Hypothalamic mechanisms of appetite regulation involve stress response and epigenetic modification

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Abstract (Academic)

Appetite regulation is primarily mediated by the hypothalamus, within which many neurotransmitters that regulate feeding are shared by the stress response circuitry. Stressors, especially those occur during critical periods of life, influence epigenetic programming and gene expression in the long-term. Therefore, the aim of this dissertation was to elucidate how hypothalamic mechanisms of appetite regulation correlate with the stress response and epigenetic modifications, using avian models and intracerebroventricular administration of various appetite-regulating factors. We first administered two methylation modifiers, S-adenosylmethionine (SAM), a methyl donor, and 5-azacytidine (AZA), a methylation inhibitor, to determine their effects on appetite. When measuring food intake immediately post-injection, SAM didn't affect fed or fasted chickens from a line selected for low bodyweight (LWS, individuals with anorexia), but suppressed feeding in fed and fasted broilers. In Japanese quail, SAM transiently induced satiety in fed but not fasted chicks. Intriguingly, AZA increased feeding in fasted LWS but decreased it in fed chicks. While it didn't affect either fed or fasted broilers, AZA induced satiety in both fed and fasted quail. These results suggests that SAM/AZA can directly affect appetite depending on genetics and nutritional state. The LWS chickens, when injected with SAM or AZA on day of hatch, didn't show increased feeding to the orexigenic stimulation of neuropeptide Y central injection on day 5 post-hatch. This suggests that epigenetic modifications occurred following SAM/AZA injection and affect appetite regulation that persisted. In other studies, we injected broilers with prostaglandin E2 (PGE2) or β-melanocyte-stimulating hormone (β-MSH) since their effects on appetite are unknown in meat-type chicks. We found that they both potently induced satiety, but the effective duration was longer in β-MSH-injected birds (up to 9 hours) than in PGE2-injected chicks (lasted for 1.5 hours). They both activated the paraventricular nucleus of the hypothalamus. The satiety induced by β-MSH mainly involved corticotropin-releasing factor and mesotocin, while the effect of PGE2 included ghrelin and brain-derived neurotropic factor. Nevertheless, all affected appetite-related factors have connections with the stress response. Thus, our results demonstrate that the hypothalamic mechanisms underlying anorexia induced by different neuroactive molecules involve the stress response and epigenetic modifications.

Abstract (Public)

Eating disorders (EDs) all involve abnormal eating behaviors and altered body weight. These aberrant conditions are associated with a change in metabolism and pose great risk to human health and animal production, and are generally characterized by two opposite outcomes, anorexia and obesity. Although affected by multiple systems within the body, appetite regulation is mainly controlled by the brain, especially the hypothalamus. Thus, it is important to understand the hypothalamic mechanisms underlying the regulation of eating behavior. In the hypothalamus, many neurotransmitters affect multiple pathways, including the stress response and those that regulate appetite. Additionally, stress, especially when occurring during early life, can influence behaviors later in life through inducing epigenetic modifications (changes to the packaging of the DNA nucleotide sequence) that alter gene expression. Therefore, the aim of this dissertation was to elucidate how hypothalamic mechanisms of appetite regulation correlate with the stress response and epigenetic modifications, using avian models. To focus on the effects within the brain, we directly injected various appetite regulating factors into the brain in each of the experiments. Previously, our group demonstrated that early-life cold exposure and delayed food supply changed DNA methylation and affected expression of appetite-related genes and food intake in a chicken line predisposed to anorexia. We herein injected chicks with one of two methylation modifiers, S-adenosylmethionine (SAM), a methyl donor, and 5-azacytidine (AZA), a methylation inhibitor, to evaluate their effects on feeding behavior. When food intake was measured immediately after injection, SAM did not affect food intake in either fed or fasted line chickens from a genetic line selected for low body weight (LWS, individuals with anorexia), but suppressed food intake in both fed and fasted broiler (meat-type chickens) chicks. In Japanese quail, however, SAM only transiently induced satiety in fed chicks but not in fasted ones.

Intriguingly, AZA increased food intake in fasted LWS chicks but decreased it in fed chicks, but AZA had no effects on food consumption in either fed or fasted broilers. Additionally, AZA suppressed food intake in both fed and fasted quail. These results suggest that SAM and AZA affect appetite differently depending on genetic background and nutritional states. LWS chickens, when injected with SAM or AZA on day of hatch, did not eat more after being injected with the potent hunger factor, neuropeptide Y, at 5 days of age. This indicates that epigenetic modifications occurred following SAM/AZA injection and had persisting effects on appetite regulation. In the other two studies, we injected broiler chicks with prostaglandin E2 (PGE2), a fatty acid-based molecule, or β-melanocyte-stimulating hormone (β-MSH), a peptide. These two molecules have been reported to regulate feeding behavior in rodents and layer-type chickens, but effects are unknown in broilers. They both potently decreased food intake in broilers, but the effective duration was much longer in β-MSH-injected birds (up to 9 hours) than in PGE2-injected chicks (lasted for 1.5 hours). They both activated the paraventricular nucleus of the hypothalamus, while β-MSH also activated the arcuate nucleus and ventromedial nucleus. We further found that the anorexia induced by β-MSH involved corticotropin-releasing factor, mesotocin, and their receptors, while the effect of PGE2 was associated with a change in ghrelin and brain-derived neurotropic factor gene expression. Nevertheless, all of these affected factors have connections with the stress response. Thus, results indicate that the hypothalamic mechanisms underlying anorexia induced by different neuroactive molecules involve the stress response and epigenetic modifications.

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Chapter 1: Introduction

Eating disorders (EDs) involve altered eating behaviors and changed body weight due to various factors including physiological, psychological, and social [1]. EDs are now prevalent all over the world, with the prevalence elevated from 3.5 % to 7.8 % during the past two decades [2]. For humans with EDs, food consuming or controlling may serve as a method to handle emotional issues, but these behaviors can easily get out of control and impose a heavy health burden [1]. Especially during the recent pandemic of COVID-19, people are frightened by the high rates of infection and mortality, which is worsened by social distancing and self-quarantine. The isolation-induced loneliness and anxiety brings huge challenges both physiologically and mentally to healthy people, let alone those with EDs [3]. Anorexia, bulimia, and binge eating are the most common EDs [2], albeit that the neurological mechanisms underlying these disorders are still not fully understood. Thus, it is urgent to figure out the pathology and find efficient therapies to prevent or cure these eating disorders.

The hypothalamus is a critical region in the central nervous system that gathers and responds to both intrinsic and extrinsic information to maintain energy homeostasis and deal with different stressors [4, 5]. Neuropeptides, neurotransmitters, and hormones existing in the brain and periphery mediate appetite and energy intake. In human, neurons in the hypothalamus receive peripheral signals, such as leptin from the adipose tissue and insulin from the pancreas, which induce a satiety tone, and ghrelin and cholecystokinin from the intestine, which stimulate eating [6]. The utilization of animal models assists in deepening our understanding of appetite regulation. Injection via both intracerebroventricular (ICV; into left lateral ventricle of brain) and intraperitoneal (IP; peripheral and assumed to enter bloodstream) routes, and genome modification by either overexpressing or knocking down or out specific genes have helped us to know the function of single or combined factors. By using these approaches, the interaction between ligand and receptors or between one and another signaling pathway can be manipulated and investigated. Take the melanocortin system as an example. ICV injection of the α-melanocyte-stimulating hormone (α-MSH) into the chicken brain induced significant anorexia, whereas a melanocortin receptor 4 (MC4R) antagonist, agouti-related peptide (AgRP), competed for the receptor with α-MSH to alleviate the reduced food intake [7]. Additionally, MC4R knockout mice were sensitive to a high-fat diet and showed continuing hyperphagic and obese phenotypes [8].

As mentioned previously, stress-like environmental stimuli are processed in the hypothalamus, which might affect appetite since the regulation of both processes shares various mediators. For instance, the corticotropin-releasing factor (CRF) is capable of inducing satiety and activating the hypothalamic-pituitary-adrenal (HPA) axis and stress response [9]. Thus, the hypothalamic mechanism of inducing satiety might also involve activation of a stress response. Besides, when stress affects individuals during some critical period, like late gestation or the early postnatal period when the brain is still developing, epigenetic modifications may occur to regulate gene expression and have a long-lasting effect, which could influence not only the hypothalamic nexus to affect appetite and the stress response [10], but other brain regions to impact emotion and reward circuitries [11].

Thus, the primary goal of this dissertation is to look for novel appetite-related factors and investigate the role of the stress response and epigenetic programming in the hypothalamic mechanisms of appetite regulation. By doing so, we gain more mechanistic insights and therapeutic targets for eating disorders and better understand the pathogenesis of these complex disorders. In this dissertation, Chapter 2 gives a more detailed literature review on how the hypothalamus regulates feeding behavior and the possible interaction with the stress response and DNA methylation (epigenetic regulation). In Chapters 3 and 4, two novel molecules were centrally (ICV) applied to different avian models to test their immediate appetite-regulating effects and longer-lasting epigenetic-modifying effects. In Chapters 5 and 6, prostaglandin E2 and β-melanocyte-stimulating hormone, respectively, were ICV injected into broilers and their anorexigenic effects studied. The anorexia induced by these two molecules likely involves stress-related mechanisms. The role of the microbiome in physiology and health, including feeding behavior and responses to stressors, is known to be important, but still not well understood. Chapter 7 reviews the relationship between the chicken brain and intestinal microbiota under heat exposure, which is connected by the microbiota-gut-brain axis. Finally, this dissertation concludes with some overall conclusions and perspectives for future research.

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Chapter 2: Literature review

2.1 Hypothalamus as the regulatory center of appetite regulation

Energy homeostasis includes two major aspects, energy intake, and expenditure, regulated by a variety of central and peripheral signals as well as internal/external factors. Although several brain regions are involved in this process, such as the hypothalamus and brain stem, the hypothalamus is known as the center, integrating signals and controlling energy balance. Within the hypothalamus, different nuclei have specific neuronal populations which produce different types of neurotransmitters and neuropeptides, either stimulating or inhibiting food intake [1]. Several hypothalamic nuclei are considered to be closely related to appetite regulation, including the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LHA) [2].

2.1.1 Appetite-related hypothalamic nuclei

Situated at the ventral part of the hypothalamus and surrounding the third ventricle, the ARC is located at a pivotal site that integrates signals from the brain and periphery [3]. Among the central nervous system compartments, the ARC serves as a communication center that builds connections between the limbic system, thalamus, hypothalamus, pituitary gland, and brain stem [3]. Although a growing number of neuropeptides and transmitters are identified within the ARC, the two major neuronal populations mediating feeding behavior are the neuropeptide Y (NPY)/agouti-related peptide (AgRP) and pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons [4]. These neurons project their axons, through which they establish networks between the ARC and other hypothalamic nuclei such as the PVN, DMN, VMH, and LHA [5]. Additionally, the ARC is susceptible to the influence of peripheral signals. The reason is that the cells constituting adjacent capillaries lack tight junctions, which makes it possible for circulating leptin and insulin to pass through the blood-brain barrier [1]. Glucose in cerebrospinal fluid can diffuse across the ependyma to the ARC [6].

The PVN is an integration nucleus receiving signals from the two functionally opposing neuron populations in the ARC and orexin neurons in the LHA and responds to these signals vigorously [6, 7]. The role of the PVN in regulating feeding has been previously demonstrated by electrolytic lesioning, the result of which is induced feeding and increased body weight in rats [8]. Corticotropin-releasing factor (CRF) originates from the PVN and functions as a versatile regulator mediating energy balance and coping with stress [9].

The VMH is known as the ‘satiety center’ because its lesioning is related to an obese phenotype in rats [10, 11]. A large amount of leptin receptors locate in the VMH, implying its function in the leptin signaling pathway [12]. Brain-derived neurotropic factor (BDNF) indicated a high expression in the VMH and interacted with the melanocortin system to decrease food consumption [13], while its deprivation led to obesity [14].

The DMN and PVN connect VMH with LHA [15]. The DMN is similar to PVN and VMH, as it also responds to NPY/AgRP neurons in ARC and has a number of leptin and insulin receptors [16, 17].

LHA is the nucleus where orexins and melanin-concentrating hormone (MCH) are generated, both of them stimulate food intake. LHA also expresses lots of NPYR5, responding to NPY signal [18]. LHA is sensitive to peripheral glucose because it contains considerable amounts of glucose receptors neurons [19].

2.1.2 Classic appetite-related neuropeptides

NPY/AgRP

The main functions of NPY in appetite are stimulating food intake, reducing energy expenditure, and inducing obesity [20]. A nanomolar level of NPY injection could stimulate food intake potently and acutely. There are five identified NPY receptors named NPYR1-5. NPY has a moderate to high affinity for all of the receptor subtypes [21]. Except for NPYR3 mainly locating in NTS, the other four receptors exist in the hypothalamus [22]. The ARC mainly projects NPY-axons to PVN. The primary physiological function of NPY/AgRP neurons in ARC may be to sense and respond to states of negative energy balance, such as starvation, by mediating feeding behavior and metabolic response. When these states occur, the activity of the neurons is increased and induces the expression of NPY before it stimulates the food intake [23]. NPY could also be released locally within ARC, binding to NPYR2 or NPYR4 acting as an inhibitory neurotransmitter [24].

The expression and effects of NPY and its receptors in the hypothalamus receive either positive or negative regulation. For example, when leptin is administrated, it decreases both food intake and the expression of NPY mRNA in the ARC and PVN [25]. Activation of the NPYR5 regulates both feeding and energy expenditure [26]. Fasting could down-regulate the expression of NPYR1 [27]. Interestingly, when NPY is knockout, mice have normal growth conditions and response to fasting [28]. The explanation might be that AgRP could compensate for the lack of NPY [29].

Central administration of AgRP significantly increases food intake and can reverse leptin-induced inhibition of feeding [30]. AgRP stimulated food intake of layer-type chicks under an ad libitum feeding condition but not broiler chicks [31]. AgRP may be regulated more robustly by changes in the metabolic state than is POMC, also be influenced by fasting [32].

Melanocortin system

The melanocortin system contains several ligand-receptors signaling pathways that converge nutritional and neural signals to regulate energy homeostasis in the central nervous system. Members of this system include a polypeptide precursor POMC, which is cleaved into several melanocortin receptor (MCR) agonists, such as α-, β-, and γ-melanocyte-stimulating hormone (MSH), and the MCR antagonist AgRP [33]. There are 5 different MCRs with a wide distribution both in the brain and periphery, mediating a variety of biological functions, such as appetite regulation, energy balance, stress responses, to name a few [34].

When neonatal chicks experienced fasting and followed intracerebroventricular injection of α-MSH, it significantly inhibited food intake in a dose-dependent manner [35]. Also, α-MSH strongly inhibited NPY-induced feeding when NPY and α-MSH were administrated simultaneously [35]. However, central injected AgRP attenuated the anorexigenic effect of α-MSH in chicks [31].

Central administration of α-MSH has an influence on the transcriptions of several appetite-related genes. It has been found that compared to vehicle-injected chicks, α-MSH injection increase the number of c-Fos immunoreactive cells in ARC, DMN, LHA, and PVN. Specifically, α-MSH treatment stimulates the expression of NPY, oxytocin receptor (OXTR), and AgRP mRNAs in the ARC. In the PVN, although c-Fos mRNA was increased by treatment with α-MSH, NPYR1 mRNA was reduced. In addition to the DMN, chicks injected with α-MSH showed higher NPY, c-Fos, and DOPA decarboxylase (DDC) mRNAs expression [36]. All these verify that α-MSH could override the orexigenic effect of NPY and reduce food intake strongly.

MC3R and MC4R, serving as the receptors of MSH, are expressed in the VMH, DMN, and ARC [37]. Both of them are involved in feeding behavior and metabolism regulation, in which they mediate reduced food intake [33, 35]. MC4R has interactions with leptin regulating feeding behavior. The negative effect of leptin on food intake and body weight is relieved by blocking MC4R [38]. On the other hand, leptin together with MC4R showed a positive effect on preventing hyperphagia [39].

CRF

CRF is a neuropeptide consisting of 41 amino acids and mediating a variety of physiological processes, including feeding behavior, stress responses, and locomotion [40-43]. In the hypothalamus, CRF is primarily produced and released from the PVN [44]. Its influence on feeding regulation will be described in this section, whereas its role in coping with stressful situations will be discussed in the following section.

Central administration of CRF in the brain induced satiety and energy expenditure in mammals [45, 46] and avian species [41, 47, 48]. In fasted rats, 1 to 10 μg dose of CRF decreased their food intake, and this effect was associated with grooming behavior [45]. The anorectic effect of CRF in chickens was potent. In young broiler chickens (2 or 4 days post-hatch), a lower dose (0.1 μg) of CRF was capable of suppressing food intake when chicks were fasted for 3 hours prior to injection [41, 49]. As for the adult chicken, 5 to 20 μg of CRF injection induced anorexia in a dose-dependent manner in layer- and broiler-type chicks at the age of 7 and 4 weeks, respectively [50]. Six-hour-fasted Japanese quail, at day 7 post-hatch, also responded to 0.1 μg of CRF and showed a decreased food consumption for 3 hours post-injection [48].

Our group further measured c-Fos (a transcription factor and biomarker indicating recent neuronal activation) immunoreactivity in the hypothalamus of broiler chickens and quail. In chickens, the ARC, PVN, VMH, and DMN were all activated by CRF injection, whereas induced activities in PVN and LHA were found in CRF treated-quail [41, 48]. The possible reason for this difference is that the long-term artificial selection on broiler chicks that are required to have better growth performance and meat production, while the Japanese quail is a species much closer to a wild-type bird. However, these studies indicated that CRF-induced satiety involves hypothalamic modification.

Subsequent measurement of appetite-related factor mRNA expressions within those activated hypothalamic nuclei indicated that in chickens, CRF injection was related to a downregulation of NPY/AgRP expression in the ARC, thus CRF attenuated the orexigenic effects of these two factors. The reduction of NPYR1 and NPYR5 levels strengthened this influence. On the other hand, CRF induced the expression of endogenous CRF and its receptors and another anorectic factor mesotocin, supporting that an anorexigenic tone was induced by exogenous CRF [41]. In comparison, CRF-injected quail had higher expression of CRF receptor sub-type 2 (CRFR2), POMC, MC4R, and c-Fos in the hypothalamus [48], suggesting that the melanocortin system involved in mediating the anorexigenic effect of CRF in Japanese quail.

Mesotocin

Mesotocin (MT), a nine amino acid peptide, is the avian equivalent to oxytocin (OXT) in mammals [51]. Like oxytocin, MT is found in the hypothalamus and peripheral organs [51].

OXT plays an important role in motion and reproduction [52], but only the effect on motor behavior appeared to be conserved in MT during evolution [51]. Nevertheless, both OXT and MT were reported to regulate appetite. Central administration of OXT could reduce food intake in rats [53] as well as in chickens [54]. Similarly, MT intracerebroventricular (ICV) injection resulted in decreased food intake in broiler and layer chickens [51, 55].

Interestingly, when hypothalamic c-Fos immunoreactivity was measured in broiler chickens treated with OXT and MT, respectively, OXT activated all five appetite-related hypothalamic nuclei (that is, the ARC, PVN, VMH, DMN, and LHA) [54], while MT only stimulated activation in the PVN and LHA [55]. Subsequent mRNA abundance in the hypothalamus indicated that CRF expression was higher in vehicle-injected chicks than OXT-injected chicks, which seemed contradictory to the result in MT-injected chicks that had higher CRFR1 expression [54, 55]. This could be explained as that OXT, one-amino-acid different than MT, may bind to different receptors in chicken. Thus, it is still possible that the CRF signaling pathway is involved in MT-induced satiety. Additionally, MC3R expression was stimulated by MT injection, so the melanocortin system might also participate in the anorectic effect of MT [55].

2.2 Unique chicken models of anorexia and obesity

The Virginia lines of chickens provide a unique avian model that chickens experiencing over 60 consecutive generations of selection divergently formed two phenotypes, the low-weight selected (LWS) and the high-weight selected (HWS) chickens, which comprised of individuals with different severities of hypophagia or hyperphagia [56]. This model is the only one of under- and over-eating behavior resulting from long-term divergent selection for body weight originated from a White Plymouth Rock founder population [57]. At eight weeks (56 days) of age, HWS acquired a 15-fold difference in body weight compared to LWS [58] with different food intake and body composition. Research revealed that the difference in food intake was a result of increased meal number instead of meal size consumed by HWS chicks compared to LWS [59]. The LWS and HWS differ in various aspects, including embryonic development [60], reproductive traits [61], immunological characteristics [62], as well as metabolism and growth [63, 64].

When NPY is administrated to both lines, LWS chicks did not respond with any magnitude of altered food intake to any dose of NPY tested, while HWS chicks responded to all doses of NPY at similar magnitudes throughout the observation [65]. This result supports the idea that there may exist different interior mechanisms between the LWS and the HWS. The c-Fos immunoreactivity was increased in both lines in the LHA and PVN [65], indicating that there are other signaling pathways in LWS overriding the orexigenic effects of NPY.

As for central α-MSH injections, the LWS line responded to all doses of α-MSH with a similar potent decrease in food intake at all observation times. However, the HWS line only responded to 600 pmol α-MSH with decreased feed intake. Increased c-Fos immunoreactivity was detected in the DMN, PVN, and VMH in both lines after α-MSH treatment; however, the magnitude of increase was greater in LWS than in HWS chicks at the PVN [66]. All these results imply that the LWS has a lower threshold of α-MSH reducing the food intake. And compared to the results of NPY central administration, we could find an opposite trend and deduce that the LWS line is sensitive to anorexigenic tone while the HWS line is susceptible to orexigenic tone.

When treated with other anorexic neuropeptides, we found that LWS chicks also showed higher sensitivities in food intake response to ICV injection of CRF [47], amylin [67], neuropeptide AF [68], insulin [69], and ghrelin [70], while HWS chicks had lower thresholds for neuropeptide S and calcitonin and calcitonin gene-related peptide [71]. Central injection of galanin [72] and gonadotropin-inhibitory hormone [73] increased food intake in both lines with similar magnitudes. Central administration of human recombinant leptin only reduced food intake in LWS but had no effects on HWS [74], while NPY [65] and obestatin [70] increased food intake in HWS but not LWS. These results collectively suggest that long-term consecutive selection for body weight altered the hypothalamic satiety mechanisms in HWS and LWS chickens.

In addition, except for the effect of a single neuropeptide, we looked at the interaction between neuropeptides. Research on human recombinant leptin indicated that leptin receptors are located on NPY/POMC neurons in the ARC. When leptin bound to its receptor, it triggered the expression of POMC and thus the level of α-MSH is increased, which then bound to MC4R and exerted a negative effect on food intake in the LWS chicken [74]. Insulin is also reported to be related to MCR [69], while amylin is interdependent with leptin [67]. These results suggest combined or compensatory effects among these neuropeptides resulting in hypophagia.

2.3 Effects of stress on appetite regulation

Stress is a physically and/or mentally threatening situation that intervenes in homeostasis. Stressful situations could be acute or chronic, depending on their magnitude and duration. When exposed to a stressor, humans or animals will respond with physiological or behavioral changes [75]. Typically, the hypothalamic-pituitary-adrenal (HPA) axis will be activated and then the adrenocorticotropic hormone (ACTH) will be produced to further stimulate the synthesis of cortisol, a glucocorticoid [76]. This will prepare the body with more glucose to fuel some related metabolic processes and respond to the stressor. Meanwhile, appetite and energy balance will be regulated under stress exposure. Many factors could influence the responses to stress, including species, sex, nutritional state, age, disease, etc [77].

2.3.1 Stress stimulates food intake

More often, humans under stress showed altered food consumption towards a greater amount and preference for more palatable food, which contains a higher portion of sugar and/or fat [78, 79]. These kinds of food are considered as ‘comfort food’ that helps to relieve stress responses and improve mood [80, 81]. Rodents, when provided access to palatable food under stress exposure, made a similar choice that sucrose intake was higher than normal chow [82]. This food preference is accompanied by behavioral and physiological changes, including less anxiety and lower plasma corticosterone level in response to pain [83, 84]. It’s worth mentioning that our group tested food preference in broiler chickens, although without stress exposure, and found that chicks had a higher intake of the high carbohydrate-based diet than high fat- and protein-based diets [85]. Future research may investigate whether stress could affect chicken’s food preference.

The mechanisms underlying the effect of comfort food intake on reducing stress response involves the brain reward pathways. Researchers found that providing a limited amount of sucrose solution daily for rats to drink significantly reduced anxiety in different behavioral tests, decreased the level of CRF in the PVN, and blunted the HPA axis in response to stress. However, when sucrose was given by gavage directly into the stomach, these beneficial effects did not occur [86]. These results suggested that the ingestion of sucrose, but not sucrose itself, was sufficient to activate the brain reward signals due to its hedonic nature.

The basolateral amygdala (BLA) is a brain region shared by the reward circuitry and stress responding pathways [87, 88]. The model of limited sucrose provision above was capable of decreasing neuron firing in the BLA induced by stress [89]. This was supported by a study that blunt HPA axis activation was not observed in rats with damaged BLA, although rats consumed a similar amount of sucrose [86]. Inside the BLA, genes that related to neuroplasticity showed altered expressions, with both pre- and pro-synaptic neural density increased [77, 90]. All these results suggested that sucrose relieved the stress response mainly through BLA synaptic remodeling and dampened HPA axis.

However, comfort food can only benefit for stress response in the short term. If individuals are obsessed in this kind of lifestyle or feeding mode in the long term, fat accumulation and obesity will follow up and in turn make the individuals more vulnerable to stress and depression [91]. Growing evidence indicates that this unlimited and prolonged consumption of high fat and sugar diets is closely related to chronic stress exposure [92, 93]. For example, high-fat diet-fed rats, rather than those provided a choice diet, typically had a higher level of basal corticosterone in comparison to rats fed with normal chow [93]. Likewise, streptozotocin-induced diabetic rats were considered to have upregulated stress response due to increased PVN CRF mRNA abundance and higher concentration of ACTH and corticosterone [93].

Most of the animal studies investigating the relationship between stress and high-fat diets used the male rats model [93], which might underestimate the impact of sex or especially the females [94]. Thus, several other studies using the female primate model largely complemented this knowledge gap. Researchers found that subordinate female macaque tended to have a stress response, such as aberrant HPA axis regulation and inflammatory response [95, 96]. When the whole group of monkeys was provided with a low-caloric diet, these subordinate individuals showed some extent of anorexia and lower body weight compared with those dominant monkeys. If both high- and low-caloric diets could be accessed, which is more similar to the environment where humans live, the dominant monkeys did not eat more than in the situation when only the low-caloric diet was available, but the subordinates did have a larger size of meal on the contrary [97, 98]. Further study found that monkeys fed in an environment with both high- and low-caloric diet had a higher level of blood cortisol when they are separated from their groupmates than those who only had access to low-caloric food [97, 99]. This finding suggested that the HPA axis is more active when individuals have sufficient calory supply. Human and rodents showed a similar reaction to these monkeys [100-102].

2.3.2 Stress induces anorexia

As previously described, although stress often stimulates food consumption, which is more common when there is a choice of diet [103, 104], it can be suppressive to food intake. There are many factors responsible for anorexia, and the neurocircuitry mediating this effect includes several brain regions, such as the prefrontal cortex (PFC), amygdala, and hypothalamus [105, 106].

Fear, especially long-term or continuing fear, is one of the factors associated with anorexia [107, 108]. It is natural for animals to be afraid of potential hazards and feed suppression under certain circumstances is an essential mechanism to adapt to the environment, although prolonged stress could tip the balance that fear overwhelms hunger [77]. The neural substrates underlying the fear-related eating disorder involve the central nucleus of the amygdala (CeA), which mediates the sensation and response to fear [105]. Lesions in CeA reversed food intake in rats in fear context [105]. LHA is another region that converges interior and exterior feeding signals, as well as signals from the amygdala and PFC [106]. The CeA, the component in the amygdala mediating fear anorexia, connects with LHA both directly and indirectly [109, 110]. Within the LHA, the suprafornical region is known as the production site of orexin, which induces food intake and regulates feeding behavior [111, 112]. CeA could directly target this region and thus influence appetite. Besides, both CeA and LHA project to regions in the brainstem, such as the nucleus of solitary tract, which is related to suppressed food intake [111, 113]. As for the PFC, the LHA and amygdala mediate hyperphagia in a satiated animal through opioid and dopamine signaling in the PFC, respectively [114, 115]. To summarize, CeA, but not BLA (related to feeding stimulation), together with PFC and LHA, forms a system contradictory to the PFC-BLA-LHA system and regulates fear-related anorexia [77].

In the hypothalamus, the PVN is another major center mediating stress-related appetite modifications. This makes sense because CRF is an important neuropeptide primarily produced in the PVN and serves as a regulator both in appetite regulation and stress response [116].

When humans or animals perceive a stressor, CRF from the PVN will first be generated and released, which will then be projected and transported to the anterior pituitary, where this neuropeptide binds to its receptor CRFR1. Once activated, CRFR1 promotes the production of ACTH, cleaved from the precursor POMC. ACTH travels to the adrenal gland to facilitate the generation of glucocorticoids, which get the body prepared for this stressor [116]. This process of HPA axis activation is not only functioning when a negative stressor presents, but plays an important role also in positive stress [117, 118].

CRF has a higher affinity for the CRFR1 than CRFR2, and it mediates stress response in the central nervous system through CRFR1 [116, 119]. In rodent models, central injection of CRF induced stress-related behaviors, such as decreased food consumption, aberrant motion, and social behavior [120-122]. Within the hippocampus, expression and distribution of CRF are much less and limited compared with the CRFR1 [123, 124]. CRF is widely proposed to be involved in cognition, with the effects being beneficial or harmful depending on the duration (short- or long-term) of its elevated expression [125, 126]. Potential mechanisms for these effects are supposed to be related to dendrite loss caused by stress [127, 128]. Additionally, the role that CRF plays in the formation of fear memory was confirmed by the administration of it and CRFR antagonists directly into the hippocampus [129-131]. As previously described, the amygdala participates in the perception of fear. And a high level of CRF was found in the CeA, whereas its receptor CRFR1 was mainly expressed in the BLA [123, 132]. Similar to the results in the hippocampus, intra-BLA injection of CRF or its receptor antagonist induced or attenuated rats' anxiety and CRFR1 knockdown in the same nucleus led to an antianxiety influence [133-135].

Compared with fear- or anxiety-related behavior changes mediated by CRF/CRFR1 under stress exposure, feeding behavior and energy homeostasis regulation are generally believed mediated by CRF/CRFR2 [136]. Regulators, such as CRF, OXT, and their receptors, of appetite and stress response, are shared by these two processes within the hypothalamus. OXT, like CRF, can be produced in the PVN, both of which are induced and released during stress exposure [137, 138]. When CRF stimulates the activation of the HPA axis, OXT will immediately strengthen the ACTH production induced by CRF [139]. Subsequent glucocorticoids synthesized in the periphery will circulate back into the brain and cause negative feedback on CRF expression by preventing transcriptional factors from binding to its promoter region [140].

Besides, OXT can directly regulate CRF neuron activity. When its endogenous concentration is high during pregnancy or chronically applied to female rats, c-Fos immunoreactivity of CRF neuron was not significant, so was the expression of CRF and subsequent ACTH and corticosterone levels [141, 142]. In another rodent model, OXT was injected into the PVN directly and CRF neuron activity was also suppressed, accompanied by alleviated anxiety and stress reaction [143]. Further study confirmed this suppressive function was indirectly mediated by gamma-aminobutyric acid (GABA), which inhibited the CRF expression [144, 145]. At the cellular level, evidence strengthened the interaction between OXT and CRF by looking at their receptors, with OXTR co-expressed on CRF neurons and vice versa [146].

Except for the interplay with CRF itself, OXT has connections with other CRF family members. Neurons in the PFC that express OXTR also produce the CRF-binding protein, which competes with the CRFR1 for CRF, thus preventing anxiety from occurring in mice [147]. In female rodents, the stress response to the loss of a partner is mediated by the CRFR2, and OXT administration could attenuate its activation and relieve the anxiety-like behavior [148].

2.3.3 Other stressors-induced anorexia

Ambient temperature, either too low or high, and food (access and composition) are environmental and nutritional stimuli that may cause stress responses in humans or animals. Especially during early life or a critical period of life, undergoing these stressors could alter brain development and functions, with beneficial or adverse influences potentially lasting for the entire lifetime [149-151].

Cold and heat stress

Chickens, despite their homeothermic nature, are vulnerable to intense temperature fluctuation during the first week post-hatch, when they only have a narrow range of thermoneutral zone [152]. However, after this period, their ability to cope with cold exposure gradually develops, following the growing feathers, increasing internal heat generation, and decreasing body surface area to body mass ratio [153]. This change was reported to be related to the switch from non-shivering to shivering heat production, with the former regulation characterized by increased oxygen consumption [153].

Cold stress is directly related to suppressed food intake in chickens. Our group previously tested a combined stressor of cold stress and food deficiency on day-of-hatch line chickens selected for body weight. We found that among all the treatment groups, only the LWS chickens didn't respond to the orexigenic effect of NPY applied on day 5 post-hatch [154]. This suggested that early life cold stress had an influence on appetite regulatory machinery. Olfati et al. (2018) reared broiler chickens in a cold environment during 3 to 6 weeks of age and found decreased food intake and body weight but increased feed conversion ratio [155].

Hypothalamus is the integration center of both appetite regulation and stress responses. Thus, much evidence focused on the hypothalamic mechanisms of cold stress in chicks. In our previous study, different hypothalamic nuclei were harvested to measure the mRNA abundance of several appetite-related factors. In the cold-treated LWS chicks, CRF and its two receptors, MC4R, and urocortin 3 (a member of the CRF family) were up-regulated in the PVN. Elevated CRFR2 was also seen in the ARC and VMH. In addition, orexigenic NPY and its receptor NPYR5 were promoted as well in the ARC and PVN, but the expression of AgRP was lower in the ARC as an exception [156]. These results suggested that cold stress might induce satiety in LWS by activating the PVN CRF signaling pathway. In comparison, when gene expressions were evaluated after NPY injection in the stressed LWS, genes that were mainly affected by or primarily mediated the stress response were AgRP and MC3R, of which the expressions were down- and up-regulated, respectively [154].

When cold stress was applied to older chicks (4 weeks of age) with cold-resistant genome background (Chinese Huainan partridge chicks), there are hundreds of genes in the hypothalamus being regulated. Genes involved in muscle activity had a lower expression in stressed chicks, whereas appetite-related genes, such as NPY, NPYR5, and cholecystokinin, were produced more. Additionally, stressed birds appeared to have higher lipid production, which indicated chicks intended to store energy against cold exposure [157].

Employing a mild and chronic cold exposure did not cause the chicks to eat less, but rather had a higher body weight. This kind of condition led to a lower expression of NPY and CART in the whole brain, which might be the reason for the similar food intake between treatments since NPY and CART have opposite effects on feeding behavior. Under this condition, expression of the lipogenic genes in the liver was reduced, suggesting that lipid synthesis was suppressed [158]. This is different from the result in the stress-resistant chicks above, but not necessarily contradictory. Maybe the peripheral fatty acids are mobilized to generate heat for the birds to adapt to the chronic cold stress. And this was partly supported by elevated levels of carnitine palmitoyl transferase 1 and mTOR in muscle, which is related to fatty acid β-oxidation [159].

Cold stress does not necessarily impair the chick's growth, although it often does [159]. Whether it causes the chicks to adapt or be more vulnerable to future stressful conditions depends on both the stressor (degree, exposed time) and the birds (age, strain, sex), to list some. However, Campderrich et al. (2019) brought a new insight to buffer early life stress by environmental enrichment, even if cold exposure indeed negatively impacted the birds. In their study, early life cold exposure made the chicks more sensitive to stressors at a later age. However, when raised in a complex environment (provided with perches and hide area), stressed birds had a less inflammatory response, better dealt with a novel stressor, and showed more resting behavior [160].

Heat stress is the other end of extreme temperature negatively influencing chicken appetite. Numerous studies have focused on this stressor and investigated its impact from various aspects. Feeding behavior is just one of them [155], but the system that regulates feeding behavior shares the signaling pathways with stress response, such as the HPA axis and the intestinal nervous and immune system. Thus, the responses induced by heat stress are systematic, and more details will be described in Chapter 7.

In 6-week-old broiler chicks, high-temperature exposure (31 and 36 °C) both suppressed food intake, reduced body weight gain and stimulated the production of corticosterone [161]. Our group also test heat stress (38 °C) on feeding behavior using younger broilers (4-day-old). Compared with the thermoneutral group, food consumption was significantly lower in heated chicks. Heat exposed chickens only responded to orexigenic NPY central administration for the first hour post-injection, whereas chicks in normal temperature had increased food intake for 3 hours post-injection. However, when injected with CRF, chickens from both groups ate less for 3 hours post-injection. Under a similar situation except that CRF was replaced by its receptor antagonist, astressin, heated chicks had a similar amount of food consumption compared with the thermoneutral group, suggesting CRF signaling is involved in the suppressive effect of heat stress. Further molecular studies supported this statement. Within the hypothalamus of the heated broilers, the PVN, LHA, and the nucleus of hippocampal commissure (NHpC) indicated enhanced c-Fos immunoreactivity. CRF mRNA abundance was elevated in the PVN, but suppressed in the NHpC, while the NPY expression was lower in the ARC [162]. All these results suggested that heat stress-induced anorexia is involved in the CRF signaling.

Food deprivation

Food deprivation is followed by hypothalamic appetite-related gene expression changes because the maintenance of energy homeostasis is modified in such a rapidly dynamic manner that guarantees the organism's survival. The responses in the hypothalamus, however, depend on avian species and the duration of food deprivation.

In chickens, 48 hours-fasted chickens had increased expression of AMP-activated protein kinase α (AMPK α) and decreased levels of glucose and insulin in the circulation, whereas 24 hours-refeeding could reverse this effect [163]. Fasting also led to a higher NPY and AgRP expression [164-166]. Other than this, fasting was related to reduced hypothalamic POMC expression in both broiler- and layer-type chickens [166-168]. In fasted line chickens, 3 hours of fasting attenuated the difference of elevated NPYR5 between the LWS and HWS but aggravated the reduction of POMC level in both lines [169]. This suggested a recovery from less energy intake was prepared by suppressed anorexigenic and promoted orexigenic signals.

In Japanese quail, birds had a higher level of NPY after 24 hours of food deprivation [170]. Food intake was stimulated in 6-day-old quail fasted for 6 hours. On day 7, 3 hours of fasting increased the expression of NPY, NPYR2, AgRP, MC3R, MC4R, and neuropeptide S but decreased CRFR1 level in quail. And refeeding for an hour after 3-hour-fasting didn't change those expressions much. This pattern was similar in quail experiencing 6 hours fasting and 1 hour refeeding [171]. Thus, Japanese quail seems to be more responsive to food deprivation than chickens, with more appetite-related factors being modified. However, the overall trend stays the same that fasting favors the expression of orexigenic genes and suppressed anorexigenic gene production, which assists the birds to maintain energy balance.

2.4 DNA methylation as a novel target for appetite regulation

Epigenetics is referred to modifications in gene activity or function without changing DNA sequences and mainly include DNA methylation, histone deacetylation, and RNA interference [172]. DNA methylation is a covalent modification involving the addition of a methyl group by DNA methyltransferases (DNMTs) to cytosines that occurs next to a guanine nucleotide, CpG. Methylation of CpG sites within the promoters of genes could lead to gene silencing [173]. In contrast, the hypomethylation of CpG sites is associated with the over-expression of some genes. The DNMT family has three major members, of which DNMT1 mainly functions to maintain the existed methylation pattern in daughter DNA strand inherited from parental DNA strand [174], whereas DNMT3a and DNMT3b initiate *de novo* methylation meaning that unmodified DNA is provided a novel pattern of methylation [175, 176]. The activity of these enzymes is active during embryo development and tends to cease by the end of differentiation. However, this does not mean that the DNA methylation pattern remains unchangeable [175]. On the contrary, DNA methylation could be modified by various factors either physiologically or pathologically, exerting life-long term effects on the organism.

In chickens, early-life stress-induced anorexia in low body weight selected chicks involves the modulation of DNA methylation. A previous study in our group found that LWS chickens, when transported from the hatcher to the laboratory on the day of hatch, had a reduced reaction to the orexigenic effect of NPY injected on day 5 post-hatch [65]. It was likely that these chicks were under stressful conditions when transported, so we mimicked the conditions by giving the birds a standardized stressor, cold stress (-20 °C for 6 minutes and 22 °C for 24 hours) with food deprivation, which is normally the situation when birds were transported at hatch. This stressor exposure caused the LWS chickens not to respond to NPY injection at a later age [154]. Appetite-related factors were affected by this combined stressor and their expressions changed accordingly, which has been described in the previous section about cold stress.

Being the classical appetite regulators, NPY and CRF were both found to have increased expression in the ARC and PVN, respectively, when the LWS chicks underwent cold exposure and delayed food supply at hatch [156]. Due to their vital roles in appetite regulation, it was not surprising that their expressions were altered under this circumstance. Thus, what interested us most was that epigenetic modification was proved a reason leading to their elevated abundance within the hypothalamus [56].

Specifically, both global DNA methylation level and the activity of DNMT were induced by stress in the ARC of LWS chickens. Although these results were not observed in the PVN, one of the CpG sites in the promoter region of the CRF gene in stressed LWS was found to have a lower methylation rate, which might affect the expression of CRF. The following experiment further verified that this altered CpG site located within the region where methyl cytosine binding domain protein 2 (MBD2) binds to the CRF gene, and stress prevented MBD2 from binding, which allowed more CRF expression. This induced CRF expression thus might set an anorectic tone in the LWS hypothalamus which overrides the hunger-stimulating effect of NPY [56].

To briefly summarize, early-life stress in chickens has an impact on their epigenetic profile and regulates their feeding behavior in later life. Stress could modify appetite-related gene expression by influencing the methylation states of CpG sites in pivotal transcriptional regions. Although our group has investigated two classical appetite-associated factors, there are more related genes and systems, such as mesotocin and melanocortin system, that could be epigenetically modified and be involved in the machinery of appetite regulation.

2.4.1 Methyl donors

Altered environmental conditions, like ambient temperature changes and environmental enrichment, when applied to organisms during prenatal or early postnatal periods, are capable of affecting epigenetic states and thus impact their development and behaviors at a later age [153, 160]. However, these influences are typically indirect by influencing the activity of enzymes, such as DNMTs, that participate in DNA methylation. Unlike these environmental factors, nutritional factors, especially diets containing methyl-donor nutrients, could directly alter the supplementation of the substrate methyl group and thereby influence the methylation process [177]. Accumulative evidence has been found in various animal models that sufficient or deficient provisions of methyl donor in parental (maternal in most studies) and/or postnatal diets have a long-lasting effect on the growth, health, and behaviors of the offspring [178-180].

There are many different methyl donors, such as S-adenosylmethionine (SAM), choline, betaine, B vitamins, that all play their important roles in the process called one-carbon metabolism [177]. This process is nothing more but a circulation where methionine and folate metabolism cycles meet one another and molecules within these cycles not only provide methyl groups (one-carbon units) to promote the synthesis of DNA, amino acids, and other compounds, but receive methyl groups to regenerate themselves to maintain the balance of this circulation [181]. For example, methionine serves as the precursor of SAM, while SAM could provide its methyl group for DNMTs to carry over and facilitate the occurrence of methylation, with itself being converted into S-adenosylhomocysteine (SAH) [177].

Sufficient supplementation of methyl donors is essential for the organisms, especially their brain development. Deficient or imbalanced supply in the diets could lead to changes in the central nervous system. For instance, pregnant mice that lack choline in the diet would give birth to offspring with abnormal hippocampus development and impaired learning and memory, due to aberrant DNA methylation at both the global level and specific genes [182]. On the contrary, oversupply of folate could reduce the risk of dementia occurrence [183]. Thus, it is highly possible that methyl-donor nutrients supply could affect neuronal development in the hypothalamus and further influence appetite regulation and energy metabolism.

Indeed, in a recent mouse study, a methyl-balanced diet served as a non-invasive method that decreased the vulnerability of young female offspring to binge eating-like behavior induced by prenatal stress during the late gestation period [184]. Binge eating is an eating disorder typically seen in females more often than in males, especially adolescent females (https://www.nimh.nih.gov), and is closely related to early-life stress. In their study, they induced the overexpress of CRF in the brain and caused prenatal stress for the fetus in the pregnant mice. This type of stress makes sense because CRF could stimulate the production of corticosterone, which renders the dams to have a limited amount of blood vessels present in the placenta and nutrient supply to the fetus [185]. And then starting at 5 weeks of age, the offspring were provided with recurrent limited access to a high-fat diet (palatable western diet, a few hours of access per day for several weeks) to develop a binge eating-like behavior. Compared with non-stressed counterparts, those stressed mice immediately showed more consumption of the western diet, although they all developed binge eating eventually [184].

To find out why stressed mice were predisposed to exaggerated eating, they focused on DNA methylation of hypothalamus genes because epigenetic changes were reported to be involved in this type of stress response and the hypothalamus is the center for appetite regulation. They found all three major DNMTs had decreased expressions in stressed mice. Further investigation found prenatal stress reduced hypothalamic expression of an enzyme, methionine synthase reductase (Mtrr), that activates the methionine synthase and maintains the normal metabolism of folate and methionine [184]. This reduction is related to an elevated level of homocysteine (Hcy) as well as hypomethylation [186]. However, they indeed found higher Hcy in the circulation but the global methylation in the hypothalamus was not affected [184]. Thus, the stress appeared to cause methylation changes to specific genes, which needed further verification. Later they used a balanced diet that provided the exact amount of balanced methyl donors for the adolescent mice, with normal chow as the control diet containing enough methyl donors but not in a balanced manner. Stressed mice fed the balanced diet then showed a normal amount of DNMTs, Mtrr, and plasma Hcy, and more surprisingly a reduction of food intake to a normal level that control mice had [184].

As for the expression of hypothalamic appetite-related genes, stressed mice had a higher abundance of AgRP but lower levels of POMC and MC4R, with no difference in MC3R, NPY, and CART, whereas the balanced diet normalized the expression of those genes in the melanocortin system [184]. This suggested prenatal stress predisposed the mice to have an orexigenic tone that stimulated their appetite when exposed to a western diet, but a balanced methyl-donor supply could rescue them from binge eating.

Choline

Choline is known for its vital role in the brain development of the fetus but is often insufficient in the supplementation for pregnant females [187]. Its deficiency correlates to a variety of issues in neuronal development and transportation, which might negatively affect cognition and memory, causing neurodegenerative diseases in later life [188, 189]. Hammoud et al. (2020) focused on the effects of its maternal supplementation on offspring's feeding behavior in rats. They divided the dams into three treatment groups, low-, recommended-, and high-choline supply. Although choline didn't affect food intake and body weight of dams during pregnancy, growth metrics and plasma hormone levels are different in their pups. Both low and high choline supply reduced leptin levels in the pups. But only high dose induced food intake after 17 weeks of normal chow feeding, whereas increased body weight gain started to show at 7 weeks of age. On the other end of energy balance, the expenditure of overall energy was less in the low dose group than the recommended and high dose groups. High choline supply in the maternal diet resulted in higher plasma free choline levels in pups at birth, but other one-carbon metabolites, such as SAM, SAH, methionine, had similar levels among groups. The high dose group also had more NPY expression than the other two groups, although POMC abundance was not affected. Taken together, supplementation of choline in gestational diets affected the feeding behavior of offspring [190]. It is important to note that a higher level of choline supply didn't always benefit pups' growth, when the dams were provided a high level of folic acid at the same time, which is more common in humans that North American women often have high folic acid consumption but insufficient choline, low level of choline supply induced a higher body weight gain during adolescence but recommended level of choline led to highest body weight gain towards adulthood [191]. In humans, high choline intake during late gestation was related to higher methylation levels in CRF and glucocorticoid receptor promoter region, lower expression of CRF in the placenta, and lower cortisol in cord plasma. Additionally, specific methyltransferase expression and global DNA methylation level were both higher in the placenta with high choline supply [192]. These results suggested choline supply during gestation could regulate the placental HPA axis activity and pup feeding behavior probably by modifying DNA methylation machinery.

Betaine

Choline affects DNA methylation through its metabolite betaine, which is involved in the SAM synthesis [193]. Studies on maternal betaine supply found hypothalamic cholesterol metabolism was influenced. First, eggs were injected with betaine, which mimicked maternal supply, and chicks hatching from these eggs showed larger body weight but less aggressive behavior at a later age. Cholesterol, cholesterol ester and enzymes that synthesize them were all increased by betaine supply. In ovo injection of betaine upregulated expression of betaine homocysteine methyltransferase (BHMT) and DNMT1, which further changed methylation level within the promoter region of genes participated in cholesterol metabolism [194]. Secondly, betaine was directly mixed in the diet for laying hens. Their first-generation offspring showed a similarly elevated level of cholesterol in the circulation and hypothalamus. Likewise, cholesterol metabolic gene expressions were generally increased. Appetite-related genes had divergent expressions, with less BDNF and CRF but more NPY in the hypothalamus. Expressions of BHMT and DNMT1 were elevated [195]. Lastly, the second generation of those betaine-supplied hens showed opposite growth performance with lower body weight and less hypothalamic cholesterol. So did the genes involved in cholesterol metabolism, which were downregulated. Abundances of NPY, BDNF, and CRF in these chicks were lower than their control counterparts.

Although BHMT level was still reduced, DNMT1 tended to express more while SAM/SAH ratio was higher in chicks from the second generation [196]. All these results indicated that maternal supplementation of betaine changes cholesterol metabolism and growth performance in the first generation of offspring but is not inheritable to the next generation. This change is related to DNA methylation at the level of methyltransferase genes.

SAM

SAM is the primary methyl donor in all living creatures, thus its availability changes could significantly alter any related biological process and following functions [197]. Many studies have been conducted on investigating methyl donor administration on feeding behavior like those described above and SAM is involved in these effects since it can be synthesized from those precursors and eventually donate its methyl group to methyltransferases. However, seldom have focused on directly providing SAM to human or animals to see if it has an effect on feeding. Bungo and Shiraishi (2010) gave layer chickens different methionine and related molecules into the brain and found that both L- and D-methionine were associated with increased food intake after injection. But SAM only tended to induce anorexia in neonatal chicks [198]. Thus, to further investigate the appetite-regulatory effect of such an important methyl donor, we applied SAM centrally to different avian models, including line chickens selected for body weight, broiler-type chickens, and Japanese quail, and detailed information can be found in the following chapters.

2.4.2 DNA methylation inhibitor

DNA methylation is mediated by DNMTs, thus the activity of DNMTs directly influences the states of methylation. There are two major molecules considered as the inhibitors of DNA methylation, 5-azacytidine (AZA) and 5-aza-2’-deoxycytidine (DAC), both of which are analogs of cytidine [199]. These two molecules were first synthesized as anti-cancer agents against leukemia [200, 201]. They can be incorporated into RNA and DNA. When it happens, they prevent DNMTs from interacting with DNA or cause damage to DNA and degrade trapped DNMTs, and thus indicated a hypomethylating property [199]. Their structure and mechanism of nucleic acid incorporation were described in detail as reviewed in [201].

Currently, there is no direct evidence that these DNA methylation inhibitors serve as regulators of appetite. But their hypomethylating effect cannot be underestimated within the hypothalamus. Environmental stress such as exposure to a drug or toxic substances during early life could result in profound epigenetic alterations in the brain [202]. Alcohol exposure is one of these stress and causes a long-lasting impairment once exposed in a developing brain [203]. POMC neurons in the ARC are sensitive to alcohol exposure in the fetus [204]. Although they can survive the toxicity of alcohol at this critical period, the function of POMC neurons is influenced and this impact lasts at a later age [205]. However, this adverse effect could be rescued by AZA injection into newborns. In brief, fetal alcohol exposure induced hypermethylation on the POMC gene, whereas AZA blocked the activity of DNMTs, reduced methylation level, and rescued the expression of POMC [205]. POMC, as the precursor of many peptides, was cleaved and produced β-endorphin, which could reduce the production of CRF and the activity of the HPA axis [206].

Like SAM, we also took AZA into consideration and tested its effect on appetite regulation in different avian models. We were curious if SAM or AZA is capable of directly affecting feeding behavior and whether the mechanism underlying possible effects is related to the epigenetic modification. More details are available in the next chapter.

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Chapter 3: Epigenetic modifiers identified as novel regulators of food intake in a unique hypophagic chicken model

Abstract

DNA methylation is an epigenetic modification that influences gene transcription, however, effects of methylation-influencing chemicals on appetite are unknown. We evaluated effects of single administration of a methyl donor, S-Adenosylmethionine (SAM), or methylation inhibitor, 5-Azacytidine (AZA), on immediate and later-age food intake in an anorexic chick model. The doses of intracerebroventricularly (ICV)-injected SAM were 0 (vehicle), 0.1, 1, and 10 µg, and AZA were 0 (vehicle), 1, 5, and 25 µg. When injected at day 5 post-hatch, there was no effect of SAM on food intake in either fed or fasted chicks, whereas AZA increased food consumption in the fasted state but decreased it in fed chicks. We then performed a single injection (same doses) at day-of-hatch (DOH) and measured food intake on day 5 in response to injection of neuropeptide Y (NPY; 0.2 µg). Irrespective of NPY, chicks injected with 1 µg of SAM ate more than others at day 5. Chicks injected with 0.1 µg of SAM did not respond to NPY, whereas all others increased their food intake. In contrast, chicks injected with AZA consumed similar amounts at day 5, but at the 5 and 25 µg doses were less responsive to NPY. In conclusion, we identified DNA methylation-regulating chemicals as novel regulators of food intake, using an anorexic avian model. AZA but not SAM affected food intake in the short-term, feeding states-dependently/with its effects depending on fast or fed states. Later, both chemicals were associated with changes in the orexigenic response to NPY, suggesting that feeding pathways might be altered through changes in methylation.

Keywords: S-Adenosylmethionine, 5-Azacytidine, Appetite, DNA methylation, Low body weight-selected line chicken, Neuropeptide Y

Introduction

Appetite regulation involves a complex interaction of pathways that are activated through the integration of signals that converge on the hypothalamus. The hypothalamus serves as the regulatory center for energy homeostasis, integrating cues from a variety of central and peripheral factors to balance energy intake and expenditure [1, 2]. Identifying novel regulators of feeding behavior and elucidating the associated molecular pathways will continue to facilitate the development of strategies to treat eating disorders.

An attractive model with which to understand appetite regulatory mechanisms are the Virginia lines of chickens. These lines, originating from a common founder population, have been continuously selected for low (LWS) or high (HWS) juvenile body weight, and after more than 60 consecutive generations of selection differ in body weight at selection age (56 days) by more than 12-fold, which is accompanied by divergent appetite phenotypes [3]. The LWS are lean and hypophagic and display varying severities of anorexia, whereas all HWS are compulsive eaters that become obese [4]. This model is the only one of under- and over-eating behavior resulting from long-term divergent selection for body weight originating from a common founder population [5].

Intracerebroventricular (ICV; into left lateral ventricle) injection of anorexigenic peptides, including α-melanocyte-stimulating hormone (α-MSH) [6], corticotropin-releasing factor (CRF) [7], amylin [8], neuropeptide AF (NPAF) [9], insulin [10] and ghrelin [11], inhibited food intake in both lines, with greater sensitivity to satiety-inducing effects in LWS than HWS. Central administration of human recombinant leptin reduced food intake in LWS but had no effect on HWS [12], while neuropeptide Y (NPY) [13] and obestatin [11] increased food intake in HWS but not LWS. These results collectively suggest that long-term selection for body weight altered the hypothalamic satiety mechanisms in HWS and LWS lines. Other than influencing feeding behavior, also observed were hypothalamic changes in c-Fos [6, 13], a marker for neuronal activity, and appetite-related peptide gene expression [14, 15], which implied molecular changes as a result of direct activation or inhibition of central feeding pathways. Moreover, the resistance of the hypophagic LWS to NPY was early-life stress-exposure-dependent [16, 17] and that the mechanism involved stress-induced magnification of anorexigenic tone originating from CRF-ergic neurons in the paraventricular nucleus of the hypothalamus. Because the effect on feeding behavior was observed at a later age than when the stressor was applied, we hypothesized the mechanism was epigenetic and occurred exclusively in the LWS because the changes were not observed in HWS [16, 17]. Indeed, this was confirmed when we identified a CpG in the promoter region of the CRF gene that was hypomethylated in response to stress in the LWS [18]. This reduced methylation disrupted binding of a transcriptional repressor, methyl domain-binding protein 2, which was associated with increased CRF mRNA in the paraventricular nucleus of the hypothalamus [18]. Thus, changes in appetite regulation can be modulated by epigenetic modifications that occur in response to environmental influences.

Epigenetics, including DNA methylation, histone acetylation and regulation of microRNAs, etc., involves changes in gene expression that do not alter the DNA sequence [19]. DNA methylation is a covalent modification requiring DNA methyltransferases (DNMTs) that catalyze the addition of a methyl group to cytosines that exist in the context of CpG dinucleotides in the DNA sequence, and is generally considered to be inhibitory with respect to effects on transcriptional regulation. S-Adenosylmethionine (SAM) is the major endogenous methyl donor [20] and is an essential component of the methionine cycle that can be generated from several dietary precursors, including folate, choline, and methionine [21, 22]. Dietary deficiencies in methyl donors led to widespread DNA demethylation or hypomethylation [23-25]. However, effects of central administration of SAM on DNMT activity and DNA methylation are unknown. 5-Azacytidine (AZA) was first identified as a chemotherapeutic drug [26]. However, at doses that do not induce cell death, once incorporated in DNA, AZA binds to DNMT1 and inhibits its activity, causing a rapid decline in DNA methylation and subsequent increases in gene transcription [27, 28].

Epigenetic-modifying chemicals thus have the potential to facilitate changes in behavior that persist over time through effects on gene regulation. Unclear, however, is whether such molecules can exert direct effects on behavior that are independent of their effects on DNA methylation. To our knowledge there are no reports of effects of methyl donors or methylation inhibitors on feeding behavior. Because we observed changes in appetite regulation in the LWS but not HWS as a result of early post-hatch environmental stressors, we hypothesize that direct administration of an epigenetic modifier has the potential to have a similar influence as stress on feeding behavior at a later age. Thus, the objective of the present study was to measure the immediate (inject at day 5 post-hatch) and long-term (inject at day of hatch and measure effects 5 days later) effects of central administration of SAM and AZA on food and water intake in LWS chicks.

Materials and methods

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Virginia Tech. The lines of chicks are from a long-term divergent selection program for either low or high body weight at 56 days of age [29]. The founding population consisted of crosses of 7 inbred lines of White Plymouth Rocks and the selection program is reviewed in [30]. All eggs were from age contemporary LWS parents from the S60 generation and were incubated in the same incubator and hatcher. After hatch, chicks were group-caged for 1 day and then transferred to individual cages in a room at a constant temperature of 32 ± 1 °C and 50 ± 5% relative humidity with 24-hours of light. Chicks had free access to a mash diet (3,000 kcal ME/kg and 21.5% crude protein) and tap water. The individual cages allowed visual and auditory contact among chicks, which were handled twice daily to adapt to handling.

Intracerebroventricular injection procedure

On the day-of-hatch (DOH) and/or day 5 post-hatch, chicks were ICV-injected using a method that does not appear to induce physiological stress [31, 32], adapted from Davis et al. (1979) [33]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed to allow for free-hand injection. Injection coordinates were based on the Kuenzel and Masson chicken stereotaxic atlas [34]. Anatomical landmarks were determined visually by using a restraining device and plastic tubing sheath. In particular, the restraining device is a piece of clay with scales that was molded with the use of a chick cadaver. The restraining device leaves the cranium exposed and coordinates the injection point at 3 mm anterior to the coronal suture and 1 mm lateral from the sagittal suture. The plastic tubing sheath over the needle was used to control injection depth (2 mm). The needle remained at injection depth in the un-anesthetized chick for 5 seconds post-injection to reduce backflow. SAM, AZA (Sigma, St. Louis, MO, USA) or chicken NPY (AnaSpec, San Jose, CA, USA) was dissolved in chicken artificial cerebrospinal fluid [35] as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the presence of dye in the lateral ventricle. Any chick without dye present in the lateral ventricle system was deleted from statistical analysis. Sex was determined visually by dissection and gonadal inspection at the time of decapitation.

Experiment 1: food and water intake in SAM-injected fed LWS chicks

On day 5 post-hatch, chicks were randomly assigned to receive either 0 (vehicle), 0.1, 1, or 10 µg of SAM, the doses of which were based on [36], by ICV injection (n = 10 in each group). After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 minutes for 180 minutes post-injection and at 360 minutes post-injection.

Experiment 2: food and water intake in SAM-injected fasted LWS chicks

Procedures were identical to Experiment 1, except that chicks were fasted for 180 minutes before SAM injection. During the fasting period, only food was withheld, while birds still had ad libitum access to water.

Experiment 3: food intake in NPY-injected LWS chicks after SAM injection

Before being group-caged on DOH, chicks were randomly assigned to receive either 0 (vehicle), 0.1, 1, or 10 µg of SAM by ICV injection (n = 20 in each group). After SAM injection, chicks were group-caged and then individually-caged as described in 2.1, where they had free access to food and water prior to NPY injection. On day 5 post-hatch, chicks were randomly assigned to receive either 0 (vehicle) or 0.2 µg of NPY (equal numbers of both treatments within each SAM-treated group) by ICV injection (n = 10 in each sub-group). After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food intake was monitored (0.01 g) at 60 minutes post-injection.

Experiment 4: food and water intake in AZA-injected fed LWS chicks

Procedures were identical to Experiment 1, except that chicks were treated with either 0 (vehicle), 1, 5, 25 µg of AZA by ICV injection (n = 28 in each group). The doses were based on a previous study [37].

Experiment 5: food and water intake in AZA-injected fasted LWS chicks

Procedures were identical to Experiment 4, except that chicks were fasted for 180 minutes before AZA injection (n = 32 in each group). Chicks had ad libitum access to water but not food when being fasted.

Experiment 6: food intake in NPY-injected LWS chicks after AZA injection

Procedures were identical to Experiment 3, except that chicks were first treated with either 0 (vehicle), 1, 5, 25 µg of AZA on DOH (n = 32 in each group). After AZA injection, chicks were given ad libitum access to food and water when being group-caged and individually-caged prior to NPY injection.

Statistical analyses

Food and water intake data for each experiment were expressed on a cumulative basis before statistical analysis. For all experiments, data were analyzed by analysis of variance (ANOVA) with SAS 9.4 (SAS institute, Cary, NC, USA) using the GLM procedure. Analyses were performed within each time point or each treatment, with the statistical model including the main effect of dose. When dose effects were significant, Tukey's method of multiple comparisons was used to separate the means. Statistical significance was set at $P < 0.05$ for all experiments.

Results

Food and water intake in SAM-injected LWS chicks

There was no effect of SAM on food or water intake at any dose or any time point in fed (Figure 3.1A and B, respectively) or fasted (Figure 3.2A and B, respectively) chicks at day 5 post-hatch.

SAM injection at DOH and NPY injection on day 5: food and water intake in LWS chicks

In chicks that received 0.1 µg of SAM on DOH, food intake was not different between vehicle and NPY treatment at 60 minutes post-injection on day 5 post-hatch (Figure 3.3A). Chicks that received the vehicle or 1 and 10 µg doses of SAM at DOH responded to NPY with increased food intake at day 5 post-hatch (Figure 3.3A), with a similar magnitude of increase at 60 minutes post-injection.

When food intake data were analyzed within the vehicle or NPY-injected groups at day 5, there was an effect of having received SAM on DOH in both groups, regardless of NPY treatment (Figure 3.3B). In both the vehicle-injected and NPY-injected chicks at day 5, there was greater food intake in chicks that had received the 1 ug SAM dose compared to those that received the vehicle at DOH (Figure 3.3B).

Food and water intake in AZA-injected fed LWS chicks

At 30 minutes post-injection food intake was not different among groups. At 60 minutes and continuing through 180 minutes, food intake was reduced (as compared to vehicle-injected) in chicks that received the 25 µg dose of AZA (Figure 3.4A). At 90 through 180 minutes post-injection, food intake was also reduced in 1 µg-injected chicks. At 360 minutes, there were no differences among groups. The only differences observed for water intake were decreases in the 1 µg-injected chicks (relative to vehicle) at 60 and 90 minutes post-injection (Figure 3.4B).

Food and water intake in AZA-injected fasted LWS chicks

When chicks were fasted prior to injection, the 25 µg dose of AZA was associated with increased food intake at 30 through 360 minutes post-injection (Figure 3.5A). The only other difference was an increase in food intake for the 5 µg-injected group at 360 minutes post-injection. There were no effects of AZA on water intake in fasted chicks at any of the time points (Figure 3.5B).

AZA injection on DOH and NPY injection on day 5: food and water intake in LWS chicks

Chicks that were injected with vehicle or 1 µg of AZA on DOH responded to NPY at day 5 with a similar increase in food intake (Figure 3.6A). Chicks that were injected with 5 or 25 µg of AZA on DOH did not respond to the ICV injection of NPY at day 5 post-hatch.

When food intake data were separated by AZA treatment within NPY treatment group, food intake was similar among AZA groups that received the vehicle on day 5 post-hatch (Figure 3.6B). However, there was an AZA dose-dependent decrease in food intake at 60 minutes post-injection in chicks that were injected with NPY at day 5, with the least amount of food consumed in response to the 5 and 25 µg doses of AZA (Figure 3.6B).

Discussion

Although it is well established that certain chemicals and nutrients can alter DNA methylation and thereby alter gene expression and ultimately behavior, the effects of direct administration of these chemicals on feeding behavior is unknown. It is also unclear whether such molecules can exert effects on feeding behavior that are independent of epigenetic-modifying effects. In the present study, we measured the effects of two such chemicals, SAM and AZA, on food and water intake using an avian model that is predisposed to anorexia. Evaluated were both direct and longer-term effects on feeding behavior. SAM and AZA were administered directly into the brain and in addition to quantifying food consumption, we evaluated the responsiveness to injection of a potent orexigenic factor to assess whether they had affected appetite regulation. Interestingly, SAM and AZA exerted different effects on food intake, both immediate and persistent, that were not independent of the feeding states of the chick. There were few effects on water intake that were likely prandial, thus this discussion will focus primarily on food intake effects and mechanisms.

SAM is the primary endogenous methyl donor and is critical for normal cellular methyl metabolism. Studies with various animal models have shown that dietary supplementation with methyl donors and participants in methyl metabolism can alter hypothalamic epigenetic regulation and thus affect gene expression and behavior [38]. Rats that consumed a high-fat diet for 5 weeks displayed changes in hypothalamic NPY and pro-opiomelanocortin (POMC) promoter methylation that were inversely correlated with mRNA abundance [39]. High-fat diets fed during pregnancy led to elevated levels of POMC DNA methylation in rat pups and an obese phenotype at a later age [40]. Palou et al. [41] demonstrated that leptin supplementation to neonatal rats decreased POMC methylation and increased POMC mRNA abundance in response to consumption of a high-fat diet, which was associated with decreases in body weight and energy intake in adults. However, to our knowledge, hypothalamic DNA methylation in the context of appetite regulation is lacking in birds. Additionally, of relevance to putative effects on appetite regulation, SAM is required for the synthesis of monoamine neurotransmitters such as norepinephrine, dopamine, and serotonin, and other cellular pathways, as reviewed in [42]. Thus, it is conceivable that provision of SAM to the central nervous system might influence feeding behavior acutely.

In the present study, SAM injection on day 5 post-hatch did not influence food or water intake of either fed or fasted LWS chicks. In a study with 3-day-old layer-type chicks in the fed state (effects were not tested in the fasted state), ICV injection of methionine but not SAM affected food intake at 30 minutes post-injection, with the high doses of methionine increasing food intake [36]. It was stated that a 100 µg dose of SAM "tended" to decrease food intake, but the difference was not significant [36]. We thus based our initial dose tests on the doses used in the layer chick study and arrived at 0.1, 1, and 10 µg doses as ones that would affect physiology without being toxic to the animal. We conclude from the data presented herein that SAM does not appear to have a direct short-term effect on feeding behavior in chicks.

However, when SAM was injected at day of hatch, we observed differences in food intake among SAM-injected groups at day 5 post-hatch, as well as responsiveness to the food intake-stimulating effect of NPY. Regardless of NPY treatment, chicks that received the middle dose of SAM ate more than other chicks and this appeared to accentuate the orexigenic response to NPY. Previously, we demonstrated that DOH stress-induced blunted responses to NPY at day 5 were due to increased CRF gene expression in the hypothalamus [16, 17] that was associated with hypomethylation at a site near the CRF promoter that disrupted binding of a transcriptional repressor [18]. It is thus tempting to speculate that because such changes were associated with

DNA methylation, provision of a methyl donor would potentially alter expression of anorexigenic factors such as CRF, leading to a more sensitized response to orexigenic factors at a later age. Regardless of NPY treatment, the greater intake of 1 μg-injected chicks relative to other SAM treatments suggests that while SAM did not have an immediate effect on food intake, it elicited changes in appetite-related pathways that were manifested at a later age.

In contrast to SAM, the methylation inhibitor AZA affected food intake within 1 hour post-injection, with opposite effects on food intake observed under different feeding states. In fed chicks, AZA suppressed food intake, whereas when chicks had been fasted for 3 hours, AZA had a stimulatory effect. This observation is intriguing because there are far fewer factors in nature that stimulate appetite than those that induce satiety. Important to note is that the orexigenic effect in fasted chicks appeared earlier and persisted for a longer duration than the anorexigenic effect in fed chicks. Typically, these types of feeding studies in the chick model are conducted within a period of 180 minutes during which there is cessation of the effect and restoration of homeostatic feeding. It is uncommon to observe an effect (following a single ICV injection) at a later time. However, because of the potential for these molecules to elicit long-term effects due to their role in regulating epigenetic modifications, we also included a 360-minute time point for data collection. Chicks that were fasted prior to injection continued to have greater food intake at 360 minutes post-injection. That the high dose elicited an increase in food intake as early as 30 minutes that continued through 6 hours suggests that the injection during the fasting state accentuated orexigenic signaling pathways while having the opposite effect during the fed state.

Consistent with this, chicks that were injected with AZA at DOH displayed a reduced responsiveness to the orexigenic effects of NPY at day 5 post-hatch. When results were analyzed within NPY treatment to tease out effects of the AZA on basal levels of food intake, there did not appear to be a difference among AZA dosages. However, in NPY-injected chicks, there was clearly a decrease in food intake in response to NPY in chicks that received the higher doses of AZA. It is enticing to conjecture that the opposite effects of DOH injection of SAM and AZA relate to methylation of appetite-associated factors, such as hypothalamic POMC or CRF. The administration of these chemicals has the potential to rewire the appetite circuitry by affecting methylation of genes encoding key factors in feeding pathways. Elucidating such mechanisms was beyond the scope of the present study but is certainly a target for future research.

Conclusions

In conclusion, we were able to identify methylation-regulating chemicals as novel regulators of appetite in animals, using a unique avian model of hypophagia, the LWS line of chicks. We evaluated effects of centrally injecting a methyl donor (SAM) and methylation inhibitor (AZA) on feeding behavior, immediately after injection, and days after injection, and observed multiple effects. While SAM did not directly affect feeding behavior after injection, it increased feeding and enhanced the orexigenic response to NPY 5 days later. Conversely, AZA had an inhibitory effect on food intake immediately after injection in fed chicks, but the opposite effect when they were fasted prior to injection. Compared with the result that SAM affected orexigenic signaling, when chicks were injected with AZA at hatch, there was decreased sensitivity to a potent orexigenic factor, NPY, 5 days later. Thus, SAM and AZA exerted different effects on food intake, with some being immediate and feeding-dependent, while others persisted for days after injection. To our knowledge, this is the first report, in any species, of such effects of these chemicals on feeding behavior.

Funding

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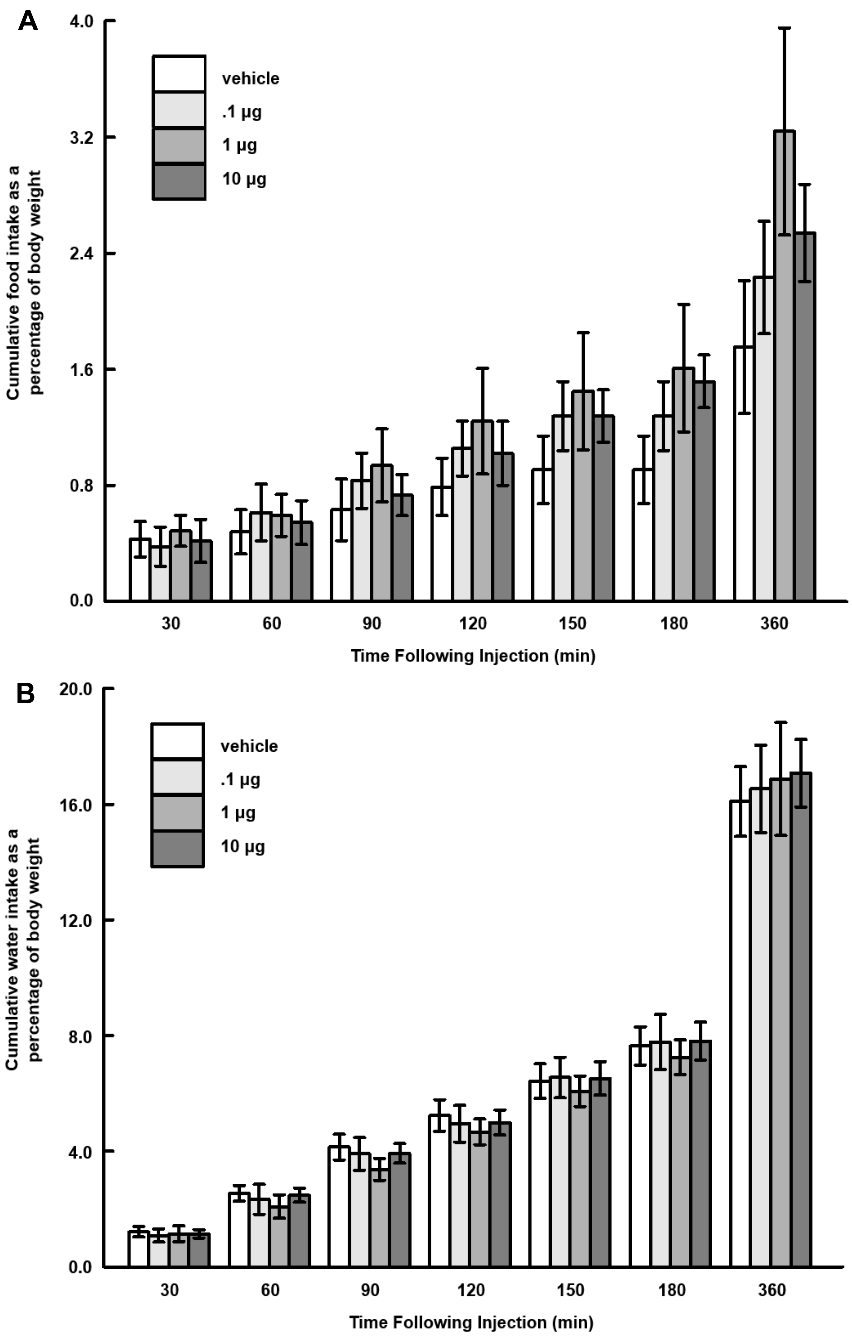

Figure 3.1. Cumulative food and water intake in SAM-injected fed LWS chicks.

Cumulative food (A, n = 9 per group) and water (B, n = 8-10 per group) intake of 5-day old fed LWS chicks that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM on day 5 post-hatch. Values represent means ± standard errors.

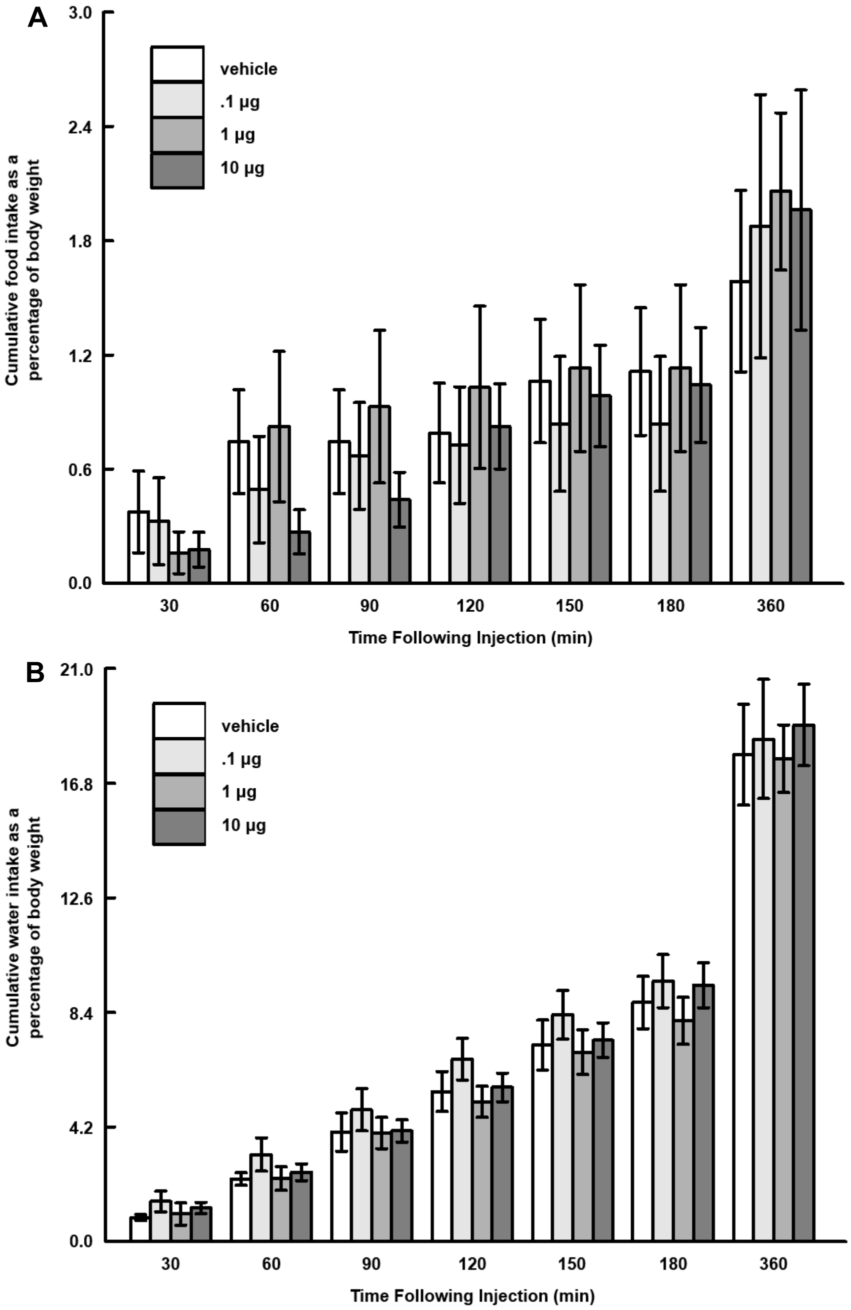

Figure 3.2. Cumulative food and water intake in SAM-injected fasted LWS chicks.

Cumulative food (A, n = 9-10 per group) and water (B, n = 9-10 per group) intake of 5-day old fasted LWS chicks that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM on day 5 post-hatch. Values represent means ± standard errors.

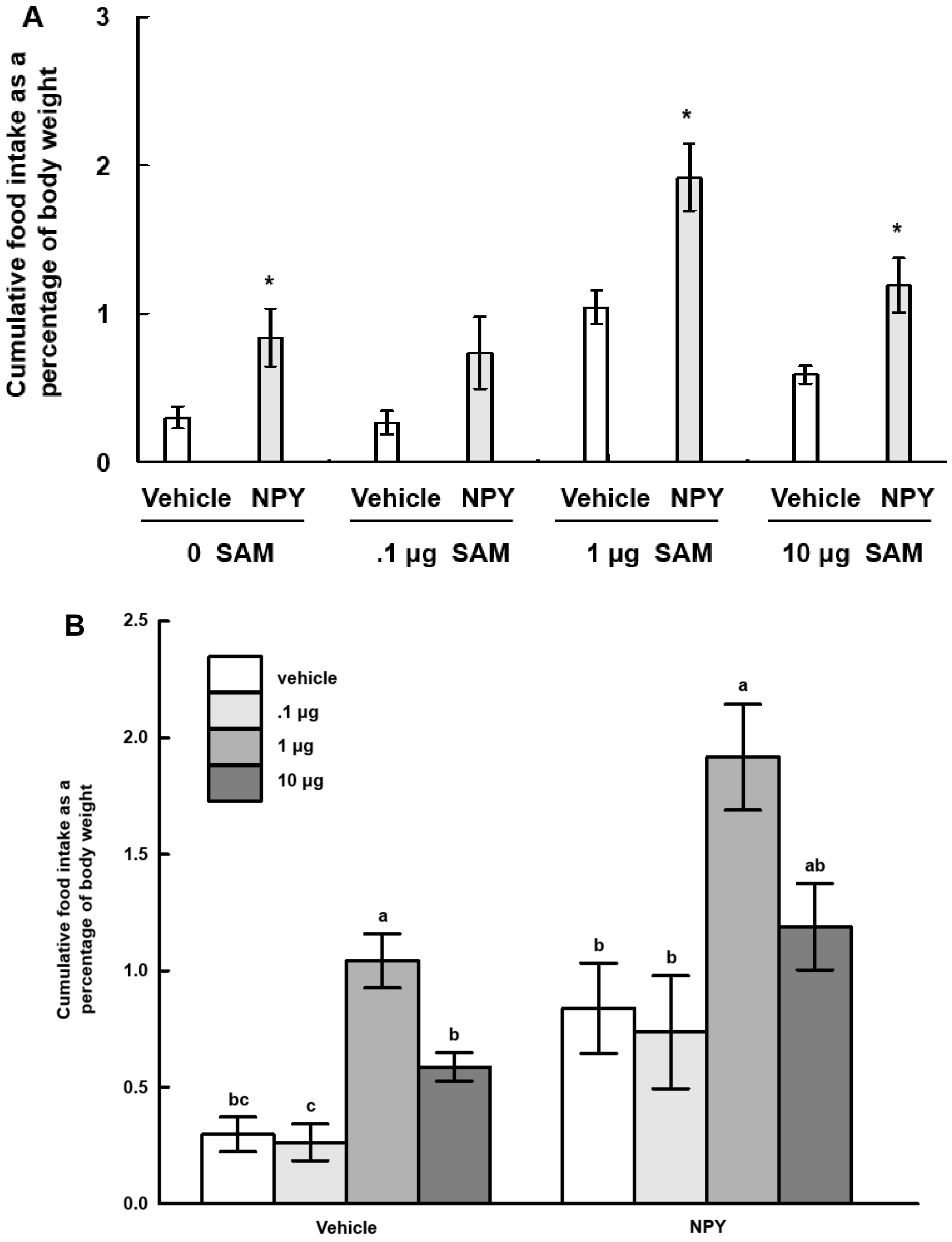
A
Cumulative food intake as a percentage of body weight
0
1
2
3
*
*
*
*
Vehicle
NPY
Vehicle
NPY
Vehicle
NPY
Vehicle
NPY
0 SAM
.1 µg SAM
1 µg SAM
10 µg SAM
B
Cumulative food intake as a percentage of body weight
0.0
0.5
1.0
1.5
2.0
2.5
vehicle
.1 µg
1 µg
10 µg
bc
c
a
b
b
b
a
ab
Vehicle
NPY

Figure 3.3. Food intake in NPY-injected LWS chicks that received SAM on the day of hatch.

Food intake at 60 minutes post-injection of vehicle or neuropeptide Y (NPY) in 5-day old LWS chicks that received a single intracerebroventricular injection of vehicle, 0.1, 1, or 10 μg of SAM on the day of hatch. Values represent means ± standard errors. *$P < 0.05$, different from vehicle within a SAM group (A). Unique letters denote a significant difference ($P < 0.05$) among SAM groups at baseline or within NPY treatment, Tukey's test (B).

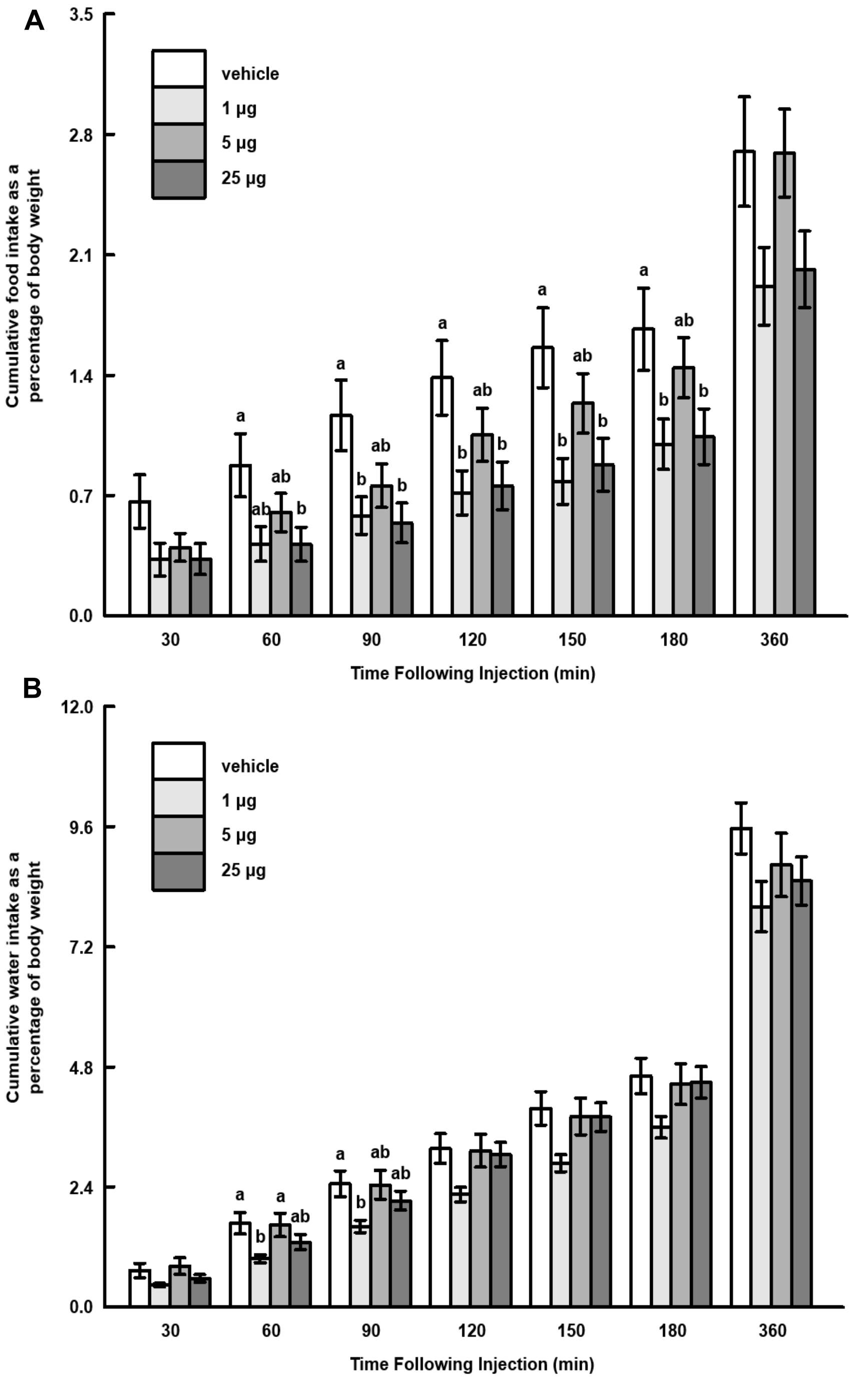
A
Cumulative food intake as a percentage of body weight
3.5
2.8
2.1
1.4
0.7
0.0
vehicle
1 µg
5 µg
25 µg
a
ab
ab
b
a
b
ab
b
a
b
ab
b
a
b
ab
b
a
b
ab
b
30
60
90
120
150
180
360
Time Following Injection (min)
B
Cumulative water intake as a percentage of body weight
12.0
9.6
7.2
4.8
2.4
0.0
vehicle
1 µg
5 µg
25 µg
a
b
a
ab
a
b
ab
ab
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 3.4. Cumulative food and water intake in AZA-injected fed LWS chicks.

Cumulative food (A, n = 21-23 per group) and water (B, n = 25-28 per group) intake of 5-day old fed LWS chicks that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 μg of AZA on day 5 post-hatch. Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

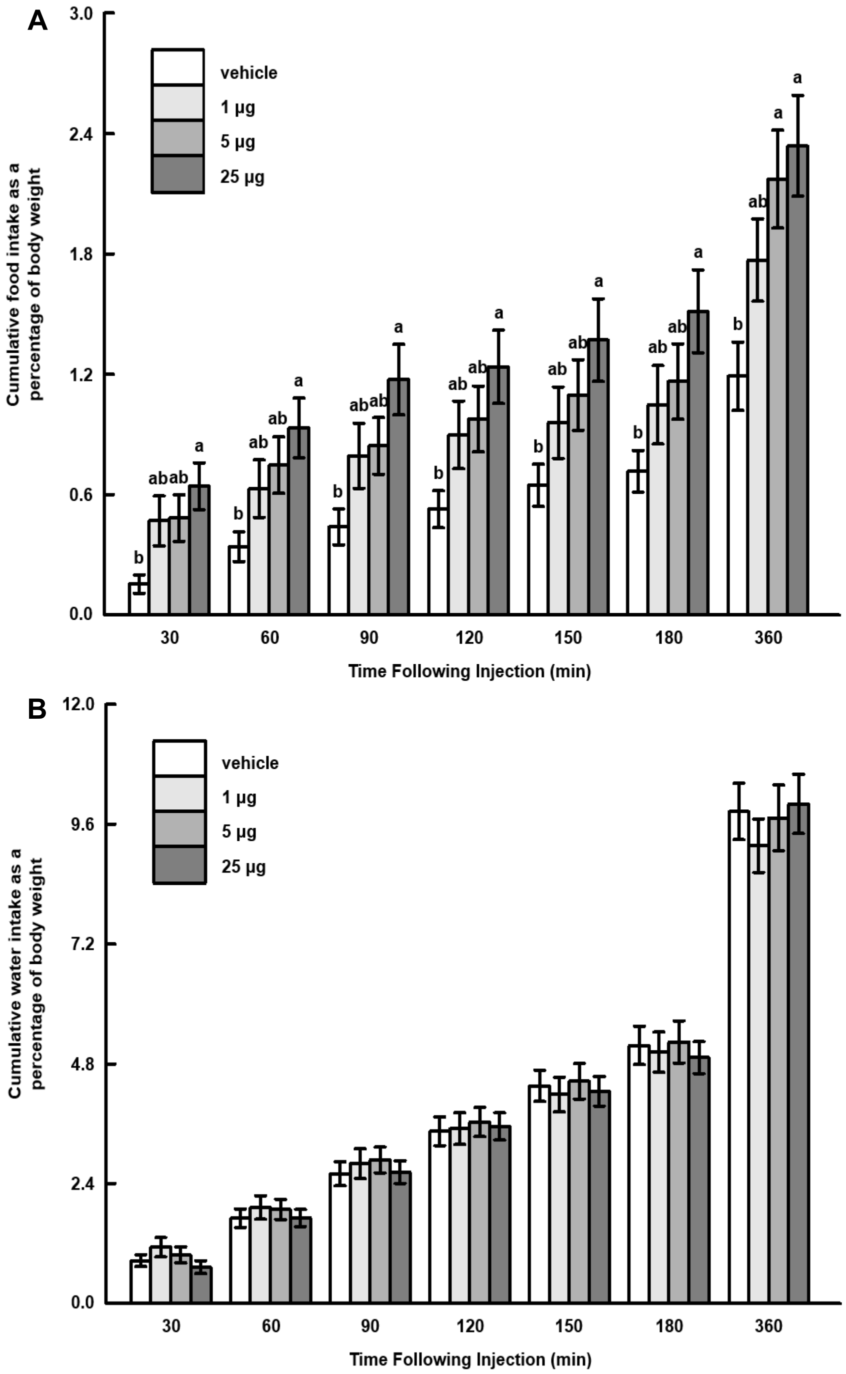
A
Cumulative food intake as a percentage of body weight
3.0
2.4
1.8
1.2
0.6
0.0
vehicle
1 µg
5 µg
25 µg
b
ab
ab
a
b
ab
ab
a
b
ab
ab
a
b
ab
ab
a
b
ab
ab
a
b
ab
ab
a
b
ab
a
a
30
60
90
120
150
180
360
Time Following Injection (min)
B
Cumulative water intake as a percentage of body weight
12.0
9.6
7.2
4.8
2.4
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 3.5. Cumulative food and water intake in AZA-injected fasted LWS chicks.

Cumulative food (A, n = 25-31 per group) and water (B, n = 30-31 per group) intake of 5-day old fasted LWS chicks that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 μg of AZA on day 5 post-hatch. Values represent means ± standard errors. Unique letters denote a significant difference (*P* < 0.05) among groups within a time point, Tukey's test.

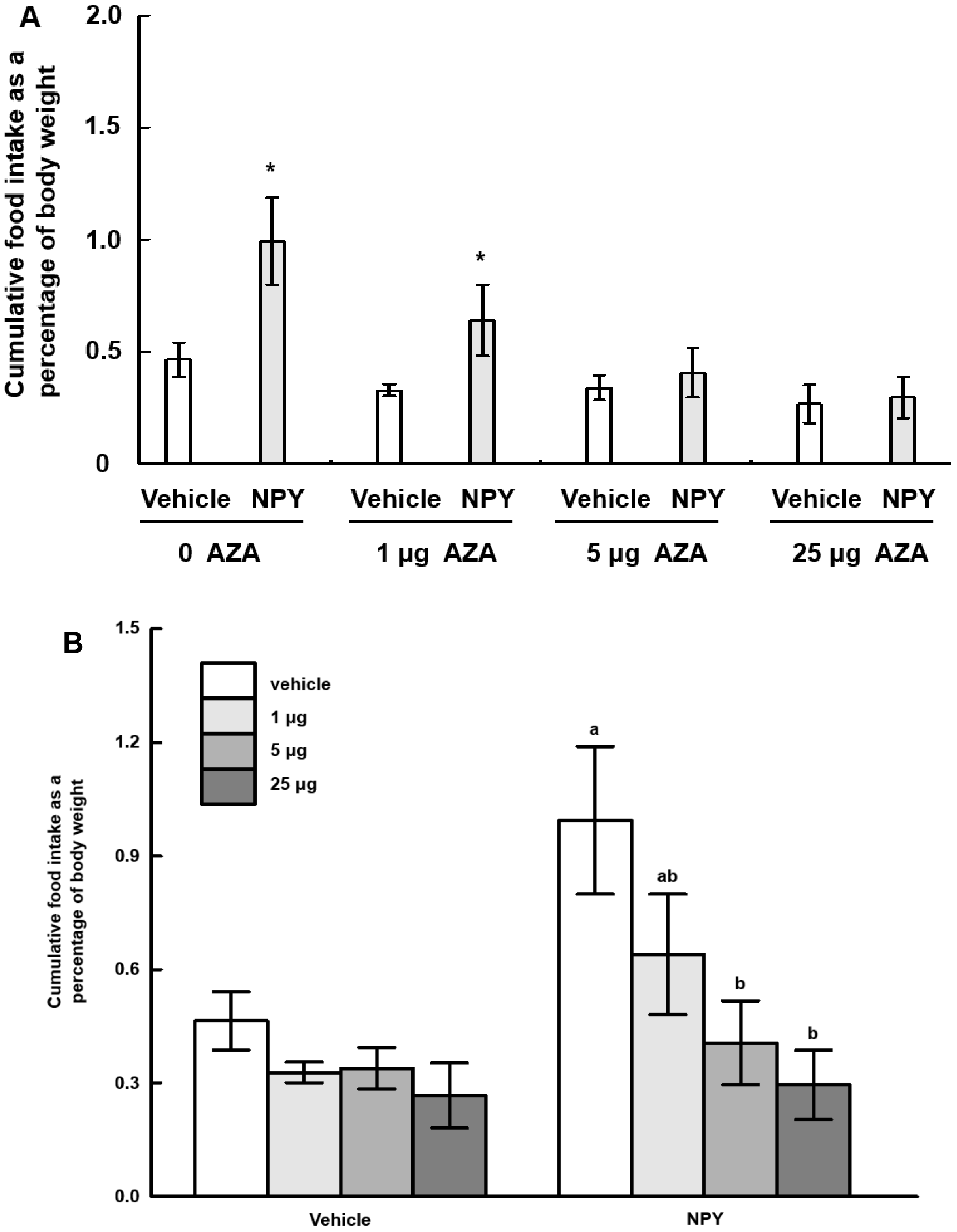
A
Cumulative food intake as a percentage of body weight
2.0
1.5
1.0
0.5
0
*
*
Vehicle
NPY
Vehicle
NPY
Vehicle
NPY
Vehicle
NPY
0 AZA
1 µg AZA
5 µg AZA
25 µg AZA
B
Cumulative food intake as a percentage of body weight
1.5
1.2
0.9
0.6
0.3
0.0
vehicle
1 µg
5 µg
25 µg
a
ab
b
b
Vehicle
NPY

Figure 3.6. Food intake in NPY-injected LWS chicks that received AZA on the day of hatch.

Food intake at 60 minutes post-injection of vehicle or neuropeptide Y (NPY) in 5-day old LWS chicks that received a single intracerebroventricular injection of vehicle, 1, 5, or 25 μg of AZA on the day of hatch. Values represent means ± standard errors. *$P < 0.05$, different from vehicle within an AZA group (A). Unique letters denote a significant difference ($P < 0.05$) among AZA groups at baseline or within NPY treatment, Tukey's test (B).

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Chapter 4: DNA methylation-modifiers reduced food intake in juvenile chickens (*Gallus gallus)* and Japanese quail (*Coturnix japonica*)

Abstract

S-Adenosylmethionine (SAM) is the major endogenous methyl donor for methyltransferase reactions, while 5-Azacytidine (AZA) is a synthetic drug that inhibits DNA methyltransferase activity. Both molecules can thus influence DNA methylation patterns in an organism and thereby affect gene expression and ultimately behavior in the long-term. Whether or not effects on behavior are exerted on a shorter time scale is unclear. The goal of this study was to explore the direct effects of SAM and AZA on feeding behavior, using chicken and Japanese quail as the animal models. Fed or 180 minute-fasted broiler chickens (at day 4 post-hatch) or 360 minute-fasted quail (at day 7 post-hatch) were intracerebroventricularly injected with SAM or AZA and food intake was measured for 360 minutes. For chickens, there was no effect of AZA, at any dose, on food intake in either fed or fasted chicks at any time point. In contrast, 1 and 10 µg doses of SAM reduced food intake in fed chicks at 60 minutes post-injection. In fasted chicks, although there were no differences for the first 30 minutes post-injection, SAM suppressed food intake during the second 30-minute period. For quail, however, AZA (25 µg dose) decreased food intake at 60 and 150 to 360 minutes post-injection in fasted birds. A reduction in food intake was also observed at 120 and 360 minutes post-injection in fed quail in response to 5 and 25 µg doses of AZA, respectively. SAM had no effect when quail were fasted, whereas 1 µg dose of SAM suppressed food consumption during the third 30-minute period. Thus, when administered directly into the central nervous system, SAM may act as a transient appetite

suppressant in both chicken and quail, whereas the direct inhibitory effect of AZA on food consumption depends on species and nutritional states.

Key words: S-Adenosylmethionine, 5-Azacytidine, Chicks, Japanese Quail, Methyl, Anorexia, Intracerebroventricular

Introduction

Appetite regulation is the result of a complex interplay between pathways that integrate at the level of the hypothalamus. Many of these pathways involve various hormones and neuropeptides originating from different organs and brain regions that communicate the energy and health status of the organism [1, 2]. Novel regulators of appetite continue to be identified. To address the mounting obesity epidemic and understand the molecular basis for eating disorders, it is critical to have a complete understanding of the physiological pathways that affect feeding behavior.

Much of the knowledge on appetite regulation in avian species was derived from studies with chickens (*Gallus gallus*). Broiler chickens have been bred for efficient meat production for over a century [3], with extensive studies focusing on improving feeding efficiency and welfare. Selection for growth-related traits led to correlated effects on feeding behavior and appetite regulation, with chickens consuming food beyond maintenance and growth energy requirements and being prone to metabolic disorders later in life. Broilers can thus serve as a model to understand compulsive eating behavior and obesity. On the other hand, Japanese quail (*Coturnix japonica*), although well-adapted to both laboratory conditions and handling procedures, is a less intensely-selected species, which may provide evolutionary perspective on physiological mechanisms that are conserved between birds and mammals.

Our group has identified a number of appetite regulators, mostly peptides, that when directly administered into the central nervous system of chicks, influence food consumption and hypothalamic physiology. For example, intracerebroventricular (ICV; into the left lateral ventricle) administration of neuropeptide Y (NPY) potently stimulated feed intake [4], while melanocortins (such as α- and β-melanocyte-stimulating hormone (MSH)) [5], corticotropin-releasing factor (CRF) [6] and mesotocin [7] exerted anorexigenic effects, to name a few. In all of those studies, changes in feeding behavior were accompanied by distinct changes in neuronal activation and gene expression of appetite-associated factors in hypothalamic nuclei, such as the arcuate nucleus and paraventricular nucleus. We have also identified novel regulators of appetite, that previously had no appetite-related roles ascribed to their function in any species, including visfatin [8] and xenopsin [9]. While most factors that exert effects on feeding behavior are proteinaceous, it is clear that other bioactive molecules have the potential to elicit behavioral effects as well.

S-Adenosylmethionine (SAM), the principal methyl donor in the body [10], is an essential component of the methionine cycle [11, 12]. It can directly influence DNA methylation and thereby affect gene expression, and there are many rodent studies focusing on the effects of methyl donors on DNA methylation as a result of dietary deficiency [13, 14] or supplementation [15, 16]. Researchers evaluated the influence of methyl analogues on feeding behavior in Leghorn chicks. Both L- and D- methionine could stimulate food intake, whereas SAM appeared to suppress feeding at a high dose (100 μg), although no significance was detected [17]. These results were controversial, since methionine could be converted into SAM and thereby provide a methyl group to other substrates, leaving the role of central-injected SAM on feeding behavior remaining unclear.

At the other end of the spectrum, there are molecules that can inhibit or prevent DNA methylation. 5-Azacytidine (AZA) was first synthesized almost 60 years ago [18], and was demonstrated to be a chemotherapeutic agent [19]. With its ability to incorporate into DNA at high concentrations, AZA inhibited DNA synthesis in tumor cells [20]. However, at low doses, AZA irreversibly binds to DNMT and inhibits its activity, leading to reductions in maintenance DNA methylation [21, 22].

The objective of this study was thus to evaluate the effects of two molecules known for their role in DNA methylation, SAM and AZA, on feeding behavior in broiler chicks and Japanese quail.

Materials and methods

Animals

Day-of-hatch Hubbard x Cobb-500 broiler chicks were obtained from a local hatchery. Chicks were group-caged the same day and then individually caged on day 2 post-hatch in a room at a constant temperature of 30 ± 2 °C and 50 ± 5% relative humidity, with 24-hour of light. Chicks were briefly handled for 5 seconds, once daily, to adapt to handling and minimize stress, and had ad libitum access to food (energy: 3,000 kcal metabolizable energy/kg and 21.5% crude protein) and water.

Japanese quail were bred and hatched in our vivarium. After removal from the hatcher, chicks were group-caged in a brooder for 4 days, then individually caged in a separate room (35 ± 1 °C and 50 ± 5% relative humidity), with a 14-hour light/10-hour dark cycle. The individual cages allowed visual and auditory contact between chicks. Quail were provided ad libitum access to a mash starter diet (energy: 2,900 kcal metabolizable energy/kg and 24% crude protein) and water. Quail handling was different from chickens and the details are given below.

Each quail was acclimated twice daily once individually caged. The acclimation procedure consisted of the chick being removed from its cage, being briefly transferred to two different Plexiglas boxes, having its head inserted into a restraining device for 5 seconds, transferred into another Plexiglas box, then placed into the restraining device once again. Upon completion of this procedure, the chick was returned to its home cage. The restraining device was a block of hardened clay, which had been molded around the head of an 8-day-old quail chick cadaver and was designed such that the entire area of the frontal bone was not obstructed and was designed with two air vents positioned to end at the nostrils. This device allowed for a free-hand ICV injection. The acclimation procedure was conducted leading up to the day of data collection.

All chicken experiments were conducted at 4 days post-hatch, whereas all quail experiments were done at 7 days post-hatch. Experimental procedures were performed according to the National Research Council Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Intracerebroventricular injection procedure

On the day of the experiment, birds were ICV-injected using a method that does not appear to induce physiological stress, adapted from [23-25]. The head of the bird was briefly inserted into a restraining device that left the cranium exposed to allow for free-hand injection. Anatomical landmarks were determined visually by using the restraining device and plastic tubing sheath. The quail restraining device coordinated the injection point at 2 mm anterior to the coronal suture and 0.75 mm lateral from the sagittal suture. The plastic tubing sheath over the needle controlled the injection depth at 1.5 mm. Injection coordinates for chickens, however, were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. The needle remained at injection depth in the un-anesthetized bird for 5 seconds post-injection to reduce backflow. Chicks were assigned to treatments at random. SAM or AZA (Sigma, St. Louis, MO, USA) was dissolved in chicken artificial cerebrospinal fluid [26] as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine the presence of dye in the lateral ventricle. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex was determined visually by dissection and gonadal inspection at the time of decapitation.

Experiment 1: food and water intake in SAM-injected fed chickens and quail

Using a randomized complete design, chicks were assigned to receive 0 (vehicle only), 0.1, 1, or 10 µg dose of SAM, the doses of which were based on a former study [17], by ICV injection (in each group: n = 10 for chicken and n = 12 for quail). After injection, chicks were returned to their individual home cages and given ad libitum access to both food and water. Food and water intake were monitored (0.01 g) every 30 minutes for the first 180 minutes post-injection and at 360 minutes post-injection. Water weight (g) was converted to volume (ml: 1 g = 1 ml). Food and water intake were calculated as a percentage of body weight to better account for the variation in body weights of quail.

Experiment 2: food and water intake in SAM-injected fasted chickens and quail

Procedures were identical to Experiment 1, except that chickens and quail were fasted for 180 and 360 minutes, respectively, before SAM injection, while access to water remained during fasting.

Experiment 3: food and water intake in AZA-injected fed chickens and quail

Procedures were identical to Experiment 1, except that chicks were treated with 0 (vehicle only), 1, 5, 25 µg dose of AZA, the doses of which were based on a former study [27].

Experiment 4: food and water intake in AZA-injected fasted chickens and quail

Procedures were identical to Experiment 3, except that chickens and quail had ad libitum access to water but no access to food for 180 and 360 minutes, respectively, prior to AZA injection.

Statistical analyses

Results are expressed as means ± standard errors. Data were analyzed using analysis of variance (ANOVA) with SAS 9.4 (SAS institute, Cary, NC, USA) using the GLM procedure within each time point, with the statistical model including the main effect of dose. When dose effects were significant, Tukey's method of multiple comparison was used to separate the means within each time point. Statistical significance was set at $P < 0.05$ for all experiments.

Results

Food and water intake in SAM-injected fed chickens and quail

At 60 minutes post-injection, food intake significantly decreased in chickens that were injected with 1 and 10 µg doses of SAM (Figure 4.1A). There were no differences detected at any other time points or on a non-cumulative basis (Figure 4.1A and 4.1B). Water intake was not affected by SAM injection in fed chicks (Figure 4.1C and 4.1D).

SAM did not affect food intake at any time points on a cumulative basis in quail, but the 1 µg dose of SAM decreased food intake during the third 30-minute period post-injection on a non-cumulative basis (Figure 4.2A and 4.2B). Water intake was not affected on a cumulative basis (Figure 4.2C), but was suppressed by 0.1 µg of SAM at 180 minutes post-injection on a non-cumulative basis (Figure 4.2D).

Food and water intake in SAM-injected fasted chickens and quail

For fasted chickens, there were no effects of SAM on food intake (Figure 4.3A). On a non-cumulative basis, however, 1 µg of SAM suppressed food intake at 60 minutes post-injection (Figure 4.3B). We also detected a difference in food intake at 360 minutes post-injection between the 1 and 10 µg SAM-injected chicks. For water intake, there was an increase in response to the 10 µg dose of SAM at 60 minutes post-injection, compared to the vehicle group (Figure 4.3C). At 120 minutes post-injection, 0.1 µg of SAM decreased water intake on a non-cumulative basis (Figure 4.3D).

SAM had no effects on quail food intake (Figure 4.4A and 4.4B) or cumulative water intake (Figure 4.4C). However, a difference in water intake was detected between the 0.1 and 1 µg doses of SAM at 120 minutes on a non-cumulative basis (Figure 4.4D).

Food and water intake in AZA-injected fed chickens and quail

Neither food nor water intake were changed in fed chickens in response to AZA injection on a cumulative basis (Figure 4.5A and 4.5C). On a non-cumulative basis, however, the 1 µg dose of

AZA increased food intake at 150 minutes post-injection (Figure 4.5B), while the 25 µg dose of AZA decreased water intake at 360 minutes post-injection (Figure 4.5D).

In fed quail, the 25 µg dose of AZA decreased food intake at 360 minutes post-injection (Figure 4.6A). On a non-cumulative basis, this effect was also observed, and quail injected with 5 µg of AZA ate less than vehicle-injected birds at 120 minutes post-injection (Figure 4.6B). Although water intake was not affected by AZA on a cumulative basis (Figure 4.6C), on a non-cumulative basis, 1 µg of AZA caused quail to drink less compared with the vehicle-injected group at 120 minutes post-injection (Figure 4.6D). Later at 180 minutes, more water was ingested in the 1 µg than the 5 µg dose group.

Food and water intake in AZA-injected fasted chickens and quail

Food and water intake were not unaffected by AZA in fasted chickens (Figure 4.7).

In fasted quail, however, the 25 µg dose of AZA decreased food and water intake from 60 to 360 minutes post-injection, although a significant difference in food intake was in comparison to the 5 µg rather than vehicle group at 90 and 120 minutes post-injection (Figure 4.8A and 4.8C). On a non-cumulative basis, quail injected with 25 µg of AZA ate less than those injected with 5 µg of AZA at 60 and 360 minutes post-injection and those in the other two groups at 360 minutes post-injection (Figure 4.8B). Vehicle- and 1 µg AZA-injected quail drank more than 5 and 25 µg AZA-injected quail at 120 and 360 minutes post-injection, respectively (Figure 4.8D). Greater water intake was observed in the 1 µg AZA group compared to the 25 µg dose of AZA at 60 minutes post-injection (Figure 4.8D).

Discussion

The purpose of this study was to evaluate the effects of central administration of two methyl-modifying compounds, SAM and AZA, on food intake in two avian models. To our knowledge, the role of such molecules in appetite regulation is relatively unstudied. We observed a short-term appetite suppressive effect of SAM. At 30 minutes post-injection, both the middle and high doses of SAM tended to suppress appetite in broilers (*P* = 0.07, this *P*-value not shown in Fig. 4.1 because it did not reach significance), while the significant decrease occurred at 60 minutes post-injection. In layer-type Leghorn chicks, a similar tendency for inhibition of feeding behavior was observed, but only in the 100 µg SAM-treated group and not at lower doses (1 and 10 µg) [17], indicating that broilers might have higher sensitivity to central administration of SAM than Leghorn chicks. This difference may be caused by different genetic backgrounds of meat- and egg-type chicks, which have been selected for unique production purposes. In quail, we did not observe any effect when birds were fasted, but decreased food consumption was transiently observed in fed quail at 90 minutes post-injection. These results are consistent with broiler chicks, although quail appeared to be less sensitive than broilers, which might be attributed to species differences and artificial selection pressure. Typically, our feeding studies in chicks terminate at 3 hours post-injection, because by this time there is a return to homeostatic feeding behavior. However, because the effect of SAM on food intake is not well studied and it is known to play a role in epigenetic regulation due to its role as a methyl donor, we also measured food intake at 6 hours post-injection, in order to determine whether there might be a longer-term effect on feeding behavior. However, no differences were detected at 360 minutes post-injection.

Fed chickens treated with SAM ate less within one-hour post-injection. Although food intake was not affected during the first 30 minutes in fasted SAM-injected chicks, it decreased significantly during the second 30-minute period in the 1 μg SAM group. There was also a tendency in both 0.1 and 10 μg SAM groups (*P* = 0.07 and 0.09 respectively, not shown in Fig. 4.2B) for food intake to be reduced at 60 minutes post-injection. Taken together, both feeding and prior fasting resulted in suppression of feeding behavior, indicating that there is an increase in anorexigenic tone.

While central injection of SAM caused a decreased in food intake in fasted chicks, it increased water consumption in the same birds, although the effective doses were slightly different. This suggests that SAM might have opposite effects on appetite and thirst regulation when chicks have undergone fasting.

For AZA treatment in chickens, we found no effects on cumulative food and water intake in either fed or fasted chicks, and only a few differences on a non-cumulative basis. These changes occurred at random time points and were not persistent, which might be caused by compensatory feeding of the birds to maintain the metabolic homeostasis or insensitivity of these broiler chicks due to selection. These results suggest that there are no direct effects of AZA on feeding behavior in broiler chicks.

Japanese quail responded to AZA more robustly, although the responses varied depending on nutritional states. The highest dose of AZA (25 μg) induced anorexia in both fed and fasted quail, but the effective duration was different. Fed quail ate less at 6 hours post-injection, whereas for fasted quail this started at 1 hour post-injection and lasted until the end of the experiment, albeit the significance disappeared at 90 and 120 minutes. A similar study on low body weight selected (LWS) chickens (unpublished work from our group) suggested that the direct effects of AZA correlated with nutritional state, with reduced food intake in fed LWS but increased food intake in fasted LWS. The discrepancy of these two studies might be attributed to species differences and long-term artificial selection. It is worth noting that the anorexigenic effect of AZA was not observed immediately after injection yet became significant in fed and fasted quail at 6 hours post-injection both on a cumulative and non-cumulative basis, suggesting that this effect might involve epigenetic changes modifying appetite-related genes' methylation and expression. Whether this persists to a later age is still unclear and warrants further investigation.

Conclusions

In conclusion, novel regulators of appetite are continuing to be identified. Here we evaluated two molecules related to methylation modification, in broiler chickens and Japanese quail. Central injection of methyl donor SAM suppressed food intake in both fed and fasted chickens and quail. In contrast, methylation inhibitor, AZA, did not affect food intake in either fed or fasted chickens, whereas it decreased food intake in fed and fasted quail, although with differences in the effective duration. Thus, SAM induces transient short-term satiety in chickens and quail, while AZA-induced anorexia depends on species and nutritional states. Both of these molecules may thus elicit direct effects on regulating feeding behavior. Further studies should involve exploration of the associated molecular mechanisms.

Funding

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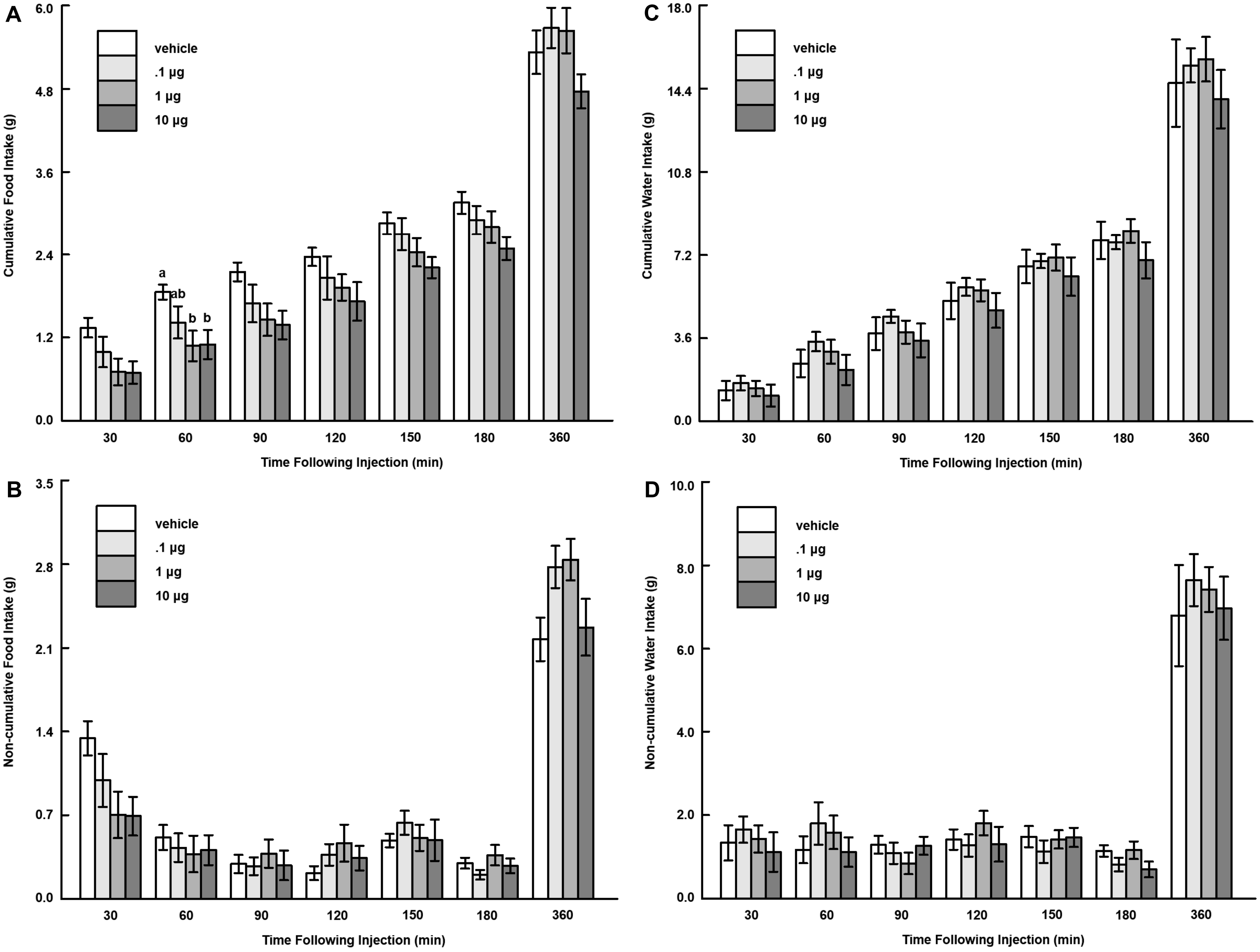
A
Cumulative Food Intake (g)
6.0
4.8
3.6
2.4
1.2
0.0
vehicle
.1 µg
1 µg
10 µg
a
ab
b
b
30
60
90
120
150
180
360
Time Following Injection (min)
B
Non-cumulative Food Intake (g)
3.5
2.8
2.1
1.4
0.7
0.0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
C
Cumulative Water Intake (g)
18.0
14.4
10.8
7.2
3.6
0.0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
D
Non-cumulative Water Intake (g)
10.0
8.0
6.0
4.0
2.0
0.0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 4.1. Food and water intake in SAM-injected fed chickens.

Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4 day-old fed broiler chicks (*Gallus gallus*) that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 μg of SAM (n = 9-10 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

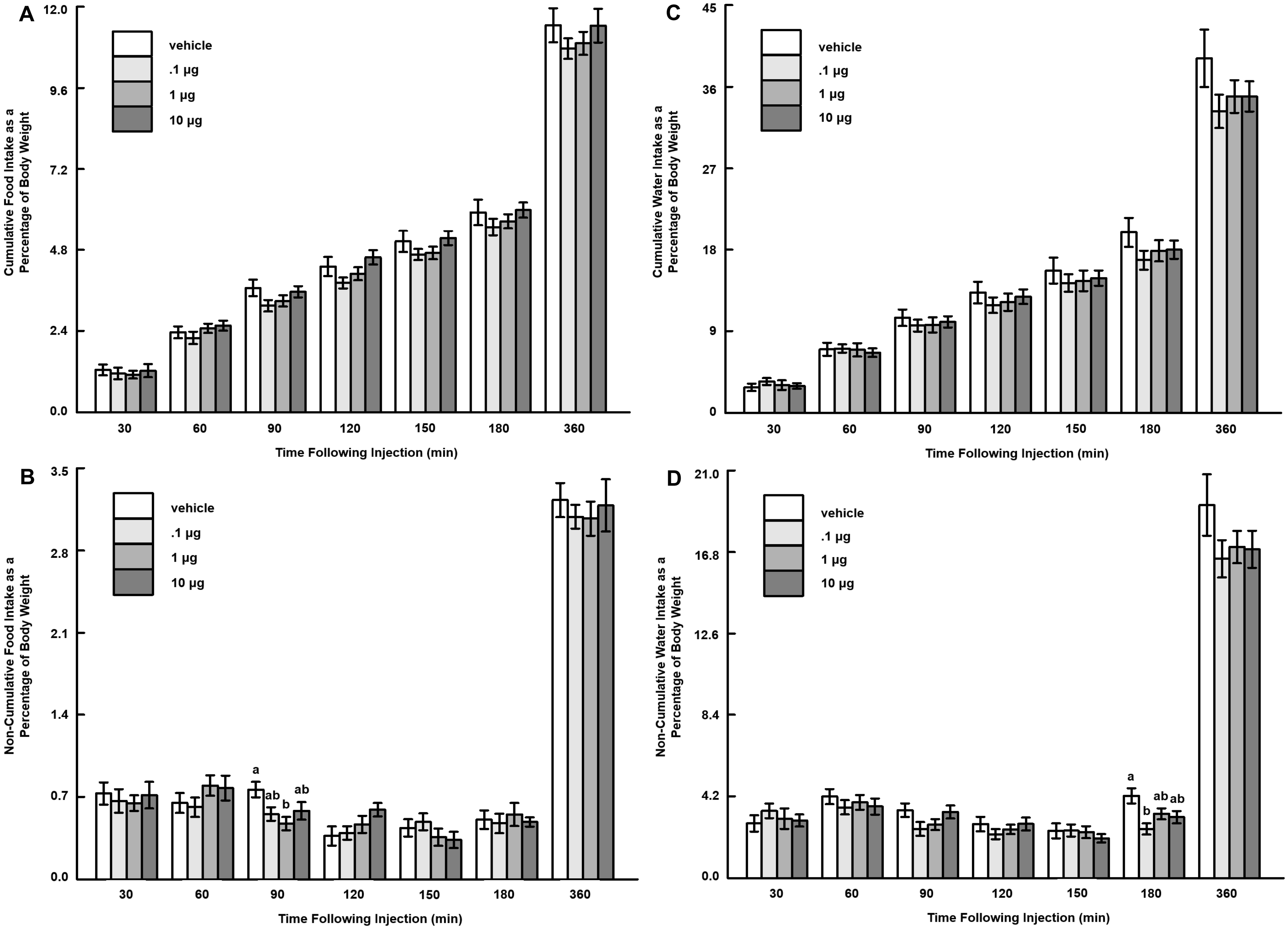
A
Cumulative Food Intake as a Percentage of Body Weight
12.0
9.6
7.2
4.8
2.4
0.0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
B
Non-Cumulative Food Intake as a Percentage of Body Weight
3.5
2.8
2.1
1.4
0.7
0.0
vehicle
.1 µg
1 µg
10 µg
a
ab
b
ab
30
60
90
120
150
180
360
Time Following Injection (min)
C
Cumulative Water Intake as a Percentage of Body Weight
45
36
27
18
9
0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
D
Non-Cumulative Water Intake as a Percentage of Body Weight
21.0
16.8
12.6
8.4
4.2
0.0
vehicle
.1 µg
1 µg
10 µg
a
b
ab
ab
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 4.2. Food and water intake in SAM-injected fed quail.

Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7 day-old fed quail (*Coturnix japonica*) that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 10-12 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 11-12 per group). Values represent means ± standard errors. Unique letters denote a significant difference (*P* < 0.05) among groups within a time point, Tukey's test.

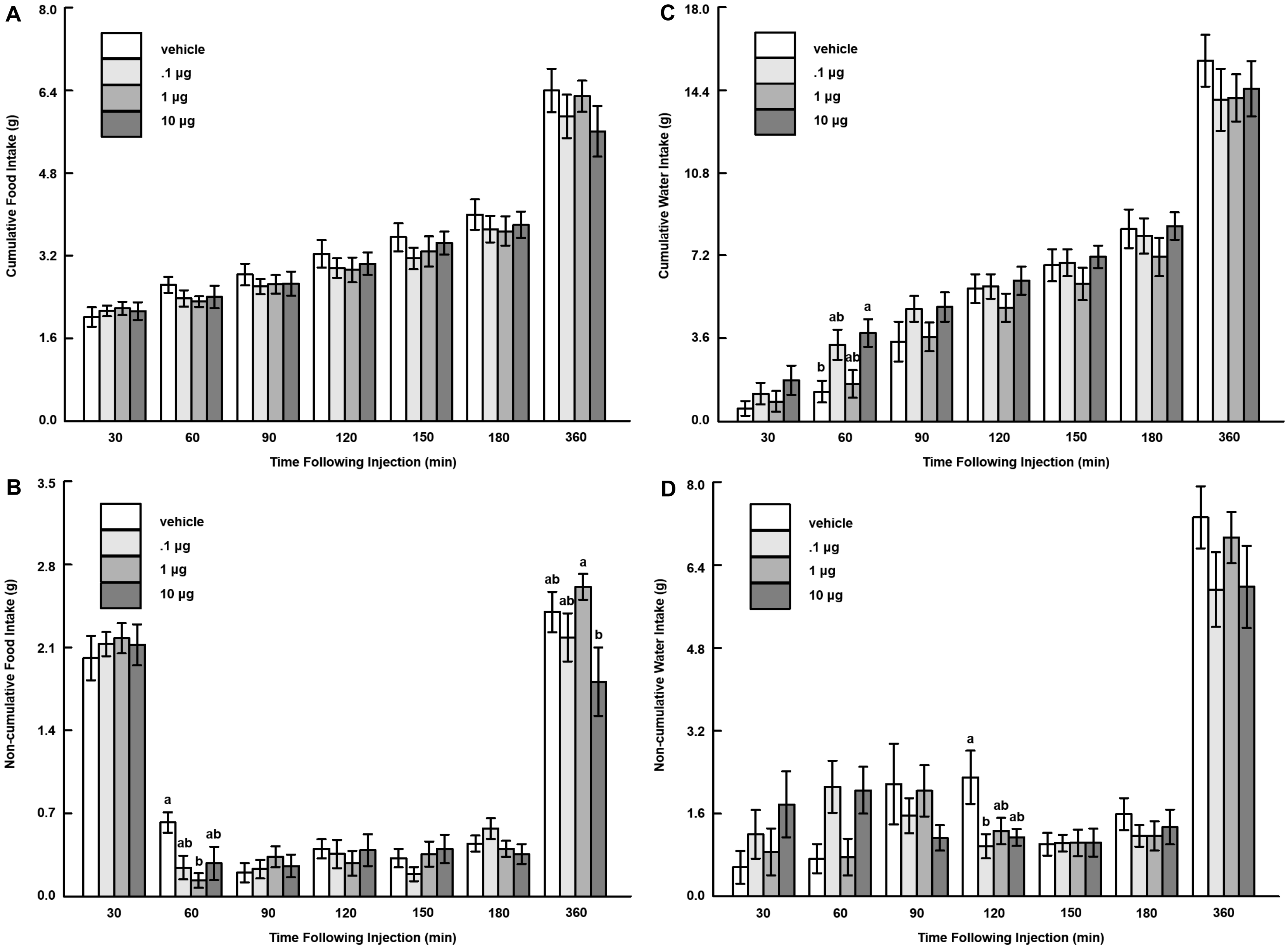
A
B
C
D
vehicle
.1 µg
1 µg
10 µg
Cumulative Food Intake (g)
Non-cumulative Food Intake (g)
Cumulative Water Intake (g)
Non-cumulative Water Intake (g)
Time Following Injection (min)
30
60
90
120
150
180
360
0.0
1.6
3.2
4.8
6.4
8.0
0.7
1.4
2.1
2.8
3.5
3.6
7.2
10.8
14.4
18.0
a
ab
b
ab
ab
ab
a
b
ab
a
b
ab
ab

Figure 4.3. Food and water intake in SAM-injected fasted chickens.

Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4 day-old fasted broiler chicks (*Gallus gallus*) that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 7-9 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

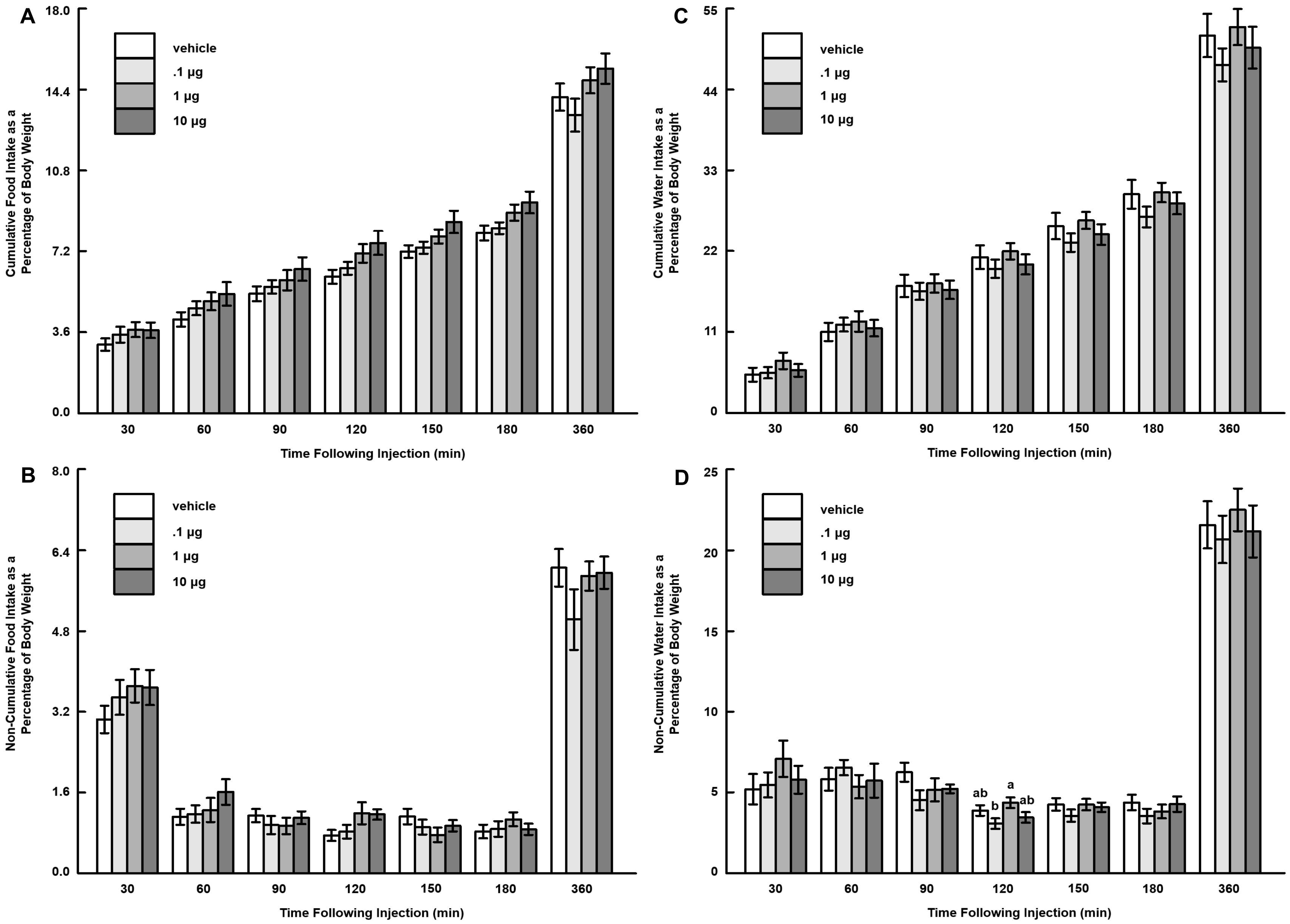
A
Cumulative Food Intake as a Percentage of Body Weight
18.0
14.4
10.8
7.2
3.6
0.0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
B
Non-Cumulative Food Intake as a Percentage of Body Weight
8.0
6.4
4.8
3.2
1.6
0.0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
C
Cumulative Water Intake as a Percentage of Body Weight
55
44
33
22
11
0
vehicle
.1 µg
1 µg
10 µg
30
60
90
120
150
180
360
Time Following Injection (min)
D
Non-Cumulative Water Intake as a Percentage of Body Weight
25
20
15
10
5
0
vehicle
.1 µg
1 µg
10 µg
ab
b
a
ab
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 4.4. Food and water intake in SAM-injected fasted quail.

Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7 day-old fasted quail (*Coturnix japonica*) that were intracerebroventricularly injected with 0 (vehicle), 0.1, 1, or 10 µg of SAM (n = 7-9 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 11 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

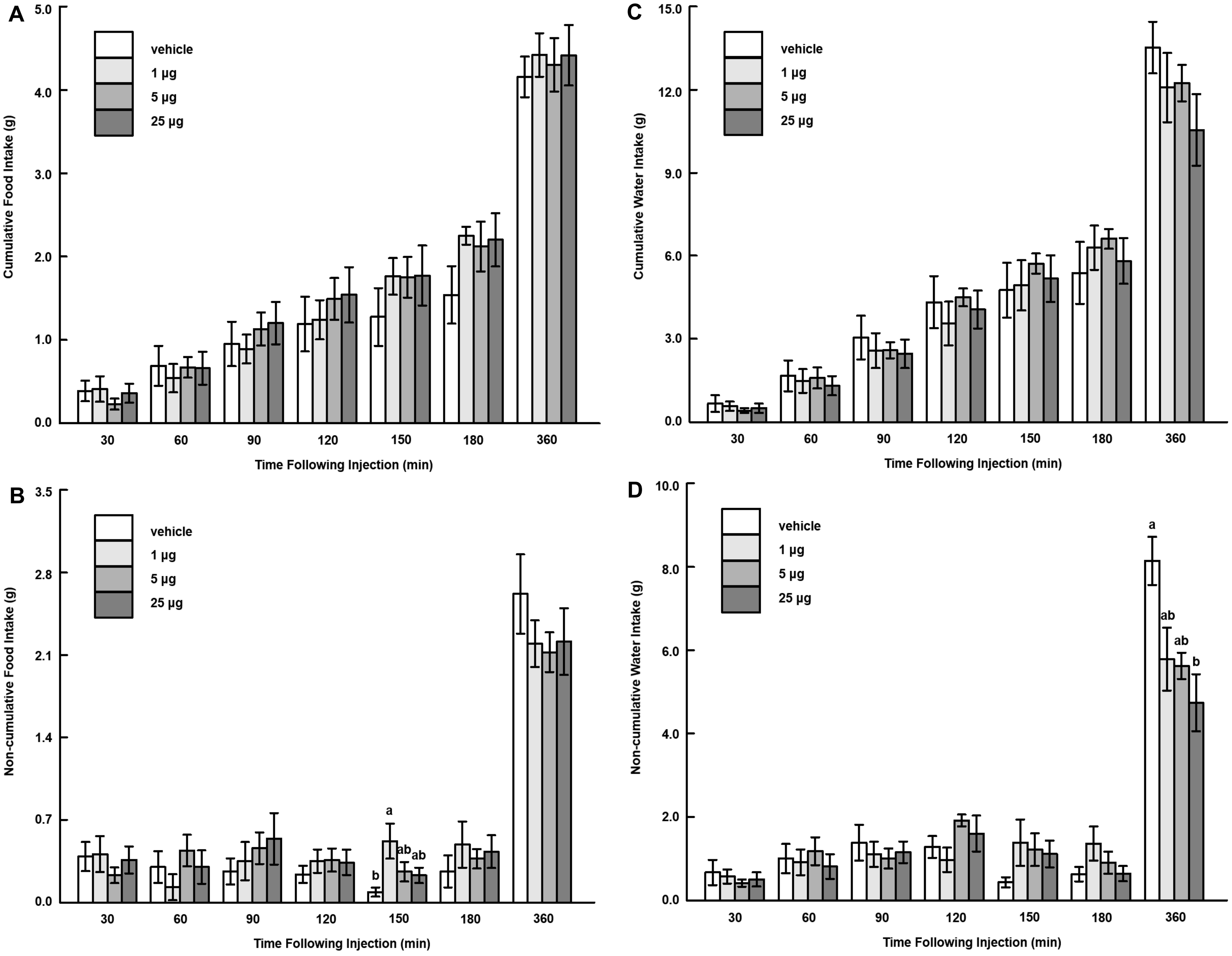
A
Cumulative Food Intake (g)
5.0
4.0
3.0
2.0
1.0
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
C
Cumulative Water Intake (g)
15.0
12.0
9.0
6.0
3.0
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
B
Non-cumulative Food Intake (g)
3.5
2.8
2.1
1.4
0.7
0.0
vehicle
1 µg
5 µg
25 µg
a
b
ab
ab
30
60
90
120
150
180
360
Time Following Injection (min)
D
Non-cumulative Water Intake (g)
10.0
8.0
6.0
4.0
2.0
0.0
vehicle
1 µg
5 µg
25 µg
a
ab
ab
b
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 4.5. Food and water intake in AZA-injected fed chickens.

Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4 day-old fed broiler chicks (*Gallus gallus*) that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 8-10 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 8-10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

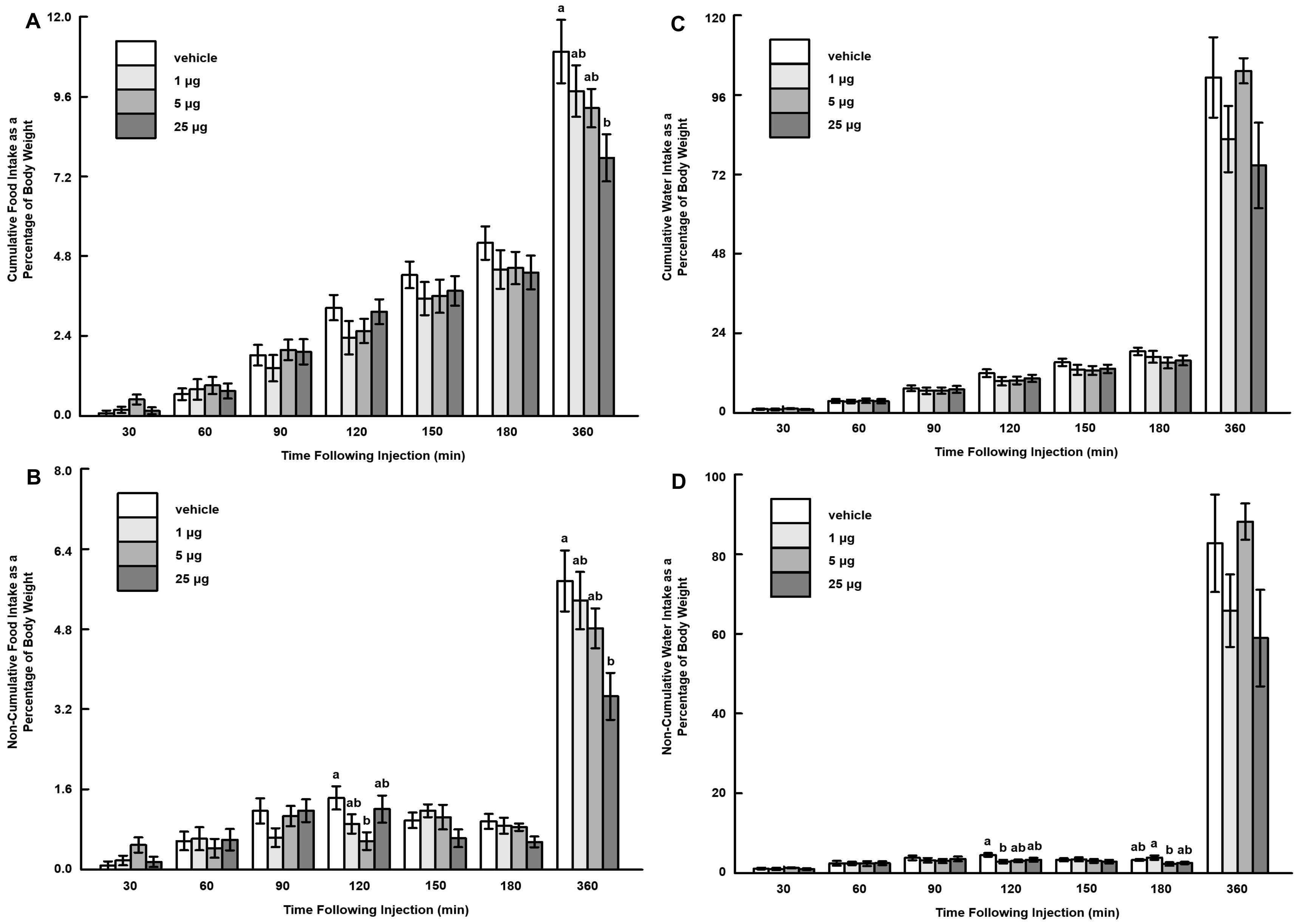
A
Cumulative Food Intake as a Percentage of Body Weight
0.0
2.4
4.8
7.2
9.6
12.0
vehicle
1 µg
5 µg
25 µg
a
ab
ab
b
30
60
90
120
150
180
360
Time Following Injection (min)
B
Non-Cumulative Food Intake as a Percentage of Body Weight
0.0
1.6
3.2
4.8
6.4
8.0
vehicle
1 µg
5 µg
25 µg
a
ab
b
ab
a
ab
ab
b
30
60
90
120
150
180
360
Time Following Injection (min)
C
Cumulative Water Intake as a Percentage of Body Weight
0
24
48
72
96
120
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
D
Non-Cumulative Water Intake as a Percentage of Body Weight
0
20
40
60
80
100
vehicle
1 µg
5 µg
25 µg
a
b
ab
ab
ab
a
b
ab
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 4.6. Food and water intake in AZA-injected fed quail.

Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7 day-old fed quail (*Coturnix japonica*) that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 9-11 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 9-11 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

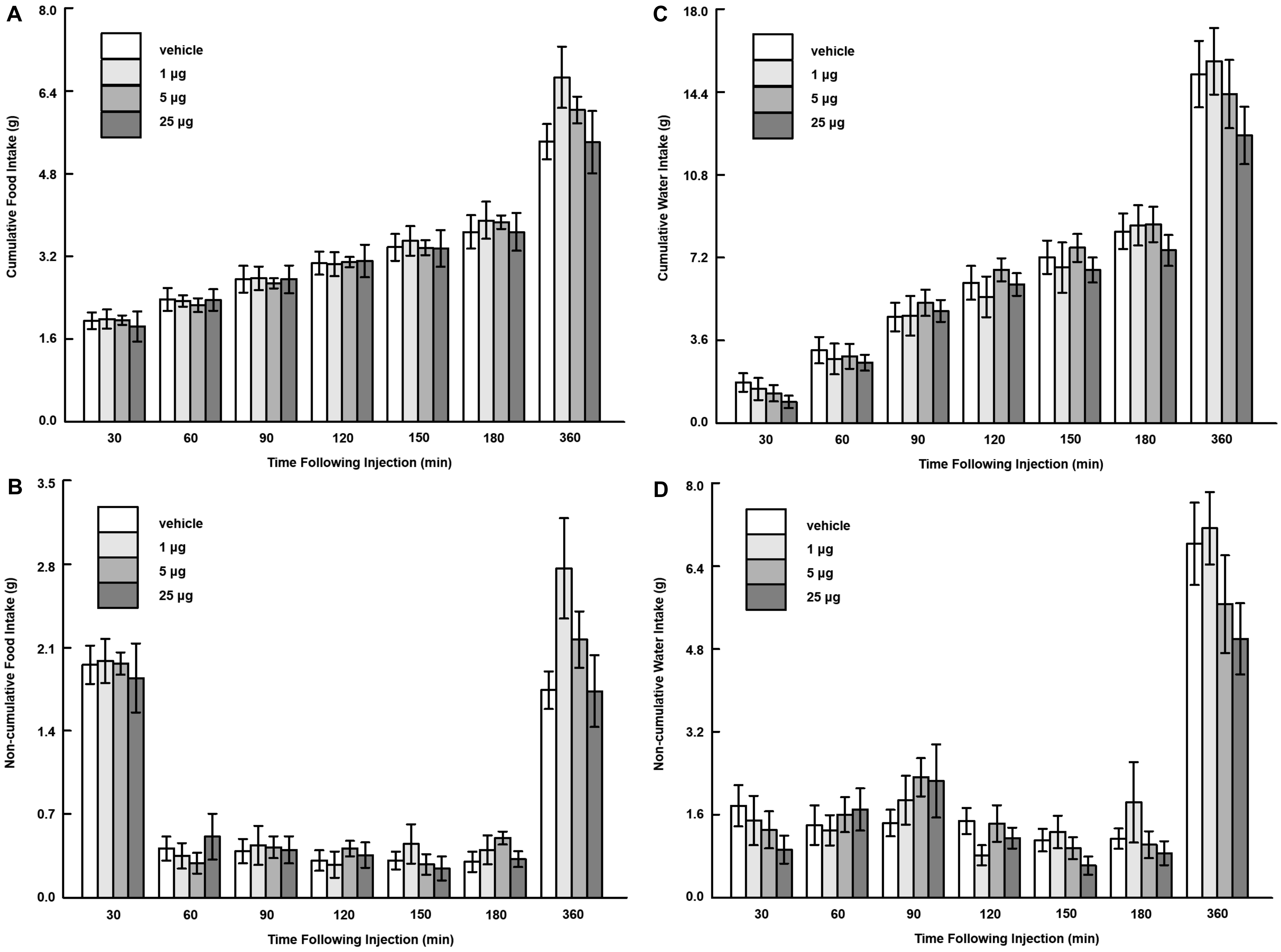
A
Cumulative Food Intake (g)
8.0
6.4
4.8
3.2
1.6
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
C
Cumulative Water Intake (g)
18.0
14.4
10.8
7.2
3.6
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
B
Non-cumulative Food Intake (g)
3.5
2.8
2.1
1.4
0.7
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
D
Non-cumulative Water Intake (g)
8.0
6.4
4.8
3.2
1.6
0.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)

Figure 4.7. Food and water intake in AZA-injected fasted chickens.

Cumulative (A) and non-cumulative (B) food intake at times post-injection of 4 day-old fasted broiler chicks (*Gallus gallus*) that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 8-9 per group). Cumulative (C) and non-cumulative (D) water intake at times post-injection of the same chicks (n = 9-10 per group). Values represent means ± standard errors.

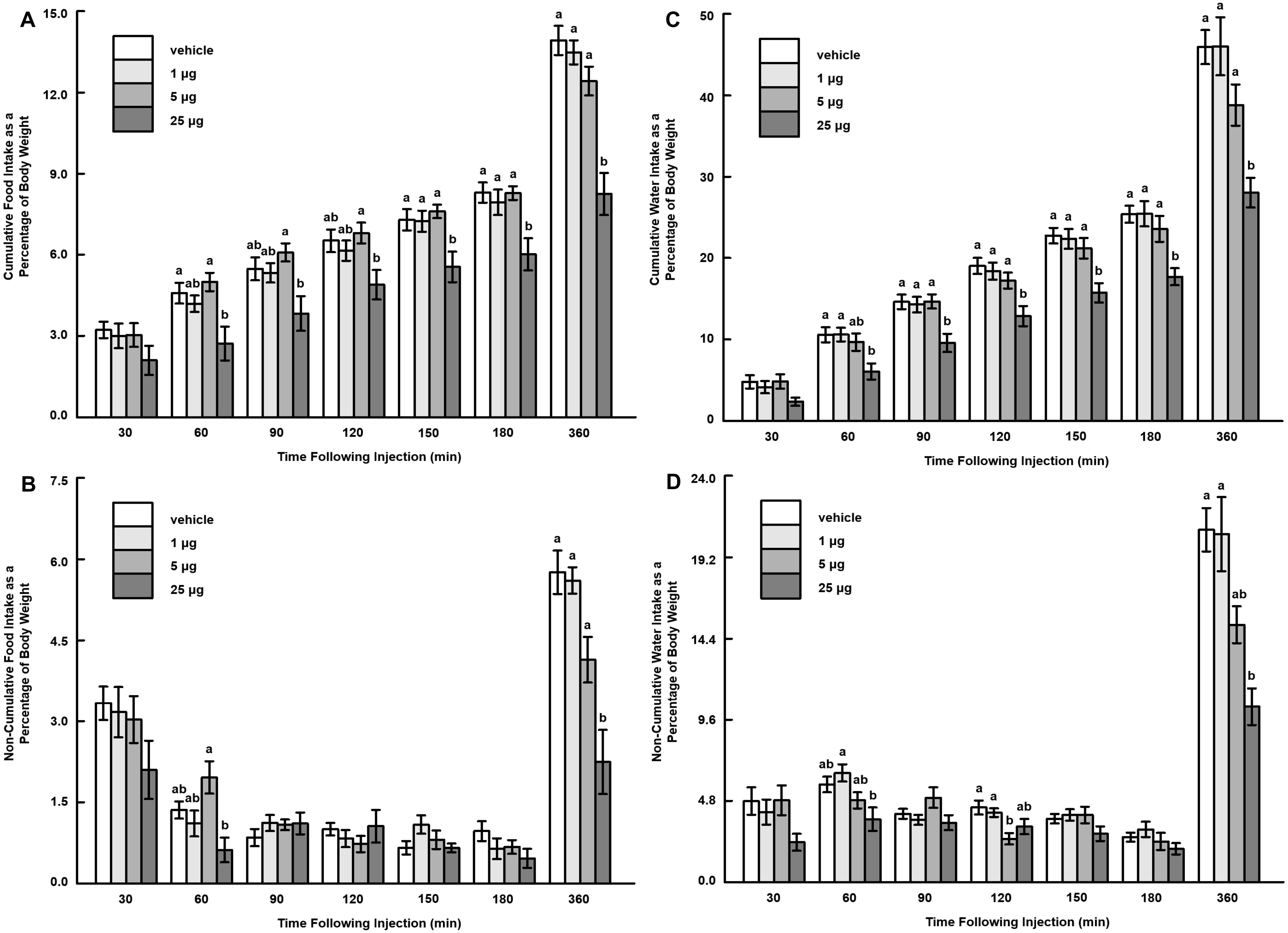A
Cumulative Food Intake as a Percentage of Body Weight
0.0
3.0
6.0
9.0
12.0
15.0
B
Non-Cumulative Food Intake as a Percentage of Body Weight
0.0
1.5
3.0
4.5
6.0
7.5
C
Cumulative Water Intake as a Percentage of Body Weight
0
10
20
30
40
50
D
Non-Cumulative Water Intake as a Percentage of Body Weight
0.0
4.8
9.6
14.4
19.2
24.0
vehicle
1 µg
5 µg
25 µg
30
60
90
120
150
180
360
Time Following Injection (min)
a
ab
b

Figure 4.8. Food and water intake in AZA-injected fasted quail.

Cumulative (A) and non-cumulative (B) food intake as a percentage of body weight at times post-injection of 7 day-old fasted quail (*Coturnix japonica*) that were intracerebroventricularly injected with 0 (vehicle), 1, 5, or 25 µg of AZA (n = 10 per group). Cumulative (C) and non-cumulative (D) water intake as a percentage of body weight at times post-injection of the same chicks (n = 10-11 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

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Chapter 5: Prostaglandin E2-induced anorexia involves hypothalamic brain-derived neurotrophic factor and ghrelin in chicks

Abstract

Central administration of prostaglandin E2 (PGE2) is associated with potent anorexia in rodents and chicks, although hypothalamic mechanisms are not fully understood. The objective of the present study was to identify hypothalamic nuclei and appetite-related factors that are involved in this anorexigenic effect, using chickens as a model. Intracerebroventricular injection of 2.5, 5, and 10 nmol of PGE2 suppressed food and water intake in broiler chicks in a dose-dependent manner. c-Fos immunoreactivity was increased in the paraventricular nucleus (PVN) at 60 minutes post-injection of 5 nmol of PGE2. Under the same treatment condition, hypothalamic expression of melanocortin receptor 3 and ghrelin mRNAs increased, whereas neuropeptide Y receptor sub-type 5 and tropomyosin receptor kinase B (TrkB) mRNAs decreased in PGE2-treated chicks. In the PVN, chicks injected with PGE2 had more brain-derived neurotrophic factor (BDNF), ghrelin, and c-Fos mRNA but less corticotrophin-releasing factor receptor 1 (CRFR1), CRFR2, and TrkB mRNA expression. In conclusion, PGE2 injection resulted in decreased food and water intake that likely involves BDNF and ghrelin originating in the PVN. Because the anorexigenic effect is so potent and hypothalamic mechanisms are similar in chickens and rodents, a greater understanding of the role of PGE2 in acute appetite regulation may have implications for treating eating and metabolic disorders in humans.

Key words: Anorexia, Prostaglandin E2, Brain-derived neurotrophic factor, Ghrelin, Hypothalamus, Paraventricular nucleus, Chicks

Introduction

Prostaglandin E2 (PGE2) is produced from arachidonic acid, a polyunsaturated fatty acid, through cyclooxygenase (COX) and PGE synthase (PGES), and has local bioactivities near production sites [1]. PGE2 is widely distributed in the brain and periphery, and binds to at least four different sub-types of G-protein-coupled E-class prostanoid receptors (EP1-EP4) [1]. Prostaglandin signaling is involved in the inflammatory response and signaling via PGE2 may lead to inflammation-induced anorexia. Through EP1 and EP3, although via distinct pathways, murine PGE2 is involved in the activation of the paraventricular nucleus (PVN) of the hypothalamus and the hypothalamic-pituitary-adrenal (HPA) axis and adrenocorticotropic hormone (ACTH) release induced by inflammatory cytokines or lipopolysaccharides (LPS) [2]. The EP3 receptor also mediates the effect of central PGE2 administration on sympathetic excitatory and febrile responses [3-5]. Systemic inflammatory responses are regulated via EP2 and EP4, although the effective sites vary greatly within the brain [6], and EP4 is the sub-type reported to mediate suppression of food intake in mice [1] and wakefulness in rats [7].

When injected directly into the brain, PGE2 induced anorexia in overnight fasted rats and mice [1, 8] and layer-type chickens that were in the fed-state [9]. However, the effect of centrally-administered PGE2 in other types of chickens remains unclear, as does the central molecular mechanism. The hypothalamus is a critical center within the central nervous system for regulating energy homeostasis, and integrates hunger and satiety signals from peripheral and other central locations [10]. Several hypothalamic nuclei, including the arcuate nucleus (ARC), PVN, ventromedial hypothalamus (VMH), dorsomedial hypothalamic nucleus (DMN), and lateral hypothalamic area (LHA), have diverse roles in sensing and transmitting these energy-related signals [11, 12]. Within and among these nuclei, various neurons project onto each other, releasing neurotransmitters with opposing and reinforcing functions in regulating energy intake. The EP4 receptor and two enzymes responsible for synthesizing PGE2 are located in the PVN [1]. Thus, the PVN is likely one of the hypothalamic nuclei involved in mediating the anorectic effect of PGE2.

Lipopolysaccharide, a bacterial endotoxin that induces an inflammatory response, stimulates PGE2 production in the blood-brain barrier endothelial cells, which could either bind to EP4 on corticotrophin-releasing factor (CRF) neurons in the PVN to induce a stress response, or bind to EP3 on serotonergic neurons in the brainstem's raphe nucleus, which releases serotonin to activate pro-opiomelanocortin (POMC) neurons in the ARC [13]. This might lead to the release of satiety-inducing melanocortins such as α-, β-, and γ-melanocyte-stimulating hormone (MSH) to directly suppress food intake [14]. Serotonin, via activation of CRF-ergic neurons in the PVN, also triggers the HPA axis, which induces the stress cascade and indirectly suppresses appetite [13]. However, the hypothalamic mechanisms underlying PGE2-associated anorexia, particularly the direct effects, are still not fully understood.

In the present study, we injected broiler-type chicks with PGE2, measured food and water intake, investigated hypothalamic c-Fos immunoreactivity, and measured mRNA abundance of appetite-related factors in both whole hypothalamus and individual hypothalamic nuclei.

Materials and methods

Animals

On day-of-hatch, Hubbard x Cobb-500 chicks were obtained from a local hatchery. Chicks were group-caged the same day and then individually caged the next day, with 30 ± 1 °C and 50 ± 5%

relative humidity and 24 hours of light. Chicks had ad libitum access to food (21.5% crude protein and 3000 kcal ME/kg) and fresh water. Chicks had visual and auditory contact with each other in the individual cages. All experiments were conducted at 4 days post-hatch. Experimental procedures were performed according to the National Research Council Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Freehand intracerebroventricular injection procedure

On the day of the experiments, chicks were injected intracerebroventricularly (ICV; lateral ventricle) using a method that does not appear to induce physiological stress [15, 16]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for freehand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 10 seconds post-injection to reduce backflow. Chicks were assigned to treatments at random. PGE2 (Tocris Bioscience, Bristol, UK) was dissolved in dimethyl sulfoxide (DMSO; final percentage: 3.5%) and artificial cerebrospinal fluid [17] as a vehicle for a total injection volume of 5 µl with 0.1% Evans Blue dye to facilitate injection site localization. We did not observe any difference in feeding or other types of behavior among groups due to the presence of DMSO, in comparison to injection with a vehicle that lacked DMSO (data not shown). After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine visually the presence of dye in the lateral ventricle. Any chick without dye present in the ventricular system was eliminated from analysis. Sex of chicks was determined visually by dissection and gonadal inspection at the time of decapitation.

Experiment 1: Food and water intake

Chicks, fasted for 180 minutes, were randomly assigned to receive 0 (vehicle), 2.5, 5, or 10 nmol of PGE2, based on previous studies [1, 9], by freehand ICV injection. After injection, chicks were returned to their individual home cages where they were given ad libitum access to both food and water. Food (0.01 g) and water (0.1 g = 0.1 ml) intake were recorded every 30 minutes for 180 minutes post-injection. Data were analyzed using the GLM procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC) and analysis of variance (ANOVA) at each time point, with the statistical model including the main effect of PGE2 treatment. When the treatment effects were significant, Tukey's method of multiple comparisons was used to separate the means within each time point.

Another set of chicks were also fasted for 180 minutes and randomly assigned to receive 0 (vehicle), 2.5, 5, or 10 nmol of PGE2 by ICV injection. Chicks were returned to their cages after injection, with free access to water, whereas food was withheld. Data collection and analysis were the same as described above.

Experiment 2: Hypothalamic c-Fos immunoreactivity

Broiler chicks, fasted for 180 minutes, were randomly assigned to receive vehicle or 5 nmol of PGE2 via ICV injection (based on the results of Experiment 1). Food was withheld after injection to prevent c-Fos immunoreactivity associated with food consumption. At 60 minutes post-injection as this is the time expected for the most robust c-Fos expression [18], chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, then perfused via the carotid artery with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) containing 0.2% picric acid at pH 7.4. Brains were removed from skulls and post-fixed for 1 hour in the same solution, after which they were blocked through a series of sucrose incubations, consisting of 20% and 30% in 0.1 M PB, until they sank [19]. Several 60 μm coronal sections that contained appetite-related nuclei based on anatomies described [20] were collected at interaural 8.0, 7.4, 6.8, or 5.4 mm in 0.02 M PB saline (PBS) containing 0.1% sodium azide using a cryostat at -10 °C. The LHA was collected at interaural 8.0 mm, the PVN at interaural 7.4, the VMH at 6.8 mm, and the DMN and ARC at interaural 5.4 mm. Sections were processed immediately after collection.

Procedures for immunohistochemistry are based on published methods [21] and steps were performed at room temperature unless otherwise indicated. Free-floating sections were pre-blocked for 1 hour with 10% normal goat serum (NGS) and 0.3% Triton X-100 in 0.02 M PBS. To inhibit endogenous peroxidase activity, sections were incubated in 1.5% hydrogen peroxide and 50% methanol in deionized water for 30 minutes. Following a 3 × 10 minutes series of incubations in wash buffer (0.05% NGS and 0.3% Triton X-100 in 0.02 M PBS), sections were incubated with rabbit polyclonal anti-c-Fos (AB429; Abcam, Cambridge, MA, USA) at a dilution of 1:5000 in PBS containing 0.3% Triton X-100, 1% NGS, and 1% blocking reagent for 48 hours under slow oscillation at 4 °C. For assay controls, the primary antibody was substituted with normal rabbit serum. Sections were then rinsed 3 × 10 minutes in wash buffer and incubated with biotinylated goat antirabbit secondary antibody at a dilution of 1:200 in PBS containing 1% NGS for 2 hours at room temperature. Following a rinse with PBS, sections were processed with avidin-biotin horseradish peroxidase complex at a dilution of 1:200. Reactions were visualized with the DAB Substrate Kit for Peroxidase (Vector Laboratories) for 10 seconds, mounted on gelatin-coated slides and cover-slipped with VectaMount (Vector Laboratories). Anatomy was confirmed using digital images from the chicken stereotaxic atlas [20], and a digital micrograph was captured for each section with a Nikon Eclipse 80i microscope, 4× objective and DS-Ri1 color camera, and images were analyzed using NIS-Elements Advanced Research Software (Nikon) and the stitching method to assemble a complete image of the section. Overlays containing the respective nucleus boundaries were digitally merged with micrographs and the number of c-Fos immunoreactive cells within each nucleus counted by an individual blind to treatment. Data were analyzed by t-tests (vehicle vs PGE2-injected within a nucleus) using the ttest procedure in SAS 9.4.

Experiment 3: Hypothalamic mRNA abundance

Chicks, fasted for 180 minutes, were randomly assigned to receive 0 or 5 nmol of PGE2 (based on Experiment 1) via ICV injection. Chicks remained fasted following injection to prevent molecular changes associated with differences in food consumption. At 60 minutes post-injection, chicks were deeply anesthetized with sodium pentobarbital via cardiopuncture, decapitated, and brains removed. The whole upside-down brain was snap frozen in liquid nitrogen for 9 seconds, which freezes the outermost portion of the brain, providing firmness while leaving the center unfrozen to permit dissection without shattering. Cuts were made visually as per the following anatomy: perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. 1.8 mm parallel to the midline two cuts were made and finally the dorsal cut was made from the anterior commissure to 0.8 mm ventral to the posterior commissure [22]. This block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Invitrogen, Carlsbad, CA, USA).

Hypothalamus was homogenized using 5 mm stainless steel beads (Qiagen, Valencia, CA, USA), TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and a Tissue Lyser II (Qiagen). The Direct-zol^{TM} RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) was then used for total RNA purification. The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a NanoPhotometer Classic (IMPLEN, Westlake Village, CA, USA).

First-strand cDNA was synthesized in 20 µl reactions from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 ℃ for 10 minutes, 37 ℃ for 120 minutes and 85 ℃ for 5 minutes. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 5.1) and validated for amplification efficiency before use (95-105%). Real-time PCR reactions were performed in 10 µl reactions that contained 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.5 µl primers (0.25 µl of 5 µM forward primer and 0.25 µl of 5 µM reverse primer), 1.5 µl nuclease-free water, and 3 µl of 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). The real-time PCR was performed under the following conditions: 95 ℃ for 20 seconds and 40 cycles of 90 ℃ for 3 seconds plus 60 ℃ for 30 seconds. A dissociation step consisting of 95 ℃ for 15 seconds, 60 ℃ for 1 minute, 95 ℃ for 15 seconds and 60 ℃ for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Experiment 4: mRNA abundance in PVN

The design was the same as for Experiment 3 except for that at 60 minutes post-injection, chicks were deeply anesthetized and then perfused via the carotid artery with 1.5 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH = 5.2).

Within 30 minutes of perfusion, the brains were sectioned in a cryostat at -10 °C into 500 µm thick coronal sections in the direction from rostral to caudal: the PVN (based on the result of Experiment 2) was collected at 7.4 mm interaural based on anatomy described [20] and punch performed. Punches were collected using sterile disposable biopsy punch instruments (1 mm, Integra York PA Inc, York, PA, USA). The punches were immediately placed in RNA lysis buffer (Norgen Biotek, Thorold, ON, Canada) with 1% beta-mercaptoethanol (Calbiochem, Millipore Sigma, Burlington, MA, USA), vortexed, snap-frozen in liquid nitrogen, and stored at -80 °C. The remaining brain section was photographed and punch accuracy verified via overlays containing the respective nucleus boundaries.

The samples were vortexed vigorously for 30 seconds and incubated at room temperature for 5 minutes before adding 70% molecular biology-grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit (Norgen Biotek). The concentration and purity of total RNA was assessed as described in Experiment 3. Subsequent reactions were performed under the same conditions as those described in Experiment 3 except that 100 ng of total RNA was used for the reverse transcription and 5-fold diluted cDNA was used for the real-time PCR.

All molecular data were analyzed using the ttest procedure in SAS 9.4. Real-time PCR data were analyzed using the ΔΔCT method with β-actin as the reference gene and the average of chicks in the vehicle group as the calibrator sample [23]. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. Statistical significance was set at $P < 0.05$ for all experiments.

Results

Effects of PGE2 on food and water intake

Chicks injected with 2.5 nmol of PGE2 ate less than control chicks during the first 90 minutes post-injection (Figure 5.1A). Those injected with 5 or 10 nmol of PGE2 consumed less food for the entire 180 minutes post-injection. Chicks injected with 10 nmol of PGE2 ate less than those injected with 2.5 nmol at all time points and those injected with 5 nmol at 90 to 150 minutes post-injection. On a non-cumulative basis, chicks injected with all doses of PGE2 ate less than vehicle-injected chicks at 30 minutes post-injection (Figure 5.1B). A compensatory increase in food intake was observed for the highest dose of PGE2 at 180 minutes post-injection.

Chicks injected with all doses of PGE2 drank less water at all time points post-injection, except that the significance was lost at 180 minutes post-injection for those injected with 5 nmol of PGE2 (Figure 5.2A). For non-cumulative water intake, all doses reduced water intake during the first 60 minutes after injection, and chicks injected with 10 nmol of PGE2 drank less until 90 minutes post-injection (Figure 5.2B). A compensatory increase in water intake occurred at 150 minutes post-injection in 5 nmol of PGE2-injected chicks.

When food was withheld after injection, chicks that received 5 and 10 nmol of PGE2 drank less at 60 and 90 minutes post-injection, whereas those treated with 2.5 nmol of PGE2 ingested less at 60 minutes post-injection (Figure 5.3A). On a non-cumulative basis, less water consumption was only observed at 60 minutes post-injection, in all PGE2-treated chicks, with no compensatory increase at a later time (Figure 5.3B).

Based on Experiment 1, 5 nmol of PGE2 was the lowest dose that decreased food intake during the entire 180 minutes of observation. Thus, this dose was used for all subsequent experiments.

Hypothalamic c-Fos immunoreactivity

PGE2 was associated with a 364% increase in the number of c-Fos immunoreactive cells in the PVN 7.4 (Figure 5.4A). There was no effect of treatment in the LHA 8.0, VMH 6.8, ARC 5.4, or DMN 5.4.

Hypothalamic mRNA

In Experiment 3, we quantified whole hypothalamic mRNA for several appetite-associated factors (Table 5.2). The mRNA abundance of melanocortin receptor 3 (MC3R) (117% increase) and ghrelin (205% increase) was greater in chicks injected with PGE2 than vehicle-injected chicks, whereas NPY receptor 5 (NPYR5) (15% decrease) and tropomyosin receptor kinase B (TrkB) (14% decrease) was lower in the PGE2-injected group than in vehicle-injected chicks. Other factors, including NPY, NPYR1, NPYR2, CRF, CRF receptor type 1 (CRFR1), CRFR2, MC4R, POMC, agouti-related peptide (AgRP), cocaine and amphetamine-regulated transcript (CART), mesotocin (MT), MT receptor (MTR), brain-derived neurotrophic factor (BDNF), c-Fos, and growth hormone secretagogue receptor (GHSR) were not affected by PGE2 injection.

mRNA abundance in the PVN

In Experiment 4, we isolated the PVN based on results of Experiment 2, and measured several appetite-associated factors (Table 5.3). The mRNA abundance of BDNF (144% increase), c-Fos (402% increase) and ghrelin (147% increase) was greater in PGE2- than in vehicle-injected chicks, while expression of CRFR1 (22% decrease), CRFR2 (38% decrease), and TrkB (13% decrease) was lower in PGE2-treated birds. Factors like NPY, NPYR1, NPYR2, NPYR5, CRF, MC3R, MC4R, CART, MT, MTR, and GHSR were not influenced by PGE2 treatment.

Discussion

Central administration of PGE2 reduces food consumption in mammals and chickens [1, 8, 9]. Levine and Morley (1981) observed a reduction of food intake after they ICV injected 24-hour-fasted rats with a wide range of doses of PGE2 (1-20 µg; 2.8-56.7 nmol) [8]. Results from another study in 18-hour-fasted mice demonstrated PGE2-induced reductions in food intake at an even lower range of doses (0.1-10 nmol) [1]. In a study with layer-type chickens, 2 or 4 µg (5.7 or 11.3 nmol) of PGE2 decreased food intake in the fed state (fasted condition was not assessed) [9]. In the present study, we administered 2.5 to 10 nmol of PGE2 to broiler-type chicks following 3 hours of fasting and observed a dose-dependent decrease in food intake, consistent with previous reports. On a cumulative basis, 5 and 10 nmol of PGE2 reduced food intake for the entire experimental period (3 hours), whereas the effect of 2.5 nmol of PGE2 lasted for 90 minutes. Compared with layer chicks that consumed very little for 90 minutes post-injection of 5.7 and 11.3 nmol of PGE2, broilers appear to be less sensitive. This difference could be explained by the different nutritional states, where metabolic changes and production of appetite-related factors are dynamically regulated to maintain energy homeostasis [24], and different genetic backgrounds, because layers and broilers have been selected for egg or meat production-related traits, respectively. However, broilers appear to be more sensitive to PGE2-related appetite effects than mice, because 10 nmol of PGE2 via central injection led to longer-lasting appetite suppression in broiler chicks. The differences between species and age of animals (4-day-old chicks vs. 7-week-old mice) could also contribute to these differential responses. On a non-cumulative basis, however, chicks injected with all doses of PGE2 ate less during the first 30 minutes post-injection. The 10 nmol dose of PGE2 tended to suppress food consumption at 90 minutes post-injection, but the difference was not statistically significant. Thus, PGE2 serves as a potent and transient suppressor of food intake in broiler chicks.

In the present study, water consumption was also evaluated, which, to our knowledge, is the first report of such in an avian species. In a similar dose-dependent manner, central injection of PGE2 led to greatly reduced water intake. On a non-cumulative basis, the anti-dipsogenic effect was transient and occurred during the first 60 minutes post-injection, although the highest dose of PGE2 suppressed water consumption until 90 minutes. To determine if this was prandial or primary in nature, water intake was measured in the absence of food. All PGE2-injected chicks drank less, supporting that the effect was primary. In rats, ICV injection of PGE2 (100 ng; 0.28 nmol) suppressed water intake induced by angiotensin II [25]. Aged rats had increased expression of hypothalamic COX and PGES, thus generating a higher level of central PGE2 coupled to decreased water consumption in response to dipsogenic stimuli [26]. Our current study did not include any of these stimuli yet we observed PGE2-induced reduced water intake in the presence and absence of food, which suggests that the effect of PGE2 on thirst regulation is evolutionarily conserved in chicks. Possible mechanisms mediating thirst regulation relate to a PGE2-induced inflammatory response [26], but investigation of this is beyond the scope of the current study.

To start to understand the molecular mechanism underlying the anorectic effects of PGE2 on feeding behavior in chicks, we quantified c-Fos immunoreactivity in various appetite-related hypothalamic nuclei. As an indicator of recent neuronal activation, the number of c-Fos immunoreactive cells was increased in the PVN, but not in other nuclei. This finding was corroborated by the real-time PCR data; c-Fos mRNA increased roughly 4-fold in the PVN of PGE2-injected chicks. The expression of c-Fos in the whole hypothalamus tended to be increased by PGE2 but the difference was not significant, which may be explained by the fact that increased expression of c-Fos in the PVN was masked by its expression in other inactivated nuclei. As reviewed, the ARC and PVN were activated and proposed to mediate the anorexigenic response to central PGE2 in mammals [13]. These two hypothalamic nuclei are important as 'first order' and 'second order' neurons, respectively, in integrating central and peripheral hormonal signals related to energy homeostasis and appetite regulation [11, 27]. In a rat study, c-Fos transcript increased in the ARC of PGE2-injected individuals, albeit not as dramatically as in the PVN [28]. We did not observe an increase in the number of c-Fos-expressing cells in the ARC in response to PGE2 injection. Interestingly, in the rat study, the increase in c-Fos expression (determined histochemically) in the ARC of PGE-2 injected animals occurred at 30 minutes post-injection and the second time point that was evaluated was 2 hours, at which point the signal had vanished, suggesting that at 1 hour, the time point at which we assessed c-Fos expression, there may not have been detectable expression. They detected the transcript, which would have been induced prior to protein translation, thus a time lag between transcriptional and translational activation would be expected. Nonetheless, c-Fos induction is very dynamic, and it is possible in our study that it was rapidly activated during the first 30 minutes post-injection and was turned off prior to our collection of samples for analysis.

In regards to activation in the PVN, our result is consistent with mammalian studies [28, 29]. The PVN is the origin of CRF production and its activation is closely related to the HPA axis and stress response [30]. That c-Fos immunoreactive neurons were colocalized within CRF- or oxytocin-immunoreactive cells in the PVN of PGE2-injected rats [28] suggests that PGE2-related anorexia involves those associated signaling pathways. Accordingly, we then measured mRNA abundance of several appetite-related factors in both the whole hypothalamus and the PVN, including MT (the avian equivalent of oxytocin), CRF, and their associated receptors. Quantities of MT and CRF mRNA were not affected in the whole hypothalamus or the PVN.

While the MT receptor (avian equivalent of the oxytocin receptor) was not affected in the whole hypothalamus or PVN, the CRF receptors were both down-regulated in the PVN in response to PGE2 administration. These results, although seemingly counterintuitive, were intriguing because they appear to complement the rat study. CRF primary transcript in the rat PVN was selectively and rapidly induced by central PGE2, and this effect lasted for 30 minutes post-injection and was almost undetectable after 2 hours [28]. Our brain tissues were collected at 60 minutes post-injection and it is thus possible that the effect of PGE2 on CRF expression was dampened by then. In rats, CRFR1 mRNA was unchanged at 30 minutes, whereas it was up-regulated at 2 hours after PGE2 injection [28]. Similar to other nucleus-specific discrepancies between rodents and chickens, it is likely that transcriptional responses are very dynamic and the change that we captured was a snapshot in the midst of other changes to eventually restore homeostatic feeding. Importantly, that expression responded to PGE2 supports that CRF signaling is likely involved in the anorexigenic response.

BDNF is a member of the neurotrophin family of secreted signaling molecules and mainly activates signaling via the TrkB [31]. BDNF and TrkB mRNAs are distributed widely among hypothalamic nuclei, including the DMN, VMH, PVN, and LHA, except that only TrkB mRNA was present in the ARC [32-35]. Most studies involving BDNF utilize rodent models. Chronic BDNF infusion to the lateral ventricle of rats led to anorexia and weight loss [36]. In mice with a BDNF deficiency, ICV injection of BDNF reversed the phenotype, causing hyperphagia, obesity, and insulin and leptin resistance [34]. When BDNF was directly infused into the PVN, resting metabolic rate and thermogenic capacity were increased, suggesting a role of BDNF in energy expenditure [37]. Expression of BDNF increased in the PVN in both rats and chicks in response to stressful stimuli [38-40], and BDNF overexpression or chronic ICV infusion elevated PVN

CRF mRNA [41, 42], indicating that BDNF regulates the HPA axis. In the present study, PVN but not whole hypothalamic BDNF mRNA was stimulated by PGE2, suggesting that in chicks it might be involved in the anorexigenic effect of PGE2. TrkB neurons in the PVN play a critical role in energy balance, as their activation leads to appetite suppression [43]. However, it is worth noting that PVN-derived BDNF effects are likely not autocrine because a large population of BDNF neurons do not express TrkB [44]. Thus, decreased expression of TrkB mRNA in the PVN and hypothalamus in our study could signal recovery from the potent anorectic tone.

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor [45]. The effects of centrally-injected ghrelin on feeding behavior are opposite in rats [46] and chicks, with food intake inhibited in neonatal chicks using both rat and chicken ghrelin [45]. The hypothalamic mechanism of ghrelin-induced anorexia in layer chicks involved the HPA axis; plasma corticosterone was increased following ICV injection of ghrelin, effects of which were abolished when ghrelin was co-administered with a CRF receptor antagonist [16]. Thus, increased whole hypothalamic and PVN-specific ghrelin mRNA suggests involvement of the HPA axis in the anorexigenic response to PGE2.

In the hypothalamus, we detected changes in the expression of two receptors of the melanocortin and NPY systems. PGE2 injection stimulated the expression of MC3R and decreased NPYR5 mRNA. Although MC3R signaling primarily regulates energy expenditure [47], it binds to the α-, β-, and γ-MSH with different affinities and might mediate their anorectic effects [11, 48, 49], although in the present study the nucleus source of expression is unclear. Among the five NPY receptors, NPYR1 and NPYR5 are thought to mediate its orexigenic effects (as reviewed in [50]), and transiently suppressed new expression of NPYR5 may contribute to the anorexigenic tone induced by PGE2.

Conclusions

In summary, the major target of exogenous PGE2-induced satiety is the PVN, which is activated and integrates afferent and efferent signals. Although CRF mRNA abundance was not changed at 60 minutes post-injection, increased BDNF and ghrelin mRNA may contribute to appetite suppression. Through measuring nucleus-specific transcriptional changes in response to PGE2 central injection, we identified potential signaling pathways that participate in and enrich the complex brain network underlying PGE2-mediated anorexia. Such information is crucial for facilitating the development of novel strategies to combat metabolic disorders.

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Table 5.1. Primers used for real-time PCR [a]

Gene	Accession No.	Sequences 5’ to 3’ (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
AgRP	NM_001031457.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
BDNF	NM_001031616.1	ACCTGGATGCTGCAAACATG/ GCGAGCTGGGTCAGAGTGA
CART	XM_003643097.3	GCTGGAGAAGCTGAAGAGCAA/GGCACCTGCCCGAACTT
c-Fos	NM_205508.1	TGTTCCTGGCAATATCGTGTTC/CTTTCCCCCCCACGTAAGA
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT/ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT/GGCCCATGTCCCATTGC
Ghrelin	NM_001001131.1	GAAGCACTGCCTAACGAAGACA/GGATGCTGAGAAGGAGAATTCCT
GHSR	XM_015291631.1	TCTGCGAGCGAAGGTGATC/AGACGGCCCAGAGGATGAG
MC3R	XM_004947236.2	GCCTCCCTTTACGTTCACATGT/GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA/GATGCCCAGAGTCACAAACACTT
MT	XM_004936280.2	TGGCTCTCTCCTCAGCTTGTTAT/GGCACGGCACGCTTACC
MTR	NM_001031569.1	TGTGCTGGACGCCCTTCT/TCCTGCGGAGCGTTGGT
NPY	NM_205473.1	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC
NPYR1	XM_015285306.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR2	NM_001031128.1	TGCCTACACCCGCATATGG/GTTCCCTGCCCCAGGACTA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA/TTGTCTTCTGCTTGCGTTTTGT
POMC	NM_001031098.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGACGAT
TrkB	XM_015280251.2	GAGACCTTGCTACCCGGAATT/ CCCAAAATCCCCAATCTTCA

[a] Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: NPY, neuropeptide Y; NPYR1, R2, and R5, NPY receptor sub-type 1, 2, and 5, respectively; CRF, corticotrophin-releasing factor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; MC3R and 4R, melanocortin receptors 3 and 4, respectively; POMC, pro-opiomelanocortin; AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; MT, mesotocin; MTR, MT receptor; BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; GHSR, growth hormone secretagogue receptor.

Table 5.2. Hypothalamic mRNA abundance ^{a}

Variable	NPY	NPYR1	NPYR2	NPYR5	CRF	CRFR1	CRFR2
Treatment							
Vehicle	1.01±0.06	1.00±0.02	1.01±0.05	1.01±0.05	1.08±0.12	1.01±0.04	1.04±0.09
PGE2	1.04±0.06	0.95±0.03	0.98±0.04	0.86±0.06	0.97±0.12	0.99±0.03	0.95±0.07
P-value	.37	.11	.32	**.03**	.26	.39	.21

Variable	MC3R	MC4R	POMC	AgRP	CART	MT	MTR
Treatment							
Vehicle	1.01±0.05	1.01±0.05	1.01±0.05	1.05±0.11	1.05±0.10	1.01±0.06	1.04±0.10
PGE2	1.18±0.06	0.95±0.04	1.05±0.08	0.99±0.12	1.18±0.07	1.10±0.08	1.21±0.25
P-value	**.02**	.17	.33	.35	.16	.20	.27

Variable	BDNF	TrkB	c-Fos	Ghrelin	GHSR
Treatment					
Vehicle	1.01±0.04	1.02±0.06	1.03±0.08	1.11±0.19	1.01±0.05
PGE2	0.92±0.07	0.88±0.05	1.32±0.17	2.27±0.29	0.91±0.06
P-value	.16	**.048**	.07	**.002**	.09

[a] At 4 days post-hatch, chicks (*Gallus gallus*) were fasted for 3 hours before intracerebroventricular injection with 0 (vehicle) or 5 nmol prostaglandin E2 (PGE2). The hypothalamus was collected at 1 hour post-injection. Data are means ± standard errors (SE) (n = 8-10 for each treatment) and *P*-values are displayed for the effect of PGE2 treatment. Abbreviations: NPY, neuropeptide Y; NPYR1, R2, and R5, NPY receptor sub-type 1, 2, and 5, respectively; CRF, corticotrophin-releasing factor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; MC3R and 4R, melanocortin receptors 3 and 4, respectively; POMC, pro-opiomelanocortin; AgRP, agouti-related peptide; CART, cocaine and amphetamine-regulated transcript; MT, mesotocin; MTR, MT receptor; BDNF, brain-derived neurotrophic factor; TrkB, tropomyosin receptor kinase B; GHSR, growth hormone secretagogue receptor.

Table 5.3. mRNA abundance in the paraventricular nucleus [a]

Variable	NPY	NPYR1	NPYR2	NPYR5	CRF	CRFR1	CRFR2
Treatment							
Vehicle	1.07±0.10	1.05±0.08	1.06±0.09	1.02±0.04	1.37±0.24	1.01±0.03	1.17±0.13
PGE2	1.04±0.06	0.98±0.06	1.03±0.06	0.93±0.04	1.45±0.21	0.79±0.05	0.73±0.11
P-value	.42	.28	.40	.09	.41	**.0003**	**.01**

Variable	MC3R	MC4R	CART	MT	MTR	BDNF	TrkB
Treatment							
Vehicle	1.08±0.10	1.14±0.12	1.54±0.23	1.34±0.20	1.03±0.07	1.02±0.06	1.02±0.05
PGE2	1.17±0.11	1.11±0.12	1.18±0.19	1.38±0.16	1.28±0.16	1.47±0.12	0.89±0.06
P-value	.28	.44	.14	.45	.08	**.0008**	**.048**

Variable	c-Fos	Ghrelin	GHSR
Treatment			
Vehicle	1.07±0.10	1.16±0.15	1.03±0.05
PGE2	4.30±0.51	1.71±0.27	1.02±0.06
P-value	**<.0001**	**.04**	.48

[a] At 4 days post-hatch, chicks (*Gallus gallus*) were fasted for 180 minutes and intracerebroventricularly injected with 0 (vehicle) or 5 nmol of prostaglandin E2 (PGE2). Then paraventricular nucleus was collected for total RNA isolation to measure mRNA abundance of neuropeptide Y (NPY), NPY receptor sub-type 1, 2, and 5 (NPYR1, R2, and R5), corticotrophin-releasing factor (CRF), CRF receptor 1 (CRFR1), CRF receptor 2 (CRFR2), melanocortin receptors 3 and 4 (MC3R and 4R), cocaine and amphetamine-regulated transcript (CART), mesotocin (MT), MT receptor (MTR), brain-derived neurotrophic factor (BDNF), tropomyosin receptor kinase B (TrkB), growth hormone secretagogue receptor (GHSR). Values are means and SEs (n = 10-18 for each treatments), with *P*-values for the effect of treatment.

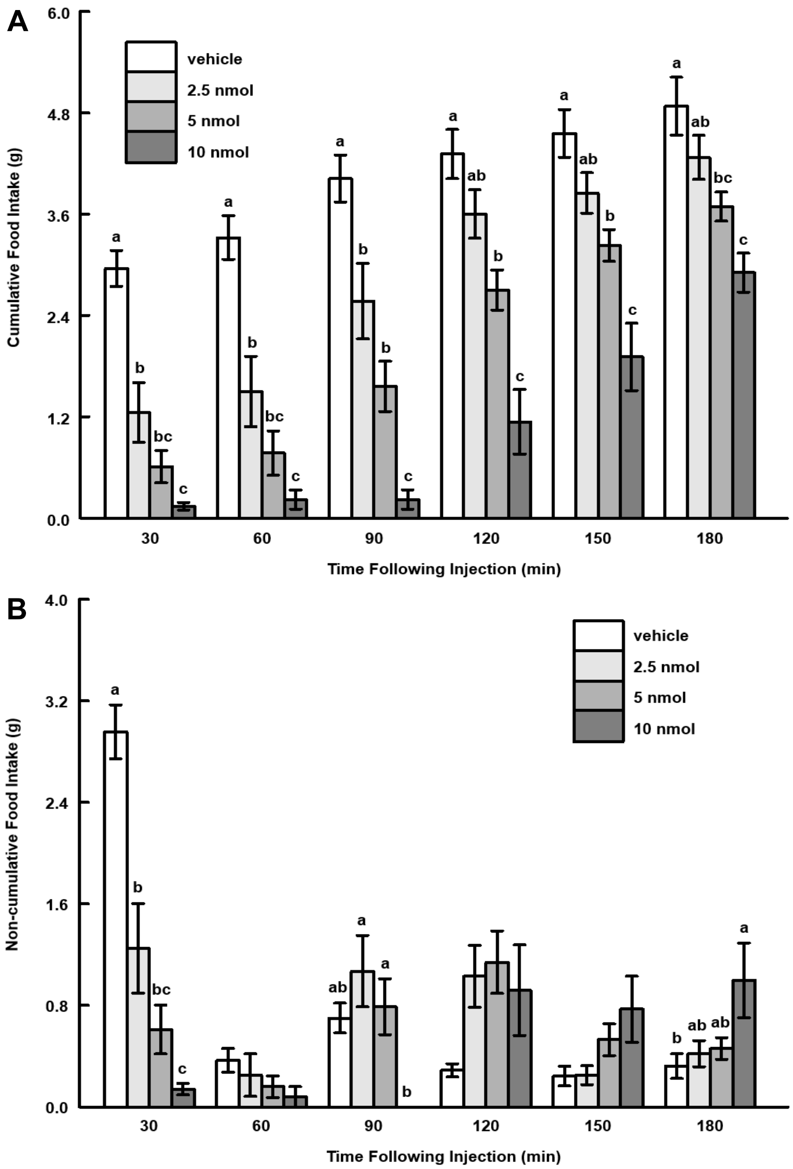

Figure 5.1. Cumulative (A) and non-cumulative (B) food intake at 30 to 180 minutes post-injection.

At 4 days post-hatch, broiler chicks (*Gallus gallus*) were intracerebroventricularly injected with 0 (vehicle), 2.5, 5, or 10 nmol of prostaglandin E2 (n = 9-10 per group). Values represent means ± standard errors. Unique letters denote a significant difference (*P* < 0.05) among groups within a time point, Tukey's test.

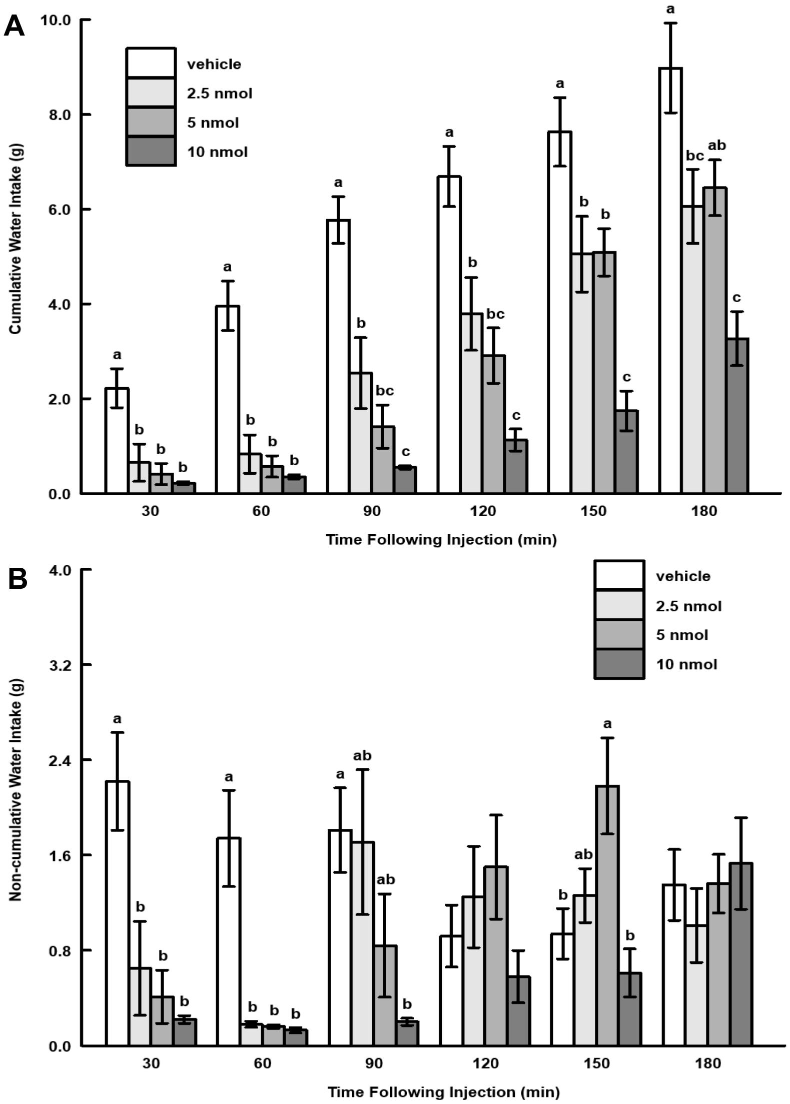

Figure 5.2. Cumulative (A) and non-cumulative (B) water intake at 30 to 180 minutes post-injection.

At 4 days post-hatch, broiler chicks (*Gallus gallus*) were intracerebroventricularly injected with 0 (vehicle), 2.5, 5, or 10 nmol of prostaglandin E2 (n = 10 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

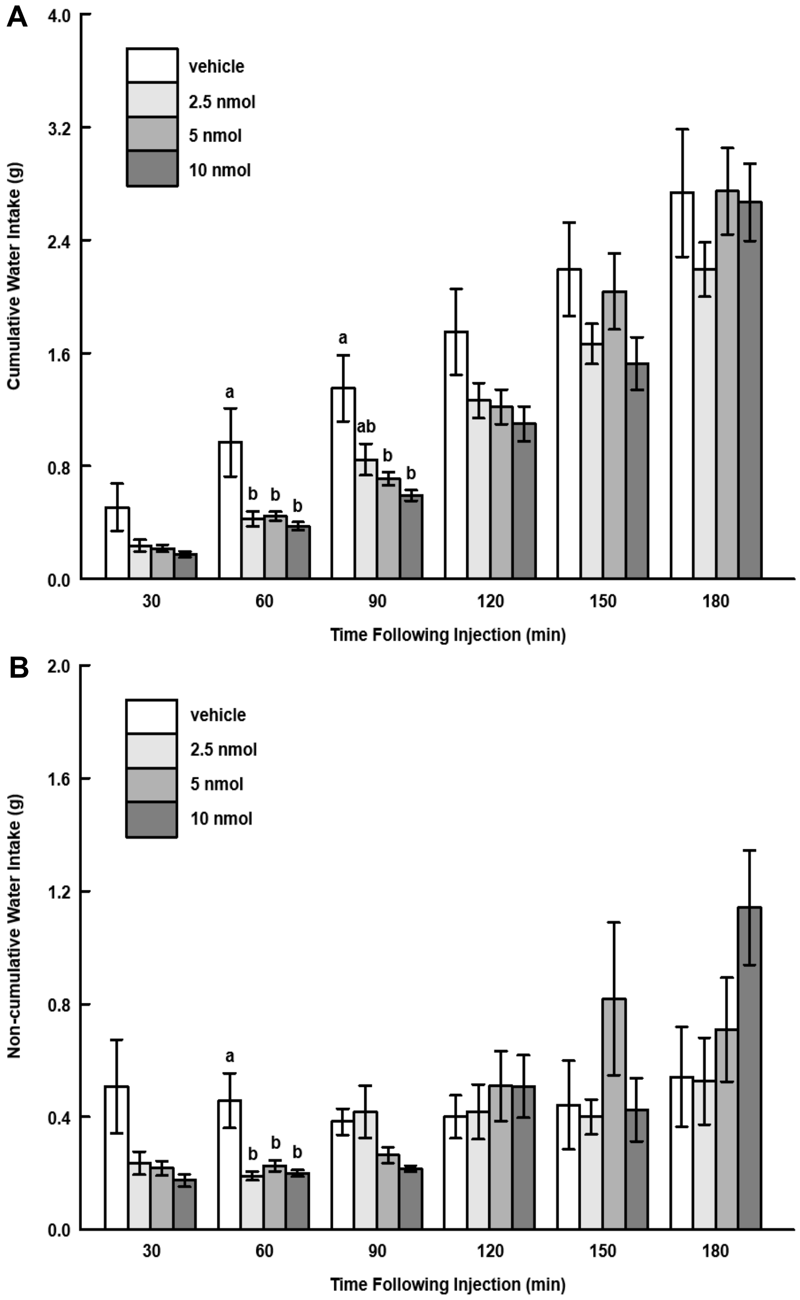
A
Cumulative Water Intake (g)
0.0
0.8
1.6
2.4
3.2
4.0
vehicle
2.5 nmol
5 nmol
10 nmol
a
b
b
b
a
ab
b
b
30
60
90
120
150
180
Time Following Injection (min)
B
Non-cumulative Water Intake (g)
0.0
0.4
0.8
1.2
1.6
2.0
vehicle
2.5 nmol
5 nmol
10 nmol
a
b
b
b
30
60
90
120
150
180
Time Following Injection (min)

Figure 5.3. Cumulative (A) and non-cumulative (B) water intake at 30 to 180 minutes post-injection with food withdrawal.

At 4 days post-hatch, broiler chicks (*Gallus gallus*) were intracerebroventricularly injected with 0 (vehicle), 2.5, 5, or 10 nmol of prostaglandin E2 (n = 11-12 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

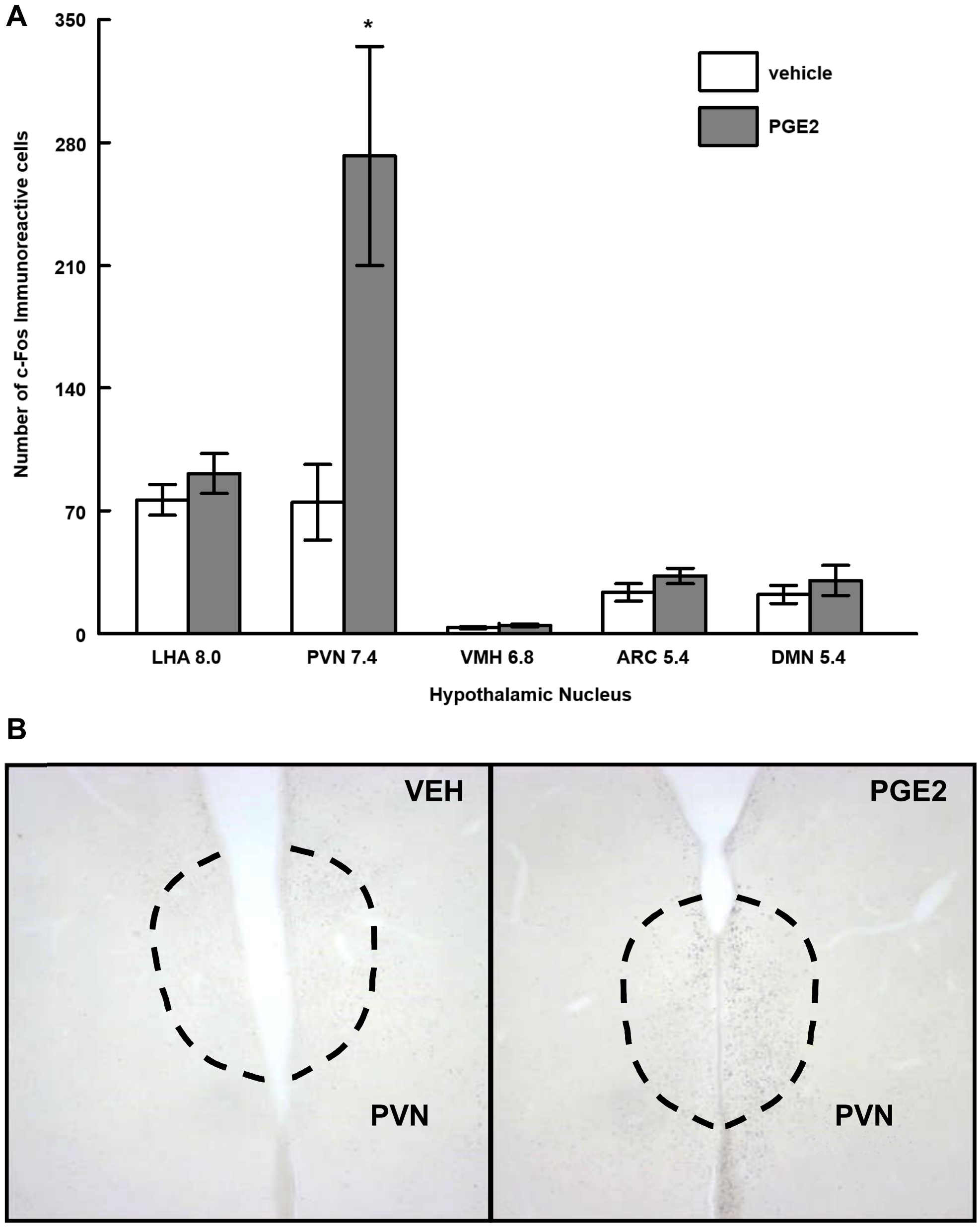
A
350
280
210
140
70
0
Number of c-Fos Immunoreactive cells
*
vehicle
PGE2
LHA 8.0
PVN 7.4
VMH 6.8
ARC 5.4
DMN 5.4
Hypothalamic Nucleus
B
VEH
PGE2
PVN
PVN

Figure 5.4. Numbers of c-Fos immunoreactive cells in different hypothalamic nuclei (A).

At 4 days post-hatch, broiler chicks (*Gallus gallus*) were intracerebroventricularly injected with 0 (vehicle) or 5 nmol of prostaglandin E2 (n = 7-10 per treatment). After 60 minutes, c-Fos immunoreactivity was detected in the lateral hypothalamus (LHA), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), arcuate nucleus (ARC), and dorsomedial nucleus (DMN) of the hypothalamus. Values represent means ± standard errors. Bar with asterisk (*) denotes a significant difference from the vehicle ($P < 0.05$). Representative photomicrographs of the PVN are shown (B).

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Chapter 6: The anorexigenic effect of beta-melanocyte-stimulating hormone involves corticotrophin-releasing factor and mesotocin in birds

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Abstract

Beta-melanocyte-stimulating hormone (β-MSH), when centrally injected, induces anorexigenic effects in rodents and chickens but its mechanism remains unclear. Thus, the primary goal of this research was to elucidate the hypothalamic mechanism using chickens. Intracerebroventricular injection of 0.3, 1.0 and 3.0 nmol of β-MSH decreased food intake for 540 minutes. Expression of hypothalamic mRNAs were affected by β-MSH injection, including corticotrophin-releasing factor (CRF) and its receptor subtype 1 (CRFR1), mesotocin (MT) and its receptor (MTR), pro-opiomelanocortin, cocaine- and amphetamine-regulated transcript (CART), growth hormone secretagogue receptor (GHSR) and neuropeptide Y (NPY) receptor subtype 5 (NPYR5). Within the arcuate nucleus, expressions of NPY, agouti-related peptide, MT and MTR were increased by β-MSH injection. β-MSH-treated chicks had more CRF, CRFR1, CRF receptor subtype 2, GHSR, NPY receptor subtype 1 (NPYR1) and NPYR5 mRNA but lower levels of CART and ghrelin, in the paraventricular nucleus. Greater amounts of mRNA for MTR, GHSR, NPYR1 and NPYR5 and less CRF expression were observed in the ventromedial hypothalamus. In conclusion, central injection of β-MSH potently reduced food intake and was associated with changes in mRNA expression of some anorexigenic factors in a hypothalamic nucleus-specific manner.

Keywords: Beta-MSH, Anorexigenic, Hypothalamic nuclei, mRNA, Chicken

Introduction

The melanocortin system involves two types of neurons within the arcuate nucleus (ARC) of hypothalamus, expressing agouti-related peptide (AgRP) or pro-opiomelanocortin (POMC), respectively [1], which affect appetite. The melanocortin peptides, including adrenocorticotropin, α-, β- and γ-melanocyte-stimulating hormones (MSH), are cleaved from the precursor peptide POMC and serve as agonists of melanocortin receptors [2]. Among these melanocortins, although α-MSH exerted a potent effect on decreasing food intake in both rats [3, 4] and chicks [5], β-MSH was reported to have higher affinity at melanocortin receptor subtype 4 (MC4R) [6] and longer lasting hypophagic effect than α-MSH [7]. Our previous study also showed the anorexigenic effect of γ2-MSH [8]. However, this effect was mainly associated with melanocortin receptor subtype 3 (MC3R), since γ-MSH has higher affinity at MC3R than α- and β-MSH [2].

β-MSH is involved in regulating body weight [9] and induces satiety in mammals [3, 10] and chicks [7, 11] by activating MC4R [4]. On chicken paraventricular nucleus (PVN) neurons, MC4R is co-expressed with corticotropin-releasing factor (CRF), and administration of MC4R agonist melanotan-II induced the expression of CRF, resulting in suppressed appetite [12]. Activated MC4R in rat PVN caused induction of c-Fos, a marker of neuronal activation, followed by oxytocin (OXT) release [13]. These findings suggested MC4R activation is associated with hypothalamic appetite-related factors regulation.

Our group previously found β-MSH potently reduced food intake in chicks and this effect was associated with appetite-related hypothalamic nuclei including the ARC, PVN and ventromedial hypothalamus (VMH) [7]. The ARC, while producing melanocortin peptides, has projections to the PVN [6]. The VMH also receives inputs from the ARC. When POMC neurons in the ARC activate MC4R in the VMH, food intake decreases [14]. Activation in these nuclei suggested a cascade type of effect associated with β-MSH, however, the hypothalamic mechanisms remain unclear in any species. We hypothesized that hypothalamic appetite-related factors, such as CRF, mesotocin (MT; avian equivalent of OXT), POMC and AgRP, are involved in this effect. Thus, we measured gene expressions at the level of the whole hypothalamus and individual hypothalamic nuclei.

Material and methods

Animals

Day-of-hatch Cobb-500 chicks were obtained from a local hatchery. Chicks were caged individually with 30 ± 1 °C and 50 ± 5% relative humidity and 24 hours of light. Chicks were provided food (21.5% crude protein and 3000 kcal ME/kg) and fresh water. Chicks had visual and auditory contact with each other. All experiments were conducted at 4 d post-hatch. Experimental procedures were performed according to the National Research Council Publication, Guide for Care and Use of Laboratory Animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

Intracerebroventricular injection procedure

On the day of experiment, chicks were injected intracerebroventricularly (ICV; into left lateral ventricle) using a method that does not appear to induce physiological stress [15, 16]. The head of chick was briefly inserted into a restraining device that left the cranium exposed and allowed for freehand injection. Injection coordinates were 3 mm anterior to the coronal suture, 1 mm lateral from the sagittal suture, and 2 mm deep targeting the left lateral ventricle. Anatomical landmarks were determined visually and by palpation. Injection depth was controlled by placing a plastic tubing sheath over the needle. The needle remained at injection depth in the un-anesthetized chick for 10 s post-injection to reduce backflow. Human β-MSH (American Peptide, Sunnyvale, CA, USA) was dissolved in artificial cerebrospinal fluid [12] as a vehicle for a total injection volume of 5 μl with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned along the frontal plane to determine visually the presence of dye in the lateral ventricle. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Sex of chicks was determined visually by dissection and gonadal inspection at the time of decapitation.

Experiment 1: Food intake

Chicks, fasted for 180 minutes, were randomly assigned to receive either vehicle, 0.3, 1.0, or 3.0 nmol of β-MSH, based on [7], by ICV injection. After injection, chicks were returned to their individual home cages and given ad libitum access to food and water. Food intake was recorded (0.01 g) every 30 minutes for 180 minutes and at 540 minutes post-injection.

Experiment 2: Hypothalamic mRNA

Chicks, fasted for 180 minutes, were randomly assigned to receive either 0 or 0.3 nmol β-MSH (based on Experiment 1) via ICV injection. Chicks remained fasted following injection to prevent molecular changes associated with differences in food consumption. At 60 minutes post-injection, chicks were anesthetized via cardiopuncture, decapitated, and brains removed. The whole upside-down brain was snap-frozen in liquid nitrogen for 9 seconds. Cuts were made visually as per the following anatomy: perpendicular to the midline suture a cut was made at the septopallio-mesencephalic tract and at the third cranial nerves. 1.8 mm parallel to the midline two cuts were made and finally the dorsal cut was made from the anterior commissure to 0.8 mm ventral to the posterior commissure [17]. This block (comprised primarily of the hypothalamus) was immediately stored in RNAlater (Invitrogen, Carlsbad, CA, USA).

Hypothalamus was homogenized using stainless steel beads (Qiagen, Valencia, CA, USA), TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA), and a Tissue Lyser II (Qiagen) and total RNA was extracted following the manufacturer's instructions. The Direct-zol^{TM} RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA) was then used for total RNA purification. The concentration and purity of total RNA was assessed by spectrophotometry at 260/280/230 nm with a Thermo NanoPhotometer Classic (IMPLEN, Westlake Village, CA, USA).

First-strand cDNA was synthesized in 20 µl reactions from 200 ng of total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) following the manufacturer's instructions. Reactions were performed under the following conditions: 25 °C for 10 minutes, 37 °C for 120 minutes and 85 °C for 5 minutes. Primers for real-time PCR were designed with Primer Express 3.0 software (Applied Biosystems) (Table 6.1) and validated for amplification efficiency before use (95-105%). Real-time PCR was performed in 10 µl reactions containing 5 µl Fast SYBR Green Master Mix (Applied Biosystems), 0.25 µl each of 5 µM primers, 1.5 µl nuclease-free water, and 3 µl 10-fold diluted cDNA using a 7500 Fast Real-Time PCR System (Applied Biosystems). The conditions for real-time PCR were 95 °C for 20 seconds and 40 cycles of 90 °C for 3 seconds plus 60 °C for 30 seconds. A dissociation step consisting of 95 °C for 15 seconds, 60 °C for 1 minute, 95 °C for 15 seconds and 60 °C for 15 seconds was performed at the end of each PCR reaction to ensure amplicon specificity.

Experiment 3: hypothalamic nucleus mRNA

The design was the same as Experiment 2 except that chicks were anesthetized and perfused via the carotid artery with 2.5 mL of RNA stabilizing buffer (16.7 mM sodium citrate, 13.3 mM EDTA, and 3.5 M ammonium sulfate; pH = 5.2). Within 30 minutes of perfusion, brains were sectioned in a cryostat at -10 °C into 500 µm thick coronal sections in the direction from rostral to caudal: the PVN, VMH and ARC were chosen because increased c-Fos immunoreactivity was observed in these nuclei [7]. Sections that corresponded to 7.4 (PVN), 6.8 (VMH) and 5.4 (ARC) interaural [18] were collected and punches performed. Punches were collected using sterile disposable biopsy punch instruments (1 mm, Integra York PA Inc, York, PA, USA), and immediately placed in RNA lysis buffer (Norgen Biotek, Thorold, ON, Canada) with 1% beta-mercaptoethanol (Calbiochem, Millipore Sigma, Burlington, MA, USA), vortexed, snap-frozen in liquid nitrogen, and stored at -80 °C. The remaining brain section was photographed and punch accuracy verified via overlays from the chicken stereotaxic atlas [18].

The punchs were vortexed vigorously and incubated at room temperature for 5 minutes before adding 70% molecular biology grade ethanol, and total RNA was isolated following the manufacturer's instructions for the Total RNA Purification Micro Kit and RNase-Free DNase I (Norgen Biotek). The concentration and purity of total RNA was assessed as described in Experiment 2. Subsequent reactions were performed under the same conditions as described in Experiment 2 except that 100 ng of total RNA was used for reverse transcription and 5-fold diluted cDNA was used for real-time PCR.

Statistical analyses

Food intake data were analyzed using the GLM procedure of SAS 9.4 (SAS Inst., Inc., Cary, NC) and analysis of variance at each time point, with statistical model including the main effect of β-MSH treatment.

All molecular data were analyzed using the Fit Model platform of JMP Pro 14 (SAS Inst., Inc., Cary, NC). Homogeneity of variance was evaluated for all variables using the Fit Y by X platform and the Unequal Variances test and Levene's test. Real-time PCR data for whole hypothalamus samples (Experiment 2) were analyzed using the ΔΔCT method with β-actin as the reference gene and the average of the chicks in the vehicle group as the calibrator sample [19]. Relative quantities calculated as $2^{-\Delta\Delta CT}$ were used for statistical analysis. Data for nucleus samples were analyzed the same as Experiment 2 except that statistical model included the main effect of β-MSH treatment within nucleus. Statistical significance was set at $P < 0.05$ for all experiments.

Results

Effect of β-MSH on food intake

During the first 180 minutes post-injection, chicks injected with β-MSH all consumed less food than the vehicle-injected chicks. In addition, chicks injected with 3.0 nmol of β-MSH ate less than the 0.3 and 1.0 nmol groups at 540 minutes post-injection (Figure 6.1).

mRNA abundance in the whole hypothalamus

In Experiment 2, we quantified whole hypothalamus mRNA expression for several appetite-associated factors. The abundance of neuropeptide Y (NPY) receptor subtype 5 (NPYR5) (125% increase), CRF (150% increase), CRF receptor subtype 1 (CRFR1) (146% increase), POMC

(124% increase), cocaine- and amphetamine-regulated transcript (CART) (159% increase), MT (123% increase), MT receptor (MTR) (147% increase), growth hormone secretagogue receptor (GHSR) (143% increase), and c-Fos (202% increase) was greater in β-MSH-injected than vehicle-injected chicks (Table 6.2). Other factors, including NPY, NPY receptor subtype 1 (NPYR1), CRF receptor subtype 2 (CRFR2), MC3R, MC4R, AgRP, and ghrelin, were not affected.

mRNA abundance in different hypothalamic nuclei

In Experiment 3, we isolated the ARC, PVN and VMH, based on the report that these regions were activated in response to β-MSH [7], and measured several appetite-associated factors. In the ARC, expression of NPY (165% increase), AgRP (362% increase), MT (505% increase) and MTR (481% increase) was greater in β-MSH-treated than in control chicks (Table 6.3). Other factors such as NPYR1, NPYR5, CRF, CRFR1, CRFR2, MC3R, MC4R, POMC, CART, GHSR, and ghrelin were not affected. In the PVN, mRNA abundance of NPYR1 (123% increase), NPYR5 (118% increase), CRF (201% increase), CRFR1 (136% increase), CRFR2 (168% increase) and GHSR (114% increase) was greater in β-MSH-treated chicks, while expression of CART (38% decrease) and ghrelin (43% decrease) were lower in β-MSH-treated chicks in the PVN (Table 6.4). Factors like NPY, MC3R, MC4R, MT, and MTR were not influenced. In the VMH, expression of NPYR1 (130% increase), NPYR5 (128% increase), MTR (182% increase) and GHSR (130% increase) was greater in β-MSH-treated chicks whereas CRF (36% decrease) expression was lower in β-MSH-injected birds (Table 6.5). Expression of other factors like NPY, CRFR1, CRFR2, CART, and MT did not change in β-MSH-injected chicks.

Discussion

In the present study we confirmed our previously reported anorexigenic effect of β-MSH on food intake in broiler chicks [7]. Since the effect remained strong at 180 minutes post-injection, we extended our observation period. Interestingly, we found the highest dose (3.0 nmol) of β-MSH continued to suppress food intake at 9 hours post-injection. These data demonstrate that β-MSH is both a fast-acting and potent satiety-related regulator, which might be explained by its high affinity at MC4R [6]. A previous study reported there was no effect when using chicken-derived β-MSH [20]. This might be explained as divergent amino acid sequences between species [21], as the human sequence was used in the present study.

We previously observed that the ARC, PVN and VMH were activated by β-MSH injection [7]. The ARC integrates diverse signals and sends projections to second-order nuclei such as the PVN and VMH [1, 14]. In the present study, we isolated these three activated nuclei and measured mRNA abundance of several appetite-related neuropeptides and their receptors. The factors were chosen based on our previous α-MSH study [22]. Our results showed that although these two MSHs both bind to MC4R, the affected factors were not the same, implying different mechanisms underlying their effects.

CRF and MT are involved in β-MSH-induced anorexia

The two effects of CRF, appetite regulation and activation of the stress response, are primarily mediated by CRFR2 [23] and CRFR1 [24], respectively. Alpha-helical CRF, a CRFR antagonist, attenuated the anorexigenic effect of CRF following microinjection into the PVN but not the VMH, indicating site-specific CRF receptor involvement in the PVN [25]. In the present study, expression of CRF, CRFR1 and CRFR2 were all increased in the PVN by β-MSH, implying CRF-CRFR pathways are involved in the regulatory effect of β-MSH. We also detected decreased CRF but not its receptors in the VMH, which supported a former report [25] that CRF does not affect food intake via the VMH. Although our results showed divergent CRF expression in the PVN and VMH, increased mRNA abundance of CRF and CRFR1 were detected at the level of whole hypothalamus. The elevated CRF expression at this level was consistent with a previous study [11]. Our results of CRF expression in different nuclei further support its role as a downstream mediator in β-MSH-induced anorexia and also highlight the utility of measuring gene expression at the nucleus level where results might differ from the whole hypothalamus.

MT interacts with CRF and mediates its anorexigenic effect. Specifically, MT expression increased in the VMH after CRF administration [26]. In turn, MT injection increased CRFR1 expression [27]. Additionally, OXT antagonist pretreatment attenuated CRF ICV injection-induced anorexia [28]. In our study, no patterns were found between CRF and MT expression within each nucleus. At the level of hypothalamus, both MT and MTR were increased following β-MSH injection, indicating this pathway is involved in β-MSH-induced anorexia.

Anorexigenic factors such as POMC, CART, ghrelin and GHSR are influenced in β-MSH treated chicks

POMC neurons receive central and peripheral signals and induce anorexia [29]. POMC derived α-, β-, and γ2-MSH all cause decreased food intake [8, 10]. In the present study, although POMC expression was not increased by β-MSH in the ARC, it was affected in the whole hypothalamus, suggesting that some other hypothalamic nucleus may contribute to POMC mRNA abundance.

CART is co-localized with POMC within the ARC [30] and induces anorexia in chicks [31]. CART-immunoreactive fibers and terminal endings are also found in the PVN and dorsomedial nucleus (DMN) [30, 32]. Interestingly, elevated CART after direct administration or viralmediated overexpression in the PVN led to increased appetite in rats [33, 34], suggesting an opposite orexigenic effect of CART in the PVN. Our data supported these reports that CART expression was inhibited by β-MSH injection within the PVN. However, whole hypothalamic CART mRNA abundance was still higher in β-MSH-injected chicks. This elevation might be related to increased expression in other hypothalamic nuclei without β-MSH-induced c-Fos immunoreactivity, i.e. the DMN, since no effects were detected within the ARC and VMH.

Central ghrelin injection decreased food intake in chickens [35]. In our study, β-MSH injection increased the expression of its receptor GHSR in hypothalamus, notably in the PVN and VMH, while ghrelin expression was reduced in the PVN.

NPY, NPY receptors and AgRP are activated to restore appetite

NPY neurons have projections from the ARC to other hypothalamic nuclei, including the PVN and VMH [36]. We showed increased NPY expression in the ARC, and increased NPYR1 and NPYR5 in the PVN and VMH, which may be a counter mechanism to restore appetite to baseline levels. In chickens, ICV injection of AgRP attenuated α-MSH-induced anorexigenic effects [5]. Our study showed that β-MSH stimulated the expression of AgRP in the ARC, which might also be a mechanism to restore homeostatic food intake.

Conclusions

In conclusion, exogenous β-MSH induces anorexia in chicks and involves CRF and MT expression changes within the hypothalamus. Several other factors, such as POMC, CART, ghrelin and GHSR, are also affected by β-MSH, suggesting that the effect of β-MSH involves other pathways, or these factors participate in other non-appetite associated responses. By detecting transcriptional changes in individual nuclei, this research provides insights to better understand hypothalamic mechanisms of β-MSH-induced anorexia.

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Table 6.1. Primers used for real-time PCR [a]

Gene	Accession No.	Sequences 5’ to 3’ (forward/reverse)
β-actin	NM_205518.1	GTCCACCGCAAATGCTTCTAA/TGCGCATTTATGGGTTTTGTT
AgRP	NM_001031457.1	GGTTCTTCAACGCCTTCTGCTA/TTCTTGCCACATGGGAAGGT
CART	XM_003643097.3	GCTGGAGAAGCTGAAGAGCAA/GGCACCTGCCCGAACTT
c-Fos	NM_205508.1	TGTTCCTGGCAATATCGTGTTC/CTTTCCCCCCCACGTAAGA
CRF	NM_001123031.1	TCAGCACCAGAGCCATCACA/GCTCTATAAAAATAAAGAGGTGACATCAGA
CRFR1	NM_204321.1	CTGCTGTCCTTGCTGGGAAT/ATCCTCCCCCGGATTGAC
CRFR2	NM_204454.1	GGATCAAATACAACACCACAAAAAAT/GGCCCATGTCCCATTGC
Ghrelin	NM_001001131.1	GAAGCACTGCCTAACGAAGACA/GGATGCTGAGAAGGAGAATTCCT
GHSR	XM_015291631.1	TCTGCGAGCGAAGGTGATC/AGACGGCCCAGAGGATGAG
MC3R	XM_004947236.2	GCCTCCCTTTACGTTCACATGT/GCTGCGATGCGCTTCAC
MC4R	NM_001031514.1	CCTCGGGAGGCTGCTATGA/GATGCCCAGAGTCACAAACACTT
MT	XM_004936280.2	TGGCTCTCTCCTCAGCTTGTTAT/GGCACGGCACGCTTACC
MTR	NM_001031569.1	TGTGCTGGACGCCCTTCT/TCCTGCGGAGCGTTGGT
NPY	NM_205473.1	CATGCAGGGCACCATGAG/CAGCGACAAGGCGAAAGTC
NPYR1	XM_015285306.1	TAGCCATGTCCACCATGCA/GGGCTTGCCTGCTTTAGAGA
NPYR5	NM_001031130.1	GGCTGGCTTTGTGGGAAA/TTGTCTTCTGCTTGCGTTTTGT
POMC	NM_001031098.1	GCCAGACCCCGCTGATG/CTTGTAGGCGCTTTTGACGAT

[a] Primers were designed with Primer Express 3.0 (Applied Biosystems). Abbreviations: AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CRF, corticotrophin-releasing factor; CRFR1 and R2, CRF receptor subtypes 1 and 2, respectively; GHSR, growth hormone secretagogue receptor; MC3R and 4R, melanocortin receptor subtypes 3 and 4, respectively; MT, mesotocin; MTR, mesotocin receptor; NPY, neuropeptide Y; NPYR1 and R5, NPY receptor subtypes 1 and 5, respectively; POMC, pro-opiomelanocortin.

Table 6.2. Hypothalamic mRNA abundance [a]

Variable	NPY	NPYR1	NPYR5	CRF	CRFR1	CRFR2	MC3R	MC4R	POMC	AgRP	CART	MT	MTR	GHSR	Ghrelin	c-Fos
Treatment																
Vehicle	1.05±0.10	1.03±0.08	1.01±0.06	1.01±0.06	1.01±0.06	1.07±0.13	1.05±0.10	0.95±0.07	0.96±0.08	1.25±0.25	1.04±0.10	1.13±0.05	1.03±0.08	1.03±0.09	1.07±0.13	1.03±0.09
β-MSH	1.22±0.06	1.05±0.07	1.26±0.14	1.52±0.10	1.47±0.21	1.17±0.08	1.12±0.11	1.05±0.05	1.19±0.08	1.32±0.14	1.65±0.11	1.39±0.12	1.51±0.12	1.47±0.14	1.17±0.13	2.08±0.17
P-value	.09	.41	**.003**	**.0006**	**.03**	.26	.32	.13	**.03**	.40	**.0004**	**.04**	**.003**	**.01**	.30	**.0001**

[a] At 4 days post-hatch, chicks (*Gallus gallus*) were intracerebroventricularly injected with vehicle or 0.3 nmol β-melanocyte-stimulating hormone (β-MSH). The hypothalamus was collected at 1 h post-injection. Values are means ± standard errors (n = 9 for each treatment). Bold numbers denote statistical significance ($P < 0.05$). Abbreviations: NPY, neuropeptide Y; NPYR1 and R5, NPY receptor subtypes 1 and 5, respectively; CRF, corticotrophin-releasing factor; CRFR1 and R2, CRF receptor subtypes 1 and 2, respectively; MC3R and 4R, melanocortin receptor subtypes 3 and 4, respectively; POMC, pro-opiomelanocortin; AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; MT, mesotocin; MTR, MT receptor; GHSR, growth hormone secretagogue receptor.

Table 6.3. mRNA abundance in the arcuate nucleus [a]

Variable	NPY	NPYR1	NPYR5	CRF	CRFR1	CRFR2	MC3R
Treatment							
Vehicle	1.26±0.24	1.11±0.11	1.25±0.17	1.52±0.38	1.02±0.05	1.62±0.32	1.03±0.07
β-MSH	2.08±0.37	1.25±0.08	1.38±0.21	1.43±0.28	1.09±0.16	2.27±0.47	0.96±0.09
P-value	**.04**	.15	.32	.43	.33	.13	.29

Variable	MC4R	POMC	AgRP	CART	MT	MTR	GHSR
Treatment							
Vehicle	1.06±0.11	1.58±0.36	2.72±1.23	1.24±0.17	3.17±1.11	1.68±0.49	1.11±0.12
β-MSH	1.18±0.12	2.60±0.49	9.85±3.72	1.51±0.37	16.02±3.85	8.08±1.92	1.01±0.08
P-value	.24	.053	**.048**	.26	**.004**	**.003**	.24

Variable	Ghrelin
Treatment	
Vehicle	1.54±0.39
β-MSH	0.89±0.17
P-value	.08

[a] At 4 days post-hatch, chicks (*Gallus gallus*) were intracerebroventricularly injected with vehicle or 0.3 nmol β-melanocyte-stimulating hormone (β-MSH). The arcuate nucleus was collected at 1 h post-injection to measure expression of neuropeptide Y (NPY), NPY receptor subtypes 1 and 5 (NPYR1 and R5, respectively), corticotrophin-releasing factor (CRF), CRF receptor subtypes 1 and 2 (CRFR1 and R2, respectively), melanocortin receptor subtypes 3 and 4 (MC3R and 4R, respectively), pro-opiomelanocortin (POMC), agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), mesotocin (MT), MT receptor (MTR), and growth hormone secretagogue receptor (GHSR). Values are means ± standard errors (n = 10-15 for each treatment), with *P*-values for the effect of treatment. Bold numbers denote statistical significance ($P < 0.05$).

Table 6.4. mRNA abundance in the paraventricular nucleus [a]

Variable	NPY	NPYR1	NPYR5	CRF	CRFR1	CRFR2	MC3R
Treatment							
Vehicle	1.05±0.08	1.02±0.05	1.03±0.06	1.48±0.34	1.08±0.10	1.05±0.09	1.08±0.10
β-MSH	1.08±0.16	1.25±0.11	1.22±0.08	2.97±0.71	1.47±0.16	1.76±0.23	1.14±0.18
P-value	.44	**.04**	**.03**	**.04**	**.02**	**.006**	.38

Variable	MC4R	CART	MT	MTR	GHSR	Ghrelin
Treatment						
Vehicle	1.06±0.09	1.18±0.17	2.70±0.84	1.22±0.17	1.03±0.06	1.23±0.16
β-MSH	1.11±0.11	0.73±0.08	1.30±0.33	1.13±0.23	1.17±0.06	0.70±0.10
P-value	.35	**.01**	.07	.39	**.046**	**.005**

[a] At 4 days post-hatch, chicks (*Gallus gallus*) were intracerebroventricularly injected with vehicle or 0.3 nmol β-melanocyte-stimulating hormone (β-MSH). The paraventricular nucleus was collected at 1 h post-injection to measure expression of neuropeptide Y (NPY), NPY receptor subtypes 1 and 5 (NPYR1 and R5, respectively), corticotrophin-releasing factor (CRF), CRF receptor subtypes 1 and 2 (CRFR1 and R2, respectively), melanocortin receptor subtypes 3 and 4 (MC3R and 4R, respectively), cocaine- and amphetamine-regulated transcript (CART), mesotocin (MT), MT receptor (MTR), and growth hormone secretagogue receptor (GHSR). Values are means ± standard errors (n = 11-17 for each treatment), with *P*-values for the effect of treatment. Bold numbers denote statistical significance ($P < 0.05$).

Table 6.5. mRNA abundance in the ventromedial hypothalamus [a]

Variable	NPY	NPYR1	NPYR5	CRF	CRFR1	CRFR2	CART
Treatment							
Vehicle	1.03±0.07	1.02±0.06	1.03±0.07	1.13±0.17	1.03±0.08	1.09±0.13	1.22±0.23
β-MSH	1.11±0.07	1.33±0.12	1.32±0.08	0.72±0.11	1.01±0.03	1.04±0.11	1.17±0.10
P-value	.22	**.02**	**.006**	**.03**	.41	.40	.41

Variable	MT	MTR	GHSR
Treatment			
Vehicle	1.89±0.48	1.14±0.17	1.00±0.09
β-MSH	2.06±0.73	2.08±0.26	1.30±0.09
P-value	.43	**.003**	**.01**

[a] At 4 days post-hatch, chicks (*Gallus gallus*) were intracerebroventricularly injected with vehicle or 0.3 nmol β-melanocyte-stimulating hormone (β-MSH). The ventromedial hypothalamus was collected at 1 h post-injection to measure expression of neuropeptide Y (NPY), NPY receptor subtypes 1 and 5 (NPYR1 and R5, respectively), corticotrophin-releasing factor (CRF), CRF receptor subtypes 1 and 2 (CRFR1 and R2, respectively), cocaine- and amphetamine-regulated transcript (CART), mesotocin (MT), MT receptor (MTR), and growth hormone secretagogue receptor (GHSR). Values are means ± standard errors (n = 11-13 for each treatment), with *P*-values for the effect of treatment. Bold numbers denote statistical significance ($P < 0.05$).

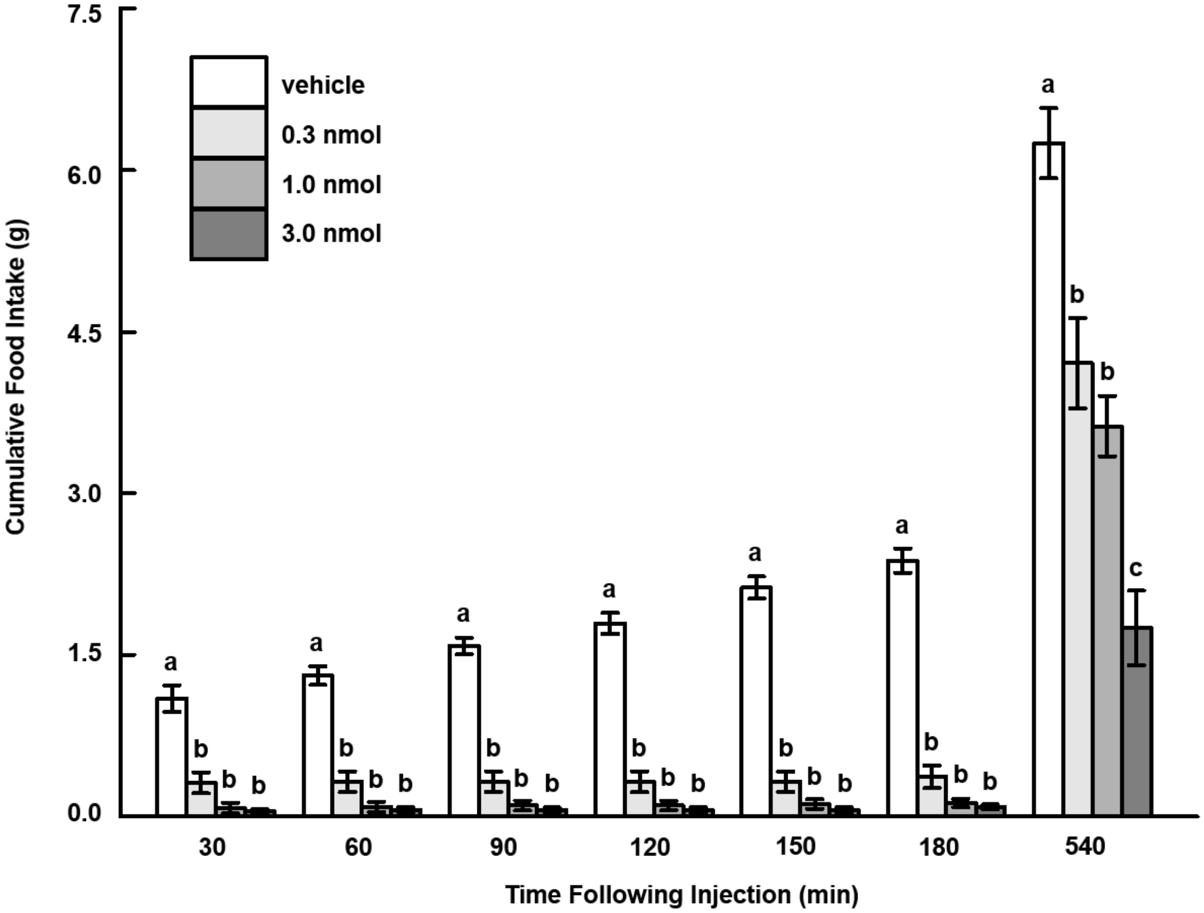

Figure 6.1. Cumulative food intake at 30 to 540 minutes post-injection.

At 4 days post-hatch, broiler chicks (*Gallus gallus*) were intracerebroventricularly injected with vehicle, 0.3, 1.0, or 3.0 nmol of β-melanocyte-stimulating hormone (n = 8-12 per group). Values represent means ± standard errors. Unique letters denote a significant difference ($P < 0.05$) among groups within a time point, Tukey's test.

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Chapter 7: The microbiota-gut-brain axis during heat stress in chickens: a review

Abstract

Heat stress is a global issue for the poultry industries with substantial annual economic losses and threats to bird health and welfare. When chickens are exposed to high ambient temperatures, like other species they undergo multiple physiological alterations, including behavioral changes such as cessation of feeding, initiation of a stress signaling cascade, and intestinal immune and inflammatory responses. The brain and gut are connected and participate in bidirectional communication via the nervous and humoral systems, this network collectively known as the gut-brain axis. Moreover, heat stress not only induces hyperthermia and oxidation at the gut epithelium, leading to impaired permeability and then susceptibility to infection and inflammation, but also alters the composition and abundance of the microbiome. The gut microflora, primarily via bacterially-derived metabolites and hormones and neurotransmitters, also communicate via similar pathways to regulate host metabolic homeostasis, health, and behavior. Thus, it stands to reason that reshaping the composition of the gut microbiota will impact intestinal health and modulate host brain circuits via multiple reinforcing and complementary mechanisms. In this mini-review, we describe the structure and function of the microbiota-gut-brain axis, with an emphasis on physiological changes that occur in heat-stressed poultry.

Key words: Microbiota-gut-brain axis, Heat stress, Hypothalamic-pituitary-adrenal axis, Chickens

Introduction

The microbiota-gut-brain axis (MGBA) has been widely investigated in human and mammalian species for decades due to its vital role in not only homeostatic maintenance but also the pathology of various neurodevelopmental and neurodegenerative disorders [1]. In addition, the importance of this axis in non-mammalian species such as chickens has been acknowledged and the potential mechanisms are being investigated. The relationship between the gut microbiome and host is considered to be mutualistic rather than commensal [2]. The host provides the microbiome with hospitable niches and undigested food, and in turn, these microorganisms metabolize and produce neuroactive components. These neuroactive molecules, such as serotonin, exert a systematic or local effect in regulating host physiological processes, by entering the circulation or interacting with enteric nervous and immune systems, respectively. Factors that act on the central nervous system, for instance via vagal afferents, influence host behaviors whereas others trigger structural and functional changes in the intestine [3]. The interactive effects between the gastrointestinal microbiota and the host could be benign or detrimental, depending on the type and magnitudes of factors, including but not limited to dietary composition, environmental stimuli, and host genetics and phenotypes [4].

Stress, by a simple definition, is the adaptive physiological and psychological response of an organism to restore homeostasis [5]. The intestinal tract is reported to be involved in responses to all kinds of stressors, including heat stress [6]. Heat stress is a major environmental challenge, and occurs when there is an imbalance in the net amount of heat energy produced by and released from an organism [7], during which the organism generates more heat than it can utilize and release. Heat stress is closely related to changes in the intestine, both structurally and functionally, and in the composition of the gut microbiota [8, 9]. Studies on the effects of heat stress on the gut microbiota in humans are lacking [10], and a variety of animal models are utilized to thoroughly investigate these effects to provide clinical insights. In this review, we focus on avian models, because heat stress has been widely reported to influence poultry meat and egg production, as well as flock health and wellbeing, through major changes in intestinal physiology and the gut microbiota [6, 11]. However, how heat stress interacts with the chicken's gut microbiota and affects the MGBA is not fully understood and requires further elucidation.

Herein, we review literature related to heat stress-induced alteration in chicken behaviors (such as feeding and social behaviors), physiological processes, intestinal integrity and the microbiota, and the immune system, with an emphasis on the relationships of these alterations to gut microbiota composition. We also review what is known regarding the use of probiotics and prebiotics as preventative and therapeutic interventions in heat-stressed animals, and discuss strategies to ameliorate the detrimental effects of high temperatures on bird behavior and health.

Microbiota-gut-brain axis

Gut microbes consist of different microorganisms such as bacteria, viruses, yeast and other fungi, and protozoa, among which the most abundant from an organismal and research study standpoint are bacteria [10], hence bacteria being the target of this review. The amount of microbiota varies dramatically between intestinal sites, from about 10^5 colony-forming units (CFU) per gram of digesta in the small intestine to around 10^{11} CFU per gram of digesta in the cecum [12, 13]. During the past decade, technological advances in profiling microbiomes within the host, from improvements in laboratory culture techniques to 16S rRNA gene sequencing and metagenomics sequencing, have enabled the study of the composition of the microbiome with greater resolution and depth. In chickens, Lactobacilli dominate several parts of the upper digestive tract, including the crop, proventriculus and ventriculus (gizzard), whereas the small intestine is mainly inhabited by *Lactobacillus*, *Enterococcus*, and *Clostridiaceae*. This prevalence of specific species is related to the function of the digestive organs, since the pH of gastric juices is relatively low, which favors domination by Lactobacilli. In the cecal tonsils, where digesta resides the longest time during digestion, and the concentration of short-chain fatty acids (SCFAs) synthesized by bacteria is greater than elsewhere in the gastrointestinal tract (GIT), the most abundant phyla are *Firmicutes*, *Bacteroides*, and *Proteobacteria* [3, 10, 12, 14].

Other than the functions of microbial products as nutrient substrates, they are capable of interacting with immune or neuroendocrine systems to regulate nervous system functions [15, 16]. For example, acetate and butyrate, two major SCFAs, are reported to participate in the maintenance of gastrointestinal tract barrier intactness, through which bacterial colonization and translocation are prevented. In addition to being taken up and used as a metabolic substrate (ATP production) by intestinal cells, particularly intestinal epithelial cells (enterocytes), SCFAs act as signaling molecules. For instance, butyrate and propionate, interact with specific G-protein-coupled receptors to regulate and maintain energy and immune homeostasis in cells and thus influence their activity, by activating pathways such as chemotaxis, apoptosis, proliferation, differentiation, and gene expression programming [17, 18]. Additionally, SCFAs are closely related to the synthesis of a variety of neuroactive molecules, such as leptin, glucagon-like peptide 1 (GLP-1), and other hormones, which can be transported through the circulation to several brain regions. Neurons in the arcuate nucleus of hypothalamus, for instance, receive signals through receptors of these neuropeptides and neurotransmitters and then regulate host's appetite [17-19]. Although the blood-brain barrier limits the penetration of many molecules into the brain parenchyma, the existence of circumventricular organs, which lack a blood-brain barrier, including the median eminence, which is located proximally to the arcuate nucleus, facilitates the receipt and integration of peripheral signals by cells in the central nervous system.

Bacteria synthesize classic neurotransmitters, such as serotonin, which can act locally or distantly through the circulation or nervous system, and as such have been referred to in the literature as “mind-altering” [20]. In the intestine, host enterochromaffin cells, a type of entero-endocrine cell, produce serotonin. While most dietary-derived tryptophan is oxidized in the liver via the kynurenine shunt, some is converted into serotonin. In fact, the majority (> 95%) of serotonin in the body is synthesized in the gut, occurring via sequential conversion of tryptophan via two enzymatic reactions. Intestinally-derived serotonin, whether of host or bacterial origin, can then act via the endocrine system or through the vagus nerve. Within the small intestine, most serotonin is released into the mucosa, and it is estimated that roughly 2% of all enteric neurons are serotonergic [21]. Via a variety of receptors, including the ionotropic 5-HT3 and metabotropic 5-HT1, 2, 4, and 7, serotonin influences gut motility (peristalsis), secretion of chemicals such as bicarbonate during digestion, vasodilation, and neuronal survival and inflammation [21].

Under normal and healthy conditions, microbial communities in the host gastrointestinal tract play an overall beneficial role. They assist in competing against pathogenic microbial taxa and maintaining intact intestinal lumen surface structures, ferment undigested polysaccharides into SCFAs, and provide additional vitamins [14]. Indeed, coprophagic species, such as rabbits and rodents recover such vitamins by consuming fecal pellets. There are detrimental effects of the gut microbiota undergoing dysbiosis, which can be initiated by but also exacerbated in response to gastrointestinal environment perturbations (temperature, pH, nutrient composition, toxins, introduction of microflora, etc.), resulting in several acute or chronic diseases in the host [10].

There is a clear relationship, for example, between intestinal diseases such as Crohn's disease and inflammatory bowel syndrome (IBS) and unbalanced SCFA production and serotonin availability in the gut [22]. Thus, maintaining a healthy gut microbial community and hospitable mucosal environment is of utmost importance to host health and well-being. Generally, a microbiota that is diverse in both composition and genetic content or is dominated by beneficial taxa is characterized as being a healthy community [10].

The gut microbiota and brain have bidirectional connections. On one hand, gut microbiota themselves are an important source of peripheral neurotransmitters and hormones. These molecules not only modulate gut functions like peristalsis, as described above, but directly communicate the intestinal state through vagal afferents to the brainstem and higher brain regions. Various stressful stimuli through peripheral and central pathways, lead to the activation of the hypothalamic-pituitary-adrenal (HPA) axis, which might further alter gut microbiota composition and activity as well as intestinal epithelial cells' function. Release of corticotropin-releasing factor (CRF) from the hypothalamus stimulates adrenocorticotropic hormone (ACTH) release from the anterior pituitary into the circulation, which then triggers the release of corticosteroids from the kidney, including cortisol by humans and corticosterone by birds from the adrenal cortex into the circulation. Corticosteroids exert a multitude of effects on the gastrointestinal tract via direct interactions with enteric muscle cells and neurons, bacteria and intestinal immune cells, leading to the release of cytokines, which via the circulation can act on the brain to affect mood, appetite, cognition, and emotion [20]. Several environmental factors, such as dietary composition and drugs, could influence activity of the MGBA through one or more pathway that feeds into one or more of these mutually reinforcing connections. For instance, appetite regulation is mediated mainly in several brain regions like the hypothalamus and brainstem. Nutrients in the gut stimulate the release of satiety factors such as cholecystokinin, and also directly affect the microbiota, which in turn regulate the concentration of cytokines and neuroactive molecules that modulate brain function [23].

Heat stress and the gut microbiota

The adaptive physiological and behavioral responses of an organism to environmental demands or pressures has been described as stress responses, by which the organism attempts to maintain or restore homeostasis [2, 10]. Stressors or stressful stimuli can vary from acute to chronic and from one time to several repetitive times, and their magnitude can be mild or severe. Additionally, the different capabilities of individuals to perceive stress result in various outcomes [24]. Individuals exposed repeatedly exposed to stressful situations appear to be more vulnerable to gastrointestinal diseases.

There exist various factors that cause changes in the intestinal microbiota of chickens. One major source of these factors is from the host itself, such as age, type and breed, sex, and sampling site in the gastrointestinal tract. Environmental factors also influence the microbiota composition, including biosecurity level, housing condition, litter, feed, temperature and location [4]. Among those environmental factors, a growing amount of evidence indicates that heat stress has significant effects on the chicken’s intestinal microbiota composition and tissue structure.

When birds are exposed to stressful factors (such as long-term exposure to sunlight, high ambient temperature and humidity, and poor ventilation), their internal energy homeostasis is disrupted and physiological alterations ensue. The transient or long-term imbalance between heat dissipation to the environment and heat production inside the animal can disturb thermostasis and eventually result in heat stress. The thermoneutral zone is the ambient temperature range where the animal efficiently regulates and maintains a constant body temperature [25]. When environmental temperature exceeds the upper critical temperature, which is the upper limit of the thermoneutral zone, animals are considered to be exposed to heat stress [26]. In general, thermoneutral zones for broiler chickens are 28~34, 25~31, 22~28, 20~25, 18~24, and 18~24 °C for each of the first six weeks of age, respectively [27].

The influence of heat stress on the composition of the gut microbiota can occur as a direct consequence of altering body temperature (hyperthermia-induced changes in cellular function) or indirectly due to an acute or gradual change in the birds' behavior, physiological status, intestinal integrity and immune system activity. Core body temperature, when elevated by exposure to high ambient temperature, surprisingly did not dramatically alter the microbiota in the cecal tonsils [13]. Another study found that compared with the thermoneutral condition, both acute and chronic heat stress led to a higher rectal temperature, with the magnitude even higher in the former situation. Rectal temperature peaked during the first two days of heat exposure, then fluctuated until it reached a plateau. After that, individuals under heat stress showed thermo homeostasis during the rest of the observation period [28]. These results suggest that core body temperature, despite being temporarily affected by heat exposure, could be adjusted promptly and exert a limited direct effect on the gut microbiota.

Heat stress induces appetite suppression

Our group demonstrated that exposure to high ambient temperatures suppressed food consumption in young broiler chickens, which was associated with changes in the activity of several appetite-regulating peptides, such as orexigenic neuropeptide Y and anorexigenic CRF, which might both have peripheral effects associated with the enteric system and HPA axis [29].

In another heat stress study, birds ate less, ingested more water, panted more often and lifted the wings much longer [30]. Typically, the reduction in food intake is sustained during heat stress and is thought to be a compensatory mechanism to reduce heat production associated with nutrient metabolism, although heat stress is associated with changes in nutrient absorption, particularly amino acids and glucose. For this reason, a multitude of heat stress studies with chickens and other species have employed dietary strategies to mitigate nutrient-induced heat production, including formulating diets to vary in macronutrient composition [31]. The GIT of chickens consumes about 7% of the energy derived from the diet, so reduced feeding, while offsetting some of the heat production in the animal, could also elicit a fast and dramatic response in the GIT, primarily jeopardizing gut integrity and mucosal immunity, which further compromises nutrient assimilation, triggers systematic inflammation, and impairs production [32, 33].

In some commercial practices, the distance of the grow-out facility from the brooder house necessitates transporting chicks over long distances after hatching and processing. Although the residual yolk sac provides a reservoir of nutrients that are resorbed into the intestine and used by the chick after hatching, delayed access to food after hatch can impair intestinal development [34], and also establishment of the microbiota composition [35]. Similar influences were observed in response to food withdrawal at a later age [36, 37]. Having no access to food, even for a period of 6 hours, allows pathogens, like *Salmonella* [36], to colonize within the gut and reshape the microbial community [37, 38]. Sequencing techniques used to evaluate the taxonomy of the gut microbiota revealed that chickens subjected to food withdrawal had altered populations in the ileum and cecum, with increased *Firmicutes* and diminished *Proteobacteria*. Overall, the major effects of food deficiency on the intestinal microbiome are on the dominant families, such as *Turicibacteraceae*, *Ruminococcaceae*, and *Enterobacteriaceae* [39]. In broiler breeders, it is common practice to restrict the amount of food consumed throughout life, in order to meet target body weights to prevent metabolic disorders and support optimal reproduction. However, as described above, such practices could negatively impact the gut microbiome which in turn could impact health of the chicken. Combined with exposure to high temperatures, restricted access to feed could have major impacts on the bacterial composition of the gastrointestinal tract and thereby affect the bird's whole-body physiology.

Heat stress also negatively affects layer-type chicks, by reducing their food consumption, egg production and quality, and increasing death rate [30, 40, 41]. In laying hens, integrity of the gut mucosa was impaired under heat exposure, resulting in limited nutrient transport across the intestinal mucosal layer [42]. Moreover, the intestinal microbiome community was modified in heat-stressed pullets and hens [8, 36, 43]. Xing et al. (2019) found that layer chicks displayed an altered microbiome composition rather than species abundance, in response to exposure to a high ambient temperature (29–35 °C), and this change was closely associated with less food consumption [13]. Another study involved exposing the layers to a cyclic temperature of 35 °C 7 hours per day, and found an increased alpha diversity, that is, the present species of the microbiome were elevated in the cecum after 2 weeks exposure, although they returned back to normal levels after 4 weeks. Additionally, the two most abundant cecal phyla, *Bacteroidetes* and *Firmicutes*, showed significantly different richness by the end of the experiment. This study suggested that heat stress started to reshape microbiota in layers at 2 weeks but the bacteria adapted to temperature change later at 4 weeks [44]. Shi et al. (2019) found slightly different results, and observed significant changes in the abundance of those two phyla starting at 1 week, although also a loss of significance by 4 weeks [45]. All of these findings suggest that the influence of heat stress on the gut microbiota depends on the magnitude and duration of heat exposure.

In layer chicks under heat exposure, there were elevated numbers of several detrimental genera, including *Escherichia*, *Shigella*, and *Clostridium*, which generate alpha-toxins and contribute to the occurrence of necrotizing enterocolitis. On the other hand, advantageous bacteria, such as *Lactobacillus* and *Ruminococcaceae*, were scarce [46]. Bacteria in the genus *Lactobacillus* groups are widely used as probiotics, since their metabolites are capable of regulating the acid-base equilibrium in the intestine, which favors the development of a beneficial but not pathogenic microbiome [47]. Some species in the *Lachnospiraceae* group are also inhibited during heat stress [48, 49]. These species produce a relatively large proportion of butyrate, which helps maintain intestinal health by facilitating epithelial development. Generally, butyrate is less abundant than other SCFA (60% acetate, 25% propionate, and 15% butyrate; at least in humans), although it serves as the major energy source for colonocytes in the large intestine and is known to affect gene expression by acting as a histone deacetylase (HDAC) inhibitor and affects signaling via activation of several G-protein-coupled receptors [50]. Many studies have demonstrated a beneficial role for butyrate in maintaining intestinal barrier integrity, and in preventing inflammation [50]. Thus, changes in the numbers of butyrate-producing bacteria could modulate the MGBA via effects of butyrate signaling on the peripheral and central nervous systems [50].

Heat stress reduces intestinal integrity

The gut microbiome environment is normally stable under healthy conditions. The intestine provides niches for bacterial to colonize and thrive, and in turn, commensal bacteria compete with pathogenic bacteria for space and nutrients to survive, and produce metabolites that boost host intestinal immunity and suppress the growth of pathogens, which collectively protect the gut epithelium. However, stressful stimuli can concurrently impair intestinal barrier integrity and alter the microbiome [51-53]. Once the mucosal layer is penetrated, intestinal pathogens have access to the host circulation and cause diseases and impair the efficiency of nutrient digestion and assimilation [54, 55].

There is evidence that the intestinal mucosa, which is susceptible to heat stress and microbiome change-induced damage and inflammation, can also adapt to the conditions to maximize nutrient assimilation in some circumstances. Heat-treated chicks had decreased plasma thyroid hormone and increased plasma corticosterone, as well as a damaged mucosal layer in the jejunum, but the ability to transport glucose across the jejunal epithelium was enhanced, which may have compensated for the lack of energy due to reduced food consumption [56]. In another study, however, when chicks experienced a higher temperature (35 °C, 5 degrees higher), their intestinal structures were significantly damaged, with reduced villus height and functional absorptive surface area, and elevated levels of blood endotoxins. These adverse impacts were not overcome by host adaptations alone, but required exogenous butyrate supply for alleviation, further demonstrating a beneficial role for butyrate in maintaining intestinal structure and function [57].

The ileum is a unique intestinal niche because of its proximity to the cecum and receipt of end-products of digestion that are not absorbed in the proximal small intestine. It is home to a larger amount of bacteria, even the pathogenic Salmonella, than the proximal small intestine and provides a rich source of nutrient substrate for fermentative activity [58]. The intestine stands as the first line of defense against invading pathogens [59]. If, for some reason, the chicken small intestinal epithelium is damaged, Salmonella adhere at impaired locations and translocate into the host, causing a systemic infection [36]. This was observed in chickens that underwent 24 hours of food deficiency or heat stress [28, 60, 61]. Treating chicks with high temperature chronically or acutely results in invading Salmonella, which are later detected in the liver, spleen, and muscles. The liver and spleen typically handle these exogenous pathogens, which are engulfed by macrophages and transported through the circulation. However, the organs that are primarily targeted by Salmonella during a systemic infection have yet to be identified [62].

Two mechanisms were proposed that mediate the effect of heat stress on the intestinal epithelium. The first is that reactive oxygen and/or nitrogen species are produced in response to high environmental temperature and increased oxidative activity, overwhelming the capacity of endogenous antioxidant systems [63]. When chicks are heat-exposed, the production of these free radical molecules provoke injury to the epithelial cell membranes, resulting in fewer tight junctions (TJ) and less expression of TJ genes. Thus, the intestinal barrier becomes permeable to paracellular entry by bacterial endotoxins. The second mechanism is that heat stress promotes the production of proinflammatory cytokines, which also damage the TJ [64]. Among those cytokines, Interleukin-2 (IL-2) and tumor necrosis factor-α (TNF-α) are among those whose concentrations in circulation are elevated in heat-stressed chicks. IL-2 is produced by T cells, and once released, it activates other types of cells like macrophages, which secrete proinflammatory cytokines, such as TNF-α, to initiate inflammation [65]. However, the secretion of IL-2 could also be stimulated by endotoxins [66], thus this mechanism might be a secondary or indirect effect.

Heat stress activates the HPA axis

The HPA axis is an essential system that integrates and mediates an organism's response to intrinsic and/or extrinsic stressors [67]. Its activation is characterized by the activation of hypothalamic CRF, the release of ACTH, and the production of corticosterone in rodents and birds [68]. Chronic and acute high ambient temperature exposure activate the HPA axis, which is usually characterized by elevated blood corticosterone in the animal. Elevations in circulating corticosterone are associated with an array of physiological responses, such as suppressed food intake and growth performance, and aberrant immune and inflammatory responses, to name a few [69].

In addition to corticosterone, activation of the HPA axis is accompanied by the generation of many other hormones, neuroactive molecules, and cytokines. These factors are shared by many systems in the body (such as the central nervous system (CNS), endocrine, and immune systems), and bidirectionally mediate systematic interplay through the binding of ligands to their receptors [70]. For example, the CNS regulates immunity primarily through the HPA axis activity and sympathetic outflow [71]. Hormones involved in the regulation are corticosterone from the HPA axis and the catecholamines from sympathetic activity. Two major catecholamines, norepinephrine and epinephrine, could further regulate the synthesis of inflammatory cytokines, with reduced levels of proinflammatory IL-12, TNF-α, and interferon γ, and enhanced expression of anti-inflammatory IL-10 and transforming growth factor β [72]. In turn, signals from visceral organs or tissues, particularly the gastrointestinal tract, can be picked up by parasympathetic inflow or sent back to the HPA axis [73]. Indeed, intestinal inflammation provides feedback to the HPA axis, which in turn regulates immune defense against pathogens [74].

Although activation of the HPA axis by heat stress is linked to intestinal immunity and inflammation [11, 75-77], few have gone so far as to investigate actual changes in the gut microflora and brain activity. Generally, beneficial commensal bacteria were less competitive, whereas pathogenic species, such as *Escherichia coli* and *Salmonella*, flourished in heat-stressed animals due to impaired intestinal integrity and function, and increased permeability [78]. In a study with broiler chickens, heat stress and/or intestinal infection with *Clostridium* and *Eimeria spp*, (bacteria and protozoal species, respectively), led to changes in concentrations of monoamines in key brain regions, including a decrease in serotonin, norepinephrine, and epinephrine in the hypothalamus, and dopamine in the mid-brain [79]. Authors speculated that these data demonstrated activation of the HPA axis via increased release of cytokines from intestinal immune cells, in response to the pathogen challenge. Monoamine concentrations and cytokine production in the small intestine were not investigated in that study. Future research should focus more on the connection between neurobiology and the gut microbiome in models of heat- and pathogen-induced intestinal dysfunction.

Alleviating the adverse effects of heat stress

A multitude of strategies have been employed to alleviate heat stress in chickens, from improvements in housing management to nutritional interventions such as varying macronutrient composition and supplementing prebiotics, probiotics, and their combination known as synbiotics [11].

Probiotics, such as live yeasts and/or *Lactobacillus* and *Bifidobacterium*, are usually the dominant beneficial bacteria in the gastrointestinal tract. Exogenous supplementation contributes to maintenance of a healthy gut via ensuring their continued establishment and proliferation, which in turn affects the HPA axis and chicken behavior or physiology [80] via immunomodulation, metabolic homeostasis, and neuroendocrine loops [81]. The most effective probiotics are usually commensal bacteria belonging to the host [82]. *Bacillus subtilis*, for example, when supplied in the broiler diet, competed with pathogens (*i.e.*, *Eimeria* spp. and *Clostridium perfringens*) for colonizing sites and nutrients, thus protecting the gut from their colonization and invasion [83]. *B. subtilis* was reported to inhibit bacterial pathogenic reproduction and promotes feed utilization by increasing microbiota diversity and promoting the proliferation of the beneficial *Lactobacillus* [84, 85]. Additionally, *B. subtilis* can stimulate the secretion of intestinal digestive enzymes to speed up nutrient metabolism when the activities of those enzymes were suppressed by chronic heat stress [86]. Longer villi and larger surface areas were observed in probiotic-supplemented chickens and protected the bird against heat exposure-induced gut dysfunction [8, 33].

Prebiotics are generally defined as food ingredients, usually a saccharide, that are not digested (or absorbed) by the host, but benefit the host by encouraging the growth of certain species of bacteria for which they serve as fermentative substrates. Common examples include fructo-oligosaccarides (FOS), manna-oligosaccarides (MOS), and inulin. Mannan-oligosaccharide is harvested from the yeast cell wall and is one of the most common prebiotics used in the poultry industry. Synbiotics are synergistic combinations of prebiotics and probiotics [87]. Both prebiotics and probiotics exert beneficial effects on animal health when supplemented into the diet [88-90]. However, their combination as synbiotics may lead to synergistic and additive effects. Synbiotics not only favor the colonization and thriving of commensal microorganisms, but activate signaling in the microbiome-gut-brain axis and microbiome-gut-immune axis to mediate systemic and local functions, which further influences host physiology and behavior

[91]. In one study, broilers were fed a normal diet or synbiotic-supplied diet and exposed to normal or high temperatures. Synbiotic supplementation not only attenuated heat stress-induced anorexia and body weight loss, but was also associated with increased preening and decreased panting and wing lifting [92]. Because of the diverse array of probiotic species and prebiotic saccharides and resulting combinatorial possibilities in a synbiotic, synbiotics can have differing effects on the gut microbiome depending on the composition. For instance, when MOS, but not FOS, was used in a synbiotic mixture, different commensal microorganisms were selectively promoted, and MOS was associated with a binding to and elimination of pathogenic bacteria from the gastrointestinal tract [88, 93].

Conclusions and implications

In summary, heat stress induces various physiological alterations that directly or indirectly regulate the intestinal microbiome community. These alterations induce changes in environmental and nutritional conditions in the gut, leading to a breach in the intestinal epithelium or barrier integrity, inflammatory states, and activation of the HPA axis and autonomic nervous system. Although growing evidence links heat stress to changes in the host brain (e.g., monoamine concentrations) and gut that are influenced by alterations in the intestinal microbiota, there are still many gaps in knowledge. For example, most studies focused on the association between heat stress and the chicken gut microbiota, but few confirmed the exact compositional changes of microbiota under different heat stress conditions (such as acute or chronic, one time or repetitive) or in response to different probiotic and prebiotic interventions in different types, breeds, and ages of chickens. In addition, various metabolites (e.g., SCFAs) and neuroactive molecules (e.g., serotonin) produced by gut microbiota under different heat stress conditions also require consideration and further exploration. Future studies should focus on utilizing more combinations of probiotics and prebiotics to improve chicken performance under heat exposure and to determine effects on microbiome composition. While it is clear that heat stress influences host and microbial physiology, it is unclear the extent to which the former is driven by the latter and vice-versa. Thus, elucidating the mechanisms that shape the physiology of the gastrointestinal tract and microbiome and how the host and microbial cells interact to drive physiology and behavior, will facilitate holistic strategies to ameliorate the effects of heat stress in animals and humans.

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Chapter 8: Epilogue

People with eating disorders have dysregulated appetite, some of which develop anorexia that is very severe and sometimes fatal, whereas others become compulsive eaters and develop an obese phenotype with metabolic disorders and other comorbidities. Although many central and peripheral factors have been reported to regulate feeding behavior, there still exist other molecules that haven’t been identified as appetite regulators, and the process of appetite regulation is not as simple as turning on/off a switch, but is complex and dynamic, involving collaborations across several systems. The central nervous system, specifically the hypothalamus, is responsible for receiving and processing signals from both extrinsic environmental stimuli, such as dietary nutrients and temperature changes, and intrinsic physiological and psychological signals. The digestive system processes food components and when they reach the gastrointestinal tract, digestible nutrient molecules stimulate the activity of gut hormones to communicate with the brain, while indigestible nutrients are utilized by colonized gut microbiota and these bacteria indirectly interact with the brain through the synthesis of short-chain fatty acids. The immune system might also be involved if toxic substances or pathogens are ingested or gut microbiota are reshaped and cause damage to the intestine.

We used different avian models in our studies. Animal models possess the advantage that some invasive approaches can be utilized to provide direct evidence, instead of measuring changes in blood or excreted samples. For example, our studies employed intracerebroventricular injection, with specific molecules being directly administered into the lateral ventricle, which can target the hypothalamus and its different nuclei to elicit a direct effect. The line chicken models we used are unique. These chicks have been selected for low or high body weight for over 63 generations and consist of individuals having different severities of anorexia or obesity, which fits our purpose perfectly. However, because the HWS chicks showed no difference among all groups when treated with SAM/AZA either on day 5 post-hatch for an immediate effect or on day of hatch to observe a later-age epigenetic effect, we did not show the data and discuss the result. It is possible that HWS chicks have been selected for a larger body weight that they are not sensitive to SAM/AZA or that they need a higher dose (have a higher threshold) to exhibit an effect. Broiler chickens were also used in our study since they have long been selected for better growth traits (not only a larger body weight) and meat production and are considered to be compulsive eaters. As a comparison, the Japanese quail did not undergo intensive artificial selection and served as a wild-type avian model, which provided information on evolution and conservation.

In this dissertation, we designed a series of experiments to investigate the effects of different neuroactive molecules on appetite regulation. First, we used two methylation modifiers, SAM and AZA. The application of such molecules is barely studied as an appetite regulator, so we included three different avian models and combined each model with different nutritional states. We found interesting and promising results that SAM/AZA have immediate effects on feeding behavior depending on species and nutritional states. Our studies are the first to provide evidence of such effects of AZA. Although only behavioral changes were shown, we brought in this novel idea that there is no specific boundary in defining which kind of molecules could affect appetite. There are many more bioactive molecules waiting to be identified as appetite regulators and join this already intricate process. Their interaction with those currently known appetite-related factors is quite promising. Future research could focus on elucidating the molecular hypothalamic mechanisms underlying the effects of SAM and AZA on appetite. It is also worth thinking that since AZA could stimulate eating in fasted LWS birds while suppressing it under the fed state, the application of AZA would be better used in individuals with anorexia when they are in a fasted state, to stimulate eating, but avoid using after a meal.

Other than the immediate effect on appetite, SAM and AZA are related to DNA methylation as a methyl donor and methylation inhibitor, respectively. Our design was to test if these two molecules could mimic the effect of early-life cold stress and nutritional deficiency on later-life feeding behavior. We found both of them, although at different dosages, were able to alter the response of chicks to NPY. Further investigations are needed to figure out which parts of the hypothalamus and appetite-related factors are affected. Like previous studies indicating a methylation level change in the CRF promoter region under stress, global- and specific gene-methylation should be targeted to uncover the mechanisms of SAM/AZA regulation. Moreover, because the process of DNA methylation is reversible, the effects of SAM/AZA on appetite are not necessarily suppressive in the long term. Our result that early-injected SAM itself could stimulate food intake at later age supports this idea. And since we provided SAM/AZA to non-stressed chicks, it is still unpredictable how later-age appetite will be modified if we provide these molecules to stressed chicks. The combination of SAM and AZA, or SAM/AZA and stress could be interesting when applied to birds. In addition to these two molecules, there are many other methyl donors and inhibitors. It shall be intriguing to administer metabolites in methionine and folate metabolism such as choline and betaine or AZA derivative 5-aza-2’-deoxycytidine to prenatal or postnatal offspring to see how appetite will be influenced. Due to the fact that these molecules could be supplemented into diets, their applications in improving animal production and promoting human health are promising.

We then injected broiler chickens with PGE2 or β-MSH and investigated their hypothalamic mechanisms. In broilers, these two molecules both potently induced anorexia, similar to the results found in rodent and layer chicks, suggesting their effects are conserved during evolution. Because broiler chickens eat compulsively, these two molecules could be regarded as potential targets against obesity. We found ghrelin and BDNF, CRF, and MT as well as their receptors were involved in the satiety induced by PGE2 and β-MSH, respectively. These data suggested that the anorexigenic effects of these two molecules involve the stress response due to the roles of the factors listed above in coping with stressful stimuli. However, to prove that these ligand-receptor signaling pathways are indeed mediating these anorectic effects, further studies should utilize their receptor antagonists to block these signals. Appetite regulation is a dynamic process, so it will be worthwhile to also sample at various time points and measure mRNA abundance following chronological order.

As mentioned above, multiple systems participate in the process of feeding behavior regulation. Although beyond the scope of the current dissertation, it is always a good idea to connect the central nervous system regulation with peripheral changes. Like cold stress, heat exposure could directly suppress food consumption and indirectly reshape the gastrointestinal environment to communicate with the brain through neural (vagus nerve) and humoral (SCFAs, cytokines, neurotransmitters, and neuropeptides) regulation. Early-life heat stress might also modify epigenetic programming and influence appetite at a later age.

To summarize, this dissertation identified AZA as a novel appetite regulator in avian species and confirmed the role of SAM in birds' feeding regulation. Their epigenetic-modifying effects also enriched our understanding of the hypothalamic mechanisms of anorexia. Future research could target these epigenetic modifiers and stress-related molecules to develop novel therapeutic strategies against eating disorders.