CORTICOTROPHIN RELEASING HORMONE MODULATION OF FEED INTAKE, GASTRIC MOTILITY, AND BEHAVIOR IN LOW AND HIGH BODY WEIGHT SELECTED LINES OF CHICKENS

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- ABSTRACT -

The effect of intracerebroventricular (ICV) injection of corticotrophin releasing hormone (CRH) and related compounds on appetite, behavior, and gastric motility in lines of chickens, one selected for low body weight (LWS) and the other high body weight (HWS), was determined. Nucleotide sequence and expression patterns of the CRHr2 receptor, involved in appetite regulation, were also determined. Some individuals of the LWS line are anorexics and many die simply from not eating while some individuals in the HWS line are compulsive eaters and exhibit obesity. CRH is a 41 residue peptide that initiates an organism's stress response and is a potent inhibitor of appetite. An ICV injection of CRH dose-dependently decreased feed intake in both lines but did not effect water intake. When CRH receptor antagonists were ICV injected an increase in feed intake in the LWS line but not in the HWS line was observed, however the appetite reducing effect of CRH was attenuated in the HWS line but not in the LWS line. The LWS line has higher concentration of corticosterone than does the HWS line. In both lines at all times treatment with CRH caused an increase in locomotion and no CRH-treated chicks from either line slept post injection. Chicks from the LWS line that were treated with CRH exhibited other anxiety related behaviors sooner than the HWS line. The LWS line showed a liner increase in crop emptying time as the dose of ICV

CRH increased. The HWS line responded with a quadratic dose response to CRH treatment. Polymorphisms in the CRHr2 receptor were found in both lines in the same positions, thus we concluded these differences do not significantly contribute to body weight differences. However, differences detected in expression patterns between lines for the CRHr2 receptor may contribute to their different body weights. We conclude that differences in the CRH system, its concentrations and differential receptor action, of these two lines may be partly responsible for their altered body weight phenotype.

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SECTION ONE
INTRODUCTORY REMARKS

Corticotropin releasing hormone (CRH) is a 41-amino acid hypothalamic neuropeptide that primarily causes release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. In addition to ACTH release, several physiological and behavioral responses are mediated by the secretion of CRH including stress responses and appetite-related and non-related behaviors. Such responses are initiated when CRH ligates with one or more of its various receptor subtypes and its associated binding protein. Several neurohormone mediators have been shown to modulate the many effects of CRH. The discussion that follows provides a basic explanation of the physiological, behavioral, and molecular aspects of CRH and its receptors, in addition to discussing the neuropeptide's effects on energy intake and body weight regulation. Also presented are the results of several experiments conducted to determine the contribution of CRH on body weight in two lines of chickens, one containing anorexic individuals and the other containing obese individuals. These results can be used to understand neurochemical pathways effecting energy balance in chickens, and may also be used to futher our understanding of body weight dysfunctions in other species, including humans.

SECTION TWO
OVERVIEW OF FEEDING BEHAVIOR

The central nervous system controls appetite associated behaviors. More specifically, Bray (1991) described the sympathetic nervous system as the principal governor of ingestion and body weight regulation. Bray (1991) concluded that most known obesities are the result of low sympathetic nervous system influence. Flier and Maratos-Flier (1998) reviewed several appetite-associated experiments and concluded it can be stimulated or inhibited through the actions of various neurotransmitters acting in the central nervous system. The first reported hypothesis that the nervous system controls ingestive behavior was based on experimentally-induced lesions in the rat brain. A "feeding center" was identified when the ventromedial hypothalamus was disrupted and these lesions caused hyperphagia resulting in obesity (Brooks, 1946; Cox and Powley, 1981). The counterpart, the satiety center, was later identified when the lateral hypothalamus of rats was destroyed. This action resulted in hypophagia and associated weight loss (Yoshida et al., 1983; Arase et al. 1987). Based on these results, early experiments designed to better understand ingestive responses were based upon intracerebroventricular (ICV) injections of neurotransmitters that had been isolated in or near the hypothalamus. The effects of these neurotransmitters were quantified by measuring ingestive response, autonomic nervous system tone, and blood-borne hormonal concentrations. These early experiments demonstrated that norepinephrine, dopamine, serotonin, neuropeptide Y, CRH, and galanin were capable of inducing appetite-associated feeding behaviors and other physiological processes.

Depriving animals of feed causes several responses including, but not limited to, increased appetite (Woods et al., 1998), reduced metabolic rate (Williams et al., 2000), reduced sympathetic nervous system activity (Sakaguchi et al., 1998), decreased heart rate and reduced blood pressure (Williams et al., 2000), and altered pituitary secretions (Dallman et al., 1993). Brady et al. (1990) and Makino et al. (1998) showed that after caloric deprivation the synthesis of various neuropeptides in the paraventricular nucleus (PVN) were altered.

One such neuropeptide, CRH, suppresses appetite when administered ICV or directly into the hypothalamic PVN (Morley and Levine, 1982; Krahn et al., 1988; Arase et al., 1989; Rothwell, 1990; Glowa et al., 1992). Britton et al. (1986) and Parrott et al. (1990) showed that ICV injections of CRH generally decreased feed consumption in rats and pigs respectively. Intravenous infusion of CRH in the rhesus monkey induced feeding activity, however, it was speculated this effect was cortisol-simulated (Kalin et al. 1983). A suppression of feed intake was observed in other unrelated species when CRH was centrally administered in rabbits (Opp et al., 1989), sheep (Ruckebusch and Malbert, 1986), and goldfish (DePedro et al, 1993). Several other neuropeptides including cholecystokinin, gastrin, and glucagon-like peptide-1 cause a reduction in ingestive behaviors when ICV injected (Denbow et al., 1982, 1999; Lecklin et al., 1998; Machidori et al., 1992; Furuse et al., 1997, 1999).

Zhang et al. (1994) genetically engineered mice to have a mutation in the gene coding for leptin (ob/ob) and found the mutation caused hyperphagia that resulted in obesity. Ahima and Flier (2000) reported that the ob/ob mutated mice are in a continuous physiological state that is comparable to mice that are being subject to long term

starvation. Several authors report that when ob/ob mutated mice are treated with leptin they resume normal feeding behavior and their obesity is reversed (Halaas et al., 1995; Pelleymounter et al., 1995; Weigle et al., 1995).

Few neuropeptide stimulators of appetite are known. Leibowitz and Brown (1980) showed that pharamomological administration of norepinephrine, when injected into the ventromedial hypothalamus and PVN, increased ingestion and body weight gain. Morris et al. (1997, 1998) demonstrated that when neuropeptide Y (NPY) is injected into the PVN it also increased feed intake. Physiological norepinephrine is released in response to the presence of NPY and results in increased appetite and body weight (Matos et al. 1996; Nishimura et al. 1996). One proposed mechanism related to the neurochemical control of appetite is depicted in Figure 2.1.

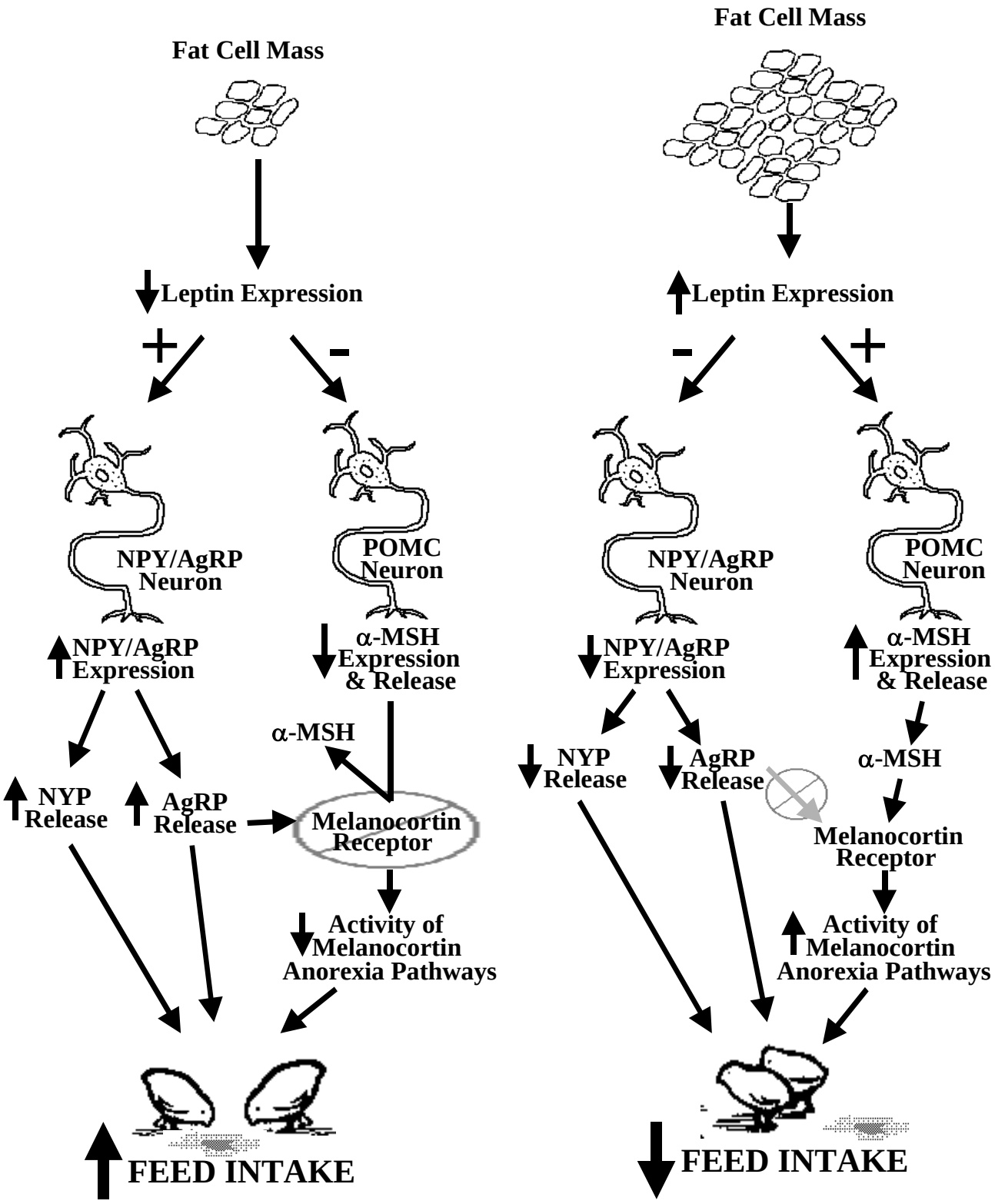


Figure 2.1. Central nervous system control of feed intake (explanation on next page)

Figure 2.1 (previous page). Central nervous system control of feed intake. Depicted on the left is a proposed neurophysiological mechanism leading to obesity. Fat cells secrete leptin in proportion to their mass resulting in stimulatory and inhibitory signals in the hypothalamus. Decreased leptin and insulin concentrations cause activation of NPY and agouti-related peptide (AgRP) releasing neurons in the hypothalamus. Release of NPY and AgRP both stimulate feed intake. The release of AgRP also blocks the binding of α-MSH to melanocortin receptors. The increase in feed intake may result in obesity. Simultaneous to NPY/AgRP neurons stimulation, proopiomelanocortin (POMC) neurons are inhibited by increasing concentrations of leptin and insulin. This inhibition results in decreased expression of α-melanocyte stimulating hormone (α-MSH). α-MSH is involved in the melanocortin induced anorexia pathway. Decreased activity of the melanocortin induced anorexia pathway, in concert with increased feed intake, may lead to obesity. Depicted on the right is a proposed neurophysiological mechanism leading to anorexia. While the same signals are involved, as on the right, their concentrations differ. An increase in fat cell mass correlates with increased leptin and insulin expression and circulating concentration, this results in inhibition of NYP/AgRP neurons while stimulating POMC containing neurons. NPY and AgRP expression are down regulated which reduces the stimulation of feed intake. Expression of one product of the POMC neuron, α-MSH, is upregulated causing activation of the melanocortin-induced anorexia pathway. Inhibition of α-MSH is lessened since AgRP concentrations are relatively lower. The combination of these affects leads to anorexia and a decrease in body weight. (Adapted from Schwartz et al. 2000).

SECTION THREE

HYPOTHALAMO-ADRENAL AXIS

The hypothalamic-adrenal axis plays a important role in various physiological and behavioral phenomena in many veterbrates. Antoni (1986) described PVN CRH containing neurons as the primary initiators of an organism's response to stress. Receptors exist in the anterior pituitary for CRH, and activation of these receptors initiates a cascade of events resulting in release of ACTH. A short negative feedback loop is established when ACTH reduces additional hypothalamic release of CRH (see Figure 3.1). The circulatory system delivers ACTH to the adrenal cortex where it functions as a cortisol secretogogue. A secondary negative feedback loop (the long loop) reduces release of both ACTH and CRH through elevated blood cortisol concentrations. Evidence for this negative feedback mechanism was supplied by Young et al. (1986), Plotsky and Sawchenko (1987), and Swanson and Simmons (1989) who showed declining corticosterone levels at the PVN and median eminence caused CRH mRNA levels to be increased.

The hypothalamic-adrenal axis is involved in many additional behavioral and physiological responses. In the avian, territorial behavior (Romero et al., 1998), immune responses (Hu et al., 1993; Trout and Mashaly, 1994), reproduction (Maney and Wingfield, 1998; Chaturvedi and Suresh, 1990 Astheimer et al., 1992), and feed intake (Furuse et al., 1997; Denbow et al., 1999) all may be effected by the dynamics of the hypothalamic-adrenal axis.

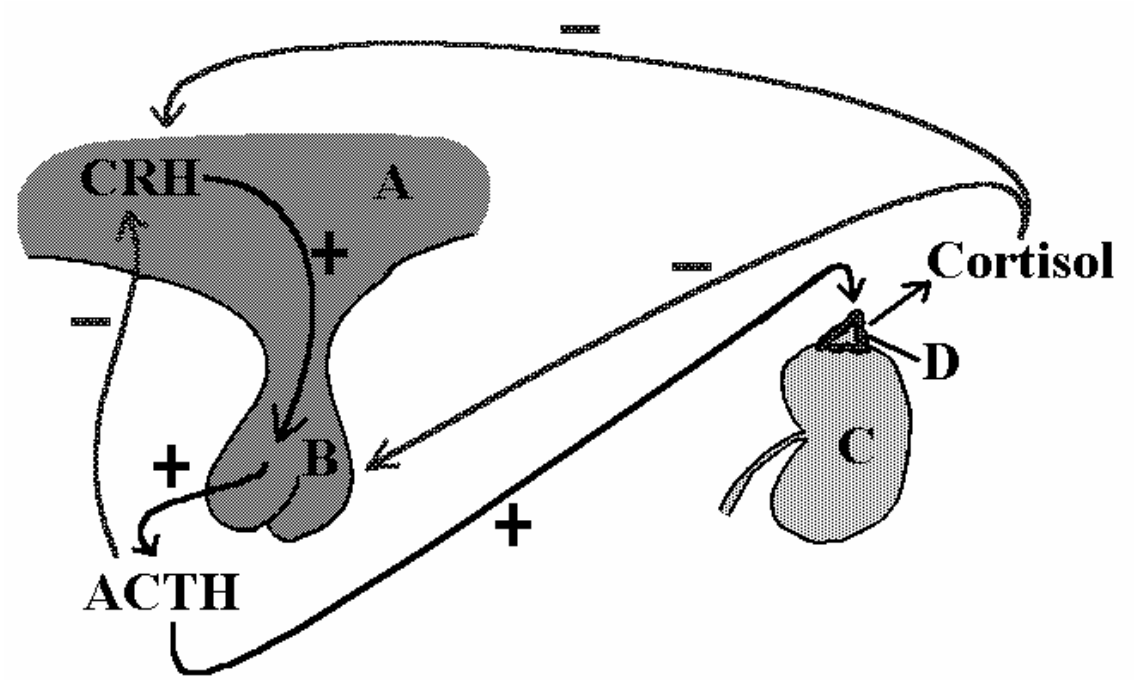


Figure 3.1. Hypothalamic-pituitary-adrenal axis. In the hypothalamus (A), CRH is synthesized and released and transported to the pituitary via the hypophyseal portal system. At the pituitary (B), CRH causes release of ACTH. ACTH travels through the circulatory system to the adrenal glands (D), located on the superior aspect of the kidney (C) in a mammal causing the release of cortisol. In the avian system, the principle glucocorticoid is corticosterone. A short negative feedback loop is established when ACTH reduces CRH synthesis and release from the hypothalamus. A secondary long feedback loop occurs when cortisol reduces ACTH and CRH release. (Adapted from Greenspan and Strewler, 1997).

Central regulation of adrenal activity differs between mammals and avians

Most studies involving CRH have been conducted using mammalian models, particularly rodents. However, mammalian and avian neurophysiology are not always parallel. CRH like immuno-reactivity has been demonstrated in several regions of the avian brain (Ball et al., 1989; Kov'acs and Westphal, 1989). In 1964, De Roos and De Roos reported that the chicken pituitary contains lower ACTH concentrations than the pituitary of mammals. Several laboratories reported that hypophysectomy in birds induced adrenal atrophy causing a reduction in corticoid production, yet the avian adrenal gland retained the capacity to produce considerable amounts of corticosterone (Miller, 1961; Nagra et al., 1963; Resko et al., 1964). Hypophysectomy in mammals resulted in termination of adrenal corticosterone production (Meij et al., 1997). Thus, avian adrenal function is more self sufficient than the mammalian system (Dulin, 1955; Newcomer, 1959). These differences between avians and mammals should be considered when reviewing literature on body weight and feeding behaviors related to the hypothalamic-pituitary-adrenal axis, and when making inferences regarding one species based on another.

SECTION FOUR
CORTICOTROPIN RELEASING HORMONE

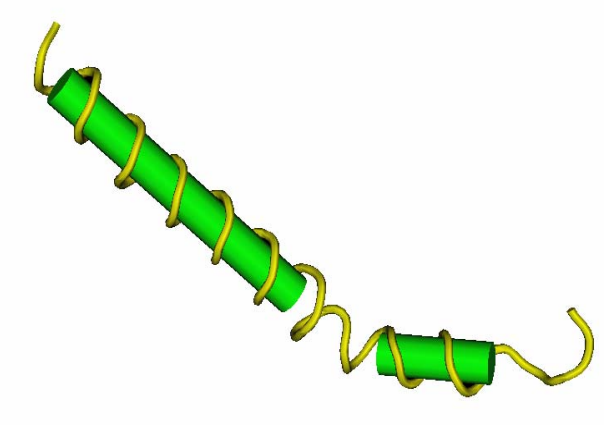


Figure 4.1. CRH Structure. (NCBI, 2002)

Corticotropin releasing hormone is a 41 residue peptide that classically governs the secretion of ACTH and other POMC derivatives from the anterior pituitary (Vale et al., 1981), in addition to various other endocrine systems (De Souza, 1987; Moreau et al., 1997). The functional neuroanatomy of CRH-containing neurons has been reviewed by Sawchenko et al. (1993). Makara et al. (1981) were the first to report the PVN as the source of CRH (see figure 4.2). Kawano et al. (1988) reported the PVN was the principle source of portal plasma CRH, and that CRH neuronal axon terminals in the median eminence have soma in the PVN. When Antoni et al. (1983) and Plotsky and Vale (1984) lesioned the PVN or its projections to the median eminence, a reduction in hypophysial portal CRH concentration was observed, in addition to a reduction in CRH immenoreactive terminals in the median eminence.

CRH has been found in other regions of the brain, however, it is most concentrated in the medial parvocellular division of the hypothalamic PVN (Sawchenko and Swanson, 1985) (see Figure 2 this section). Neurons in the paraventricular nucleus that contain CRH are primarily involved in the regulation of the pituitary-adrenal axis (Antoni, 1986). CRH is unequally distributed in the brain of mammals (Olschowka et al.,

1982; Swanson et al., 1983; Palkovits et al., 1989) and avians (Jozsa et al., 1984; Ball et al., 1989; Viglietti-Panzica and Panzia, 1991). Frankel et al. (1967) showed that the chicken corticotropic area lies in the medio-basal hypothalamus. Peczely (1969), through *in vitro* studies, found that the site of CRH production is in the supraoptic area in the pigeon. Jozsa et al. (1984) demonstrated CRH containing perikarya in the paraventricular, preoptic, and mammillary nuclei of the hypothalamus, and in some extrahypothalamic areas including the nuclei dorsomedialis, dorsolateralis thalami, nucleus accumbens septi, lobus parolfactorius, periaqueductal gray of the mesencephalon, and nucleus oculomotorius ventralis in the domestic fowl. CRH nerve fibers and terminals existed in the external zone of the median eminence and the organum vasculosum of the lamina terminalis (Jozsa et al. 1984).

Silverman et al. (1989), through an immunocytochemical study, demonstrated that CRH-containing neurons synapse with non-CRH related neurons within the PVN. A majority of the terminals formed axo-dendritic synapses, of which greater than 85% were symmetrical. Some axo-somatic terminals were also observed and parvicellular and magnocellular neurons were innervated with mostly symmetrical synapses. Finally, Silverman et al. (1989) found CRH-synthesizing neurons synapse on other CRH containing neurons and dendrites in the aforementioned areas.

Sawchenko et al. (1993) reported CRH neurons have the capacity to express a variety of neuropeptides including angiotensin II, cholecystokinin, enkephalin, neurotensin, prolactin-inhibiting hormone, vasopressin, and vasoactive intestinal polypeptide. It is not surprising, given the diversity of CRH-related neuron products, that one third of the cells that constitute the PVN are capable of synthesizing CRH (Swanson

and Kuypers, 1980). Neurons, which primarily secrete other neuropeptides, can also be induced to synthesize CRH. For example, Young (1986), Dohanics et al. (1990) and Imaki et al. (1992) demonstrated a systemic salt load challenge can also increase CRH mRNA and peptide concentrations in oxytocinergic neurons.

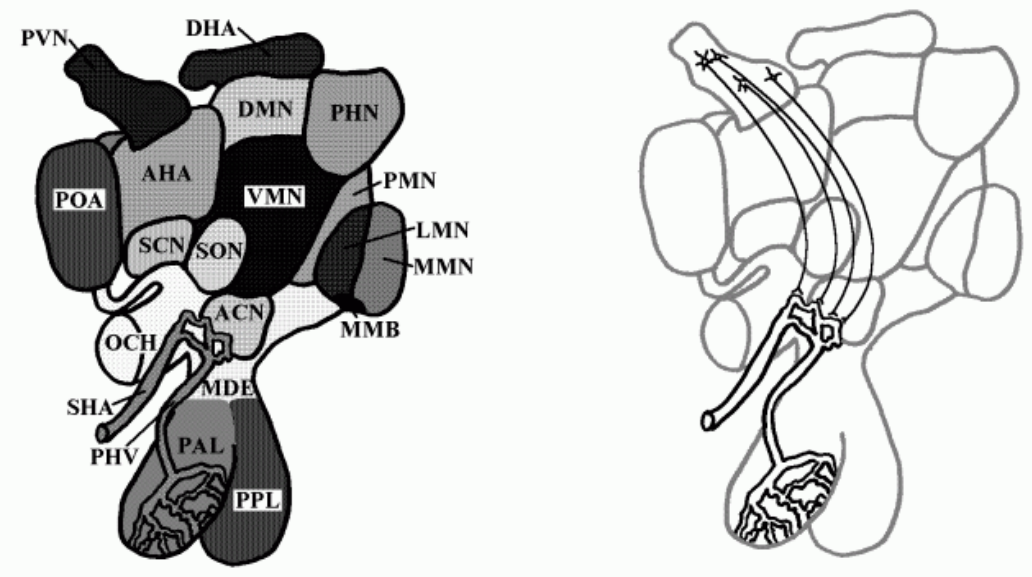


Figure 4.2. Human hypothalamic CRH-secreting neurons. Left: AHA, anterior hypothalamic area; CAN, arcuate nucleus; DHA, dorsal hypothalamic area; DMN, dorsomedial nucleus; LMN, lateral mamillary nucleus; MDE, median eminence; MMB, mammillary body; MMN, medial mammillary nucleus; OCH, optic chiasm; PAL, pituitary anterior lobe; PHN, posterior hypothalamic nucleus; PHV, portal hypophysial vessel; PMN, premammillary nucleus; POA, preoptic area; PPL, pituitary posterior lobe; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; SHA, superior hypophysial artery; SON, supraoptic nucleus; VMN, ventromedial nucleus. Right: Located in the PVN are CRH neuron soma which have axons that terminate on the portal hypophysial vessel system at the MDE. (Adapted from Greenspan and Strewler, 1997)

A single gene encodes CRH on chromosome 8q13 in the human (Arbiser et al., 1988). The gene contains two exons spaced 686-800 bp by an intron. Exon two codes completely for the prepro-CRH precursor peptide (187-196 residues long, Majzoub et al. 1993). Transcription of the CRH gene may begin at one of 11 sites upstream of the coding sequence, and one of these site predominates (Robinson et al., 1989; Adler et al., 1990). Located 30 base pairs upstream of the predominant start site resides the TATA box, and 63 base pairs upstream from that is a CAAT sequence. CRH gene expression up-regulation occurs after a single stimulatory event with a lag time of 1 to 4 hours (Harbuz et al., 1990; Kollack et al., 1990). Herman et al. (1992) found that CRH neurons can respond with a rapid increase in cellular activation and synthesis of the CRH peptide.

The synthesis and release of CRH is not limited to the central nervous system. Several reports indicate that CRH originates from several non-neuronal peripheral sources including the placenta, testis, ovary, gut, immune system, adrenals, and heart (Sasaki et al., 1988; Yoon et al., 1988; Stephanou et al., 1990; Crofford et al., 1992; Kawahito et al., 1994; Muglia et al., 1994). CRH gene transcription is activated by several signals including catecholamines, serotonin, acetylcholine, interleukin 1 and 6, and may be inhibited by glucocorticoids and γ-aminobutyric acid (GABA), through protein kinase-A, -C, and glucocorticoid second messenger systems (Majzoub et al., 1993). Additionally, Richard et al. (1996) showed that the expression of CRH receptor transcript was reduced in the VNM of obese rats.

Lowry (1993) reported the recovery and description of a protein from the plasma from pregnant humans which had the capability to bind and reversibly neutralize CRH. This protein, CRH binding protein, is expressed in the central system (Potter et al., 1991).

Sawchenko et al. (1993) speculated that the CRH binding protein might be released in locations where it modifies local synaptic, autocrine, or paracrine actions of CRH. For example, CRH binding protein is expressed in the anterior pituitary corticotrophs and may regulate the activity of CRH at that level.

Mediation of CRH secretion

Many substances effect CRH release from the hypothalamus (see Table 1 this section). Due to the abundance of available information, the description which follows only superficially describes the major mediators of CRH synthesis and release. Acetylcholine increased CRH synthesis and release (Calogero et al., 1988; Tsagarakis et al., 1988), and this action appears mediated via muscarinic receptors (Grossman et al., 1993). Catecholaminergic and CRH neurons are in proximity within the PVN. It is not disputed that catecholamines effect CRH release; however, the nature of the effects are controversial (Al-Damluji, 1988). Most conclude that catecholamines facilitate CRH release.

Serotonin is also known to induce CRH release (Tsagarakis et al., 1989a; Calogero et al., 1989). Hillhouse et al. (1975) reported that cholinergic antagonists reduced serotonin concentration that also induced the CRH release. However, Buckingham and Hodges (1977) and Calogero et al. (1989) were unable to replicate this effect. Grossman et al. (1993) reviewed the actions of GABA on CRH and conclude that it inhibited CRH, whereas GABA antagonists increase the stress response mediated by CRH. GABA is co-released with CRH and may serve as a CRH saturation avoidance mechanism.

Leptin increases CRH expression (Schwartz et al., 1996) and concentration (Uehara et al., 1998) in the rat hypothalamus. Antagonists of CRH attenuated the inhibitory effect on feed intake caused by leptin (Uehara et al., 1998; Gardner et al., 1998). Brady et al. (1990) showed that decreasing leptin concentration during fasting was directly correlated to declining CRH concentration. Kochavi et al. (2001) reported

that rats with a null mutation of the leptin receptor did not experience a difference of CRH potency compared to wild type rats that lack the null mutation.

White (1993) has shown that NPY regulates the hypothalamic-pituitary-adrenal axis. Soma of CRH secreting neurons are in proximity to NPY fibers in the PVN (Wahlestedt et al., 1987; Li et al., 2000). Milner et al. (1993) reported that a CRH pathway extends from the PVN to the C1 rostral ventrolateral medulla, which has a high concentration of NPY containing soma. Morris et al. (1998) also showed that CRH stimulated NPY secretion in the PVN in a dose-dependent manner. NPY increased CRH mRNA expression in the rat hypothalamus (Suda et al., 1993). Several authors report that NPY administration caused the secretion of CRH in the hypothalamus (Haas et al., 1989; Tsagarakis et al., 1989b; Hastings et al., 2001). Liu et al. (1994) showed NPY increased hypophyseal-portal plasma CRH concentration, and Wood et al. (1998) reported that NPY-induced CRH release suppressed feeding behavior.

Table 4.1. Neuropeptides which directly modulate CRH activity.

Stimulatory	**Inhibitory**
Angiotensin II	α-melanocyte-stimulating hormone
Interleukin 1	Atrial natriuretic peptide
Interleukin 6	Corticotropin-like intermediate lobe peptide
Endothelin (?)	Corticotropin releasing hormone
Leptin	Dynorphin/β-endorphin
Neuropeptide Y	Somatostatin 1-28
	Substance P

Adapted from Grossman et al., 1993; Hastings et al., 2001.

SECTION FIVE
CRH RECEPTORS

Presently three types of CRH receptors have been demonstrated. Complimentary DNA that binds one type of CRH receptor has been cloned from the human (Chang et al., 1993), rat (Perrin et al., 1993), and mouse (Vita et al., 1993; Xiong et al., 1995), and these receptors are designated type A, or CRHr1 with 98% sequence identity and found throughout the central nervous system. A secondary CRH receptor subtype has been cloned (Lovenberg et al., 1995; Stenzel et al., 1995; Perrin et al., 1995) from the heart, but not pituitary, of rats and mice which binds urotensin 1 and sauvarine, two potent CRH receptor agonists, with greater affinity than CRH. This receptor is called type B, or CRHr2. Chen et al. (1993) reported that the human CRHr1 was identical to CRHr2 except that it contained a 29 amino acid insert that may be due to alternative splicing of a single gene.

The two subtypes are located in different tissues and have different binding properties (Vale et al., 1997). Three splice variants exist for CRHr2, CRHr2α, CRHr2β, and CRHr2γ, (Lovenberg, et al., 1995; Gottowik et al., 1997). The CRHr2α receptor is located solely in the central nervous system, while the CRHr2β exists in the atria and ventricles of the heart (Perrin et al., 1995).

Jinxing et al. (1996) cloned a CRH receptor from the chicken which had higher affinity for urotensin and sauvagine than for CRH, a high degree of sequence conservation with the mammalian type CRHr1 but functions more closely to the mammalian type CRHr2.

Arai et al. (2001) reported a third CHR receptor, CRHr3, that was identified in the catfish. The CRHr3 receptor was found expressed primarily in the pituitary and

urophysis, a hormone storage and secretion organ of the caudal neuroscecretory system of bony fishes.

Smith et al. (1998) and Timpl et al. (1998) reported that stimulation of CRHr1 induces the ACTH response to stressors. Pozzoli et al. (1996) found that CRH and glucocorticoids attenuate expression of CRHr1 in anterior pituitary cell cultures. CRHr2 is involved in coping mechanisms for stress such as anxiolysis, anorexia, hypertension, and cardioprotection (Kishimoto et al., 1995; Bale et al., 2000; Coste et al., 2000; Hashimoto et al., 2001). Eghbal-Ahmadi et al. (1997) demonstrated that when a gravid rat is deprived of feed, the infant rat has altered CHRr2 mRNA in the VMH. Sensory input and feed intake also effect CRHr2 mRNA in the hypothalamus and amygdala of immature rats (Eghbal-Ahmadi et al., 1999). Nazarloo et al. (2002) demonstrated that nutritional stress, as a result of starvation, caused a decrease in CHRr2β mRNA in the rat cardiovascular system.

CRH receptor agonist

Lederis (1983) and Britton et al. (1984) reported that sauvagine and urotensin 1 have similar biological activities as does CRH-induced ACTH release, behavioral effects, and vasodilatation. Urotensin 1 is obtained from the urophysis, a hormone storage organ of bony fishes, and shares structural homology with CRH (Vale et al., 1981; Lederis et al., 1982). Lederis et al. (1983) reported urotensin 1 is a more efficacious secretogogue of ACTH than CRH, which may indicate a change in the hemodynamic action of urotensin 1 in the mammalian GI tract during evolution from fishes to mammals.

Makino et al. (1997) and Yamamoto et al. (1998) have shown urocortin decreases feed intake by activating CRH receptors while not inducing either strong anxiety-like behaviors or physiological responses. Urocortin is related to urotensin with 63% sequence identity, and to CRH with 45% sequence identity (Vaughan et al., 1995). Urocortin has a higher affinity for the CRHr2 than the CRHr1 receptor (Vaughan et al., 1995; Yamamoto et al., 1998). Benoit et al. (2000) speculated that urocortin might be the endogenous ligand for the CRHr2 receptor.

Molecular and physiological properties of CRH receptors

The binding characteristics of CRH to its receptors have been described by several authors (DeSouza and Kuhar, 1986; Aguilera et al., 1987; De Souza and Nemeroff, 1990; Grigoriadis et al., 1993). Radiolabeled CRH analogs binding to CRH receptors in the rat brain and pituitary were dependent on time, temperature, and tissue concentration. The binding was reversible, saturable, and displayed high affinity. Chrousos et al. (1985) reported the plasma half life of CRH could be described by two components in the primate. The half-life of the first component was 17.1 min, and the second was 198.0 min. Down regulation of the receptor also is a control of CRH bioactivity. Childs et al. (1986) and Webster et al. (1991) incubated anterior pituitary cells with CRH and found a reduction of CRH receptors within several hours to 1 day. It was hypothesized this effect was necessary for rapid counter-regulation, via homologous desensitization, to prevent over stimulation of CRH receptors at CRH receptor sites within the CNS (Spiess et al. 1989).

CRH receptors exist in the brain (DeSouza et al., 1984), pituitary (Wynn et al., 1983), adrenals (Udelsman et al., 1986), spleen (Webster and De Souza, 1988), sympathetic nervous system (Udelsman et al., 1986), peripheral tissues (Dave et al., 1985), and aorta (Dashwood et al., 1987). Several functional studies have been conducted to determine the effects of CRH receptor activation (Dave et al., 1985; Webster et al., 1989; Ulisse et al., 1990; Audhya et al., 1991). The family of CRH receptors have significant homology to other G-protein receptors (Xiong et al., 1995) including calcitonin (Lin et al., 1991), secretin (Ishihara et al., 1991), vasoactive intestinal polypeptide (Ishihara et al., 1992, Lutz et al., 1993), parathyroid hormone (Juppner et al., 1991; Abou-Samra et al., 1992), growth hormone releasing hormone (Mayo, 1992; Lin et al., 1992; Gaylinn et al., 1993), glucagon (Jelinek et al., 1993), glucagon-like peptide (Thorens, 1992), and pituitary adenylate cyclase-activating polypeptide (Ishihara et al., 1992; Pisegna and Wank, 1993; Inagaki et al., 1994). CRH induces its effect when it binds to cell surface receptors that are coupled to G_s-protein and adenylate cyclase (Labrie et al., 1982a, b, 1993; Chen et al., 1986). The receptor is coupled to a GTP-binding protein (Perrin et al., 1986) and mediates the CRH-stimulated increase in intracellular cAMP concentration (Bilezikjian and Vale, 1983).

SECTION SIX
PHARMACOLOGICAL ADMINISTRATION OF CRH AND NON-INGESTIVE BEHAVIORS

CRH is a modulator of various biological responses that are unrelated to the pituitary-adrenal axis. Rothwell (1990) and Richard (1993) documented the thermogenic and anorectic actions of CRH. Receptor antagonists for CRH block conditions that induce CRH-associated anorexia including the thermogenic actions of fenfluramine (Le Feuvre et al., 1991), in addition to the anorectic effects of resistant stress (Shibasaki et al., 1988), treadmill running (Rivest and Richard, 1990), estradiol (Dagnault et al., 1993), and caffeine effects (Racotta et al., 1994) in rodents. Brown et al. (1982) demonstrated that central administration of CRH increased the concentration of plasma ACTH, epinephrine, and norepinephrine. Rivest and Rivier (1995) showed that CRH has deleterious effects on reproductive performance.

CRH caused several responses when administered into the cebero-ventricular system that included increased sympathetic outflow (Brown et al., 1982; Fisher et al, 1982). Increased sympathetic tone corresponds to elevated mean arterial pressure and heart rate, in addition to increasing glucose and glucagon levels (Jurgen-Lenz et al., 1987). When CRH was injected into the third cerebroventricle plasma glucocorticoid levels were increased and stress and anxiety-related behaviors were more apparent in rats (Heinrichs, et al., 1997). Taste (Gosnell et al., 1983; Heinrichs et al., 1991) and place aversion (Cador et al., 1992) were also observed after central CRH administration. Central injection of CRH induces brown adipose tissue thermogenesis in rats (LeFeuvre et al., 1987) and inhibits digestive activity (Taché et al., 1990). Additionally, when CRH

was administered to rats (Britton et al., 1982; Benoit et al., 2000), mice (Contarino et al., 2000), chicks (Denbow et al., 1999; Furuse et al., 1997) and marsupials (Hope et al., 2000), feed intake was reduced.

SECTION SEVEN
PHYSIOLOGICAL EFFECT OF CRH ON FEED INTAKE

CRH is a participant in feed intake and energy balance (Richard et al., 1996; Rothwell, 1990). Krahn and Gosnell (1988) showed that exogenous CRH administered into the PVN reduced feed intake and subsequently reduced body weight. Krahn and Gosnell (1988) reported that injection of CRH into the lateral hypothalamus, ventromedial hypothalamus, globus pallidus, or striatum of rats did not effect feed intake.

Rothwell (1990) hypothesized that some obesities may be the consequence of decreased central CRH response. Several experiments in rodents during which obesity was prevented, reduced, or reversed by adrenalectomy (York and Godbole, 1979; Castonguay et al., 1986; Romsos et al., 1987; Tokunaga et al., 1989; Tokuyama and Himms, 1989; Ouerghi et al., 1992) support this hypothesis.

An increase in thermogenesis was observed by Richard (1993) after ICV administration of CRH in rats. Huang et al. (1998) and Seeley et al. (1996) demonstrated that feed restriction reduced expression of CRH, while involuntary overfeeding and satiation caused increased concentration of CRH mRNA in the PVN. Richard et al. (1996) showed that CRH mRNA levels were decreased in the PVN of obese Zucker fa/fa rats while Huang et al. (1998) showed that expression of CRHr2 is reduced in ob/ob mice. Additionally Brady et al. (1990) and Makino et al. (1998) demonstrated that caloric deprivation caused altered CRH synthesis in the paraventricular nucleus. Cabanac and Richard (1995) reported that CRH administration lower the body weight threshold at which feed deprived rats start to hoard feed.

A wide range of CRH concentrations stimulated duodenal bicarbonate secretion through the release of beta endorphin from the pituitary (Lenz, 1989), which decreases gastric acid secretion. Glowa et al. (1992), after reviewing several experiments where vagotomy, adrenalectomy, cervical cord transection, and ganglionic blockage were used to reverse CRH inhibitory effects, concluded the sympathetic nervous system mediated the CRH response. Glowa et al. (1992) also reported vasopressin and opiate antagonists blocked the response in the canine; but, not in rats. Gosnell et al. (1983) caused a conditioned response with saccharin and CRH administration. After treatment, when exposed to feed laced with saccharin alone, rats exhibited reduced feed intake.

SECTION EIGHT
CORTICOTROPIN RELEASING HORMONE INHIBITION OF FEED INTAKE: GASTRIC MECHANISM INVOLVEMENT

The stress response and CRH

When an animal is in a stressful situation, a reflexive and articulated series of physiological and behavioral modifications are initiated to benefit the individual. The sympathetic nervous system and the hypothalamic pituitary adrenal axis are responsible for such modifications. CRH causes the release of ACTH and other proopiomelanocortin derivatives from the adenohypophysis (Vale et al., 1981). Corticosteroid hormones, specifically glucocorticoids, are released in response to ACTH stimulation, and help the individual resist and cope with stressors. The modulation of endocrine (Vale et al., 1981), autonomic (Brown and Fisher, 1985) and behavioral (Koob et al., 1993) responses to stress are initiated by CRH release from the hypothalamus. When stressful situations occur, vegetative functions such as digestion are diminished to provide blood supply for essential stress-induced responses (Tamer et al., 1997). Using rodents and primates, it has been shown that CRH receptor activation in the CNS triggers most behavioral, neuroendocrine, autonomic, immunologic, and visceral responses to stress (Habib et al., 2000; Koob and Heinrichs, 1999; Webster et al., 1997).

Gastric modulation

In the late 1970's it was determined that peptides acting in the brain caused changes in gastrointestinal function (Burks, 1978; Bueno and Ferre, 1982; Tache et al., 1990). Several peptides have been shown to induce such effects (Tache et al., 1993,

1999, 2001). Central injection of CRH inhibited both gastric and intestinal motility in dogs (Bueno and Fioramonti, 1986; Lee and Sarna, 1997). Intravenous infusion of CRH inhibited gastric emptying in rats (Tache et al., 1999), mice (Webster et al., 1996), and dogs (Nozu et al., 1999). The effects of intravenous CRH injection are most likely not due to a central nervous system action (Banks et al., 1996) due to the presence of the blood brain barrier. Martins et al. (1997) demonstrated that radiolabled CRH passed from the brain through the blood brain barrier and could cause effects in peripheral organs.

The central actions of CRH on gastric motility are mediated through the vagal pathway. Kihara et al. (2001) reported that rats which had undergone truncal vagotomy did not show altered gastroduodenal motility after ICV injection of urocortin. However, in the same study, mechanical sympathectomized rats responded to treatment. Greater than 90% of preganglionic neurons that project to the stomach (Shapiro et al., 1985) have a role in vagal regulation of gastric contractility (Heymann-Monnikes et al., 1991). Owens and Nemeroff (1991) reported that the effects of CRH on gastrointestinal motility may be due to a reduction in parasympathetic impulses. Additionally, in mice sympathetic blockage prevented or attenuated ICV CRH gastric emptying, and caused inhibition of small and large intestine transit (Lenz et al., 1988; Tache et al., 1993).

In rats, peripheral urocortin dose-dependently delayed gastric emptying of a non-nutrient meal with greater magnitude than CRH, and the effect was found to be mediated through CRHr2 (Nozu et al., 1999). Nozu et al. (1999) showed that both CRH and urocortin inhibited gastric emptying in rats and the effects were totally reversed by astressin, a CRHr1 and 2 antagonist. Nozu et al. (1999) also reported that urocortin had a

2.3-fold greater effect than CRH on inhibition of gastric emptying, and antalarmin and NBI027914, selective CRHr1 antagonists, had no effect on CRH or urocortin modulation of gastric motility.

Iwakiri et al. (1996) was the first to report the presence of CRH binding sites in gastrointestinal smooth muscle cells, specifically in the guinea pig cecum. Iwakiri et al. (1996) also reported that CRH receptor antagonists inhibited CRH-induced relaxation of these smooth mucle cells. Tamer et al. (1997) showed that gastric emptying was inhibited by both central administration of CRH and from swim stress in rats. Lee et al. (1997) used dogs to find that delay in gastric emptying was not due to changes in gastric or pyloric motility, but rather changes in frequency of duodenal contractions. Kihara et al., (2001) showed that peripheral and central urocortin interrupted fasted motor patterns of gastrodudenal motility in rats. However, when administered to fed rats, there was a decrease in antrum and an increase in duodenum motility. Overall transit time was decreased (Kihara et al. 2001).

The role of CRH in human irritable bowel syndrome (IBS) has been investigated. Fukudo et al. (1989) found CRH-induced motility in the descending colon in control and IBS suffering patients. Fukudo et al. (1989) conclude that human intestinal motility is controlled in part by CRH, and patients with IBS have an exaggerated response to CRH. Saunders et al. (2002) showed that in rats peripheral CRH induces watery diarrhea primarily through the activation of CRHr1.

Mechanism of Action

Hanani et al. (1992) showed that CRH caused excitation of myenteric neurons in a section of guinea pig ileum. It was also reported that contraction was due to the action of acetylcholine release (Holt et al., 1984). Lei et al. (1983) reported CRH had a direct vasodilator action, at high concentrations, in pre-contracted mesenteric beds in rats. The direct inhibitory action of CRH on isolated cecal smooth muscle cells occurred through an adenylate cyclase system and cAMP-dependent protein kinase (Iwakiri et al., 1996).

SECTION NINE
RESEARCH OBJECTIVES

After reviewing the literature the following questions were adressed:

1. Does central administration of CRH differentally effect appetite in chickens that have undergone low (LWS) or high (HWS) selected linear body weight?
2. Does central administration of CRH receptor agonists and antagonists affect appetite differently in these lines?
3. Is gastric motility differentially effected by central CRH in these lines?
4. Are behaviors not associated with ingestion differentially affected by central CRH in these lines?
5. Is the CRH system hyper- or hypo-active in these lines?
6. Do polymorphisms exist between the genes coding for CRH and its receptor in these lines?
7. Is the expression of the CRH receptor different in these two lines?

These experiments will advanced understanding of the actions of CRH in these lines, which in turn, can be used to better understand the mechanism of CRH in the regulation of body weight and feed intake in non-body-weight-selected chickens. These results will add to the literature and may contribute to the creation of a pharmacological agent, which can reverse or prevent obesity in animals including humans.

These questions were addressed by using intracerebroventricular injections of CRH and related compounds into the two lines and monitoring various respones. In addition molecular techniques were utilized to determine genetic differences between the two lines in regard to the CRH system.

SECTION TEN
ANIMAL MODEL

The animal models used in these experiments are the result of long-term genetic selection (Siegel, 1962; Dunnington and Siegel, 1996). Seven inbred lines of White Plymouth Rock chickens formed a segregating gene pool. From this pool chickens with heavier (HWS) or lower (LWS) body weights at 8 weeks of age were selected to develop a high and a low body weight select line. After the first generation individual phenotypic selection was practiced within each closed line for body weight at 8 weeks of age. Eight dams and 48 sires and dams were selected to generate each of the first four generations. Generations 5 to 25, and 25 thereafter utilized 12 and 48, and 14 and 56 sires and dams, respectively.

Abdominal fat pads are heavier in the HWS than the LWS line of chickens (Dunnington et al., 1986). The LWS line exhibits natural anorexia (Zelenka et al., 1988), and delayed (Dunnington and Siegel, 1996), or prevented sexual maturity (Dunnington et al., 1983, 1984). The HWS line chickens are compulsive eaters (Siegel et al., 1984). A feed restriction program must be implemented after 8 weeks-of-age for the high weight line, otherwise, egg production is impaired and premature death occurs. Since feed consumption exceeds skeletal and lean body weight needs, fat deposition increases resulting in obesity in the HWS line. This condition is accompanied by undesirable reproductive complications (Katanbaf et al., 1989; Liu et al., 1994).

Metabolism, gauged by feed efficiency and oxygen consumption, is different between lines (Owens et al., 1971). LWS line chicks have higher rates of oxygen consumption, while HWS line chicks have more efficient feed utilization. Onset of lay is

delayed in the high weight line, while in the low weight line a proportion never reach sexual maturity. However, LWS line pullets can be induced to cycle with force feeding (Siegel and Dunnington, 1987). LWS line females do not consume enough feed *ad libitum* to attain a threshold of body weight, body composition, or both, which are essential to commence egg production (Zelenka et al., 1987). A portion of the LWS line chicks do not eat, and therefore die within the first week post hatch (Dunningotn and Siegel, 1997). The work of Burkhart et al. (1983) showed that alterations in the satiety mechanisms in these birds may explain the resulting obese and anorexic conditions. This population of body weight selected chickens serves as a unique model to study eating disorders in other species (Zelenka et al, 1987), including humans.

SECTION ELEVEN
CENTRALLY INJECTED CORTICOTROPHIN RELEASING HORMONE AND UROCORTIN DIFFERENTIALLY EFFECT APPETITE IN LINES OF CHICKENS ONE CONTAINING ANOREXIC AND THE OTHER OBESE INDIVIDUALS

Abstract

The effect of intracerebroventricular (ICV) injection of corticotrophin releasing hormone (CRH) and urocortin (a CRH type 2 receptor agonist) on feed and water intake in lines of chickens that contains some anorexic and the other some obese individuals was determined. An ICV injection of either CRH or urocortin dose-dependently decreased feed intake in chicks previously fasted for 180 min. The magnitude of feed intake suppression after treatment with both peptides was greatest in the low weight line, and supports the hypothesis that the CRH type 2 receptor is involved in anorexic conditions. Water intake was not effected by treatment. These results suggest that CRH and urocortin act differently within the central nervous system in lines of chickens containing obese and anorexic individuals while having no effect on water intake.

Introduction

The ability to develop novel strategies to treat eating disorders by either increasing or decreasing food intake is facilitated by an understanding of the natural satiety and hunger mechanisms in organisms with appetite related altered phenotypes. Presented here are experiments conducted to determine the effects of two related appetite suppressing neuropeptides on feeding behavior in two lines of chickens selected for 46 consecutive generations for high or low body weight at 56 days of age. These lines of chickens originated from a common base population (Dunnington and Siegel, 1996) and contain individuals that exhibit anorexia (low weight line, LWS) and obesity (high weight line, HWS).

Corticotrophin releasing hormone (CRH) is a 41 amino acid peptide that was first found to govern the secretion of adrenocorticotrophin in sheep, and thus was said to be the initiator of the stress response of an organism (Vale et al., 1981). Later, it was found that the release of CRH also causes secretions from other endocrine systems in rats (De Souza, 1987; Moreau et al., 1997). CRH has also been shown to be a participant in feed intake and energy balance mechanisms in rats (Richards et al., 1996; Rothwell, 1990; Richard et al., 2002). Denbow et al. (1999) demonstrated that ICV injections of CRH decreased feed intake in broiler- and layer-type chickens while having no effect on either water intake or body temperature. Zhang et al. (2001) reported that CRH also decreases appetite in 1-day old broiler chicks.

Urocortin, a 40 amino acid peptide related to CRH, was first isolated in rats (Vaughan et al.,1995) and its physiological effects have been recently reviewed by Oki and Hironobu Sasano (2004). When injected centrally in rats urocortin causes a more

potent appetite-suppressing effect than CRH (Spina et al., 1996, Skelton et al., 2000). This effect (Smagin et al., 1998) is induced by stimulation of the CRH type 2 receptor (CRHr2). Donaldson et al. (1996) reported that in mammalian systems urocortin has a higher affinity than CRH for CRHr2. CRHr2 mediates chronic anorexia (Reyes et al., 2001). Urocortin is less efficacious than CRH in reducing appetite in 1-day old chicks (Zhang et al., 2001). Expression patterns of uorcortin in the rat brain overlap with regions of CRHr2 (Smagin et al., 1998).

The purpose of the present experiment was to compare the effects of central administration of CRH and urocortin on ingestive behaviors in two lines of chickens, one selected for low the other high body weight. Other animal models exist to study body weight dysfunctions (Richard et al., 1996) through disruption of a single gene. However, our model is the polygenic product of divergent phenotypic selection and may better show synergisms and interactions of neurotransmitter pathways that have arisen during natural and artificial selection.

Materials and Methods

Animal Model

Our animal model is the result of a long-term divergent selection (46 consecutive generations) for high or low body weight at 56 days of age. Currently, these chickens differ in body weight at selection age by approximately nine fold (200 vs. 1820 g). For a review of the selection program see Dunnington and Siegel (1996) and Siegel and Wolford (2003), and are a study in evolution see Hill (2005). According to Dunnington and Siegel (1997), some of the LWS chickens exhibit anorexic behaviors and many die as

neonates simply from not eating even when chicks are bedded in mash feed. Siegel et al. (1984) reported that birds in the HWS line are compulsive eaters and exhibit obesity. Therefore, these lines can serve as models to study anorexia and obesity, and may provide insights into the physiology of eating disorders in other species (Zelenka et al., 1987), including humans. Chicks used in these experiments were from the 46th generation of selection.

Husbandry

All chicks were housed in individual cages (200 x 130 x 100 mm) with individual feed and water containers, and exposed to continuous lighting and a room temperature of 28^{o}C. The chicks had visual and auditory contact with each other. Cages were designed to impede perching on the feed and water containers. Chicks were handled twice daily to reduce stress associated with holding during the injection procedure. Injections were performed at 4 days post hatch. Using young chicks is advantageous for LWS line because individuals with anorexic tendencies may still be alive at this age. Four days of age was chosen for these experiments because the yolk sac is absent (personal observation), and the birds have had time to learn to eat and drink from the feeders.

Intracebroventrical Injections

Chicks were injected using an method adapted from Davis et al. (1979). Prior to injection, cranial down was trimmed. While manually holding the chick's body, the head was placed in an apparatus that restricted movement but allowed breathing. Once the head was held stationary, the injection was made 1 mm lateral from the centerline

running anterior to posterior, and 3 mm anterior to the coronal suture located by palpation using a microsyringe with a 26 gauge needle. The depth of injection was controlled by placing a sheath over the needle so that only 4 mm of needle (measured from the midpoint of the bevel) was exposed below the sheath. The injection was made slowly and the needle left in position for 15 sec after the syringe had been emptied to reduce flow back upon needle removal. After practice and before conducting these experiments, the technician was able to deliver dye into the ventricle system with 96% accuracy.

Peptides and Localization

Ovine CRH was purchased from Sigma Chemical Company (St. Louis, MO, USA). Rattus urocortin was purchased from American Peptide Company, Inc. (Sunnyvale, CA, USA). All treatments were assigned at random and were injected dissolved in artificial cerebrospinal fluid (aCSF) in a total injection volume of 5 μL with 0.6% Evans Blue dye to facilitate injection site localization. Other laboratories report using 0.1% Evans Blue for experiments lasting 120 min, however we found 0.6% was the lowest concentration still present 180 min after injection in these lines. After this time, the chick was decapitated and the head quick frozen in liquid nitrogen. The head was sectioned along the frontal plane to determine site of injection. Any chicks where dye was found outside of the ventricle system were eliminated from analysis.

Quantification of Feed and Water Intake

Chicks were restricted from feed 180 min prior to treatment as described by Zhang et al. (2001). Water was available *ad libitum*. After injection, feed and water consumption were recorded (0.01 g) from time 0 to 180 min at 30 min intervals.

Statistical Analysis

Cumulative feed and water intake were evaluated with a repeated measurement analysis of variance (ANOVA) using the mixed effect modeling procedure, SAS Proc Mixed (1999) with line, sex, and the interaction between them as main variables. Kenward and Roger approximation (1997) was used to determine degrees of freedom. To account for non-heterogeneous variance of the urocortin cumulative feed intake data were transformed to square roots. Treatment effects were partitioned into linear and quadratic contrasts to determine the dose–response relationships at each time period and to determine the shape of response for each dose over time. Significance implies $p \leq 0.05$.

Results and Discussion

In both lines CRH caused a dose-dependent reduction in feed intake (see Figure 1.11). The treatment effect on feed intake response was linear in both lines, but quadratic only in LWS birds. In both lines the shape of individual treatment response tested positive for a linear response while the LWS line also tested positive for a quadratic response. The quadratic response in LWS aCSF treated birds arises from two distinct copious feeding periods that occurred during 0 to 30 and 60 to 90 min post injection.

This responcse may arise from the 180 min fasting interval prior to injection and may be related to maximal crop filling and feed transit into the proventriculus. The HWS birds may be able to store a greater amount of feed in their crop, and thus may continue gastric loading longer into the 180 min fasting period than the LWS. This pattern may also account for the absence of feed intake in the HWS line 60 min after 0.4 μg and 30 mins-post injection after 0.2 μg CRH. It should be noted that the high and low dose of CRH blocked this pattern in LWS, and the middle dose delayed the second copious feeding period by 30 min. A quadratic type response to a neurotransmitter is typical, because higher doses do not necessarily cause a greater response, and may desensitize the receptor (Changeux et al., 1992).

The magnitude of appetite suppression after treatment with CRH was greatest in the LWS line and accounts for the significant line by treatment interaction. Feed consumption after 0.1 μg and 0.2 μg CRH over the entire data collection period followed a similar trend, while the 0.4 μg dose nearly totally blocked appetite after the initial copious feeding period 0 to 30 min post injection. The suppression of appetite was attenuated in HWS birds that were treated with 0.1 μg CRH and also contributes to the line by treatment interaction. These results demonstrate that feed intake is differentially affected by ICV treatment in these lines. Differences in affinity for the receptor or differences in cellular down stream signaling may account for these observed alterations. Water intake was not affected by CRH treatment in the LWS, however the response was quadratic after 60 min in HWS birds (see Figure 2 this section). Due to the lack of a line by treatment interaction for water intake it appears that the effect of CRH is specific for feed intake in these two lines.

Urocortin caused a dose-dependent decrease in feed intake in both lines (see Figure 11.3 this section). Cumulative feed intake after treatment with aCSF was similar across the CRH and urocortin experiments in LWS birds, however, HWS birds in the urocortin experiment ate more during the copious feeding period from 0 to 30 min post injection. Following this first feeding period, the slopes of the cumulative feed intake are similar. The most efficacious dose of urocortin in LWS line was 1.6 μg, while in the HWS line 0.8 and 1.6 μg have similar efficacy. This effect is most likely due to receptor desensitization (Changeux et al., 1992).

Apparently the LWS line is more susceptible to the influance of urocortin because appetite after treatment with 1.6 μg nearly totally blocked appetite after the initial feeding period from 0 to 30 min post treatment. This result was similar to that observed in the CRH feed intake trial. Since larger doses of urocortin were required to reduce appetite (0.1 μg CRH = 21 nM; 0.4 and 0.8 μg urocortin = 84 and 169 nM), CRH appears to be a more powerful satiety signal than is urocortin in both lines. Although urocortin effects were linear in both lines, there was also a quadratic response in the HWS after 120 min. The quadratic response is the result of the middle dose being more efficacious after 120 min than the high dose and contributes to the line by treatment interaction after 120 min. There was no line by treatment interaction for water intake after ICV urocortin in these lines. Urocortin does not appear to affect thirst in these lines of chickens (Fig 11.4).

Bray et al. (1991) suggested that most obesities are caused by a decrease in activity of the sympathetic nervous system. Our results compliment those of Kuo et al. (2001) who demonstrated that HWS birds have higher parasympathetic tone while LWS birds have higher sympathetic tone. Brown et al. (1982) showed that ICV CRH increased

sympathetic nervous system output through enhanced secretion of both epinephrine and norepinephrine. CRH also modulates the activity of the parasympathetic nervous system (Brown and Fisher, 1985). Sympathetic nervous system activity is associated with hypophagia and weight loss after lateral hypothalamus lesioning (Yoshida et al., 1983; Garofalo et al., 1996).

Okamoto et al. (2001) reported that anorectic effects of leptin are mediated by CRH in the hypothalamus in rats. Recently Kuo et al (unpublished) reported that central injection of leptin reduces feed intake in the LWS, but not in the HWS line. The influence of leptin on the already respectively hyper-responsive CRH central satiety system may in concert contribute to the low body weight and anorexia in the LWS line.

Our results are in agreement with those of Furuse et al. (2001) that in chicks the appetite reducing central effects of CRH ars stronger than that of urocortin. However the doses of urocortin Furuse et al. (2001) used to reduce appetite were much lower than our lowest effective dose. Since urocortin has a higher affinity for the CRHr2 than type 1 (Kobb and Heinricks, 1999), and non-weight selected chickens respond at a much lower dose, selection for both high and low body weight may have resulted in a reduction of function of CRHr2. CRHr2 likely mediates appetite suppression induced by ICV CRH (Pelleymounter et al., 2000). Although urocortin binds to CRHr2 with higher affinity than does CRH in mammalian systems, we demonstrated that ICV CRH more potently decreases appetite than urocortin. This effect can be explained by the work of Hotta et al., (1999) that showed stimulation of brain CRHr1 suppresses appetite in the absence of CRHr2 input. Additionally, anxiety-like behaviors, mediated through CRHr1, may reduce feeding behavior due to incompatibility with feeding (Richard et al., 2002).

Chronic and acute anorexia are mediated by CRHr1 and CRHr2 respectively (Reyes et al., 2001; Zorrilla et al., 2003). Expression of CRHr2 mRNA is reduced in obese rats and those that have been fasted (Richard et al., 1996; Timofeeva and Richard, 1997). The results of the experiment support that both CRHr1 and r2 involvment in the incidence of anorexia.

The results reported here show that both central administration of CRH and urocortin reduces appetite in genetic lines of chickens that contain anorexic and obese individuals, and that the magnitude of feed intake reduction for both peptides is greater in the LWS line than in the HWS line. The reduction in appetite seen in HWS chicks after urocortin treatment may indicate a loss of function in CRHr2 because other laboratories (Zhang et al., 2001) report results with much lower doses. Since LWS birds also require a higher dose of CRH than reported by Zhang et al. (2001), alterations in both lines at the level of CRHr2 may have occurred during divergent selection for body weight. Our results support the notion of targeting both CRHr1 and r2 for treatment of body weight dysfunctions, especially those associated with anorexia.

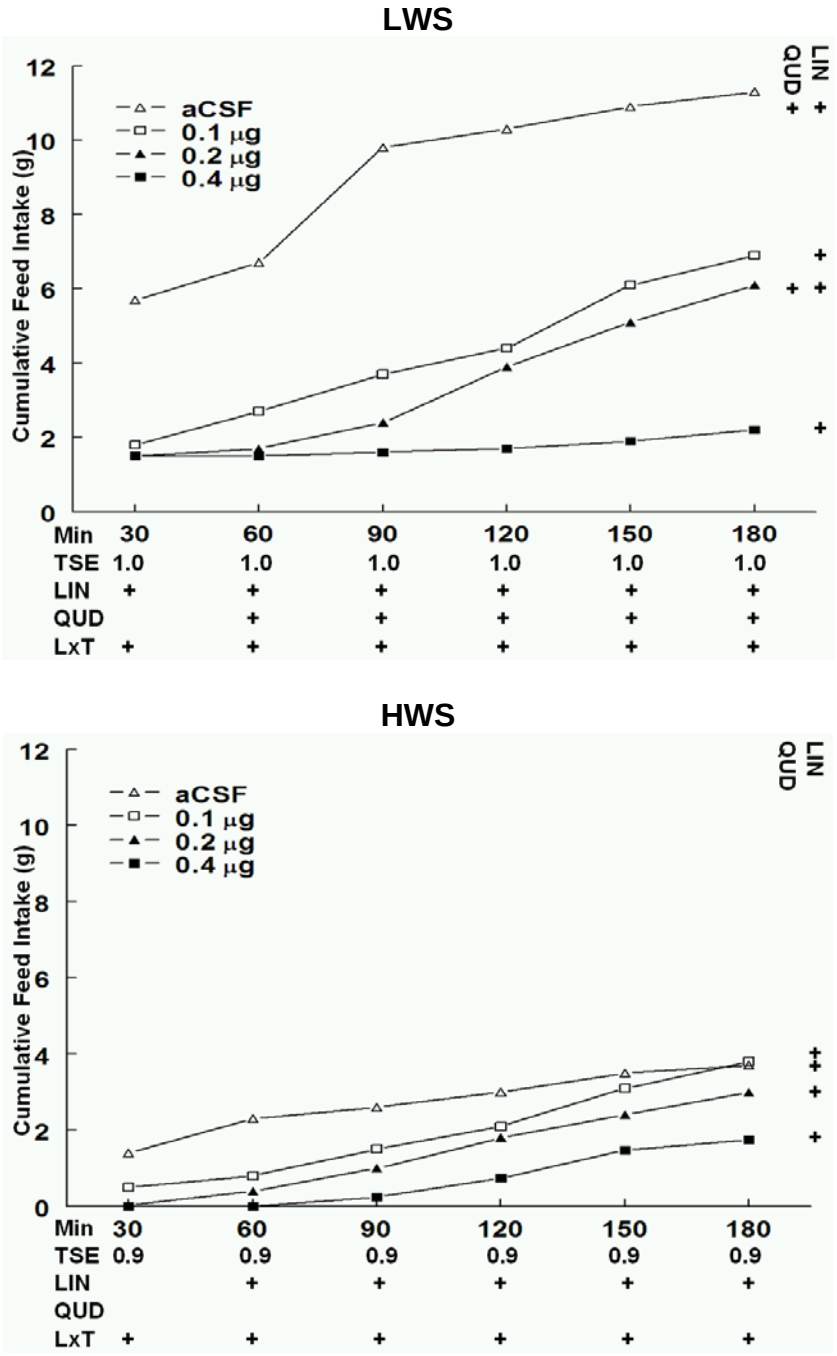


Figure 11.1. Cumulative feed intake from lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of CRH. Min, minutes post injection; TSE, standard error of the treatment mean; LIN, linear contrast; QUD, quadratic contrast; LxT, line by treatment interaction; aCSF, artificial cerebrospinal fluid (control); + $P \geq 0.05$.

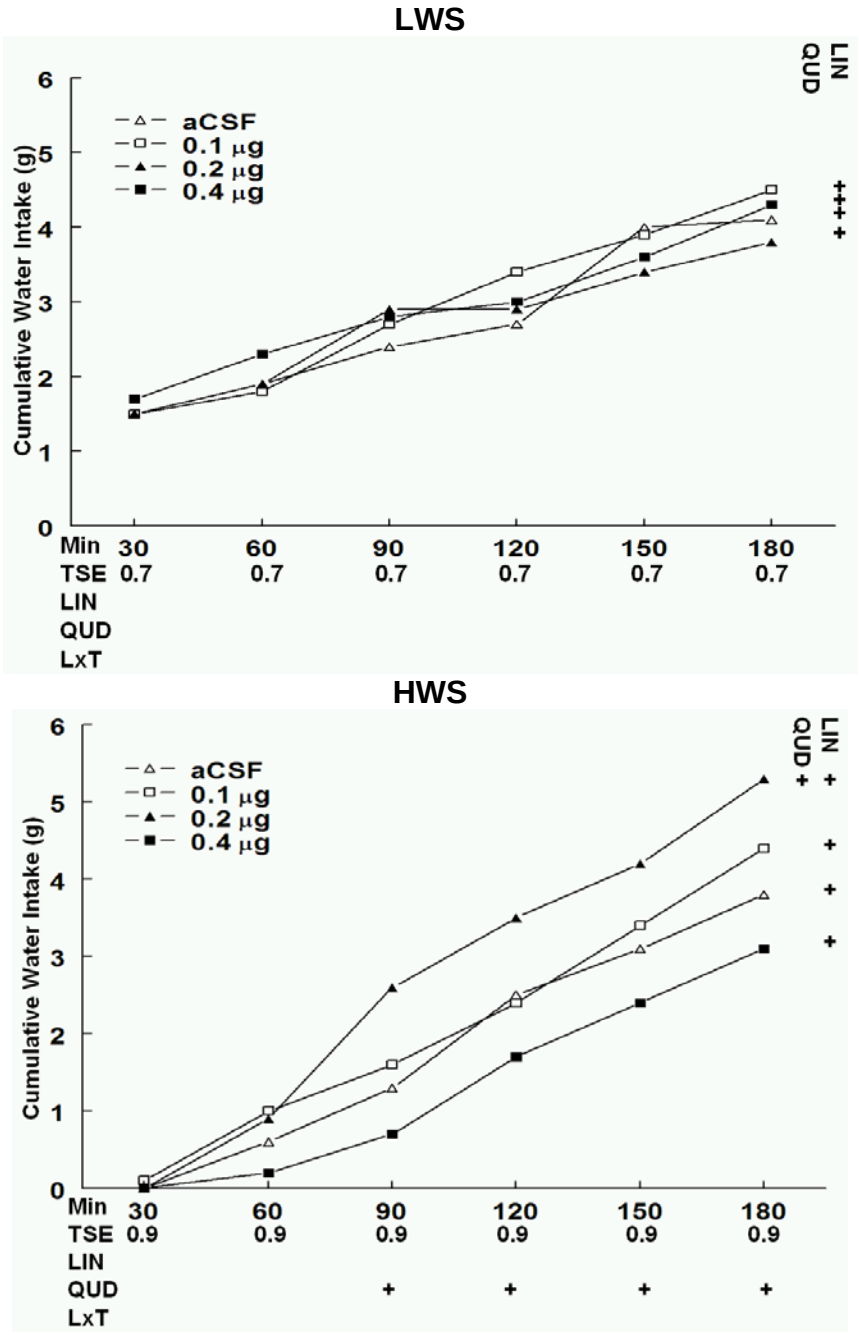


Figure 11.2. Cumulative water intake from lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of CRH. Min, minutes post injection; TSE, standard error of the treatment mean; LIN, linear contrast; QUD, quadratic contrast; LxT, line by treatment interaction; ; aCSF, artificial cerebrospinal fluid (control); + $P \geq 0.05$.

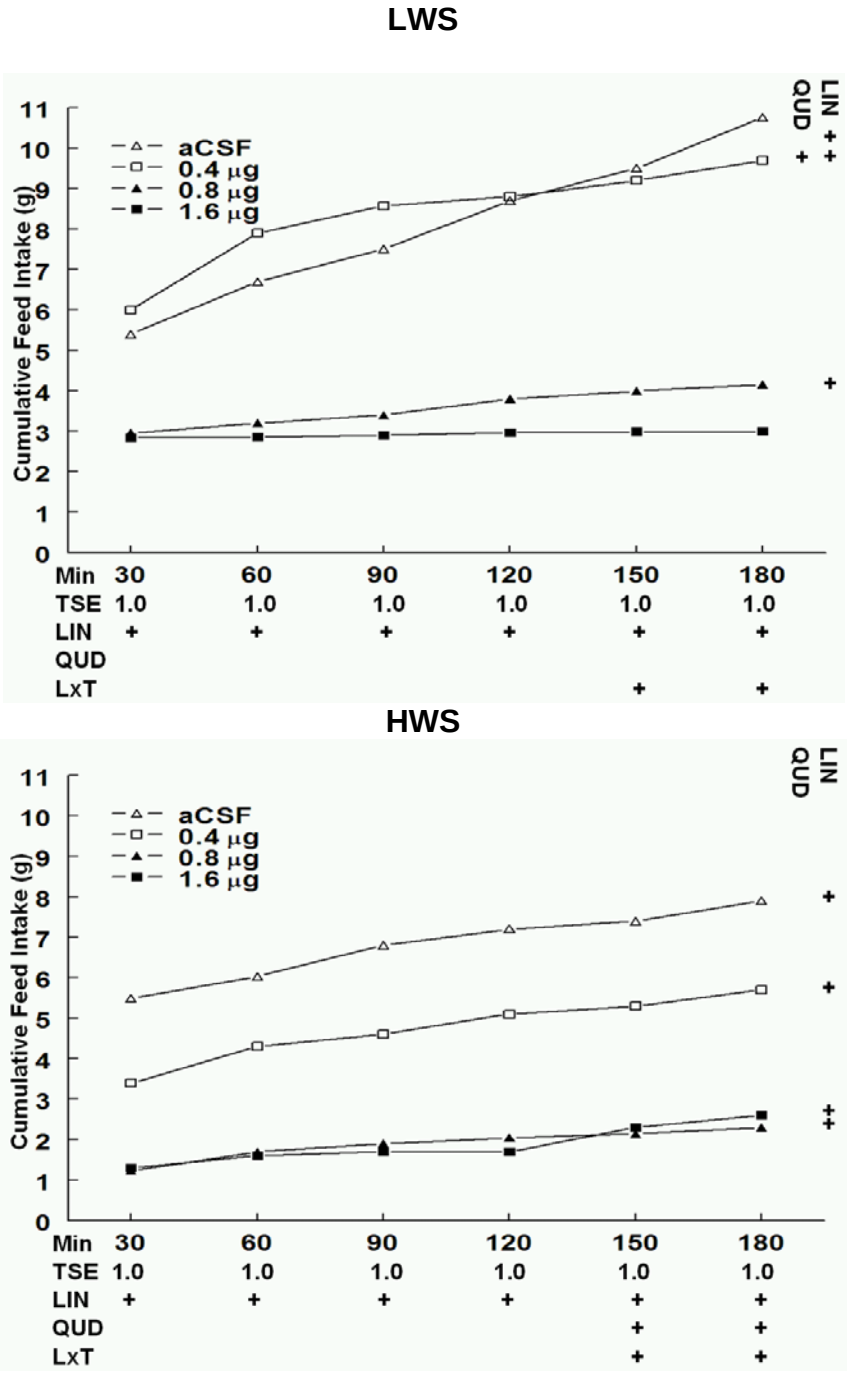


Figure 11.3. Cumulative feed intake from lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of urocortin. Min, minutes post injection; TSE, standard error of the treatment mean; LIN, linear contrast; QUD, quadratic contrast; LxT, line by treatment interaction; ; aCSF, artificial cerebrospinal fluid (control); + $P \geq 0.05$.

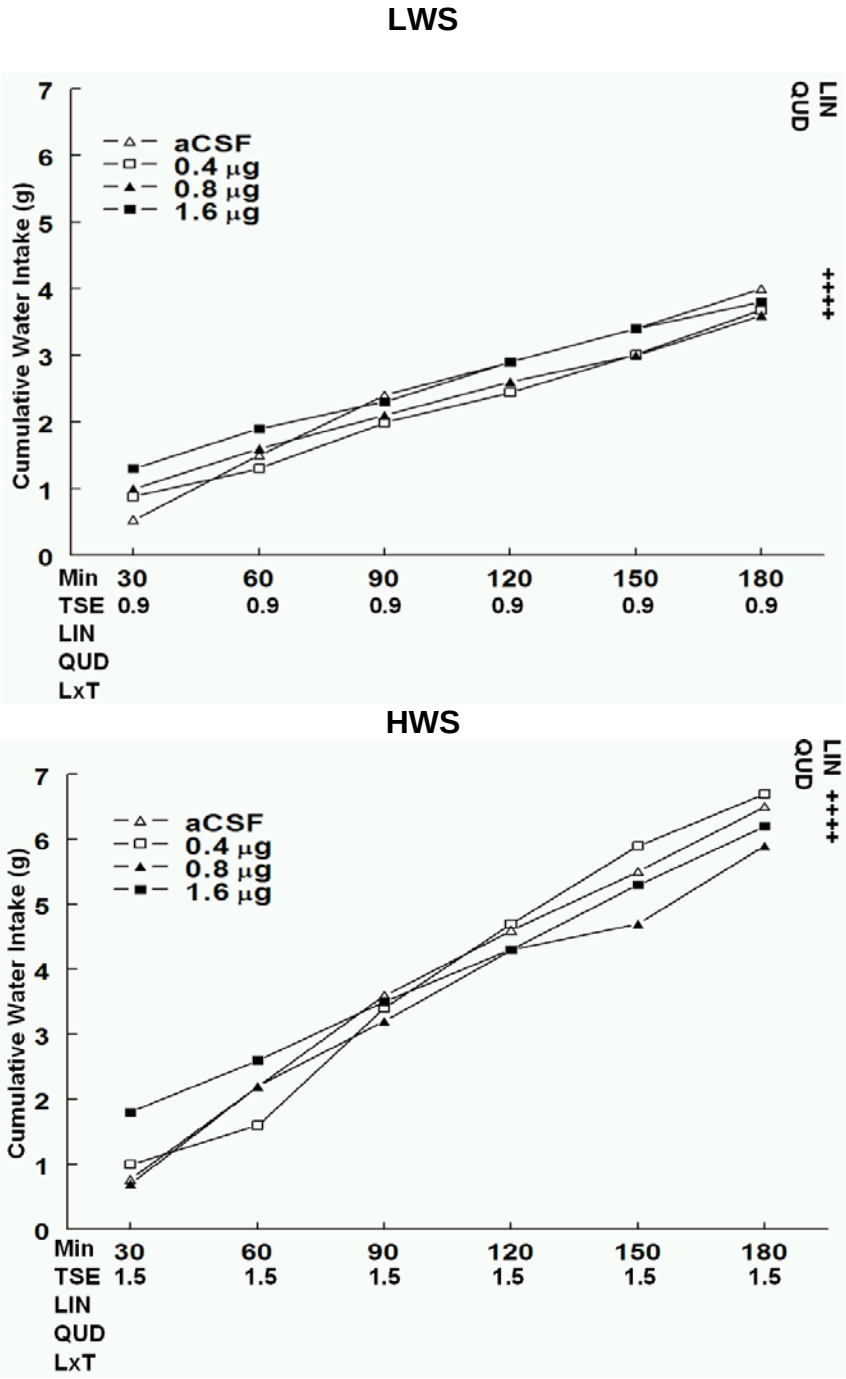


Figure 11.4. Cumulative water intake from lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of urocortin. Min, minutes post injection; TSE, standard error of the treatment mean; LIN, linear contrast; QUD, quadratic contrast; LxT, line by treatment interaction; ; aCSF, artificial cerebrospinal fluid (control); + $P \geq 0.05$.

SECTION TWLEVE
CENTRALLY INJECTED CORTICOTROPHIN RELEASING HORMONE RECEPTOR ANTAGONISTS AND BINDING PROTEIN ANTAGONIST DIFFERENTIALLY EFFECT APPETITE IN LINES OF CHICKENS ONE CONTAINING ANOREXIC AND THE OTHER OBESE INDIVIDUALS

Abstract

The effects of corticotrophin releasing hormone (CRH) receptor antagonists, astressin, α helical corticotrophin releasing fragment (9-41), and corticotrophin releasing factor fragment 6-33, on feed intake were evaluated in two lines of chickens that have been selected from a common base population for high (HWS) and low (LWS) juvenile body weight. The HWS line contains some obese individuals and the LWS line some anorexic individuals. All antagonists increased feed intake in the LWS line but not in the HWS line. The three antagonists attenuated the appetite reducing effects of CRH in the HWS, but not in the LWS, line. The concentration of corticosterone was higher in the LWS than the HWS line. We conclude that differences in the CRH system, its concentrations and differential actions dependant on receptor type, between lines may contribute to their differences in growth and feed intake.

Introduction

The central nervous system regulates ingestive behaviors. More specifically, Bray (1991) described the sympathetic nervous system as the principal governor in regulation of feed intake and hence body weight regulation. Bray (1991) concluded that most known obesities are the result of low sympathetic nervous system influence. Flier and Maratos-Flier (1998) reviewed appetite-related studies and concluded appetite can be stimulated or inhibited through the actions of various neurotransmitters. One such neurotransmitter, corticotrophin releasing hormone (CRH), a component of the sympathetic nervous system, suppresses feed intake when administered intracerebroventriculary (ICV) or directly into the hypothalamic paraventricular nucleus of rodents (Morley and Levine, 1982; Krahn et al., 1988; Arase et al., 1989; Rothwell, 1990; Glowa et al., 1992), chickens (Denbow et al., 1999), and young chicks (Zhang et al., 2001).

CRH, a 41 amino acid peptide (Vale et al., 1981) that initiates an organism's response to stress, is a potent secretogogue for adrenocorticotropic hormone (ACTH) and other proopiomelanocortin gene driven residues (Vale et al., 1983; Owens and Nemeroff, 1991). ACTH causes the release of glucocorticoids that exert a negative feedback on CRH synthesis and secretion (Gulyas et al., 1995). In addition to the hypothalamic-pituitary-adrenal axis, CRH affects the autonomic nervous system, immune system, and causes alterations in behavior (Dunn and Berridge, 1990).

Three distinct types of CRH receptors have been isolated. Complimentary DNAs that bind one type of CRH receptor have been cloned from the human (Chang et al., 1993), rat (Perrin et al., 1993), and mouse (Vita et al., 1993; Xiong et al., 1995). This

receptor is designated CRHr1 and shares a 98% sequence identity among species. A secondary receptor subtype of CRHr2 cloned from rats (Lovenberg et al., 1995) and mice (Stenzel et al., 1995) binds urotensin 1 with stronger affinity than CRH. Arai et al. (2001) identified a third CHR receptor, CRHr3 in the catfish. Information on the biological effects of CRHr3 activation is limited.

Smith et al. (1998) and Timpl et al. (1998) reported that CRHr1 induces the release of ACTH in response to stressors. CRHr2 is involved in coping mechanisms for stressors such as anxiolysis, anorexia, hypertension and cardioprotection (Kishimoto et al., 1995; Bale et al., 2000; Coste et al., 2000; Hashimoto et al., 2001). Eghbal-Ahmadi et al. (1997) demonstrated that when a gravid rat was deprived of feed, the infant rat had altered CHRr2 mRNA in the ventromedial hypothalamus. Intake of feed also affects CRHr2 mRNA expression in the hypothalamus and amygdala of rats (Eghbal-Ahmadi et al., 1999). Nazarloo et al. (2002) showed that with starvation there was a decrease in CRHr2 mRNA in the rat cardiovascular system.

Lowry (1993) described a protein obtained in plasma from pregnant humans which had the capability to bind and neutralize CRH. This protein, CRH binding protein (CRH-BP), is expressed in the brain (Potter et al., 1992) and binds to CRH with similar affinity as does CRH to CRH receptors (Behan et al., 1995). Behan et al. (1995) reported that CRH-BP is found in non-CRH producing neurons, and that CRH-BP's biological function is uncertain. One possible action may be to shield the maternal pituitary gland from increased CRH concentration during late pregnancy (Campbell et al., 1987; Sasaki et al., 1987). In non-pregnant systems it has been speculated that CRH-BP binds and

prevents CRH from accessing its receptors, and serves as a CRH reservoir (Behan et al., 1995; Karolyi et al., 1999).

The present experiment was designed to compare the contribution of central CRHr1 and CRHr2 receptors and CRH-BP on feed intake in lines of chickens that have undergone long-term selection for high and low juvenile body weight (Dunnington and Siegel, 1996). These lines are known to differ in feed intake and body weight and include obese and anorexic individuals.

Materials and Methods

Animals and Husbandry

Our animal model is the result of a long term divergent selection (47 consecutive generations) for high or low body weight at 56 days of age. Eggs were obtained from age contemporary parents and incubated in the same machine. Upon hatch chicks were transferred to individual cages (200 x 130 x 100 mm) with individual feed and water containers and were exposed to continuous lighting, and 28^{o}C room temperature. The chicks had visual and auditory contact with each other. Cages were designed to impede perching on the feed and water containers. Chicks were handled twice daily to reduce stress associated with the injection procedure. Injections were performed at 4 d post hatch. Using young chicks is advantageous for experiments involving the LWS line because individuals with anorexic tendencies may still be alive at this age. Four days of age was chosen for these experiments because the yolk sac is absent (personal observation), and the birds have had time to learn to eat and drink from the feeders.

Currently these chickens differ in body weight at selection age by approximately nine fold (200 vs. 1820 g). For a review of this selection program see Dunnington and Siegel (1996) and Siegel and Wolford (2003). According to Dunnington and Siegel (1997), some of the low-weight select (LWS) line exhibit anorexic behaviors and many die as neonates simply from not eating even when chicks are bedded in mash feed. Siegel et al. (1984) reported that birds in the high-weight line (HWS) are compulsive eaters and exhibit obesity. Therefore, these lines served as unique models to study anorexia and obesity and may provide insights into the physiology of eating disorders in other species (Zelenka et al., 1987), including humans. Chicks used in these experiments were from the 47th generation of selection.

Three CRH receptor antagonists were used in these experiments. Astressin, a competitive antagonist, has similar affinity for CRHr1 and CRHr2 receptors (Rivier et al., 2002) and inhibits CRH-induced behavioral effects in rats (Maecker et al., 1997). The antagonist, α-helical CRH (9-41), has a significantly higher affinity for CRHr2 than CRHr1 (Perrin et al., 1999). CRH fragment (6–33) competitively binds the CRH-BP, but not CRH receptors (Behan et al., 1995, Gulyas et la., 1995).

Intracebroventrical Injections

Chicks were injected using a method adapted from Davis et al. (1979). Prior to injection, cranial down was trimmed. While manually holding the chick's body, the head was placed in an apparatus that restricted movement but allowed breathing. Once the head was held stationary, the injection was made 1 mm lateral from centerline running anterior to posterior, and 3 mm anterior to the coronal suture located by palpation using a

microsyringe with a 26 gauge needle. The depth of injection was controlled by placing a sheath over the needle so that only 4 mm of needle (measured from the midpoint of the bevel) was exposed below the sheath. The injection was made slowly and the needle left in position for 15 sec after the syringe had been emptied to reduce flow back upon needle removal. After practice and before conducting these experiments, the technician was able to deliver dye into the ventricle system with 96% accuracy.

Peptides and Localization

Ovine CRH, α helical corticotrophin releasing fragment (9-41, [αCRH]), astressin and corticotrophin releasing factor fragment 6-33 (BPF) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All treatments were assigned at random and were injected dissolved in artificial cerebrospinal fluid (aCSF) for a total injection volume of 5 μL with 0.6% Evans Blue dye to facilitate injection site localization. After the experiment, the chick was decapitated and the head quick frozen in liquid nitrogen. The head was sectioned along the frontal plane to determine site of injection. Any chicks where dye was found outside of the ventricle system were eliminated from analysis.

Quantification of Feed Intake

Chicks were restricted from feed 180 min prior to treatment as described by Zhang et al. (2001). Water was available *ad libitum*. After injection, feed consumption was recorded (0.01 g) from time 0 to 180 min at 30 min intervals.

Corticosterone Assay

To ascertain stressor effects, chicks from each line were assigned to 1 of 2 treatment groups. In the first groups chicks were immediately decapitated; in the second group each chick was restrained by hand 10 min prior to decapitation. Blood samples were collected after decapitation in EDTA-coated tubes and centrifuged for 15 min at 3000 x G and the supernatants stored at -80^{o}C until analysis. Corticosterone concentration in the plasma samples were determined by radioimmunoassay using a commercially available kit (MP Biomedicals, Orangeburg, NY).

Statistical Analysis

Cumulative feed and water intake were evaluated with a repeated measurement analysis of variance (ANOVA) using the mixed effect modeling procedure, SAS Proc Mixed (1999) with line, sex, and the interaction between them as main variables. Kenward and Roger approximation (1997) was used to determine degrees of freedom. To account for non-heterogeneous variance, feed intake data were transformed to square roots. Nonorthogonal contrasts were used for obtaining all pairwise comparisons among sample means at each time period. Significance implies $P \leq 0.05$.

Results and Discussion

There was a line by treatment interaction for feed intake at all time periods from 60 min on. Therefore we analyzed within line and treatment. CRH reduced feed intake in both lines at all observation times (see Figures 12.1, 12.2, and 12.3 this section). Treatment with αCRH, that has a higher affinity for CRHr2 than CRHr1, caused a significant increase in feeding after 150 min in LWS but not in HWS chicks (see Figure

12.1). During the observation period αCRH did not block the appetite reducing effect of CRH in the LWS line, however it did antagonize CRH's appetite suppressing effects in the HWS line.

The non selective CRH receptor antagonist astressin, while having no effect in the HWS line (see Figure 12.2), stimulated feed intake in the LWS line after 120 min. Although astressin did not antagonize the appetite reducing effects of CRH at any time in the LWS line, it did antagonize the effect of CRH-induced appetite suppression after 60 min in the HWS line.

CRH fragment (6–33), which competitively binds the CRH-BP, caused a profound increase in feed intake after 90 min in the LWS line (see Figure 12.2). This effect was not observed in the HWS line. BFP did not antagonize the appetite reducing effects of CRH in the LWS line, however, after 90 min its effect was observed in the HWS line.

The above results lead to hypothesis that the LWS line had higher physiological concentrations of CRH than the HWS line. To evaluate this hypothesis the activity of the hypothalamic-pituitary axis in these lines was measured by quantifying corticosterone concentrations. Concentration of cosrticosterone was higher in non-stressed LWS line chicks (0.536 ± 0.093 μg/mL, n=15) than in non-stressed HWS line chicks (0.334 ± 0.038 μg/mL, n=16). After both lines were subjected to restraint the treatment effect was significant with the percent increase due to stress being 351% (1.711±0.144 μg/mL, n=15) for the LWS line, and 353% (1354±0.067 μg/mL, n=16) for the HWS line.

Our results compliment those of Denbow et al. (1999) and Zhang et al (2001) in that CRH treatment caused a reduction in feed intake. However, the effects of the three

antagonists used here, or of any CRH related antagonists, have not been investigated in 4-day post hatch chicks. These data support the hypothesis that differences may exist involving CRH receptors between these two lines. Either line may have receptor mutations that affect binding of the two receptor antagonists to their respective receptors that may result in differential affinity as compared to mammalian species. This possibility may explain why the antagonists stimulate feed intake when administered alone, but are unable to reverse the appetite suppressing effects when administered in tandem with ovine CRH in the LWS line.

The responses observed after treatment with both receptor antagonists were similar, except that the stimulation of feed intake occurred earlier in the LWS line after treatment with astressin than with α-CRH. Because astressin targets both CRHr1 and CRHr2 with similar affinity in mammals, the effect observed in the LWS line may be due to synergistic contributions of both types of receptors to suppress appetite. The contribution of CRHr1 in appetite suppression in the HWS may not be as strong as is in the LWS line because appetite antagonism was delayed 90 min in the HWS line after astressin compared to α-CRH.

Controversy over the mechanism of BPF action makes interpretation of our data more difficult. The stimulation of feed intake after BPF administration in the LWS line occurred sooner and was greater than that detected after treatment with the receptor antagonists (see figure 12.3). If the administration of BPF accelerates metabolism of the chick's natural CRH, this would be supported the observation that the LWS line has higher concentrations of natural CRH. BPF causing displacement of natural CRH resulting in larger quantities being available to the CRH receptors was not supported by

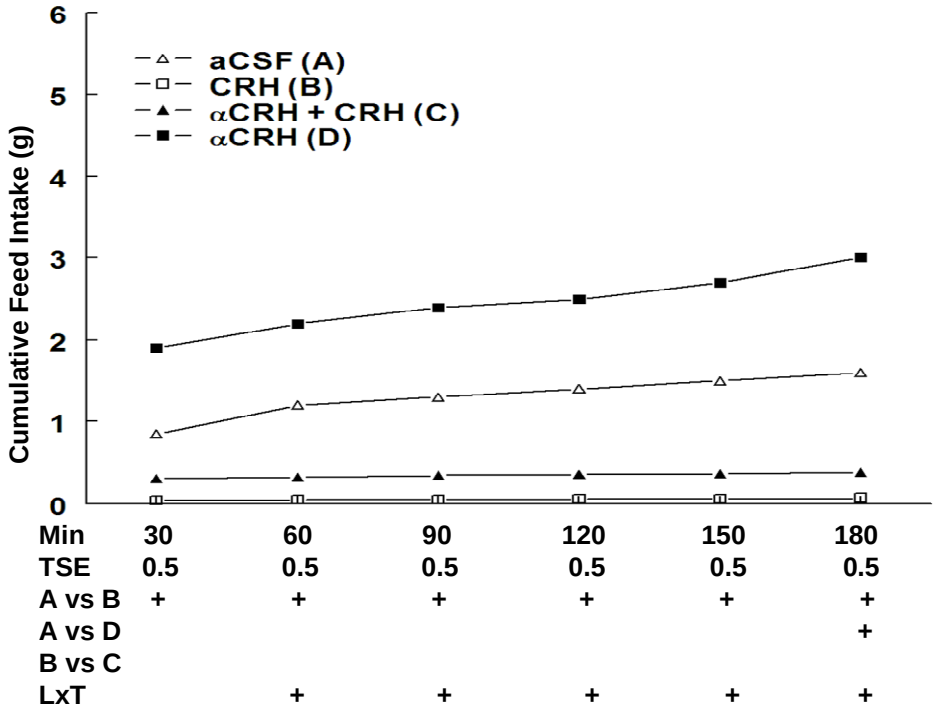
our assay data. However, the appetite suppression antagonism observed after 90 min in the HWS line supports the thesis that BPF administration is accelerating CRH metabolism and reducing availability of natural CRH to its receptors.

In conclusion, these results demonstrate differences in the CRH signaling system between these lines of chickens. Since these lines differ in basal concentrations of physiological corticosterone, we assume this is correlated to differential concentrations of natural CRH. Appetite is affected by CRH receptor antagonists alone, yet these antagonists did not attenuate CRH's suppression of feed intake in the LWS line. Conversely, whereas solo administration of the CRH receptor antagonists used here did not effect appetite in the HWS line, they reduced the satiety signal caused by central administration. These data support that differences in the CRH system of these two lines may contribute to differences in their feed, and hence altered body weights. These results may also further our understanding of the contribution of the CRH system in body weight dysfunctions in other species, including humans.

Other animal models exist to study body weight dysfunctions (Richard et al., 1996) through disruption of a single gene, however our model is the polygenic product of divergent phenotypic selection and may better show synergisms and interactions of neurotransmitter pathways that have arisen during natural and artificial selection per the infinitestinal model.

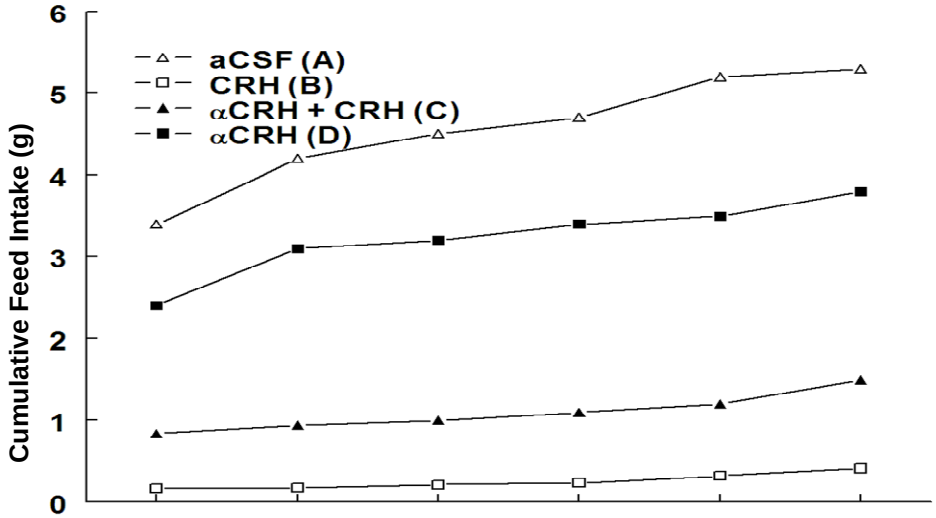
Figure 12.1 (next page). Cumulative feed intake from lines of chickens selected for low (top) or high 56 day body weight (bottom) following intracerebroventricular injection of artificial cerebrospinal fluid, CRH, αCRH + CRH, or αCRH. aCSF, artificial cerebrospinal fluid; Min, minutes post injection; TSE, standard error of the treatment mean; LxT, line by treatment interaction; + $P \geq 0.05$.

LWS



Min	30	60	90	120	150	180
TSE	0.5	0.5	0.5	0.5	0.5	0.5
A vs B	+	+	+	+	+	+
A vs D						+
B vs C						
LxT		+	+	+	+	+

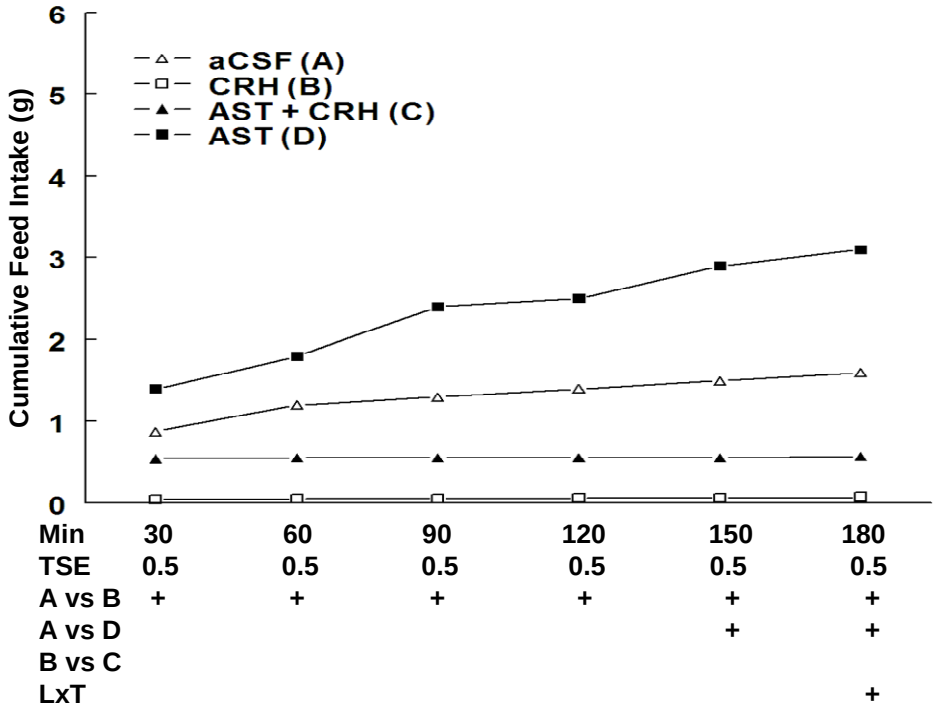
HWS



Min	30	60	90	120	150	180
TSE	0.5	0.5	0.5	0.5	0.5	0.5
A vs B	+	+	+	+	+	+
A vs D						
B vs C	+	+	+	+	+	+
LxT		+	+	+	+	+

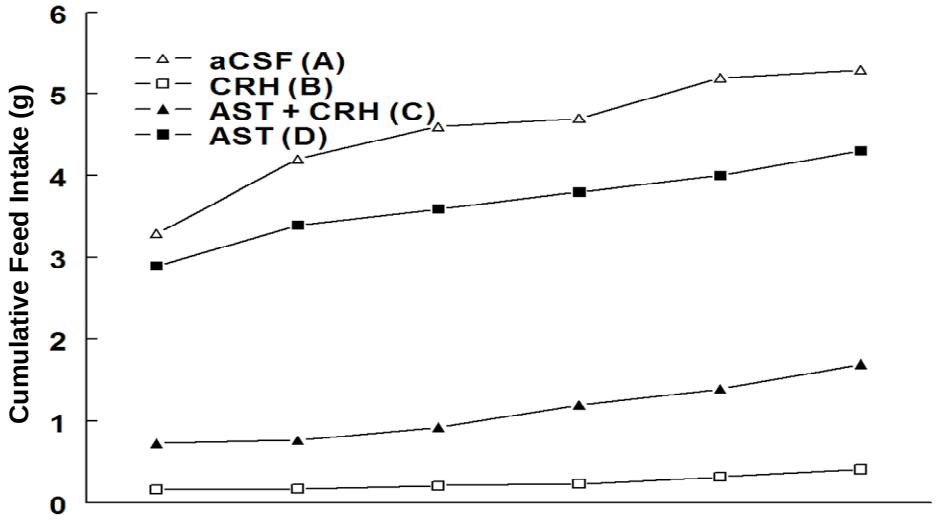
Figure 12.2 (next page). Cumulative feed intake from lines of chickens selected for low (top) or high 56 day body weight (bottom) following intracerebroventricular injection of artificial cerebrospinal fluid, CRH, astressin + CRH, or astressin. aCSF, artificial cerebrospinal fluid; AST, astressin; Min, minutes post injection; TSE, standard error of the treatment mean; LxT, line by treatment interaction; + $P \geq 0.05$.

LWS



Min	30	60	90	120	150	180
TSE	0.5	0.5	0.5	0.5	0.5	0.5
A vs B	+	+	+	+	+	+
A vs D					+	+
B vs C						
LxT						+

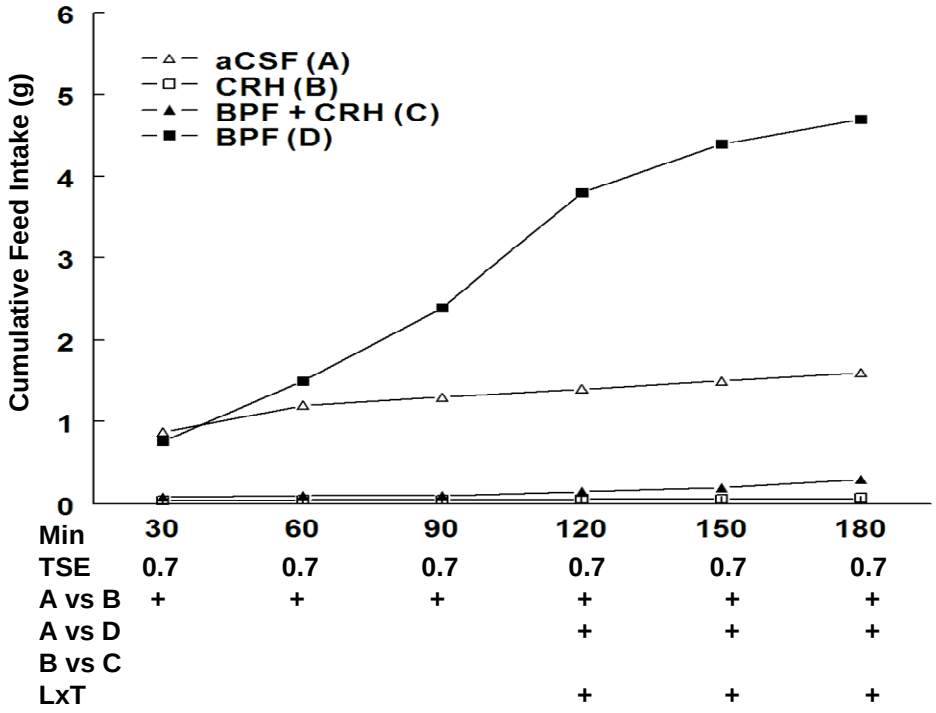
HWS



Min	30	60	90	120	150	180
TSE	0.5	0.5	0.5	0.5	0.5	0.5
A vs B	+	+	+	+	+	+
A vs D						
B vs C			+	+	+	+
LxT						+

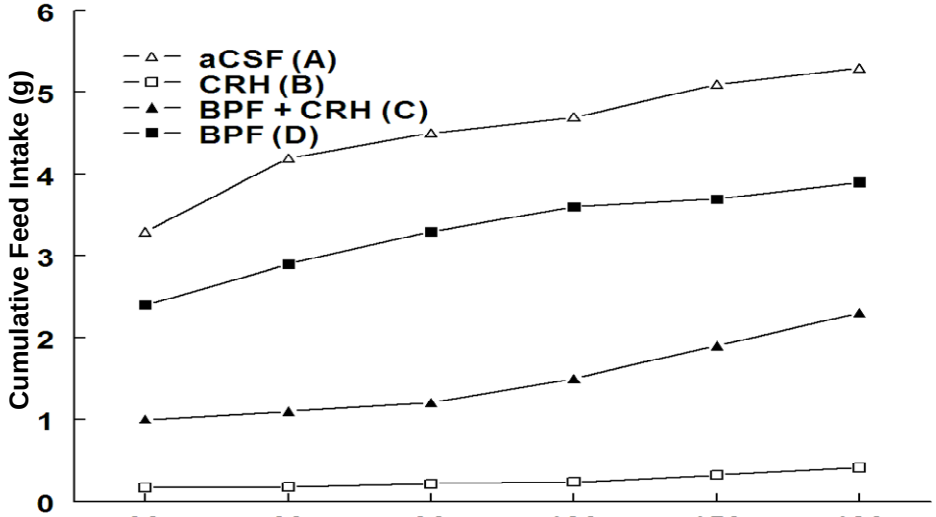
Figure 12.3 (next page). Cumulative feed intake from lines of chickens selected for low (top) or high 56 day body weight (bottom) following intracerebroventricular injection of artificial cerebrospinal fluid, CRH, CRH binding protein fragment + CRH, or CRH binding protein fragment. aCSF, artificial cerebrospinal fluid; BPF, CRH binding protein fragment; Min, minutes post injection; TSE, standard error of the treatment mean; LxT, line by treatment interaction; + $P \geq 0.05$.

LWS



Min	30	60	90	120	150	180
TSE	0.7	0.7	0.7	0.7	0.7	0.7
A vs B	+	+	+	+	+	+
A vs D				+	+	+
B vs C						
LxT				+	+	+

HWS



Min	30	60	90	120	150	180
TSE	0.7	0.7	0.7	0.7	0.7	0.7
A vs B	+	+	+	+	+	+
A vs D						
B vs C				+	+	+
LxT				+	+	+

SECTION THIRTEEN
CORTICOTROPHIN RELEASING HORMONE CAUSES DIFFERENT BEHAVIORS AND ALTERED GASTRIC MOTILITY IN LINES CONTAINING OBESE AND ANOREXIC CHICKENS

Abstract

Two unique lines of chickens that contain some anorexic and some obese individuals were used to study the effects of intacerebroventricular injection of corticotrophin releasing hormone (CRH) on behavior and gastric motility. The line with anorexic individuals exhibited a higher frequency of stress related behaviors and decreased crop empting after central administration of CRH. Chickens from the line with obese individuals had accelerated crop emptying in response to central administration CRH. We conclude that the differential responses to central CRH may, in part, be responsible for the differences in differences in body weights of these lines.

Introduction

Corticotrophin releasing hormone (CRH), is a 41 amino acid peptide that was first described by Vale et al. (1981), and initiates an organism's endocrinological response to stress. According to Nozu et al. (1999) central CRH plays a key role in integrating the efferent components of behavioral, endocrine, autonomic, and immune responses to stress. CRH centrally modifys ingestive, sexual, and locomotor behaviors, cardiovascular function, and gastric secretion in mammalian species (Pappas et al., 1985). The effects of CRH on stress responses in chickens are similar to mammals (Panksepp et al., 1997; Zhang et al., 2001; Ohgushi et al., 2001).

Stressful stimuli alter gastrointestinal motor and secretory functions (Narducci et al., 1985). Stimuli such as fear, anger, and stress decrease gastric secretion, blood flow and emptying (Enck and Holtmann, 1992). CRH is released during stressful circumstances (Plotsky and Vale, 1984) and is involved in the alteration of gastrointestinal motor function (Tache et al., 1993; Martinez et al., 1997). Gastrointestinal responses to central and peripherally administered CRH have been shown in several mammalian species (Pappas et al., 1985; Castagliuolo et al., 1996; Fukudo et al., 1998; Santos et al., 1999). Peripheral CRH administration inhibited gastric contractility and emptying, and slowed small intestate transit while stimulating colonic motility, transit, and fecal pellet output in rodents (Williams et al., 1987; Lenz et al., 1988; Sheldon et al., 1990; Gue et al., 1991; Martinez et al., 1997; Martinez et al., 1999). Central administration reduced gastric emptying (Tache et al., 1987; Lenz et al., 1988a). Central CRH influences gastric emptying via modulation of the parasympathetic nervous system (Tache et al. 1987).

In addition to effects on the gastrointestinal system, CRH reduces feed intake in mice (Contarino et al., 2000), chickens (Denbow et al., 1999), marsupials (Hope et al., 2000), and other species. CRH activates the sympathetic nervous system and causes increased locomotor activity, enhancement of arousal, and induction of aggression in rats (Shibasaki et al., 1991). CRH also enhances arousal in chickens (Zhang et al., 2001).

Levine et al. (2005) reported that obese humans spent more time in a seated posture than lean individuals. Earlier Levine et al. (1999) reported that when lean humans overate, an increase in exercise activity thermogenesis prevented an increase in body weight, and Bray (1991) reported that most obesities are the result of low sympathetic nervous system activity. The experiments presented here we used chickens from lines containing obese and anorexic individuals. The anorexic containing line has increased, while the obese containing line has reduced, sympathetic nervous system output (Kuo et al., 1999). These unique lines of chickens were used to study the effects of central corticotrophin releasing hormone (CRH) on behavior and gastric motility. CRH decreases appetite and stimulates activity of the sympathetic nervous system (Arase et al., 1989). The hypothesis was that central CRH would hyperactivate the sympathetic nervous system in chickens from the line containing anorexics and that reduced sedimentary behaviors would contribute to their lower body weights. In contrast those from the line with obese individuals may have a hypoactive sympathetic response to CRH that would increase sedimentary behaviors.

Materials and Methods

Animal Model

Our animal model is the result of a long-term divergent selection (47 consecutive generations) for high (HWS) or low (LWS) body weight at 56 days of age. Currently these lines of chickens differ in body weight at selection age by approximately nine fold (200 vs. 1820 g). For a review of this selection program see Dunnington and Siegel (1996) and Siegel and Wolford (2003). According to Dunnington and Siegel (1997), some individuals in the LWS line exhibit anorexic behaviors and many die as neonates simply from not eating even when bedded in mash feed. Siegel et al. (1984) reported that HWS line birds are compulsive eaters and exhibit obesity. Therefore, these lines serve as models to study anorexia and obesity and can provide insights into the physiology of eating disorders in other species (Zelenka et al., 1987), including humans. Chicks used in these experiments were from the 47th generation of selection.

Husbandry

All chicks were maintained under continuous lighting, at 28^{o}C. Chicks were group caged for the crop emptying experiment. For the behavior experiment chicks were caged individually (20 x 30 cm) without visual contact with each other from the day of hatch to reduce isolation stress during testing. Chicks were handled twice daily to reduce stress associated with the injection procedure for both experiments. Injections were performed at 5 d post hatch.

Intraceberoventrical Injections

Chicks where injected using a method adapted from Davis et al. (1979). Prior to injection, cranial down was trimmed. While manually holding the chick's body, the head was placed in an apparatus that allowed breathing but restricted movement. Once the head was held stationary, the injection was made 1 mm lateral from centerline running anterior to posterior, and 3 mm anterior to the coronal suture located by palpation using a microsyringe with a 26 gauge needle. The injection depth was controlled by placing a sheath over the needle so that only 4 mm of needle (measured from the midpoint of the bevel) was exposed below the sheath. The injection was made slowly and the needle left in position for 15 sec after the syringe had been emptied to reduce flow back upon needle removal. After practice and before conducting these experiments, dye was delivered into the ventricle system with 96% accuracy.

Peptides and Localization

Ovine CRH was purchased from Sigma Chemical Company (St. Louis, MO, USA). All treatments were assigned at random and were injected dissolved in artificial cerebrospinal fluid (aCSF), as described by Anderson et al. (1972) that also served as a control. The total injection volume was 5 μL with 0.6% Evans Blue dye added to facilitate injection site localization. After the experiment, the chick was decapitated and the head quickly frozen in liquid nitrogen. The head was sectioned along the frontal plane to determine site of injection. Any chicks with dye found outside of the ventricle system were eliminated from analysis.

Crop emptying experiment

Chicks from both lines, that had been fasted 12 h and with *ad libitum* access to water, were centrally injected with either 0 or 0.4 μg CRH at random. Immediately following injection the chick was weighed (0.01 g) and gavaged with a slurry containing 50% water 50% ground to powder chicken feed infused into the crop. Position of the tube was confirmed by palpation. The crop was filled to approximately 90% of its capacity. A sample of the slurry was also collected at the time of gavage. After gavage the chick was weighed again to determine the weight of slurry administered. Chicks that vomited were removed from the experiment. Ninety min post gavage the chick was anesthetized with Halothane (Halocarbon, River Edge, NJ) and the lower followed by the upper esophagus was clamped, and the crop was removed in its entirety. Crop contents were recovered. Slurry samples and crop contents were dried overnight at 60^{o}C and then weighed. Percent recovery was calculated on a dry matter basis. The percent of dry matter gavaged was calculated from the slurry sample collected at the time of gavage.

Behavior Experiment

Ad libitum fed and watered chicks from both lines were centrally injected with 0 or 0.4 μg CRH at random. After injection chicks were placed into a clear observation chamber (190 x 190 cm). This chamber was enclosed by a secondary chamber that restricted the birds visual field to only inside the secondary chamber. The observation chamber floor was divided into 9 equally sized squares with alternating black and white colors that had chicken feed scattered upon it. All activities of the chicks were monitored automatically and recorded on VHS tape for subsequent analysis from 0 to 30 min post-

injection with respect to the following 9 behavioral categories: number of steps, escape attempts, jumps, and pecks, the time spent standing, sitting, sleeping, and preening and locomotion. Locomotion was quantified by determining the number of times the chick moved from one colored square to another recorded by an overhead camera.

Statistical analysis

Percent recovery data were analyzed using the GLM procedures of SAS (1999) by line and dose of CRH. Treatment effects were partitioned into linear and quadratic contrasts to determine the dose dependant response. Behavior data were analyzed using the Mann-Whitney U test. Significance implies $P < 0.05$.

Results and Discussion

In the crop emptying experiment the aCSF-treated LWS and HWS lines had similar percent content crop empyting rates (see Figure 13.1). The LWS line showed a liner dose-dependant increase in crop emptying in response to central CRH. The HWS line responded with a quadratic dose-dependant response to CRH treatment. The doses of 0.1 and 0.2 μg CRH have similar efficacy while the high dose, 0.4 μg reduced recovery to a level comparable to aCSF treatment.

Treatment with CRH had no effect on the cumulative time spent sitting or preening in either line (see Figure 13.2). In both lines treatment with CRH caused an increase in the cumulative number of steps and locomotion (see Table 1 this section). Chicks that had been treated with aCSF from both lines slept, however, no CRH-treated chicks from either line slept post injection. Chicks from the LWS line that were treated

with CRH jumped more by 20 min post injection than did HWS CRH-treated chicks (see Table 13.1). None of the aCSF treated chicks attempted escape, however, some of the LWS line tried to escape within the first 5 min while the first escape attempt in the HWS line was delayed until after 10 min (data not shown). The time spent standing was not affected by treatment in the LWS line, but was increased in CRH-treated HWS line chicks after 20 min (data not shown). The cumulative number of pecks was different between aCSF treated LWS and HWS line chicks at times 15 and 20 (see Table 13.2). The HWS line pecked more during these intervals. Pecking was not effected by CRH treatment in the LWS line, but was reduced after 5 min in the HWS line.

Ohgushi et al. (2001) reported that chicks treated with CRH displayed freezing behavior, an effect not observed in the present experiment. The LWS line chicks appeared to respond to central CRH treatment with more anxiety-related behaviors than the HWS chicks as indicated by increased jumps and attempting escape sooner. This result may be explained by the LWS line's increased sympathetic nervous system output (Kuo et al., 1999) and because the LWS line has higher concentrations of corticosterone than does the HWS line (Cline, unpublished data). Although it was anticipated that the HWS line would have reduced locomotion compared to the LWS line, but this behavior was similar for both lines.

In an experiment conduced by Zhang et al. (2001) chicks that were centrally injected with CRH moved more, vocalized loudly, and were excited. Although grooming was increased in rats by central administration of CRH (Britton et al., 1982), this effect was not observed in chicks by Zhang et al. (2001) nor in the present experiment. Zhang et al. (2003) found that centrally treated CRH chicks were excited as evidenced by

increased spontaneous activities. Chicks in the present study exhibited more stress-related behaviors after CRH treatment. Zhang et al. (2003) also demonstrated that CRH-treated chicks attempted escape, as did our CRH-treated chicks.

The results obtained here are not consistent with those of Levine et al. (2005) who reported that obese humans spent a greater percentage of time in a seated posture compared to lean individuals. Time spent sitting was similar for the LWS and HWS lines, and the opposite effect from what was expected occurred for standing, where the HWS stood more than LWS. This effect may at first seem contradictory, however, when considering that the LWS chicks changed from a sitting to standing posture more frequently, this repeated change of behavior may burn more calories than maintaining a continuous posture.

The data from the crop emptying experiment demonstrate that the LWS line is more sensitive to the effects of central CRH than the HWS line. Decreased gastric transit is correlated with decreased appetite. The longer the GI system remains distended the longer satiety signals such as cholecystokinin and glucagon-like peptide-1 (Beglinger and Degen, 2004) will be released and affect appetite. A decreased transit time in response to CRH would also limit the rate of nutrient absorption. The HWS line with faster transit may have increased nutrient absorption simply because more nutrients pass through the gut.

Growth as measured by body weight at a chronological age (56 d in this experiment) is a highly complex trait. Genetic selection for low or high body weight will result in combinations of genes which directly and indirectly will influence acquisition and allocation of resources via behavior and metabolic processes. From these results we

conclude the differential responses to central CRH may, in part, be responsible for the body weights of these lines. This experiment demonstrates that chickens from lines differing in growth also differ in magnitudes of behaviors which and supports in that reguard the findings of Levin et al. (2005), and demonstrates differential gastric motility patterns between lines after central CRH treatment.

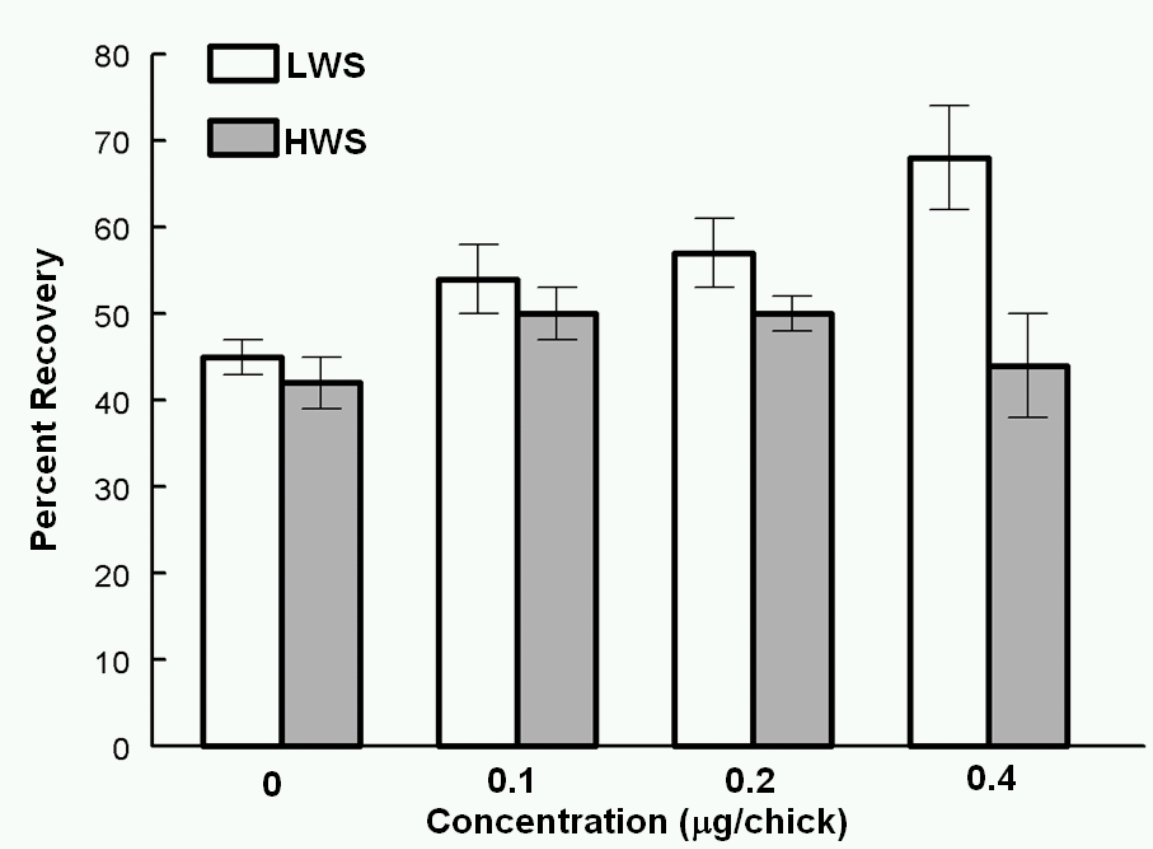


Figure 13.1. Percent crop content recovery (± SEM) 90 min post gavage in lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of artificial cerebrospinal fluid (aCSF) or 0.4 μg CRH.

Table 13.1. Behavior Quantification.

	Cumulative Jumps		Cumulative Steps		Cumulative Locomotion	
	aCSF	CRH	aCSF	CRH	aCSF	CRH
			Time 5			
LWS	0 ± 0	0.29 ± 0.18	1 ± 1[a]	83 ± 26[b]	1 ± 1[a]	19± 7[b]
HWS	0 ± 0	0.14 ± 0.14	18 ± 8[a]	88 ± 28[b]	3 ± 2[a]	22 ± 6[b]
			Time 10			
LWS	0 ± 0	0.43 ± 0.20	2 ± 1[a]	203 ± 49[b]	1 ± 1[a]	53 ± 14[b]
HWS	0.14 ± 0.14	0.14 ± 0.14	53 ± 25[a]	232 ± 49[b]	9 ± 5[a]	57 ± 13[b]
			Time 15			
LWS	0 ± 0	0.71 ± 0.36	4 ± 2[a]	318 ± 65[b]	1 ± 1[a]	87 ± 18[b]
HWS	0.14 ± 0.14	0.14 ± 0.14	109 ± 47[a]	379 ± 63[b]	21 ± 11[a]	93 ± 19[b]
			Time 20			
LWS	0 ± 0[a]	2.14 ± 1.18[b]	11 ± 7[a]	444 ± 85[b]	2 ± 1[a]	134 ± 25[b]
HWS	0.14 ± 0.14	0.29 ± 0.18	154 ± 70[a]	540 ± 82[b]	31 ± 17[a]	135 ± 28[b]
			Time 25			
LWS	0 ± 0[a]	2.86 ± 1.30[b]	27 ± 14[a]	571 ± 108[b]	4 ± 3[a]	181 ± 32[b]
HWS	0.14 ± 0.14	0.43 ± 0.30	184 ± 83[a]	721 ± 107[b]	36 ± 22[a]	181 ± 36[b]
			Time 30			
LWS	0 ± 0[a]	3.57 ± 1.39[b]	43 ± 22[a]	717 ± 129[b]	8 ± 4[a]	240 ± 41[b]
HWS	0.14 ± 0.14	1.00 ± 0.72	207 ± 95[a]	874 ± 120[b]	41 ± 24[a]	225 ± 42[b]

[a,b] Values with different superscripts differ ($P < 0.05$) within line between treatments per behavior.
Number of cumulative jumps, steps, and locomotion in lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of artificial cerebrospinal fluid (aCSF) or CRH. Values are means ± standard error.

Table 13.2. Number of cumulative pecks in lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of artificial cerebrospinal fluid (aCSF) or CRH. Values are means ± standard error.

	aCSF	CRH
	Time 5	
LWS	1 ± 1	0 ± 0
HWS	13 ± 7	1 ± 1
	Time 10	
LWS	1 ± 1	1 ± 1
HWS	54 ± 28[a]	1 ± 1[b]
	Time 15	
LWS	1 ± 1[c]	1 ± 1
HWS	116 ± 43[ad]	1 ± 1[b]
	Time 20	
LWS	3 ± 2[c]	2 ± 2
HWS	147 ± 58[ad]	1 ± 1[b]
	Time 25	
LWS	26 ± 17	2 ± 2
HWS	170 ± 72[a]	1 ± 1[b]
	Time 30	
LWS	26 ± 17	3 ± 2
HWS	217 ± 109[a]	3 ± 1[b]

[a,b] Values differ ($P < 0.05$) within line between treatments.
[c,d] Values differ ($P < 0.05$) between lines within treatment.

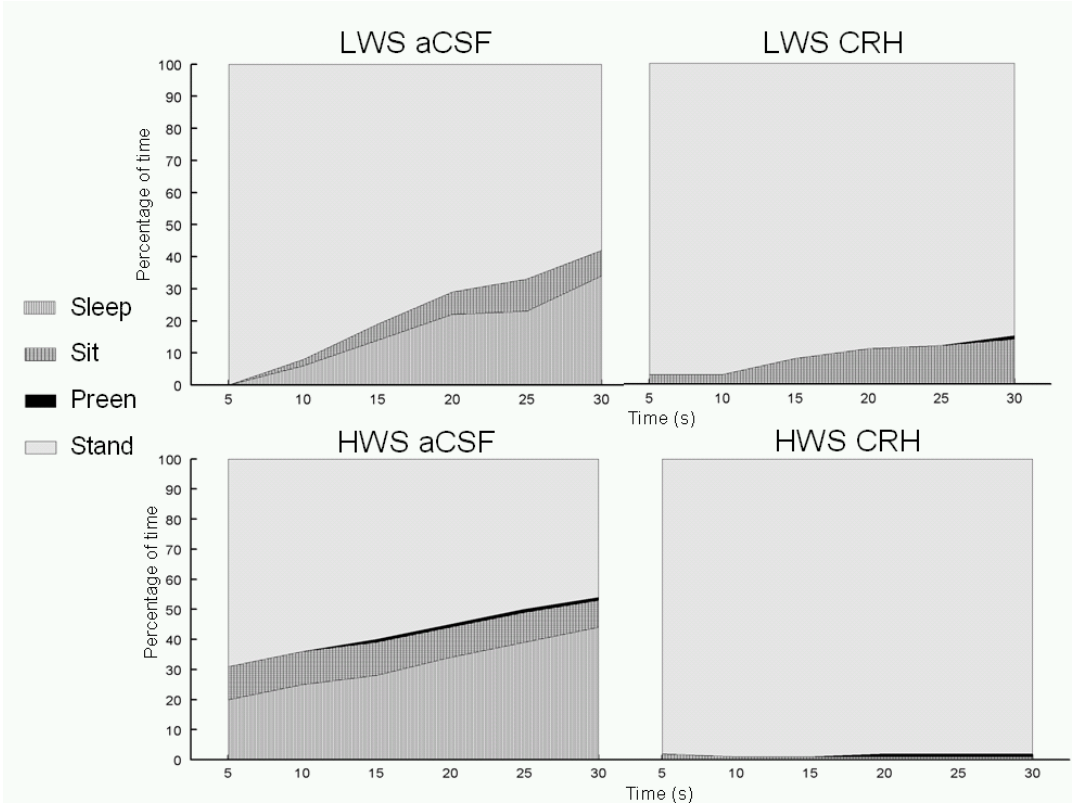


Figure 13.2. Ethograms depicting time-related behaviors in lines of chickens selected for low (LWS) or high (HWS) 56 day body weight following intracerebroventricular injection of artificial cerebrospinal fluid (aCSF) or CRH.

SECTION FOURTEEN
MOLECULAR ASPECTS OF CORTICOTROPHIN RELEASING HORMONE RECEPTOR TYPE TWO IN LINES OF CHICKENS ONE CONTAINING ANOREXIC AND THE OTHER OBESE INDIVIDUALS

Abstract

Corticotrophin releasing hormone receptor type 2 (CRHr2) is involved in appetite modulation. This experiment was designed to compare nucleotide sequences for the CRHr2 gene and its expression patterns in 17 organs from two lines of chickens, one selected for low body weight (LWS) that contains some anorexic individuals, the other selected for high body weight (HWS) that contains some obese individuals. Four polymorphisms in the nucleotide sequence that differ from the chicken genome project were found in both LWS and HWS lines. We also observed that CRHr2 was expressed in all organs tested, and that expression pattern differences exists between lines, sexes, and within lines and sexes. These differences in expression patterns may contribute to the altered body weight of these lines.

Introduction

Corticotrophin releasing hormone (CRH), a 41 residue peptide, is involved in stress reactivity (Vale et al., 1981). CRH governs the release of adrenocorticotropin hormone, which causes the release of glucocorticoids (Vale et al., 1983; Turnbull and Rivier, 1997). In addition to the hypothalamic-pituitary-adrenal axis, CRH effects several other body systems and processes (Dunn et al., 1990). Once such effect was demonstrated by Denbow et al. (1999) where CRH was found to be a potent inhibitor of appetite in chickens.

Three types of CRH receptors have been isloated. Complimentary DNA that bind a CRH receptor has been cloned from the human (Chang et al., 1993), rat (Perrin et al., 1993), and mouse (Vita et al., 1993; Xiong et al., 1995) and these receptors are designated CRHr1 with 98% sequence identity. A secondary CRH subtype, CRHr2, has also been cloned (Lovenberg et al., 1995; Stenzel et al., 1995; Perrin et al., 1995) from rodents that binds urotensin 1 and sauvarine with greater affinity than CRH. Chen et al. (1993) reported the human CRHr1 was identical to CRHr2 except that it contained a 29 amino acid insert that may be due to alternative splicing of a single gene. The two subtypes are located in different tissues and have different binding properties (Vale et al., 1997). A third subtype, CRHr3, was identified in the catfish where it is expressed in the pituitary and urophysis (Arai et al. 2001). CRHr3 has structurally greater similarity to CRHr1 than CRHr2 (Arai et al., 2001).

Smith et al. (1998) and Timpl et al. (1998) reported that CRHr1 induces the ACTH response to stresses. Pozzoli et al. (1996) found that CRH and glucocorticoids attenuate expression of CRHr1 in anterior pituitary cell cultures. Others report CRHr2 is

involved in coping mechanisms for stress such as anxiolysis, anorexia, hypertension and cardioprotection (Kishimoto et al., 1995; Bale et al., 2000; Coste et al., 2000; Hashimoto et al., 2001). Eghbal-Ahmadi et al. (1997, 1999) demonstrated that feed deprivation and sensory input alters CHRr2 mRNA concentration in the ventromedial hypothalamus.

Bray (1991) described the sympathetic nervous system as the principal governor of ingestion and body weight regulation. Bray (1991) concluded that most known obesities are the result of low sympathetic nervous system influence. Kuo et al. (2001) demonstrated that the low weight select (LWS) line has higher sympathetic output than do the high weight select (HWS) chicken. Moreover we recently found (Cline et al., unpublished data) that the LWS line has higher corticosterone concentrations under both normal and stressful circumstances. CRH is an activator of the sympathetic nervous system, and recently we demonstrated differences in appetite, gastric motility, and other behaviors after central CRH administration in both LWS and HWS lines (Cline et al., unpublished data). Since peripheral CRHr2 mRNA is affected by starvation (Naxarloo et al., 2002) we hypothesize that differences in the nucleotide sequence for CRHr2 between the HWS and LWS chickens or tissue-specific gene expression patterns may differ for CRHr2 between lines.

Materials and Methods

Animal Model

Our animal model is the result of a long-term divergent selection for high or low body weight at 56 days of age. Currently, these chickens differ in body weight at selection age by approximately nine fold (200 vs. 1820 g). For a review of the selection

program see Dunnington and Siegel (1996) and Siegel and Wolford (2003). According to Dunnington and Siegel (1997), some of the LWS line exhibit anorexic behaviors and many die as neonates simply from not eating even when chicks are bedded in mash feed. Siegel et al. (1984) reported that individuals in the HWS line are compulsive eaters and exhibit obesity. Therefore, these lines serve as models to study anorexia and obesity, and can provide insights into the physiology of eating disorders in other species (Zelenka et al., 1987), including humans. Chickens used in these experiments were from the 45th generation of selection.

Sequencing

Whole blood was collected from 10 LWS males, 10 LWS females, 10 HWS males, and 10 HWS females at 225 days-of-age. Genomic DNA was isolated using a DNA purification system (Gentra Systems). Utilizing the alignment of UI-R-BU0-apa-a-07-0-UI.s1 UI-R-BU0 Rattus norvegicus cDNA clone (Genbank 8486402), *Gallus gallus* mRNA for putative corticotropin-releasing hormone (Genbank GGA557031) and the reported chicken genomic sequence information for *Gallus gallus* clone WAG-77D19 (Genbank AC092081) 5 sets of primers were designed to amplify 5 non overlapping regions of CRHr2 coding regions in these lines. Samples were sequenced by fluorescent BigDye-terminator chemistry using an ABI3700 automated DNA sequencer (Perkin-Elmer, Foster City, CA).

Gene expression

Total RNA from brain, adrenals, proventriculus, gizzard, pancreas, gonad, lung, fat, kidney, muscle, crop, duodenum, cecum, large intestine, jejunum, liver, and ileum were isolated using the Tri-Reagent protocol (Molecular Research Center, Inc. Cincinnati, OH). Synthesis of single-strand cDNA, and reverse transcriptase (RT) reactions were carried out using random primers (Promega, Madison WI), MMLV reverse transcriptase (Promega, Madison WI), and 1 μg of total RNA in 50 μL reactions.

Left (GGAGTATCGATGCCTTCGG) and right (CAGAAGAAGTTGGTCACCACAA) primers were designed according to the alignments described in the sequencing section to amplify the chicken CRHr2 receptor. Amplification products were analyzed from both lines and sexes (n = 5 per line per sex). Real time PCR was performed (DNA Engine Opticon, MJ Research, Inc, Waltham, MA) to analyze CRHr2 gene expression, using a QuantiTech TM SYBERr Green PCR kit (Qiagen, La Jolla, CA). Amplification was carried out in a total volume of 25 μl containing 1x SYBER Green master mix, 0.3 mM of each primer, RNase free water and 1 μl of cDNA. Each sample was amplified with CRHr2 related or beta actin primers which served as an external control.

Analysis

Data were analyzed based on cycle threshold, the cycle number at which fluorescence is discernable from noise. This point appears during the exponential phase of the PCR reaction and is inversely proportional to the initial number of template molecules in the sample. Results were expressed as relative expression. The cycle

number for CRHr2 expression cycle threshold was calculated and normalized with the beta actin cycle threshold (Kuo et al., 2005; Meijerink et al., 2001). Data were analyzed using the GLM procedures of SAS (SAS, 1999) with line, sex, and the interaction between them as main variables. When interactions between line and sex were significant comparisons were made within line and sex. Results are reported as mean ± SE. Significance implies $P \leq 0.05$.

Results and Discussion

Four polymorphisms were detected between the lines used in this experiment and the chicken genome. All base pair positions described are in reference to the *Gallus gallus* chromosome 2. Both lines had a single polymorphism located between base pairs 3753564 and 5 where the sequence product had an insertion of a cytosine. Our sequence product has a cytosine whereas the chicken genome had an adenine at position 3753633. Additionally, between positions 3752464 and 5 our sequence product had an insertion of a cytosine. Finally, our sequence product had the deletion of two bases at positions 3727070 and 1.

The results of the gene expression experiment are summarized in Tables 14.1-14.3. CRHr2 was expressed in both lines in all organs tested. With respect to the digestive system line by sex interactions were found for the crop (LWS♂ > LWS♀); the duodenum (LWS♂ < LWS♀), and the cecum (LWS♂ < HWS♂, see Table 14.1). Males from both lines had lower expression than did females from both lines at the ileum (see Table 14.2). The HWS line had lower expression in the jejunum and liver than did the

LWS line (see Table 14.3). No differences were detected in the pancreases, gizzard, or proventriculus.

Differences were also detected in organs not related to the digestive system. Line by sex interactions were found in the muscle (HWS♀ > LWS♀); the kidney (LWS♀ > LWS♂); and in the fat (HWS♀ > LWS♀). Differences in the sexes were found for lung and gonad where females had higher levels than did the males. There were no differences in expression levels found in the adrenal or brain.

Jinxing et al. (1996) cloned a CRH receptor from the chicken which had higher affinity for urotensin and sauvagine than for CRH, a high degree of sequence conservation with the mammalian type CRHr1 but functioned more closely to the mammalian type CRHr2. The CRHr2 receptor is highly conserved through evolution and was cloned in the chicken recently by DeGroef et al. (2004). DeGroef et al. (2004) reported that chicken CRHr2 is composed of 412 residues and is a 7-transmembrane G protein coupled receptor and has 78 to 80% homology to mammalian CRHr2. This group also reported that although CRHr1 and r2 were expressed in the chicken brain, CRHr2 was more widely expressed in the periphery. Consistent with our findings DeGroef et al. (2004) found CRHr2 was expressed in every organ investigated.

Kuo et al. (unpublished data) recently described polymorphisms in the leptin receptor for the lines used here. Since leptin is a secretagogue for CRH, these differences may differentially modulate the CRH system in these lines. We conclude because the polymorphisms detected here were present in both lines these alterations were present in the base population and their contribution to the altered body weight phenotypes was miminal. The many expression pattern differences between sexes and the line x sex

interactions also do not easily lead to an involvment of the changes in body weight of these lines. Differences between lines detected in the jejunum may be one mechanism that decreases gastric transit time in the LWS line. Scharrer (1999) reported that fatty acid oxidation in the liver serves as a satiety signal. Since CRHr2 expression patterns differ at the liver between lines, this difference may influence ingestion via signals arising from the liver in response to the CRH system. However the action of CRHr2 at the liver has not been investigated in any species.

In conclusion we have observed 4 polymorphisms in the nucleotide sequence of the LWS and HWS lines that differ from the chicken genome. Since the differences exist in both lines, they probably do not contribute to the body weight differences between lines. We showed CRHr2 is expressed in both lines in all organs tested, and that expression pattern differences exist between lines, sexes, and within lines and sexes. These differences in expression patterns may contribute to the altered body weight of the two lines.

Table 14.1. Means ± SE of CRHr2/beta actin cycle threshold where line x sex interactions were significant.

Cecum		Male		Female
	LWS	2.16 ± 0.02	NS	2.17 ± 0.01
		*		NS
	HWS	1.97 ± 0.02	NS	2.18 ± 0.04
Crop		Male		Female
	LWS	1.71 ± 0.19	*	2.18 ± 0.01
		NS		NS
	HWS	2.03 ± 0.01	NS	1.94 ± 0.02
Duodenum		Male		Female
	LWS	2.14 ± 0.06	NS	1.97 ± 0.02
		NS		NS
	HWS	2.23 ± 0.02	*	1.95 ± 0.02
Fat		Male		Female
	LWS	2.09 ± 0.03	NS	1.76 ± 0.10
		NS		*
	HWS	2.06 ± 0.04	NS	2.15 ± 0.05
Kidney		Male		Female
	LWS	2.00 ± 0.02	*	2.16 ± 0.05
		NS		NS
	HWS	2.14 ± 0.04	NS	2.11 ± 0.02
Large Intestine		Male		Female
	LWS	1.97 ± 0.03	*	2.02 ± 0.05
		*		NS
	HWS	2.15 ± 0.03	NS	2.11 ± 0.02
Muscle		Male		Female
	LWS	2.09 ± 0.03	*	1.12 ± 0.03
		NS		*
	HWS	2.06 ± 0.04	NS	2.15 ± 0.05

* Means differ
NS Means do not differ

Table 14.2. Means ± SE of CRHr2/beta actin cycle threshold where sex interactions were significant.

Gonad	Male		Female
	1.48 ± 0.05	*	1.77 ± 0.06
Illeum	Male		Female
	2.14 ± 0.02	*	2.06 ± 0.03
Lung	Male		Female
	2.06 ± 0.03	*	2.14 ± 0.02

* Means differ

Table 14.3. Means ± SE of CRHr2/beta actin cycle threshold where line interactions were significant.

Liver	LWS		HWS
	2.06 ± 0.03	*	2.14 ± 0.02
Jejunum	LWS		HWS
	2.01 ± 0.03	*	2.14 ± 0.09

* Means differ

SECTION FIFTEEN
SYNTHESIS

This dissertation consists of a series of experiments designed to determine the contribution of the CRH system on appetite and body weight in two lines of chickens, one containing anorexic individuals and the other obese individuals. These lines which originated from a common base population, had undergone long term selection for juvenile body weight. The results can be used to explain neurological pathways effecting energy balance in chickens, and may also be used to understand body weight dysfunctions in other species including humans.

In the first set of experiments, the effect of ICV injection of CRH and urocortin on feed and water intake in the LWS and HWS lines was investigated. An ICV injection of either CRH or urocortin dose-dependently decreased feed intake in chicks previously fasted for 180 min. The magnitude of feed intake suppression after treatment with CRH was greater in the LWS than HWS line, and a higher concentration of urocortin was required to reduce appetite in the LWS than the HWS line. Water intake was not effected by treatment. From these results it was hypothesized the LWS birds were more sensitive to the effects of central CRH administration, and that CRH type 2 receptor activation was different in these lines. The LWS line may have a loss of CRHr2 function because higher concentration of urocortin was necessary to cause the same response as was observed in the HWS line. The results from this experiment demonstrate that CRH and urocortin act differently within the central nervous system in the LWS and HWS lines while having no effect on water intake.

Based on those results a second set of experiments explored the effects of the CRH receptor antagonists astressin, α helical CRH fragment (9-41) and CRH fragment 6-33 on feed intake in the LWS and HWS lines. All antagonists, when administered alone, caused an increase in feed intake in the LWS, but not in the HWS line. The three antagonists attenuated the appetite reducing effect of CRH in the HWS line but not in the LWS line. It was hypothesized that the LWS line had higher concentrations of natural CRH. Thus the activity of the CRH system was measured in each in line by assaying plasma corticosterone concentrations. The concentration of corticosterone was higher in the LWS than the HWS line under both normal and stressful circumstances. The percent incrase due to stress was similar in both lines. It was concluded that differences in the CRH system, primarily due to hyperactivity of the LWS line's hypothalamic-pituitary-adrenal axis and altered receptor activity, between these two lines, may be partly responsible for the altered body weight phenotypes observed in these lines.

During the course of the above series of experiments, it was observed that the LWS line seemed to respond to central CRH with increased stress-related behaviors. It was hypothesized that alterations in the hyperactive CRH system in the LWS line may, in synergism with the satiety signal exerted directly by CRH, reduce appetite. Thus, the effects of central CRH on behaviors of these lines was investigated. While both lines responded to treatment with increased anxiety-associated behaviors, the LWS line jumped more and attempted to escape sooner. Suggesting that they were more responsive than the HWS line. Pecking was decreased in the HWS, but not LWS line. This effect was not observed in the LWS line because the control birds did not peck. It was concluded that LWS chickens were more stressed in the observation chamber resulting in

reduced pecking even after the control treatment. This observation was supported by the finding the LWS line has higher concentrations of corticosterone during both normal and stressful circumstances. It was concluded from this experiment that the hyperactive CRH system in the LWS also reduced appetite by increasing anxiety-like behaviors that are competitive with ingestive behaviors.

It was also hypothesized that the hyperactive CRH system in the LWS line may influence the transit time of feed through the alimentary canal. Alterations in alimentary canal transit time may effect appetite through activation of stretch receptors, and by increased satiety and hunger signals being released from the gastric system traveling to the central nervous system. Thus, both lines were injected ICV with CRH and crop emptying was monitored. CRH decreased crop emptying in both lines. However, the LWS line responded with a linear dose-dependant decrease in crop emptying in response to central CRH while the response in the HWS line was quadratic. Thus, the HWS line had accelerated crop emptying in response to increasing concentrations of central CRH compared to the LWS line. It was concluded that the differential gastric responses to central CRH may, in part, contribute to the differences in body weights of these lines. If transit is slower in the LWS line, they simply would consume less feed because distension of the alimentary canal would increase the release of satiety signals, such as insulin like growth factor and CCK, and they would have a lengthened sensation of satiation.

The results of our experiments to this point could be interpreted that differences in the concentration of natural CRH in concert with alterations in the activity of its associated receptors may be partly responsible for differences in body weight between

lines. To test this hypothesis, experiments were designed to compare nucleotide sequences for the CRHr2 gene and its expression patterns in 17 organs from the two lines. We found four polymorphisms in the nucleotide sequence of the LWS and HWS lines that differ from the chicken genome project. Since the differences exist in both lines, it was concluded they do not contribute to the body weight differences between lines. We also showed CRHr2 is expressed in both lines in all organs tested, and that expression pattern differences exist between lines, sexes, and within lines and sexes. These differences in expression patterns may contribute to the differences in body weight between these lines.

To summarize this study, it is concluded that central CRH reduces appetite in both lines. The increase in body weight of the HWS line is not due to the absence of CRH activity (see Fig. 15.1). LWS chickens have higher concentration of corticosterone, and it is hypothesized they also have higher CRH concentrations. The LWS line is more susceptible to the effects of environmental stressors and gastric transit is slowed in the LWS line. Reduced gastric transit would result in the gastrointestinal tract being distended for a greater amount of time and would cause increased release of satiety signals from the small intestine such as CCK and GLP1. The CRH system may be hyperactive in the LWS line resulting in more satiety signals reaching the lateral hypothalamus, including CRH itself and corticosterone. In short, the LWS has stronger CRH related satiety signals reaching the central system than does the HWS line. Also, it can be concluded that the HWS line has a less active CRH signaling system which may contribute to their heavier body weight.

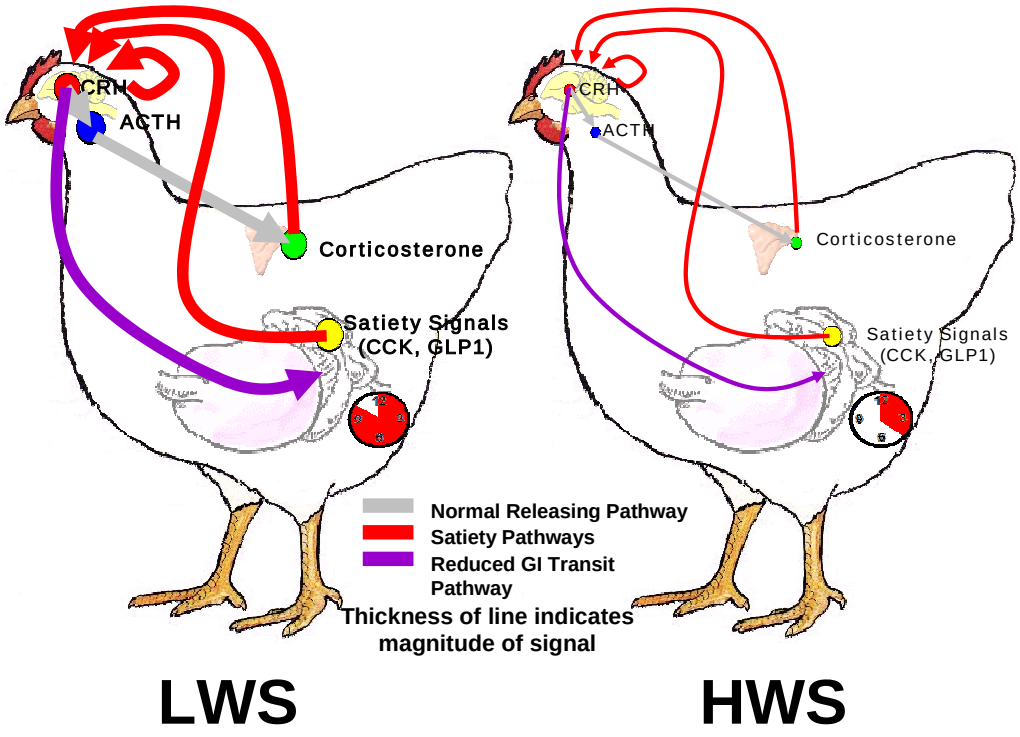


Figure 15.1. Proposed CRH systems in LWS and HWS lines of chickens related to their altered body weight. The LWS line (left) has greater concentration of CRH that results in greater release of ACTH from the pituitary. CRH at the level of the hypothalamus increases activation of the satiety center of the brain. Increased concentration of ACTH is correlated to increased concentration of corticosterone. Corticoserone is also a satiety signal. The increased CRH concentration also is responsible for decreased transit time through the alimentary canal, and increases the time it is distended. This effect results in increased release of gut-derived satiety signals that target the central system. In short, the LWS line has stronger satiety signals compared to the HWS line. The HWS line (right) undergo the same cascades, however, the concentration of signals is reduced, and the magnitude of gastric motility reduction is not as strong, resulting in fewer satiety signals from the gut.

SECTION SIXTEEN
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SECTION SEVENTEEN
CIRRICULUM VITAE
MARK ANDREW CLINE

Professional Employment

2005	**Assistant Professor of Biology**, Department of Biology, Radford University
2002-2005	**Instructor of Biology**, Department of Biology, Radford University
2002	**Instructor of Biology**, Upward Bound Program, Virginia Polytechnic Institute and State University
2002	**Instructor of Anatomy**, Governor's School, Virginia Polytechnic Institute and State University
2002	**Instructor of Agriculture Technology**, Department of Agriculture Technology, Virginia Polytechnic Institute and State University
1999-2001	**Graduate Teaching Assistant**, Department of Animal Sciences, Virginia Polytechnic Institute and State University

Education

2005	Doctor of Philosophy, Animal Science: Physiology - Virginia Polytechnic Institute and State University, Blacksburg. Area of specialization – Neurochemical regulation of behavior. Completion date: May 2005.
2002	Masters of Science, Animal Science: Physiology - Virginia Polytechnic Institute and State University, Blacksburg. Area of specialization – Manipulative reproductive physiology.
1999	Bachelors of Science, Animal Science, minor: Biology - Virginia Polytechnic Institute and State University, Blacksburg.
1996	Associate of Arts and Sciences - Blue Ridge Community College, Weyers Cave, Virginia.

Teaching Experiences

Fall 2004 **Concepts of Biology HONORS**, (Radford University, BIOL 105) lecture & laboratory, 4 credits, 25 students

This course was conducted totally electronically. Students came to class with a wireless laptop computer and electronic textbook.

An introduction to the basic processes of life and science. Emphasis was on scientific investigation and processes common to most organisms including humans: cellular structures and functions, mechanisms of inheritance, and mechanisms of adaptation. Applications were made to genetic technologies, human disorders, and bioethics.

Fall 2004 **Human Anatomy & Physiology** (Radford University, BIOL 322), lecture & laboratory, 6 credits, 50 students
The purpose of this course was to acquaint the student with the structure of the human body and the physiological mechanisms used to maintain homeostasis. Lecture devoted to physiological processes, while the laboratory was the major vehicle for presenting structure.

2004 **Principles of Biology II** (Radford University, BIOL 102), lecture & laboratory, 4 credits, 26 students
This course focused on the study of structures, functions, and interrelations of plants and animals to teach biological principles, accuracy in observation, independence in interpretation, and structure and function of the human body.

2004 **Human Structure & Function I** (Radford University, BIOL 310), lecture & laboratory, 4 credits, 26 students
Focused on the structure of the human body and the physiological mechanisms used to maintain homeostasis. Specifically, this first part of a two semester sequence, included cellular physiology, histology, integumentary system, osteology, nervous system and special senses, muscular system, cardiovascular system, and the respiratory system.

2002-2003 **Principles of Biology I**, (Radford University, BIOL 101) lecture & laboratory, 4 credits, 75 (Fall 2002) and 152 students (Fall 2003)
This course was an introduction to scientific methodology and unifying principles that affect all life, including the structure of cells, how organisms acquire and use energy, how genetic information is inherited, how an organism is affected by its living and nonliving environment, and how species evolve.

2003 **Human Structure & Function II** (Radford University, BIOL 311), lecture & laboratory, 4 credits, 56 students
Focused on the structure of the human body and the physiological mechanisms used to maintain homeostasis. Specifically, this second part of a two semester sequence, included hematology, gastrointestinal system, liver and spleen, temperature regulation, renal system and body fluids, reproduction and endocrinology.

2003 **Introduction to Higher Education** (Radford University, UNIV 100), lecture, 1 credit, 18 students
This course explored the meaning and value of a comprehensive liberal arts education, taught problem solving and decision-making processes, and promoted academic success through selected readings, presentations, discussions, and experiential learning opportunities.

2002 **Biology** (Virginia Polytechnic Institute and State University, Upward Bound program), 16 students
This course dealt with the study of animal and plant anatomy and physiology, ecology, and biological chemistry. This six-week course was part of the Upward Bound program and was intended to encourage and prepare disadvantaged high school students to pursue a college education.

2002 **Anatomy and Physiology** (Governor's School), 28 students
This short course dealt with basic anatomy and physiology of domestic animals and was an introduction to physiological research. Students enrolled in this course were high school seniors that entered college the following semester.

2001 **Animal Agriculture** (Virginia Polytechnic Institute and State University, AT 0164), lecture & laboratory, 4 credits, 50 students
This course dealt with the study of animal products, production methods and management systems for beef, sheep, horses, dairy, swine, goats, and poultry. Classroom instruction, demonstrations, and hands-on experience with livestock and poultry were a powerful learning tool for this course.

Directed Undergraduate Research Courses

2002-2005 **Directed Study and Research** (Radford University, BIOL 491), 2-3 credits, 9 students
Students were given first exposure to research by assisting in various pre-planned experiments. Results were disseminated at the Radford University Undergraduate Research Forum and/or the Virginia Academy of Science Meeting, and/or the National Conference on Undergraduate Research.

2004 **Undergraduate Research** (Radford University, BIOL 492), 3 credits, 1 student
The student was extensively involved in planning, orchestrating, and analysis of an animal behavior experiment.

2003 **Final Honors Project** (Radford University, BIOL 488), 2 credits, 1 student
The student conducted a literature search and prepared a manuscript based upon a feeding behavior experiment. Results were disseminated at the Annual Mid-Atlantic Regional Conference of Undergraduate Scholarship.

Invited Lectures

2004-2005 **Neurochemical Regulation** (Virginia Polytechnic Institute and State University, ALS 2304), 3 credits, 40 students
Topic covered: Synaptic transmission and the blood brain barrier.

2002-2003 **Animal Anatomy and Physiology** (Virginia Polytechnic Institute and State University, ALS 2304), 4 credits, 112 students
Topics covered: Respiration, muscle physiology, and the endocrine system.

1999-2000 2002 **Animal Agriculture** (Virginia Polytechnic Institute and State University, AT 0164)
Topics covered: Reproductive technologies, animal handling, animal health, and reproductive cycles and manipulation.

Graduate Teaching Assistant

2002 **Principles of Biology**, (Polytechnic Institute and State University, BIOL 1106), 3 credits, 200 students
This course dealt with the study of animal and plant anatomy and physiology, ecology, and animal behavior.

1999-2001 **Animal Anatomy and Physiology**, (Virginia Polytechnic Institute and State University, ALS 2304), laboratory instructor, 90 (1999), 60 (2000), & 60 (2001) students
This course dealt with the study of various aspects of animal anatomy and physiology.

Undergraduate Teaching Assistant

1997-1998 Undergraduate Teaching Assistant: Animal Anatomy and Physiology (Polytechnic Institute and State University, ASL 2304), 60 (1997) & 90 (1998) students
This course dealt with the study of various aspects of animal anatomy and physiology.

Graduate Mentoring of Undergraduate Research at Virginia Tech

2002-2004 Corticotropin releasing hormone modulation of feed intake in *Gallus gallus* (ALS 4994) 4 students

2002 Leptin modulation of feed intake in *Gallus gallus* (ALS 4994) 1 student

2001 Validation of luteinizing hormone assay (ALS 4994) 1 student

2000-2001 Efficacy of three synthetic GnRH products via their induced luteinizing hormone profile in *Ovis aries* (ALS 4994) 2 students

2000 Estrous synchronization via prostaglandin $F_{2\alpha}$ and GnRH in *Ovis aries* (ALS 4994) 1 student

1999 Relationship between nutritional status and superovulation in *Ovis aries* (ALS 4994) 1 student

1999 Ultrasonography after superovulatory treatment in *Ovis aries* (ALS 4994) 1 student

Professional Presentations by Directed Undergraduates

2005 Smith, Marissa and Mark Cline. Effect of intracerebroventricular administration of enterostatin on ingestive behaviors in goldfish (Carrasius auratus). Paper presented at the 83rd Annual Virginia Academy of Science Meeting, Harrisonburg, VA.

2005 Nandar, Wint and Mark Cline. Effect of intracerebroventricular administration of amylin on feed and water intake in chicks. Paper presented at the 83rd Annual Virginia Academy of Science Meeting, Harrisonburg, VA.

2005 Twimasi, Catherine and Mark Cline. Effect of intracerebroventricular administration of enterostatin on feed intake in chicks. Paper presented at the 83rd Annual Virginia Academy of Science Meeting, Harrisonburg, VA.

2005 Nandar, Wint and Mark Cline. Effect of intracerebroventricular administration of amylin on feed and water intake in chicks. Paper presented at the National Conference on Undergraduate Research, Lexington, VA.

2005 Twimasi, Catherine and Mark Cline. Effect of intracerebroventricular administration of enterostatin on feed intake in chicks. Paper presented at the National Conference on Undergraduate Research, Lexington, VA.

2005 Smith, Marissa and Mark Cline. Behavioral effects following intracerebroventricular administration of agouti-related peptide in juvenile Gallus gallus. Paper presented at the National Conference on Undergraduate Research, Lexington, VA.

2005 Nandar, Wint and Mark Cline. Effect of intracerebroventricular administration of amylin on feed and water intake in chicks. Paper presented at the 14th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2005 Twimasi, Catherine and Mark Cline. Effect of intracerebroventricular administration of enterostatin on feed intake in chicks. Paper presented at the 14th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2005 Smith, Marissa and Mark Cline. Behavioral effects following intracerebroventricular administration of agouti-related peptide in juvenile Gallus gallus. Paper presented at the 14th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2005 Smith, Marissa and Mark Cline. Behavioral effects following intracerebroventricular administration of neuropeptide Y in juvenile Gallus gallus. Poster presented at Experimental Biology 2005.

2005 Smith, Marissa and Mark Cline. Behavioral effects following intracerebroventricular administration of neuropeptide Y in juvenile Gallus gallus. Poster presented at American Physiological Society Undergraduate Poster Session, San Diego, CA.

2004 McKinney, J. L., M. A. Cline, D. M. Denbow and P. B. Siegel. Urocortin reduces ingestive behavior in obese and anorexic juvenile chickens. Paper presented at the 82nd Annual Virginia Academy of Science Meeting, Richmond.

2004 Smith, M. L., M. A. Cline, D. M. Denbow and P. B. Siegel. Effect of central injection of corticotrophin releasing hormone on gastric motility in obese and anorexic chickens. Paper presented at the 18th National Conference of Undergraduate Research, Indiana University-Purdue University, Indianapolis.

2004 Howard, B. A., M. A. Cline, D. M. Denbow and P. B. Siegel. Inhibition of feed intake after central injection of corticotrophin releasing hormone in obese and anorexic juvenile chickens. Paper presented at the 13th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2004 McKinney, J. L., M. A. Cline, D. M. Denbow and P. B. Siegel. Urocortin modulation of feeding behavior in obese and anorexic juvenile chickens. Paper presented at the 13th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2003 Pariser, M., M. Cline, A. Y. Kuo, D. Denbow and P. Siegel. Effects of intracerebroventricular injection of urocrotin in obese and anorexic models. Paper presented at the 5th Annual Mid-Atlantic Regional Conference of Undergraduate Scholarship, Sweet Briar College, Sweet Briar, Virginia.

2003 Brooks, N., M. Cline, D. Denbow and P. Siegel. Urotensin I induced inhibition of feeding behavior. Paper presented at the 12th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2003 Smith, M. L., M. A. Cline, A. Y. Kuo, D. M. Denbow and P. B. Siegel. Corticotrophin releasing hormone modulation of gastric emptying in obese and anorexic chickens. Poster presented at the 12th Annual Undergraduate/Graduate & Student Engagement Forum, Radford University.

2001 Umgarger, S., M. Cline and J. Hall. Efficacy of synthetic GnRH analogs for estrous synchronization in sheep. Poster presented at the 17th Annual Research Symposium of Virginia Polytechnic Institute and State University.

1999 King, L. M., M. A. Cline, G. S. Lewis and M. L. Wahlberg. Effects of nutritional status and superovulatory treatment on ovulation rate in sheep. Paper presented at the John Lee Pratt Animal Nutrition Senior Research Scholarship Symposium, Virginia Polytechnic Institute and State University.

Research Experience (Lead Investigator)

2002-2005	Corticotrophin releasing hormone and associated peptides modulation of feed intake and body mass in obese and anorexic chickens
2002-2004	Corticotrophin releasing hormone modulation of gastric motility in obese and anorexic chickens
2004	Corticotrophin releasing hormone and associated peptides affect on behaviors in obese and anorexic chickens
2000-2001	Comparison of ovulation synchronization protocols utilizing synthetic GnRH
2001	Efficacy of two synthetic GnRH analogs via induced luteinizing hormone profiles
1999-2000	Site of embryo deposition during embryo transfer in *Ovis aries*
1997	Determination of ovulation after injection of PMSG or P.G. 600®

Collaborative Experiments

2004-2005	Cloning of the CRH gene from obese and anorexic chickens, USDA
2003	Leptin modulation of body weight regulation in *Gallus gallus,* VA Tech
2003	Gherlin modulation of body weight regulation in *Gallus gallus,* VA Tech
1998	Oxytocin induced cervical dilation for artificial insemination in *Ovis aries,* VA Tech
1997	Induced cervical dilation: evaluation of the effects on fertilization rates and embryonic development in *Ovis aries,* VA Tech
1997	Uterine response to multiple exposures of *Escherichia coli* and *Arcanobacterum pyogenes* in *Ovis aries* and *Sus scrofa,* VA Tech

Professional Publications and Presentations

King, J., K. Terry, M. Cline, E. Oakes, P. Christensen, N. Sigmon, L. LeMay and S. Gentry. Teaching and Learning in a Wireless Environment with Tablet PCs: Advantages and Limitations. Abstract submitted to Syllabus 2005.

Cline, M. A., M. L. Smith, A. Y. Kuo, D. M. Denbow and P. B. Siegel. Central corticotrophin releasing hormone induces different behaviors in lines with obese and anorexic chicks. Abstract submitted to Experimental Biology 2005.

Kuo, A. Y, C. M. Ashwell, M. P. Richards, S. M. Poch, M. A. Cline, P. B. Siegel and D. M. Denbow. in press. Ghrelin gene sequence and expression in lines of chickens selected for high and low body weight. Physiology and Behavior.

Kuo, A. Y., M. A. Cline, E. Warner, P. B Siegel and D. M. Denbow. *in press.* Effects of human recombinant leptin on food and water intake in line of chickens selected for high and low body weight. Physiology and Behavior.

Hall, J. B., W. D. Whittier, J. Myers, M. A. Cline and D. Cuddy. *in press*. GnRH based estrus synchronization systems for beef cows. Journal of Animal Science.

Cline M. A., J. B. Hall and W. D. Whittier. 2002. Efficacy of commercial GnRH preparations for use in the Ovsynch synchronization protocol: beef cattle LH profile. NCR-87 2001 Annual Report.

Cline, M. A., J.B. Hall and W.D. Whittier. 2002. Efficacy of synthetic GnRH analogs for estrous synchronization. Joint Meeting of American Dairy Science Association and American and Canadian Societies of Animal Science, Quebec, Canada.

Whittier, W.D., J. B. Hall, A. Britt and M. A. Cline. 2002. Effect of GnRH dose used in the Ovsynch system on AI pregnancy rates in beef cows. Annual Meeting of the American Association of Bovine Practitioners, Madison, Wisconsin.

Cline M. A., J. B. Hall and W. D. Whittier. 2001. Efficacy of commercial GnRH preparations for use in the Ovsynch synchronization protocol: beef cattle field trials. NCR-87 2001 Annual Report.

Cline M. A., J. N. Ralston, R. C. Seals and G. S. Lewis. 2001. Intervals from norgestomet withdraw and injection of equine chorionic gonadotropin or P.G. 600® to estrus and ovulation in ewes. Journal of Animal Science. 79:589-594.

Cline M. A., J. N. Ralston, R. C. Seals and G. S. Lewis. 1997. Determination of ovulation time in sheep after injection of PMSG or P.G. 600®. Journal of Animal Science. 75(Supplement 1):25.

Cline M. A., J. N. Ralston, R. C. Seals and G. S. Lewis. Determination of Ovulation Time in Sheep After Injection of PMSG or P.G. 600®. Paper presented at the Southern Section of the American Society of Animal Science Annual Meetings, Feb. 1998.

Professional Affiliations, Organizations and Societies

American Association for Higher Education
American Association for the Advancement of Science
American Physiological Society
Association of College and University Biology Educators
The Human Anatomy and Physiology Society
Virginia Academy of Science