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Central Administration of Neuropeptide Y (NPY) and Vasotocin 4 Receptor (VT4R/V1aR) Antagonists on Food Intake and a Stress Response in Chicks

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Central Administration of Neuropeptide Y (NPY) and Vasotocin 4 Receptor (VT4R/V1aR) Antagonists on Food Intake and a Stress Response in Chicks.

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Abstract

The objective of this study was to determine the central effect of the VT4R antagonists (SR-49059 and H-5350 (Manning compound)) on corticosterone (CORT) levels during stress and food intake. A 22-gauge stainless steel guide cannula was surgically implanted into the lateral ventricle of the birds. A preliminary study was done to test the antagonists and their role on food intake. Birds were injected with saline, NPY (4µg), NPY (4µg)+SR-49059 (250ng), or NPY (4µg)+Manning compound (250ng). Birds injected with saline had the lowest 1h food intake (17.7g±1.6). Birds injected with NPY+saline had a significantly higher intake (27.1g± 1.0), which was enhanced when birds were injected with NPY+SR-49059 (44.6g±2.6) or with NPY+ Manning (35.9±2.8) compound. These findings were followed up with an acute immobilization stress study. Before immobilization, birds were injected with saline, SR-49059 (250ng), or Manning compound (250ng). Acute stress included wrapping the birds in a harness and leaving them in an unfamiliar cage for 30 minutes. The treatments included no stress, stress, stress+SR-49059, stress+Manning compound. Sample size ranged from 5 to 8 birds per treatment. Blood samples were collected and plasma quantified for CORT by RIA. Results showed that the two antagonists + stress significantly lowered CORT levels when compared to the stress group ($p<0.05$). A third study was conducted to determine the role of the antagonists alone on food intake. The antagonists increased food intake compared to the control (saline) birds, but did not increase food intake more than birds injected with the antagonist+NPY ($p<0.05$). In summary, there appears to be an interaction between NPY induced food intake and the vasotocinergic system on the feeding response in birds. The two antagonists have a greater than additive effect on food intake when given with NPY.

Acknowledgments

I owe many thanks to my advisors, Dr. Wayne J. Kuenzel and Dr. Seong W. Kang for supporting me throughout my study. They have provided me with knowledge, training, and encouragement, and were always willing to help when needed at the farm.

My colleagues, Gurueswar and Aman, have continually given up their time to help me with my data collection. I would not have been able to complete my work without them.

Lastly, I would like to thank my family and friends who have helped me and encouraged me along the way, and God for allowing me this opportunity.

Dedication

I would like to dedicate this thesis to my parents. They are the strongest, hardest working people I know. They have always been my heroes and the most influential people in my life.

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I. Introduction

The response of poultry to stress and their regulation of food intake throughout their life span are both very important to the poultry industry. Stress can lead to disease, which is not only bad for the bird, but is also a major issue for the producer due to a loss of efficiency in the conversion of feed to body weight gain. One of the main problems is when a bird is diseased or chronically stressed, meat yield and egg production declines. Therefore, it is relevant to examine the neural regulation of stress in poultry and how it impacts food intake so that issues like this are less prevalent throughout the poultry industry. Arginine vasotocin (AVT) is a neuropeptide hormone that is involved in the neuroendocrine stress response in birds. AVT is one of the major neuropeptides in the hypothalamo-pituitary adrenal (HPA) axis. The HPA axis is a complex system that controls reactions to stress, as well as regulates many body processes (digestion, immune system, mood, energy expenditure, etc). The HPA is composed of the hypothalamus, which releases corticotropin-releasing hormone (CRH), vasopressin (mammals)/vasotocin (non-mammalian vertebrates), the anterior pituitary gland which releases adrenocorticotropic hormone (ACTH), and the adrenal gland which releases glucocorticoids (cortisol, corticosterone) and catecholamines (epinephrine and norepinephrine). The effects of vasopressin and vasotocin neurons are mediated by G protein coupled receptors (GPCRs), which belong to the rhodopsin-like receptor family. In mammals, there are three vasopressin receptors: V1a, V1b, and V2. In the avian species, there are four known vasotocin receptors: VT1, VT2, VT3, and VT4 (Cornett, et.al., 2013). When a bird is stressed AVT levels will increase, which causes the stress hormone, corticosterone (CORT) to rise (Cornett et al., 2013). As stated previously, CRH is also released when the hypothalamus is activated due to a stressor or

stimulus. Studies have shown that when AVP and CRH are given together or separately they greatly increase ACTH release (Gillies et al., 1982; Madison et al., 2008). When centrally injected, AVT significantly reduces food intake (Tachibana et. al., 2013), and Neuropeptide Y (NPY) has been shown to increase vasopressin and CORT levels in rats (Leibowitz et. al., 1988). NPY is well known as a potent inducer of food intake. In this thesis, chickens were centrally injected with two different antagonists for the vasotocin subtype 4 receptor (VT4R), homologous to the mammalian V1a receptor, to test the effects of the antagonists on CORT release and food intake, as well as to rank the antagonists' efficacy in vivo, based on results from a previous in vitro study (Jayanthi et al., 2014). The purpose of this study was to develop an in vivo procedure for screening potential blockers of the VT4R/V1aR by determining how effective those blockers are in inhibiting the binding of agonists to the V1aR, such that the negative effects of stress that decrease food intake are blocked.

Literature Review

A. Growing Demands of the Poultry Industry to Meet Consumer Needs

The poultry industry continues to grow more and more rapidly. In the United States alone, per capita consumption of chicken has risen from 30 to 80 lbs. over the last 50 years or so. With that in mind, beef consumption per capita has fallen, while pork intake has remained relatively constant over the past 5 decades.

According to the US Poultry and Egg Association (2013), the combined value of poultry production is over $40 billion per year. Throughout the 1940's, the poultry industry began to start producing larger flocks of broilers, which lead to the development of processing plants capable of handling the larger scale of poultry produced/farm. Overall the result was the eventual large-scale commercialization we see today in the production of broiler and turkey meat. The average per capita consumption of broiler meat consumed in 2012 was approximately 55 lbs, turkey meat consumed was 13 lbs, beef consumed was 51 lbs, and pork consumed was 42 lbs. (Figure 1a) (www.poultryegginstitute.org, 2014). These data show that overall, poultry (broilers and turkeys) was consumed more than beef or pork (approximately 68 lbs for total poultry consumed).

B. Recent Progress in the Broiler Industry

Beginning in the 1950s, poultry scientists, focused upon ways to select birds that were more efficient in utilizing diets available while nutritionists were constructing tables detailing the modern birds' requirements for energy, protein, specific amino acids, minerals and vitamins. This desire for increased efficiency lead to larger housing units, greater densities of birds per house, and genetic selection of specific breeds of birds for meat and eggs to name a few of the changes. With these changes, came a need for scientists of all disciplines to ensure each sector of the poultry industry was running smoothly. Throughout the years, physiologists, geneticists,

veterinarians, behaviorists, molecular biologists, etc., have been working to make sure that as the poultry industry changes to meet the demands of consumers, birds produced could handle the environments and diets created for them. In order to create a uniform bird, these poultry scientists developed parent stock to ensure uniformity within the industry. The males and females are typically third generation offspring and are not genetically selected for the same traits. The modern bird has changed drastically over the last fifty years (Figure 1b) (http://heritagefoodsusa.com). The modern broiler has been selectively bred to consume a larger volume of protein and therefore, gain weight more rapidly. These birds are raised in specific, highly controlled environments, combined with unrestricted access to high protein feed and artificial lighting conditions to stimulate growth and achieve desired body weight by 4-8 weeks (depending on the type of bird and what it is raised for) (www.poultryegginstitute.org, 2014).

C. Stress in Poultry

With all of the changes that have been made over the years to the environment and to the birds themselves, there come some negative outcomes. One of these issues is stress, which can affect food intake, as well as meat yield, egg laying, etc. With that in mind, two of the main factors that are very important in the poultry industry are stress and food intake. Stress should be minimized, while food intake efficiency should be maximized. With the increased density and body weights of these birds, problems have arisen pertaining to stress and food intake. Since these two factors are so important, it is imperative to perform studies to find ways to decrease stress and maximize feeding efficiency. The poultry industry is continuing to grow and the demand for poultry will continue to increase as the world continues to be more and more populated.

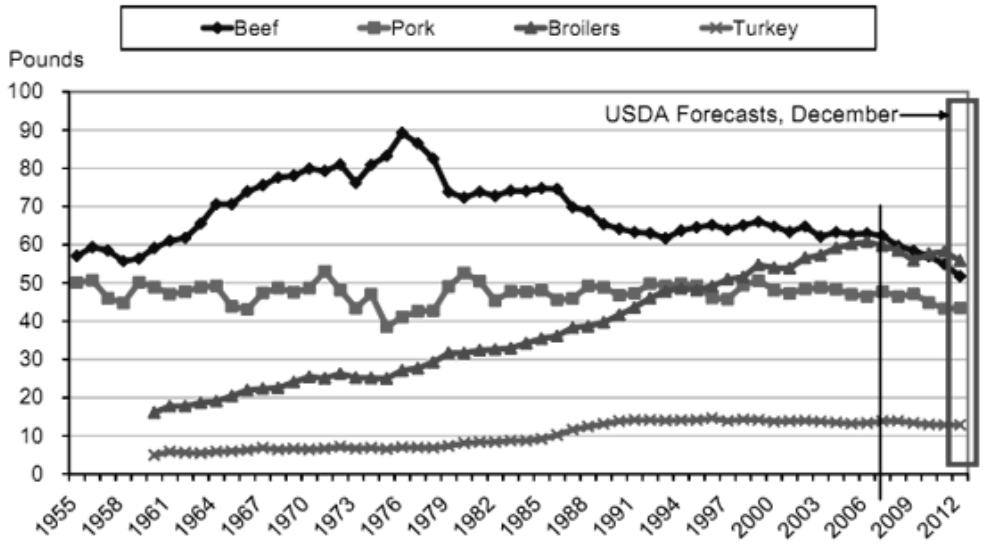

Figure 1a. U.S. meat and poultry consumption per capita from 1955-2012.

Figure 1b. The visual difference in size and number of days needed to reach each size from a bird raised in 1950 and a bird raised in 2008.

D. Stress

Stress can be a physiological or psychological reaction to environmental challenges, which can cause a response to an emotional or physical threat. When an organism is stressed, all activities that are not of importance at the time for survival are shut off. These include feeding, the reproductive activity, and responses of the immune system. This is known as the "General Adaptation Syndrome" (Selye, 1936). These processes are shut off to aid the animal to flee the stressful or harmful situation. All of the organism's energy is used to get away. This is known as an initial response to a stressor. The stress response has been initiated at this point. At the start of the stressor, the hypothalamo-pituitary-adrenal (HPA) axis is triggered. This begins at the hypothalamus of the brain, where corticotropin-releasing hormone (CRH), and arginine vasotocin (or arginine vasopressin in mammals) is released to the anterior pituitary. The anterior pituitary releases adrenocorticotropic hormone (ACTH), which is transported via the cardiovascular system to the adrenal glands. The adrenal glands release glucocorticoids (cortisol in humans, corticosterone in birds and rodents) and catecholamines (epinephrine and norepinephrine) into the blood stream. Plasma corticosterone (CORT) or cortisol is the primary glucocorticoid used as a measure of the neuroendocrine response to stress in the HPA axis of vertebrates (Madison et. al., 2008). Glucocorticoids provide the body with energy via stimulating fatty acid release, inhibiting protein synthesis, enhancing glucose utilization, stimulating the liver to synthesize glucose from protein, suppress both pain perception and the immune system. It takes anywhere from fifteen to sixty minutes after a stressful event for glucocorticoid levels to reach their maximum levels. The body is made to endure this in acute circumstances. However, when a stressor continues, the organism enters a stage of resistance. When glucocorticoid levels remain high, energy expenditures remain high at the expense of an

animal's productivity. Specifically, these costs to the organism include, suppression of reproductive processes, weight loss, and diminished inflammatory and immune responses. This means that the stress response does not shut off. Therefore there are always high levels of glucocorticoids. In monkeys (Olive baboons), high levels of cortisol has been shown to be toxic to hippocampal neurons (Sapulsky et.al., 2004). The hippocampus contains a high density of receptors for cortisol and is believed to play a role in the negative feedback loop that decreases cortisol production. In the study, degeneration of the hippocampus blocked the ability to inhibit cortisol production, which led to the eventual death of the monkeys. This same type of stress related degeneration of hippocampal neurons has been documented in humans as well. Therefore, long-term stress can have a negative impact on organisms. This is the same in the poultry industry, which is why it is so important to study the effects of stress on poultry. There are several advantages to using the chicken as a model to study the effects of the HPA axis during stress. The chicken allows for serial blood sampling due to body size, as well as a larger blood volume to enable more than one assay to be performed if needed when compared to rodents and wild avian species utilized in stress research. In addition to these advantages, it is possible to raise and maintain birds under uniform conditions to minimize variability among birds throughout the study. All of these advantages make the chicken an excellent model in studying stress research.

E. Types of Stressors Impacting Poultry

Psychological/Psychogenic Stressors

Psychological stress is an organism's response to an outside stimulus or stressor. Fear is a common psychological stress in birds. In poultry, fears may occur due to a perception of a threat from humans or predators. Birds have a tendency to exhibit fear by running away, jumping or acting flighty, running away from the predator, and calling out to alert others in the flock. It's hard to determine a difference between fear and stress in poultry because birds seem to act similarly with both, and stress sometimes causes fear and vice versa. Social stress stems from relationships with other birds. One of the earlier behaviors demonstrated is the pecking order that involves an important learning process. Mating stress when sexual maturity develops is also stressful to birds. It includes the stress of searching for and finding a mate, and the competition that takes place among males. Additionally there is the learning process of interacting appropriately with an individual of the opposite sex. Physiological (and psychological) stress also occurs when a bird undergoes immobilization stress, where they are wrapped in a harness to keep their wings immobilized.

Physical Stressors

Any type of environmental stressor is generally regarded as a physical stressor. This can include a change in lighting (birds are very sensitive to changing light due to their photoperiods), or a change in ventilation in order to keep ammonia levels as low as possible. Lighting schedule is related to feed consumption in birds. For example, broilers are grown under a continuous light schedule in order to maximize food consumption and growth rate. The main stress in the poultry industry that falls under climatic stress is heat stress. However, cold stress can occur too, which is why it is so important to keep birds in temperature-controlled barns (especially when

performing experiments or trials). Ventilation is a major focus in managing poultry. Inappropriate environments such as extreme weather conditions, overcrowding, insufficient or broken equipment can lead to poor ventilation in a poultry house. Poor ventilation can result in litter and health problems (Arjona, 1988). Like most organisms, a change in diet or a shortage of food and water will have an impact on birds. This can cause them a lot of stress if not tended to appropriately. However, nutritional needs will change based on the type of feed regimen being fed. With the many stressors that can affect the bird and lead to issues within the poultry industry it is highly important to study stress and work towards reducing it as much as possible.

F. Corticotropin Releasing Hormone and Arginine Vasotocin Neurons Initiate Stress Hormone Release

Corticotropin Releasing Hormone (CRH)

CRH is produced within the paraventricular nucleus of the hypothalamus. It is released in response to a stimulus or stressor. Once released, CRH is carried to the anterior lobe of the pituitary to stimulate adrenocorticotropic (ACTH) release. ACTH stimulates the synthesis of glucocorticoids (cortisol in mammals and corticosterone in non-mammalian species). The receptors for CRH, CRHR1, CRHR2 are found in the central nervous system and periphery (Bale et al., 2000). There are many studies that show that CRH is imperative in the stress response. A study done with mice lacking a CRHR1, showed that the medulla of the adrenal gland is atrophied and stress-induced release of adrenocorticotropic hormone (ACTH) and corticosterone is reduced. Their results demonstrated a key role of the CRHR1 in mediating the stress response

and anxiety-related behavior (Timpl et al., 1998). Another study showed that mice generated to be deficient for the CRH receptor 2 (CRHR2), displayed a greater anxiety-like behavior and are hypersensitive to stress. These mice also had decreased food intake (Bale et al., 2000). There are also many studies that show that there is a relationship between CRH in food intake. CRH, when centrally injected, has been shown to decrease food intake in Sprague-Dawley rats (Arase et al., 1988). A study done in rats showed that orexin (a peptide which stimulates food intake) was found to stimulate the release of CRH. The orexin-stimulated CRH was then blocked by a neuropeptide Y (NPY) antagonist. The experimenters concluded that the effect of orexin on food intake may be complex because of a link between orexin and CRH and orexin and NPY (Ida et al., 2000). Lastly, CRH has also been shown to reduce food intake in chickens. Central injection of CRH causes a decrease in food intake in chicks in both broiler and Leghorn chickens. The experimenters concluded that CRH acts via the central nervous system to decrease food intake, but does not affect water intake or body temperature in these birds (Denbow et al., 1998).

Arginine Vasotocin

Arginine vasotocin (AVT), known as vasopressin (AVP) in mammals, is a neuropeptide hormone that is involved in diverse functions. The neuropeptide hormone, AVT is produced by neurons in the hypothalamus. Large, magnocellular neurons project to the posterior pituitary gland (neurohypophysis) where their neurosecretions (AVT) are released directly into the blood stream. Arginine vasotocin secreted from magnocellular neurons is a major endocrine regulator of water balance and osmotic homeostasis, contraction of blood vessels, and reabsorption of water in the kidneys (McCormick and Bradshaw, 2006). Arginine vasotocin released into the

peripheral circulation is best known as antidiuretic hormone.

The AVT is also produced by parvocellular (small-sized) neurons that is released into the median eminence and transported by portal capillaries to the anterior pituitary where it binds to receptors on cells that release ACTH. The ACTH is then carried to the adrenal gland for the release of corticosterone, the stress hormone in birds, in the classical neuroendocrine axis, discussed previously. There are some functions of AVT that appear to result from that peptide being released from both magnocellular hypothalamic neurons and parvocellular hypothalamic neurons including social and sexual behavior in mammalian and non-mammalian vertebrates. These behaviors in birds include primarily singing, mating behavior, aggression, and courtship/bonding behavior (Goodson, et al., 2005). Other behavioral studies in that laboratory have shown AVT plays an integral role in social hierarchy and pair bonding behavior in several non-mammalian vertebrates (Goodson et al., 2005). Social hierarchy occurs naturally in most species, and arises when members of a social group interact to establish a ranking system within the group. This often results in aggressive behavior in order to establish a ranking order. Pair bonding is a strong fondness between two con-specific animals that in many cases leads to monogamy (Young, 2003; Castro and Matt, 1997). A study was done with zebra finches that showed increased levels of AVT were linked to an increase in aggressive, competitive behavior in non-paired male finches. However, once paired, the male zebra finches exhibited a more defensive behavior (Kabelik et. al., 2009). Likewise, aggressive behavior was decreased in male and female zebra finches following central injection of the Manning compound (250 ng), used as an arginine vasotocin antagonist (Goodson et. al, 2004).

In female Syrian hamsters, arginine vasopressin (AVP) has been shown to stimulate aggression after being injected into the anterior hypothalamus, and when injected with a

vasopressin 1A receptor (V1aR) antagonist the aggressive behavior ceased (Gutzler, 2010). An experiment on neural responses to territorial challenge and nonsocial stress was conducted in sparrows. Researchers found that injection of a V1aR antagonist (250ng, Manning compound) significantly reduced aggressive and stress-like behaviors (Goodson et.al., 2004). These studies support a link between mammalian and non-mammalian behavior via a AVP/AVT receptor.

G. Receptors of AVT (VT2 and VT4) shown to be involved in the HPA axis

Just as there has been less research done on the role of AVT in the HPA axis compared to CRH, similarly there has been less emphasis on the role of receptors involved in that mediate the effects of AVT, particularly in birds. Two AVT receptors recently investigated in our laboratory are believed to be responsible for the release of corticosterone. Functionally, the avian vasotocin subtype 2 receptor (VT2R) (Jurkevich et al., 2005; 2008; Kuenzel et al., 2013) and the avian vasotocin subtype 4 receptor (VT4R) (Selvam et al., 2013; Kuenzel et al., 2013) are thought to be involved in the neuroendocrine hypothalamo/pituitary/adrenal axis stress response in birds and are shown to be localized in corticotropes, which in birds occur explicitly in the cephalic region of the anterior pituitary. When cloned, the avian VT4R showed to have a 69% sequence homology with the human V1aR (Selvam et. al., 2013).

H. Evidence Showing that the V1aR and VT4R inhibits the Orexigenic Effect of Neuropeptide Y (NPY)

As cited earlier, CRH has been shown to reduce food intake in mammalian and non-mammalian studies. In addition to those studies, elevated NPY and reduced CRH gene expression were found to be a compensatory physiological response to restore food intake,

primarily in food-restricted and food-deprived animals (Brady, et. al., 1990). CRH and NPY act in the hypothalamus to influence energy homeostasis and may both mediate the anorexic effect of leptin (Uehara, et. al., 1998). Another study concluded that the relationship between the NPY system and the HPA axis is complex and includes positive feedback between NPY and CRH (Mastorakos and Zapanti, 2004). These articles show that there is a relationship between NPY and CRH, and between NPY and the HPA axis in general.

In previous studies, central administration of AVT was shown to significantly reduce food intake in chicks (Figure 2) (Tachibana et. al., 2013). Additionally, serum vasopressin levels were found to be high in rats when injected with NPY (Figure 3; Leibowitz et. al., 1988). In the same study, injections of NPY also increased corticosterone (CORT) serum levels (Figure 4; Leibowitz et.al., 1988). Vasopressin has even been shown to reduce food intake in pygmy goats when injected intraperitoneally (IP) (Meyer, et.al., 1989). CORT has also been thought to be a major antagonist to insulin functions. Glucocorticoids and insulin have been shown to widely oppose each other in mammals in regard to energy balance (Strack, et al., 1995). This is because glucocorticoids stimulate hyperphagia, while insulin inhibits feeding. This antagonism occurs at tissues where insulin operates its primary storing actions by decreasing plasma glucose levels (Remage-Healey et. al., 2001). However, birds are thought to use fat as an energy source more so than sugar. Because fat yields twice the amount of energy as carbohydrates or protein, this preference for lipids as the primary energy use is much more feasible for organisms adapted for flight. In white-crowned sparrows, feeding decreases when plasma lipids are elevated, but feeding is insensitive to changes in plasma glucose levels (Boswell et. al., 1995). Likewise, a study involving modification of circulating blood glucose levels in chickens was performed to discover if a glucostatic mechanism exists for food intake in birds. The experimenters used

insulin, glucagon, glucose, and fructose to modify the circulating levels of glucose, however, none of these resulted in any significant alterations in food intake over a four-hour period. They concluded that there is either not a glucostatic mechanism of food intake control or (if there is a mechanism) it does not operate in a manner that is easily detectable using protocols that are useful in mammals (Smith and Bright-Taylor, 1973). Stress has been shown to decrease plasma triglycerides in rats (Hershock and Vogel, 1989) and ACTH administration to domestic fowl elevates CORT levels and leads to increased fatty acids (Heald et.al., 1965). Likewise, in Japanese quail, both ACTH and a synthetic glucocorticoid administration caused an increase in plasma fatty acids (Bray, 1993). Therefore, food intake and NPY release (or lack of) could be working based on the V1aR and fat intake.

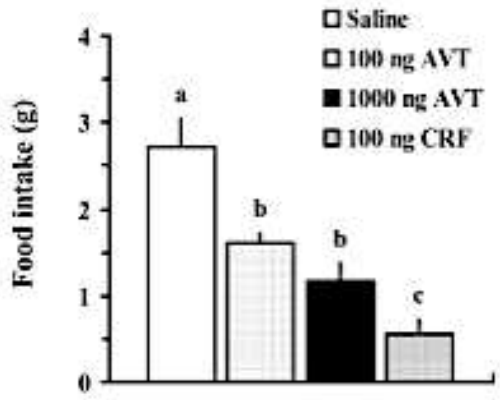

Figure 2. Food intake in chicks following central injection of AVT at two doses (100ng and 1000ng) and CRF (100ng) (Tachibana et. al., 2013).

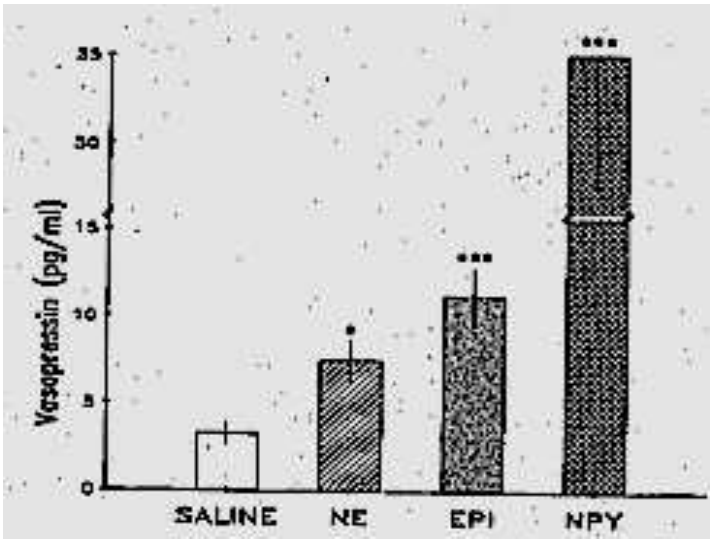

Figure 3. AVP serum levels in rats following central injection of saline, Norepinephrine (NE), Epiniphrine (EPI), and Neuropeptide Y (NPY) (Leibowitz, et. al,, 1988).

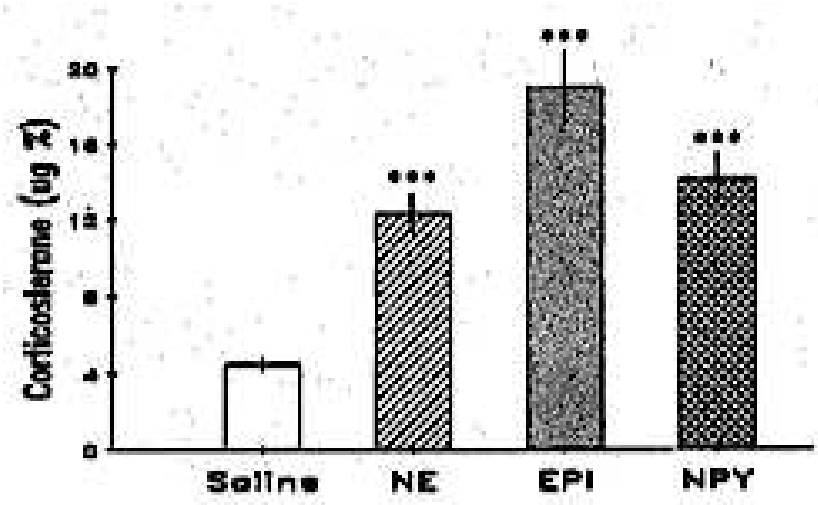

Figure 4. Corticosterone (CORT) levels in rats following central injection of saline, Norepinephrine (NE), Epiniphrine (EPI), and Neuropeptide Y (NPY) (Leibowitz, et. al., 1988).

I. Feeding Response of NPY

Neuropeptide Y (NPY) is known to be one of the most effective compounds to stimulate food intake in both mammalian and non-mammalian species (Kuenzel et. al., 1987). In the autonomic system it is produced mainly by neurons of the sympathetic nervous system and serves as a strong vasoconstrictor and also causes growth of fat tissue. It is produced in various

locations in the brain, however, the primary source of NPY is the arcuate nucleus of the hypothalamus. In addition to inducing food intake, when injected intracerebroventricularly (ICV), it potentiates blood levels of adrenocorticotropic hormone (ACTH), CORT, and arginine vasopressin (Leibowitz et. al., 1988). In other studies, it has also been shown to play a role in storage of fat as energy, reduces stress and anxiety, reduces pain perception, affects circadian rhythm, and lowers blood pressure (Tatemoto, 2004). A study was done using genetically obese rats to demonstrate the role of NPY in eating disorders including obesity (Dryden et.al., 1995). The study revealed four main determinants that contributed to obesity in rats: an increase in glucocorticoid concentrations in plasma, decreased sensitivity or resistance to insulin, mutation of leptin receptors, and an increase in NPY mRNA and NPY release. In an adrenalectomized rat study, glucocorticoids stimulated and insulin inhibited NPY mRNA and food intake (Strack et. al., 1995). The researchers concluded that effects of corticosterone and insulin on food intake may be mediated, in part, through regulation of hypothalamic NPY synthesis and secretion. When glucocorticoids are released and levels remain high, gluconeogenesis is stimulated. Subsequently, this causes an increase in blood glucose that activates the release of insulin to help regulate glucose levels by storing it as glycogen in the body tissues. Furthermore, high levels of glucocorticoids have also been shown to cause an increase of NPY. There have also been numerous studies to show that stress can stimulate NPY release and, depending on their diet, can cause a higher fat accumulation on their body (Kuo et. al., 2007). Injection of NPY into the supraoptic nucleus of unanesthetized rats has also been shown to increase plasma vasopressin when compared to controls, and the experimenters concluded that NPY might directly excite vasopressin-containing neurons and thereby cause secretion of vasopressin (Willoughby and Blessing, 1987). As stated previously, CRH and AVT are both released from the hypothalamus

once a bird is stressed. Injection of corticotrophin releasing factor (CRF) into the right lateral ventricle of chickens caused a significant decrease in food intake in both fed and over night-fasted broilers and Leghorns (Furuse et al., 1997; Denbow et.al., 1999). This has also been shown in white-crowned sparrows (Richardson et. al., 2000). Therefore, CRH and AVT have both been shown to decrease food intake when injected centrally. Interestingly, when NPY is centrally injected into the brain of rats, the rats exhibit anxiolytic-like behavior (Britton et. al., 2000). A study done where NPY was injected into the paraventricular nucleus (PVN) of rats undergoing corticosterone replacement therapy showed that there was a reduction in feeding compared to the control rats undergoing NPY PVN injections. The experimenters concluded that hypothalamic NPY-induced feeding response is largely dependent upon circulating corticosterone levels, and that no other adrenal or pituitary hormone is essential (Stanley et.al., 1989). These findings could conclude that NPY modulates an animal's behavior in response to a stressful stimulus. In comparison, the results of a study done in Japanese quail showed that CORT can stimulate food intake following a period of food deprivation (Wall and Cockrem, 2009). These studies, as well as many others, show that there is a relationship between stress and food intake. AVT and CORT are included in this relationship, and understanding more about this stress and food intake relationship is important. There are numerous data suggesting that NPY plays a crucial role in activating the hypothalamo-pituitary-adrenal (HPA) axis. NPY Y_5 is a well-known feeding receptor. When an NPY Y_5 subtype agonist was administered centrally to rats, there was a significant increase in plasma ACTH and CORT compared with CSF administration (Figure 5; Kakui and Kitamura, 2007). This gives increasing evidence that there is a relationship between the stress and feeding systems.

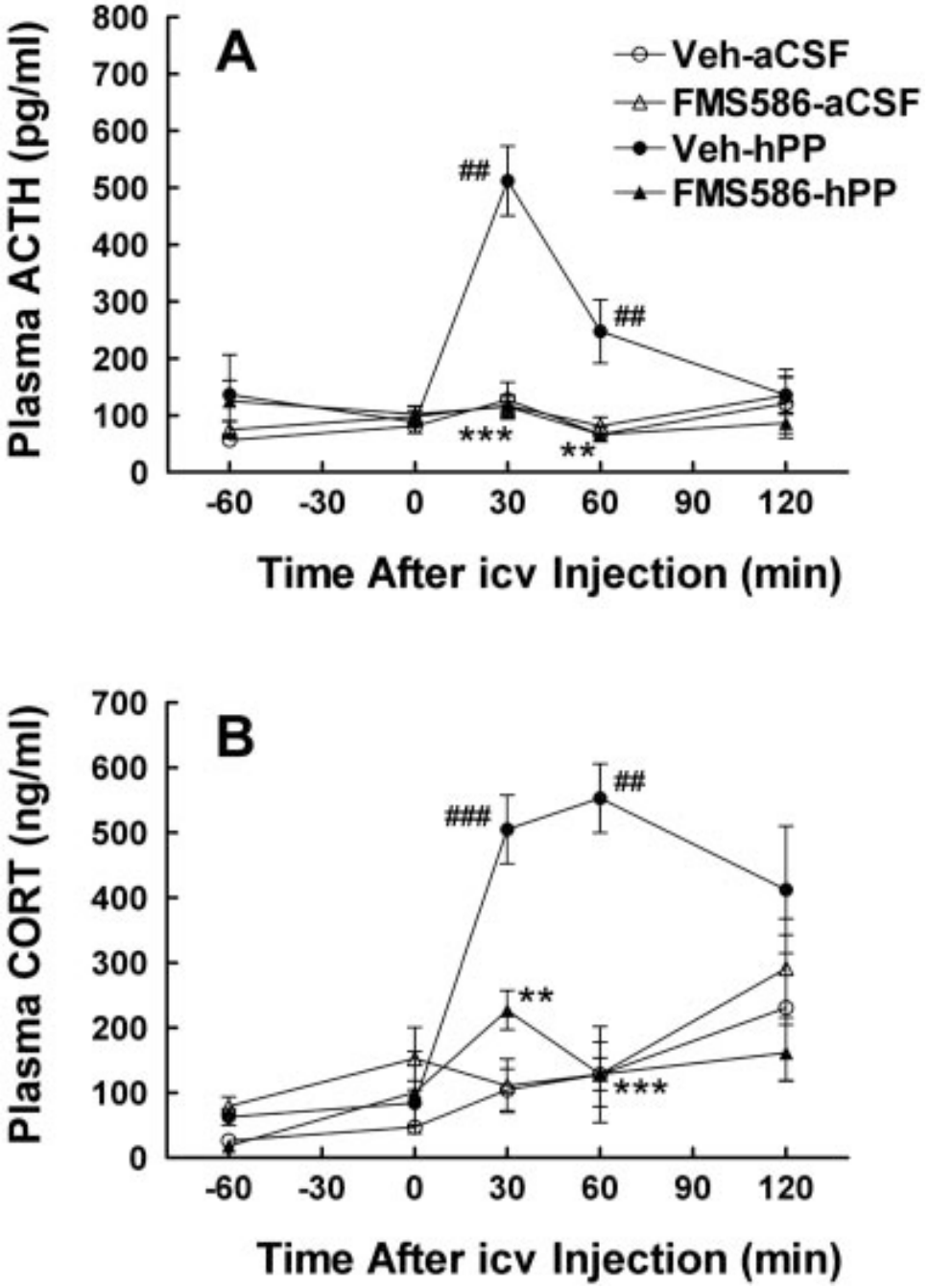

Figure 5. Effect of Y5 selective agonist (hPP, 100 pmol, icv) and/or Y5-selective antagonist (FMS586, 25 mg/kg, po) on plasma ACTH (A) and CORT (B) levels in conscious male Fisher rats (Kakui and Kitamura, 2007).

J. Blockers of the VT4R/V1aR

An in vitro study was conducted in which antagonists were screened to map their potential binding sites to the VT4R (Jayanthi et. al., 2014) (Figure 8). The four antagonists screened were SR-49059 (Figure 9), OPC-21268, H-6722, and H-5350 (Manning compound) (Figure 10). Based on this study, the non-peptide antagonist, SR-49059 showed the strongest binding affinity to the pocket or binding site of the VT4R, based upon 3D modeling of the VT4R. Importantly a high agreement was shown between the modeling study and results of an in vitro experiment using primary pituitary cells and monitoring the expression of POMC hnRNA after application of each to the four receptor blockers and a cocktail of CRH and AVT that simulated a stress response. The peptide antagonist, Manning compound showed the weakest binding affinity to the VT4R. However, in avian studies, the Manning compound is widely used as a VT4R antagonist, and SR-49059 is more widely used in mammalian studies as a V1aR antagonist (Serradeil-Le Gal et. al., 1993). The VT4R is known to be homologous to the vasopressin receptor 1a (V1aR) in mammals. In a study done in 2009, V1aR deficient mice had a greater food intake compared with the wild type mice that had functional V1aRs (Figure 6; Aoyagi et. al., 2009). The mice also exhibited hyperglycemia and hyperleptinemia. Based on their results, it was concluded that AVP reduced food intake when compared to saline controls (Figure 7), and the orexigenic effect of NPY was even more enhanced in the V1aR deficient mice than in the wild-type mice. Additionally, when a V1aR antagonist was centrally administered, the food intake of the wild-type mice greatly increased food intake as well. The results suggest that AVP could suppress the NPY-induced orexigenic effect via the V1aR, and that blockade or inhibition of the AVP and V1aR signal resulted in enhanced NPY-induced food intake. Therefore, AVP and the V1aR appear to be involved in appetite regulation as an

anorexigenic factor for the NPY-induced orexigenic effect. Considering that the avian VT4 and mammalian V1a receptors are regarded as homologous (Selvam, et.al., 2013), appropriate VT4R antagonists could increase food intake in an avian species by blocking AVT binding to the VT4R.

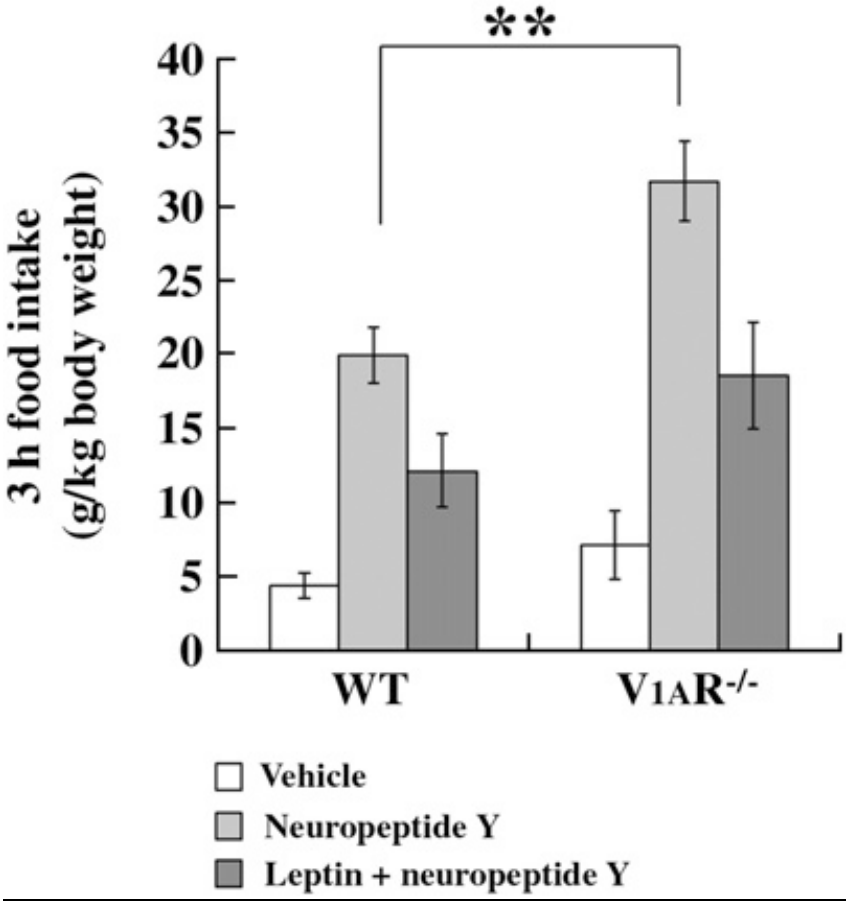

Figure 6. Alteration of neuropeptide Y-induced food consumption in V1aR−/− mice. WT and V1aR−/− mice were treated with i.c.v. administration of the vehicle (0.9% NaCl), 300 pmol/body of neuropeptide Y, or neuropeptide Y plus 300 pmol/body of leptin under the same feeding conditions. Food consumption for 3 h was measured after administration and expressed as the ratio of the amount of food intake (g)/body weight (kg). Values are the mean±S. E.M. **$\mathbf{P}$<0.01 vs. WT mice, determined by the unpaired Student's **t**-test (Aoyagi, et. al., 2009).

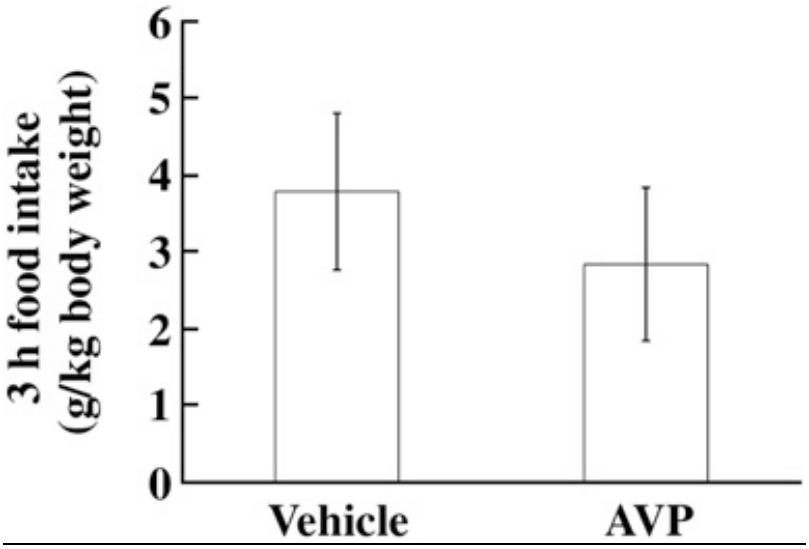

Figure 7. Effect of AVP treatment on food intake in WT mice. WT were treated with i.c.v. administration of the vehicle (0.9% NaCl), or 300 pmol/body of AVP under similar feeding conditions. Food consumption for 3 h was measured after administration and expressed as the ratio of the amount of food intake (g)/body weight (kg). Values are the mean ±S. E. M. Note that with such a large SEM, there is no difference between the two groups of mice. These data are not showing that AVP significantly reduced food intake in mice (Aoyagi et. al., 2009).

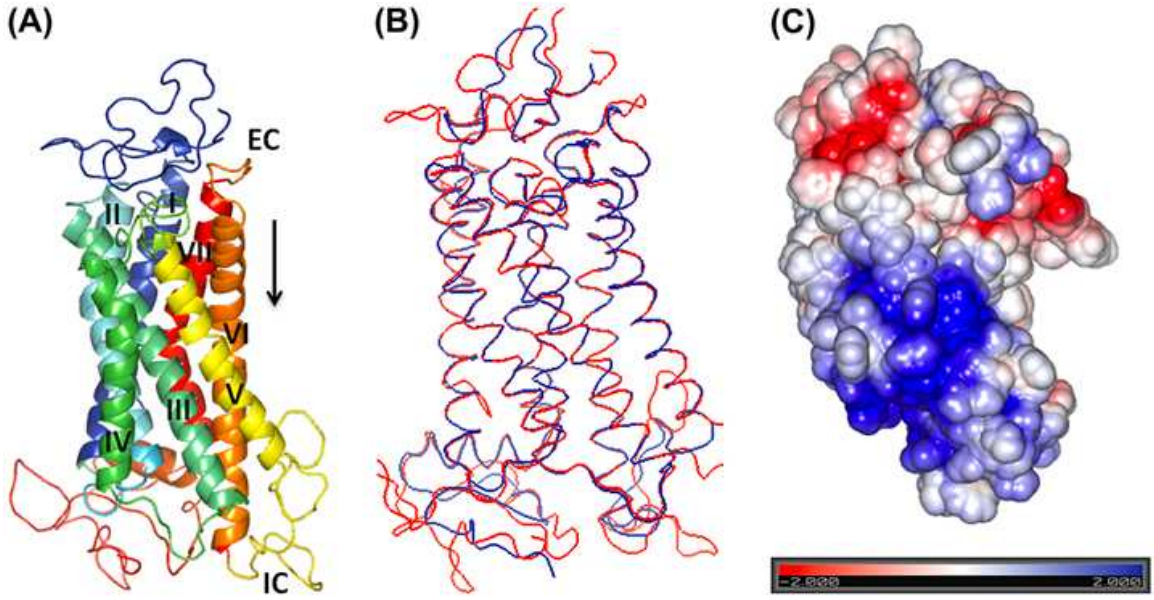

Figure 8. A 3D homology structure of the VT4R (A) Homology Model built using the template 1JFP/1U19 (bovine rhodopsin). Seven transmembrane helices (TM-I-VII), each shown with a different spectral colour are labelled with Roman numbers. EC – Extracellular side and IC -

intracellular side of the receptor. (B) Superimposition of the template (1U19) shown in red and target (VT4R) shown in blue structures are represented by ribbon diagram. (C) Electrostatic potential map of VT4R positively and negative charged residues are represented in blue and red, respectively (Jayanthi et.al., 2014).

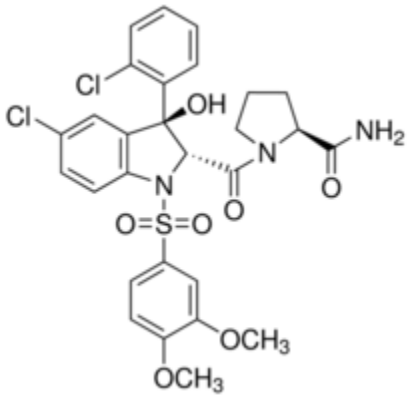

Figure 9. Structure of SR-49059. Molecular weight is 620.50 (Jayanthi et. al., 2014).

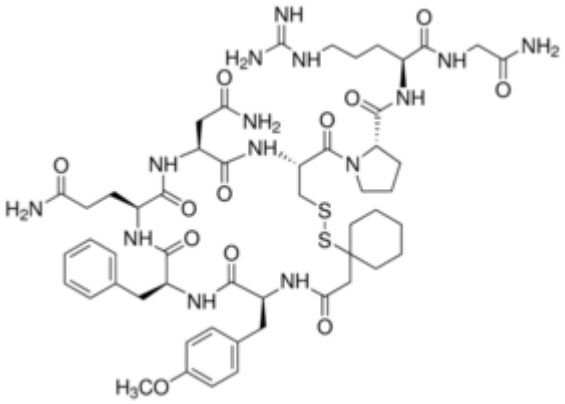

Figure 10. Structure of the Manning compound. Molecular weight is 1151.36 (Jayanthi et. al., 2014).

K. Hypothesis/Objectives

The first objective of the study was to determine whether NPY administration in broilers not only increases food intake but also increases plasma levels of the stress hormone, corticosterone.

A second objective was to ascertain whether effective blockers of the avian VT4R, homologous to the mammalian V1aR would effect a greater increase in food intake compared to NPY administration alone.

Our hypothesis was that administering NPY and reputed specific blockers of the avian VT4R/V1aR could be a useful *in vivo* procedure for screening the effectiveness of potential V1aR blockers to inhibit the avian V1aR based upon the augmented food intake measured compared to NPY administered alone.

II. Materials and Methods

A. Facilities and Animals

Male broiler chicks were obtained from a commercial hatchery on day one of hatch. Chicks were raised in battery brooder cages for their first 2 weeks and thereafter were randomly distributed to cages (two per cage). Environmental temperature was set at 32ºC from the day of hatching and was dropped 3ºC per week to reach approximately 23ºC, where it was maintained until the end of experiments. Birds were fed a standard broiler diet of chick starter feed ad libitum and maintained with a photoperiod of 16 h of light and 8 h of darkness each day. All of the procedures and experimental protocols for use in chickens were approved by the University of Arkansas Institutional Animal Care and Use Committee (Appendix B).

B. Cannulation

At three weeks of age the birds were deeply anesthetized and their heads positioned in a stereotaxic instrument to implant a guide cannula (Plastics One, Roanoke, VA) at the following

coordinates (1.0 mm anterior to the lambda suture mark on the skull and +0.8 mm lateral to midline) to target the dorsal region of the lateral ventricle in order to perform intracerebroventricular (ICV) injections. The depth of the guide cannula was 3.0 mm from the dorsal reading of the skull. Birds were allowed to recover post-surgery for at least 2 days prior to being tested for cannula position using the angiotensin II drinking response (Maney and Wingfield, 1998; Takei 1977). This drinking response is done to test the cannula position. Confirmation can be seen if the birds go to water within two minutes after injection of angiotensin II. At least two days were allowed after angiotensin II injections to begin experiments. All of the methods and materials were approved by the University of Arkansas Institutional Animal Care and Use Committee.

C. Immobilization stress

Chicks underwent immobilization stress for one hour following ICV injections. Immobilization stress included wrapping the birds in a harness to prevent wing movement and prevent standing, while still having full access to water. This immobilization stress is considered an acute stress. Control birds were placed back in their home cage and did not undergo immobilization stress.

D. VT4R/V1aR Antagonists and NPY

Antagonists used in this study were SR-49059 and H-5350 (Manning compound). The compounds were decided upon based on a previous *in vitro* cell culture study and 3-D modeling of the avian V1aR (Jayanthi et al., 2014). Doses were determined based on prevalence and effectiveness found in the literature review. Both antagonists were obtained from Sigma Aldrich

or Bachem, depending on availability of the compounds.

E. Data collection

Immobilization stress experiment, avian blockers and food intake

In all experiments, birds were randomly selected for each treatment group. Only birds that met the requirements following the angiotensin II response method were used for this study. The first experiment included four treatment groups: saline, saline with immobilization stress, SR-49059 (250ng or 4.03×10^{-10} mol) with immobilization stress, and Manning compound (250ng or 2.17×10^{-10} mol) with immobilization stress. The total number of birds per treatment were n=8. Immobilization stress was performed over a 30 minute period. One of the blockers or saline was first administered ICV and the bird was quickly secured in a harness that prevented the bird from moving its wings or standing. After 30min of immobilization, the bird was released from its harness and a blood sample was taken within two minutes from its brachial vein. The heparinized blood sample was either placed immediately on ice or in a refrigerator until all blood samples were collected for that day.

Each bird was then placed back in their cage in order to determine individual food intake for one hour (g/hr). This allowed a determination of the effect of each antagonist on food intake in comparison to that of the saline control group. The four treatments compared included a saline only (n=8), and three groups restrained and given either SR-49059 (250ng, n=8), Manning compound (250ng, n=8) or saline ICV.

NPY experiment, avian V1aR blockers and food intake.

A dose-response experiment was conducted with NPY to determine an optimum NPY dosage. The objective was to determine the dose of NPY that would consistently stimulate food

intake significantly greater than a control group given saline. It was important not to induce a maximum food intake response, since the goal was to determine the interaction of NPY with each of the avian V1aR blockers that were found to reduce the stress response of birds subjected to a psychological stressor, immobilization. The initial dosage selected was based on a previous study in which 9µg was shown to induce a near maximum food intake response in broiler chicks (Kuenzel, et.al., 1987). The treatment groups were saline (n=6), 1µg NPY (n=6), 3µg NPY (n=6), 4µg NPY (n=4), and 7µg NPY (n=6). Food intake (g) was recorded for one hour immediately following ICV administration.

Once the initial experiments with NPY were completed to find the appropriate dose of NPY, a food intake study was designed to examine the interaction of NPY with the effective V1aR antagonists. The treatment groups include: saline alone, saline and NPY (4µg), SR-49059 (250ng) and NPY (4µg), and the Manning compound (250ng) and NPY (4µg).

Lastly, blood samples were collected from birds injected with saline, saline and immobilization stress, SR-49059 (250ng), SR-49059 (250ng) and immobilization stress, NPY (4µg), and NPY (4µg) and immobilization stress. This was done to determine the level of stress being inflicted or blocked from the injection of these compounds.

Immediately following ICV injections, birds from each of the four treatment groups were returned to their cages and food intake determined for one hour. Thereafter, birds were removed from their cages, blood samples were taken using syringes with heparin and their feeders weighed to determine food intake. All blood samples taken were immediately cooled either on ice or placed in a refrigerator.

At the end of each sampling day, blood samples were taken back to the laboratory and centrifuged at 1200 g. Plasma was removed and frozen at -80C until assayed for corticosterone

utilizing a radioimmunoassay.

F. Radioimmunoassay

Plasma samples from the immobilization stress and NPY plus blocker experiments were quantified for corticosterone (CORT) by radioimmunoassay (RIA) (Madison et. al., 2008). Blood samples were taken following 30-minute immobilization stress, or (in non-immobilized groups) was taken 30 minutes following ICV injections. All samples were assayed in duplicate. The primary antibody for CORT was purchased from Fitzgerald Inc. (Concord, MA, USA). The secondary antibody and I^{125} tracer were purchased from MP Biomedicals Inc. (Orangeburg, NY, USA).

G. Statistical analysis

One-way ANOVA was used to determine a level of significance among treatment groups. LSD was used to determine differences among the means. All data are presented as mean ± SEM and significance level utilized was $p < 0.05$.

III. Results

Our objective in this study was to examine the possible interaction of the stress hormone, corticosterone (CORT), and food intake. Recent studies showed that an effective mediator of the neuroendocrine hypothalamo-pituitary-adrenal (HPA) axis was an avian vasotocin receptor, the VT4R (Kuenzel et al., 2013; Selvam et al., 2013). Four blockers of the homologous receptor of the avian VT4R, the mammalian V1aR were found efficacious in inhibiting the expression of the VT4R *in vitro*, at the level of the anterior pituitary (Jayanthi et al., 2014). We wished to determine whether those receptor blockers might also be effective in the live animal. The first experiment examined how effective the top ranked blocker, SR49059 and a second blocker utilized in several past avian studies, the Manning compound, would be at decreasing the level of plasma CORT following 1h of a psychogenic stressor, immobilization. Results showed that indeed both the Manning compound and SR49059 reduced released CORT, however, only the SR49059 data were significantly reduced CORT levels ($p < 0.05$; Fig. 1).

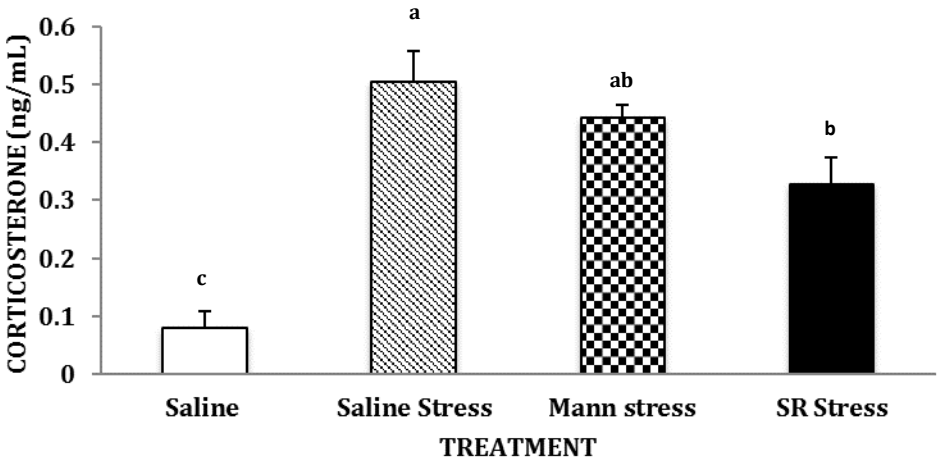

FIGURE 1. Corticosterone levels in controls and immobilized birds with and without VT4R/V1aR Blockers. Intracerebroventricular injections of the Manning compound (250ng/4µL), SR49059 (250ng/4µL) or saline (4µL). Histograms show means and error bars indicate + standard error of the mean (SEM). Letters indicate significant differences ($p < 0.05$). Sample size, n = 8 birds/trt.

A. Establishing a dose for SR49059

A review of literature indicated that SR49059 was mainly used for mammalian studies, whereas the Manning compound was used more in avian studies (Goodson et. al.,2004; Goodson et. al. 2005). The concentration of the SR49059 utilized in our first stress study (250ng) was based upon experiments performed primarily in rodents (Stojicic et. al., 2008; Milutinovic-Smiljanic et. al., 2013). The concentration of SR49059 was not consistent throughout mammalian studies. Although the level worked well, no previous studies were found where this particular blocker was utilized in avian species. Therefore a dose-reponse study was performed to ascertain whether a lower dose would also be effective prior to initiatiating the planned food intake experiments utilizing both VT4R blockers. The preliminary study utilizing a dose of NPY known to stimulate food intake in chickens coupled with two concentration levels of the SR49059 blocker was performed. Results obtained are shown in Fig. 2.

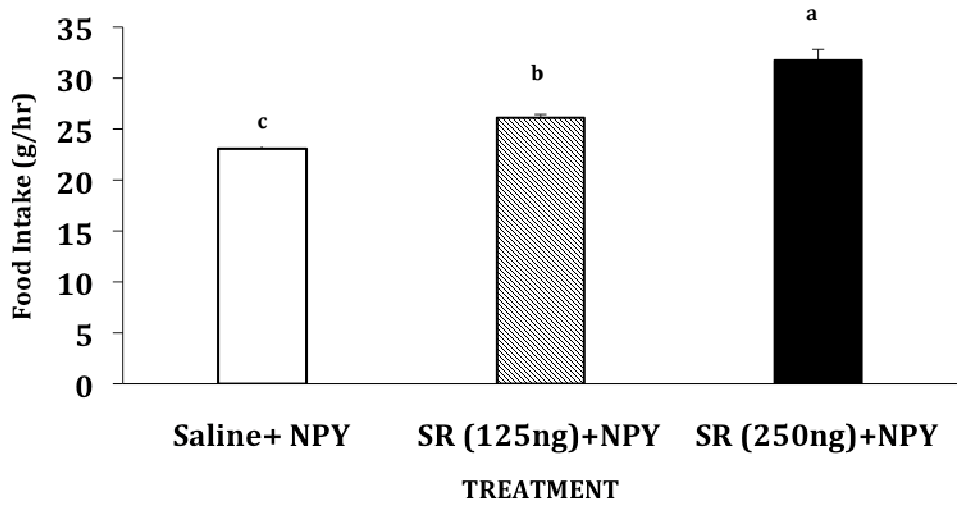

FIGURE 2
Food intake (g/hr) in male broilers injected ICV with SR49059 (125ng/4u) and NPY (4ug/4uL), SR49059 (250 ng/4uL) and NPY (4ug/4uL), or saline (4uL) and NPY (4ug/4uL). Histograms show means and error bars indicate + standard error of the mean (SEM). Letters indicate significant differences ($p < 0.05$). Sample size, n = 4 birds/trt.

From these results, we concluded that the 250ng SR49059 group increased food intake the most and was significantly higher in doing so than the other two groups. As expected, the lower dose of SR49059 showed less of an impact on food intake compared to the saline+NPY group. Because all individual birds within the higher dose (250ng) treatment group showed consistently higher food intake compared to the NPY controls, we chose to use that dose in our research. Based on studies done by Goodson et. al. (2004; 2005) in birds, there is much evidence to show that 250ng of the Manning compound is an optimum dose to use for that blocker.

B. Establishing a dose for neuropeptide Y (NPY)

Neuropeptide Y is still regarded as one of the most effective orexigenic compounds that occurs naturally in vertebrates (Kuenzel et al., 1987). It was important to find a dose that stimulated food intake, however, not maximally in the event that blocking the avian V1aR might

actually augment the effectiveness of this compound. A dose-response experiment was therefore designed utilizing 1µg, 3µg or 7µg of NPY each given in a volume of 4µL. A control group was given 4µL of saline.

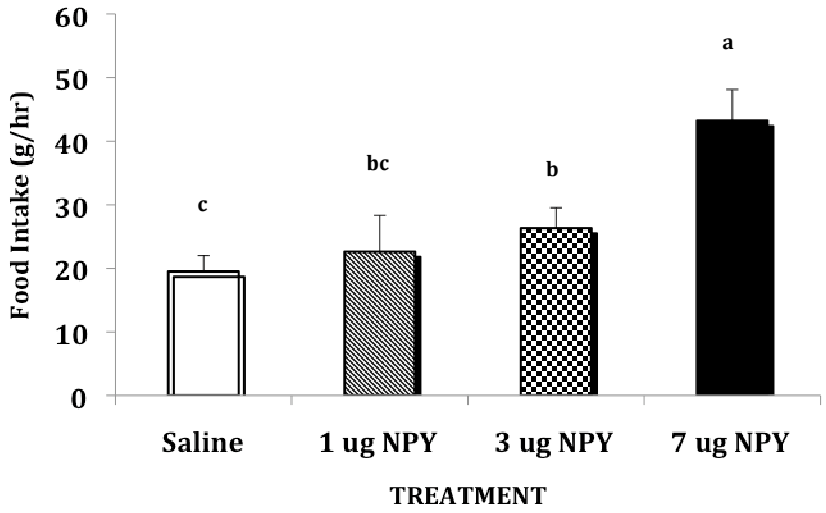

FIGURE 3a

a) Food intake (g/hr) in male broilers (n=6 birds per treatment) injected ICV with 1 µg neuropeptide Y (NPY), 3 µg NPY, 7 µg NPY, or saline (control). (b) Whiskers indicate +standard error of mean (SEM). Different letters above columns indicate significant differences ($p<0.05$).

Following ICV injection of NPY showed that all three doses (1µg, 3µg, and 7µg) were significantly different from the saline control group. They all significantly increased food intake compared to the control. The 1µg and 3µg groups were not significantly different from one another, however, the 3µg group did stimulate increased food intake when compared to the 1µg group. The 7µg group showed the highest food intake and more than doubled food intake

compared to the saline controls. Because there was such a difference on the 3µg and 7µg groups, we chose to test a 4µg of NPY group to compare food intake with the 3µg and 7µg groups. This was done so that we could use an NPY dose that was not to full capacity and would still be able to increase food intake if the addition of the ICV injection of the VT4R antagonists augmented the effects of NPY.

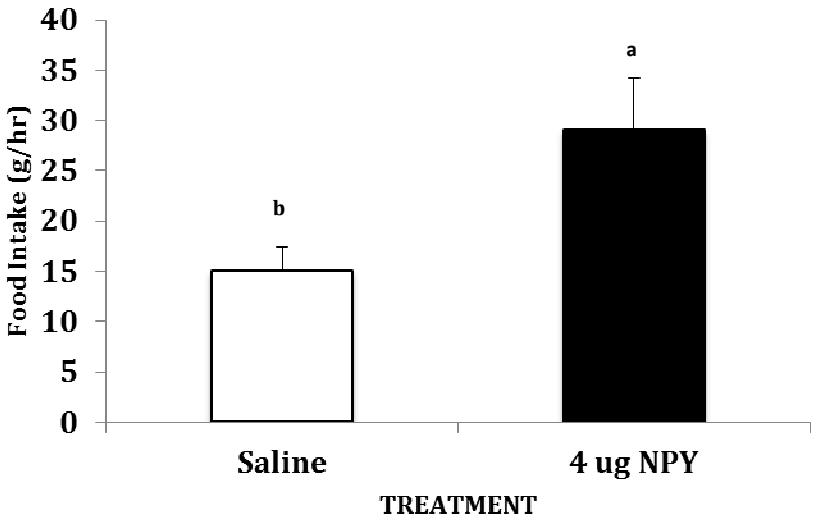

FIGURE 3b

(a) Food intake (g/hr) in male broilers (n=4 birds per treatment) injected ICV with 4 µg neuropeptide Y (NPY) or saline (control). (b) Error bars indicate +standard error of mean (SEM). Different letters above columns indicate significant differences ($p<0.05$).

C. Testing the blockers ability to increase food intake

The next step was to determine whether any of the two blockers of the avian V1aR used in the first experiment affected food intake when administered alone. Each of the two inhibitors of the vasotocin/vasopressin receptor subtype VT4R/V1aR was administered intracerebroventricularly (ICV) into the lateral ventricle of the chick brain and food intake was

measured over a one-hour period. The two blockers, SR-49059 and Manning compound, both significantly increased food intake when administered individually via ICV injections compared to saline injected controls (Figure 4).

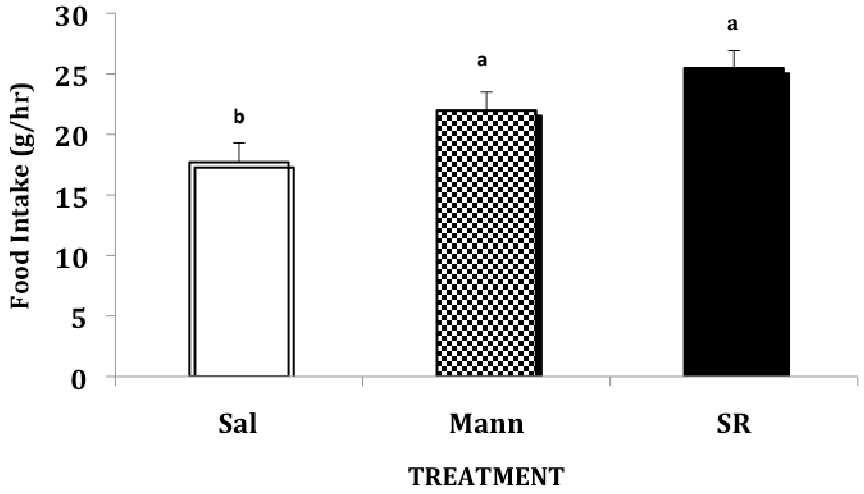

FIGURE 4.
Food intake (g/hr) in male broilers (n=8 birds per treatment) injected ICV with 250 ng SR49059, 250 ng Manning Compound, or saline (control). (b) Whiskers indicate +standard error of mean (SEM). Different letters above columns indicate significant differences ($p<0.05$).

The doses of each were 250ng per bird. The group injected with SR-49059 increased food intake more than the Manning group, however, the SR-49059 was not significantly higher than the Manning group. Both antagonist groups showed significantly higher food intake when compared to the saline control group ($p < 0.05$). A table (Table 1) expressing the food intake data in g/ kg body weight can be found in Appendix 1.

D. Testing effects of NPY on food intake when coupled with blockers of the avian V1aR

The next study compared the interaction of ICV injection of NPY and each antagonist regarding their ability to increase food intake compared with an ICV injection of NPY alone. As expected, the saline control group showed a significantly lower food intake than the other groups (Fig. 5).

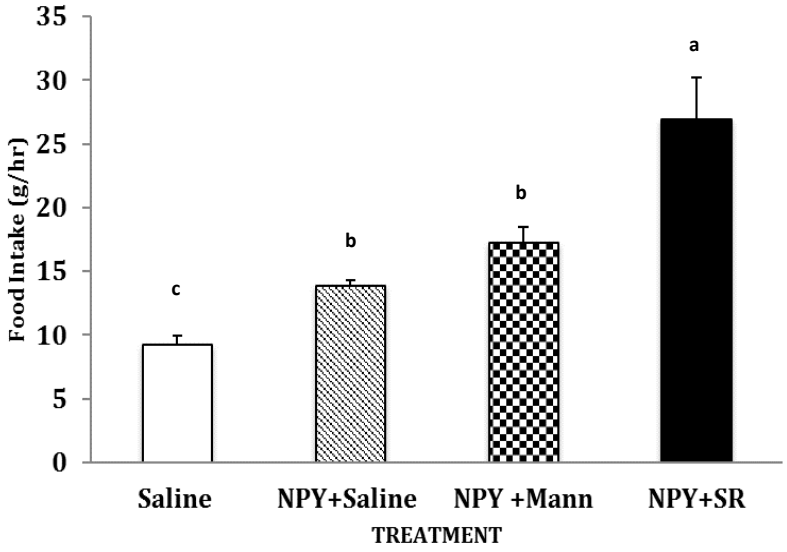

FIGURE 5

(a) Food intake (g/hr) in male broilers (n=8 birds per treatment) injected ICV with 250 ng Manning Compound and 4 µg neuropeptide Y (NPY), 250 ng SR49059 and 4 µg NPY, saline and 4 µg NPY, or saline (control). (b) Whiskers indicate +standard error of mean (SEM). Different letters above columns indicate significant differences ($p<0.05$). (This figure corresponds with Table 4 in Appendix A.)

Tables 1-5, expressing food intake on the basis of grams of intake/kg body weight can be found in Appendix A.

The NPY alone and NPY and Manning compound groups were not significantly different. However, overall the NPY and Manning compound group did show greater food

intake than the NPY alone group. The NPY and SR-49059 group showed a significant increase in food intake when compared to the other three groups.

E. The effect of the SR49059 V1aR blocker and NPY on Plasma Corticosterone in Unstressed and Stressed Birds

Since the SR-49059 group was able to reduce CORT concentrations lower than the saline + stress and Manning compound + stress groups (Fig. 1) and the SR-49059 compound coupled with NPY showed the highest food intake response (Fig. 5), we chose to examine the CORT concentrations among saline, saline + stress, SR-49059, SR-49059 + stress, NPY, and NPY + stress groups. In the three groups that were not stressed (Fig 6a), the lowest CORT concentrations were the saline control and the SR-49059 control groups, with no significance between the two. However, they are both significantly different from the unstressed group given NPY (Fig. 6a). Among the three groups that were stressed (Fig. 6b), the saline control group and the NPY had the highest plasma concentrations of CORT and they were not significantly different from each other. In contrast, the birds administered SR-49059 and then stressed showed significantly lower CORT levels than the saline and NPY groups that were subjected to immobilization stress (Fig. 6b). Of interest, the SR49059 group + stress displayed CORT levels not different from the unstressed birds given NPY alone (Fig. 6).

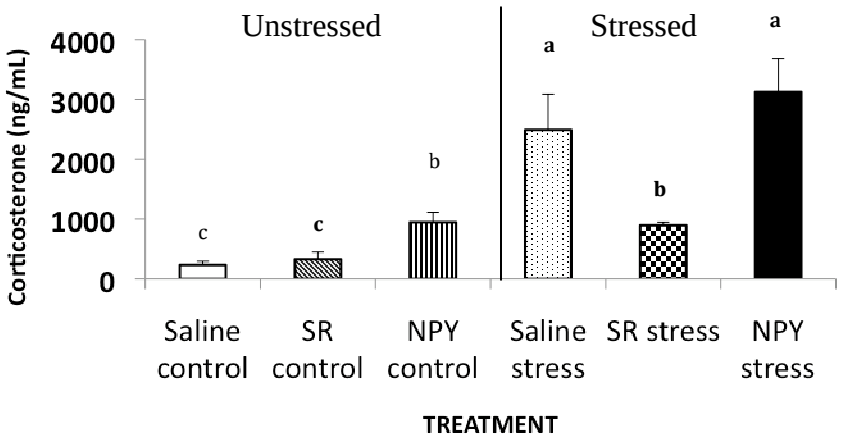

FIGURE 6a and 6b
(a) Plasma corticosterone (ng/ml) in males without and with immobilization stress following central administration of saline, SR49059 or NPY.
n=8 birds per treatment; injected ICV with 250 ng SR49059, 4µg of neuropeptide Y (NPY), or saline (control). (b) Whiskers indicate +standard error of mean (SEM). Different letters above columns indicate significant differences ($p<0.05$).

IV. Discussion

Here we confirm that antagonists of the VT4 receptor (VT4R) reduce stress-induced hypothalamic-pituitary-adrenal (HPA) axis responses as measured by corticosterone (CORT) concentrations. From these studies we have shown that there is a relationship between NPY and the stress response in part via the VT4 receptor in chicks. We were able to see a decrease in CORT levels via the SR-49059 and the Manning compound VT4R antagonists, as well as see an increase in food intake with the co-administration of NPY and the blockers. There could also have been a relationship between the other vasotocin and corticotropin releasing hormone receptors and would be worth looking into in future studies. However, based on the results from this study, there is evidence to conclude that the AVT receptor VT4R and NPY are related in terms of food intake. These results follow with previous studies that there is a relationship between NPY and the HPA axis (Uehara et. al., 1998; Mastorakos and Zapanti, 2004). There have also been studies that state that AVT reduces food intake in chicks (Tachibana et. al., 2013). Previous central injection studies have shown that when NPY is injected into the paraventricular nucleus (PVN) of the brain, an increase in AVP and CORT can be seen, with a greater increase being seen in AVP (Leibowitz et. al., 1988). This leads us to conclude from the study that NPY is able to stimulate AVP possibly via a vasopressin receptor. From the current study, we showed that central injection of NPY with a VT4R antagonist was able to significantly increase food intake above the level of NPY alone. This leads us to conclude that NPY is acting via the VT4R to achieve maximum food intake. However, food intake could possibly be increased even more by using antagonists to other vasotocin receptors as well as the VT4R. The antagonists alone were also able to increase food intake more than saline alone. This backs up the data that shows

an increase of food intake with the antagonists in unison with NPY. However, when the antagonists were administered and a restraint stress was induced, a decrease in CORT was seen, which we would expect based on previous studies (Nagarajan et.al., 2014). Based on the results from this study, it appears that NPY and AVT are both activated when augmentation of food intake occurs. Hence, when the negative effects of AVT on food intake are blocked by antagonists, the positive effects of NPY were shown to be enhanced.

Based on the stress data shown from this study, injection of NPY in combination with stress increased CORT when compared to the stress alone group. This is an interesting finding. These data further suggest that NPY not only plays a major role in food intake, but also has (or can have) involvement in the stress response via the VT4R and CORT. However, NPY could also be acting on CRH (Ida et. al., 2000) and should be further investigated to get a better grasp of this phenomenon. AVP and CRH have been shown to reduce food intake in mammals (Leibowitz et. al., 1988; Arase et. al., 1988) as well as in chicks (Tachibana et. al., 2013; Denbow et. al., 1998). In addition, elevated NPY and reduced CRH gene expression were found to restore food intake (Brady et. al., 1990), and both NPY and CRH are thought to both mediate the anorexic effect of leptin (Uehara et. al., 1998). These studies, as well as the data collected here show that there is some sort of balancing mechanism going on between CRH and AVT with NPY in regards to the HPA axis and food intake. With respect to CORT release, it is expected that if AVP or AVT is secreted there will in turn be a release of CORT. However, it is interesting to note that NPY also increases CORT. This leads to the idea that CORT may be either antagonizing or working with NPY. It is a possibility that since the VT4R antagonists used in this study seem to work with NPY to increase food intake (and AVT is known to

decrease food intake) the antagonists seem to, in some way, overcome the interaction between CORT and NPY thereby optimizing the effects of NPY.

Based on previous studies suggesting that birds use fat for energy more so than sugar, there could be an interaction between circulating lipid levels and NPY. When a bird is stressed their plasma triglycerides are decreased (Hershock and Vogel, 1988). Likewise, administration of ACTH elevates CORT levels in domestic fowl, which leads to increased fatty acids (Heald et. al., 1965). This will lead to a decrease in food intake and therefore could be an underlying reason that this is happening possibly via NPY and AVT and CORT. In addition, obesity in rats has been linked to an increase in NPY mRNA and NPY release resulting in increased glucocorticoids (Strack et. al., 1995). Importantly, the hypothalamic NPY-induced feeding response has been shown to be largely dependent upon circulating CORT levels (Stanley et. al., 1989). This further supports evidence that there is a fundamental metabolic interaction between these compounds.

More specifically, the current data suggest that there is a link between NPY and the VT4 receptor, not just a relationship between AVT, CORT and NPY. The mammalian homolog to the VT4R, V1aR, has been studied for both food intake and stress data in mammalian studies. V1aR deficient mice had a greater food intake compared to wild-type mice that had functional V1aRs and exhibited hyperglycemia and hyperleptinemia (Aoyagi et. al., 2009). They also found that AVP reduced food intake and that the orexigenic effect of NPY was even more enhanced in the V1aR deficient mice. In addition, when a V1aR antagonist was administered via ICV injection, food intake in the wild-type mice also greatly increased. This truly shows a direct relationship between AVP and NPY, which is supporting data found here in the current study. Hence in a broiler chick naturally stimulated to eat, its AVT may normally function to suppress the full

effects of NPY, while any blockade of AVT function would result in an enhanced NPY-induced food intake.

As shown in the present study as well as in a previous studies (Jayanthi et. al., 2014), the VT4R antagonist SR-49059 was shown to block or decrease AVT more effectively than the Manning compound. The previous data were based on a 3D modeling experiment, showing that SR-49059 had the highest binding affinity to the VT4R of the four antagonists screened. On the other hand, the Manning compound showed the weakest binding affinity of the four. The binding affinity data were in agreement with a previous in vitro study using pituitary cells. The current in vivo study using the same two antagonists supports the previous findings a and provides new findings that the SR-49059 was more effective than the Manning compound for enhancing feeding and decreasing stress-related CORT release. Hence, the procedure utilized in this study could be used as an effective technique *in vivo* to screen antagonists for their efficacy in blocking the avian VT4R.

Therefore, results herein support our hypothesis that effective blocking of the avian VT4R/V1aR would reduce CORT as well as enhance food intake following administration of NPY. Further, we discovered that the antagonist, SR-49059, was more effective at decreasing CORT and increasing food intake than the Manning compound, which were in agreement with the previous modeling and in vitro studies. From this study as well as those previously cited, there appears to be a strong antagonism between NPY and CRH as well as an antagonism between AVP/AVT and NPY. There are more studies showing an antagonism of CRH and NPY than AVP/AVT and NPY. From this study, it is obvious that a strong relationship of NPY to the HPA axis exists, especially in regards to AVT and the VT4R. Future studies should be done to test more blockers as they are synthesized to see how effective this in vivo technique is at

screening blockers, as well as to look more deeply into the difference in the CRH and NPY relationship and the AVP/AVT and NPY relationship. Future studies may also want to look into the effects of antagonism of the VT4R when a repeated restraint stress is applied. This study was done using an acute stress consisting of 1h of restraint. There may be an attenuated mechanism that occurs where the birds do not show as high levels of CORT after injected with NPY, and may show higher levels of CORT after injected with a blocker. In addition to these suggestions, it may also be worthwhile to look into the avian CRH receptors and other AVP/AVT receptor blockers to look more deeply into the effects of these blockers with NPY in regards to food intake. With these studies (besides measuring CORT), other glucocorticoids/mineralocorticoids, NPY, AVT, and brain samples should be taken to get a better idea of interactions between NPY and the HPA-axis regarding stress and food intake.

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VI. Appendix A.

TABLE 1. Food Intake for 1 Hour Following ICV Administration of Antagonists

Treatment	F.I. (g/kg of [1]B.W.)	n	SEM
Saline[2]	9.0[a]	8	1.6
SR49059	13.0[b]	8	1.52
Manning	11.5[b]	8	1.43

The saline group is significantly different than the antagonists group. SR49059 and the Manning Compound are not significantly different. Differences based on $p<0.05$.
[1]Abbreviations: BW = body weight; FI = Food Intake; ICV = intracerebroventricular injections;
[2] Sterile physiological saline. All ICV injections were 4µL in volume; SR49059 (250ng); Manning compound (250ng).

TABLE 2. Food Intake for 1 hour following ICV administration of saline and NPY (4µg), SR49059 (125ng) and NPY (4µg), or SR49059 (250ng) and NPY (4µg)[1]

Treatment	F.I. (g/kg of B.W.)	n	SEM
Saline[2]+NPY (4ug)	8.5[c]	4	0.48
SR(125ng)+NPY (4ug)	10.0[b]	4	0.37
SR(250ng)+NPY(4ug)	11.9[a]	4	0.29

All three groups showed to be significantly different from one another. Differences based on $p<0.05$.
[1]Abbreviations: BW = body weight; FI = Food Intake; ICV = intracerebroventricular injections;
[2] Sterile physiological saline. All ICV injections were 4µL in volume;

TABLE 3. Food Intake for 1 hour Following ICV Administration of Saline or NPY at one of 3 doses[1]

Treatment	F.I. (g/kg of B.W.)	n	SEM
Saline[2]	8.4[c]	6	2.5
1 ug NPY	10.6[bc]	6	5.7
3 ug NPY	13.2[b]	6	3.4
7 ug NPY	18.25[a]	6	4.9

The saline group is significantly different from both the 3µg and 7µg NPY groups. The 1µg of NPY group is only different from the 7µg group, and the 7µg NPY group is significantly different from all of the other groups. Differences based on $p<0.05$. [1]Abbreviations: BW = body weight; FI = Food Intake; ICV = intracerebroventricular injections;
[2] Sterile physiological saline. All ICV injections were 4µL in volume;

TABLE 4. Food Intake for 1 hour following ICV administration of saline or 4ug of NPY[1]

Treatment	F.I. (g/kg of B.W.)	n	SEM
Saline[2]	5.7[b]	4	2.37
4 ug NPY	10.4[a]	4	5.24

The two groups are significantly different from one another. Differences based on $p<0.05$.
[1]Abbreviations: BW = body weight; FI = Food Intake; ICV = intracerebroventricular injections;
[2] Sterile physiological saline. All ICV injections were 4µL in volume;

TABLE 5. Food Intake 1 hour following ICV administration of saline, NPY (4µg) and saline, NPY (4µg) and Manning Compound (250ng), or NPY (4µg) and SR49059 (250ng)[1]

Treatment	F.I. (g/kg of body weight)	n	SEM
Saline[2]	5.1[c]	8	0.77
NPY+Saline	8.0[b]	8	0.43
NPY+Manning compound	9.0[b]	8	1.25
NPY+SR49059	14.5[a]	8	3.23

The saline and NPY+SR49059 groups are significantly different from the other three groups. There is no difference between the NPY+Saline and the NPY+Manning groups. Differences based on $p<0.05$.
[1]Abbreviations: BW = body weight; FI = Food Intake; ICV = intracerebroventricular injections;
[2] Sterile physiological saline. All ICV injections were 4µL in volume; SR49059 (250ng); Manning compound (250ng); NPY (4µg).

VII. Appendix B.

UNIVERSITY OF
ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Wayne Kuenzel

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: February 5, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date: **February 3, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13030- "Neuroendocrine studies addressing stress, reproduction and behavior in poultry".** You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **02-03-2016**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian