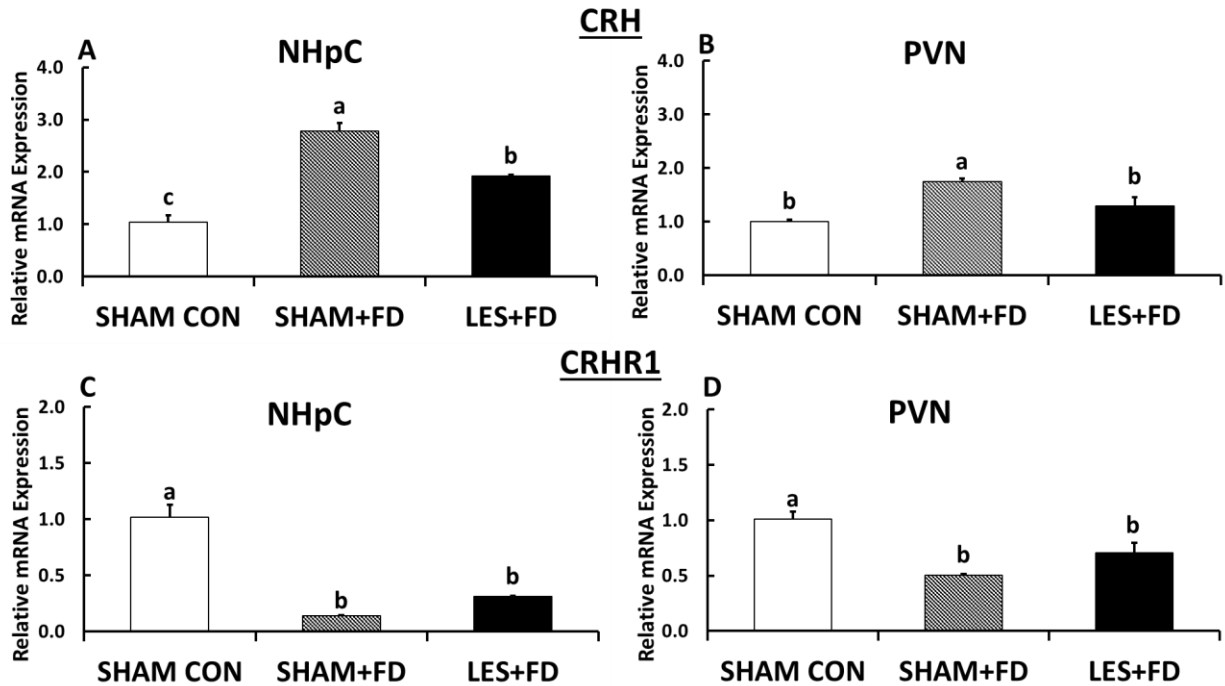
**Fig. 3.** Relative gene expression of CRH within the A. nucleus of the hippocampal commissure (NHpC) and B. paraventricular nucleus (PVN), and relative gene expression of corticotropin releasing hormone receptor 1 (CRHR1) in the C. NHpC and D. PVN. SHAM CON (n=10) subjected to surgical procedures but no passage of electrical current. SHAM+FD (n=6) subjected to sham surgery along with 2h of food FD stress. LES+FD (n=10) subjected to electrical lesioning of the NHpC along with 2h of FD stress. Data were set as fold changes of relative expression levels using the $2^{-\Delta\Delta Ct}$ method after normalization. All data were analyzed using the Tukey-Kramer HSD test. Different letters above columns for each neural structure indicate significant differences ($p < 0.05$). Error bars represent standard errors.

### 3.2 Gene expression of CRH and CRHR1 within the NHpC and PVN

Corticotropin releasing hormone (CRH) expression within the NHpC was expressed as expected with a significant decrease in the LES+FD group compared to the SHAM+FD group (Fig. 3A) due to the partial electrolytic ablation of the structure, although it does not reach the level of the SHAM CON group ($p < 0.05$). The SHAM+FD was significantly upregulated in comparison with the SHAM CON as expected with a 2h food deprivation stress response ($p < 0.05$). Within the paraventricular nucleus (PVN), CRH expression unexpectedly follows the

same pattern as the NHpC with the SHAM CON and LES+FD groups showing no statistical

difference (Fig. 3B) (p > 0.05).  The SHAM+FD group was significantly elevated due to 2h of

food deprivation compared to both the SHAM CON and LES+FD groups (p < 0.05).  In past

studies, corticotropin releasing hormone receptor 1 (CRHR1) within the NHpC was shown to be

downregulated during stress (Kadhim et al, 2019).  Both the SHAM+FD and LES+FD groups

follow this pattern (Fig. 3C) as they showed significantly decreased CRHR1 mRNA compared to

SHAM CON birds (p < 0.05).  In contrast, CRHR1 within the PVN was previously found

upregulated during FD stress (Kadhim et al, 2019).   Also, the SHAM+FD and LES+FD groups

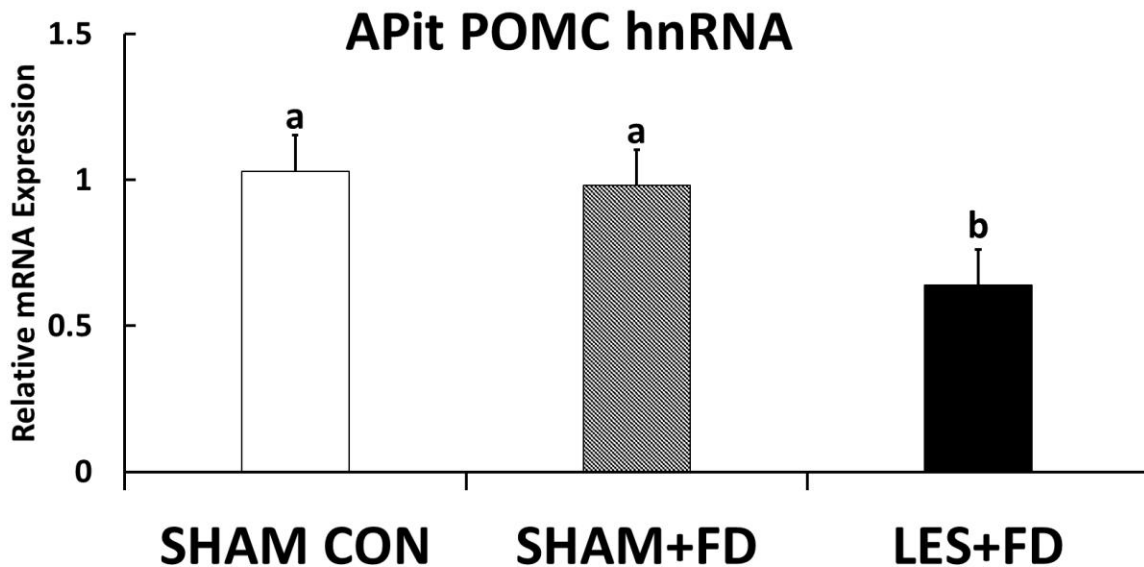showed significantly decreased CRHR1 mRNA compared with the SHAM CON group (Fig. 3D

(p < 0.05).



**Fig. 4.**  Relative gene expression of heteronuclear proopiomelanocortin (POMC hnRNA) within the anterior pituitary (APit).  SHAM CON (n=10) birds were subjected to surgical procedures but no passage of electrical current.  The SHAM+FD (n=6) group was subjected to sham surgery along with 2h of FD stress.  The LES+FD (n=10) birds were subjected to lesioning of the NHpC and 2h of food deprivation stress.  Data were set as fold changes of relative expression levels using the $2^{-\Delta\Delta Ct}$ method after normalization.  All data were analyzed using Tukey-Kramer HSD test.  Different letters above columns indicate significant differences (p < 0.05).  Error bars represent standard errors.

### 3.3 APit POMC gene expression

Anterior pituitary (APit) proopiomelanocortin heteronuclear RNA (POMC hnRNA) expression was measured, as the heteronuclear sequence codes for the pre-prohormone POMC that is later spliced to produce adrenocorticotropin (ACTH), the pituitary stress hormone. Measuring POMC hnRNA would determine whether lesioning the NHpC would decrease pituitary function. For the LES+FD group, a significant downregulation occurred during the 2h FD stress (Fig. 4) compared to both the SHAM CON and SHAM+FD groups (p < 0.05).
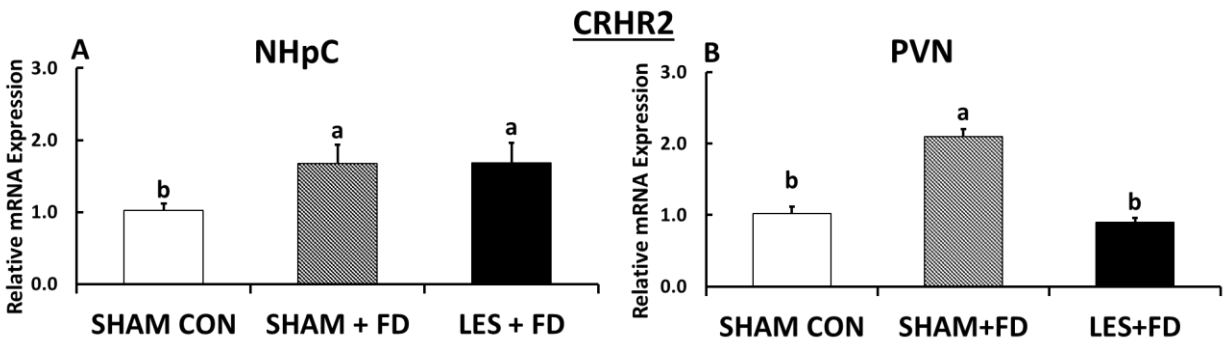


**Fig. 5.** Relative gene expression of corticotropin releasing hormone receptor 2 (CRHR2) within the **A**. nucleus of the hippocampal commissure (NHpC) and **B**. paraventricular nucleus (PVN). SHAM CON (n=10) birds were subjected to surgical procedures but no passage of electrical current. The SHAM+FD (n=6) group was subjected to sham surgery along with 2h of FD stress. The LES+FD (n=10) birds were subjected to lesioning of the NHpC and 2h FD stress. Data were set as fold changes of relative expression levels using the $2^{-\Delta\Delta Ct}$ method after normalization. All data were analyzed using Tukey-Kramer HSD test. Different letters above columns for each anatomical structure indicate significant differences (p < 0.05). Error bars represent standard errors.

### 3.4 CRHR2 expression in the NHpC and PVN

Within the NHpC an upregulation of CRHR2 occurred within the SHAM+FD and LES+FD groups in comparison to the SHAM CON group (Fig. 5A) following 2h FD stress (p < 0.05). Within the PVN, the SHAM+FD CRHR2 group can also be seen upregulated in comparison to the SHAM CON group similarly to the NHpC (Fig. 5B) (p < 0.05). Interestingly,

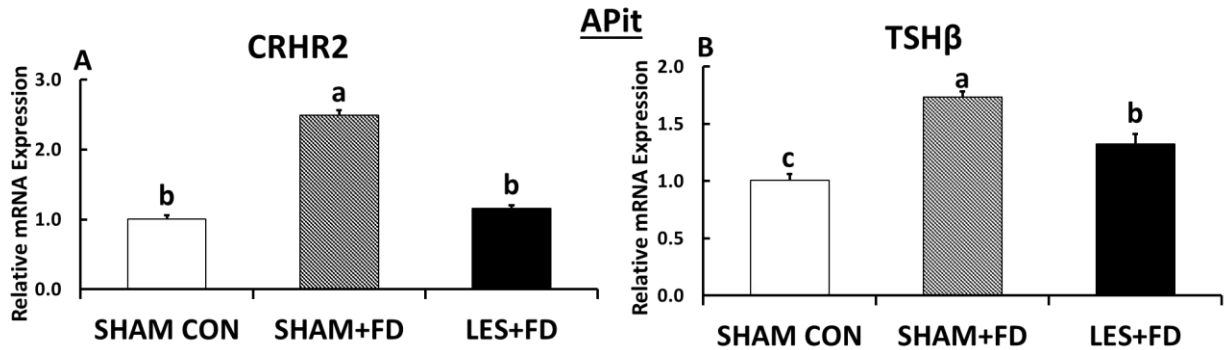The PVN LES+FD CRHR2 group can be seen at basal levels in comparison with the SHAM CON group (Fig. 5B) ($p > 0.05$).



**Fig. 6.** Relative gene expression of **A.** corticotropin releasing hormone receptor 2 (CRHR2) and **B**. thyroid stimulating hormone (TSHβ) within the anterior pituitary (APit). The SHAM CON (n=10) group was subjected to surgical procedures but no passage of electrical current. SHAM+FD (n=6) birds were subjected to sham surgery along with 2h of FD stress. The LES+FD (n=10) group was subjected to lesioning of the NHpC and 2h FD stress. Data were set as fold changes of relative expression levels using the $2^{-\Delta\Delta Ct}$ method after normalization. All data were analyzed using Tukey-Kramer HSD test. Different letters above columns indicate significant differences ($p < 0.05$). Error bars represent standard errors.

### 3.5 APit CRHR2 and TSHβ expression

CRHR2 expression within the APit for the LES+FD group was found to be significantly downregulated compared to the SHAM+FD birds ($p < 0.05$) and nonsignificant compared to SHAM CON birds. The SHAM+FD group illustrates highly significant expression compared to both the SHAM CON and LES+FD groups ($p < 0.05$). For thyroid stimulating hormone (TSHβ) mRNA, a similar pattern to CRHR2 was observed. Although the LES+FD group was significantly upregulated compared to the SHAM CON group ($p < 0.05$), it was downregulated when compared to the SHAM+FD group ($p < 0.05$). The SHAM+FD group was significantly upregulated compared to SHAM CON ($p < 0.05$).
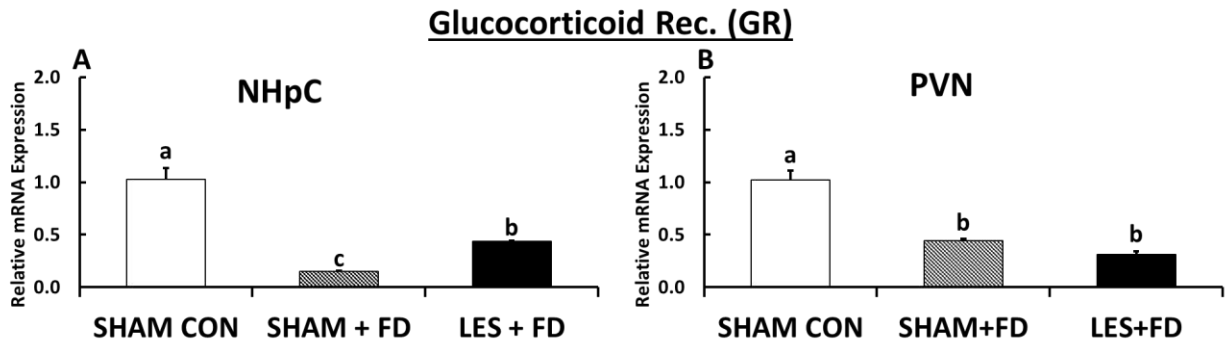
**Fig. 7.** Relative gene expression of glucocorticoid receptor (GR) within the **A**. nucleus of the hippocampal commissure (NHpC) and **B**. paraventricular nucleus (PVN). The SHAM CON (n=10) birds were subjected to surgical procedures but no passage of electrical current. The SHAM+FD (n=6) group was subjected to sham surgery along with 2h of FD stress. The LES+FD birds (n=10) were subjected to lesioning of the NHpC and 2h FD stress. Data were set as fold changes of relative expression levels using the $2^{-\Delta\Delta Ct}$ method after normalization. All data were analyzed using Tukey-Kramer HSD test. Different letters above columns indicate significant differences ($p < 0.05$). Error bars represent standard errors.

### 3.6 GR within the NHpC and PVN

Glucocorticoid receptor (GR) was measured within the NHpC and PVN to observe the adrenocortical feedback mechanism during a 2h FD stress. Both the SHAM+FD and LES+FD groups in the NHpC were significantly downregulated compared to the SHAM CON group (Fig.7A) ($p < 0.05$), although the LES+FD group was significantly elevated from the SHAM+FD group ($p < 0.05$). Within the PVN, the SHAM+FD and LES+FD groups both showed downregulation compared to the SHAM CON birds (Fig. 7B) ($p < 0.05$).

Glucocorticoid receptors within the APit were also measured to observe the adrenocortical feedback mechanism during 2h FD stress. The LES+FD group illustrates a significant downregulation of the receptor compared to both the SHAM CON and SHAM+FD groups (Fig 8; $p < 0.05$). The SHAM+FD group was also significantly downregulated during 2h FD stress compared to the SHAM CON group (Fig 8; $p < 0.05$).
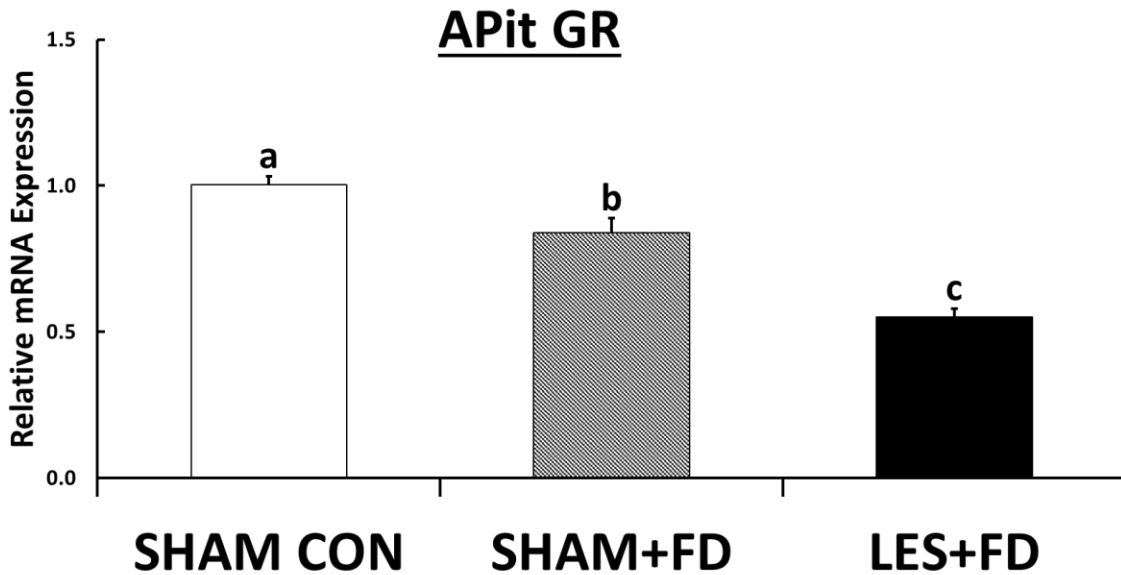
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**Fig. 8.** Relative gene expression of glucocorticoid receptor (GR) within the anterior pituitary (APit). The SHAM CON birds (n=10) were subjected to surgical procedures but no passage of electrical current. The SHAM+FD group (n=6) was subjected to sham surgery and 2h FD stress. The LES+FD birds (n=10) were subjected to lesioning of the NHpC and 2h FD stress. Data were set as fold changes of relative expression levels using the $2^{-\Delta\Delta Ct}$ method after normalization. All data were analyzed using Tukey-Kramer HSD test. Different letters above columns indicate significant differences (p < 0.05). Error bars represent standard errors.

Glucocorticoid receptors within the APit were also measured to observe the adrenocortical feedback mechanism during 2h FD stress. The LES+FD group illustrates a significant downregulation of the receptor compared to both the SHAM CON and SHAM+FD groups (p < 0.05). The SHAM+FD group was also significantly downregulated during 2h FD stress compared to the SHAM CON group (p < 0.05).

## 4. Discussion

### 4.1 Blood corticosterone decreases due to CRH reduction in NHpC and PVN

The 41-residue peptide, CRH, contained within the hypothalamic paraventricular nucleus (PVN) of vertebrates has been regarded as the first important neuropeptide activated in the

neuroendocrine stress response due to its stimulation of ACTH released from corticotropes

within the APit (Vale et al, 1981). Recent evidence in avian species suggests that CRH neurons

within the septal NHpC play a critical role within the avian HPA axis as early activation of

NHpC CRH mRNA levels occurred concurrently with increased plasma CORT levels at 2h of

food deprivation (FD) stress (Nagarajan et al, 2017). The present study measured plasma CORT

at 2h FD to observe the effects of partial ablation through electrolytic lesioning of NHpC CRH

neurons at the height of their activation. The 2h time point was critical for this study due to data

showing a 65% increase of CRH that peaked at 2h of FD. In contrast, gene expression of CRH

in the PVN was just beginning to rise and did not peak until hours later (Kadhim et al., 2019).

The question addressed was, does the NHpC drive the early stress response of the

neuroendocrine HPA axis? If so, would lesioning the NHpC followed by subjecting birds to 2h

FD affect the normal function of the traditional HPA axis? As seen in the plasma CORT

response to 2h FD stress in birds with partial lesions of the NHpC, there was a significant

decrease in CORT levels. Notably, this level of CORT decrease directly corresponds with the

decrease of CRH mRNA in the NHpC. Unexpectedly, the PVN which did not sustain any

lesions to its structure in the LES+FD group, showed a significant decrease in CRH mRNA.

Importantly, the major receptor of CRH, CRHR1, showed significant down regulation in both the

NHpC and PVN. The cascade effect of targeted lesions to the NHpC causing decreased gene

expression of its CRH and CRHR1 as well as those of the PVN provide additional data for the

role of the NHpC in the initiation of the neuroendocrine stress response. Indeed, decreased

activity of both the NHpC and PVN effected a decrease in expression of corticotropes in the APit

as shown by decreased expression of POMC hnRNA (Fig. 4), which is critical for ACTH

production that is transported to the avian adrenal gland to stimulate CORT hormone production

and release.  The POMC hnRNA reduction clearly illustrates that CRH levels being transported into the APit have been significantly reduced from the NHpC and/or PVN having a direct effect on blood CORT levels.  In addition to providing data of the role of the NHpC in early activation of the avian neuroendocrine HPA axis, the concurrent decrease in PVN gene expression suggests possible neural connectivity between the NHpC and PVN.  Further studies will be needed to verify this relationship.

**4.2 NHpC influence on PVN gene expression**

The NHpC has been shown in the past to be involved in early activation food deprivation stress, preceding responses observed in the PVN (Nagarajan et al, 2017).  Evidence in this study shows that the NHpC may also directly influence PVN activation during food deprivation stress. After lesioning, the NHpC illustrated decreased CRH due to physical destruction of the cells along with decreased CRH mRNA expression within in the PVN, although no visual damage was noted within the PVN during sectioning of each brain within the LES + FD group. Although 2h is not peak PVN CRH expression, the LES+FD group shows a clear reduction in expression compared to the positive control group SHAM+FD.  Another uncharacteristic response was that of CRHR1 in the PVN that illustrated decreased mRNA levels, which contrasts with previous data showing CRH and CRHR1 increase during FD stress (Kadhim et al, 2019).  This lack of CRH and CRHR1 mRNA within the PVN points to a reduced response within the nucleus for the LES+FD group of birds.  The lack of PVN activation during this 2h food deprivation stress suggests that the NHpC not only has an early activation during stress, but also may directly be involved in the activation and/or regulation of the PVN CRH and CRHR1 mRNA expression suggesting a possible connection between the two structures.

## 4.3 Glucocorticoid receptor effects on HPA axis feedback regulation

The regulation of the final product of the HPA axis is essential to the health of the animal as excessive CORT levels can become detrimental during chronic stress.  Glucocorticoid receptors (GR), within the hypothalamus and APit are known to act suppressively during a stress response when CORT regulation is needed. (Sapolsky et al, 2000).  GR within the APit have been found to regulate CORT through negative feedback suppression of POMC through a negative glucocorticoid response element (nGRE), which is located on POMC's promoter region (Subramaniam, et al, 1998).  This negative feedback can be seen within the NHpC lesion group of birds.  Specifically, the GR within the NHpC, PVN and APIT demonstrates behavior similar to that of long-term chronic exposure to glucocorticoids which causes downregulation (Yuan et al, 2016; Dickens et al., 2009).  This evidence is further supported by the POMC hnRNA data as it demonstrates that the GRs have begun inhibiting transcription in the APit for the NHpC lesion group of birds.  Lesioning of the NHpC had a clear effect on the feedback mechanisms as the GR receptor mRNA within the LES+FD groups was found to be significantly downregulated which is abnormal due to the lower concentration of plasma CORT. This demonstrates that the NHpC could be a vital structure in neuroendocrine stress regulation.
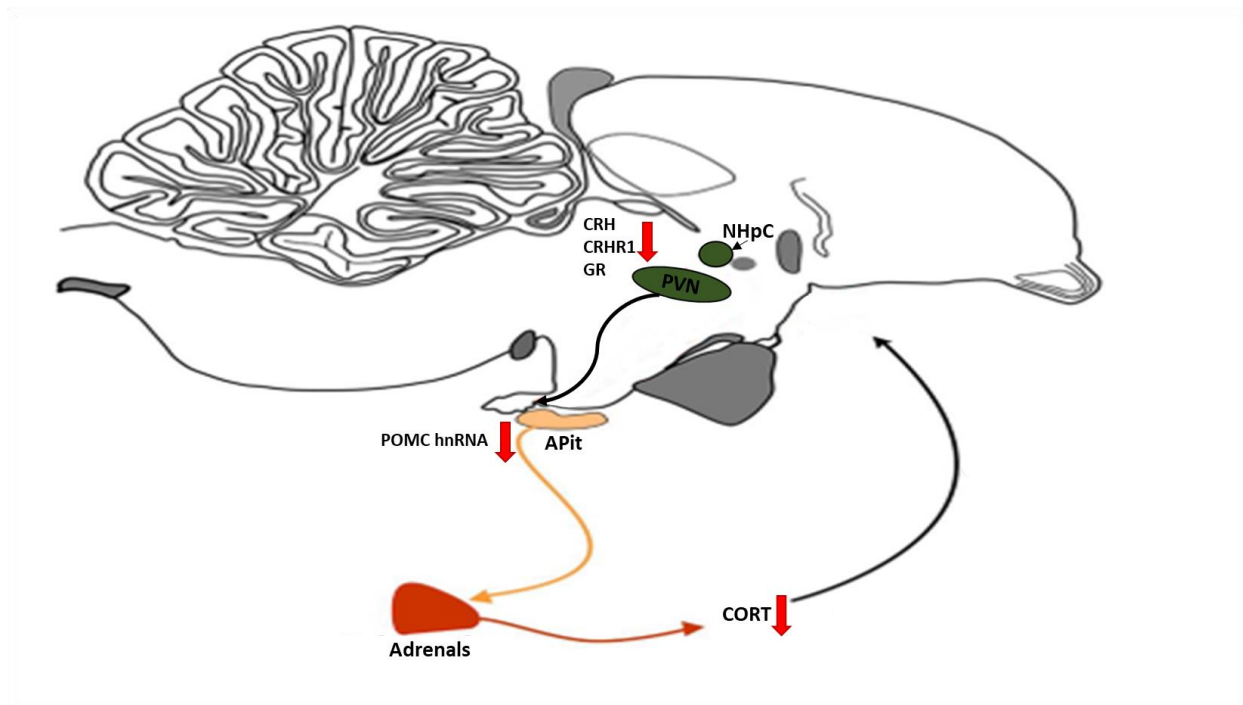
**Fig. 9.** This figure is a summarization of the LES+FD group of birds result's displaying the overall effects of the lesions within the NHpC for the hypothalamo-pituitary-adrenal axis system. Red arrows to the left of the NHpC, PVN and APit represent decreased mRNA levels. The genes present are as CRH/CRHR1/GR within the NHpC, CRH/CRHR1/GR within the PVN and POMC hnRNA within the APit. CORT is also depicted within the blood followed by a red arrow representing a decrease.

## 4.4 Similarities of the central nucleus of the amygdala to the NHpC

In this study PVN mRNA expression of several critical neurohormones and their receptors for both the HPA and HPT axes were found to be significantly downregulated during the 2h food deprivation stress post NHpC CRH neuron lesioning. The significant effects on the PVN suggest that a connection between the NHpC and PVN and that the NHpC could be critical in the responsiveness of the PVN during early short-term acute food deprivation stress. The PVN has been shown in previous studies to be indirectly connected to extra-hypothalamic CRH dense structures in mammals such as the central nucleus of the amygdala (CeA), which was

found to be possibly related to HPA activation in stress through the connection of the bed nucleus of the stria terminalis (BNST) (Choi et al., 2008), which is directly connected to the PVN. Another study of the CeA in which CRH neurons were silenced through injection of a 21-mer double -stranded RNA oligonucleotide which interfered with CRH RNA synthesis concluded that the nucleus is involved in the endocrine response of the HPA during behavioral stress (Callahan et al, 2013). Similarly, the knock down of NHpC CRH neurons certainly implies involvement in endocrine HPA activity as the lesioning leads directly to the significant decreased responses in the PVN and APit leading to the final significant decrease of CORT in the blood.

This study utilized food deprivation stress to observe the changes in the endocrine function of the HPA. Although food deprivation was used it was only until a 2h timepoint leading to the possibility that 2h of FD stress is acting as a psychological stressor. One study that lesioned the CeA, demonstrated the structure was involved in inhibiting fear conditioned startle responses (Hitchcock and Davis, 1991). It is possible that the NHpC extra-hypothalamic CRH is acting on the HPA during psychologically and early neuroendocrine stress.

**4.5 NHpC CRH involvement in Hypothalamo-Pituitary-Thyroidal axis**

CRH is not only an important neurohormone involved in the HPA axis but has been linked to the thyroid system with the release of thyroid stimulating hormone (TSH) produced by thyrotropes within the APit (De Groef et al., 2005a). A study with bird embryos concluded that when injected with ovine CRH, plasma CORT and TSH increases as early as 1 h after injection (Meeuwis et al., 1989). The mechanism of this release is mediated through CRHR2 located on thyrotropes within the avian APit, hence allowing CRH to directly bind to the CRHR2 to regulate TSH release into the blood (De Groef et al., 2003). This finding provides direct

evidence that CRH can be selectively regulated to influence both corticotropes and thyrotropes within the APit illustrating the connection between the adrenocortical and thyroid systems (De Groef et al., 2005b). Another regulating factor linking the HPA axis and HPT axes is prepro-TRH178-199, a TRH preprohormone, that has been found to demonstrate corticotropin release-inhibiting properties (Redei et al., 1995). A restraint stress study in rats involving the injection of prepro-TRH178-199 concluded that ACTH at the level of APit was inhibited demonstrating the connection between prepro-TRH peptides and acute stress (McGivern et al., 1997). Furthermore, CRHR2 mRNA expression within the PVN illustrates upregulation in non-lesioned groups during FD stress (Fig. 5B) along with upregulation of CRH mRNA (Fig. 3B). In contrast, the LES+FD birds demonstrated downregulation of both CRHR2 mRNA expression and CRH mRNA expression in the PVN showing a close relationship between CRH and CRHR2 in that structure. Indeed CRH, CRHR1 and CRHR2 were shown to be positively regulated within the PVN during several sampling times during 8h of FD of chickens (Kadhim et al., 2019). Previously, a mammalian restraint stress study showed a similar relationship between CRH and CRHR2 within the PVN (Greetfeld et al., 2009). A similar pattern holds true within the APit between CRHR2 and TSHβ in the LES+FD group. Both CRHR2 and TSHB mRNA were significantly lower in the LES+FD group compared to the SHAM+FD birds. The similar expression of PVN and APit CRHR2 mRNA suggests that at the level of the brain, in the PVN, CRHR2 may in some manner affect activity of CRHR2 a on pituitary thyrotropes thereby linking the PVN not only with the HPA axis but the HPT axis as well (Geris et al., 1996).

Thyroid stimulating hormone beta subunit (TSHβ) mRNA has been measured within the chicken APit thyrotropes as early as E5 (Van AS et al, 2000; Nakamura et al, 2004). Measurements of TSHβ in the current study show significant upregulation in the non-lesioned

stress group and the opposite for the lesion group. This expression follows CRHR2 expression which is responsible for the binding of CRH and activating the release of TSH from thyrotropic cells. The lesioning of the NHpC resulted in downregulation of CRHR2 within the PVN and APit along with downregulation of TSHβ within the APit suggesting that CRH neurons within the NHpC could be vital to the function of the HPT axis during acute FD stress. This expression demonstrates that TSHβ is effectively disrupted within the NHpC LES+FD groups illustrating the importance of the CRH neurons within this septal nucleus.

Evidence of HPT axis activation during stress has been found within mammals although findings have been conflicting. One study that observed decreased levels of $T_3$ and $T_4$ in the periphery with repeated foot shock stress in rats (Helmreich et al., 2005). Another study utilizing rats found that 2 min immobilization with repetition after 3 min induced increased levels of thyroid hormone secretions (Turakulov et al., 1994). It has also been found that acute and chronic noise stress in male rats showed increased levels of TSH and $T_3$ in serum (Armario et al., 1984). Armario argues that the discrepancies with HPT axis activation during stress are due to the different effects of physical verses psychological stressors. Armario states that stressors of psychological nature tend to increase HPT axis activity whereas physical-type stressors tend to decrease HPT axis activity. This could explain why our data of an early acute FD stress is activating the HPT axis during 2h of FD stress. Another recent study in chickens utilizing another form of avian CRH, CRH2, a proposed 5th member of the CRH family (40-aa with 60.41% shared identity to CRH), was injected into APit cells to observe the peptide's functionality (Bu et al., 2019). Interestingly, CRH2 has not been found in mammals. The peptide was found to stimulate TSHβ and was 15-fold more potent in activating CRHR2 than CRHR1.

Additionally, high concentrations of CRH2 effect ACTH release. This demonstrates another connection between the adrenocortical and thyroid systems in birds.
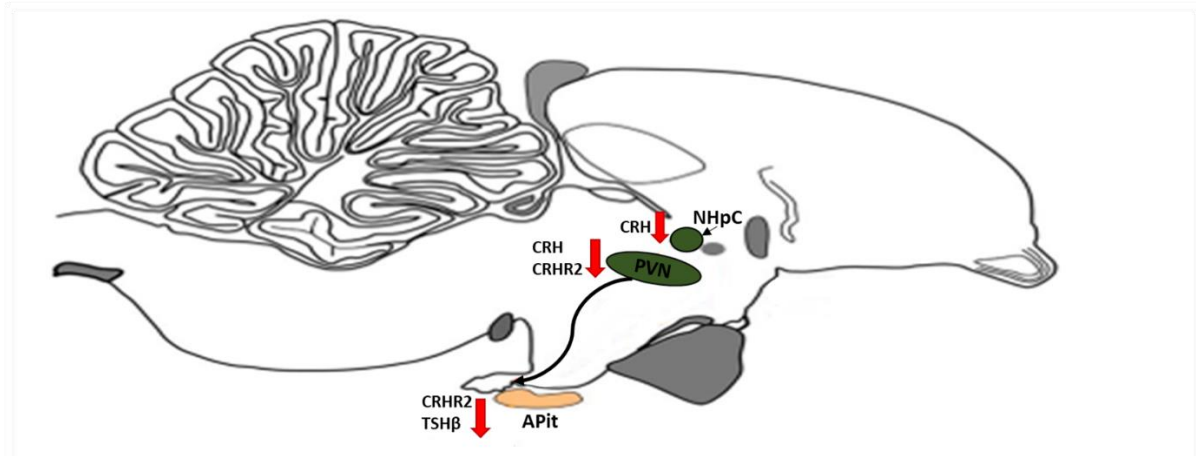


**Fig. 10.** This figure is a summarization of the LES+FD group of birds result's displaying the overall effects of the lesions within the NHpC for the hypothalamo-pituitary-thyroid axis system. Red arrows to the left of the NHpC, PVN and APit represent decreased mRNA levels. The genes present are as CRH within the NHpC, CRH/CRHR2 within the PVN and CRHR2/TSHβ within the APit.

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**Conclusion**

In conclusion, decreased CORT concentrations following NHpC ablation strongly suggest that CRH neurons within the NHpC play a critical role in the avian HPA axis utilizing FD as a stressor. Knock down of NHpC CRH neurons also had significant effects on PVN gene expression, specifically, PVN CRH mRNA expression was significantly decreased at 2h of FD within the NHpC lesion group. Data suggest a possible neural connection between the NHpC and PVN as well as NHpC activity being necessary for PVN function. Interestingly, NHpC CRH mRNA regulation appeared to be connected to TSHβ mRNA expression within APit suggesting that CRH within the NHpC could be important for HPT axis function in FD stress. CRHR2 located on thyrotropes within the APit are known to be involved in the HPT axis, and it is also possible that CRHR2 within the PVN is a necessary receptor in the thyroid system due to displaying similar regulation. Thus, CRH produced by the NHpC and/or PVN may be driving simultaneous responses of the HPA and HPT axes, opening the possibility that the HPT axis is involved in the avian neuroendocrine stress response.