DIFFERENTIAL REGULATION OF APPETITE IN LINES OF CHICKENS SELECTED FOR HIGH AND LOW JUVENILE BODY WEIGHT: THE ROLE OF β-MSH

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ABSTRACT

Melanocortins play a key role in appetite regulation across species. One such melanocortin, beta-melanocyte stimulating hormone (β-MSH) is receiving increasing attention for its anorexigenic effects. In chicks selected for low (LWS) and high (HWS) juvenile body weight, β-MSH differentially decreased food intake and HWS chicks may be more sensitive to its effects. Both lines responded similarly to β-MSH with decreased water intake. While whole blood glucose concentrations and ingestive and non-ingestive behaviors (sit, stand, preen, perch, deep rest, jumps, escape attempts, feed pecks, defecations, and total distance traveled) were not affected in either line, β-MSH increased corticosterone in LWS chicks but not HWS chicks. However, despite the increase in corticosterone concentration in LWS, astressin, a corticotrophin releasing hormone (CRH) receptor antagonist, did not attenuate the effects of β-MSH in either line suggesting that the altered stress response may not be acting via CRH receptors. When β-MSH was co-administered with HS014, a highly selective antagonist for the melanocortin 4 receptor, only LWS responded with an attenuated response to β-MSH suggesting that the differential response may in part be due to altered receptor affinity or binding resulting from the selection process. To investigate the roles of the hypothalamus and hindbrain in the differential food intake response, an experiment was designed where chicks were injected targeting either the lateral or 4th ventricle utilizing a novel freehand

injection procedure. Chicks from both lines responded similarly to β-MSH following both lateral and 4th ventricle injections. Together, these data suggest that alterations in the b-melanocortinergic appetite regulation system may be in part responsible for the differential body weights of the LWS and HWS lines.

[Adaptations of chapters II, III, and IV have been published in Neuroscience Letters, Journal of Neuroendocrinology, and Behavioural Brain Research, respectively]

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AgRP	agouti-related peptide
α-MSH	alpha-melanocyte stimulating hormone
ARC	arcuate nucleus
β-MSH	beta-melanocyte stimulating hormone
CART	cocaine and amphetamine related transcript
CRH	corticotrophin releasing hormone
DMN	dorsomedial nucleus
DMX	dorsal motor nucleus of the vagus
γ-MSH	gamma-melanocyte stimulating hormone
IL-6	interleukin-6
IN	infundibular nucleus
MC1-5R	melanocortin receptors 1-5
MTII	melanotan II
NPY	neuropeptide Y
PHN	periventricular nucleus
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus

CHAPTER I

LITERATURE REVIEW

Obesity has become a worldwide epidemic and the incidence is continuing to rise daily. Currently rivaling smoking as the most prevalent actual cause of death in the US, obesity now claims over 450,000 lives each year [1]. It is also associated with a myriad of comorbid conditions including sleep apnea, asthma, dislipidemia, hypertension, and diabetes [2].

Energy Balance Regulation

Body weight is maintained by a balance between energy intake and energy expenditure. Appetite, and thus typically food intake, is affected by a variety of central and peripheral signals. Food intake is not only affected by hunger and satiety, but also by energy partitioning and nutrient absorption. Body weight disorders, whether anorexia or obesity, can result from imbalances among one or several of these components, which can make pharmacological and behavioral regulation of them complex [reviewed in 3].

The Melanocortin System

In mammals, two main appetite-related neuronal populations exist within the arcuate nucleus (ARC). Neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons are located medially, bordering the third ventricle, while proopiomelanocortin (POMC) neurons are localized laterally [4]. Arcuate POMC neurons exhibit both insulin [5] and leptin [6] receptors to receive peripheral hunger, satiety, and adiposity signals. Within the ARC, the NPY/AgRP neurons also have melanocortin receptors for autocrine and paracrine regulation of appetite [7]. Arcuate

AgRP and POMC neurons can project to downstream hypothalamic and/or brainstem nuclei with nuclei expressing melanocortin 3 and/or 4 receptors (MC3/4R). These neuronal populations can then affect energy balance and body weight as a whole by altering energy expenditure, food intake, or both.

Leptin is a satiety hormone secreted by adipocytes [8] that can enter the hypothalamus at the ARC because of the lack of functional blood brain barrier (BBB) [9]. Insulin, on the other hand, though secreted in proportion to adiposity from the pancreatic beta cells [10], decreases body weight [11] and food intake [12] following central administration. Removing insulin receptors from neurons results in hyperphagia and increased body weight [13]. Insulin is thought to be able to cross the BBB based on data showing proportional concentrations of central and plasma insulin [14]. However, the effects of insulin are attenuated following administration of a nonselective MC3 and MC4R antagonist. This suggests that melanocortin receptors may have a key role in the downstream insulin pathway [5]. In both broiler and layer-type chicks, insulin decreases food intake, and increases expression of cocaine and amphetamine related transcript, POMC, and corticotrophin releasing hormone (CRH) but did not affect the expression of AgRP and Neuropeptide Y (NPY) mRNA in the hypothalamus [15]. Previous studies, however, have shown a link between NPY and insulin where centrally administered NPY actually increased insulin concentration [8].

Melanocortin Ligands

Released from the arcuate nucleus, POMC is cleaved by prohormone convertases into multiple smaller bioactive molecules including ACTH, α–, β–, and γ–MSH, the endorphins (α–, β– and

γ–endorphin), corticotrophin-like imtermediate peptide, and the lipotropins (β– and γ–lipotropin). These smaller bioactive peptides then act as ligands for MC1-5R. The hypothalamic POMC neurons project to brainstem nuclei where a separate population of POMC neurons are also known to exist in mammals [16].

In response to stress, corticotropin releasing hormone is secreted into the hypophyseal portal blood system from the hypothalamus. ACTH is then cleaved from POMC and released from the pituitary gland into circulation where it stimulates secretion of glucocorticoids from the adrenal gland [17] which mediate the stress response. In both mammals [18] and chickens [19] ACTH binds to the MC2R, also known as ACTHR.

In mammals, α– and β–MSH are nonselective for the MC3 and MC4R. However, γ–MSH selectively binds to MC3R [20]. Comprised of the first 13 residues of the ACTH sequence, α–MSH decreases food intake and increases neuronal activity in areas of the hypothalamus, hindbrain, and amygdala when centrally administered in rodents [21]. In broiler chicks, α–MSH decreases food intake and pecking while increasing the amount of time spent sitting but not affecting other behaviors [22]. When broiler chicks are centrally administered α–MSH + NPY (an endogenous orexigenic peptide), feed intake is still decreased while sitting behavior is increased compared to control-treated or α–MSH-treated chicks [22]. Thus, at similar concentrations, α–MSH is a more potent anorexigenic signal than NPY is an orexigenic signal in broiler chicks [22].

In rodents, β–MSH decreased food intake, but only under specific fasting conditions [23, 24]. Following a 48 hour fast, centrally administered β–MSH did not affect feed intake [25] which may be due to the prolonged fast (compared to decreased food intake reported after 24 hour fasts) or the dose used in this study which was the lowest effective dose used by other laboratories. This study also used a low number of animals for a behavioral study thus more animals may have reduced the variance and allowed an effect to be seen. In mammals, β–MSH binds MC4R with higher affinity than α–MSH in both human and rat cell lines [26] suggesting that it may be the main endogenous melanocortin receptor agonist. However, in chickens, α-MSH binds to MC4R with greater affinity than does β–MSH [19].

In broiler chicks, β–MSH decreased food and water intake while increasing plasma corticosterone concentrations [27]. Chicks also had increased c-Fos expression in the ventromedial hypothalamus, and paraventricular, periventricular, and infundibular [synonymous with mammalian arcuate nucleus] nuclei indicating increased neuronal activation in these areas [27]. Behaviors which may be competitive with food intake such as jumps, locomotion, sleeping, and preening were not increased by β–MSH thus the effect of β–MSH on appetite in broiler chicks is likely primary [27].

Synthetic ligands, especially selective ones, enable investigation of the specific roles of receptors within a given system. HS014, a cyclic synthetic compound (cyclic [AcCys11, D-Nal14, Cys18, Asp-NH(2)22]-β-MSH(11-22) [28] is highly selective for MC4R in rodents, having a 300 fold greater affinity for MC4R than the endogenous ligand, α–MSH [29], and a 20 fold greater

affinity for MC4R than MC3R [30]. HS014 increases short-term food intake with dose-dependent effects lasting from 1 to 4 hours when centrally administered in male rats [30].

Melanocortin Receptors

MC1-5R are 7-transmembrane G protein-coupled receptors [31] that are distributed throughout the brain and periphery. In mammals, MC3 and MC4R are found in both the hypothalamus and hindbrain [32, 33]. Within the mammalian hypothalamus, MC4R has been located in the lateral hypothalamus, paraventricular, periventricular, supraoptic, ventromedial, dorsomedial, arcuate, anterior hypothalamic, and ventral premammilary nuclei [32], many of which have been associated with appetite regulation. In the mammalian brainstem, MC4R has been located in the nucleus of the solitary tract, intermediolateral nucleus [34], parabrachial nucleus [32], and the dorsal motor nucleus of the vagus (DMX) which has the highest MC4R expression of any brain nuclei [32]. Mammalian MC3R have an analogous expression pattern within the mediobasal hypothalamus and hindbrain but has lower hindbrain expression than that of MC4R [34].

In chickens, some controversy exists as to the expression of MC3R within the brain. Ling et al. [19] reported a lack of central MC3R expression in chickens, but did report the presence of MC3R in the adrenal gland, similar to mammalian expression. However, recent work by Ka et al. [35] reported the presence of central MC3R in chickens. The presence of central MC3R is supported by the anorexigenic effect in broiler chicks following central administration of the MC3R agonist, γ-MSH.

In mammals, MC4R activation stimulates adenylyate cyclase and leads to an increase in intracellular cAMP [36]. MC4R is strongly associated with appetite regulation, though it also plays a role in the regulation of energy expenditure [37], MC4R mutations are the most common type of monogenic mutations linked to obesity [38], and these mutations account for up to 6% of human morbid obesity cases [39]. These mutations can, however, cause either constitutive action or inactivation of MC4R. Thus, the ultimate obesity resulting can be caused by various mechanisms. In the case of MC4R inactivation, α–MSH cannot bind MC4R which leads to hyperphagia [40], decreased energy expenditure [41], and increased adiposity. However, the authors do not suggest potential mechanisms behind the obesity resulting from constitutive MC4R activation, typically caused by a Leu250Gln mutation [42]. Based on current literature and knowledge of melanocortinergic appetite regulation, a constitutive action mutation of MC4R should result in hypophagia and possibly anorexia.

Activation of mammalian MC3R stimulates phospholipase C [43] while increasing free cytosolic calcium concentrations [44]. Prior treatment with a nitric oxide synthase inhibitor prevents α-MSH and γ-MSH from increasing sexual behavior [45], which also indicates a role for nitric oxide in the MC3R pathway. MC3R is primarily related to regulation of energy expenditure [46] and its role in appetite regulation is controversial in mammals [23, 24, 47, 48]. MC3R knockout mice exhibit altered energy partitioning which may be responsible for their inability to self-regulate food intake and/or energy expenditure in response to their increased adiposity [46].

CHAPTER II

GAMMA (2)-MELANOCYTE STIMULATING HORMONE DECREASES FOOD INTAKE IN CHICKS

Abstract:

The role of gamma melanocyte stimulating hormone (γ-MSH) in appetite regulation is controversial in mammals and to our knowledge unreported within the avian class. Thus, the present study was designed to determine the effects of intracerebroventricularly (ICV) administered γ2-MSH on food intake using Cobb-500 chicks as models. In Experiment 1, chicks that received ICV γ2-MSH decreased their food intake throughout the 180 min observation period and plasma glucose concentration was not affected. Water intake was also decreased in ICV γ2-MSH-treated chicks, but only from 30 to 90 min post injection. In Experiment 2, food pecking efficiency was decreased in ICV γ2-MSH treated chicks and the amount of time spent sitting was increased. Other behaviors were not significantly affected by ICV γ2-MSH including distance traveled, the number of jumps, escape attempts, defecations, food pecks, exploratory pecks, and the amount of time spent standing, preening, perching, or in deep rest. These data suggest that γ2-MSH is associated with anorexigenic effects and because of γ-MSH's selectivity, implicates the melanocortin 3 receptor in appetite regulation.

Key words: anorexigenic; chick; melanocortin; gamma-MSH

Though appetite regulation is governed by many complex and redundant pathways, the melanocortin system is thought to play a primary role. Within the brain, α-, β-, and γ-melanocyte stimulating hormone (MSH) are cleaved from proopiomelanocortin by prohormone

convertases. γ-MSH is highly selective for the melanocortin 3 receptor (MC3R) versus MC4R in both mammals and chickens [19]. Of the melanocortins, γ-MSH is the least studied and effects of γ-MSH in the avian class are unreported to our knowledge. The 3 subtypes of γ-MSH, γ1, γ2, and γ3, have varied physiological effects in rodents. γ2-MSH, the peptide investigated in the present study (Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly), induces a cataleptic state in rats [49] thus the effects on food intake may be masked. γ1-MSH, however, while not endogenous in rats, decreases food intake [25].

Both α- and β-MSH have anorexigenic effects [22, 27, 50] in chicks. Further, in genetically selected lines of low and high weight chickens containing anorexic or obese individuals, the low weight chicks were more sensitive to α-MSH's hypophagic effect [51]. The present study was designed to determine if γ2-MSH also affected food intake.

Cobb-500 chicks (*Gallus gallus*) were obtained from a commercial hatchery on the morning of hatch from parental stocks of 30–34 weeks of age. They were caged individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity with *ad libitum* access to a mash diet (20% crude protein and 2685 kcal ME/kg) and tap water. Experiments were conducted between 12:00 and 16:00 h, performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals, and were approved by the Radford University Institutional Animal Care and Use committee.

Chicks were injected using a method adapted from Davis et al [52]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for a free-hand injection to be performed. 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-anaesthetized chick for 5 s to reduce backflow. Chicks were assigned to treatments at random. γ2-MSH (American Peptide, Sunnyvale, California, USA) was dissolved in avian artificial cerebrospinal fluid [53] as a vehicle for a total injection volume of 5 μL with 0.06% Evans Blue dye to facilitate injection site localization. After data collection, the chick was decapitated and its head sectioned coronally to verify injection site. Data from chicks without dye present in the lateral ventricle system were eliminated from statistical analysis. After decapitation, sex was visually determined through presence of an ovary or testes via dissection.

In experiment 1, chicks, fasted for 180 min, were randomly assigned to receive either 0 (vehicle only), 1.5, 3.0, or 6.0 nmol γ2-MSH by ICV injection. After injection, chicks were returned to their individual cages and given *ad libitum* access to both food and water. Food intake was monitored (measurement accuracy = 0.01 g) every 30 min for 180 min post injection and was analyzed by repeated measures ANOVA using the MIXED procedure of SAS. The model included dose, time, and the interaction of dose with time. Sex was not significant, and was removed from the model. Kenward-Rogers approximation was used for denominator degrees of freedom, and the error structure was UN R RCORR. As a post hoc test, pair-wise comparisons were made using Bonferroni probabilities. Six to 9 chicks per dose were available for the analysis.

Trunk blood was collected from chicks immediately after the 180 min food and water intake reading. Whole blood glucose concentration was determined in duplicate using the OneTouch Basic glucose measurement system (Lifescan, Milpitas, CA, USA) with sensitivity in

the range 20–600 mg ⁄ dl. Blood glucose data were analyzed using ANOVA via the GLM procedure of SAS.

Experiment 2 was conducted to determine whether the alterations in food intake were due to increases in behaviors that are competitive with ingestion. Chicks were kept in individual cages with auditory but not visual contact with each other, and were randomly assigned to receive either vehicle or 3.0 nmol γ2–MSH by ICV injection. Following a 180 min fast, chicks were injected and immediately placed in a 290 × 290 mm acrylic recording arena with food and water containers in diagonal corners. Chicks were simultaneously and automatically recorded from three angles for 30 min post injection on DVD and data were analyzed in 5 min intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). At 30 min post injection, food intake was measured. Locomotion (m traveled), the amount of time spent standing, sitting, preening, perching, or in deep rest, and the number of jumps, steps, feeding and exploratory pecks, and escape attempts were quantified. Food pecks were defined as pecks within the food container, whereas any other pecks were counted as exploratory. Deep rest was defined as the eyes closed for greater than 3 s, starting 3 s after eye closure. Preening was defined as trimming or dressing of down with the beak. Due to heterogeneous variance, behavior data were analyzed by the Mann–Whitney U test using the NPAR1WAY procedure of SAS. Pecking efficiency at 30 min post-injection was calculated by dividing food consumed by number of food pecks for each chick. Pecking efficiency and food intake were analyzed by ANOVA using the GLM procedure of SAS. Nine vehicle and10 γ2-MSH-treated chicks were available for analysis.

In Experiment 1, ICV γ2-MSH injection was associated with reduced food intake (Figure 2.1). Chicks which received 3.0 and 6.0 nmol γ2-MSH had a similar magnitude of reduced food

intake at all times, whereas those treated with 1.5 nmol only reduced food intake at 180 min following injection. γ2-MSH induced hypophagia is similar to that of α- **[22, 50]** and β-MSH **[27]** when tested in chicks. However, the threshold of hypophagia for ICV α-MSH appears to be much lower; Kawakami et al., [54] demonstrated a 50% reduction in food intake 30 min after a 24 pmol ICV α-MSH injection, whereas our lowest does of 1.5 nmol was not affective at 30 min post injection and 3.0 nmol caused only a 29% reduction in food intake. The threshold of hypophagia was also lower for β-MSH; 0.3 nmol caused an approximate 40% reduction in food intake **[27]**. Thus, γ2-MSH appears to be a less potent regulator of satiety than α- and β-MSH in chicks. This may be due to the specificity of γ-MSH for MC3R whereas α- and β-MSH nonspecifically bind both MC3R and MC4R. In chickens, γ1-MSH binds MC3R with higher affinity than α- and β-MSH, respectively [19]. However, the affinity for γ2-MSH to chicken MC3R has not been reported to our knowledge. In humans, γ1-MSH also has the greatest affinity for MC3R, but β-MSH has a higher affinity than does α-MSH [55]. The present data supports presence of central MC3R in chickens **[35]** because of γ-MSH's selectivity for this receptor. This is contrary to previous reports that chicks lack central MC3R [19].

In Experiment 1, water intake was also affected (Figure 2.2). Chicks in the 3.0 and 6.0 nmol treatment groups reduced their water intake from 30 to 90 min post injection. However, after 90 min post injection, water intake was not affected by ICV γ2-MSH. This is different from the sustaining antidipsogenic effect of β-MSH in chicks after all doses tested [27]. However, water intake was not influenced by α-MSH in either high or low weight chicks [51]. Thus, α-MSH may have less of an effect on drinking than β- or γ-MSH. Also in Experiment 1, whole blood glucose concentration was measured but was not affected by γ2-MSH at 180 min post

injection. Glucose concentrations were 304.1 ± 14.7, 320.9 ± 27.0, 279.5 ± 5.4, and 316.1 ± 18.2 mg/dl for 0, 1.5, 3.0, and 6.0 nmol γ2-MSH, respectively.

In the present study, chicks treated with 3.0 nmol γ2-MSH spent more time sitting than vehicle-treated chicks at 15 min post injection (Table 2.1). Other behaviors such as the total distance traveled, the number of feed and exploratory pecks, jumps, escape attempts, and defecations, and the amount of time spent standing, perching, preening, or in deep rest were not affected by γ2-MSH. The increased amount of time spent sitting may be counter balanced by non-significant decreases in multiple other timed behaviors. However, pecking efficiency was decreased by γ2-MSH; 2.5 ± 0.28 and 2.1 ± 0.23 mg/peck for control and γ2-MSH treated chicks, respectively ($P = 0.023$). Though food intake was decreased, the number of food pecks was not different between control and γ2-MSH treated chicks, which is accounted for by the decreased peck efficiency of the γ2-MSH treated chicks.

In chicks, central α-MSH increases the time spent sitting and decreases time spent standing while not affecting other behaviors [22]. β-MSH, when centrally administered, decreased the number of steps taken, the total distance traveled, and the amount of time spent standing [27]. Thus, the behavioral response to γ2-MSH in chicks is similar to the response to other melanocortins, especially that of α-MSH.

Because behavioral effects associated with stress in birds were not affected by γ2-MSH, along with the lack of effect on other behaviors competitive with food intake, suggest that both the anorexigenic and antidipsogenic roles of γ2-MSH in chicks are not secondary to alterations in other behaviors tested. α- [50]and β-MSH [27] likely influence appetite via alterations in hypothalamic-pituitary-adrenal axis signaling which may also be the case for γ-MSH, however further studies are required to determine this.

Thus, the mechanisms through which γ-MSH affects food intake require further investigation. Though primarily associated with energy expenditure, whereas MC4R is primarily associated with food intake, these data support a role for MC3R in appetite regulation in chicks, which is controversial in rodents [23, 24, 47, 48].

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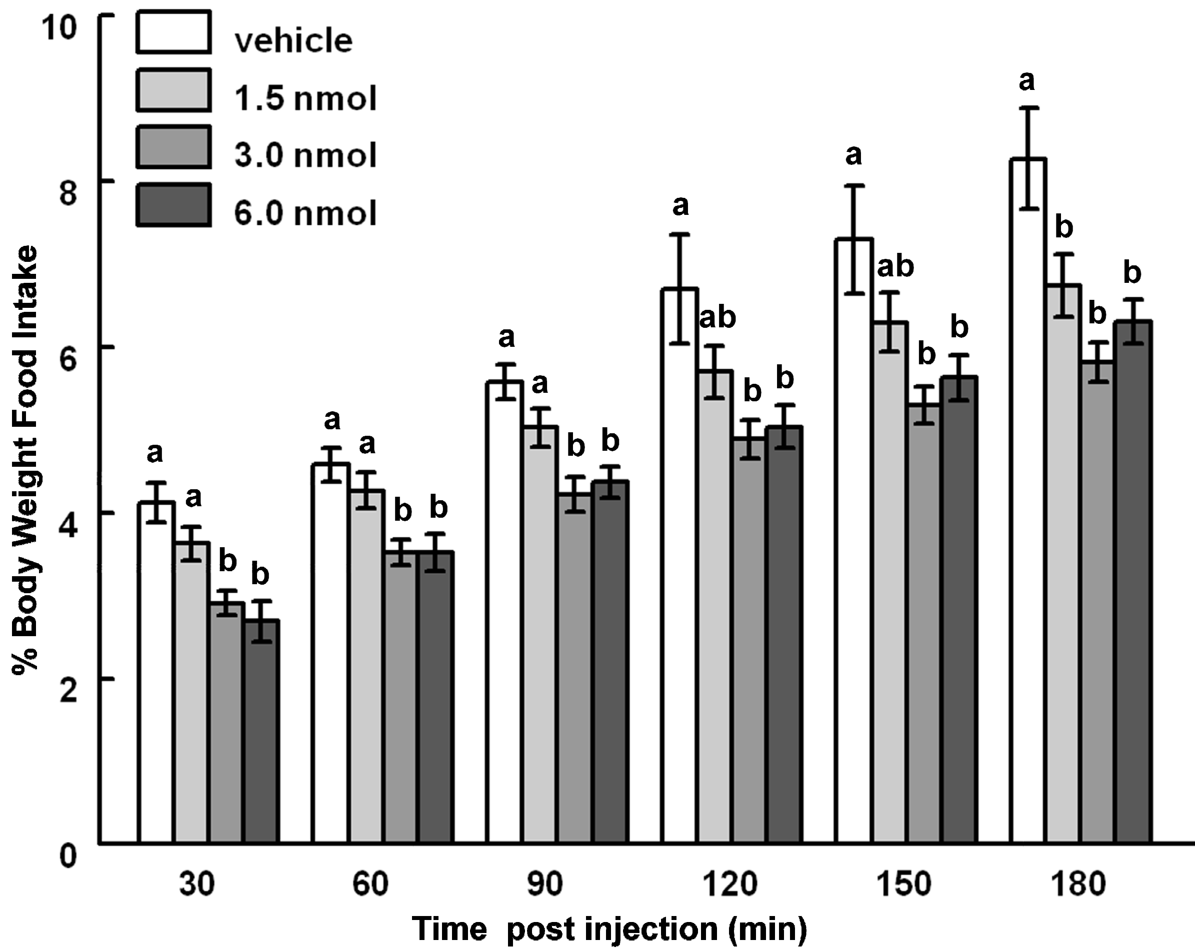

Figure 2.1. Cumulative food intake following intracerebroventricular injection of γ2-MSH (Experiment 1; 6-9 chicks per dose). Values are the means ± SE; bars with different superscripts are different from each other within a time point ($P < 0.05$).

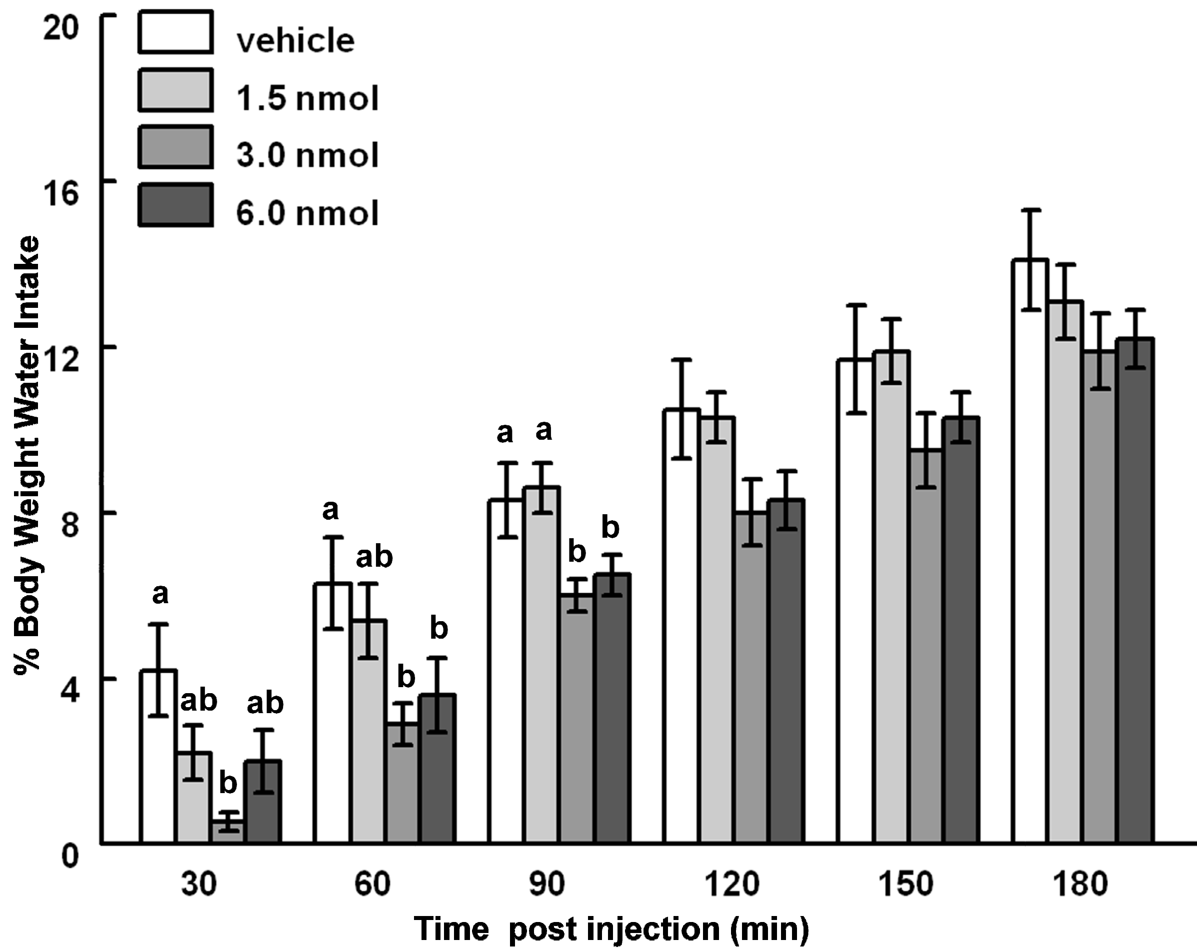

Figure 2.2. Cumulative water intake following intracerebroventricular injection of γ2-MSH (Experiment 1; 6-9 chicks per dose). Values are the means ± SE; bars with different superscripts are different from each other within a time point ($P < 0.05$).

Parameter	Treatment	Time post injection (min)					
		5	10	15	20	25	30
Food pecks (n)	0	277±67.7	666±119	1057±144	1288±192	1482±215	1650±264
	γ2-MSH	246±83.2	533±108	739±139	1000±178	1170±173	1257±16
Exploratory pecks (n)	0	19.8±11.3	20.6±11.3	22.7±11.6	24.3±11.4	30.7±10.8	34.9±11.7
	γ2-MSH	33.4±20.3	34.5±20.1	37.6±20.0	37.8±20.0	43.8±20.0	52.7±18.7
Defecations (n)	0	0.2±0.1	0.7±0.1	0.7±0.1	0.9±0.1	1.3±0.2	1.5±0.4
	γ2-MSH	0.3±0.2	0.7±0.3	0.8±0.3	1.0±0.2	1.0±0.2	1.2±0.4
Distance (m)	0	1.4±0.6	2.3±0.7	0.3±1.1	4.7±1.5	5.8±1.7	6.9±2.0
	γ2-MSH	0.5±0.2	0.7±0.2	1.8±0.3	3.1±0.8	4.1±0.9	5.3±1.0
Jumps (n)	0	0.5±0.4	1.5±0.8	4.5±3.3	5.2±3.8	7.2±4.7	8.2±4.7
	γ2-MSH	0.4±0.4	0.5±0.4	2.1±1.3	4.3±2.2	5.0±2.1	7.0± 8
Escape attempts (n)	0	0.4±0.3	1.1±0.5	3.0±2.1	3.4±2.4	4.5±3.0	5.4±3.1
	γ2-MSH	0.4±0.4	0.4±0.4	1.3±1.0	3.3±1.6	4.4±1.8	6.1±2.6

Table 2.1. Count-type behaviors following intracerebroventricular injection of γ2-MSH in chicks (Experiment 2). Nine vehicle and 10 γ2-MSH treated chicks per treatment group were available for analysis. Values are the means ± SE. * significantly different (*P* < 0.05) from vehicle within an observation time.

Parameter	Treatment	Time post injection (min)					
		5	10	15	20	25	30
Stand time (s)	0	299±0.1	588±9.3	877±9.2	1153±15	1419±28	1686±52
	γ2-MSH	271±26	540±56	807±85	1058±114	1307±118	1531±127
Sit time (s)	0	0	0	1.2±1.2	24.5±13.4	52.3±25	55.4±26
	γ2-MSH	7.6±6.5	7.6±6.5	10.0±6.4 *	14.9±7.0	29.0±14	60.1±38
Deep rest time (s)	0	0	0	0	6.0±6.0	9.5±6.6	37.3±31
	γ2-MSH	20.0±19.8	50.0±49	80.0±79	121±109	156±110	186±114
Preen time (s)	0	0	0	0.1±0.1	3.9±2.8	6.4±5.1	6.4±5.1
	γ2-MSH	0.1±0.1	0.5±0.3	0.5±0.3	0.8±0.4	0.8±0.4	12.3±11
Perch time (s)	0	0	10.8±9.2	10.8±9.2	11.2±9.3	12.0±9.4	13.1±9.7
	γ2-MSH	0	0.9±0.9	2.0±2.0	3.2±2.4	5.1±3.4	8.5±6.8

Table 2.2. Mutually exclusive timed behaviors following intracerebroventricular injection of γ2-MSH in chicks (Experiment 2). Nine vehicle and 10 γ2-MSH treated chicks per treatment group were available for analysis. Values are the means ± SE. * significantly different ($P < 0.05$) from vehicle within an observation time.

CHAPTER III

β-MELANOCYTE STIMULATING HORMONE POTENTLY REDUCES APPETITE VIA THE HYPOTHALAMUS IN CHICKS

Abstract

The melanocortin system interactively with other appetite-related systems plays a significant role in appetite regulation. The appetite-related effects of once such melanocortin, β-MSH, are well documented in rodents; however, its effects in avians are not thoroughly understood. Thus, I designed a study to determine the effects of central β-MSH on feed and water intake, plasma corticosterone concentration, both ingestive and non-ingestive behaviors, and hypothalamic neuronal activation using Cobb-500 chicks. β-MSH-treated chicks responded with decreased feed and water intake; however when water intake was measured independently of feed, water intake was not affected. β-MSH-treated chicks also had increased plasma corticosterone concentration and increased *c-Fos* reactivity in the periventricular, paraventricular (PVN), and infundibular (IN) nuclei, and the ventromedial hypothalamus (VMH), however the lateral hypothalamus was not affected. The effect on feed intake is primary since behaviors that may be competitive with feed intake were not increased in β-MSH-treated chicks. Based on these results I conclude that β-MSH causes anorexigenic effects primarily via stimulation of satiety-related hypothalamic nuclei in chicks.

Key words: appetite, β-MSH, behavior, chick, corticosterone, feeding

Introduction

Melanocortins, first reported to change skin color in frogs [1], also play a significant role in energy balance regulation. Melanocortin peptides are derived from proopiomelanocortin (POMC) and include ACTH, α-, β- and γ-MSH. The 5 melanocortin receptors are G-protein coupled that cause activation of $G\alpha_s$ that leads to increased concentration of intracellular cAMP [reviewed in 2]. Melanocortin receptors are associated with numerous physiological processes [3], one of the most prominent being energy balance regulation. β-MSH, a melanocortin overlooked in avian appetite biology, reduces feed intake without affecting water intake in fasted rats [4] through increased mediobasal hypothalamic neuronal activity [5]. β-MSH binds to both the melanocortin 4 receptor (MC4R), which is primarily associated with the regulation of feed intake [4], and the melanocortin 3 receptor (MC3R), which is primarily regulating energy expenditure [6]. In the present study I used a model which does not express MC3R, but rather only expresses MC4R in the brain [7].

The MC4R receptor has been implicated in human erectile dysfunction [8] and pain [9] in addition to a number of body weight dysfunctions. Farooqi et al. [10] found that heritable mutations in MC4R cause human hyperphagia and cause obesity. In the agouti mouse the over expressed agouti protein [11,12] causes antagonism of hypothalamic MC4R which leads to its obese phenotype [13,14,15]. Additionally, gene-targeted disruption of MC4R causes hyperphagia, hyperinsulinemia and hyperglycemia in mice [15]. Thus, manipulation of MC4R is a logical target for the reversal of body weight dysfunctions in a range of species.

The anorexigenic and other behavioral effects of α-MSH have been documented in chicks. Central α-MSH potently reduces feed intake in chicks [16] while causing behavioral effects that may be competitive to ingestion [17]. In rats, α- and β-MSH bind the MC4R with

similar affinity [18]. However, information on the effects of β-MSH in the avian class is lacking. I hypothesized that β-MSH, like α-MSH, would cause anorexigenic effects in chicks. Thus, I measured feed and water intake, plasma corticosterone concentration, ingestive and non-ingestive behaviors, and hypothalamic neuronal activation following central β-MSH administration in broiler type chicks.

Experimental procedures

Animals

Day of hatch Cobb-500 broiler chicks from breeders 30 to 40 weeks of age were obtained from a commercial hatchery. They were caged individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity with *ad lib.* access to a mesh diet (20% crude protein and 2864 kcal/kg metabolizable energy) and water.

I.c.v. injection procedure

Chicks were injected using a method adapted from Davis et al. [19]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for free-hand 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 for 10 s post injection to reduce backflow. β-MSH was dissolved in artificial cerebrospinal fluid for a total injection volume of 5 µL with 0.1% Evans Blue dye to facilitate injection site localization. Following data

collection, the chick was decapitated and the head sectioned along the frontal plane to determine site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis. Numbers of chicks in each experiment are provided in the results section. Following decapitation, sex was visually detected by dissection.

Exp 1: Effect on feed and water intake

Chicks, fasted for 180 min, were assigned at random to receive 0, 0.30, 1.00 or 3.00 nmol β-MSH (American Peptide, Sunnyvale, CA, USA) by ICV injection. Following injection, chicks were returned to their individual cages and given *ad lib.* access to both feed and water. Feed and water intake were monitored (0.01 g) every 30 min for 180 min post injection concurrently. Data were analyzed using ANOVA at each time point. The model included β-MSH concentration, sex and the interaction of sex with β-MSH concentration. As a post-hoc analysis, β-MSH concentration effects were partitioned into linear and quadratic contrasts to determine concentration relationships at each time period. Statistical significance was set at $P < 0.05$ for all exp. Water weight (g) was converted to volume (ml; 1 g = 1 ml).

Exp 2: Plasma corticosterone concentration

Chicks from Exp 1 were decapitated 180 min after injection and blood was collected into microcentrifuge tubes containing 0.06 mg EDTA. Microcentrifuge tubes were immediately centrifuged at 3,000 × *g* for 10 min and the supernatant was collected. Plasma corticosterone

concentrations were determined using a commercially available enzyme immunoassay kit (Correlate-EIA, Assay Designs Inc., Ann Arbor, MI, USA). The intra-assay precision was 8.6%. Data were analyzed in the same manner as in Exp 1, but at only the single time point.

Exp 3: Effect on water intake

The procedures were identical to those in Exp 1 except that chicks were not fasted prior to injection, and feed was restricted during the observation period.

Exp 4: Behavior

Chicks, after 1 d post hatch, were kept in individual cages with auditory but not visual contact with each other. Chicks, fasted for 180 min, were assigned at random to receive either 0 or 0.3 nmol β-MSH ICV Following injection, chicks were immediately placed in a 290 x 290 mm acrylic recording arena with feed and water containers in diagonal corners. Chicks were simultaneously and automatically recorded from 3 angles for 30 min post injection on DVD and were later analyzed in 5 min intervals using ANY-maze behavioral analysis software (Stoelting, Wood Dale, IL). Feed consumption was quantified at 30 min post injection. Additionally, locomotion (cm traveled), the amount of time spent standing, sitting, preening, or in deep rest, and the number of steps, jumps, feed or exploratory pecks, drinks, and escape attempts were quantified. Feed pecks were defined as pecks within the feed container whereas any other pecks were counted as exploratory. Drinks were defined as the chick dipping its beak in water then

raising and extending its head to swallow. Deep rest was defined as the eyes closed for greater than 3 s, starting 3 s after eye closure. Data were analyzed with a Mann-Whitney U test.

Exp 5: Immunocytochemistry

Chicks, fasted for 180 min, were assigned at random to receive either 0 or 0.30 nmol β-MSH ICV and then were given *ad lib.* access to both feed and water post injection. Thirty min after injection, chicks were deeply anesthetised with an intraperitoneal injection of sodium pentobarbital (30 mg/kg body weight) and decapitated. The brain was immediately fixed with a 2% paraformaldehyde, 0.1% gluteraldehyde solution via a carotid artery. The head was positioned in a stereotaxic instrument and the brain sectioned frontally according to Kuenzel and Masson [20]. The blocked brain was placed in 20% sucrose in phosphate buffered saline for 40 h at 4°C. Using a cryostat, 40 μm sections were cut from areas of the brain that contained the lateral hypothalamus, IN, periventricular nucleus, PVN, and VMH and mounted on poly-L-lysine coated slides. Sections were incubated with anti-*Fos* polyclonal antibody (1:600, v/v; Sigma, St. Louis, MO, USA) for 48 h at 4 °C and then with an alkaline phosphatase-conjugated secondary monoclonal antibody (1:600 v/v; Sigma) at room temperature for 2 h. The secondary antibody was visualized using alkaline phosphatase substrate kit III (Vector Laboratories Ltd., Burlingame, CA, USA). The number of reactive cells was counted from the injected side of the brain in an area 200 μm^2 located in the center of each respective nucleus, according to coordinates based on Kuenzel and Masson [20]. Two sections were counted and averaged to arrive at the value for each chick. Data were analyzed by two tailed *t*-test.

Results

Exp 1: Feed intake and water intake

Chicks responded to ICV β-MSH with decreased feed intake (Fig 3.1). This effect was significant at all observation times. The highest concentration, 3.00 nmol was most efficacious at reducing feed intake. As time progressed, the magnitude of treatment divergence from control increased; there was not compensatory feed intake post injection. Feed intake was not affected by sex or a sex by concentration interaction. Water intake was also reduced by ICV β-MSH (Figure 3.2). However, the responses did not plateau as did feed intake, but rather had less slope than did the control group. The effect on water intake was significant by 60 min post injection; later than the effect on feed intake. Water intake was not affected by sex or a sex by β-MSH concentration interaction. For this experiment, 9 to 10 chicks per β-MSH concentration were available for the analysis.

Plasma corticosterone concentration

The range of β-MSH concentrations injected caused a linear increase in plasma corticosterone concentration (Figure 3.3). The highest concentration, 3.0 nmol β-MSH, was associated with the highest plasma corticosterone concentration. The β-MSH-induced increase in plasma corticosterone was not affected by sex or a sex by β-MSH concentration interaction.

Effect on water intake in feed-restricted chicks.

When chicks were feed restricted, central β-MSH did not affect water intake (Figure 3.4). For this experiment, 9 to 10 chicks per β-MSH concentration were available for the analysis.

Behavior

β-MSH-treated chicks responded with decreased feed pecks during the observation period (Table 3.1). β-MSH chicks consumed less feed than controls (0.5 ± 0.50 g vs. 2.73 ± 0.26; $P < 0.05$); however, exploratory pecks were not affected. One non-β-MSH-treated drank during the last 5 min of observation and no other chicks drank. Locomotion was affected by treatment; β-MSH treated chicks stepped less and traveled less distance at each observation time. Jumps and escape attempts were not affected by treatment. Treatment with β-MSH decreased time spent standing after 20 min post injection. However, other timed behaviors, sit, deep rest, and preen were not affected by central β-MSH injection. Ten control and 8 β-MSH treated chicks were available for the analysis.

Immunocytochemistry

The lateral hypothalamus was not affected by ICV β-MSH (Figure 3.5). However, β-MSH-treated chicks had pronounced activation of the infundibular nucleus (IN), periventricular nucleus, paraventricular nucleus (PVN) and ventromedial hypothalamus (VMH). c-*Fos* reactivity was most increased in the periventricular nucleus followed by IN, PVN and VMH (385, 314, 309 and 204% of control reactivity respectively). Data from 6 chicks per treatment were available for the analysis.

Discussion

The anorexigenic effect of β-MSH measured in Exp 1 is similar with that of rodents [18,4]. Since treatment divergence had occurred by the first observation time, β-MSH exerted its

effect within 30 min of injection, and is a fast-acting satiety-related peptide in chicks. Additionally, since the magnitude of treatment divergence increased over time, β-MSH exerts a sustaining feed intake suppressing effect in chicks. Thus, β-MSH may be a long-term modulator of feed intake regulation in chicks. β-MSH has higher affinity for the MC4R than does α-MSH [21]. When Kawakami et al. [22] injected α-MSH in chicks there was compensatory feed intake post injection, unlike the effect I observed.

I hypothesized the effect on water intake in Exp 1 was not a direct effect of β-MSH, hence the design of Exp 3. This thesis was supported when an effect on water intake was not detected in Exp 3. This finding is similar to rodents as Brown et al. [23] reported that melanocortins do not affect thirst in rats. The decreased water intake in Exp 1 was due to decreased feed intake; thus the effect on water intake was secondary. Simply put, when the animal eats less it tends to drink less. I also hypothesized that the effect on feed intake was behavior specific. Exp 4 was designed to determine if β-MSH caused behaviors, other than ingestion, that may be competitive with feed intake, and thus contribute to the anorexigenic effect. The behaviors that were affected are not competitive with feed intake. Thus, I designed Exp 5 to determine β-MSH's hypothalamic mechanism.

Activation of the chick's IN, periventricular nucleus, PVN and VMH nuclei of the hypothalamus is similar to the rat [5] since the IN in chicks is homologous to the arcuate nucleus of mammals [24]. Together with the feed intake data this may be interpreted as the appetite-related effects of β-MSH have been conserved during vertebrate evolution. In mammals, MC4R is expressed in the arcuate nucleus [25] that has projections to the PVN which also expresses MC4R [26]. The VMH [14] and periventricular nucleus [25] also contain MC4R receptors. The

IN, PVN, and VMH are classically associated with satiety perception. Treatment with β-MSH in the chick may have concurrently activated these 4 nuclei, or a cascade type effect may have been initiated. Since the IN has projections to the periventricular nucleus and PVN, such a cascade may have been initiated directly at the IN. Additionally, the PVN also contains a population of CRH-secreting neurons [27]. Thus, β-MSH stimulation of the PVN may be responsible for the increased plasma corticosterone detected in Exp 2. Since activation of the HPA is associated with decreased feed intake in chicks [28], activation of the PVN may caused secondary satiety signals to be released that contribute to the overall anorexigenic effect.

Despite that antagonism of MC4R in ring doves [29] and chicks [16] caused an increase in avian feed intake, β-MSH did not affect the lateral hypothalamus, a nucleus classically associated with hunger [30, 31]. Thus in chicks, β-MSH may directly cause the perception of satiety without affecting the perception of hunger. Due to the dramatic activation of satiety-related nuclei and the PVN which may activate the HPA, I conclude the anorexigenic related effects of β-MSH are primarily hypothalamic in origin.

In conclusion, the results of our study provide evidence that β-MSH causes physiological responses in chicks associated with an increase in the magnitude of satiety perception. β-MSH decreased feed intake and although water intake was reduced when feed was available, this effect was secondary to the reduction in feed intake. The effects on feed intake are behavior specific; other behaviors unrelated to ingestion do not compete with feed intake. The anorexigenic effects of β-MSH are mediated at the hypothalamus; in particular the IN, periventricular nucleus, PVN and VMH are involved. Thus, I conclude that central β-MSH causes anorexigenic effects via the

hypothalamus in chicks and its appetite-related effects have been conserved during divergent evolution of chicks and rodents.

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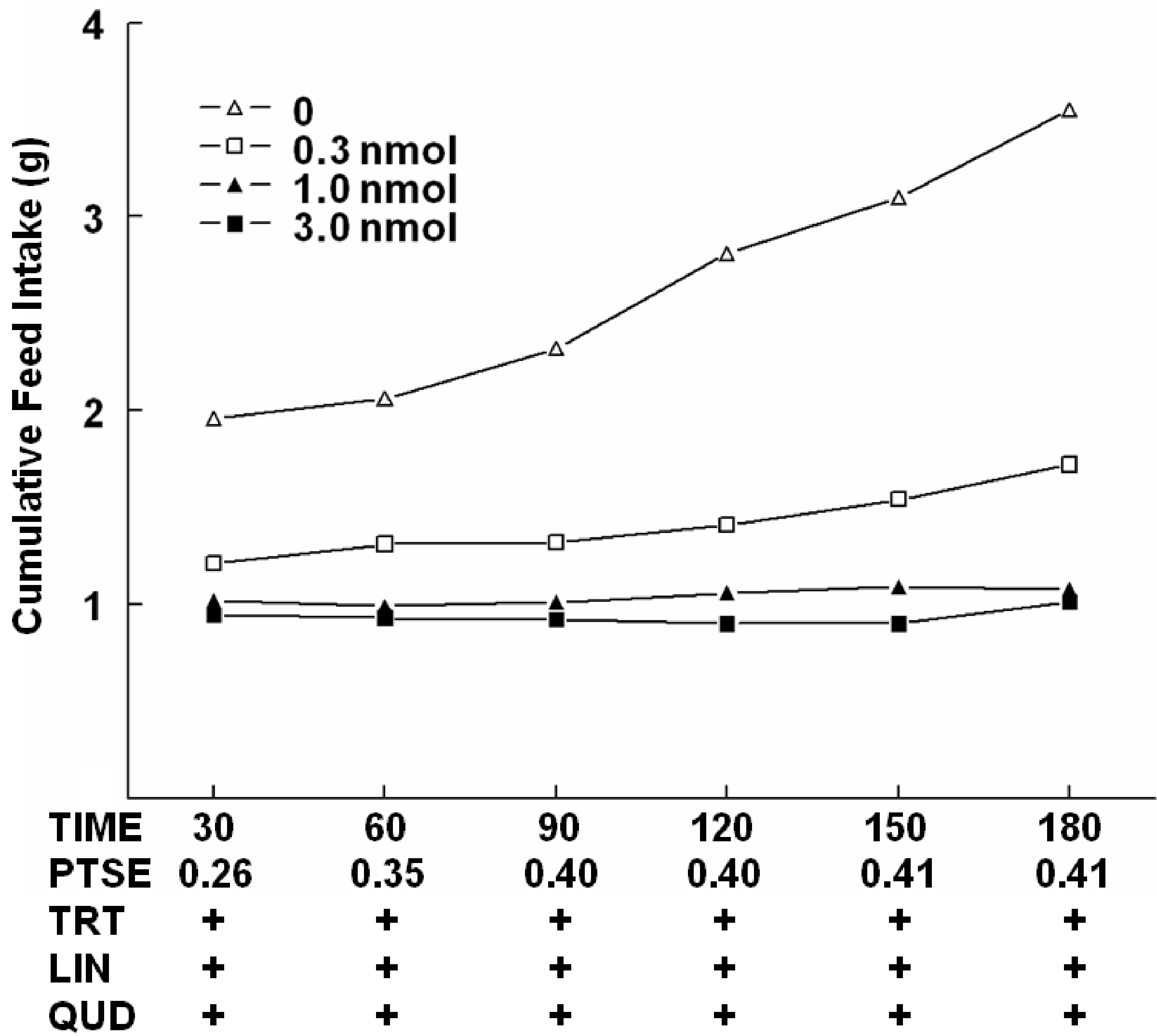

Figure 3.1. Cumulative feed intake following ICV injection of β-MSH (Exp 1). LIN, linear contrast; TIM, time post injections in minutes; PTSE, pooled standard error of the treatment mean; TRT, treatment effect; QUD, quadratic contrast; +, $P \leq 0.05$.

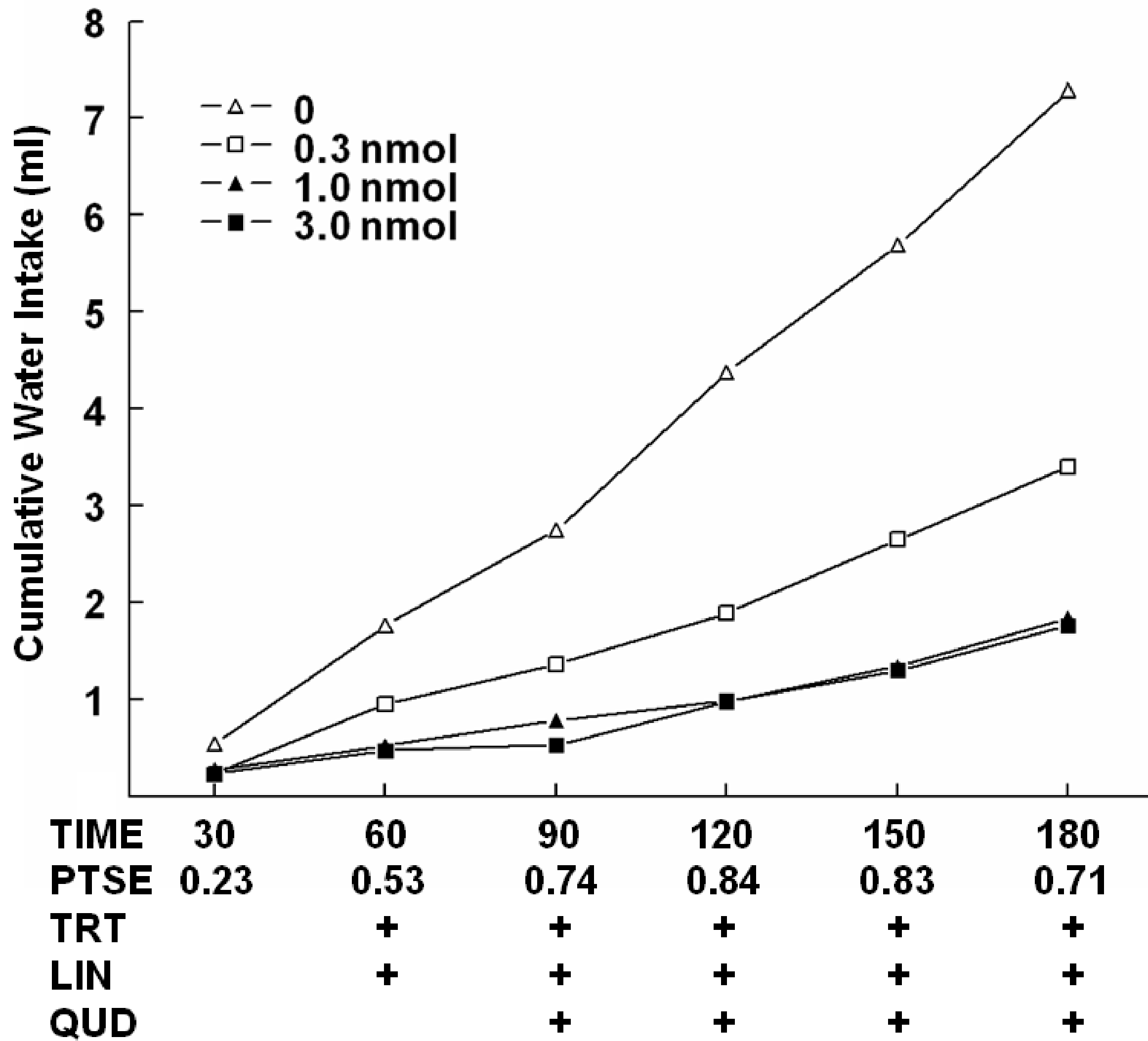

Figure 3.2. Cumulative water intake following ICV injection of β-MSH in fed chicks (Exp 1). LIN, linear contrast; TIM, time post injections in minutes; PTSE, pooled standard error of the treatment mean; TRT, treatment effect; QUD, quadratic contrast; +, $P \leq 0.05$.

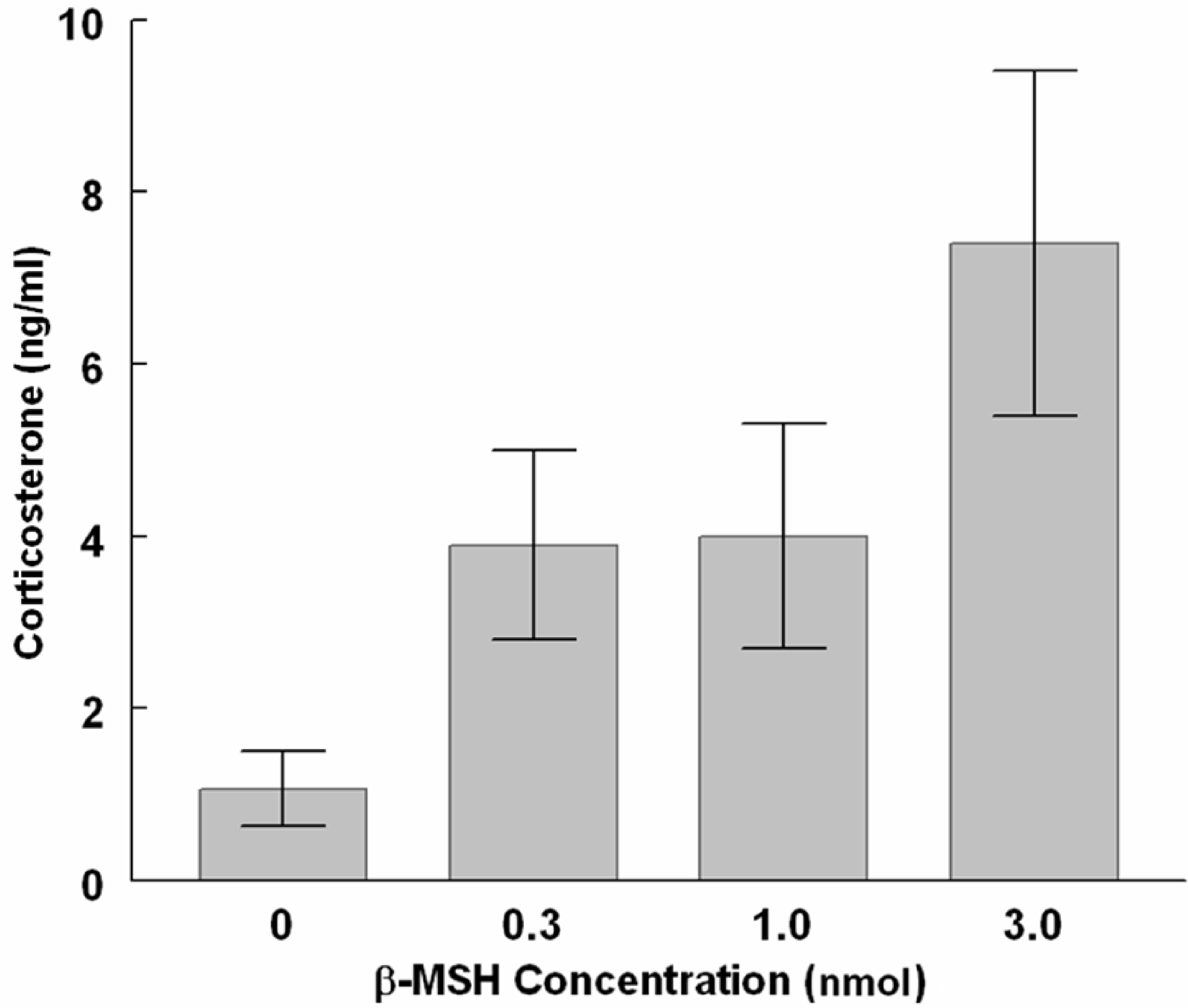

Figure 3.3. Plasma corticosterone concentrations 180 min after ICV injection of β-MSH (Exp 2).

These data test significant for a linear type response. Values are means ± S.E.M.

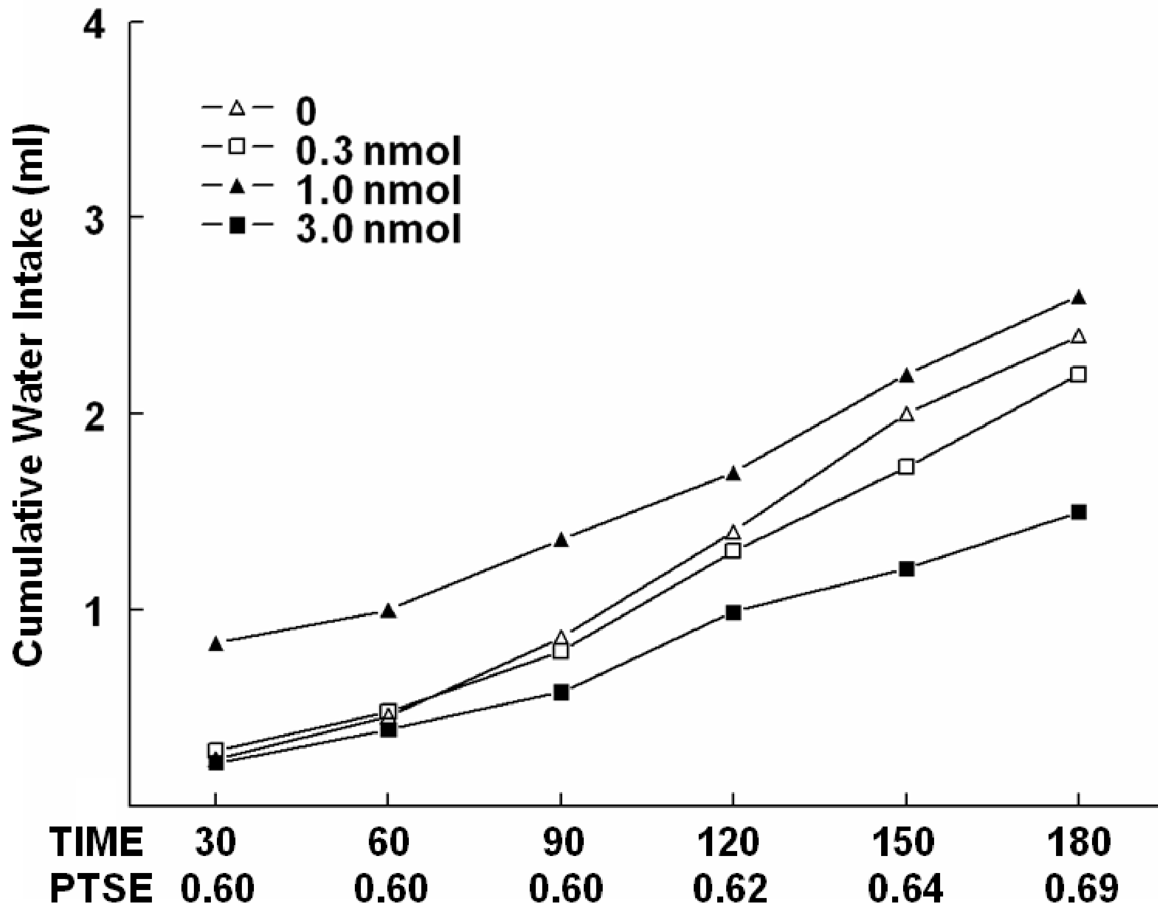

Figure 3.4. Cumulative water intake following ICV injection of β-MSH in fasted chicks (Exp 3). No effect was detected.

Parameter	Treatment	Time post injection (min)					
		5	10	15	20	25	30
Feed pecks	0	179.8±44.3	526.8±80.3	731.8±115.7	760.2±117.9	775.7±119.7	868.6±126.2
	β-MSH	10.1±10.0*	15.8±10.7*	47.0±36.6*	94.6±83.5*	139.8±128.4*	146.0±134.6*
Exploratory pecks	0	0.6±0.4	1.4±0.7	1.5±0.6	2.3±0.7	2.8±0.7	6.0±1.6
	β-MSH	0.3±0.2	0.4±0.3	3.4±2.1	3.5±2.1	3.5±2.1	3.8±2.1
Drinks	0	0	0	0	0	0	0.2±0.2
	β-MSH	0	0	0	0	0	0
Steps	0	51.3±11.6	88.1±21.1	122.0±29.2	168.5±34.3	206.2±41.8	240.1±44.7
	β-MSH	25.1±10.4*	40.3±18.8*	59.5±28.0*	62.3±27.9*	62.8±27.8*	67.0±27.6*
Distance (cm)	0	124.6±33.3	183.8±46.4	254.1±67.9	339.9±82.9	411.9±98.0	472.3±102.1
	β-MSH	47.5±23.8*	72.8±37.2*	111.0±56.0*	165. 6±74.4*	165.6±74.4*	171.1±73.4*
Jumps	0	0.5±0.4	0.6±0.4	0.6±0.4	0.8±0.4	0.9±0.4	1.3±0.5
	β-MSH	0.5±0.3	0.5±0.3	0.6±0.3	0.6±0.3	0.6±0.3	0.8±0.4

Escape	0	1.3±0.8	1.9±1.0	3.4±1.7	4.4±2.0	5.2±2.3	5.7±2.6
Attempts	β-MSH	0.38±0.38	0.38±0.38	0.38±0.38	0.38±0.38	0.38±0.38	0.38±0.38
Stand time	0	274.4±16.7	531.2±23.2	718.8±57.0	907.6±91.4	1045.9±143.7	1247.7±132.6
(s)	β-MSH	228.5±34.6	402.7±71.2	540.8±109.9	630.8±143.7	680.1±173.2*	738.3±209.5*
Sit Time	0	5.3±3.9	48.4±21.2	160.0±59.7	221.7±74.0	322.7±86.0	363.4±91.5
(s)	β-MSH	47.9±23.8	134.9±48.6	253.5±83.2	399.9±120.1	579.7±161.3	751.4±208.9
Deep rest time	0	3.6±3.6	3.6±3.6	4.3±3.6	52.4±29.0	111.2±46.0	168.3±60.3
(s)	β-MSH	23.0±17.3	61.3±54.1	100.1±91.3	163.7±127.8	233.9±169.8	304.1±213.4
Preen time	0	0	0	0.22±0.5	1.6±1.1	3.4±1.9	3.6±2.1
(s)	β-MSH	0	0.2±0.2	0.48±0.48	0.5±0.5	0.8±0.8	0.8±0.8

Table 3.1. Changes in behaviors of chicks after central injection of β-MSH (Exp 4). Values are means ± S.E.M. Significance from control is indicated by (*) which implies $P \leq 0.05$

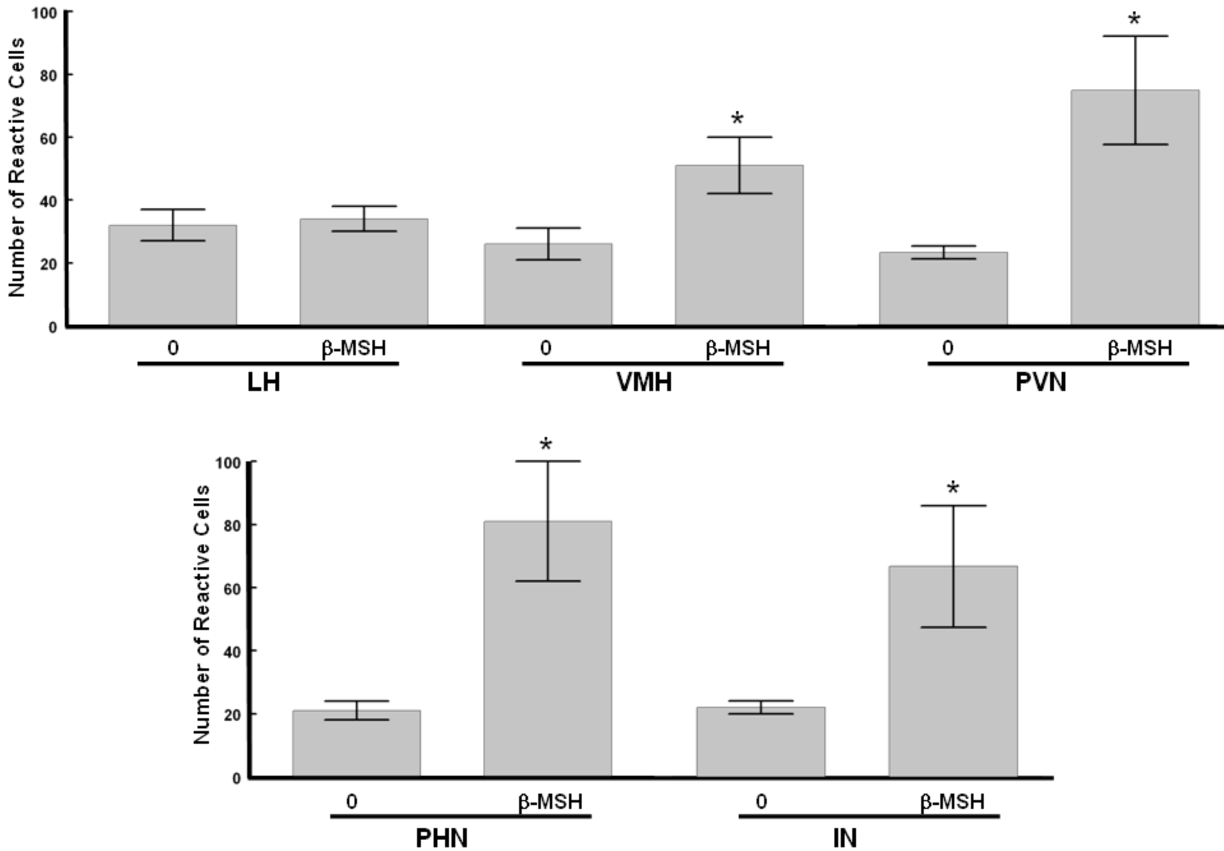

Figure 3.5. Effect of ICV injection of β-MSH on the number of reactive cells in the chick hypothalamus (Exp 5). (*) = different from control ($P \leq 0.05$). Values are means ± S.E.M.

CHAPTER IV

THE THRESHOLD OF INSULIN-INDUCED HYPOPHAGIA IS LOWER IN CHICKS SELECTED FOR LOW RATHER THAN HIGH JUVENILE BODY WEIGHT

ABSTRACT

Chicks genetically selected for low juvenile body weight had a lower threshold of central insulin-induced decreased food and water intake and whole blood glucose concentration than those selected for juvenile high body weight. Plasma corticosterone concentration was increased but not differently between lines. Therefore, selection may have affected insulin sensitivity which may have then contributed to their hypo- and hyperphagia and differential body weights.

Insulin is secreted by pancreatic beta cells in proportion to peripheral adiposity [10] and exerts its effects on energy homeostasis via stimulation of the central melanocortin system in both rodents [5] and chicks [56]. In rodents, central insulin induces hypophagia [12] and decreases body weight [11] when centrally administered. Additionally, insulin receptor knockout animals have increased body weight and adiposity [13].

In chicks, food intake decreases following central administration of insulin similarly to that in mammals [15]. In addition, central insulin increases corticotrophin releasing hormone (CRH) mRNA expression in chicks [7] indicating a potential role of the hypothalamic-pituitary-adrenal axis in the effects of central insulin.

To our knowledge, effects of insulin have not been investigated in polygenic models of obesity yet most human obesities do not result from a single gene defect. The lines of chickens used in this study were from a long-term divergent selection experiment for low (low weight select line; LWS) or high (high weight select line; HWS) body weight at 56 days of age. Chicks from the LWS line are hypophagic even when bed in mash feed, and chicks from the HWS line must be placed on feed-restriction diets by 8 weeks of age. Thus, I measured food and water intake in these lines as a response to central insulin. Further, I investigated the effects of central insulin on whole blood glucose and plasma corticosterone concentrations.

The founder population of HWS and LWS Plymouth White Rock chickens consisted of crosses of 7 partially inbred lines and the selected lines have been maintained as closed populations. There is now more than a 9-fold difference in body weight between these lines at selection age. Review of the selection program may be found in Dunnington and Siegel [57], Siegel and Wolford (2003) and Le Rouzic et al. [58]. The LWS and HWS lines exhibit hypo- and hyperphagia, respectively. Eggs obtained from age contemporary parents from S_{50} generation parental stocks were incubated in the same machine. After hatch, chicks were group caged for 2 d, then individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity where they had *ad libitum* access to a mash diet (20% crude protein, 2,685 kcal ME/kg) and tap water. The individual cages allowed visual and auditory contact with other chicks. Chicks were handled twice daily to adapt to handling. All trials were conducted between 11:00 and 16:00 h using 4 d post hatch chicks. This age was chosen to allow for time for the chicks to learn to eat from the feeders and for the yolk sac to be absorbed. Data were recorded from both lines concurrently, and injections were performed sequentially, LWS, HWS, LWS, HWS and so forth. Experimental procedures were performed according to the National Research Council

publication, Guide for Care and Use of Laboratory Animals and were approved by the Radford University Institutional Animal Care and Use Committee.

Chicks were injected using a method adapted from Davis et al. [52] that does not appear to induce physiological stress [59]. The head of the chick was briefly inserted into a restraining device that left the cranium exposed and allowed for free-hand 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. Chicks were assigned to treatments at random. Human recombinant insulin (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in artificial cerebrospinal fluid (aCSF) in a total injection volume of 5 μL with 0.1% Evans Blue dye to facilitate injection site localization. After data collection, chicks were decapitated and heads sectioned coronally to determine site of injection. Any chick without dye present in the lateral ventricle system was eliminated from analysis.

Chicks, fasted for 180 min were randomly assigned to receive vehicle (aCSF), 0.02, 0.17 or 1.72 nmol insulin by intracerebroventricular (ICV) injection. There were 8, 9, 9, and 10 LWS and 5, 5, 6, and 5 HWS chicks at each of the respective dosages available for analysis. The average body weight was 25.28 ± 1.57 g and 47.82 ± 2.00 g for LWS and HWS chicks, respectively. After injection, food and water consumption was recorded (± 0.01 g) for 180 min at 30 min intervals. Data were analyzed using two-way analysis of variance (ANOVA) at each time point. There was no effect of sex thus the reduced model included line, insulin dose and the line by insulin dose interaction. When the interaction was significant ($P < 0.05$), data were analyzed within each line for the effect of insulin dose using Tukey's method of multiple

comparisons. Water weight (g) was converted to volume (ml; 1 g = 1 ml), and both food and water consumption were normalized by body weight to account for the inherent differences in consumption between the lines. Significance implies $P \leq 0.05$. To illustrate the inherent difference in food intake between the lines, when food intake is not converted to a body weight basis LWS control chicks consumed 1.15 ± 0.98 g and HWS control chicks 2.70 ± 0.72 g of food by 180 min post injection.

At the conclusion of food and water intake data collection, whole blood glucose was measured in duplicate using OneTouch Ultra blood glucose meters. Trunk blood was also collected and centrifuged to obtain plasma, and corticosterone concentrations were determined by ELISA immunoassay (Cayman Chemical Company, Ann Arbor, MI). Intra-assay variance was 6.62%.

Central insulin significantly decreased food intake in both lines of chicks at the 0.17 and 1.72 nmol doses at all observation times. By 180 min post injection there was an approximate 80% reduction in food intake in the LWS chicks and 50% reduction in HWS chicks treated with insulin compared to control-treated chicks within each line (Figure 4.1). LWS chicks also responded to 0.02 nmol insulin whereas those from line HWS did not. The magnitude of hypophagia after central insulin was greatest at 30 min post injection in HWS chicks with decreasing magnitude thereafter. However, the magnitude increased as a function of time in LWS chicks. This is because HWS chicks treated with effective doses of insulin continued to consume food between injection and 60 min but did not thereafter. LWS insulin-treated chicks did not consume considerable amounts of food 30 min following injection. It is therefore likely that the hyperphagia in HWS chicks might be due to the low insulin sensitivity in the central appetite regulatory system.

Shiraishi et al. [56] and Schwartz et al. [12] demonstrated that the anorexigenic effects of insulin are mediated by interactions with the melanocortin system. Thus this is also likely the case in the LWS and HWS lines. Recently, Cline et al. showed that the LWS chicks have a lower threshold of response to α-melanocyte stimulating hormone, a potent melanocortin receptor ligand, than do HWS chicks [51]. Therefore, the increased sensitivity to central insulin in the LWS chicks may be associated with differences in melanocortinergic signaling pathways between the lines; a thesis that warrants further investigation.

Another possible explanation is that there are differences in the abundance or the function of brain insulin receptor in LWS and HWS chicks. For example, that LWS chicks were more sensitive to its anorexigenic effects may be due to up-regulated central insulin receptors or a gain of function in these receptors because the endogenous insulin concentration should be lower. Alternatively, a loss of function or receptor desensitization may have occurred in the HWS line and be responsible for this difference in sensitivity to central insulin. However, these hypotheses are beyond the scope of the present study.

Water intake also decreased in both lines. However, a line by dose interaction was significant with HWS responding at lower doses than LWS chicks (Figure 4.2). This effect was similar to that reported for responses to α-melanocyte stimulating hormone [51], neuropeptide S [60], and corticotrophin releasing factor [61] in these lines. It is therefore likely that there are similar differences not only in the feeding regulatory system, but also in the drinking regulatory system of these two lines of chickens.

Whole blood glucose concentrations were also decreased in both lines (Figure 4.3), although more so in LWS than HWS chicks resulting in a significant line by dose interaction. This likely is a direct effect of insulin on glucose secretion, or it may primarily be attributed to

the decreased food intake and be a secondary effect of insulin. Because the separation of treatment means followed similar patterns in the food intake and glucose concentration means, the effect of insulin on glucose appears more likely secondary to the decreased food intake.

Based on data indicating increased CRF mRNA in chicks following central insulin [15], and that these lines respond differentially to central CRF [15], I also measured plasma corticosterone concentrations. Both lines responded similarly to central insulin, and there was no line by dose interaction. Corticosterone concentrations were significantly increased by only the 1.72 nmol dose (Figure 4.4) whereas effects on food intake occurred at much lower doses. This pattern indicates that the mechanism of insulin's anorexigenic effect at lower doses (0.02 nmol and 0.17 nmol) is likely not related to the activation of the hypothalamic-pituitary-adrenal axis in HWS and LWS chicks.

These body weight selected lines provide polygenic models for anorexia and obesity and may offer better insights into body weight disturbances in other species. They exhibit differential effects of central insulin on food intake, water intake, and glucose concentrations. These results suggest that the decrease of the anorexigenic effect of insulin in HWS chicks might be one of the causes for the difference in body weight between the LWS and HWS lines.

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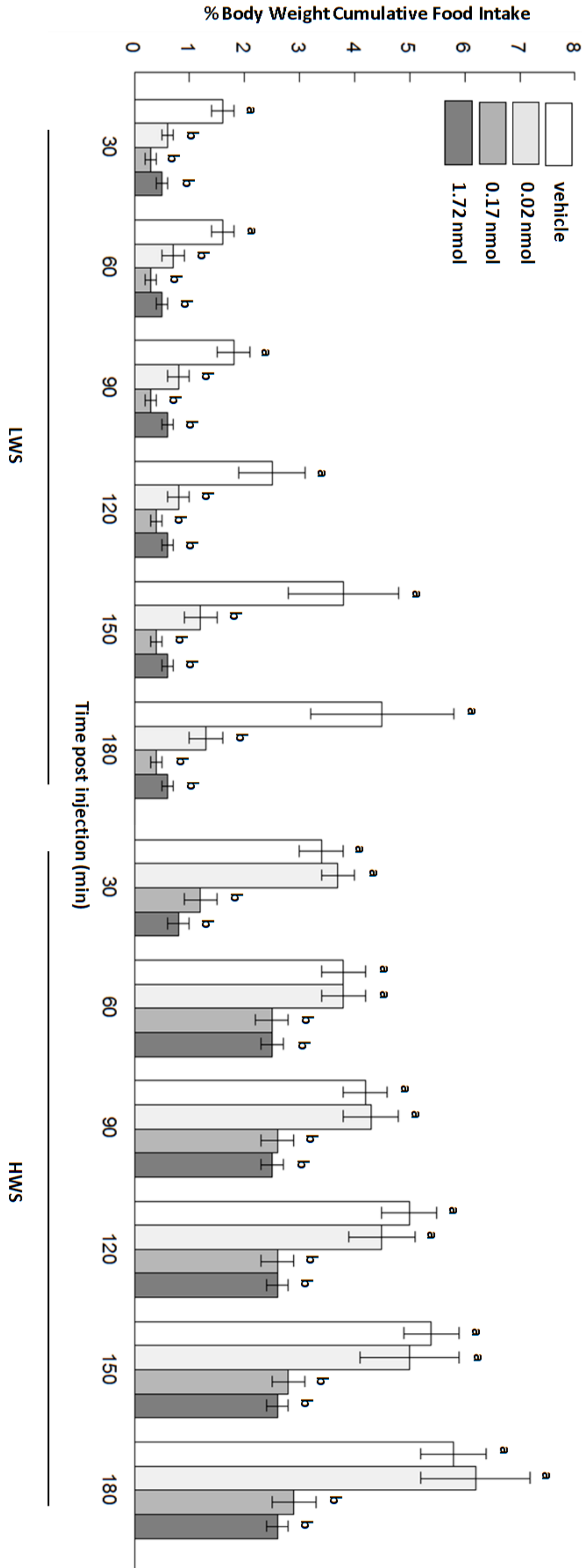
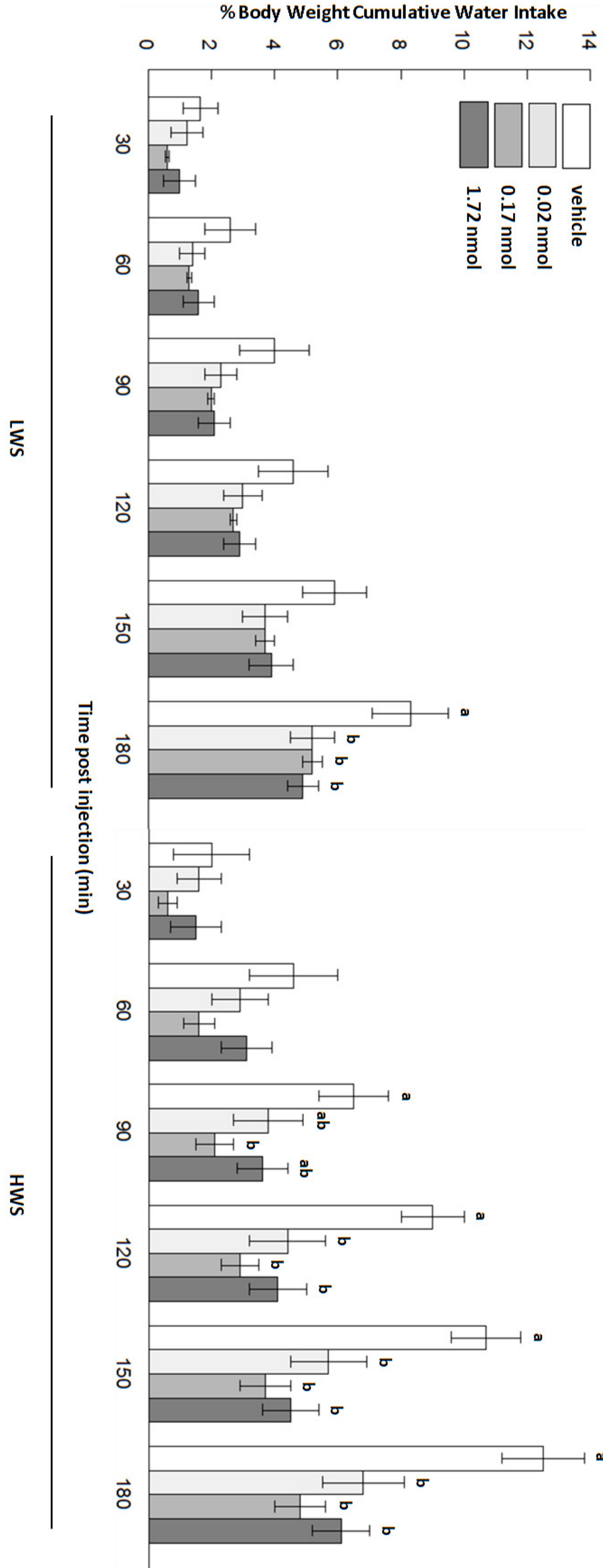

% Body Weight Cumulative Food Intake
vehicle
0.02 nmol
0.17 nmol
1.72 nmol
Time post injection (min)
LWS
HWS

Figure 4.1. Cumulative food intake expressed as percent body weight following intracerebroventricular injection of insulin in low (LWS) and high (HWS) body weight of chicks. Values are means±SE; bars with different letters are different from each other within a time point ($P < 0.05$).



% Body Weight Cumulative Water Intake
0
2
4
6
8
10
12
14
vehicle
0.02 nmol
0.17 nmol
1.72 nmol
30
60
90
120
150
180
LWS
HWS
Time post injection (min)
a
b
ab

Figure 4.2. Cumulative water intake expressed as percent body weight following intracerebroventricular injection of insulin in low (LWS) and high (HWS) body weight lines of chicks. Values are means±SE; bars with different letters are different from each other within a time point ($P < 0.05$).

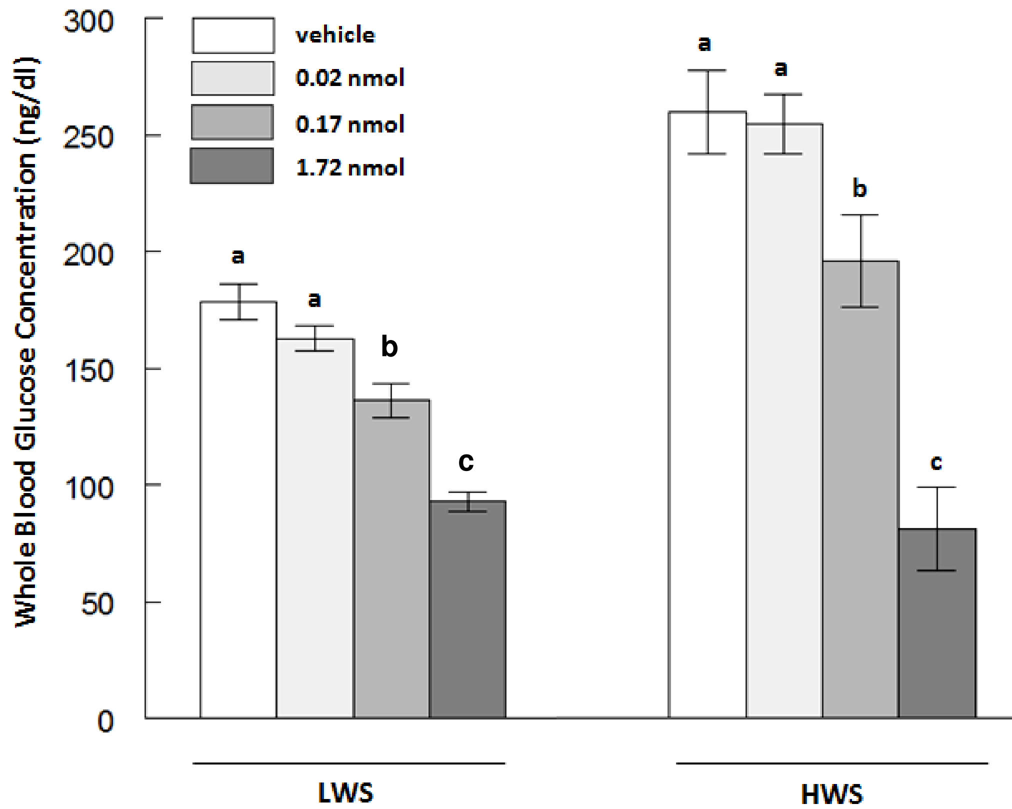


Figure 4.3. Whole blood glucose concentrations following intracerebroventricular injection of insulin in low (LWS) and high (HWS) body weight lines of chicks. Values are means±SE; bars with different letters are different from each other ($P < 0.05$).

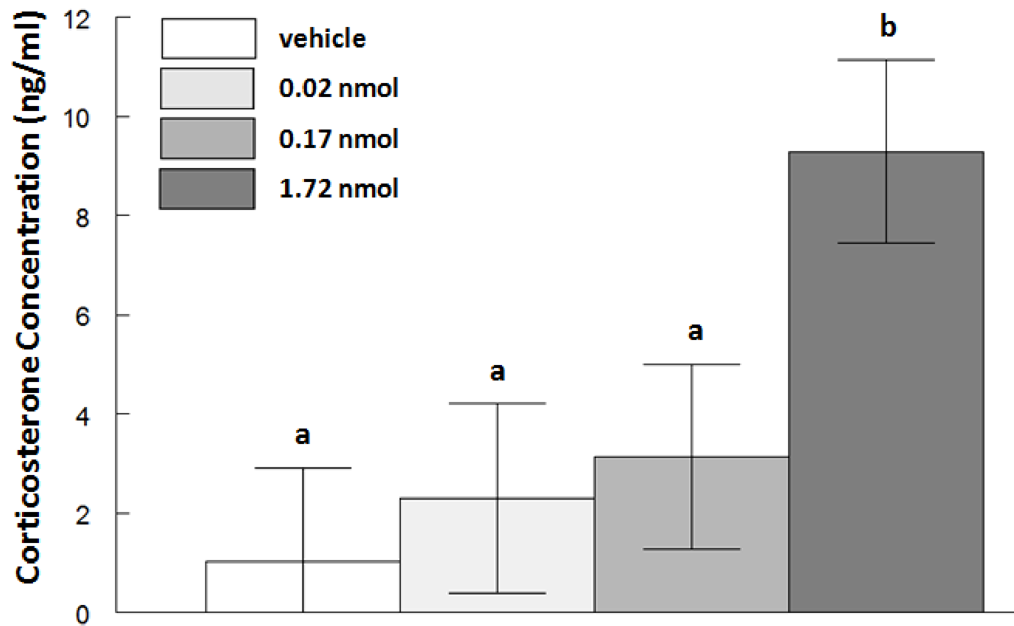


Figure 4.4. Plasma corticosterone concentrations following intracerebroventricular injection of insulin in pooled low (LWS) and high (HWS) body weight lines of chicks. Values are means±SE; bars with different letters are different from each other ($P < 0.05$).

CHAPTER V

DIFFERENTIAL RESPONSES TO β-MELANOCYTE STIMULATING HORMONE IN HIGH AND LOW WEIGHT LINES OF CHICKS

Animal Model

Chicks used in the following experiments are the result of long-term divergent phenotypic selection for juvenile body weight for 51 consecutive generations. The founder population of HWS and LWS Plymouth White Rock chickens consisted of crosses of 7 partially inbred lines and the selected lines have been maintained as closed populations. Currently the lines differ in weight by approximately 9 fold. Chicks in the HWS line are obese, hyperphagic and have larger fat pads than those of LWS chicks [62]. These chicks must be placed on a feed-restriction diet by 8 weeks of age to prevent premature death and continue egg production. The LWS line contains some true anorexics which will not consume a sustainable amount of feed during the first Iek post hatch.

In addition to differing in food intake, these lines also differ in oxygen consumption and energy expenditure, likely leading to the increased adiposity found in the HWS [63]. This increased adiposity in HWS chicks, and the lack thereof in LWS chicks, may be responsible for the impairment in laying in both lines. Supporting the idea that decreased food intake was at least in part responsible for impawered reproduction in the LWS line, Siegel and Dunnington [64] reported that LWS chicks could be force-fed to stimulate sexual maturity.

Unlike most animal models of anorexia and obesity which result from single-gene knockouts, these lines provide a unique polygenic model of body weight disorders. The use of these models enables a more complete investigation into natural hunger and satiety mechanisms.
On day of hatch, chicks were group housed with *ad libitum* access to mash feed and water. Chicks were moved to individual cages at 2 days post hatch to reduce novel environment and isolation stress during experiments. Chicks used for behavior experiments had auditory, but not visual contact with each other and chicks used for all other experiments had auditory and visual contact with each other and individual feed and water containers. Unless otherwise stated, chicks had *ad libitum* access to a poultry starter diet (Southern States, 20% crude protein) and water.

Introduction

Much research supports the key role of the melanocortin system in food intake and body weight regulation. In fact, a mutation in the melanocortin 4 receptor (MC4R) is the most common monogenic cause of obesity in humans [38]. The melanocortin system also includes 4 other receptors (MC1-3R and MC5R) as well as several ligands, most of which are products of enzymatic cleavage of pro-opiomelanocortin by prohormone convertases. In rodents, while MC4R plays a predominant role in food intake regulation, MC3R plays a predominant role in regulating energy expenditure. In chicks, the role of MC3R is less understood. While some laboratories report presence of central MC3R in chicks, others suggest a lack of MC3R [19, 35]

One of the more interesting features of the melanocortin system is its self-regulation of an endogenous antagonism. While some melanocortin ligands such as Agouti-related peptide

(AgRP) increase food intake, others such as α-, β-, and γ-melanocyte stimulating hormone (MSH) decrease food intake. In both broiler and layer type chicks, α-MSH decreases food intake [50, 54]. In lines of chicks resulting from long-term divergent selection for body weight (Siegel Lines), α-MSH has a stronger affect in low weight chicks (LWS) than high weight chicks (HWS) [51].

Based on previous data on differential melanocortin signaling in LWS and HWS chicks, the present study was designed to investigate whether β-MSH signaling was also altered by body weight selection in the lines. To address this question, food and water intake were measured following β-MSH injection. To determine the mechanism underlying differences in food intake following β-MSH, whole blood glucose was measured and ingestive and non-ingestive behaviors were quantified. Because other melanocortins have affected the hypothalamic-pituitary-adrenal stress response, corticosterone concentration was measured as well as food intake following a co-injection of β-MSH and astressin, a CRH receptor antagonist. Experiments designed to investigate the role of the MC4R and hindbrain in the differential food intake response were also conducted with a coadministration of β-MSH and HS014, a highly selective MC4R antagonist, and a comparison of food intake effects following injection on β-MSH into the lateral and 4th ventricles, respectively.

Materials and Methods

Animals

Eggs, obtained from S_{51} generation parental lines were group incubated in the same machine. After hatch, chicks were group caged for 2 days then caged individually in a room at 30 ± 2 °C and 50 ± 5% relative humidity. Unless otherwise indicated, chicks had *ad libitum* access to tap water and a mash diet (20% crude protein, 2,685 kcal ME/kg). The individual cages for all but the behavioral experiments allowed visual and auditory contact with other chicks. Individual cages for the behavioral experiment allowed auditory but not visual contact between chicks. Chicks were handled daily to decrease handling stress during experiments. Data were collected from both lines concurrently and treatments were randomized. Experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and were approved by the Radford University Institutional Animal Care and Use Committee.

ICV injection procedure

Chicks were injected using a method adapted from Davis et al. [17] that does not appear to induce physiological stress [18]. The head of the chick was briefly inserted in a restraining device that exposed the cranium and allowed for free-hand 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 26 gauge needle affixed to a 10 µL Hamilton syringe. To reduce backflow, the needle remained at the injection depth for 5 s post-injection. β-MSH (American Peptide Company, Sunnyvale, CA),

astressin (American Peptide Company, Sunnyvale, CA), and HS014 (Sigma-Aldrich, St. Louis, MO) were dissolved in artificial avian cerebrospinal fluid [19] as a vehicle for a total injection volume of 5 μl with 0.1% Evans Blue dye to facilitate injection site localization at the conclusion of each experiment. In the food intake experiment, following data collection, chicks were decapitated, their heads sectioned frontally to determine injection site, and in experiment 1, trunk blood was collected. In all experiments, any chick without dye present in the ventricle system was eliminated from analysis.

β-MSH effects on food and water intake and glucose and corticosterone concentrations

In Experiment 1, chicks from both lines were fasted for 180 min prior to injection. Chicks were randomly assigned to receive 0 (vehicle only), 0.01, 0.1 or 1.0 nmol β-MSH by ICV injection. Following injection, chicks were returned to individual cages and given *ad libitum* access to food and water, with individual containers weighed every 30 min for 180 min post injection. Data were analyzed using analysis of variance (ANOVA) at each time point. The model included line, dose, and the line by dose interaction. When the interaction was significant ($P < 0.05$), data were analyzed within each line using Tukey's method of multiple comparisons as a post hoc test. The LWS and HWS lines consume inherently different amounts of food thus food and water intake data were divided by body weight. This conversion was made by dividing the food consumed by each chick by its body weight at time of injection, and multiplying by 100. Trunk blood was collected from chicks immediately after the 180 min food and water intake reading. Whole blood glucose concentration was determined in duplicate using the One Touch Basic glucose measurement system (Lifescan, Milpitas, CA, USA) with a sensitivity range of 20 - 600

mg⁄dl. Blood glucose data were analyzed similar to food and water intake data.   Trunk blood was centrifuged to obtain plasma which was used to determine corticosterone concentrations (Cayman Chemical Company Corticosterone ELISA). Glucose and corticosterone data were analyzed similarly to food and water intake data.

β-MSH effects on ingestive and non-ingestive behaviors

Chicks were injected similarly to Experiment 1 with vehicle or 0.1 nmol β-MSH and immediately placed in a clear plexiglass arena with pre-weighed food and water in diagonal corners and recorded automatically on DVD from 3 angles for 30 min post injection. Count-type behaviors (number of feed pecks, jumps, escape attempts, and defecations) mutually exclusive timed behaviors (standing, sitting, preening, perching, and deep rest) and the total distance traveled were analyzed using AnyMaze Behavioral Analysis Software (Stoelting, Wood Dale, IL, USA) on a cumulative basis in 5 min intervals. Data were subjected to a one-way non-parametric Mann Whitney-U test due to heterogeneous variance.

Food and water intake after β-MSH + HS014

In Experiment 3, the experimental procedures were identical to those used in Experiment 1 except that chicks were randomly assigned to receive vehicle, β-MSH, 0.1 nmol HS014, or a dual injection of 0.1 nmol β-MSH + 0.1 nmol HS014. Following injection, food and water intake were recorded for 180 min. Data were analyzed as in Experiment 1.

Food and water intake after β-MSH + astressin

In Experiment 4, the experimental procedures were identical to those used in Experiment 1, except that chicks were randomly assigned to receive vehicle, 0.1 nmol β-MSH, 6.0 nmol astressin, or a dual injection of 0.1 nmol β-MSH + 6.0 nmol astressin. Following injection, food and water intake were recorded for 180 min. Data were analyzed as in Experiment 1.

Food and water intake after 4th ventricle injection

In Experiment 5, chicks were either injected with vehicle or 0.1 nmol β-MSH as in Experiments 1-4, or were freehand injected targeting the 4th ventricle. Chicks receiving hindbrain injections were placed in the same restraining apparatus previously described and injections were made immediately between the cerebrum and cerebellum. The needle was left in place 10s to reduce backflow. Chicks displaying abnormal motor behaviors or not consuming food were removed from the Experiment. Following injection chicks were placed back in their individual cages with *ad libitum* access to food and water. Food and water intake were measured at 30 min intervals for 180 min post injection. At the conclusion of the data collection, chicks were decapitated and the cranium sectioned frontally to determine injection site. Chicks lacking dye in the targeted ventricle (lateral or 4th ventricle) were removed from the analysis. Data were analyzed similarly to Experiment 1.

Food and water intake after γ-MSH

In Experiment 6, chicks from both lines were fasted for 180 min prior to injection. Chicks were randomly assigned to receive 0 (vehicle only), 1.5, 3.0 or 6.0 nmol γ-MSH by ICV injection.

Following injection, chicks were returned to individual cages and given *ad libitum* access to food and water, with individual containers weighed every 30 min for 180 min post injection. Data were analyzed using analysis of variance (ANOVA) at each time point. The model included line, dose, and the line by dose interaction. When the interaction was significant ($P < 0.05$), data were analyzed within each line using Tukey's method of multiple comparisons as a post hoc test. The LWS and HWS lines consume inherently different amounts of food thus food and water intake data were divided by body weight. This conversion was made by dividing the food consumed by each chick by its body weight at time of injection, and multiplying by 100.

Results

Chicks from both lines responded to central β-MSH with decreased food intake though the LWS chicks had a higher sensitivity in Experiment 1 (Figure 5.1). Both lines responded to β-MSH with similar decreases in water intake (Figure 5.2) while whole blood glucose concentrations were not affected (Figure 5.3). Plasma corticosterone concentration was higher in vehicle-treated LWS than HWS and only LWS responded to central β-MSH with an increased corticosterone concentration (Figure 5.4).

In Experiment 2, there were no significant line by treatment interactions. However, LWS inherently had a greater number of feed pecks and escape attempts than HWS while traveling less and having a lower number of defecations and jumps (Table 5.1). LWS chicks also spent less time standing than HWS chicks and no other timed behaviors were inherently different

between lines (Table 5.2). In response to β-MSH, both lines decreased the number of feed pecks similarly (Table 5.1).

HS014 attenuated the anorexigenic effect of β-MSH in LWS, but not in HWS chicks (Experiment 3; Figure 5.5) but did not affect water intake (Figure 5.6). Astressin did not attenuate the anorexigenic effects of β-MSH in either line (Experiment 4; Figure 5.7). When comparing food intake following β-MSH injections in the lateral and 4th ventricles, both lines responded similarly and food intake was reduced similarly in HWS and LWS chicks injection sites in LWS and HWS chicks (Figure 5.8) while water intake (Figure 5.9) was not affected (Experiment 5).

In experiment 6, γ-MSH did not affect food intake (Figure 5.10) in fasted or fed LWS or HWS chicks at a wide range of doses. Water intake was also not affected by γ-MSH in LWS or HWS chicks (Figure 5.11).

Discussion

Similar to its anorexigenic effect in broiler-type chicks [27] and rats [25], β-MSH decreased food intake in both LWS and HWS chicks. In both lines food intake was decreased at all observation times. LWS chicks responded similarly to all doses of β-MSH tested, while HWS chicks exhibited a dose-dependent response. That HWS chicks were more sensitive to these effects

was inconsistent with effects found following administration of α-MSH [22], suggesting a specific alteration in the β-MSH system as opposed to the melanocortin system in its entwerety. Together, these data may indicate an alteration in melanocortinergic signaling or receptor binding affinity for β-MSH in high and low weight individuals which may be contributing, at least in part, to their differential body Weights. Because β-MSH binds MC3R and MC4R and .MC4R mutations are the most common monogenic cause of obesity known in humans [38], Experiment 3 focused on the role of MC4R in this differential response to β-MSH.

In Experiment 3, HS014 was co-administered with β-MSH. HS014, a highly selective MC4R antagonist [30], attenuated the response to β-MSH in LWS but not HWS chicks. This suggests that MC4R is involved in the β-MSH response in LWS, but that the MC4R in HWS may have a lower affinity or altered downstream response to β-MSH. HS014 treatment alone decreased food intake in HWS to the same food intake as vehicle-treated LWS chicks. Thus the altered mechanism of response to β-MSH in HWS is likely downstream of MC4R.

To investigate the mechanism underlying β-MSH induced increased corticosterone concentration, astressin was administered with β-MSH to antagonize the CRH receptors and determine their role in the response. However, although β-MSH increased corticosterone concentrations in LWS, astressin did not attenuate the anorexigenic effect of β-MSH, indicating that effect on corticosterone is likely not mediated via CRH receptors and this pathway warrants further research.

The lack of stimulation of behaviors that would be competitive with food intake indicate that the anorexigenic response to β-MSH is likely primary rather than secondary to behavioral modifications. In broiler-type chicks, β-MSH similarly did not stimulate anxio-like or adverse behaviors [27] thus β-MSH may have higher potential as a pharmacological treatment option for body weight disorders compared to other appetite-related signals which alter food intake but also stimulate competitive or active behaviors likely resulting in a lack of a net effect on body weight. [65].

In experiment 6, γ-MSH, which is highly selective for MC3R in both mammals and chickens [19], did not affect food intake in fasted or fed HWS or LWS chicks at a wide range of doses. Although γ-MSH decreased food intake in broiler-type chicks, it's response in rodents is controversial with laboratories reporting hypophagic [25], normophagic [23], and hyperphagic [66] responses. γ-MSH can induce a cataleptic state in rats [49] which may account for the varied food intake responses in rodents. That γ-MSH had no effect on food intake in LWS or HWS chicks suggests that alterations in this system may have occurred through the selection process in both lines.

In summary, central β-MSH was found to have anorexigenic effects in both LWS and HWS chicks. Neither line responded with altered glucose concentrations or behaviors following central β-MSH. LWS chicks had an increase in corticosterone concentration although co-

administration of astressin and β-MSH did not attenuate the effects seen in β-MSH treated chicks. Because injections in the lateral and 4th ventricle elicited similar responses to β-MSH, both the hypothalamus and hindbrain may be partly responsible for the effects of central β-MSH. These data suggest that β-MSH may play a role in the differential body weights of LWS and HWS chicks.

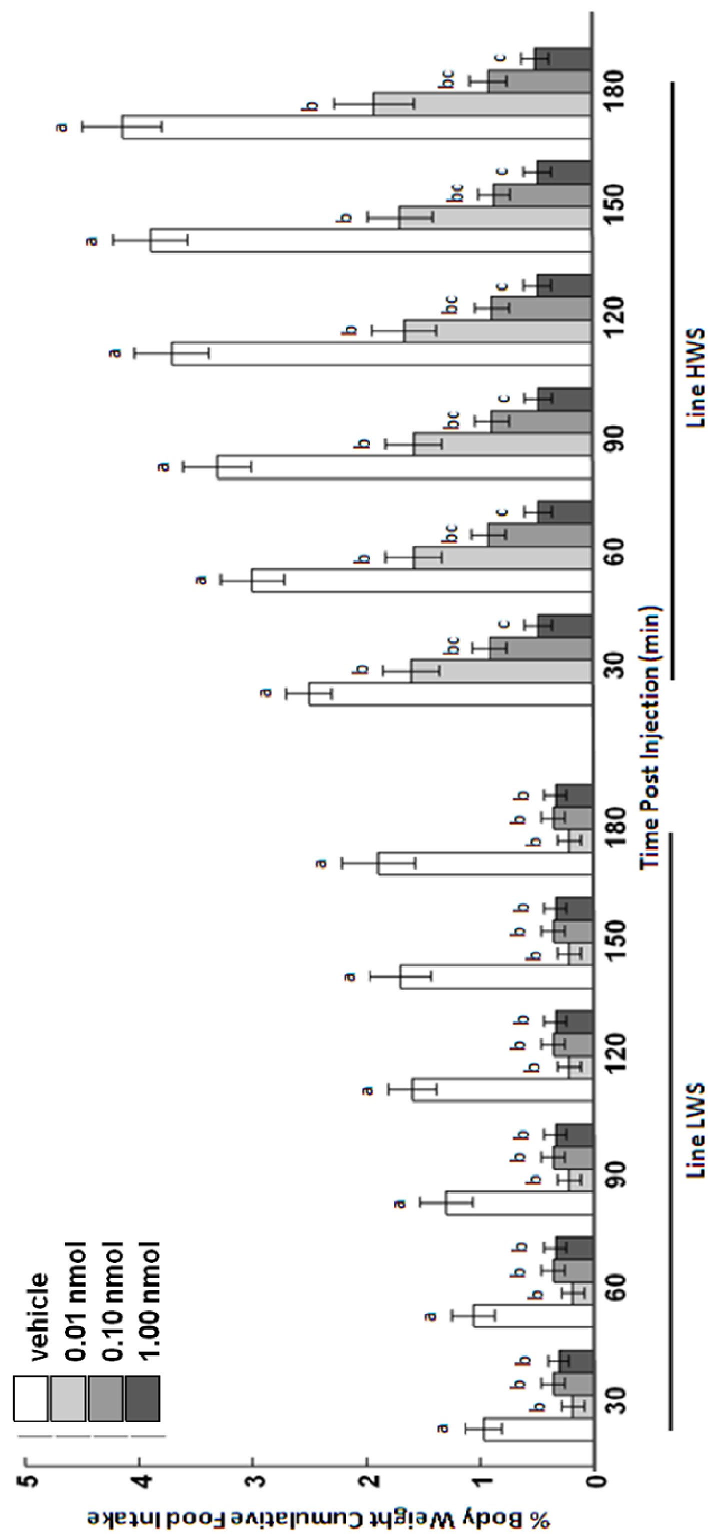


Figure 5.1. Effects of central β-MSH on cumulative food intake in LWS and HWS chicks. Superscripts indicate significant differences (P <0.05). Values expressed as means ± SEM.

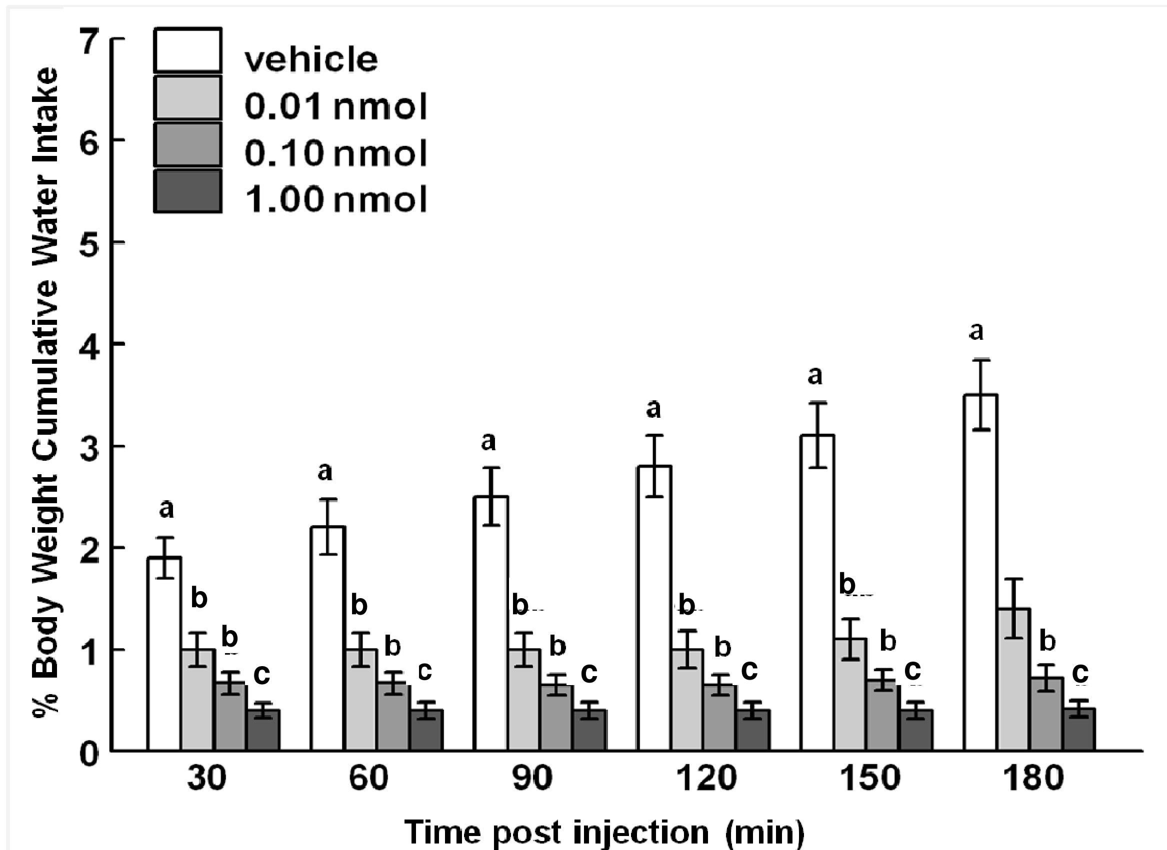


Figure 5.2. Effects of central β-MSH on cumulative water intake in pooled LWS and HWS chicks. Superscripts indicate significant differences (*P* <0.05). Values expressed as means ± SEM.

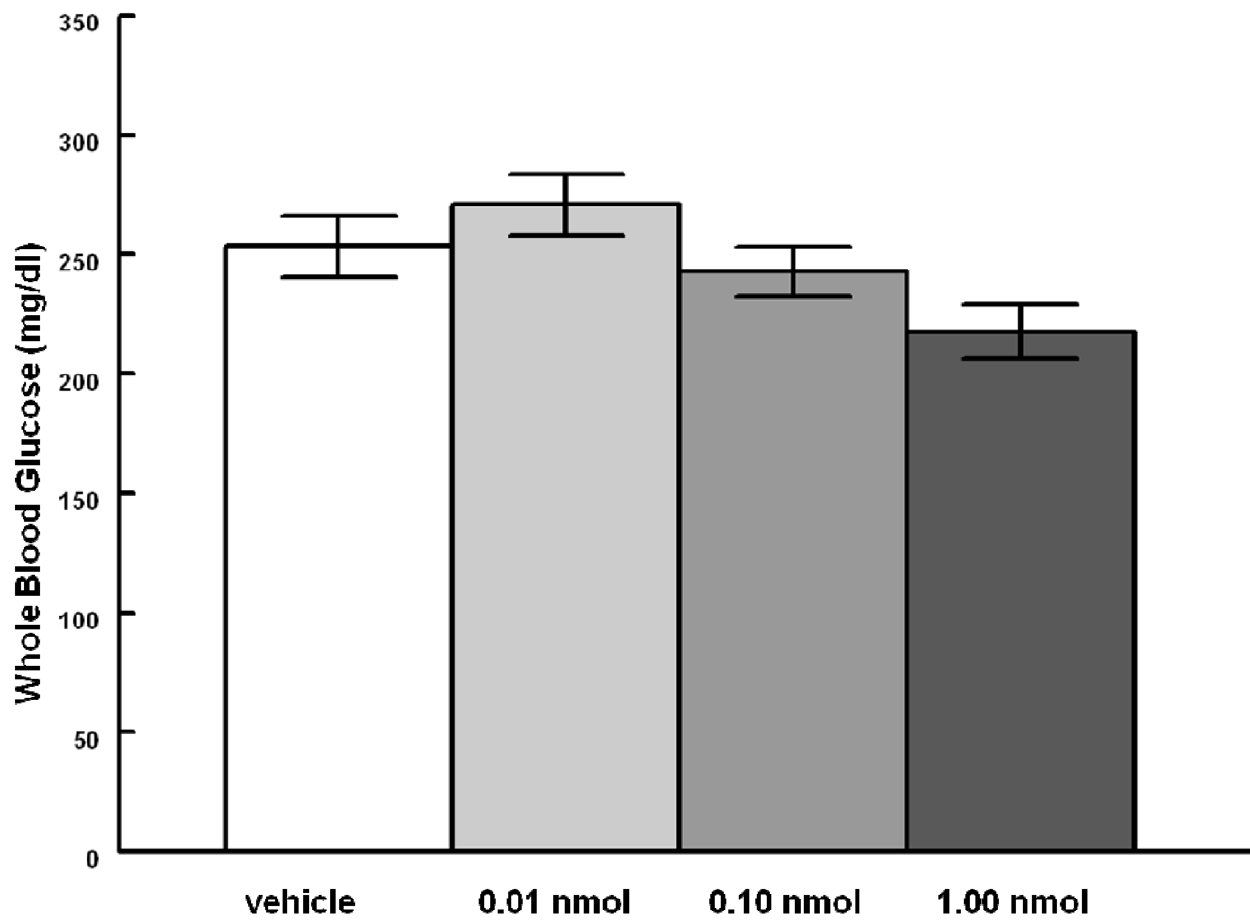


Figure 5.3. Effects of β-MSH on whole blood glucose concentration in LWS and HWS chicks. Due to a lack of line by treatment difference, the lines were pooled. Glucose concentration was not affected by any dose of β-MSH tested. Values expressed as means ± SEM.

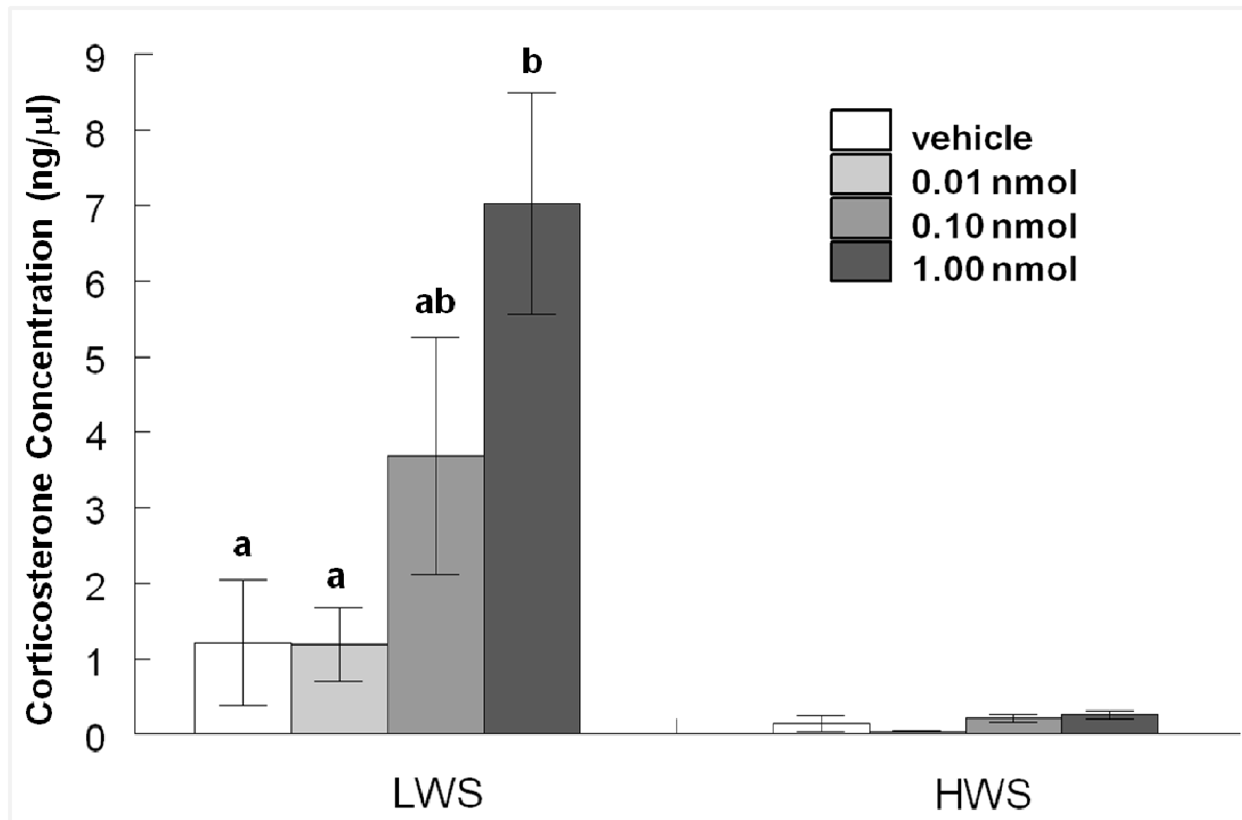


Figure 5.4. Effects of central β-MSH on corticosterone concentration in LWS and HWS chicks. Superscripts indicate significant differences ($P < 0.05$). Values expressed as means ± SEM.

Post-injection time (min)		Feed Pecks		Defecations		Escape Attempts		Distance Traveled		Jump	
		Vehicle	β- MSH	Vehicle	β- MSH	Vehicle	β- MSH	Vehicle	β- MSH	Vehicle	β- MSH
5	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	P>0.05		P>0.05		P>0.05		P>0.05		**P<0.05**	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	61.6±32.0	4.90±3.65	0.0±0.0	0.0±0.0	0.30±0.30	0.30±0.21	0.65±0.28	2.70±2.10	0.10±0.10	0.0±0.0
	HWS	41.0±17.2	33.6±22.8	0.20±0.13	0.30±0.15	0.20±0.20	0.60±0.26	1.71±0.43	2.70±0.79	0.30±0.30	0.6±0.33
10	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	P>0.05		**P<0.05**		**P<0.05**		P>0.05		**P<0.05**	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	84.5±41.5	8.3±6.95	0.00±0.00	0.10±0.10	0.30±0.30	0.30±0.21	0.96±0.93	4.10±3.20	0.10±0.10	0.0±0.0
	HWS	115±60.6	75.4±38.3	0.20±0.13	0.40±0.16	0.40±0.26	1.7±0.95	3.30±0.73	4.20±1.20	1.10±0.82	0.90±0.34
15	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	P>0.05		**P<0.05**		**P<0.05**		P>0.05		**P<0.05**	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	98.8±48.1	8.4±7.05	0.00±0.00	0.10±0.10	0.30±0.30	0.40±0.22	1.30±0.59	5.40±4.20	0.10±0.10	0.0±0.0
	HWS	153±85.6	102±49.8	0.20±0.13	0.60±0.22	0.50±0.34	2.3±1.09	5.20±0.96	5.50±1.51	2.20±1.48	1.10±0.37
20	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	P>0.05		**P<0.05**		**P<0.05**		**P<0.05**		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	113±55.5	10.5±9.13	0.0±0.0	0.10±0.10	0.30±0.30	0.70±0.49	1.70±0.74	6.60±4.80	0.10±0.10	0.50±0.40
	HWS	270±89.4	120±63.15	0.20±0.13	0.60±0.22	1.20±0.91	2.70±1.34	7.30±1.20	6.80±2.0	3.40±2.34	1.30±0.47
25	TRT	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	P>0.05		**P<0.05**		**P<0.05**		**P<0.05**		**P<0.05**	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	153±70.26	11.7±10.3	0.10±0.10	0.10±0.10	0.30±0.30	1.20±0.98	2.50±0.87	7.20±5.00	0.10±0.10	1.0±0.89
	HWS	380±110	132±65.8	0.50±0.22	0.80±0.29	1.40±0.89	3.30±1.43	9.20±1.51	9.30±2.85	4.70±3.17	2.80±1.03
30	TRT	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		**P<0.05**		**P<0.05**		**P<0.05**		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	15.9±126	15.8±14.4	0.10±0.10	0.20±0.13	0.30±0.30	1.30±0.98	3.80±1.19	7.63±5.12	0.50±0.40	1.0±0.89
	HWS	15.8±14.4	161±73.2	0.50±0.22	0.90±0.31	1.40±0.89	3.80±1.64	10.7±1.74	11.8±3.44	5.70±3.95	3.6±1.40

Table 5.1. Effects of central β-MSH on count-type behaviors in LWS and HWS chicks. Values expressed as means ± SEM.

Post-injection time (min)		Standing		Perching		Sitting		Resting		Preening	
		Vehicle	β-MSH	Vehicle	β-MSH	Vehicle	β-MSH	Vehicle	β-MSH	Vehicle	β-MSH
5	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	169±47.1	147±46.7	10.7±10.5	0.49±0.40	0.20±0.13	0.0±0.0	0.30±0.30	25.2±25.2	0.0±0.0	0.0±0.0
	HWS	299±0.11	275±12.9	0.09±0.09	0.16±0.16	0.0±0.0	5.52±5.52	0.0±0.0	19±12.7	0.0±0.0	0.0±0.0
10	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	295±86.1	575±12.6	33.9±23.8	21.3±21.2	0.50±0.26	16.9±16.9	29±29.2	55.2±55.2	2.75±1.10	1.68±129
	HWS	260±87.7	480±54.3	0.09±0.09	0.76±0.60	0.50±5.61	43.4±34.9	14±10.9	74.5±49.4	4.04±3.91	0.82±316
15	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	419±128	370±129.8	61.5±49.2	41.4±41.3	0.70±0.36	36.6±36.5	55.5±50.7	85.6±85.1	5.88±218	1.68±0.68
	HWS	819±452	662±94.2	0.09±0.09	1.09±0.69	35.6±35.6	96.6±68.9	40.0±29.7	138±81.4	4.32±4.19	0.82±0.82
20	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	536±69.0	472±166	90.2±76.5	42.2±42.1	0.70±0.36	83.2±66.7	89.1±80	115±115	8.06±6.22	1.68±0.68
	HWS	1088±166	845±131	0.99±0.89	1.38±0.69	65.6±65.6	130±96.2	40±29.7	222±105	4.5±4.18	0.89±0.81
25	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	659±213	567±202	100±86.6	42.8±42.6	1.76±0.96	138±97.8	133±110	145±145	12.7±6.19	6.32±2.58
	HWS	1348±953	1068±154	1.13±0.88	6.82±5.19	95.6±95.6	169±123	49±190	252±113	4.69±4.16	2.47±1.68
30	TRT	P>0.05		P>0.05		P>0.05		P>0.05		P>0.05	
	LIN	**P<0.05**		P>0.05		P>0.05		P>0.05		P>0.05	
	L x T	Ns		Ns		Ns		Ns		Ns	
	LWS	763±243	668±239	101±86.5	42.8±42.6	20.8±14.2	190±130	184±134	166±157	14.9±7.20	7.8±3.01
	HWS	1563±123	1290±184	3.70±2.52	14±10.8	113±109	188±137	82±47.6	292±122	9.1±8.5	2.47±1.68

Table 5.2. Effects of central β-MSH on mutually exclusive timed behaviors in LWS and HWS chicks. Values expressed as means ± SEM.

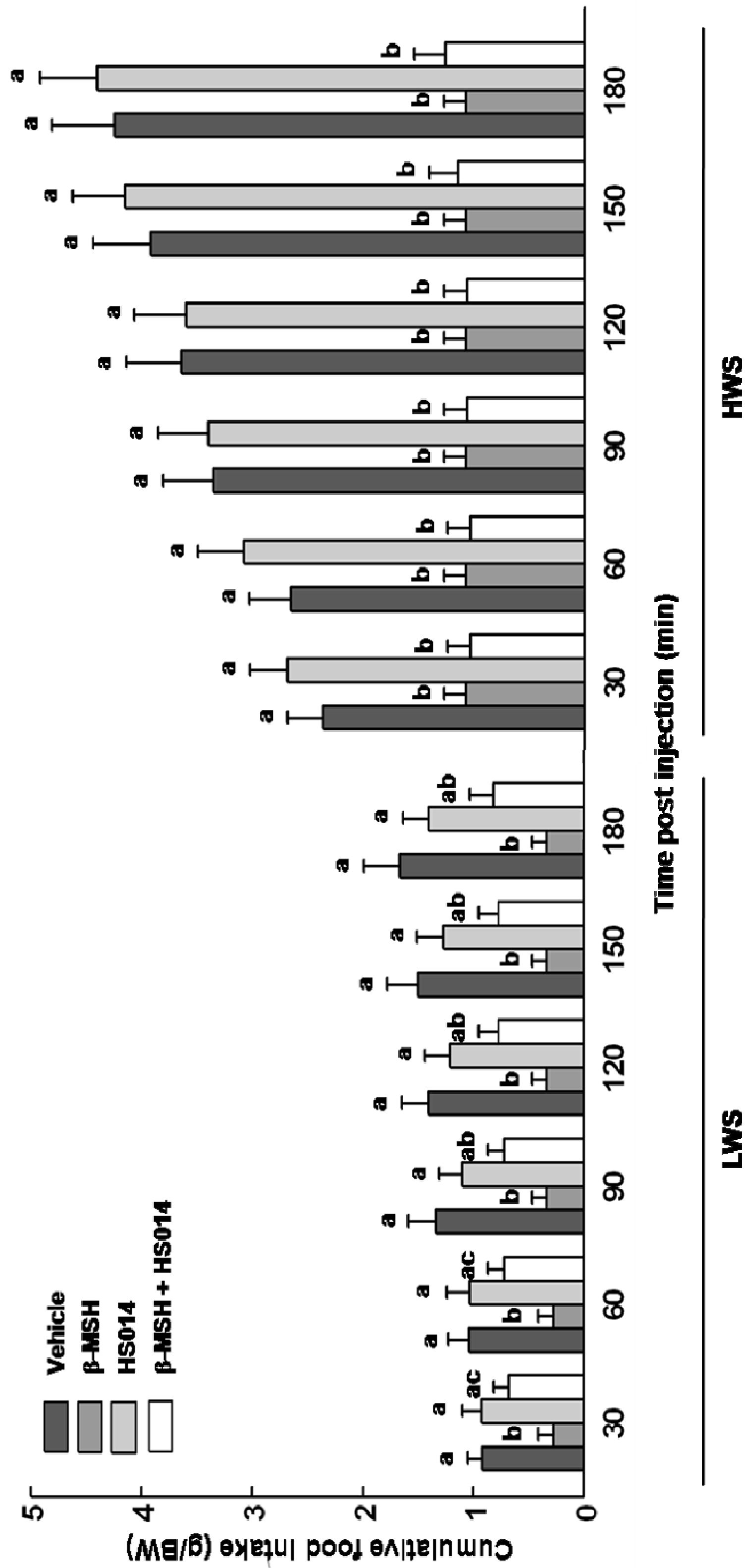


Figure 5.5. Effects of central β-MSH and HS014 on cumulative food intake in LWS and HWS chicks. Superscripts indicate significant differences ($P < 0.05$). Values expressed as means ± SEM.

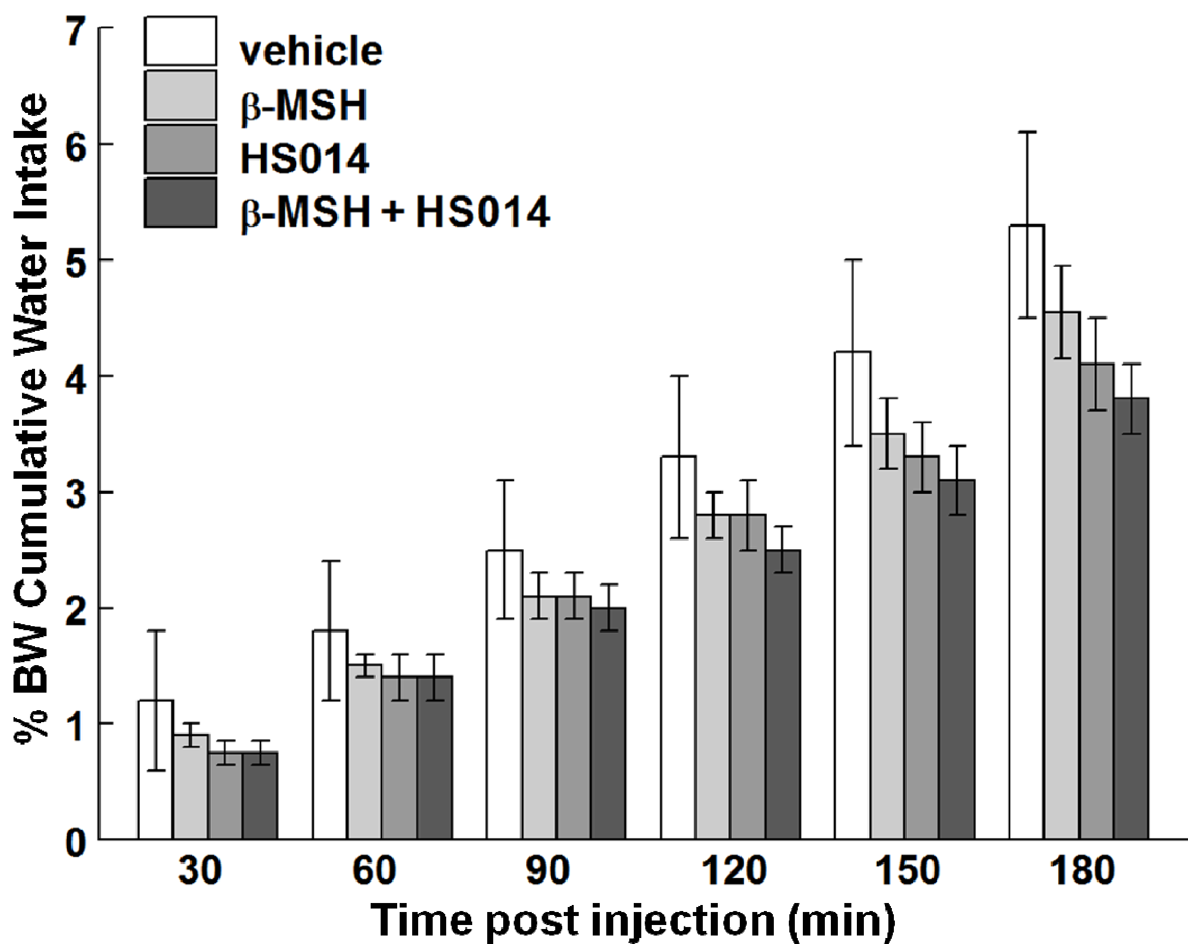


Figure 5.6. Effects of β-MSH + HS014 on cumulative water intake in LWS and HWS chicks. Water intake was not affected differentially in LWS and HWS chicks thus the lines were pooled and there was no overall effect on water. Values expressed as means ± SEM.

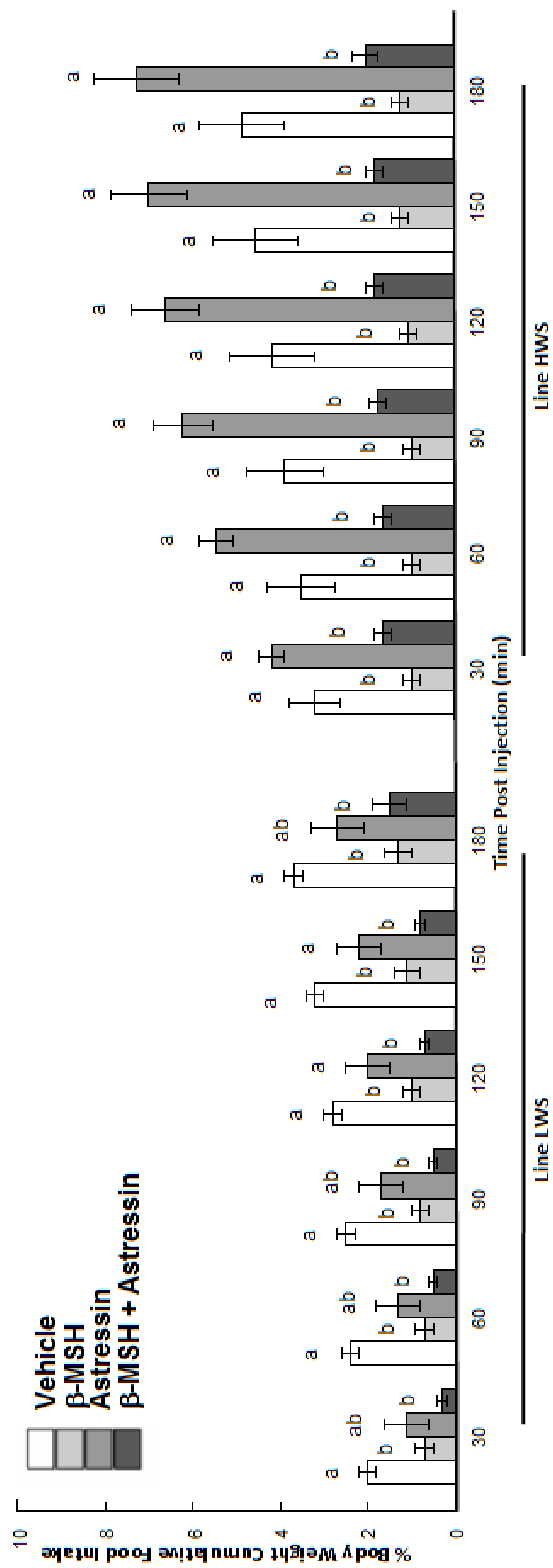


Figure 5.7. Effects of central β-MSH and astressin on cumulative food intake in LWS and HWS chicks. Superscripts indicate significant differences ($P < 0.05$). Values expressed as means ± SEM.

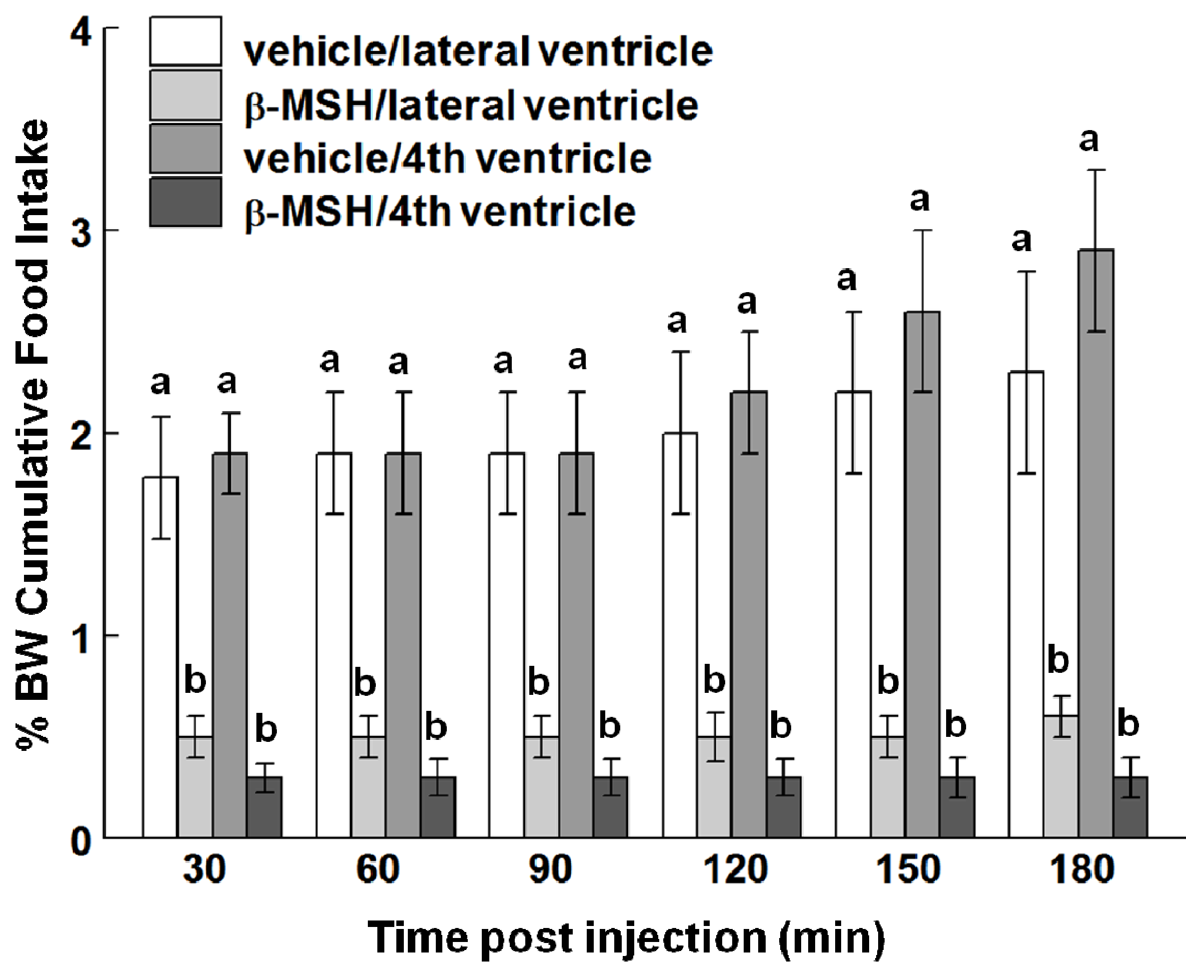


Figure 5.8. Effects of β-MSH on cumulative food intake in pooled LWS and HWS chicks. Superscripts indicate significant differences ($P < 0.05$). Values expressed as means ± SEM.

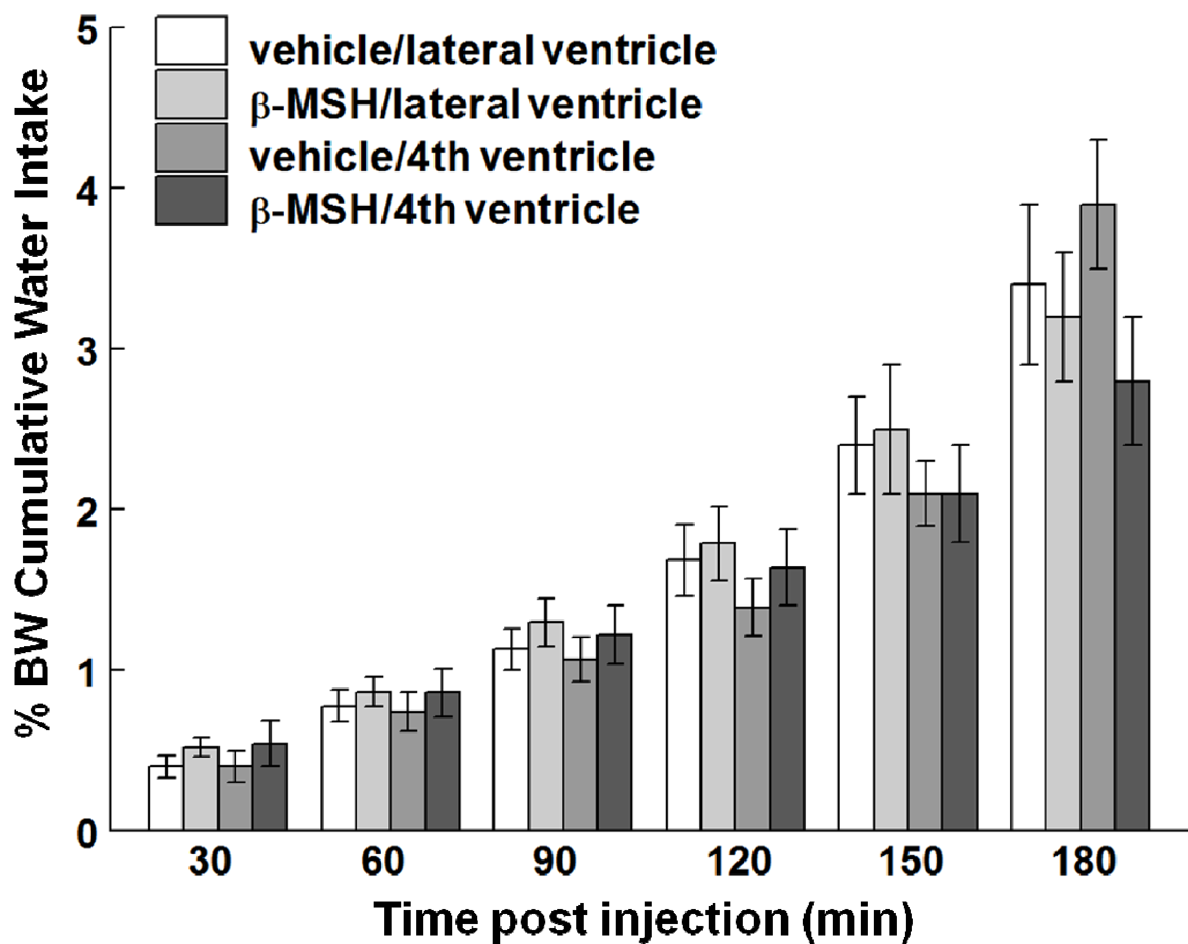


Figure 5.9. Effects of β-MSH on cumulative water intake following lateral or 4th ventricle injections. Water intake was not affected by β-MSH following either injection. Values expressed as means ± SEM.

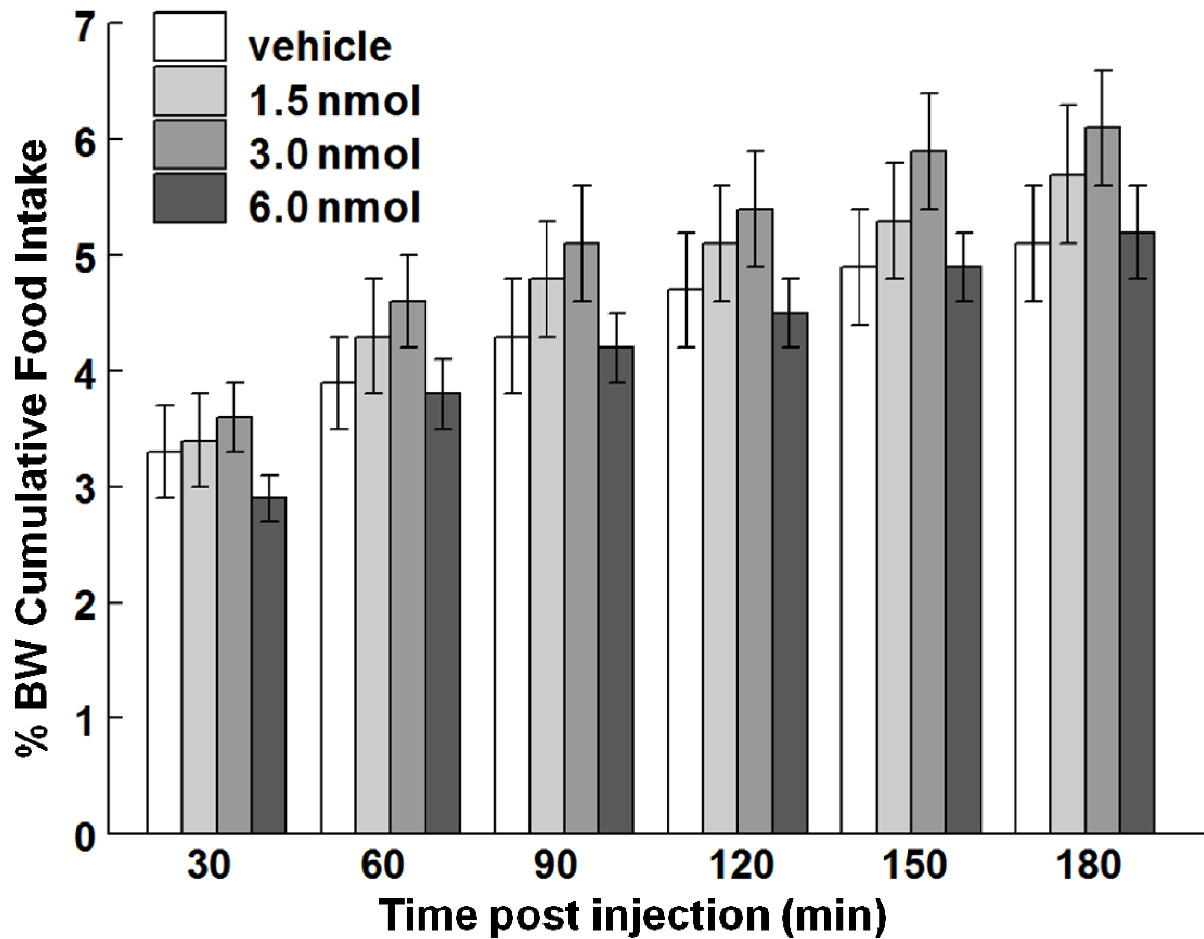


Figure 5.10. Effects of γ-MSH on cumulative food intake in fasted LWS and HWS chicks. Food intake was not affected by γ-MSH in either line at a wide range of doses or in fasted or fed conditions (data for other doses and fed condition not shown). Values expressed as means ± SEM.

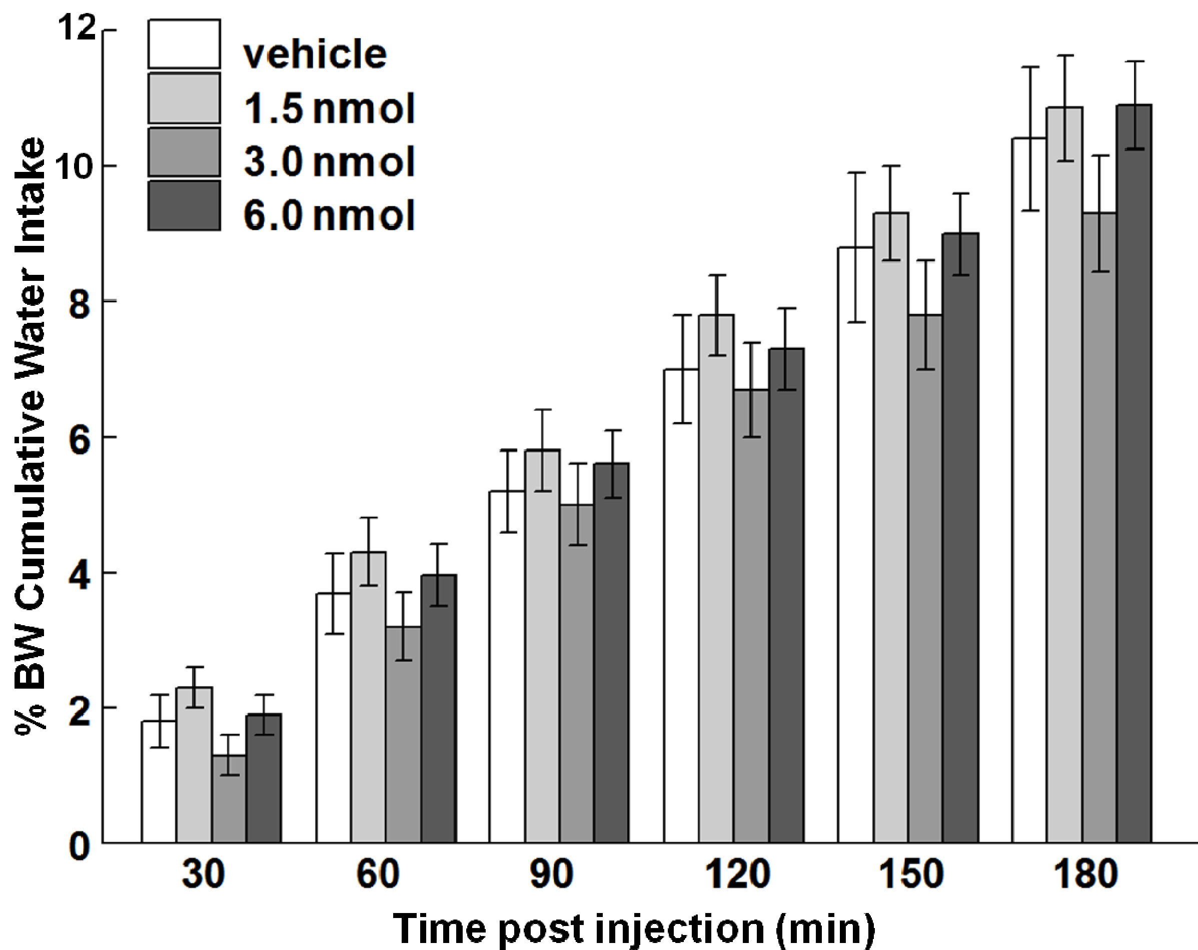


Figure 5.11. Effect of γ-MSH on cumulative water intake in LWS and HWS chicks. Water intake was not affected by γ-MSH in either line at a wide range of doses or in fasted or fed conditions (data for other doses and fed condition not shown). Values expressed as means ± SEM.

CHAPTER VI

SYNTHESIS

The present series of experiments was designed to elucidate differences in melanocortinergic appetite regulation in lines of chicks selected for high and low body weight. These lines result from long-term divergent selection for juvenile body weight and serve as unique polygenic models of anorexia and obesity.

Several appetite-related peptides have differential effects in these LWS and HWS lines, including other melanocortin or melanocortin-related peptides. One such peptide, insulin, was anorexigenic in both LWS and HWS chicks though LWS chicks are more sensitive to its effects on both food intake and whole blood glucose concentration [67]. Insulin accesses the hypothalamus via the arcuate nucleus, where the blood brain border is absent, and binds to receptors on both AgRP/NPY and POMC/CART neurons thus influencing the melanocortin system. That LWS chicks were more sensitive than HWS ones to the effects of centrally administered insulin suggests that the endogenous insulin signaling system may have been altered through the selection process.

The differential insulin signaling in the lines may be partly responsible for the differential melanocortin signaling. In the present study, when β-MSH was centrally administered, food intake was decreased in both lines, and the LWS may be more sensitive to the hypophagic effects.

Chicks of the LWS line have inherently higher corticosterone concentration than those of the HWS line. Based on present and recently published data, one hypothesis for this difference may involve cytokines, specifically IL-6. IL-6 is secreted from macrophages and adipocytes. They can cross the blood brain barrier, stimulate lipolysis in muscle and adipose tissue, and bind IL-6 receptors located in most tissues, including the zona reticularis and zona fasciculata of the adrenal gland [68]. Chicken and mammalian IL-6 receptors share 40% homology and a similar distribution [69]. Stimulation of these adrenal cells by IL-6 results in a decreased secretion of corticosterone. Corticosterone inversely feeds back on IL-6 secretion [68]. In HWS chicks with increased adiposity, there likely is also an increase in IL-6 which may account for their innately low corticosterone concentration. In addition, IL-6 is known to be a pro-inflammatory cytokine that stimulates adipocyte inflammation and is implicated in development of pre-diabetes and type-II diabetes [70]. The adipocyte inflammation caused by IL-6 may partly explain the altered responses to insulin and leptin in the HWS line.

The binding of MC2R and MC5R stimulates IL-6 production in differentiating adipocytes [65]. Administering β-MSH, which primarily binds MC3R and MC4R, may negatively feedback on POMC neurons and suppress the release of other POMC-derived ligands, thus effectively decreasing the stimulation of the other MCR's. The lack of stimulation of these receptors would result in a decrease in IL-6 and thus an increase in corticosterone. This may explain why the LWS respond to central β-MSH with increased corticosterone concentration through a

mechanism other than the CRH pathway since astressin, a CRHR antagonist, did not attenuate the effects of β-MSH.

Although β-MSH binds to both MC3R and MC4R, but the specific roles of each receptor in the hypophagic response are less understood. Thus, experiments were designed to investigate these receptors in the differential responses in LWS and HWS chicks. Unlike broiler-type chicks, neither line altered their food intake in response to central γ-MSH in fasting or non-fasting conditions at a wide range of doses. γ-MSH is selective for MC3R and the presence of this receptor is controversial in chicks. However, γ-MSH did elicit a hypophagic response in broiler-type chicks suggests that chicks do have MC3R or that γ-MSH has evolved to bind a different receptor in chicks. To investigate the role of MC4R, HS014, a highly selective MC4R antagonist, was coadministered with β-MSH. HS014 attenuated the hypophagic response in LWS, but not HWS chicks. This suggests that the differential response to central β-MSH in the lines may be due to differential receptor binding or affinity or differential mechanisms downstream of MC4R.

It is well established in mammals that an independent population of POMC neurons reside in the hindbrain, particularly the NTS. This POMC population is able to regulate food intake and energy expenditure independently from the hypothalamus, as evidenced by studies with decerebrate models lacking communicative pathways between the hindbrain and hypothalamus. To investigate the role of the hindbrain in the differential response to β-MSH in LWS and HWS chicks, an experiment was designed to compare the responses to β-MSH following injection into

the lateral or 4th ventricle. Chicks injected in either location responded similarly suggesting that both the hypothalamus and hindbrain can mediate the effects of β-MSH and the hypothalamus is not essential. This is because injections in the lateral ventricle will reach both the hypothalamus and hindbrain whereas injections in the 4th ventricle should not reach the hypothalamus due to cerebrospinal flow patterns.

Overall, the series of experiments included in this dissertation investigated the mechanisms underlying differential food intake responses in lines of chicks selected for low and high body weight. Though both LWS and HWS chicks had reduced food intake, the HWS chicks may be more sensitive to the effects of central β-MSH. Neither line had altered whole blood glucose concentration or behaviors in response to central β-MSH. Although LWS chicks had increased corticosterone concentrations, astressin did not attenuate the effects of β-MSH in either line thus the response may not be mediated via CRH receptors, and may be working through a mechanism such as IL-6. Together, these data provide background that could be used to develop strategies for pharmacological treatments for body weight disorders, particularly because HWS may be more sensitive to the effects and neither LWS nor HWS respond with adverse behaviors.

CHAPTER VII

LITERATURE CITED

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