ABSTRACT

Title of Document:	IDENTIFICATION OF NOVEL MECHANISMS IN THE HYPOTHALAMUS LINKED TO ADIPOSITY.
	Mardi S. Byerly, Ph.D., 2007
Directed By:	Professor Tom E. Porter, Department of Animal and Avian Science

Body composition is a trait regulated by many different genes, giving rise to the severity of the phenotype in a continuous manner. We propose to investigate genetic interactions regulating obesity by focusing on genetic heretibility and genes that interact with Brain-Derived Neurotrophic Factor (BDNF) or thyroid hormone. We have utilized a novel model of obesity, genetically selected chicken lines, because differences in abdominal fat between the lines are based on genotype alone, independent of environmental influences. In order to identify novel gene networks that might give rise to the obese phenotype, hypothalamic gene expression was characterized using microarray technology. Hypothalamic neuronal cell cultures and *in vivo* manipulations were performed to verify that BDNF interacts with the hypothalamic-pituitary-thyroid axis, while simultaneously identifying novel genes that may interact together or independently of BDNF. A hypothetical model suggesting how BDNF and thyroid hormone reciprocally modulate genes in the hypothalamus already known to contribute to energy regulation was developed.

IDENTIFICATION OF NOVEL MECHANISMS IN THE HYPOTHALAMUS LINKED TO ADIPOSITY.

By

Mardi S. Byerly

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2007

Advisory Committee:
Professor Tom E. Porter, Chair
Professor Mary Ann Ottinger
Professor Thomas W. Castonguay
Professor Timothy H. Moran
Professor William R. Jeffery

Dedication

I dedicate this work and thesis to those who have loved me endlessly and supported me to reach this accomplishment, specifically, my family, my life long best friend, Stephanie Balmer, and my dog, Dexter.

A person can only be so lucky to have a best friend like Stephanie and I had the fortune of finding her friendship at the early age of 8. Stephanie is a passionate person with love and joy oozing out of her to the degree that it infects those around her. She is amazing, beautiful and has influenced me in so many ways. I remember spending our lunch hours during junior high huddled over books reading about calories, dieting and food. What else would an aspiring ballet dancer and her best friend do during that time? Her dedication, joy, sense of fashion and style has impacted my life in every way.

My other best friend is my brother, Robert Byerly – a true genius that walks every step in his life with earnest dedication, a man who has my respect, admiration and a role model throughout all the days of my life. I have followed his footsteps, trusted in him and headed his advice. My brother amazed me by always earning straight A's, while I received that darn C in penmanship, becoming a valedictorian, and delivering great speeches that left audiences emotionally moved. Now he is a father of two beautiful and joyful boys, Tommy and Michael, and a husband to an amazing, beautiful woman, Michelle. He is a force of power that causes people to be silent when he speaks, yearning to hear the next profound thought that will exit his mouth. I am fortunate to have him as a brother, for he always makes me laugh, making my stomach ache we laugh so hard, since no one knows me better.

I dedicate this to my parents, Gail and Robert, who raised me right. They gave me the strength to believe in myself and instilled in me the knowledge to persevere, so I can always keep trying. Their love for me is undeniable and I am blessed to have such great characteristics from both of them. My mother is strong, intelligent and loving in that she always says it simple and straight. I thank her for my strength to believe in myself and to fight for what I know I can achieve. My father is also intelligent, charismatic, gentle, loving, and a get-along-with-the-world type of guy who will always pick me up when I fall down, tell me to try again and to never stop trying. In some ways, my parents are opposite, but the combination of these opposites provides a force that should not be reckoned with. Thank you, mom and dad, for raising me and always loving me unconditionally.

Lastly, I dedicate this to my most loyal companion over the last 10 year, Dexter the best dog that ever walked the face of the earth, at least this is my opinion. I thank him for always being there, for wagging his tail and being so happy to see me every day, every morning when I open my eyes, and every night when I fall asleep. He has made my life easier while completing this dissertation. He is everything great I ever imagined a dog could be.

Acknowledgements

I would like to acknowledge all my friends, across the US, who have made my time in graduate school so enjoyable. They know who they are and they know that I care very much about all of them. I will miss them as we all move on and move away, but the good times will always live in my memory and close to my heart.

I would also like to acknowledge my committee members. I am so lucky to have brilliant enjoyable people to guide me through this time in my life, both here at the University of Maryland as well as Purdue University. Dr. Tom Porter, while I traveled along the road to complete my research in your lab I often felt confused about the path that I was being led down, but now at the end of the path I look back and I truly believe that you did an amazing job in guiding me and more importantly in helping me grow so that I have confidence and believe in myself as a scientist. You were always there for me to ask a question and were an excellent mentor. Dr. Ed Fox, I thank you for you friendship and for taking me under your wing to teach me everything about science in a crash course. Thank you so much for allowing me to ask questions with no judgment in return. Dr. William Jeffery, I am grateful that you guided me in my "side" projects and showed me that it is possible to be genuinely interested in research unrelated to obesity and food intake. Thank you for teaching me about eye development and giving me projects to work on. Every moment in your lab has been enjoyable and I am grateful for the opportunity. Lastly, I would not have been able to complete this work without the help and support of my collaborators. I would like to thank Larry A. Cogburn, University of Delaware, and those at Institute

Nationale de la Recherches Agrinomique, Jean Simon, Michel Duclos and Elisabeth Lebihan-Duval.

Table of Contents

Chapter 1: The Obesity Epidemic and Eating Disorders

Introduction

The obesity epidemic around the globe has risen 3-fold or more over the past 20 years; currently, one billion adults are overweight with 300 million of them being obese[1]. In several developed countries the cost of health related issues associated with obesity encompasses 2-6% of total health related expenses, with some areas being as high as 7%[2]. Elucidation of mechanisms that regulate food intake and body weight will allow development of novel treatments to alleviate the severity of health care costs and improve the quality of life for the obese. In addition, twenty-two million children under the age of 5 are classified as obese, with the number having doubled since 1980[2]. Obesity results from an imbalance between food intake and energy expenditure or a lack of physiological feedback required to maintain an appropriate level of food intake and body weight[3]. Lesions of the lateral nuclei of the hypothalamus result in obesity and lesions of either the ventromedial or paraventricular nuclei result in an abnormal increase in food intake, suggesting that the hypothalamus exerts significant control in regulation of food intake and body weight[4,5]. Therefore, we propose to examine hypothalamic gene expression during development of obesity in a genetically selected fat and lean animal model.

Eating disorders often arise from the following three factors: 1) environmental influences alone, 2) genetics alone or 3) environment acting on genetic predisposition that increases an individual's risk for expressing an eating disorder. Examples of

eating disorders are obesity, anorexia nervosa, bulimia nervosa and binge eating disorder[6,7]. Eating disorders encompass disturbances in energy balance which are caused by or result in food intake alterations that are aberrant or compulsive in nature and genetically are polygenic and multifactorial (a number of genes each with small effects that contribute to the severity of the phenotype)[3]. Obesity is considered an eating disorder because over eating (hyperphagia) is one trait associated with the phenotype with intake usually being compulsive in nature, not easily controlled and initiated by cues in the surrounding environment. Anorexia and bulimia are similar to obesity in that they have extremes in food intake and obsessions[6]. Increased or decreased food intake and obsessive behaviors resemble addictive behavior in that it is modulated by neurochemical abnormalities and the risk for developing such disorders is most often determined by an underlying genetic predisposition that increases an individual's susceptibility to develop the disorder. It has been shown that alterations in neurotransmitters, neuropeptides and neuroendocrine functions can contribute to these differences[8].

Obesity, hereditability and genetics

Hereditability is a factor associated with obesity since the likelihood of developing this phenotype increases when a biological relative is obese: twins, 50-80%; intermediate family, 30-50%; adoption, 10-30%[9]. Genetic implications associated with obesity are further supported by the fact that the phenotype appears as a continuously distributed phenotype, providing evidence that the severity of the phenotype depends on which alleles segregate together, as well as the number of genes associated with the phenotype[10-18]. Similar to humans, hereditability of

adiposity is also present in the chicken model of obesity[19]. The Fat and Lean lines used in our studies are an ideal model for investigating neural gene expression regulating food intake, metabolism, and body weight in the hypothalamus since intercrossing of the two lines results in a normal distribution of these phenotypes, indicating that the phenotypes arise because of quantitative traits and not from a spontaneous, deletional or point mutation[19,20].

Quantitative traits are phenotypes that arise from genetic influences that reside on multiple genes, and a model such as this is a powerful tool to investigate genetic interactions[19]. One rat model utilizing quantitative traits was bred to be susceptible to dietary-induced obesity (DIO) or to be diet resistant (DR) for developing obesity, allowing investigation of a rodent model to determine how environment and genotype interact to influence the susceptibility for obesity[21]. To our knowledge this is the only known quantitative trait model of obesity present in the rodent species. Thus, the Fat and Lean lines of chicken might currently be the only known animal model of obesity arising from genotype alone, more specifically, adiposity that develops independently of environmental influences. We strongly believe that the chicken is an excellent but under utilized model organism for studies of weight regulation as well as an ideal model to investigate genetic interactions that influence expression of the obese or lean phenotypes.

The Brain and Obesity

The hypothalamus is quite important in modulating energy balance throughout the entire animal, but it is not the only brain region implicated in the control of eating disorders. For example, intrinsic activation, arising from internal stimuli like hunger,

activates the hypothalamus, insula, amygdala, anterior cingulated, thalamus and brainstem[22]. On the other hand, satiety activates the orbitofrontal cortex (OFC), bilateral occipital cortex, inferior temporal cortex and posterior temporal region. Extrinsic factors, such as palatable foods, activate the amygdala and OFC[22] and have been shown to also activate the gustatory cortex, striatum, midbrain, subcallosal region and caudomedial orbitofrontal cortex[7].

So, how do these brain regions differ in an obese relative to a non-obese individual? It has been suggested that obese individuals have decreased electrical activation in the hypothalamus after a meal, relative to a lean individual[7]. The activation in the prefrontal cortex and the limbic/paralimbic areas also demonstrate differences between obese and lean individuals. For example, the prefrontal cortex influences other areas of the brain in order to suppress food intake, with the prefrontal cortex actually suppressing activity in the hypothalamus (i.e. lateral hypothalamus), thalamus, limbic system (olfactory cortex, amygdala, and hippocampus) and the basal ganglia[23]. Obese individuals differ from lean individuals given that they have increased activation of the prefrontal cortex and decreased activation in the limbic/paralimbic system. To elaborate a bit further, it has been shown that after a meal, posterior cingulated, amygdala, insular cortex and primary ingestive cortex activity is different between lean and obese individuals[23] and strikingly, this activity in 2 out of the 4 regions (the posterior cingulate and the amygdala) could return to normal if obese individuals returned to normal weight. Not all brain regions return to normal, it suggesting that the areas that remain abnormal might contribute to the

rebound effect of weight gain that most obese individuals experience after losing weight.

The nucleus accumbens and the ventral tagmental areas have both been implicated in eating disorders and addiction, which may function to reinforce the eating disorder through positive rewards via dopamine receptors[24]. The ventral striatum and the prefrontal cortex are regions known to regulate incentive learning and reinforcement mechanisms associated with positive rewards through dopamine receptors[25]. However, it should be noted that addictive behaviors to drugs, termed unnatural since this is a learned behavior, are different than addictive behaviors to food due to the nature of the reward; food addiction is termed natural since the behavior is driven by internal physiological cues. It is the natural rewards, like food, that may work independent of dopaminergic signals in this area, but the unnatural rewards, induced by drugs, work via dopaminergic signaling pathways. This will be described in more detail later. Thus, alterations in dopaminergic working in other brain regions than this and endorphin signaling in these brain regions may give rise to the eating disorders that have addictive properties.

Anorexia and bulimia involve alteration to the serotonergic signaling pathway in the frontal, temporal, cingulated and parietal regions[6]. Although both eating disorders have alterations in the same signaling system, there are differences between the two. First, Kaye et.al.[6] (2005) demonstrated similarities between anorexia and bulimia by showing reduced serotonin 2A receptor activity in the subgenual cingulate, mesial temporal (amygdala), and parietal cortical regions. Differences between anorexic and bulimic individuals were then demonstrated by showing that

serotonin 1A receptor functions in bulimia, but it is the 2A receptor that functions in anorexia[6]. The brain region that likely modulates both the anorexic and bulimic phenotype is likely the amygdala signaling pathway to the cingulate. This alteration in serotonin signaling relates to addictive behavior, given that it can also influence extremes of impulse control, perhaps giving rise to a genetic susceptibility that might influence the development of an eating disorder[26] through addictive processes that resemble reward deficiency syndrome[24].

Obesity, food intake and neuropeptides in the hypothalamus

The hypothalamus is a primary modulator of metabolism, food intake and body weight. It has been previously shown that ablating specific nuclei within the hypothalamus can result in altered food intake[4,5]. For example, hunger has been associated with the lateral nuclei and satiety with both the paraventricular (PVN) and ventromedial (VMH) nuclei. Similar to mammals, lesions in the VMH of the chickens result in an obese phenotype[27]. Given this, it is probable that neural circuitry regulating food intake and body weight in the hypothalamus is evolutionarily conserved. Hypothalamic nuclei contain either orexigenic/anabolic neuropeptides (i.e. Neuropeptide Y, NPY; Agouti-related protein AGRP) or anorexigenic/catabolic neuropeptides (i.e. proopiomelanocortin, POMC) and regulate body weight, metabolism and food intake via expression of these neuropeptides. For example, increasing leptin levels, a hormone released from adipose tissue, increases activity and neuropeptides located in orexigenic neurons of the PVN, result in increased food intake[8].

Schwartz et.al. (2000) reviewed how this neuronal circuitry functions. Normally, decreased leptin, as well as insulin, inhibits POMC signaling leading to a decrease in α-melanocyte-stimulating hormone (αMSH) levels, a product of POMC cleavage by POMC converting enzymes[28]. Decreased food intake results when αMSH decreases melanocortin (MC) receptor signaling, since αMSH is a ligand for the Melanocortin-4 receptor (MC4R). Working through another pathway, the decreased leptin and insulin levels activate the NPY/AGRP neuron by increasing production and release of AGRP. This results in increased food intake since AGRP is an inverse agonist for MC4R, preventing further binding of αMSH to the MC receptor and resulting in the opposite phenotype [2 9]. The melanocortin pathway is unique in the sense it has a naturally occurring agonist (αMSH) and inverse agonist (AGRP) that serve to modulate signaling.

Brain-Derived Neurotrophic Factor (BDNF) and Obesity

One neuropeptide we would like to focus on is BDNF because it has been implicated in the etiology of eating disorders[30-35]. BDNF mutant mice (+/-) were first created to investigate its role in sensory neuron development[36]. However, it was noticed that both obesity and hyperphagia were phenotypes present in BDNF haploinsufficient mice[32,34]. Of even greater interest, conditional mutants lacking BDNF only in the brain after birth, mutants that eliminate confounding developmental effects, demonstrate an even greater obesity than the haploinsufficient mice, suggesting that modulation of body weight is directly related to BDNF expression in the brain[35]. BDNF has also been suggested to be part of a mechanism

underlying mature-onset obesity due to increased intake, implying that it may play a role in the development of obesity over time due to hyperphagic intake[30].

Altered levels of BDNF have been associated with anorexia, obesity, bulimia[31,37], and alcoholism[38]. Three BDNF variants and a polymorphism were identified by screening patients with obesity, eating disorders and attention deficit hyperactivity disorder[31]. Mechanisms that contribute to obesity and alcoholism appear to be influenced by decreased BDNF expression, since it has been shown that decreased levels of BDNF lead to development of obesity[35] and increased behavioral response to alcohol exposure[38]. On the other hand, opposite changes in BDNF expression were observed in humans with obesity and anorexia, with decreased levels in obese humans and increased levels in anorexics[37]. It is plausible that the molecular signals that respond to increased or decreased levels of BDNF could contribute to the development of these specific phenotypes. For example, endorphin levels are increased in an anorexic state[39] and this increase in opioids could be interacting with or modulating BDNF expression. This idea has been supported since an opioid receptor agonist increased levels of BDNF[40]. In addition to this, BDNF interacts with other signaling pathways to mediate these addictive phenotypes. For example, BDNF has been shown to regulate expression of the dopamine 3 receptor, a receptor in the nucleus accumbens that has been associated with addictive behaviors[41]. Lastly, polymorphisms and variants in the BDNF gene are associated with development of the obese, anorexic or bulimic phenotype[31]. However, it remains to be determined exactly how these polymorphisms and variants contribute to development of the phenotype. For example, are they contributing independent of environmental

influences or do these variants create a genetic susceptibility that induces the phenotype after experience.

It is plausible that BDNF might induce a phenotype only after experience, given that Fox and Byerly[30] demonstrated that BDNF haploinsuffecient mice (heterozygous for the BDNF gene) do not exhibit hyperphagic intake (increased intake) or obesity if they are fed a well balanced diet as young animals, but at 8 months of age the animals over consume food and are obese regardless of the diet they consume. Even though young BDNF haploinsufficient mice do not over consume food on a well balanced diet until later in life, environmental influences can induce the hyperphagic intake in the young animals if they are given a high-fat diet. However, the obese phenotype still did not develop at the earlier age even when the high-fat diet was over consumed for a two week period of time. Given this, it is plausible that the high-fat diet and the decreased BDNF expression interact with another signaling system that induced hyperphagic intake without the obese phenotype being present, such as the melanocortin system[42].

Neuroendocrine system and obesity

Thyroid Hormone

Thyroid hormones are produced in the thyroid gland, with thyroxine (T_4) and low levels of triiodothyronine (T_3) forming within thyroglobulin[43]. Thyroid hormones are known to modulate many aspects of metabolism, primarily by increasing basal metabolic rate after fasting or refeeding. This process contributes to energy homeostasis by matching energy intake and energy expenditure over time. If thyroid

hormones are in excess (hyperthyroid), this leads to negative energy balance and weight loss because the basal metabolic rate is increased relative to energy intake. When thyroid hormone levels are minimal (hypothyroid) this results in weight gain because basal metabolic rate is decreased relative to energy intake resulting in a positive energy balance. Interestingly, euthyroid morbidly obese individuals are not able to maintain this balance and have an overall positive energy balance despite slightly elevated levels of T_3[44]. Thyroid hormones are also considered tissue growth factors because they contribute to such things as neuronal development and protein synthesis.

Thyroid hormone deiodinases are enzymes that convert T_4 to T_3, as well as inactivate the thyroid hormones[45]. Type I deiodinase is present in the periphery and converts T_4 to T_3, Type II deiodinase is in the brain and converts T_4 to T_3 with 1000 times greater affinity than Type I[46]. The TRH neurons located in the paraventricular nucleus (PVN) of the hypothalamus respond to T_3 levels either in the periphery or from other brain regions, since there is very low, if any, Type II deiodinase present in the PVN[47]. Tanycytes are glia located along the inferior portion of the third ventricle and are innervated by peptidergic and aminergic neurons[48-50]. Tanycyte cells are believed to be the structure that links ventricular cerebrospinal fluid (CSF) to the arcuate nucleus of the hypothalamus, suggesting that this anatomical structure is key in linking neuroendocrine events with CSF[49]. Tanycytes also have a high concentration of Type II deiodinase and are thought to be the primary source in the brain signaling to TRH neurons by converting T_4 to T_3, as well as the arcuate nucleus

and in lower concentrations in the ventromedial nucleus, dorsomedial nucleus and lateral hypothalamus[51].

Brain-Derived Neurotrophic Factor and Thyroid Hormone

Hyperthyroidism has been associated with increased food intake. There is currently a small body of literature providing evidence that BDNF may interact with or be modulated by the hypothalamic-pituitary-thyroid axis. Since decreased levels of BDNF and increased levels of thyroid hormone (T_3) have both been associated with hyperphagia, we are proposing to further investigate the interaction between BDNF and the HPT axis. It has previously been shown that hypothalamic neurons treated with BDNF enhance the expression of thyrotropin releasing hormone (TRH) mRNA[52]. Adult neurons in the band of Broca treated with T_3 have suppressed BDNF expression[53]. Recently it has been shown that BDNF can regulate expression of pre-pro-TRH in the PVN[54]. Additionally, this interaction may also involve neuropeptides known to modulate food intake and energy expenditure, such as NPY. Increased levels of T_3 result in increased expression of NPY, along with CART and POMC[55], and BDNF and TrkB signaling have also been demonstrated to modulate expression of NPY in the hypothalamus[56].

Glucocorticoids, leptin and insulin

Glucocorticoids are hormones that regulate many different aspects of physiology altered by stress. The cortex of the adrenal gland is responsible for synthesizing glucocorticoids, as well as aldosterone. One physiological parameter modulated by glucocorticoids is metabolism, stimulating gluconeogenesis to maintain

energy balance, under chronic food and fluid deprivation. Glucocorticoids modulate expression of CRH levels in the hypothalamus, due to negative feedback, and excessive glucocorticoid levels result in hyperglycemia, insulin insensitivity, weight gain, and obesity[43].

Insulin was the first hormone identified to reduce food intake[57]. Another signal that also reduces food intake is Leptin, which is released from adipocytes. Both of these hormones circulate at levels that correspond to the amount of adipose tissue and are referred to as adiposity signals[8]. These adiposity signals modulate neuropeptide gene expression in the hypothalamus by suppressing anabolic signals and increasing catabolic neuropeptide signals. The increased levels of leptin and insulin result in decreased NPY and AGRP neuropeptide levels with a simultaneous increase in POMC levels in the arcuate nucleus of hypothalamus. For example, this decreased NPY/AGRP signals result in decreased production of NPY and decreased release of NPY onto neurons in the PVN, resulting in decreased food intake. This mechanism would normally serve to modulate energy balance by subsequently decreasing plasma insulin, leptin and body weight[58], but malfunctions in the signaling often occur in individuals with an obese phenotype.

Glucocorticoids are another hormone that modulate neuropeptide signaling in the hypothalamus, such as NPY/AGRP and POMC. Increased levels of glucocorticoids trigger the following responses in the brain: 1) decreased CRH expression due to negative feedback[43], 2) altered activity of POMC neurons responding to changes in leptin and insulin levels[59,60], 3) and if glucocorticoids are chronically in excess adiposity signals will be altered, resulting in increased

lipogenesis, decreased lipolysis, decreased insulin sensitivity (due to decreased gluconeogenesis) and increased glucose utilization[43]. Glucocorticoids are known to functionally antagonize some of insulin's peripheral effects with decreased levels of glucocorticoids resulting in increased insulin sensitivity[61].

Brain-Derived Neurotrophic Factor and Glucocorticoids

BDNF has been shown to interact with the hypothalamic-pituitary-adrenocortical (HPA) system. The Val66Met polymorphism in the BDNF gene has been associated with impaired glucose metabolism and the anorexic phenotype[62,63] as well as the HPA axis dysregulation in depressed patients[64]. However, the majority of the focus is on BDNF regulation in the hippocampus in relation to stress and learning[65-67]. Very little research focuses on BDNF expression in the hypothalamus, but it has been shown that immobilization stress does increase BDNF mRNA and protein expression in the hypothalamus[68-70].

Neurotransmitters and Eating disorders

Eating disorders include obsessive thoughts and a compulsion to consume, similar to drug cravings[71] and eating disorders are often co-expressed with addictive behaviors in this form. Compulsive eating stimulates the reward pathway, the dopamine pathway, similar to drugs of abuse[24]. However, addictive mechanisms for drugs may function differently from addictive behavior associated with food intake since there are two types of rewards that interact with this pathway, natural and unnatural rewards. Natural rewards involve satisfaction of physiological drive, such

as food, sex and other pleasurable activities, whereas unnatural rewards are learned, such as drugs, gambling and alcohol. The differences between individuals that may cause them to seek out one type of behavior over the other may be attributable to a genetic component that I will discuss later, as well as dopaminergic signaling in different brain regions.

Dopamine

Gene-environment interactions can also increase the likelihood to develop an eating disorder, but presently relatively few studies have examined this. Environmental influences can alter responses to food cues, cognition, emotion, drive and reward in the following circumstances: 1) television ads or billboards may activate the drive to seek the rewarding aspects of food[72] and 2) childhood maltreatment may induce over consumption and obesity[73]. Cues can induce behavioral stimuli through operant or classical conditioning and can be associated with palatable foods or addictive drugs. These cues may act on the Dopamine system to predict rewards by increasing the behavioral response necessary for obtaining the reward[25].

The Dopamine reward incentive pathway, as well as the opioid signaling pathway, may contribute toward compulsive overeating in obese individuals by reinforcing the behavior, thus contributing toward repetitive behavior. It is also likely that environmental cues may have activated the expression of the disorder due to a genetic risk/susceptibility for inducing the compulsive overeating. For example, it has been shown that abusive or neglectful situations could serve to trigger events that lead to this behavior and phenotype. The association between childhood trauma and

severe obesity was examined in extremely obese patients about to undergo bariatric surgery, and 69% of obese patients self-reported childhood maltreatment, suggesting a positive correlation between the two[73].

Other environmental factors that could act on the genetic susceptibility are natural rewards and drugs of abuse since they both activate the dopaminergic system, however activating this system is not essential for 'liking' a food[74]. Dopamine deficient mice, lacking the ability to make dopamine, have a preference for the sweet tastes of sucrose as demonstrated by increased lick rates when they gained exposure to the sweet source[74], suggesting that the sweet taste reward reinforces behavior independently of dopaminergic signaling. However, in unnatural rewards that involve addiction towards drugs of abuse, dopaminergic signaling is a key pathway involved with the addictive behavior. Perhaps instead of working through the dopamine system this 'liking' of food functions through the opioidergic or serotonergic signaling processes that I will explain below.

The reward pathway (i.e. the dopamine (DA) pathway) has been shown to have a significant genetic component involving genetic and environmental influences that contribute to behavioral choices. It has been suggested that DA may regulate anticipation of a given reward, more so than mechanisms that respond to receiving the reward itself. The dopamine D2 receptor (DRD2) may play a large role in heritability of the impulsivity, appetite and reward aspect of eating disorders. Two polymorphisms of this gene have been associated with increased BMI and obesity[24,75], with expression of DRD2 being decreased in individuals with obesity, similar to reward deficiency syndrome which also underlies alcoholism[76]. Not

surprisingly, this gene has also been associated with many other behavioral disorders like alcoholism, smoking, personality, Tourette syndrome and gambling. However, it is the variants and polymorphisms of this gene and how their alleles pair together that give rise to the varied phenotypes attributable to this one gene.

Opioidergic Signaling

Endorphins are associated with aspects of food intake consumption like being satisfied, satiated and aspects of reward (i.e., sedation, rest and bliss)[24], as well as increasing food intake when an agonist is given and decreasing food intake when an antagonist is given[77]. Endorphins are also associated with determining palatability of food and how an individual feels when they consume the food[78] and might be involved with eating disorders involving hyperphagic intake, similar to obesity, bulimia and binge eating disorder. Sugar-dependent animals show similar alterations in dopamine and opioid mRNA levels in the ventral striatum as morphine-dependent animals, suggesting that neuronal functions might be similar for both types of addiction in this brain region[79]. The ventral striatum also contains opioid receptors (mu, delta and kappa) that regulate palatability and the positive response one feels after consuming some foods, and in the nucleus accumbens the opioid receptor functions to regulate sucrose, salt and saccharin appetite in water-deprived animals[25]. Desiring the acquisition of the positive feeling associated with consuming certain food may be strong enough to then bypass the energy balance set point determined by the arcuate nucleus of the hypothalamus[25] and ultimately result in body weight that is greater than the normal set point.

Demonstrating a relationship between food intake and opioid levels, highly palatable foods appear to produce an analgesic effect in human females eating chocolate chip cookies compared to an unpalatable food, thereby providing further evidence supporting the positive effect opioids can have when certain foods are consumed[71,80]. Also, bulimic individuals have been shown to decrease the binging episode if they are administered an opiate blocker, naloxene[71,81]. Lastly, it has also been suggested that opioidergic signaling has altered functions in anorexia. For example, increased levels of β-endorphin were measured in the anorexic state[77]. Perhaps, it is the increased level of endorphins that induce a pleasant feeling in the anorexic individual, providing a possible explanation for why they do not feel as good when they consume food or increase weight because this would lead to a decrease in the amount of endorphins.

Serotonergic Signaling

Brain serotonin signaling interacts with many different systems, such as the opioid system and brain-derived neurotrophic factor, which have been associated with depression, consumption of foods for taste and pleasure, obesity and food cravings. Loss of the serotonin 2C receptor leads to the development of obesity, whereas increasing the serotonin precursor, tryptophan, is thought to be associated with carbohydrate cravings [71]. Given that low levels of serotonin are expressed in depressed individuals, it is plausible that carbohydrate consumption that leads to increased serotonin precursor production would drive individuals to consume more carbohydrates in order to self medicate because it results in a pleasurable feeling, perhaps explaining why obesity and depression are often co-expressed together.

It has been observed that anorexic and bulimic individuals have altered serotonergic functioning in the frontal, cingulated, temporal and parietal regions of the brain[6]. Kaye et.al.[6] have reported that different serotonin receptor types are associated with anorexia and bulimia. Anorexic individuals have reduced 5-HT 2A receptor functions, whereas bulimic individuals have increased 5-HT1A receptor function. Further evidence to support the role of serotonin in bulimia demonstrated that an increased urge to binge occurs when bulimic women were given a tryptophan deficient drink[71].

From the Macroscopic and Microscopic to Perception and Cognition

Eating disorders are a complex disease arising from environmental as well as physiological cues. It is most likely the physiological enforcement (i.e. satiety, reward, sensations induced by the release of opioids) following environmental cues such as visual or smell cues, billboards, driving past fast food places or restaurants that alter our neurochemistry to drive our behaviors and thoughts. At some point the environmental and physiological cues integrate to produce phenotypes and behavioral patterns, but we do not know what the mechanism of integration is as of yet and to determine when these psychological cues turn into the neurological and molecular cues is something that would be beneficial. Genetic susceptibility provides an explanation for why people integrate the same environmental or physiological cue differently. The cost of food is another example of environmental cues that influence food intake behavior and behavioral choices. Lowering the costs of healthy foods at a grocery store and increasing the costs of unhealthy food does result in people choosing to eat more healthy (personal communication, Allan Geliebter). The taste,

texture and palatability of the food consumed are also properties that influence food consumption behavior. Even more, these properties often lead to activation of the dopamine or opioid pathway, which is one way behaviors are repeated and reinforced. These are just the tip of the iceberg for examples integrating factors that influence our psychology to subsequently influence our physiology, or vice versa.

Rationale

I am particularly interested in investigating regulation of Brain-Derived Neurotrophic Factor (BDNF) since it has been implicated in the etiology of human eating disorders, specifically obesity and anorexia[31,33,37,63]. Obesity has also been associated with endocrine abnormalities. One example being suppressed levels of serum triiodothyronine (T_3) in obese Zucker rats relative to the lean rats[82]. Given this, I am are proposing to use a selectively bred Fat line of animal having 255% more adipose tissue overall relative to a Lean line. The Fat line also has decreased levels of T_3 relative to a Lean line during the fed state and during development[83]. This is a useful model for identifying evolutionarily conserved processes regulating body composition due to alterations in the hypothalamic-pituitary thyroid (HPT) axis.

We have obtained access to Fat and Lean lines of chicken selectively bred for 21 generations based on adiposity or leanness. Selective breeding allowed for segregation of alleles that regulate expression of these phenotypes. More importantly, the contribution provided by our model of obesity stems from the fact that the adipose state in these lines develops independently of environmental influences (i.e. a high fat or high calorie diet), suggesting that development of

adiposity is based on genotype differences alone. This is an extremely important dissociation because it is often experimentally difficult to separate out physiological alterations arising from increased adipose mass without having simultaneous alterations in food intake. Therefore, we believe we have a powerful model to better elucidate genetic interactions related to adiposity. With this model, we are proposing to use gene expression to functionally map the circuitry in the hypothalamus related to either a high or low adipose state, and with this we would like to elucidate and validate hypothalamic gene expression that influences development of either an obese or lean phenotype, specifically focusing on BDNF interactions with the hypothalamic-pituitary thyroid (HPT) axis. My overall hypothesis is that BDNF in the hypothalamus regulates expression of the obese or lean phenotype by interacting with the HPT axis (i.e. thyrotropin releasing hormone, TRH) as well as modulating expression of other genes known to regulate food intake, metabolic rate and body weight via signals that arise from Tyrosine Receptor Kinase B (TrkB) activation, the main mediator of BDNF signaling. My specific objectives were to complete the following: 1) Utilize microarray technology to identify genes and expression patterns during the divergence of adiposity, without simultaneous alterations in food intake. 2) Determine which anorexigenic and orexigenic genes are altered during the divergence of adiposity independent of food intake alterations. 3) In primary hypothalamic neuronal cultures, characterize changes in mRNA levels for BDNF, TrkB, and TRH in response to Triiodothyronine (T_3) or BDNF treatment. 4) Determine effects of hyperthyroidism and hypothyroidism *in vivo* on hypothalamic expression of BDNF, TrkB, TRH, and candidate genes previously shown to be

differentially expressed in the Fat and Lean lines, and to determine how glucocorticoids modulate expression of anorexigenic and orexigenic neuropeptides in the brain alone or in the presence of T_3.

Chapter 2: Transcriptional profiling of hypothalamic gene expression during the development of adiposity in genetically selected fat and lean animals

Abstract

The hypothalamus controls many physiologic processes that can lead to excess adiposity, including metabolic rate, nutrient partitioning, and food intake. To elucidate genetic interactions within the hypothalamus that may control development of excess body fat, we characterized global gene expression patterns in the hypothalamus before and after divergence of visceral fat in Fat or Lean genetic lines of chickens selectively bred for high or low percent body fat. These animal lines were chosen because adiposity diverges between the two lines in the absence of environmental influences such as differences in food intake. Gene expression patterns during the development of adiposity were investigated using cDNA microarrays representing about half the genome. GeneCluster and GeneOntology were then used to identify biological trends and generate hypotheses associated with divergence of adiposity between the two lines. A model derived from the data suggests that Tumor necrosis factor α (TNFα) signaling may interact with other genes known to modulate body fat composition. Additionally, three genes involved in glycolysis were shown to be expressed at lower levels in the Fat line prior to the divergence of adiposity. These data support the hypothesis that genotype may lead to the development of an "obese" phenotype through transcriptional or metabolic processes within the hypothalamus. Further validation of our findings will allow for identification of how these novel candidate genes influence hypothalamic function to control the development of an "obese" phenotype.

Introduction

In humans and other animals, heritability and genetic susceptibility are factors that contribute to differences in body composition and development of an obese phenotype [9]. The hypothalamus maintains energy homeostasis by exerting significant control over food intake and metabolic rate [4,5]. Previously, it has been difficult to dissociate genes that regulate the development of adiposity from genes that also regulate food intake, because these alterations often occur simultaneously. However, Fat and Lean lines of chicken selectively bred to be fat or lean develop adiposity without having simultaneous alterations in food intake [19,84,85]. These divergently selected animal lines provide a novel model to dissociate and identify genes that contribute to genetically based alteration in visceral adiposity and those that induce development of the fat or lean phenotypes. In this study, global gene expression patterns within the hypothalamus of the Fat and Lean line animals were determined using cDNA microarrays before and after divergence in levels of body fat between the lines. The gene expression data were then clustered using self-organizing maps (SOMs) to identify: 1) biologically relevant patterns in the data set, 2) phenotypic marker genes distinguishing fat and lean animals, and 3) neighborhood marker genes centered around specific genes of interest that have been identified in transgenic or knockout mice to contribute to the obese or lean phenotype. A novel hypothesis for potential interactions among genes regulating the susceptibility to obesity was then developed.

Currently there are more than 600 genes identified to be associated with obesity, and new genes are emerging each day [16]. Here we utilize a genomic screen to identify novel candidate genes and biological pathways. Insight into mechanisms

regulating obesity can be improved by 1) dissociating the mechanisms that regulate obesity from those that regulate alterations in food intake, 2) identifying multiple factors simultaneously present in the genetic background that contribute to the development of obesity and 3) including a sampling time when no differences in adiposity are present, thus providing a baseline to compare physiological alterations before and after the onset of excessive adiposity. The present study has included all of these features in its design. In order to provide insight into the dynamics of biological processes and pathways associated with the divergence of adiposity, we performed an analysis using well defined biological processes based on Gene Ontology (GO) categories and cluster analysis in order to develop two novel hypotheses suggesting that genes regulating TNFα signaling also interact with other genes known to modulate body composition and genes known to regulate glycolysis may contribute within the hypothalamus to regulate adiposity.

Materials and Methods

Animals and tissue preparation.

Genetically bred fat and lean chicken lines developed at institut scientifique de recherche agronomique (INRA, station de recherches avicoles, Nouzilly, France) were used in this study (Leclercq et al., 1980). These chickens underwent genetic selection for about 21 generations by selectively breeding those animals exhibiting the greatest and lowest amount of abdominal fat at similar body weight at 9 weeks of age. Males were kept and reared in floor pens (4.4 x 3.9 meters). Fat (n=16) and Lean (n=16) chickens were reared together in the same pen to eliminate environmental

differences. They were given *ad libitum* access to food and water throughout the study using conventional starter (0-3 weeks) and grower (3-11 weeks) pellet diets. The light/dark cycle was: 24 hr of light for the first two days post-hatch and then 14 hr of light and 10 hr of dark thereafter.

Animals were sacrificed at 1, 3, 5 and 7 weeks of age at INRA, with the hypothalamus being immediately dissected and snap frozen in liquid nitrogen (n=4 for each age and group) and stored at –80 ^{o}C until further processing. Visceral fat, which is the predominating fat source in the chicken[86,87], was dissected out of the abdominal region using a scapel. All procedures were handled in accordance with the Institutional Animal Care and Use Committees at INRA, the University of Delaware and the University of Maryland.

Primer design and validation.

All primers were designed using Primer Express (v 2.0, Applied Biosystems) and designed using a homologous region of chicken cDNAs when aligned with the same gene sequence for the mouse. Primers were 18-30 nucleotides in length with a melting temperature between 58-64 ºC or 69-72 ºC. Primer sequences are provided in Table 1. PCR products were between 100 and 150 base pairs.

RNA isolation, cDNA preparation and quantitative PCR (qPCR).

Individual tissue samples were homogenized and total RNA extracted with Rneasy Midi kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Extracted RNA was quantified using UV absorbance (260/280) and integrity verified using a Bioanalyzer (Agilent Technologies, Palo Alto, CA). One μg RNA was used to create the cDNA using the stated protocol for Superscript III reverse transcriptase

GENE	Sense primer	Antisense primer
Cyto b	5'TTCACATCGGACGAGGCCTAT3'	5'CACAAAGGCGGTGGCTATGA3'
EF1α	5'GTGTGTGGAGAGCTTCTCCCAGTAC3'	5'CGCTCTTCTTCTCCACGTTCTTG3'
ND	5'GCACTGCTGGAATTTGCTGAA3'	5'GCCCAAGAAGCTGAAGGTCTT3'
Phosg	5'CACCCCTTCTTCAGCACCAT3'	5'ATGGTGTCCTTCAGGCTCTCA3'
SPOCK2	5'GAAGAGCGAAGCAGCACAGT3'	5'CCGAAGGAGAAGTTCTCGAAG3'
SNAP-α	5'AAGCCCACGAGGAGCAGAAC3'	5'GCGAAGCAACATGGTTGTGA3'
5-HTP	5'AATCCGTATACTCAGAGTGTGCAGAT3'	5'AGGGCATCGCTGACAATGTC3'
Neuro Enolase	5'AGATGACCCAAGCCGCTACA3'	5'GGTCAAAGGGATCCTCAATGG3'
CEBP	5'GGATGAAAATGCTGGGTCCAA3'	5'CGCCGCTCTAATTCCCACTTG3'
Induced by TNF	5'ACAACGCCGTCACCCTATTC3'	5'GTTCCCCAGGAAGAGGACGAA3'
Phoshogluc isomerase	5''GCCCAACCAATTCCATCATG3'	5'CCAGACAACCCCCTGAACAA3'
GHRHR	5'CCTTGGCATTCGGCTTTATTTAG3'	5'TCAGGAAACAGTAGAGGAGTGCTACA3'
ADAM12	5'TCAGATGAAATGAAGCAGTCA3'	5'GTGCTCTTCCCAACTGCAAGA3'
GAPDH	5'AAGGAGTGAGCCAAGCACACA3'	5'TCACTGCAGGATGCAGAACTG3'
B-actin	5'CCCAAAGCCAACAGAGAGAAG3'	5'ACCATCACCAGAGTCCATCAC3'

Table 1. qPCR primers used for verifying expression profiles.

(Invitrogen, Carlsbad, CA) and an oligo(dT) primer. Blank cDNA was made as a negative control to measure genomic DNA contamination in total RNA samples as described, but with no reverse transcriptase added. mRNA levels were quantified using 2µl of diluted cDNA (1:200) in a 20µl qPCR reaction using Sybr green realtime quantitative PCR master mix [2X PCR buffer containing 100 mM KCl, 20 mM Tris-HCl (pH 9.0), 0.2% triton-X 100, 3.8 mM $MgCl_2$, 0.12 U/µl Taq Polymerase, 400 nM dNTPs, 40 nM fluorescein (Invitrogen), and SYBR Green I Nucleic Acid Gel Stain (Invitrogen) diluted 1:10,000] and analyzed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) . In all cases, a three-step PCR cycle was used: initial denaturation at 95 C for 3 min followed by 40 cycles of 94 ^{o}C for 45 sec, 60 ^{o}C for 45 sec and 72 ^{o}C for 45 sec followed by a melt curve analysis to verify that one product was amplified.

Microarray processing and data analysis.

RNA samples were analyzed with cDNA microarrays, representing more than half the genome (14,053 genes), using a reference design. The microarrays used were the Del-Mar 14K Chicken Integrated Systems microarray, and a description of these arrays along with the annotations for each cDNA available in the Gene Expression Omnibus (GPL 1731). Aliquots of all RNA samples were pooled for use as an internal reference, with experimental samples (25 µg) labeled with Cy3 and the pooled reference RNA (25 µg) labeled with Cy5 during first strand cDNA synthesis. Microarrays were run for four ages for either the Fat or Lean line (Fat line: 4 ages, n=4 samples/age, Lean line: 4 ages, n=4 samples/age). The University of Maryland Biotechnology Institute's Microarray Core Facility labeled the cDNA samples with

either Cy3 or Cy5, hybridized the microarrays, and scanned the images for processing (http://www.umbi.umd.edu/~cbr/macore/macorestart.htm, Rockville, MD).

Data were analyzed using TM4 microarray suite from The Institute for Genome Research (TIGR). Images were processed using Spotfinder, and data were Lowess normalized without background correction and regularized for standard deviation using Midas. Background expression was determined from 8 spots of salmon testes DNA and the mean pixel intensity for the spots calculated. The spots having a mean pixel intensity less than background were deleted from the analysis. Out of 19,200 total spots on the microarray, 14,063 genes comprising about half the chicken genome were represented. Data points were deleted if more than half the data points were not present. From this, statistics were ran on 6,041 data points and significant differences in gene expression were identified for 790 genes ($P<.05$) by ANOVA using the Statistical Analysis System (Statistical Analysis System, v.8.02, SAS Institute, Cary, NC). The 790 significant genes were preprocessed in Genecluster version 2.1.7 (Broad Institute, Cambridge, MA) by normalizing expression levels to have a mean=0 and variance=1 and then clustered by SOMs to identify 18 different patterns of expression between the two genetic lines (3x6 arrangement). qPCR was performed to confirm the microarray results and included all samples used for Cy3 labeling and the pooled RNA used for the Cy5-labeled reference. All data for both qPCR and microarray are presented with the mean data point equal to one, in order to place both data sets on the same scale.

Statistical analysis and graphical display of data.

PCR products were verified for the appropriate size with dissociation curve analysis and gel electrophoresis. The qPCR output data provided a Ct value for the threshold cycle. The Ct value represents the threshold cycle, which is achieved when the amount of amplified product reaches a fixed threshold for fluorescence due to binding of SYBR green to the double-stranded PCR product. Data were first transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency (GeNorm v3.4) [88]. The ΔΔCt value was generated using geNorm software and methods [88]. Briefly, the data were first transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to two housekeeping genes, β-actin and GAPDH.

All comparisons of values for qPCR were made using a two-way ANOVA (line by week) with a Pdiff post-hoc analysis used to identify individual differences between the groups at different ages (Statistical Analysis System, v.8.02, SAS Institute, Cary, NC). Values reported are means $\pm$ SEM, and for all statistical tests $P<$ 0.05 was required for significance. Graphpad was used to construct all graphs (Graphpad Prism Version 4.0, Graphpad Software, Inc.).

Results

Exploratory data analysis for hypothalamic gene expression: general phenotype features

We have identified 561 genes differentially expressed by line and 442 genes significant for line-by-age interactions using a two-way ANOVA. Additionally, 77

genes were differentially expressed between lines having at least 160% expression difference during the development of adiposity. A table including all means, SEM, and P-values and all of the normalized and non-normalized microarray results are available in the Gene Expression Omnibus (GSM231561-231590). A subset of genes have been shown to correlate with either the Fat or Lean phenotype, suggesting that 21 generations of selective breeding resulted in segregation of alleles for genes expressed in the hypothalamus that correlate with the Fat and Lean phenotypes. Genes most closely correlated with expression specific to either the Fat or Lean line are shown (Fig 1a, lean line: weeks 1, 3, 5, and 7; and 1b, fat line: weeks 1, 3, 5 and 7; 40 each). Self-Organizing Maps (SOMs) clustering allows identification of fundamental patterns of gene expression for a phenotype of interest [89], and this analysis was applied to the genes found to be expressed at significantly different levels between the lines (Fig. 2). Genes differentially expressed between the Fat and Lean lines were organized in this manner in order to identify biologically relevant clusters to develop novel hypotheses for the divergence of either the fat or lean phenotype. Each cluster represents the mean of all genes within the cluster, and expression levels were normalized to have a mean = 0 and SD = to 1 across time points [89]. The data are presented in a 3x6 SOMs. We verified the accuracy of the expression patterns for eight clusters, 13 genes, plus GAPDH and β-actin, using qPCR (Table 2 and Fig. 3).

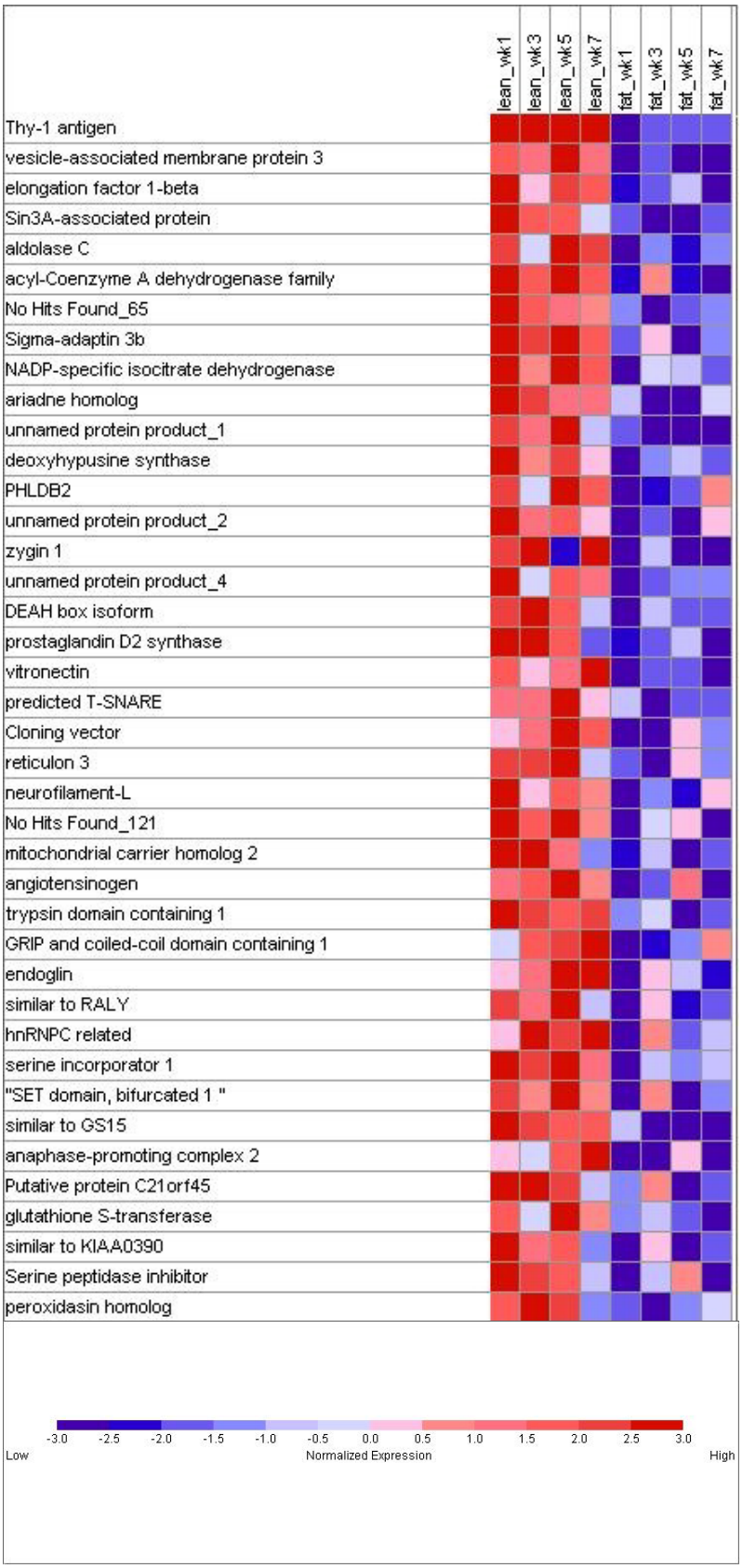

lean_wk1
lean_wk3
lean_wk5
lean_wk7
fat_wk1
fat_wk3
fat_wk5
fat_wk7
Thy-1 antigen
vesicle-associated membrane protein 3
elongation factor 1-beta
Sin3A-associated protein
aldolase C
acyl-Coenzyme A dehydrogenase family
No Hits Found_65
Sigma-adaptin 3b
NADP-specific isocitrate dehydrogenase
ariadne homolog
unnamed protein product_1
deoxyhypusine synthase
PHLDB2
unnamed protein product_2
zygin 1
unnamed protein product_4
DEAH box isoform
prostaglandin D2 synthase
vitronectin
predicted T-SNARE
Cloning vector
reticulon 3
neurofilament-L
No Hits Found_121
mitochondrial carrier homolog 2
angiotensinogen
trypsin domain containing 1
GRIP and coiled-coil domain containing 1
endoglin
similar to RALY
hnRNPC related
serine incorporator 1
"SET domain, bifurcated 1 "
similar to GS15
anaphase-promoting complex 2
Putative protein C21orf45
glutathione S-transferase
similar to KIAA0390
Serine peptidase inhibitor
peroxidasin homolog
-3.0 -2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0
Low
Normalized Expression
High

Figure 1a.

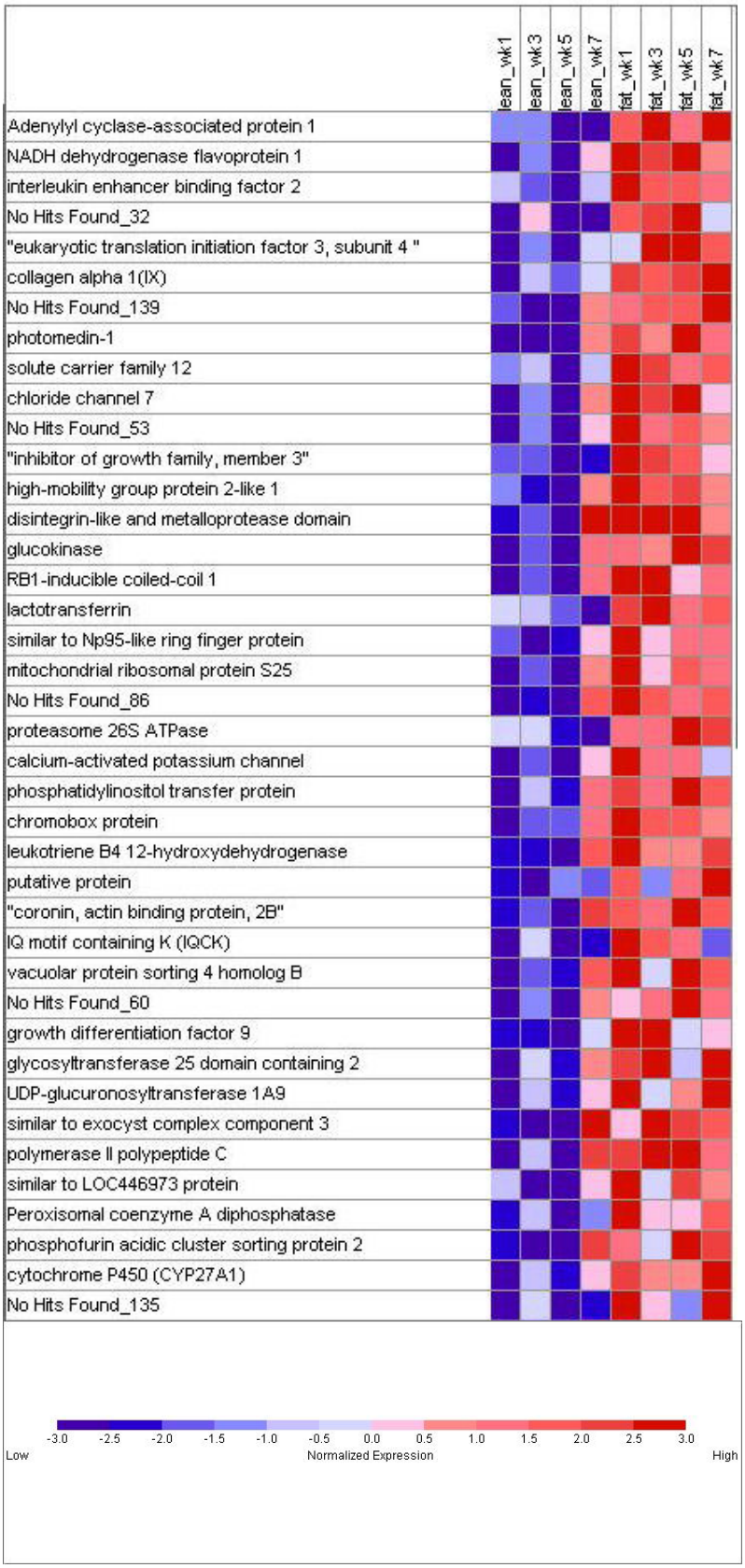

lean_wk1
lean_wk3
lean_wk5
lean_wk7
fat_wk1
fat_wk3
fat_wk5
fat_wk7
Adenylyl cyclase-associated protein 1
NADH dehydrogenase flavoprotein 1
interleukin enhancer binding factor 2
No Hits Found_32
"eukaryotic translation initiation factor 3, subunit 4 "
collagen alpha 1(IX)
No Hits Found_139
photomedin-1
solute carrier family 12
chloride channel 7
No Hits Found_53
"inhibitor of growth family, member 3"
high-mobility group protein 2-like 1
disintegrin-like and metalloprotease domain
glucokinase
RB1-inducible coiled-coil 1
lactotransferrin
similar to Np95-like ring finger protein
mitochondrial ribosomal protein S25
No Hits Found_86
proteasome 26S ATPase
calcium-activated potassium channel
phosphatidylinositol transfer protein
chromobox protein
leukotriene B4 12-hydroxydehydrogenase
putative protein
"coronin, actin binding protein, 2B"
IQ motif containing K (IQCK)
vacuolar protein sorting 4 homolog B
No Hits Found_60
growth differentiation factor 9
glycosyltransferase 25 domain containing 2
UDP-glucuronosyltransferase 1A9
similar to exocyst complex component 3
polymerase II polypeptide C
similar to LOC446973 protein
Peroxisomal coenzyme A diphosphatase
phosphofurin acidic cluster sorting protein 2
cytochrome P450 (CYP27A1)
No Hits Found_135
-3.0 -2.5 -2.0 -1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5 3.0
Low
Normalized Expression
High

Figure 1b.

Figure 1. **Hypothalamic expression patterns that correlate with either the Fat or Lean phenotype.** a) 40 genes most closely correlated to be upregulated in the Lean line relative to the Fat line before (week 1), during (week 2 and 5) and after the development of adiposity (week 7). b) 40 genes most closely correlated to be upregulated in the Fat relative to Lean line over weeks 1-7, before, during and after the divergence of adiposity. All genes are differentially expressed between lines ($P<.05$) and ranking is calculated based on the difference of the means for each line scaled by the sum of the standard deviations $(x_1-x_2)/(x_1^2+x_2^2)^{1/2}$. Genes in red represent high expression levels and genes in blue represent low expression levels.

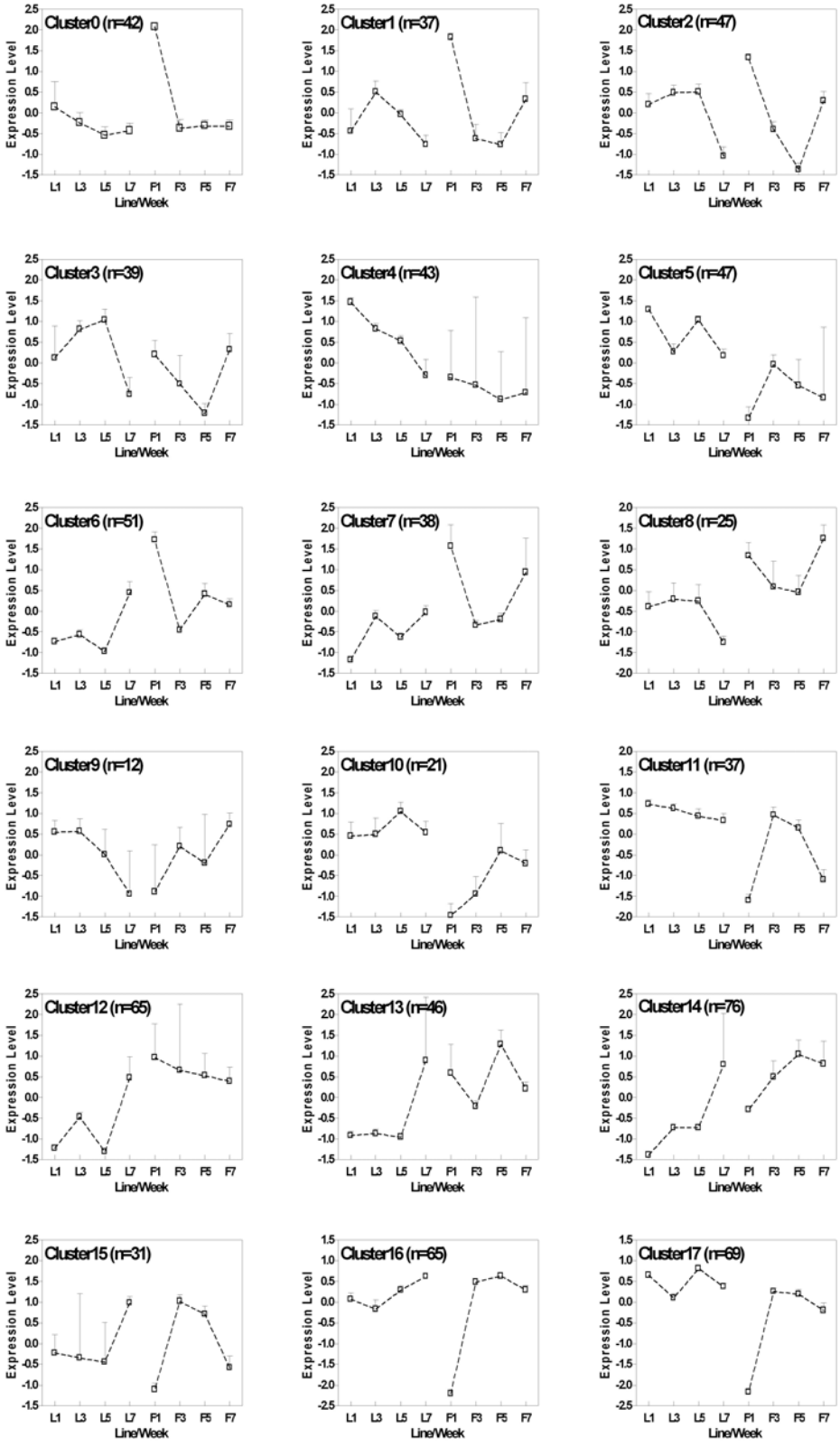

Cluster0 (n=42)
Cluster1 (n=37)
Cluster2 (n=47)
Cluster3 (n=39)
Cluster4 (n=43)
Cluster5 (n=47)
Cluster6 (n=51)
Cluster7 (n=38)
Cluster8 (n=25)
Cluster9 (n=12)
Cluster10 (n=21)
Cluster11 (n=37)
Cluster12 (n=65)
Cluster13 (n=46)
Cluster14 (n=76)
Cluster15 (n=31)
Cluster16 (n=65)
Cluster17 (n=69)
Expression Level
Line/Week
L1 L3 L5 L7 P1 F3 F5 F7

Fig 2. **Self-organizing maps (SOMs) analysis demonstrating biologically relevant clusters and expression patterns between the fat and lean lines.** 3x6 SOM with 18 clusters (cluster0-cluster17) using 791 genes, with 561 significant by lines and 442 significant for the line-by-age interaction ($P<.05$). The number of genes included in each cluster is provided in the upper left corner, and values reported are means ± SEM.

GENE	Spot	Cluster
Cytochrome b (Cyto b)	17687	5
Elongation factor 1 α (EF1α)	16106	17
No description (ND)	17744	3
Phoshpoglycerate mutase (Phosg mut)	6482	17
Sparc/osteonectin (SPOCK2)	6854	10
Soluble NSF-attachment protein (SNAP α)	16210	16
5-tryptophan hydroxylase (5-HTP)	7185	6
Phosphoglucose isomerase	73	16
Neural Enolase	13011	17
CCAAT/enhancer-binding protein (CEBP)	19069	12
Induced by TNF alpha (TNF AP)	10372	14
Growth hormone releasing hormone receptor (GHRHR)	11448	6
Metalloprotease 1	13505	6

Table 2. Verification of expression profiles by qPCR was performed on the above genes from different clusters.

Hypothalmic gene expression profiles during the development of adiposity divergence.

The hypothalamus has been shown to be an area of the brain that modulates body weight and adiposity. We analyzed gene expression for Fat and Lean lines at 1, 3, 5 and 7 of age to measure differences in gene expression correlating with the development of the phenotypes. There were no differences in adiposity at week 1 of age, providing a baseline to compare physiological alterations before and after the onset of excessive adiposity. Differences in adiposity developed by week 3 in the Fat relative to Lean line. Thus, we sought to investigate which genes were differentially expressed prior to and/or after the divergence of adiposity (Table 3a and b). Criteria for an upregulated or down regulated gene were as follows: 1) gene expression must be significantly different by line or line-by-age as identified by the microarray (P<.05, 790 genes total), 2) Fat line > Lean line was termed upregulated and Fat line < Lean line was termed downregulated, and 3) at the age of interest there must be no overlapping error bars between the data points, as presented in the SOMs cluster data (Fig. 2). Nine clusters contained gene expression patterns being upregulated at week 1 in the Fat line, prior to any divergence in adiposity between the Fat and Lean lines (Fig. 2, clusters: 0, 1, 2, 6, 7, 8, 12, 13, 14, n=427 genes), eight clusters contained gene expression patterns being downregulated in the Fat line at the same age (Fig. 2, clusters: 4, 5, 9, 10, 11, 15, 16, 17, n=325 genes) and one cluster had no difference between the lines at this age. After adiposity differences began to emerge between

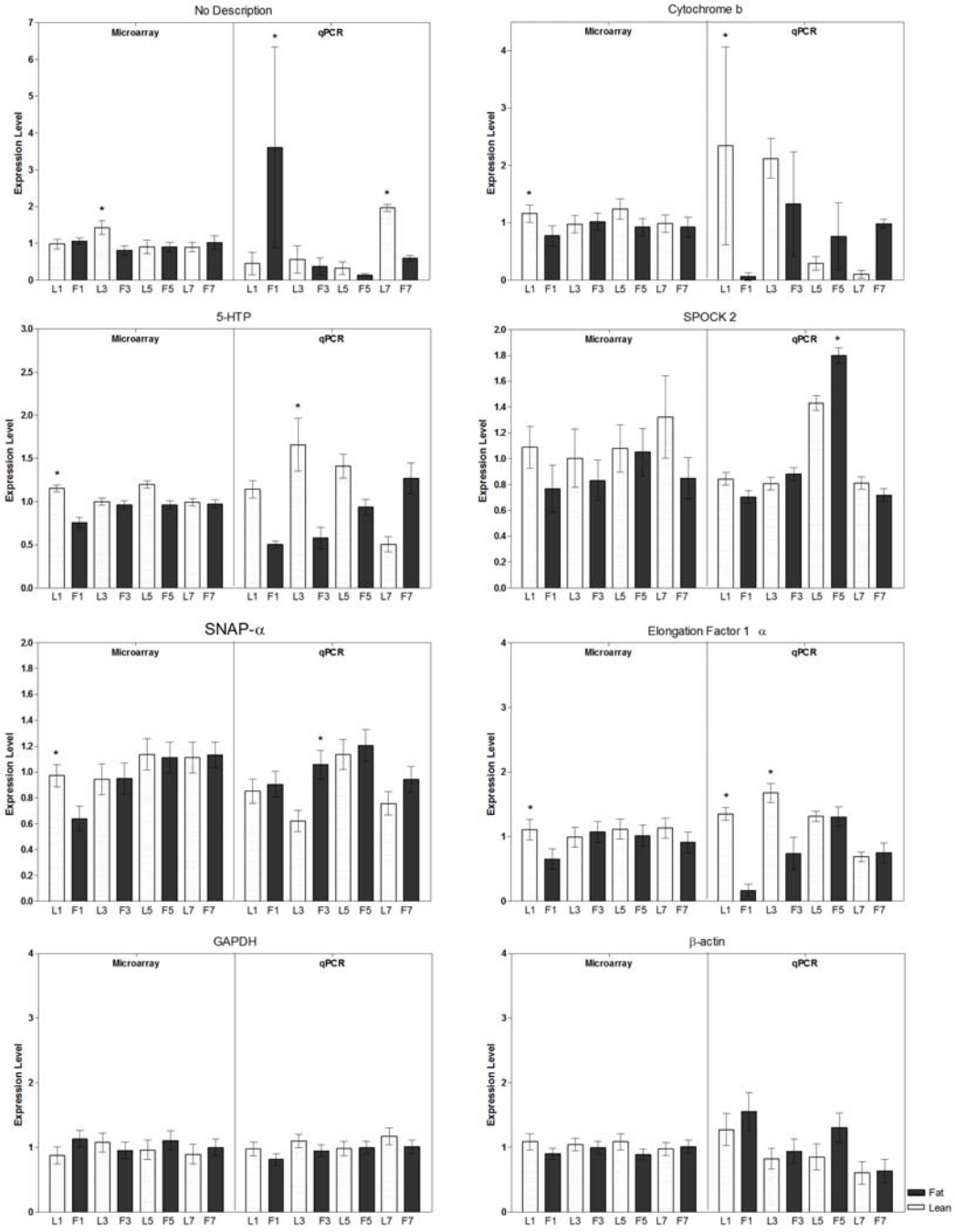

No Description
Cytochrome b
5-HTP
SPOCK 2
SNAP-α
Elongation Factor 1 α
GAPDH
β-actin
Microarray
qPCR
Expression Level
L1 F1 L3 F3 L5 F5 L7 F7
Fat
Lean

Fig 3. **qPCR verification of SOMs clusters.** QPCR results demonstrate a correspondence with the reported microarray data. Microarray data are shown on the left and qPCR data are presented on the right of each graph. Microarray data have been converted from a log value, compressing the data points to be centered around the value of 1. Fat line is represented with the closed bar and Lean line with the open bar. Values reported are means ± SEM, $P<0.05$. * denotes differential expression between the lines at the same age ($P< 0.05$).

Table 3a. **The total number of upregulated and downregulated genes in the Fat relative to Lean line at week 1 or week 3.**

	upregulated		downregulated	
	Wk 1	Wk 3	Wk 1	Wk 3
Fat vs Lean	427 (54%)	321 (40%)	325 (41%)	144 (18%)
Transcription – DNA dependent	31 (7.6%)	22 (6.8%)	19 (5.8%)	12 (8.3%)
signal transduction	29 (6.8%)	18 (5.6%)	15 (4.6%)	6 (4.1%)
transcription	24 (5.6%)	17 (5.3%)	15 (4.6%)	11 (7.6%)
protein biosynthesis	12 (2.8%)	12 (3.7%)	17 (5.2%)	2 (1.4%)
transport	15 (3.5%)	9 (2.8%)	15 (4.6%)	10 (6.9%)
biological process unknown	17 (4.0%)	4 (1.2%)	11 (3.4%)	9 (6.2%)
metabolism	9 (2.1%)	4 (1.2%)	10 (3.1%)	2 (1.4%)
carbohydrate metabolism	4 (.01%)	5 (.02%)	4 (.01%)	1 (.01%)
lipid metabolism	5 (1.2%)	4 (1.2%)	4 (1.2%)	2 (1.4%)
glycolysis	0 (0.0%)	4 (1.2%)	5 (1.5%)	0 (0.0%)

*Gene numbers represent genes obtained using a p-value < .05, 790 genes total. Biological processes on the left obtained from GO analysis.

Table 3b. **The total number of upregulated and downregulated genes in the Fat relative to Lean line at week 5 or week 7.**

	upregulated		downregulated	
	Wk 5	Wk 7	Wk 5	Wk 7
Fat vs Lean	372 (47%)	160 (20%)	266 (34%)	223 (28%)
Transcription – DNA dependent	23 (6.2%)	11 (6.8%)	17 (5.4%)	15 (6.7%)
signal transduction	24 (6.4%)	7 (4.4%)	10 (3.8%)	11 (4.9%)
protein biosynthesis	8 (2.1%)	3 (1.9%)	11 (4.1%)	12 (5.4%)
transport	14 (3.8%)	10 (6.2%)	13 (4.9%)	9 (4.0%)
biological process unknown	12 (3.2%)	9 (5.6%)	10 (3.8%)	9 (4.0%)
metabolism	5 (1.3%)	5 (3.1%)	10 (3.8%)	4 (1.8%)
carbohydrate metabolism	6 (1.6%)	4 (2.5%)	3 (1.1%)	3 (1.3%)
lipid metabolism	5 (1.3%)	4 (2.5%)	3 (1.1%)	2 (0.1%)
glycolysis	2 (0.01%)	0 (0.0%)	3 (1.1%)	4 (1.8%)

*Gene numbers represent genes obtained using a p-value < .05, 790 genes total. Biological processes on the left obtained from GO analysis.

the two lines, week 3, fewer clusters exhibited upregulated expression patterns (Fig. 2, five clusters: 12, 13, 14, 16, 17, n=321 genes) and even fewer exhibited downregulated expression patters in the Fat relative to Lean line (Fig. 2, four clusters: 1, 2, 3, 10, n=144 genes) (Table 3a). Many more genes were upregulated or downregulated in the Fat relative to Lean line at week 1 of age relative to week 3 of age. This is likely attributable to developmental processes or neural circuitry being established during this age. This also suggests that the development/establishment of these neural processes could contribute to the differential gene expression observed and, more importantly, to the differential phenotype observed between the two lines. Given this, we identified groups of functionally related genes to better determine what processes could modulate this effect.

GO processes at the gene level that are specific for either the fat or lean phenotype

In order to better understand the functionality of the expression patterns related to the development of adiposity, we utilized the Gene Ontology system (GO). The GO system allows for the following three main classes of processes to be distinguished between the Fat and Lean lines: biological processes, molecular functions and cellular processes. We chose to focus our analysis on biological processes, since we would like to identify how selective breeding altered genetic expression resulting in changes in biological processes that lead to the fat or lean phenotypes.

Out of all significant genes ($P<.05$) and genes with the greatest fold difference of expression between the two lines (82 genes, 160% or greater fold difference), regulation of transcription and signal transduction were processes that encompassed

the largest number of genes differentially expressed between the two lines, but glycolysis was the process most strikingly different during the development of adiposity (Fig. 4). Glycolytic genes comprised a very small number of all significant genes (2.1%), but encompassed a larger percentage of genes with the greatest fold difference (9.1%) (Fig. 4). This suggests that genes known to regulate glycolysis within the hypothalamus may have played a large role in driving the difference between the two phenotypes.

At week 1 of age, there were 5 glycolytic genes downregulated and no genes upregulated in the Fat relative to Lean line (Table 4). All of these genes are involved in catalyzing a different step of glycolysis, suggesting that prior to the divergence of adiposity the Fat line may have had a lower level of glucose metabolism in the hypothalamus relative to the lean line, which corresponds with the overall decreased level of plasma glucose in the Fat line (Table 5)[90]. After adiposity differences began to diverge between the lines at week 3 of age this trend reversed. The same genes that were downregulated at week 1 were upregulated at week 3 in the Fat relative to Lean line (Table 3a), suggesting that differences were present prior to the divergence in adiposity and reversed once adiposity differences began to emerge between the two lines. Aldolase C was the only gene involved in glycolysis not different at this point in time. This pattern continued at week 5 of age with 2 genes regulating the first two steps in glycolysis still being upregulated (Phosphoglucose isomerase and Hexokinase 1) and 3 genes regulating the 4^{th}, 8^{th} and 9^{th} steps in glycolysis being

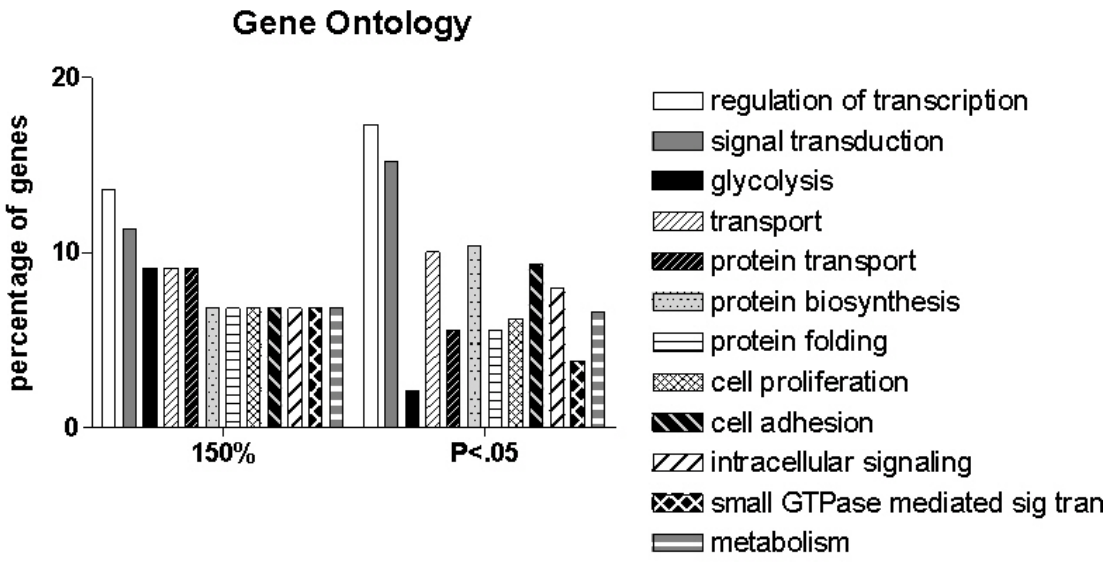

Gene Ontology
percentage of genes
20
10
0
150%
P<.05
regulation of transcription
signal transduction
glycolysis
transport
protein transport
protein biosynthesis
protein folding
cell proliferation
cell adhesion
intracellular signaling
small GTPase mediated sig tran
metabolism

Figure 4. **Gene Ontology (GO) analysis for differentially expressed genes in the hypothalamus.** Genes on the left exhibit GO distribution for only the genes with 150% or greater fold difference between the Fat and Lean lines and genes on the right show the GO distribution for all genes significantly different between the lines.

Table 4. **Genes involved in glycolysis**

Spot	Microarray Gene	Function	Glycolysis step # (out of 10)
4930	Hexokinase 1	converts glucose to glucose-6-phosphate	1
73	Phosphoglucose isomerase	interconversion of glucose-6-phosphate and fructose-6-phosphate	2
3836	Aldolase C	cleaves fructose-1-phosphate to dihydroxyacetone phosphate and D-glyceraldehyde	4
6482	Phosphoglycerate mutase	converts 3-phosphoglycerate to 2-phosphoglycerate	8
13011	Neural Enolase	converts 2-phosphoglycerate to phosphoenolpyruvate	9

Table 5. **Phenotypic data for Fat and Lean lines of chicken.**

Age (wk)	1	3	5	7	9	11
Body weight (g)						
Fat	115 ± 4.0	544 ± 21	1297 ± 37*	1983 ± 36	2693 ± 91	3222 ± 154
Lean	123 ± 3.0	551 ± 16	1204 ± 21*	1964 ± 40	2787 ± 53	3281 ± 302
Abdominal fat (g)						
Fat	0.5 ± 0.1	13 ± 1.0*	38 ± 2.0*	88 ± 3.0*	124 ± 8.0*	150 ± 20*
Lean	0.4 ± 0.1	5 ± 0.5*	15 ± 2.0*	31 ± 2.0*	54 ± 8.0*	59 ± 10*
Abdominal Fat:body weight (%)						
Fat	0.4 ± 0.1	2.3 ± 0.1*	2.9 ± 0.2*	4.4 ± 0.2*	4.6 ± 0.3*	4.6 ± 0.5*
Lean	0.3 ± 0.1	1.0 ± 0.1*	1.2 ± 0.1*	1.6 ± 0.1*	1.9 ± 0.3*	1.8 ± 0.3*
Plasma Insulin (ng/mL)						
Fat	1.3 ± 0.3	1.3 ± 0.3	1.7 ± 0.3	1.6 ± 0.2	1.5 ± 0.4	1.4 ± 0.2
Lean	0.9 ± 0.2	1.3 ± 0.2	1.7 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.1
Plasma Glucose (mg/100mL)						
Fat	232 ± 6.0	237 ± 3.0*	225 ± 6.0*	41 ± 8.0	231 ± 5.0	222 ± 7.0
Lean	246 ± 5.0	254 ± 4.0*	245 ± 6.0*	249 ± 4.0	233 ± 4.0	232 ± 6.0

Mean ± SEM, n=8, *$p<0.05$ in a one-way ANOVA comparing Fat and Lean lines of the same age.

downregulated (Aldolase C, Phoshpoglycerate mutase and Neural Enolase). By week 7 of age, when the divergence in adiposity had started to stabilize between the two lines (Table 5), the expression pattern of most genes regulating glycolysis were similar to the patterns observed at week 1 of age, with four of the genes being downregulated and no change between the lines for Aldolase C (Table 3b). To verify expression patterns for some of the glycolytic genes, we ran qPCR on the following three genes: Phosphoglucose isomerase, Phoshpoglycerate mutase and Neural Enolase (Fig. 5). No other biological processes demonstrated such pivotal and distinct changes in expression during the development of adiposity. These results suggest that genes regulating glycolysis could promote divergence of the fat or lean phenotype.

Next, we chose to annotate SOMs cluster 17 with GO processes in order to determine which biological processes were upregulated and downregulated during adiposity development (Table 6). We chose cluster 17 because there were two genes regulating steps 8 and 9 of glycolysis in this cluster, Neural enoloase and Phosphoglycerate mutase. Thus, we wanted to further identify other biological processes downregulated that might be biologically relevant to the glycolytic pathway. Of the 69 genes in the cluster all genes were linked to a GO term. Presented in table 6 are biological processes having at least 2 genes in a category, unless it was involved with metabolism. Interestingly, the genes in this cluster were mainly identified to be involved with protein synthesis and metabolism. In order to confirm

Table 6 **Gene Ontology (GO) analysis for cluster 17**

Biological Process	# of Genes	Gene Name
Protein Biosynthesis	5	Seryl-tRNA synthetase **Eukaryotic translation EF1α** Ribosomal protein L17 Ribosomal protein L35A Ribosomal protein L26
Regulation of transcription DNA dependent	6	non-POU domain containing Calreticulin No Hits Found multiple endocrine neoplasia 1 PQBP-1b/c TSC-22
G-protein receptor Signaling	3	G protein-coupled receptor 123 Amyloid-like protein 2 precursor tubulin beta 3
Proteolysis	3	*ADAM 12* F-box only protein 3 No Hits Found
Protein Polymerization	3	tubulin alpha 1 tubulin beta 3 tubulin beta 5
Protein Folding	2	Calreticulin Calnexin
Glycolysis	2	**Phosphoglycerate mutase 1** **Neural enolase**
Carbohydrate Metabolism	1	*Klotho secreted isoform*
Metabolism	3	**Phosphoglycerate mutase 1** Phospholipid-transporting ATPase Retinol dehydrogenase 11
Intracellular protein transport	3	**Golgi SNAP receptor complex** Soluble NSF-attchment protein alpha adaptor-related protein complex 2
Cell adhesion	3	*ADAM 12* Beta-amyloid precursor protein Glucose Regulated Protein, 95-KD (Grp94)
Microtubule-based movement	3	tubulin alpha 1 tubulin beta 3 tubulin beta 5

Bold denotes qPCR verification for microarray data. *Italic* denotes a homologues gene associated with the development of obesity.

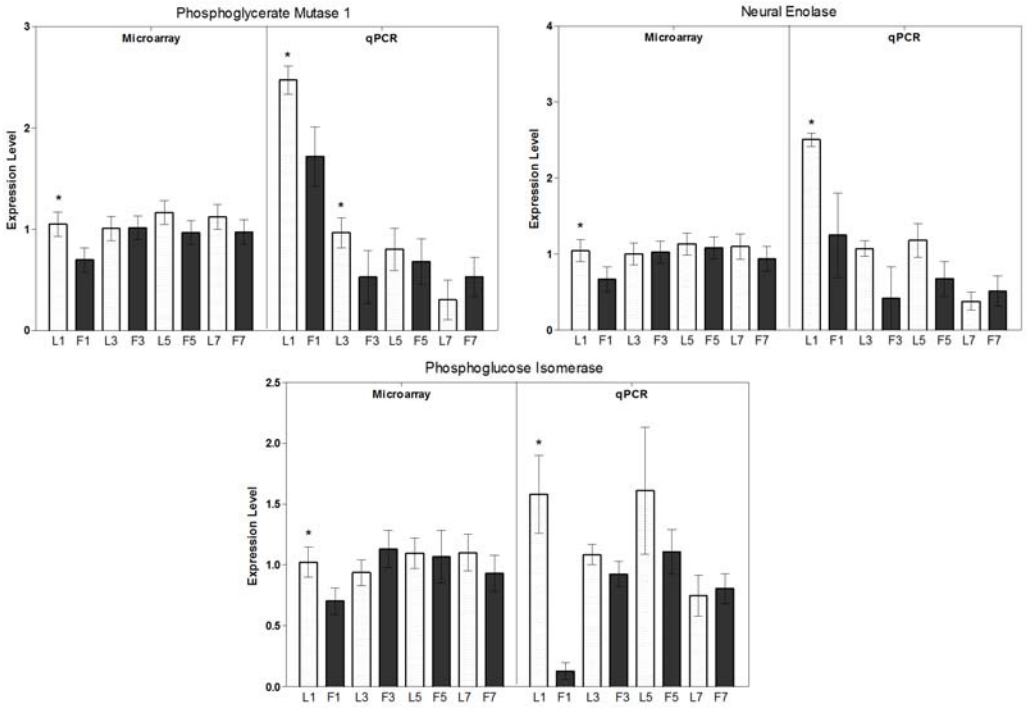

Phosphoglycerate Mutase 1
Microarray
qPCR
Expression Level
L1 F1 L3 F3 L5 F5 L7 F7
Neural Enolase
Microarray
qPCR
Expression Level
L1 F1 L3 F3 L5 F5 L7 F7
Phosphoglucose Isomerase
Microarray
qPCR
Expression Level
L1 F1 L3 F3 L5 F5 L7 F7

Figure 5. **Expression pattern verification for genes involved in glycolysis, Table 3c and Table 6.** Data expression patterns are similar for both the microarray (left) and qPCR (right). Fat line is represented with the closed bar and Lean line with the open bar. The log scale microarray data has been converted, which compresses the data points around the value of 1. Values reported are means ± SEM, $P<.05$. * denotes differential expression between the lines at the same age.

the gene profiles observed in the cluster, we performed qPCR for five of the genes (Fig. 3, 5, and 7), with two of these being involved in glycolysis, Neural Enolase and Phoshpoglycerate mutase (Table 4 and Fig. 5), as well as one gene involved with intracellular transport and one involved with protein biosynthesis (Fig. 3). In most cases, gene expression profiles determined from the microarray analysis were confirmed by qPCR.

Biological processes and adiposity divergence.

More than 600 genes have been associated with body weight and adiposity in other species, with 166 of these genes being linked directly to body composition or metabolism by knockout and transgenic mouse technologies [16]. To assess their potential role within the hypothalamus leading to the fat and lean phenotypes, we compared these 166 genes with the 790 genes identified by the microarray analysis. From this we identified 24 genes that were homologous or directly related to the biological signaling of the gene. We then selected a small number of these genes to pursue further. The selection for these genes was based on having high similarity to a gene family known to modulate body composition with the gene itself not having yet been identified to be linked to body composition (Table 7). 5 out of the 6 genes had predicted gene expression differences in the Fat and Lean line, all except Metalloprotease 1. From this, we verified expression levels for three genes with qPCR, CEBPζ, a gene induced by TNF α (TNF AP) and Metalloprotease 1 (Fig.6). Since the genes may be functioning in a similar manner as the knockout or transgenic mouse, mechanisms created by allelic segregation, we then ran a marker specific

Table 7. **Genes identified in the microarray and their homologues in knockout or transgenic mice.**

Spot	Microarray Gene	Knockout gene/ Transgenic	Phenotype	Microarray Expression
10372	TNFα induced protein	TNFα overexpression	obese	Fat Line ↑
16446	Zinc Finger Protein 36	ZFP36 -/-	obese	Fat Line ↓
13505	Metalloprotease 1	ADAMS TS -/-	lean	Lean line ↓
17139	ADAM12 precursor	ADAMS TS -/-	lean	Lean line ↑
5763	Isocitrate Dehydrogenase	IDPc overexpression	obese	Fat Line ↑
19069	CEBP ζ	CEBP α -/-	lean	Lean line ↓

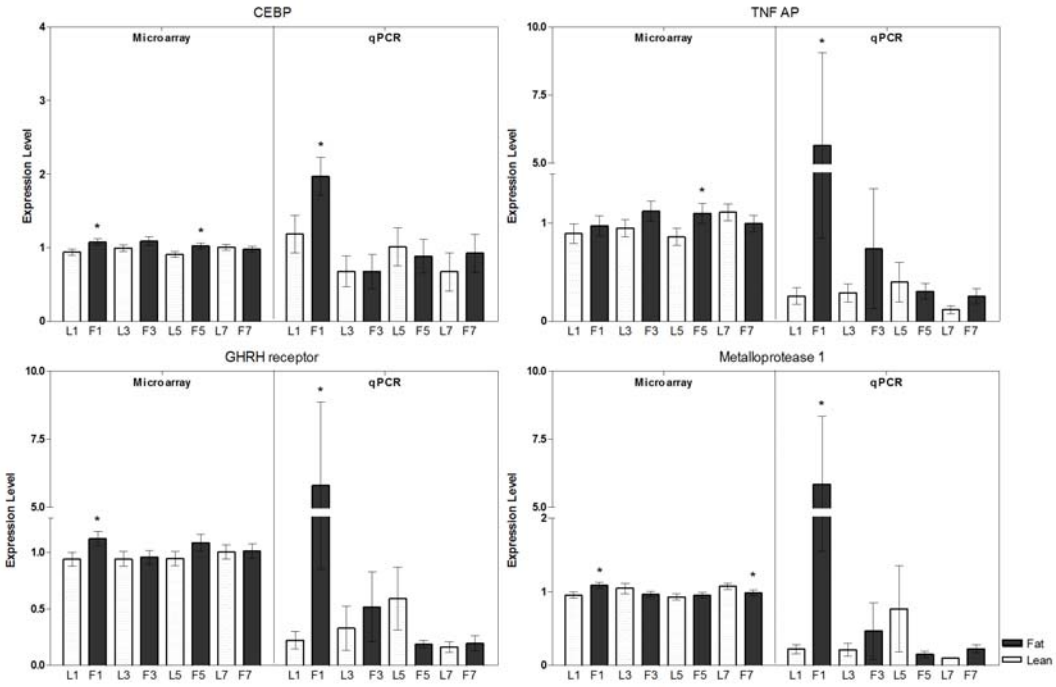

CEBP
TNF AP
GHRH receptor
Metalloprotease 1
Microarray
qPCR
Expression Level
L1 F1 L3 F3 L5 F5 L7 F7
Fat
Lean

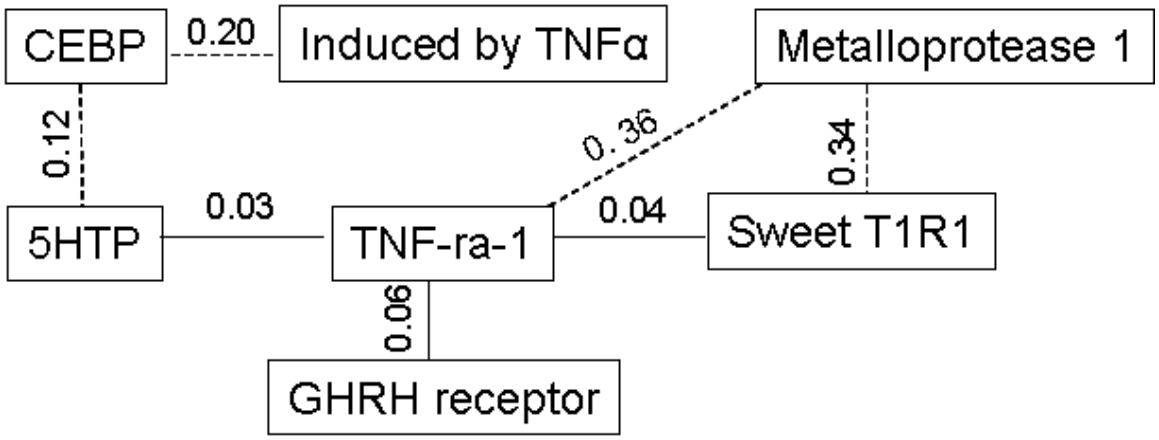
Figure 6. **Expression pattern verification for genes identified by comparison of significant genes.** Increased expression patterns for the Fat line relative to Lean at week 1 of age are increased for all genes presented in our hypothetical model. These trends confirm predictions in Table 5. Two of these genes have been identified in published lists identifying knockout or transgenic mice with alterations in body composition, CEBP and TNF AP, and two genes were identified using the KNN algorithm, GHRH receptor and Metalloprotease 1 (Fig 7a). Fat line is represented with the closed bar and Lean line with the open bar. The log scale microarray data has been converted, which compresses the data points around the value of 1. Values reported are means ± SEM, $P<.05$. * denotes differential expression between the lines at the same age.

analysis (KNN algorithm) using these genes (Table 7) to generate a hypothetical model. The marker specific analysis allows identification for genes that may be biologically relevant with one another, based on similarity of expression profile.

Of the candidate genes listed in Tables 4 and 7, one gene at a time was used as a marker for analyzing the microarray data, as well as to determine the following: 1) genes which are most closely correlated with one another, 2) potential candidate genes that may be an upstream regulator or downstream target of gene expression and 3) genes that are candidates for interacting with genes involved with glycolysis or genes previously identified through the transgenic/knockout comparison. We chose to run this analysis on genes already confirmed to be involved with regulating the development of adiposity or body weight in other species (Tables 8-12) or for genes involved with glycolysis (Tables 13-17), and our results are rather intriguing. From this data we propose two hypothetical models based on the gene expression patterns (Fig. 7a and b).

From the data we show that genes involved in TNFα signaling are linked to microarray expression patterns (Fig. 7a). For example, TNF receptor associated factor 1 (TRAF-1) is potentially related biologically to many genes already identified to modulate body composition, such as the following: the rate limiting factor for serotonin synthesis 5-tryptophan hydroxylase (5-HTP), growth hormone releasing hormone receptor (GHRHR), sweet taste receptor T1R1, and metalloprotease 1 (Table 7). This model also suggests that CEBPζ is biologically relevant to 5-HTP and

a)


CEBP
0.20
Induced by TNFα
Metalloprotease 1
0.12
0.36
0.34
5HTP
0.03
TNF-ra-1
0.04
Sweet T1R1
0.06
GHRH receptor

b)

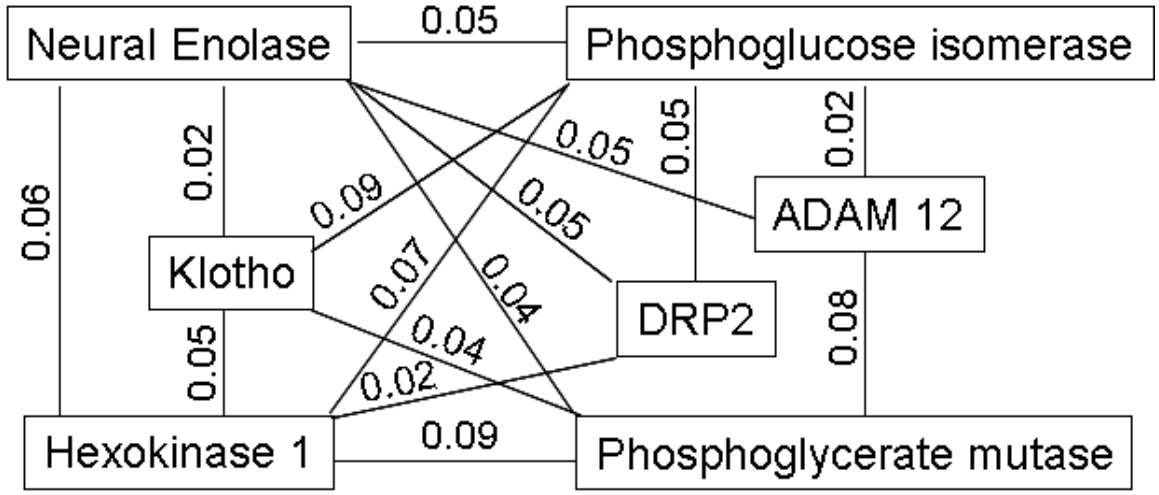

Neural Enolase
0.05
Phosphoglucose isomerase
0.06
0.02
0.05
0.05
0.02
0.09
0.05
ADAM 12
Klotho
0.07
0.04
DRP2
0.04
0.08
0.05
0.02
Hexokinase 1
0.09
Phosphoglycerate mutase

Figure 7. **Potential genetic interactions identified by specific gene marker analysis.** a) Genes known to modulate body composition (Table 3) are identified as being linked to the TNFα singalling processes. b) Genes known to modulate glycolysis are linked to Klotho, Dihydropyrimidase protein-2 (DRP2), and ADAM 12. These are key candidate genes known to modulate body composition that may interact upstream or downstream of one another. Differential gene expression occurs primarily at week 1 of age, as identified by qPCR. The dashed lines indicate that the genes correlated are > 0.10 and are less likely to be biologically relevant, based on microarray data.

Table 8 Marker selection analysis for TNF receptor associated factor 1

Gene Name	Spot	Score
tumor necrosis factor type 1	15016	0
MAP kinase phosphatase-1	14632	0
preprocarboxypeptidase A	2215	0.01
protein tyrosine phosphatase	1679	0.01
No Hits Found_49	5980	0.02
immunoglobulin-like receptor	5532	0.02
ezrin	17732	0.02
UDP-glucuronosyltransferase 1A9	9610	0.02
similar to Heat shock protein HSP 90-alpha	5677	0.02
Sk-tropomodulin	13748	0.02
Unknown protein_4	2775	0.02
Tryptophan 5-monooxygenase	7185	0.03
Cytochrome P450	17362	0.03
KIAA0232 protein	8499	0.03
chromosome 20 open reading frame 149	13481	0.03
No Hits Found_99	4689	0.03
ankyrin repeat	5336	0.03
acid alpha glucosidase	12981	0.03
No Hits Found_85	15679	0.03
Peroxisomal coenzyme A diphosphatase	4869	0.03
No Hits Found_80	11711	0.04
vacuolar protein sorting 4 homolog B	498	0.04
Similar to LIM domains	16825	0.04
Serum albumin precursor	13896	0.04
similar to Np95-like ring finger protein	10599	0.04
Acylphosphatephosphohydrolase	14313	0.04
hypothetical protein MGC38361	13731	0.04
No Hits Found_58	16838	0.04
No Hits Found_88	14208	0.04
aldo-keto reductase	2889	0.04
mitochondrial ribosomal protein S25	2875	0.04
Unknown protein _3	2888	0.04
putative sweet taste receptor T1R1	17155	0.04
No Hits Found_129	9825	0.04
hypothetical protein_3	12224	0.04
No Hits Found_3	10789	0.05
LAP	12407	0.05
No Hits Found_64	17248	0.05
ribosome-binding protein	14626	0.05
No Hits Found_46	16241	0.05
cytochrome P450 (CYP27A1)	5763	0.05
No Hits Found_29	12453	0.05
Creatine kinase	15098	0.05
target of Jun 3	474	0.05
No Hits Found_6	10653	0.05
No Hits Found_54	15755	0.05
No Hits Found_31	12392	0.06
GHRH receptor	11448	0.06
Ig gamma chain	11778	0.06
AI182287	7560	0.06

Table 9 Marker selection analysis for metalloprotease 1

Gene Name	Spot	Score
Metalloprotease 1	13505	0
MOX-2 protein	10314	0.16
No Hits Found_24	11934	0.17
Tnf receptor-associated factor 2	12802	0.19
hypothetical protein _1	17246	0.24
No Hits Found_64	17248	0.24
proline-rich protein	15447	0.24
RIKEN cDNA 5430432P15	16539	0.24
c6.1a protein	3113	0.25
Estrogen-regulated protein	18505	0.25
No Hits Found_131	2550	0.25
No Hits Found_98	1999	0.26
chromosome 20 ORF 149	13481	0.26
No Hits Found_95	15307	0.26
meiosis-specific MutS	16256	0.26
Ig gamma chain	11778	0.26
interferon regulatory factor 5	16603	0.28
FLJ00039 protein	6119	0.28
DKFZP586A0522 protein	1980	0.28
coronin 7	6094	0.29
selenoprotein SelM	5038	0.29
No Hits Found_83	6267	0.3
No Hits Found_112	1341	0.31
novel glycoprotease	17485	0.31
translation termination factor 1	15127	0.31
septin 6	13333	0.31
No Hits Found_85	15679	0.31
similar to RIKEN cDNA	18314	0.31
5-aminolevulinic acid synthase	13255	0.32
unnamed protein product _7	18984	0.32
No Hits Found_54	15755	0.32
Similar to LIM domains	16825	0.32
DNA-directed DNA polymerase	16612	0.32
No Hits Found_80	11711	0.32
similar to CG3714-PA	11017	0.32
No Hits Found_3	10789	0.33
No Hits Found_105	6711	0.33
immunoglobulin-like receptor	5532	0.33
No Hits Found_101	9678	0.33
unnamed protein product_13	18002	0.33
putative sweet taste receptor T1R1	17155	0.34
hypothetical protein MGC38361	13731	0.34
fast myosin heavy chain isoform 2	12332	0.34
heat shock protein 40	17417	0.35
zinc finger protein 1	14057	0.35
outer dense fiber of sperm tails 2	10349	0.35
CREATINE KINASE	15098	0.35
tumor necrosis factor type 1	15016	0.36
No Hits Found_88	14208	0.36
N-deacetylase/N-sulfotransferase	10917	0.36

Table 10 Marker selection analysis for Isocitrate Dehydrogenase

Gene Name	Spot	Score
Isocitrate dehydrogenase	16230	0
Putative protein C21orf45	346	0.03
visinin-like 1	11435	0.04
No Hits Found_67	14042	0.04
unnamed protein product_9	5958	0.05
poly(A) binding protein	1670	0.05
eukaryotic initiation factor 5	11520	0.05
No Hits Found_130	16172	0.05
similar to LPS-responsive	11025	0.05
No Hits Found_5	17423	0.05
RIKEN cDNA 1110066L09	17279	0.05
serine incorporator 1	9694	0.05
ATP synthase alpha subunit_1	8133	0.06
DEAH box isoform	2736	0.06
No Hits Found_84	4336	0.06
ATP synthase alpha subunit	13508	0.06
prenyl protein protease	9139	0.06
NEURONAL PROTEIN 3.1	4912	0.06
HSPC288	13041	0.06
GAPDH	14661	0.07
deoxyhypusine synthase	16197	0.07
similar to RALY	1194	0.07
trypsin domain containing 1	10806	0.07
No Hits Found_34	2861	0.07
vesicle associated protein	5128	0.07
agCP3452	8686	0.08
hnRNPC related	6730	0.08
Lipid Transfer ProteinII	12459	0.08
unnamed protein product_1	4379	0.08
RIKEN cDNA _1	7775	0.08
similar to GS15	7790	0.08
No Hits Found_25	7453	0.08
mitochondrial ribosomal prtn S23	17989	0.09
No Hits Found_116	14562	0.09
No Hits Found_15	11568	0.09
ankyrin 2	17734	0.09
Nesprin-2	843	0.09
pre-mRNA splicing factor_2	14844	0.09
extensin	17131	0.09
Alpha1,2-Mannosidase	13854	0.09
No Hits Found_127	18905	0.1
Rho-related	1196	0.1
RIKEN cDNA 2610209N15	4982	0.1
mitochondrial ribosomal prtn L17	16194	0.1
hypothetical protein_8	5165	0.1
hypothetical protein_7	5205	0.1
Lipoate synthase	6863	0.1
pre-mRNA splicing factor	9696	0.1
No Hits Found_70	4448	0.1
endocrine neoplasia 1	12899	0.1

Table 11 Marker selection analysis for Induced by TNF alpha (TNF AP)

Gene Name	Spot	Score
induced by tumor necrosis factor alpha	10372	0
c-src tyrosine kinase	10037	0.03
SPT3-associated factor	2884	0.03
similar to exocyst complex component 3	6894	0.03
splicing factor 3a	10344	0.04
No Hits Found_60	1724	0.04
Ribonuclease CL2	13900	0.04
GATA-2	5044	0.05
LIM-domain protein CRP1	13465	0.05
disintegrin-like and metalloprotease domain	4482	0.05
IGF-binding protein 2	3240	0.05
Tropomyosin 1 alpha chain	17149	0.05
eukaryotic translation initiation factor 3, s6	628	0.05
Similar to CGI-67 protein	14406	0.05
FLJ22611-like protein	3250	0.05
vesicle-associated membrane protein	16240	0.05
No Hits Found_33	10679	0.05
nucleotide pyrophosphatase 3	593	0.06
type V preprocollagen alpha	4453	0.06
Unknown protein_1	3921	0.06
Rho GTPase activating protein 26	11565	0.06
TROPONIN I	2751	0.06
FLJ00315 protein	17077	0.06
muscle specific gene	8010	0.06
hypothetical protein _3	10193	0.06
origin recognition complex	2327	0.06
polymerase II polypeptide C	2825	0.07
No Hits Found_7	4878	0.07
glucokinase	1753	0.07
hypothetical protein FLJ11200	15682	0.07
heat shock protein 40	17417	0.07
Tcf-3 co-repressor CtBP	161	0.07
phosphatidylinositol transfer protein	9927	0.07
RB1-inducible coiled-coil 1	7105	0.08
Ferredoxin-NADP(+)reductase	1322	0.08
PTEN induced putative kinase 1	15035	0.08
NADH dehydrogenase flavoprotein 1	5452	0.08
Collagen alpha 1	5378	0.08
chloride channel 7	530	0.08
Creatine kinase	15098	0.08
Arp23	9681	0.08
ATP synthase beta chain	1743	0.08
coronin, actin binding protein, 2B	14087	0.09
eukaryotic translation elongation factor 1_2	15734	0.09
BLOCK 25	7848	0.09
hypothetical protein	16484	0.09
Apolipoprotein A-I	12316	0.09
Calpactin I light chain	2210	0.09
No Hits Found_72	1662	0.09
ATP/GTP-binding protein	8012	0.09

Table 12 Marker selection analysis for CEBPζ

Gene Name	Spot	Score
CEBPζ	19069	0
RB1-inducible coiled-coil 1	7105	0.05
ATP synthase beta chain	1743	0.05
KIAA1892 protein	4417	0.05
NADH dehydrogenase flavoprotein 1	5452	0.06
RIKEN cDNA _4	3763	0.06
IQ motif containing K (IQCK)	244	0.07
heat shock protein 40	17417	0.07
N-deacetylase/N-sulfotransferase	10917	0.07
Unknown protein _5	357	0.07
CREATINE KINASE	15098	0.08
disintegrin-like and metalloprotease domain	4482	0.08
polymerase II polypeptide C	2825	0.09
Ferredoxin-NADP(+)reductase\	1322	0.09
interleukin enhancer binding factor 2	9723	0.09
Tropomyosin 1 alpha chain	17149	0.09
Unknown protein_1	3921	0.09
eukaryotic translation initiation factor 3, s6	628	0.1
mitochondrial ribosomal protein S25	2875	0.1
No Hits Found_86	4050	0.1
chloride channel 7	530	0.1
origin recognition complex	2327	0.1
Unknown protein _3	2888	0.11
phosphatidylinositol transfer protein	9927	0.11
No Hits Found_54	15755	0.11
induced by tumor necrosis factor alpha	10372	0.12
No Hits Found_85	15679	0.12
ADP-ribosylation-like factor 6	13499	0.12
Tryptophan 5-monooxygenase	7185	0.12
novel glycoprotease	17485	0.12
hypothetical protein FLJ11200	15682	0.12
importin 13	1294	0.12
Similar to LIM domains	16825	0.13
target of Jun 3	474	0.13
inhibitor of growth family, member 3	13496	0.13
immunoglobulin-like receptor	5532	0.13
Rho GTPase activating protein 26	11565	0.13
Cytochrome P450	17362	0.13
hypothetical protein_4	17208	0.14
No Hits Found_7	4878	0.14
No Hits Found_60	1724	0.14
coronin, actin binding protein, 2B	14087	0.14
infertility-related sperm protein	6576	0.14
Acylphosphatephosphohydrolase	14313	0.14
photomedin-1	7902	0.14
Tcf-3 co-repressor CtBP	161	0.15
Peroxisomal coenzyme A diphosphatase	4869	0.15
glucokinase	1753	0.15
No Hits Found_139	4526	0.15
leukotriene B4 12-hydroxydehydrogenase	680	0.15

Table 13 Marker selection analysis for phosphoglucose isomerase

Gene Name	Spot	Score
phosphoglucose isomerase	73	0
tetraspan 3	17122	0.01
retinoblastoma tumor suppressor	9201	0.02
ACTIN, CYTOPLASMIC TYPE 5	4161	0.02
nuclear distribution gene C	17031	0.02
endocrine neoplasia 1	12899	0.02
ADAM 12	17139	0.02
tubulin, beta 5	2954	0.03
Similar to macropain	13235	0.03
unnamed protein product	3853	0.03
LEP100 protein	14458	0.04
beta-amyloid precursor	3906	0.04
ubiquitin-like 3	18010	0.04
No Hits Found_116	14562	0.04
eukaryotic translation elongation factor 1_1	16106	0.05
ERO1-like	9129	0.05
POL-like	15506	0.05
Neural enolase (NSE)	13011	0.05
EST H08032.1	13326	0.05
vesicle associated protein	5128	0.05
Similar to hypothetical protein	14095	0.05
Dihydropyrimidinase related protein-2	12821	0.05
tubulin, beta	9603	0.05
KIAA0605 gene product	13929	0.05
malignant liposarcoma	6875	0.06
synapsin Iib	14818	0.06
vesicular fusion protein NSF	9664	0.06
adenosinetriphosphatase	13232	0.06
hexokinase1	10230	0.07
gamma-synergin	14853	0.07
Ubiquitin thiolesterase 1	13281	0.07
unnamed protein product _13	2687	0.07
similar to Eferin	15094	0.07
pre-mRNA splicing factor_1	14854	0.07
unnamed protein product_17	6569	0.07
No Hits Found_20	15371	0.07
Piccolo protein	6717	0.07
ankyrin 2	17734	0.07
nuclear transcription factor Y	4189	0.08
chaperonin containing TCP-1	17165	0.08
T-cell lymphoma tumor antigen	9876	0.08
QkI-2	16051	0.08
No Hits Found_71	6082	0.08
Regulatory protein TSC-22	5214	0.08
HSCO protein	12939	0.08
pre-mRNA splicing factor_2	14844	0.08
Selenophosphate synthetase 2	13258	0.08
Sodium pump 3_1	2045	0.09
klotho secreted isoform	3653	0.09
unnamed protein product	15622	0.09

Table 14 Marker selection analysis for hexokinase 1

Gene Name	Spot	Score
hexokinase1	10230	0
gamma-synergin	14853	0.01
beta-amyloid precursor	3906	0.02
Ubiquitin thiolesterase 1	13281	0.02
NDRG	4966	0.02
vesicular fusion protein NSF	9664	0.02
Dihydropyrimidinase related protein-2	12821	0.02
No Hits Found_20	15371	0.03
adenosinetriphosphatase	13232	0.03
valosin-containing protein	1746	0.03
tubulin, beta 5	2954	0.03
LEP100 protein	14458	0.03
ubiquitin-like 3	18010	0.04
ERO1-like	9129	0.04
nuclear ribonucleoprotein L	11670	0.04
EGF-response factor 2	16446	0.04
SNAP-beta	16210	0.04
Sodium pump 3_1	2045	0.05
klotho secreted isoform	3653	0.05
Dynactin	16239	0.05
unnamed protein product_17	6569	0.05
synapsin Iib	14818	0.05
phosphate carrier protein	12265	0.06
unnamed protein product _6	16147	0.06
transcription elongation factor B	1676	0.06
Neural enolase (NSE)	13011	0.06
Unknown protein_5	1831	0.06
retinoblastoma tumor suppressor	9201	0.06
phosphoglucose isomerase	73	0.07
Similar to macropain	13235	0.07
nuclear distribution gene C	17031	0.07
QkI-2	16051	0.07
Selenophosphate synthetase 2	13258	0.07
unnamed protein product	3853	0.07
sphingosine kinase 1	3703	0.07
ACTIN, CYTOPLASMIC TYPE 5	4161	0.07
unnamed protein product _12	2170	0.07
POL-like	15506	0.07
unnamed protein product	15622	0.07
endocrine neoplasia 1	12899	0.08
ankyrin repeat and SOCS box protein	14767	0.08
Sodium pump 3	8105	0.08
SET protein	11620	0.08
KIAA0605 gene product	13929	0.08
EST H08032.1	13326	0.08
tetraspan 3	17122	0.08
malignant liposarcoma	6875	0.08
similar to phosphoglycerate mutase	6482	0.09
No Hits Found_68	7604	0.09
similar to Eferin	15094	0.09

Table 15 Marker selection analysis for phosphoglycerate mutase

Gene Name	Spot	Score
similar to phosphoglycerate mutase	6482	0
Sodium pump 3_1	2045	0.02
Sodium pump 3	8105	0.02
Amyloid-like protein 2	12582	0.03
SNARE	10056	0.03
transcription elongation factor B	1676	0.03
beta-amyloid precursor	9607	0.03
eukaryotic translation elongation factor 1_1	16106	0.04
Sodium pump 3_2	11249	0.04
klotho secreted isoform	3653	0.04
eukaryotic translation elongation factor 1	16149	0.04
fusion protein attachment protein	8244	0.04
pre-mRNA splicing factor	9696	0.04
Neural enolase (NSE)	13011	0.04
Regulatory protein TSC-22	5214	0.05
alpha 1 type III collagen	5511	0.05
similar to Amyloid beta A4	1900	0.05
No Hits Found_128	4568	0.05
hypothetical protein _8	487	0.06
RNA-binding protein FUS	16410	0.06
extensin	17131	0.06
PP2A-alpha	3986	0.06
pre-mRNA splicing factor_2	14844	0.06
unnamed protein product	3853	0.06
No Hits Found_56	12987	0.06
Na/K ATPase_1	2049	0.06
beta-amyloid precursor	3906	0.07
calmodulin 2	14543	0.07
Similar to protein kinase C	6549	0.07
No Hits Found_20	15371	0.07
F-box only protein 3	1309	0.07
VAMP	8081	0.07
Unknown protein	14486	0.08
poly(A) binding protein	1670	0.08
ADAM 12	17139	0.08
ACTIN, CYTOPLASMIC TYPE 5	4161	0.08
EST H08032.1	13326	0.08
No Hits Found_68	7604	0.08
vesicle associated protein	5128	0.08
adenosinetriphosphatase	13232	0.08
malignant liposarcoma	6875	0.08
Na/K ATPase_3	4949	0.08
endocrine neoplasia 1	12899	0.08
hexokinase1	10230	0.09
agCP3452	8686	0.09
synapsin Iib	14818	0.09
Na/K ATPase	1902	0.09
Pleiotrophin	8479	0.09
Unknown gene product	17829	0.09
phosphate carrier protein	12265	0.09

Table 16 Marker selection analysis for Neural Enolase

Gene Name	Spot	Score
Neural enolase (NSE)	13011	0
malignant liposarcoma	6875	0.01
klotho secreted isoform	3653	0.02
PP2A-alpha	3986	0.02
ACTIN, CYTOPLASMIC TYPE 5	4161	0.02
No Hits Found_20	15371	0.02
Sodium pump 3_1	2045	0.02
RNA-binding protein FUS	16410	0.03
beta-amyloid precursor	3906	0.03
eukaryotic translation elongation factor 1_1	16106	0.03
Regulatory protein TSC-22	5214	0.03
unnamed protein product	3853	0.03
synapsin Iib	14818	0.03
EST H08032.1	13326	0.03
tubulin, beta 5	2954	0.03
Unknown protein_3	17661	0.03
endocrine neoplasia 1	12899	0.03
vesicle associated protein	5128	0.03
No Hits Found_116	14562	0.04
No Hits Found_56	12987	0.04
transcription elongation factor B	1676	0.04
PQBP-1b/c	3258	0.04
similar to phosphoglycerate mutase	6482	0.04
tubulin, beta	9603	0.05
ADAM 12	17139	0.05
POL-like	15506	0.05
phosphoglucose isomerase	73	0.05
tetraspan 3	17122	0.05
No Hits Found_68	7604	0.05
beta-amyloid precursor	9607	0.05
Amyloid-like protein 2	12582	0.05
Dihydropyrimidinase related protein-2	12821	0.05
alpha 1 type III collagen	5511	0.06
similar to hypothetical protein_1	8410	0.06
gamma-synergin	14853	0.06
No Hits Found_47	5021	0.06
hexokinase1	10230	0.06
retinoblastoma tumor suppressor	9201	0.06
ubiquitin-like 3	18010	0.06
SNARE	10056	0.06
similar to Amyloid beta A4	1900	0.06
serum deprivation response protein	13473	0.06
unnamed protein product _6	16147	0.07
vesicular fusion protein NSF	9664	0.07
SET protein	11620	0.07
KIAA0605 gene product	13929	0.07
Similar to macropain	13235	0.07
Sodium pump 3	8105	0.07
fusion protein attachment protein	8244	0.07
Fas-ligand associated factor 3	7452	0.07

Table 17 Marker selection analysis for Aldolase C

Gene Name	Spot	Score
aldolase C	3836	0
PHLDB2	1283	0.07
NADP-specific isocitrate dehydrogenase	5860	0.07
unnamed protein product_4	15664	0.07
vesicle-associated membrane protein 3	1740	0.07
neurofilament-L	4896	0.09
Thy-1 antigen	5098	0.09
elongation factor 1-beta	2131	0.09
serine incorporator 1	9694	0.1
vitronectin	11696	0.11
RIKEN cDNA 1200015A22	3363	0.12
glutathione S-transferase	10	0.12
pleiotrophin	8479	0.13
deoxyhypusine synthase	16197	0.13
SET domain, bifurcated 1	9877	0.13
Na/K ATPase_2	17653	0.14
No Hits Found_25	7453	0.14
SNARE	10056	0.14
Na/K ATPase_1	2049	0.15
No Hits Found_34	2861	0.15
alpha 1 type III collagen	5511	0.15
cytochrome b	17687	0.15
Sigma-adaptin 3b	5121	0.15
Similar to protein kinase C	6549	0.16
unnamed protein product_2	2694	0.16
acyl-Coenzyme A dehydrogenase family	16554	0.16
tryptophan 5-monooxgenase	1732	0.16
No Hits Found_84	4336	0.16
Tyrosine kinase receptor	5022	0.16
hypothetical protein _8	487	0.17
endoglin	14721	0.17
Na/K ATPase_3	4949	0.17
EH-domain containing 3	4323	0.17
VAMP	8081	0.18
SH3BGRL3-likeprotein	2800	0.18
Unknown protein	14486	0.18
hydroxyproline-rich glycoprotein	10403	0.18
Amyloid-like protein 2	12582	0.18
Na/K ATPase	1902	0.18
translation elongation factor 1 alpha_1	1751	0.18
trypsin domain containing 1	10806	0.19
Alpha1,2-Mannosidase	13854	0.19
unnamed protein product_1	4379	0.19
Cloning vector	4125	0.19
unnamed protein product_9	5958	0.19
unknown	1805	0.2
Sodium pump 3	8105	0.2
F-box only protein 3	1309	0.2
Transthyretin precursor (Prealbumin)	8007	0.2
beta actin	5104	0.2

TNF AP gene expression. We ran qPCR to verify expression profiles for four of these genes, CEBPζ, TNF AP, Metalloprotease 1, and GHRHR (Fig. 6). All but TNF AP were confirmed by qPCR. However, for TNF AP there was a 10-fold difference in mRNA levels between the Fat and Lean line at week 1, which closely correlated with expression of the other three genes evaluated.

Glycolysis was a major biological process altered between the Fat and Lean lines, with five out of the ten genes involved with converting glucose into pyruvate identified in the microarray analysis (Table 4). Using the KNN algorithm, we identified four out of the five genes as being closely linked, which isn't surprising since they are all key genes involved in glycolysis (Fig. 7b and Tables 13-17). From this analysis, we identified Klotho, DRP2 and ADAM12 as genes that may interact with the glycolytic pathway in the hypothalamus during the divergence of adiposity between the Fat and Lean lines. Expression profiles for these three genes remains to be confirmed by qPCR.

Discussion

In this quantitative trait "obesity" model, microarray analysis revealed dynamic changes in gene expression within the hypothalamus before and after the development of adiposity. A number of the genes identified with the microarray analysis have been previously shown by transgenic or knockout mouse technology to directly regulate adiposity or body weight. We also found that genes involved in glycolysis were dramatically downregulated in the Fat line relative to the Lean line before the development of adiposity, week 1 (Fig. 3a and Fig. 5). Our study also contributes novel information by identifying the following: 1) 40 genes that correlate

with expression of either the fat or lean phenotype (Fig. 1), 2) downregulation of genes involved in glycolysis in the Fat line relative to lean is observed during the phase prior to adiposity divergence as well as the phase after adiposity has diverged and begun to stabilize between the lines, suggesting that it plays a key role in determining genetic susceptibility, and 3) many of the genes which have been shown to be associated with adiposity and body weight through transgenic or knockout techniques are also associated with genetically induced obesity via genetic selection of animal lines.

Glycolysis and the development of adiposity.

We have identified differences in genetic selection for genes known to modulate 5 out of the 10 steps involved with glycolysis. Glycolytic gene expression was downregulated in the hypothalamus of the Fat relative to Lean line at week 1 of age, prior to any notable differences in adiposity between the lines. In a similar manner, it has been shown that an enhancement in hepatic glycolysis has also been associated with a reduction in obesity [91], which parallels the upregulation in the lean line or downregulation in the fat line of glycolytic genes. We found it remarkable that 5 out of the 10 genes for enzymes regulating glycolysis were identified from the microarray. Expression patterns were confirmed for three genes using qPCR (Fig. 5). We have also identified three genes which are potentially upstream regulators, downstream targets or genes simply expressed in parallel with four of the genes involved with glycolysis, Klotho, DRP2 and ADAM12.

Klotho is a gene that has been associated with regulating energy metabolism. Mice lacking Klotho gene expression have almost no white adipose tissue, but normal

levels of brown adipose tissue[92]. Surprisingly, we observed increased levels in the Lean line relative to the Fat line before adiposity began to diverge, week 1. However, it is plausible that another parameter, like glucose levels could predominantly regulate Klotho gene expression. For example, the C1818T polymorphism in exon 4 of this gene has been associated with higher fasting plasma glucose level than individuals without the polymorphism[93]. The Lean line does have higher levels of glucose, so it is plausible that a similar polymorphism is present in the chicken Klotho gene.

We have also demonstrated potential links between adiposity, DRP2 and ADAM 12. The disintegrin and metalloprotease with thrombospondin motifs 1 [94] have been directly associated with modulating body composition [95] and are present in the ADAM 12 gene. Expression patterns of this gene are opposite than predicted at week 1, increased expression in the Lean relative to Fat line, since mice lacking this motif have been shown to have a lean phenotype. In regards to DRP2, also known as CRMP2, the gene is associated with Alzheimer's disease[96,97] and is known to be involved with development of the nervous system[98]. This is the first report associating the function of this gene with energy regulation, specifically glycolysis and processes contributing to the divergence of the fat or lean phenotype, suggesting that further investigation for the role of DRP2 in glycolytic metabolism is warranted.

Homologous genes in knockout or transgenic mice were identified by the microarray analysis

Many genes have been identified to be involved with regulating body composition through genetic manipulations. Analysis of the microarray data identified some of the same genes previously known to modulate adiposity, and the

data support the hypothesis developed for which genes interact, upstream or downstream, of one another. TNFα associated genes were another family of genes that had expression differences between lines. TNFα is a cytokine secreted from many cell types, including adipocytes, and has been shown to act on the hypothalamus to suppress appetite [99]. We demonstrated an upregulation for genes related to TNFα signaling in the Lean relative to Fat line, potentially explaining the decreased adiposity in the Lean relative to Fat line.

The TRAF-1 molecule has been associated with gene expression for the sweet taste receptor (T1R1), which is also present in the lining of the small intestine and is associated with glucose uptake [100]. The Fat and Lean lines have differences in glucose levels, with the Fat line having lower plasma glucose and higher plasma insulin relative to the lean line [90]. Since expression of T1R1 and TRAF-1 are lower in the Fat relative to Lean line, this suggests that T1R1 in the hypothalamus may be associated with TRAF-1 to modulate glucose uptake in the neuron, in a similar manner as demonstrated in the intestine. TRAF-1 was also associated in our current study with GHRHR and 5-HTP, the rate-limiting enzyme for serotonin production. Both of these genes have been previously implicated in regulating body composition[101,102] and indirect relationships have been shown to exist between TNFα and GHRH or 5-HTP. For example, by blocking serotonin receptors the antitumor effects of TNFα were attenuated [103]. We demonstrate here that decreased levels of 5-HTP mRNA correlate with decreased levels of TRAF-1 mRNA in the Fat relative to Lean line. In regards to GHRH, mice with deficits in GHRH signaling were not able to increase mRNA expression for GHRHR or TNFα after virus treatment, suggesting that a direct

relationship between the two genes may exist [104]. Therefore, future investigations to further characterize these interactions are warranted.

Our analysis also suggests that TNF AP may interact with CEBPζ, CEBPα and β have been associated with adipocyte differentiation [105], and here we show that hypothalamic CEBPζ may also be associated with the divergence of adiposity in the Fat and Lean lines, with CEBPζ mRNA levels being greater in the Fat relative to Lean line. Lastly, Metalloprotease 1 was also identified in the present study as a candidate gene associated with TRAF-1. Interestingly, it has been found that tissue inhibitor of metalloprotease 1 is elevated in obese children, resulting in lower levels of metalloprotease 1 [106]. This trend corresponds with the increased levels of metalloprotease 1 mRNA observed in the hypothalamus of the Fat line relative to Lean line at week 1 of age. It has also been shown that TNFα elevates the levels of a disintegrin and metalloprotease with thrombospondin motifs 1 [94], which has been directly associated with modulating body composition [95], which in combination with our current findings suggests that TNFα may interact with or regulate expression of metalloprotease 1 to modulate body composition.

Allelic segregation and genetic influences

The data presented here further support the possibility that genotype may influence development of the obese phenotype by regulating hypothalamic expression patterns of genes in ways not previously predicted. This may occur by allelic segregation occurring in genes presented in this paper or it may be due to alleles of the unidentified genes that modulate expression for the genes identified here. Furthermore, it is possible that differential expression in the hypothalamus of genes

related to TNFα signaling processes, CEBP and glycolysis in the Fat relative to Lean line prior to the onset of adiposity serve to establish a set point for long-term regulation of body fat stores during a critical developmental period. As adiposity develops and the adipose mass set point evolves and matures over the first 9 weeks, these genes may function to establish homeostatic mechanisms by differentially modulating one of the following: 1) metabolism, 2) nutrient utilization or 3) body composition set point. Furthermore, during a critical period shortly after hatch/birth, expression patterns of TNFα signaling processes and glycolytic genes may serve to regulate development of the fat or lean phenotype, or this may occur in parallel. The altered gene expression at week 1 of age, prior to any differences in adiposity, may modulate neuronal synaptic development to regulate the long-term body fat "set-point" at this age, which is further supported by the fact that neural pathways between nuclei in the hypothalamus are not established until postnatal day 8 in the mouse [107]. Furthermore, we have identified a large set of genes that are differentially regulated due to the allelic segregation from 21 generations of selective breeding and which may potentially interact to regulate energy homeostatic mechanisms.

Quantitative trait animal models are useful for identifying novel candidate genes that regulate energy homeostasis. For example, one rat model was bred to be susceptible (DIO) or resistant (DR) to dietary-induced obesity, and has been very useful for elucidating how environment and genotype interact to influence the development of obesity [21]. Here we present a model that allowed us to study genotype-regulated differences during development of adiposity, independent of environmental variables, suggesting that this chicken model has the potential to

elucidate the underpinnings of genotype induced human obesity. Furthermore, this study has a high relevancy for human implications since the chicken genome has a greater synteny with the human genome than does the mouse [108,109]. Thus, here we utilized microarray analysis to investigate potential polygenic expression patterns and identify novel candidate genes that may contribute to the development of genotype induced obesity which may have relevancy with potential clinical implications.

Chapter 3: Differential Expression of Anorexigenic and Orexigenic Genes in Fat and Lean Lines

Abstract

Gene interactions in the hypothalamus may regulate development of obesity, since this brain region modulates food intake, metabolic rate and body weight. We characterized gene expression in the hypothalamus during development of adiposity in fat and lean chicken lines, selectively bred for high and low percent body fat that diverges independent of environmental influences. Using quantitative real time PCR we determined gene expression patterns before and after differences in adiposity diverge between the lines for 14 orexigenic and anorexigenic genes. Neuropeptide Y (NPY) mRNA was decreased and Pro-opiomelanocortin (POMC) mRNA was increased in the fat relative to the lean line, consistent with known responses to adipose signals. Genes demonstrating an interesting expression profile were Thyrotropin Releasing Hormone (TRH), Brain-Derived Neurotrophic Factor (BDNF), Tyrosine kinase receptor B (TrkB), and Leptin Receptor (LEPR) since they demonstrated differences in expression between the lines prior to any differences in adiposity (week1). Thus, these genes might be key anorexigenic and orexigenic neuropeptides that modulate the divergence of an obese phenotype prior to alterations in levels of adiposity. Surprisingly, differential expression between or within lines for Melanocortin-4 receptor mRNA was not observed. This suggests that genotype may influence development of the obese phenotype or interact with hypothalamic gene expression patterns regulating anorexigenic and orexigenic genes which may program an initial set point to establish long-term regulation of body fat stores.

Introduction

In humans and other species, heritability influencing genetic susceptibility contribute to the development of an obese phenotype [9]. Increased adiposity may arise from genotype variations, which contribute to susceptibility for developing the phenotype, and environmental factors, such as diet, exercise, and social factors [110]. Altering neuropeptide expression in the hypothalamus may disrupt the mechanisms that serve to regulate energy homeostasis, thereby altering the ability to maintain a constant level of energy stores over time in the form of fat [8,111]. More specifically, neuropeptide expression in the hypothalamus has been identified as a key regulator of body weight and food intake processes based on lesion studies that ablate either the ventromedial hypothalamus or the lateral hypothalamus [4,5]. Although both genetic and environmental factors contribute to disrupting energy homeostasis, alterations in environmental factors are not always essential to disrupt energy regulation, and genetic factors alone can give rise to an obese phenotype [112].

Many neuropeptides in the hypothalamus have been implicated in energy homeostasis, some increasing fat deposition (orexigenic/anabolic) and some decreasing fat deposition (anorexigenic/catabolic). Several candidate neuropeptide-containing pathways in the hypothalamus have emerged as mediators for energy homeostasis. Under normal conditions, these peptides function in a complimentary fashion to maintain a dynamic equilibrium of energy homeostasis with orexigenic pathways that serve to increase food intake, increase fat deposition and decrease energy expenditure (e.g. Neuropeptide Y, NPY; Agouti-related protein, AGRP; Orexin, ORX; and melanin concentrating hormone, MCH) and anorexigenic

pathways that decrease food intake, decrease fat deposition and increase energy expenditure (e.g. Corticotropin releasing hormone, CRH; Thyrotropin releasing hormone, TRH; Cocaine- and amphetamine-regulated transcript, CART; and Proopiomelanocortin, POMC) [8,113-115]. Although glucocorticoids have an anabolic effect on adipose tissue, we have chosen to define CRH as a catabolic neuropeptide based on regulation by adipose singals. CRH mRNA levels increase in order to decrease energy intake as leptin and insulin levels increase[8]. Although monogenic alterations can result in food intake and/or body weight disregulation, it remains to be determined which polygenic alterations in anorexigenic and orexigenic gene expression contribute to altered phenotypic states like obesity.

Novel candidate genes that regulate energy homeostasis can be identified by using quantitative trait animal models selectively bred for a phenotype. Rats bred to be susceptible (DIO) or resistant (DR) to dietary-induced obesity have been very useful for elucidating how environment and genotype interact to influence the development of obesity[21]. Here we present a chicken model that allows us to elucidate genotype induced obesity, independent of environmental variables. Furthermore, the chicken genome has more synteny with the human genome than a rodent species[108,109], further supporting how relevant and useful these data are for potential clinical implications by identifying more mechanisms that contribute to obesity susceptibility based on genotype.

Therefore, we utilized Fat and Lean chicken lines in order to determine anorexigenic and orexigenic gene expression that correlated with the development of adiposity. Selective breeding for fat and lean phenotypes over 21 generations

allowed segregation of alleles regulating body composition in these lines[15]. More importantly, these phenotypes develop independently of environmental and behavioral influences (i.e. a high fat or high calorie diet, level of food intake) [19,84,85], suggesting that development of adiposity is based on genotype differences alone. Neuropeptides in the hypothalamus are known to modulate energy homeostasis in the same manner in both chickens, rodents and humans[116]. Here we dissociate genetic from environmental influences on adiposity by utilizing Fat and Lean lines of chickens, which allows us to identify genes that regulate genetic susceptibility for body composition [19,84]. This is an important dissociation because it is often difficult to separate genetic influences regulating increased adipose mass without simultaneously altering food intake.

In order to better elucidate the underlying genetic mechanisms that contribute to the development of the "obese" phenotype, independent of altering food intake, we have characterized anorexigenic and orexigenic gene expression in the hypothalamus prior to and after differences in adiposity in this unique animal model. We predicted that increased expression of anorexigenic neuropeptides, such as POMC, CRH, TRH and CART, with simultaneous suppression of orexigenic neuropeptides such as NPY, AGRP, ORX and MCH would be observed in the fat relative to the lean line in order to compensate for increased adipose fat, since previous work has demonstrated that increased adipose mass results in increased expression of POMC and decreased expression of NPY and AGRP [8]. However, if the predicted compensatory patterns of expression for either the anorexigenic or orexigenic genes are not observed, then it is plausible that these genes could contribute to the underlying mechanisms that result in

either a fat or lean phenotype. Prior to this study, it was unknown whether differences in anorexigenic and orexigenic neuropeptide expression would develop prior to or after divergence in adiposity. It also remained to be determined whether differences in some or all of these genes would be observed independent of alterations in food intake. Our results demonstrate that 8 out of the 14 genes tested have altered expression, which occurs independently of altered food intake, as previously demonstrated in this model of adiposity[19,84,85]. Four of the genes were expressed in a direction different from that predicted and two of these genes were differentially expressed prior to the onset of adiposity divergence between the two lines. These results help to elucidate the mechanisms that may contribute to development of genetically based adiposity.

Materials and Methods

Animals and tissue preparation.

Genetically selected Fat and Lean chicken lines, developed at INRA, were used [19]. Selective breeding for about 21 generations occurred by choosing animals with the greatest and lowest amount of abdominal fat with similar body weight at 9 weeks of age. Males from each line were kept and reared in floor pens (4.4 x 3.9 meters). Fat (16) and Lean (16) chickens were reared together in the same pen to eliminate environmental differences. They were given *ad libitum* access to food and water at all times using conventional starter (0-3 weeks) and grower (3-11 weeks) pellet diets. The light/dark cycle was: 24 hr of light for the first two days and then 14 hr of light and 10 hr of dark thereafter.

Hypothalami were collected from Fat and Lean animals at weeks 1, 3, 5 and 7 of age in order measure differences in gene expression correlating with the development of the phenotypes. Adiposity differences diverged between the Fat and Lean line by week 3 of age. There were no differences in adiposity at week 1 of age, providing a baseline to compare physiological alterations before and after the onset of excessive adiposity. Animals were sacrificed with the hypothalamus being immediately dissected and snap frozen in liquid nitrogen (n=4 for each age and group) and stored at –80 ºC until further processing. Commercial coated-tube radioimmunoassays were used to measure plasma levels of thyroid hormones (T_3 and T_4) (MP Biomedicals, Solon, OH). All procedures were handled in accordance with the Institutional Animal Care and Use Committee at the University of Maryland, University of Delaware and the Institute de Nationale Recherches Agronomique.

Primer design and validation.

All primers were designed using Primer Express (v 2.0, Applied Biosystems, Foster City, CA) and designed using a homologous region of chicken cDNAs when aligned with the same gene sequence for the mouse. Primers were 18-30 nucleotides in length with a melting temperature between 58-64 ºC or 69-72 ºC. The PCR product was between 100 and 150bp. Forward and reverse primer sequences are listed in Table 1.

GENE	Sense primer	Antisense primer
AGRP	5'AGAGCGGACCGTGAGGACACTT3'	5'GTTGGCATTTCCTCCCAAAGGA3'
AMPK	5'TCTCCGCGGTGGATTACTGT3'	5'AGCAGCACGTTCTCTGGTTTC3'
BDNF	5'TGGGTAACAGCAGCGGAGAA3'	5'TATTGCTTCAGTTGGCCTTTAG3'
CART	5'CACCTGCCCGAACTTCTTCTCGTA3'	5'CCCGAGAGAAGGAGCTGATCGA3'
CCKr	5'CGCACCGTCACCAACTCTTT3'	5'GAAGACGAAGGTGCCCATGA3'
CRH	5'CACAGCAACAGGAAACTGATGGAAA3'	5'AAAGAGGTGACATCAGAGCAGCACTATG3'
LEPR	5'AAAACCCAGAGCGTAGCGTCCAA3'	5'TTGCTTACGCGATCGTTCACAAG3'
MC4R	5'CGGGAGGCTGCTATGAACAA3'	5'AGCTGATGATGCCCAGAGTCA3'
MCH	5'GATTCCAGACATGACTTCTCAAATCATGGT3'	5'TCAGTGTCAGCTGGAAAAGCAATGG3'
NPY	5'GGGAAAGCACAGAAAACATTCC3'	5'AAATCCCATCACCACATCGAA3'
Orexin	5'CACGCTGAGAAGGACCTGACCAA3'	5'CCAGGGCCACAGGGAGGTATTTAA3'
POMC	5'AGGGACCTCAGGGATCATCAA3'	5'TGTTCAAGGGCAGGTTGGA3'
TRH	5'AGCATCTTTTGGAGACATTCAG3'	5'CAGCTCCAGGTAGTTGACAAGGT3'
TrkB	5'GTCCTGGGTGCTCACTAACCTT3'	5'TTATGGTTAACGAGGCAGGATTC3'
GAPDH	5'AAGGAGTGAGCCAAGCACACA3'	5'TCACTGCAGGATGCAGAACTG3'
B-actin	5'CCCAAAGCCAACAGAGAGAAG3'	5'ACCATCACCAGAGTCCATCAC3'

Table 1. Primer sequences used for qPCR.

RNA isolation and cDNA preparation.

Individual tissue samples were homogenized and total RNA extracted with Rneasy Midi kits (Qiagen, Valencia, CA) according to the manufacturer's protocol. Extracted RNA was quantified using UV absorbance (260nm) and using a bioanalyzer (Agilent Technologies, Palo Alto, CA). 1μg total RNA was used to create the cDNA using the stated protocol for Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo(dT) primer. Blank cDNA was made as described in order to have a negative control which measures genomic DNA contamination with total RNA samples, but with no reverse transcriptase added. mRNA levels were quantified using 2μl of diluted cDNA (1:200) in a 20μl quantitative real time PCR (qPCR) reaction using invitrogen recombinant Taq, 2X PCR buffer containing 100 mM KCl, 20 mM Tris-HCl (pH 9.0), 0.2% triton-X 100, 3.8 mM $MgCl_2$, 0.12 U/μl Taq Polymerase, 400 nM dNTPs, 40 nM fluorescein, and SYBR Green I Nucleic Acid Gel Stain diluted 1:10,000 (all Invitrogen, Carlsbad, CA) and analyzed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) . In all cases, a three-step PCR cycle was used: initial denaturation at 95 ºC for 3 min followed by 40 cycles of 94 ºC for 45s, 60 ºC for 45s and 72 ºC for 45s, followed by melting temperature analysis at 94 ºC in order to verify that one PCR product was amplified. PCR products were verified by dissociation curve analysis and gel electrophoresis for the appropriate size.

Statistical analysis and graphical display of data

The qPCR output data provided a Ct value for the threshold cycle. The Ct value represents the threshold cycle, which is achieved when the amount of amplified

product reaches a fixed threshold for fluorescence due to binding of SYBR green to the double-stranded PCR product. Given that each cycle is on a log scale the data were transformed to a ΔCt value by subtracting the blank Ct value from the sample Ct value. The ΔΔCt value was generated using geNorm software and methods [88]. Briefly, the data were first transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to two housekeeping genes, β-actin and GAPDH.

Differences in gene expression for all experiments were tested using a two-way analysis of variance (ANOVA), with main effects of Line and Age as well as the Line-by-Age interaction tested, using the Mixed procedure in SAS, with post-hoc comparisons by age performed using the PDIFF procedure (Statistical Analysis System, v.8.02, SAS Institute, Cary, NC). Values reported are means ± SEM, and $P < 0.05$ was required for statistical significance.

Results

Phenotype difference between the Fat and Lean lines

Phenotype differences between the Fat and Lean lines were observable for abdominal fat and the abdominal fat to body weight ratio (%), but no differences between the lines were apparent for body weight (Table 4, Chapter 2). The abdominal fat levels were not different between the two lines at week 1 of age, but differences

began to emerge by week 3 and maximal divergence was observed by week 7, reaching a plateau thereafter. By 7 weeks of age the Fat line had 266% more abdominal fat than the Lean line, which is a 2.8 fold difference in abdominal fat to body weight ratio (Table 4, Chapter 2). We observed differences in T_3 levels prior to any differences (week 1) in adiposity and immediately after adiposity began to diverge between the lines (week 3). The modestly increased T_3 levels (Fig. 1a) are similar to morbidly obese euthyroid humans [44], as well as previous observations in the Fat and Lean lines [83]. We did not observe any difference in T_3 at weeks 5 and 7 (Fig. 1a), and there were no differences observed for T_4 between the lines (Fig 1b).

Hypothalamic anorexigenic and orexigenic gene expression between the Fat and Lean lines

We screened hypothalamic genes (Figs. 2 and 3) known to modulate energy homeostasis in the same manner as in mammals [116] and identified 8 genes which differed according to increased adiposity: BDNF, TrkB, TRH, LEPR, POMC, AGRP, NPY and CRH (Fig. 2 and 3). Overall, 8 of the 15 genes tested were differentially expressed between the two lines, with 6 out of the 8 genes being classified as anorexigenic genes.

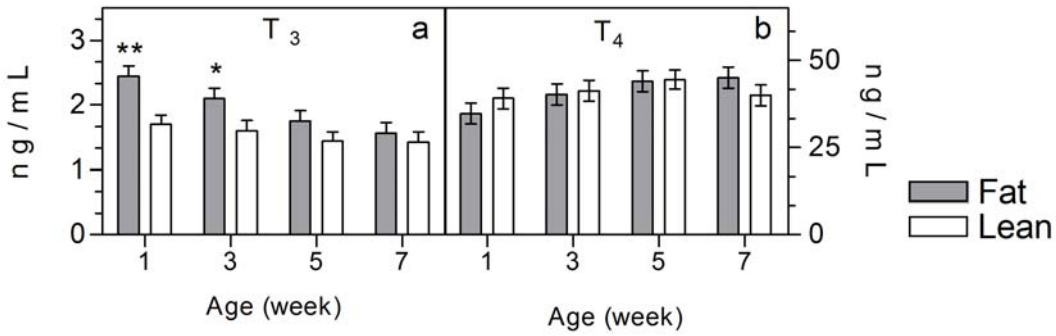

T 3
a
T4
b
**
*
n g / m L
n g / m L
0
1
2
3
0
25
50
1
3
5
7
1
3
5
7
Age (week)
Age (week)
Fat
Lean

Figure 1. **Plasma Thyroid Hormone levels in Fat and Lean Lines during development of adiposity. a-b.** Endogenous Triiodothyronine levels (T_3) (**a**) and endogenous thyroxing levels (T4) in Fat and Lean lines at weeks 1, 3, 5 and 7 (n = 4). *p<0.05 and **p<0.01 fat relative to lean line for the same age.

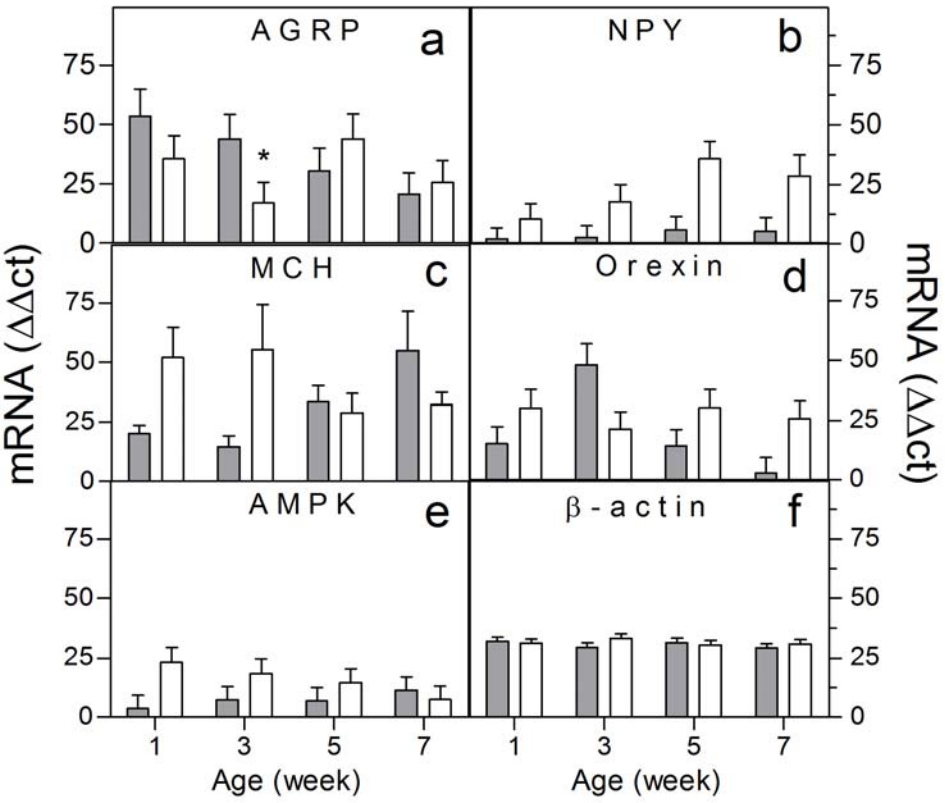

AGRP
a
NPY
b
MCH
c
Orexin
d
AMPK
e
β-actin
f
mRNA (ΔΔct)
mRNA (ΔΔct)
*
0
25
50
75
1
3
5
7
Age (week)
Age (week)

Figure 2. **Anabolic gene expression in the hypothalamus of Fat and Lean lines. a-f,** Quantification of hypothalamic AGRP (**a**), NPY[1] (**b**), MCH (**c**), Orexin (**d**), AMPK (**e**), and β-actin (**f**) mRNA levels from Fat (filled bar) and Lean (open bar) lines weeks 1, 3, 5 and 7 of age (n = 4) by qPCR analysis. [l]Denotes an overall difference between lines. * $p<0.05$, Fat relative to Lean line for the same age.

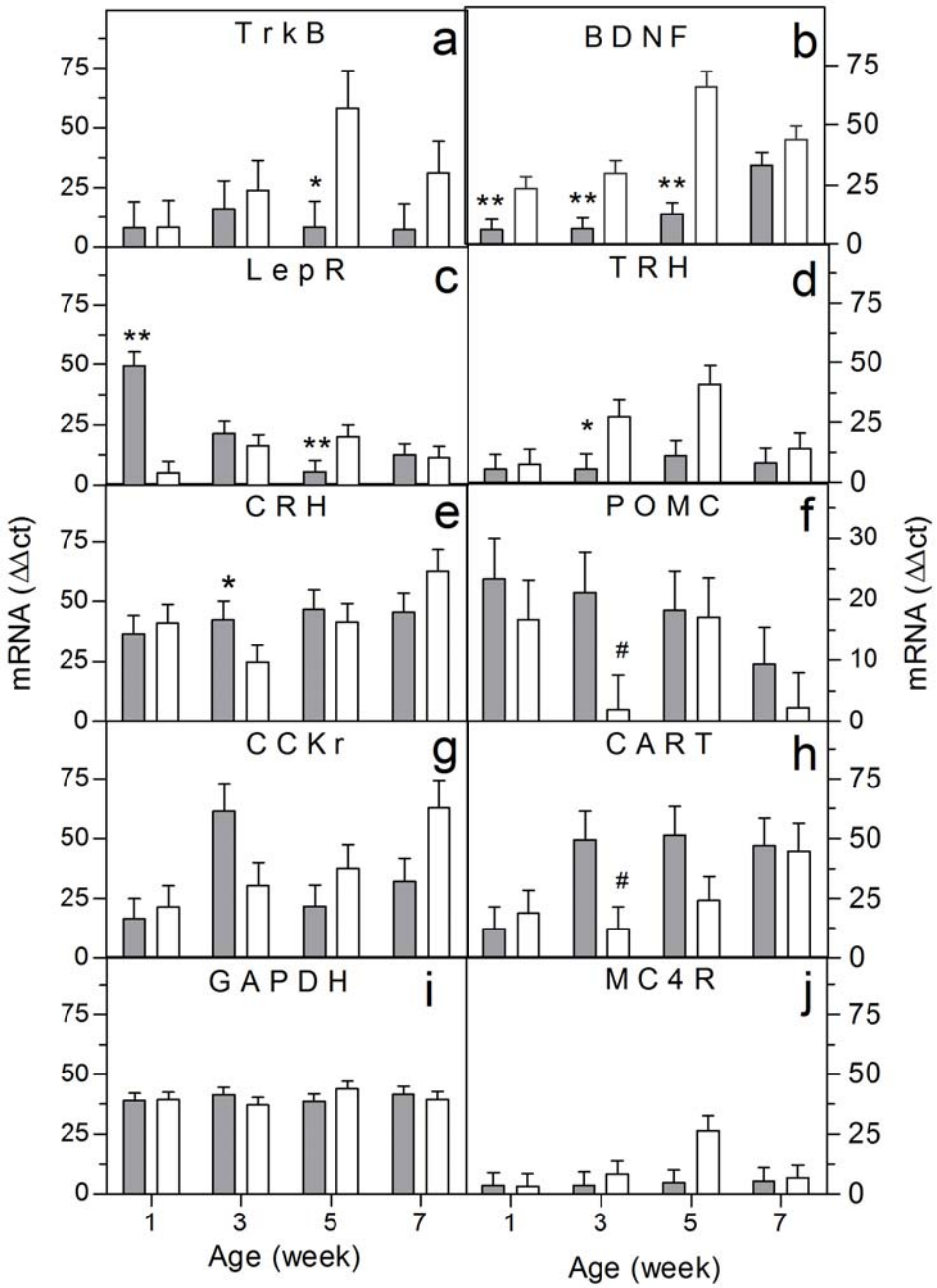

TrkB
a
BDNF
b
LepR
c
TRH
d
CRH
e
POMC
f
CCKr
g
CART
h
GAPDH
i
MC4R
j
mRNA (ΔΔct)
mRNA (ΔΔct)
Age (week)
Age (week)
1
3
5
7

Figure 3. **Anorexigenic neuropeptide mRNA levels in Fat and Lean Lines during development of adiposity. a-j.** TrkB[1] (**a**), BDNF[1] (**b**), LEPR (**c**), TRH[1] (**d**), CRH (**e**) POMC[1] (**f**), CCKr (**g**), CART (**h**), GAPDH (**i**), and MC4R (**j**) by qPCR from Fat (filled bar) and Lean (open bar) lines weeks 1, 3, 5 and 7 of age (n = 4). * $p<0.05$, **$p<0.01$, # $p<0.055$ Fat relative to Lean line for the same age. [1]Denotes an overall difference between lines.

The two orexigenic genes differentially expressed between the lines were AGRP and NPY. AGRP expression was greater in the Fat line relative to the Lean line at the time point when adiposity began to emerge, week 3 of age (Fig. 2a). Surprisingly, NPY gene expression was not expressed in the predicted direction, being greater overall in the Lean line relative to the Fat line (Fig. 1b). There were no differences in gene expression for MCH, Orexin, and AMPK (Fig 2c-e, respectively). β-actin and GAPDH gene expression after normalization were not different between the lines (Fig. 2f and 3k, respectively).

There were six anorexigenic genes differentially expressed between the two lines. Prior to and after the onset of increased adiposity, BDNF was decreased in the Fat line (Fig. 3b), suggesting that a reciprocal relationship may exist *in vivo* between plasma T_3 levels and hypothalamic BDNF expression. Additionally, we also observed subsequent suppression of TrkB mRNA at weeks 3 and 5 (Fig. 3d) and TRH at week 5 (Fig. 3a) in the Fat relative to Lean Line. LEPR mRNA was increased in the Fat relative to Lean line at week 1, and by week 5 LEPR was decreased in the Fat line (Fig. 3c). Interestingly, the profile of LEPR correlated with TrkB expression at week 5 of age, suggesting a potential biological interaction between the genes since expression profiles are similar. POMC gene expression was greater in the Fat relative to Lean line over all time points, which was expected (Fig. 3f). CART gene expression had a trend toward being greater in the Fat relative to Lean line, however it was not significant (Fig. 2g, p=.056). MC4R and CCK receptor were not significantly different between the two lines (Fig. 3g and j, respectively).

Discussion

In this quantitative trait model of adiposity, with no simultaneous alterations in food intake [84,117-119], we detected differences in anorexigenic and orexigenic gene expression before and after differences in adiposity began to diverge between the lines. Increased adiposity developed by week 3 in the Fat relative to Lean line, with no differences in adiposity at week 1 of age. We identified one hormone, T_3, and 8 genes which differed according to increased adiposity: BDNF, TrkB, TRH, LEPR, POMC, AGRP, NPY and CRH (Fig. 1). CART only approached significance ($p=.055$) (Fig. 1h). The modestly increased T_3 levels resemble those in morbidly obese euthyroid humans [44], as well as previous observations in the Fat and Lean lines[83].

During energy homeostasis, anorexigenic and orexigenic genes function to maintain steady state levels of adipose tissue. As adipose stores increase, anorexigenic signals also increase and orexigenic signals decrease. Thus, if these signals are working in a normal physiological fashion we would expect anorexigenic expression to be increased and orexigenic expression to be decreased in the Fat relative to the Lean line. However, increased adiposity often results when these signals become miss regulated and this appears to be the case for the Fat and Lean lines. An example of miss regulation is mice lacking the ability to express an anorexigenic gene, BDNF, in the brain after development. These mice are unable to properly compensate for increased adipose mass and become about three times overweight relative to a mouse expressing BDNF in the brain[35], with adult onset obesity also appearing in heterozygous mice [30]. Therefore, hypothalamic gene

expression levels based on genotype, not the environment, may occur due to a point mutation or polymorphism in the same gene or another gene that modulates expression of the anorexigenic/orexigenic gene, to compensate for the increased adiposity. Here we show that T_3 as well as BDNF and LEPR mRNA levels are different between the lines prior to any adiposity differences. We also show that NPY, POMC, LEPR and T_3 expression levels are in the direction opposite to that predicted by prior characterization of gene function, suggesting dysregulation of these genes in this model of adiposity.

No differences between the lines were observed for MC4R, MCH, Orexin, CCKr, and AMPK, possibly because there were no differences in food intake between the two lines or because altered expression of these genes is not necessary to induce differences in adiposity in this quantitative trait model. CART expression values had a trend toward significance. Differences in hypothalamic gene expression between the Fat and Lean lines were observed in both the predicted direction and the direction not predicted. Differences in the predicted direction for catabolic/anorexigenic neuropeptides were observed for BDNF at weeks 1, 3, and 5, TrkB at week 5, TRH at week 3, and for LEPR at week 5 of age, with the Lean line having greater mRNA expression levels relative to the Fat line. Overall POMC expression, LEPR at week 1 of age and T_3 at weeks 1 and 3 of age were not in the predicted direction, being greater in the Fat line relative to Lean line. However, since POMC mRNA translation results in 5 different protein products, due to enzymatic cleavage, the αMSH levels in the hypothalamus may remain unchanged. Predicted differences for anabolic/ orexigenic neuropeptides were observed for AGRP, with the Fat line being greater

than the Lean line. Surprisingly, NPY, which is also an anabolic gene, was expressed at greater levels in the Lean line relative to the Fat line. It is probable that gene expression levels for anabolic and catabolic neuropeptides not in the predicted direction (dysregulation) could contribute to physiological alterations that regulate development of either the fat or lean phenotype. Alterations in genetic expression in the Fat relative to Lean line may be attributable to the following physiological changes: 1) thyroid hormone feedback from or to the hypothalamus, 2) adiposity signals (i.e. leptin or insulin), 3) fuel partitioning and metabolic pathways, and 4) polygenic expression patterns attributable to allelic segregation during the selective breeding over 21 generations.

Hypothalamic regulation and adipose signals

Signals arising from adipose tissue, such as leptin and insulin, have been known to alter neuropeptide expression in the hypothalamus [120] and the data presented here also suggest that adiposity signals, and the corresponding hypothalamic receptor, may influence the development of these phenotypes. For example, binding of leptin to the LEPR has been shown to up-regulate POMC mRNA expression and down-regulate NPY mRNA expression [121]. Given that LEPR mRNA levels are increased in the Lean relative to Fat line at week 5 this suggests that LEPR signaling may also be upregulated which would result in upregulate POMC and downregulate NPY gene expression at this age. However, our findings suggest that LEPR signaling processes may not be functioning as predicted, since POMC levels are increased and NPY levels are decreased at all ages in the Fat relative to Lean line. It is also possible that the POMC and NPY gene sequences have a mutation that

prevents them from responding to the leptin receptor signal or that there is another unknown protein that out competes the LEPR signal. In support of this hypothesis, it has been shown that liver leptin levels are increased 1.7 fold in the Fat line relative to the Lean line [122], but a controversy about the validity of this identified chicken leptin sequence exists [123]. Regardless, the avian LEPR has been accurately sequenced [124].

As adiposity diverged between the two lines, interesting expression profile differences between the lines were observed for LEPR and BDNF. At week 1 of age LEPR expression was increased and BDNF expression decreased in the fat relative to lean line. Decreased expression of BDNF has been associated with development of an obese phenotype [32,35]. However, it has not been previously shown that decreased BDNF expression contributes to the development of the obese phenotype without alterations in food intake, and our results indicate that hypothalamic BDNF gene expression may affect accumulation of body fat without altering food intake. Additionally, it was surprising that LEPR mRNA levels switched directionality over time as adiposity diverged between the two lines. Therefore, it is plausible that LEPR gene expression may be regulated at week 1, prior to differences in adiposity, by a mechanism independent of adiposity signaling and by week 5 gene expression may be mediated by adiposity signals. The gene expression levels may also be representative of leptin signaling via LEPR. The possibility that LEPR signaling may not be able to elicit its normal anorectic action at week 1 of age is further supported by the observation that leptin does not exert an anorectic effect until after postnatal day 9 in the mouse [125].

Metabolism and fuel partitioning

Metabolism is likely to be a major contributing factor to the different phenotypes between the lines, as differences in body fat occur in the absence of differences in food intake. Gene expression for TRH and CRH were different between the lines, which suggests that there may be differences in the hormonal feedback circuitry, especially since plasma T_3 levels were different. In fact, it has been previously shown that thyroid hormone levels are different between the Fat and Lean lines, with T_3, but not T_4, being greater in the Lean line at week 9 of age, but not at week 16 or 34 [83]. Additionally, corresponding with the increased levels of T_3 in the Fat relative to Lean line at weeks 1 and 3, maximal divergence in fattening occurs between the two lines over developmental weeks 3–7, previously shown and present data[84,117]. Furthermore, T_3 was decreased in the Fat relative to the Lean line in the fed state and refed state after a fast, but not in the fasted state alone [119]. However, it must be noted that differences in thyroid hormone levels between the lines are minimal and are not sufficient on their own to account for differences in body fat between the lines. In contrast to TRH, CRH was significantly decreased in the Fat relative to Lean line (Fig. 3e), which is surprising since both corticosterone levels and corticosterone sensitivity remained unchanged between the two lines, including fed and fasted states [118,119]. However, it has been shown that decreased T_3 levels can modulate expression of CRH levels [126], which is a plausible explanation for why CRH mRNA levels are different in the hypothalamus despite no alterations in plasma corticosterone between the Fat and Lean lines.

The Fat line has lower plasma glucose and higher plasma insulin levels relative to the Lean line between weeks 3 and 5 (Table 4, Chapter 2), and around 6 and 11 months of age as which may contribute to metabolic alterations influencing the fat or lean phenotype between the lines [90]. This pathway also has the potential to contribute to the differential expression observed for BDNF and LEPR at week one, since increased glucose levels are present in the Lean line from hatch, with no alteration in insulin at this age [117]. Furthermore, the glucose-insulin imbalance is partly attributable to altered signaling in the liver, but not muscle tissue [127], suggesting that metabolic utilization and fuel partitioning in the liver may contribute to phenotype differences and hypothalamic gene expression patterns.

Hyperphagia and energy utilization, such as metabolizability or expenditure (i.e. basal metabolic rate, maintenance and dietary induced thermogenesis) [84,85,117-119] are not factors contributing to phenotype differences between the lines. However, differences in metabolic pathways and fuel partitioning might be a mechanism that contributes to phenotype differences. For example, both lipid and protein metabolism are different between the lines [128] given that the Fat line showed decreased ability to retain protein relative to the Lean line, which could be in part due to differences in nitrogen metabolism [85]. It has also been shown that lipogenesis was higher in the Fat relative to the Lean line when animals are fed a low fat diet [118]. In fact, these differences in fuel partitioning and lipogenesis could contribute to differences in hypothalamic gene expression, since it has been previously shown that a high-fat diet can decrease BDNF levels in the brain [91,129-131], suggesting that future characterization

of interactions between dietary properties and hypothalamic BDNF expression in the Fat and Lean lines are well deserved.

Allelic segregation and genetic influences

The data presented here further support that genotype may influence development of adiposity by regulating hypothalamic expression patterns of anorexigenic and orexigenic genes in ways not previously predicted. Furthermore, it is possible that differential expression of hypothalamic BDNF and LEPR in the Fat relative to Lean line prior to the onset of adiposity might serve to establish a "set point" for long-term regulation of body fat stores during a critical developmental period. As adiposity develops and the adipose mass set point evolves and matures over the first 7 weeks, these genes function to establish homeostatic mechanisms by differentially modulating one of the following: 1) metabolism, 2) nutrient utilization and 3) body composition. Furthermore, during a critical period shortly after hatch/birth expression patterns of hypothalamic anorexigenic and orexigenic neuropeptides may serve to regulate development of the fat or lean phenotype, which is further supported by the fact that neural pathways between nuclei in the hypothalamus are not established until postnatal day 8 in the mouse [107]. Lastly, we have identified a small set of genes that are differentially regulated due to the allelic segregation from 21 generations of selective breeding, and this finding elucidates one potential polygenic expression pattern that may contribute to the fat or lean phenotype.

Here we dissociate genetic from environmental influences on obesity by utilizing Fat and Lean chickens selectively bred for phenotype over 21 generations.

We propose that genetic factors influencing body composition involve interactions between BDNF and thyroid hormone. *In vivo* data presented here demonstrate that alterations in BDNF and T_3 during progression of adiposity may be one mechanism of genetic susceptibility for an obese phenotype.

Fat and Lean line data suggest that BDNF or T_3 levels could serve as the primary signaling factor to initiate hypothalamic gene expression during the different stages of adiposity development, since before and at the onset of adiposity divergence (week 1 and 3), plasma T_3 was increased and hypothalamic BDNF expression was decreased in the Fat relative to Lean line. Additionally, at week 3 hypothalamic TRH mRNA levels were suppressed in the Fat line, suggesting that either T_3 could inhibit expression of TRH in the Fat line (Fig. 1e) or increased BDNF expression in the Lean line could increase expression of TRH (Fig. 1f). Although at first glance it might seem surprising that TRH levels were not elevated until week 5, it should be noted that plasma T_3 levels need to increase 1.6 fold in order to reduce pro-TRH levels [47]. Also, Deiodinase 2 is not present in the paraventricular nucleus where TRH neurons are located, which suggests that TRH neurons may only be responding to plasma T_3 or to other brain regions that first convert T_4 to T_3 (e.g. tanycytes) [45]. Finally, at week 5 of age, hypothalamic BDNF mRNA levels continued to increase in the Lean relative to Fat line. It is still unknown whether BDNF or T_3 initiates the differential expression for TRH, TrkB, or LEPR genes.

Chapter 4: BDNF and Thyroid Hormone: A Tipping Point That May Regulate Levels of Body Fat

Abstract

Genetically related obesity results from altered states of energy metabolism, often arising from defects in hormonal and neuropeptide signaling [8]. Alterations in food intake [21], inherited genetic factors [9] and hormone levels contribute to energy homeostasis and obesity. Previously, we utilized an animal model to identify candidate genes associated with the divergence of adiposity in genetically selected Fat and Lean animal lines, independent of alterations in food intake. Differences in candidate gene expression occurred simultaneously with alterations in both plasma thyroid hormone levels and Brain-Derived Neurotrophic Factor (BDNF) expression in the hypothalamus of the brain. Differential expression of BDNF contributes to both obesity and anorexia in humans [33,37,63]. Conditional mutant mice lacking BDNF in the brain after development also demonstrate severe obesity [35]. Additionally, modestly elevated levels of thyroid hormone, triiodothyronine (T_3), have been observed in morbidly obese euthyroid humans [44]. The interactions between thyroid hormone and BDNF that contribute to energy stored as fat have not been extensively studied and are currently unknown. We confirmed interactions between BDNF and the hypothalamic-pituitary thyroid axis *in vitro* and identified leptin receptor (LEPR), Pro-opiomelanocortin (POMC), Agouti related protein (AGRP) and 5-tryptophan hydroxylase (5-HTP) as additional targets of BDNF or T_3 in cell culture. These genes

may interact with BDNF and T_3 to modulate development of a genetically induced fat or lean phenotype.

Introduction

Disrupting energy homeostasis leads to an obese phenotype affecting about 100 million adults and 22 million children worldwide [2]. This condition arises from multiple factors, including metabolism, genetics, food intake and energy expenditure [9,21]. Both BDNF and T_3 have been implicated in the etiology of obesity [30,35,37,82]. BDNF haploinsufficient mice or mice with conditional depletion of BDNF in the brain after birth become obese [32,35]. BDNF modulates expression of thyrotropin releasing hormone (TRH) [52] in the hypothalamus, and neurons treated with T_3 suppress BDNF gene expression in the band of Broca [53]. Therefore, further characterization of T_3 and BDNF interactions in the hypothalamus is needed. We propose that T_3 decreases expression of BDNF, TRH and the primary signaling factor for BDNF, tyrosine kinase receptor B (TrkB), whereas BDNF protein acts reciprocally to increase gene expression for BDNF, TRH and TrkB.

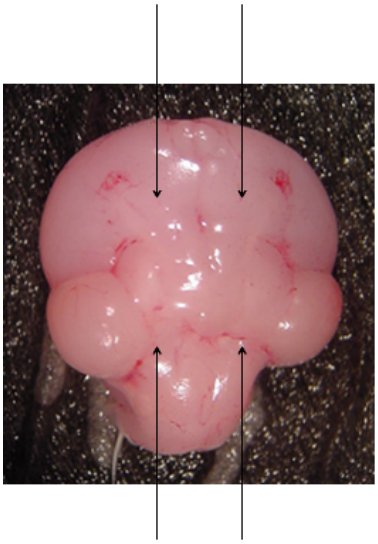
The expression levels of these two pathways correlate with development of the fat or lean phenotype in genetically selected Fat and Lean lines of chicken (Chapter 3). We observed that BDNF levels are decreased in the Fat relative to Lean line at week 1, 3 and 5 of age, as well as increased T_3 levels in the Fat line relative to the Lean line at weeks 1 and 3. This correlation suggested that interactions between the two pathways may modulate development of the phenotypes. Thus, we tested these interactions in primary hypothalamic cell culture (in vitro) and by directly treating embryos over 48-hours with T_3 or methimazole (MMI), a thyroid hormone synthesis inhibitor.

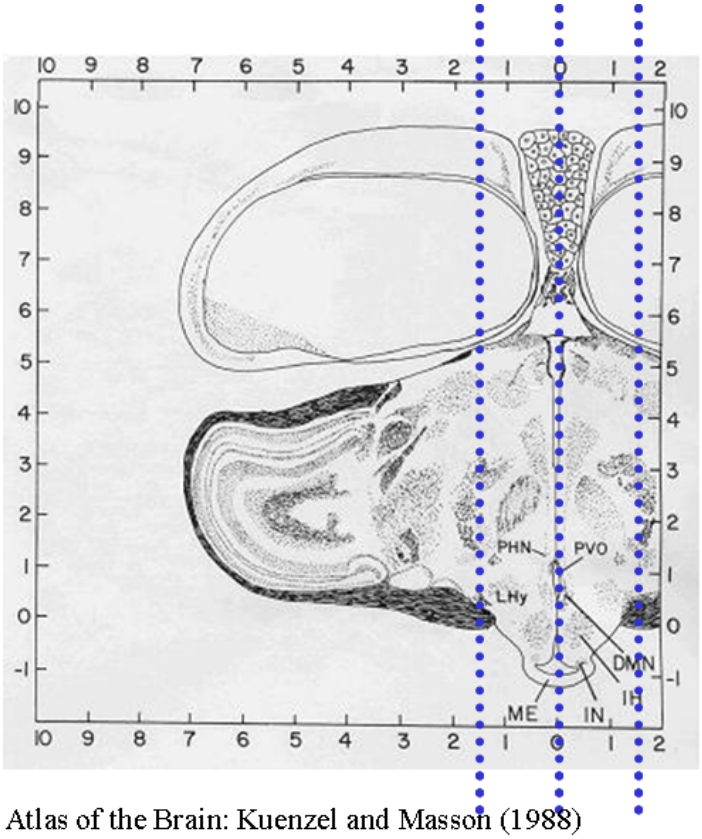
To test interactions of BDNF and T_3 directly, we first assessed whether T_3 was able to alter hypothalamic BDNF, TrkB and TRH gene expression and then determined whether BDNF was able to reciprocally modulate expression of these genes *in vitro*. Then we determined whether the BDNF protein or T_3 could modulate expression of anabolic and catabolic gene expression in the hypothalamus, genes that are known to be associated with regulating energy homeostasis, as well as genes identified via microarray analysis to be differentially expressed between the Fat and Lean lines. Finally, we treated chicken embryos during the last days of development with either T_3 or methimazole, a thyroid synthesis inhibitor, in order to determine whether thyroid hormone is also able to modulate hypothalamic gene expression *in vivo*.

Materials and Methods

Dissections and neuronal cell culture

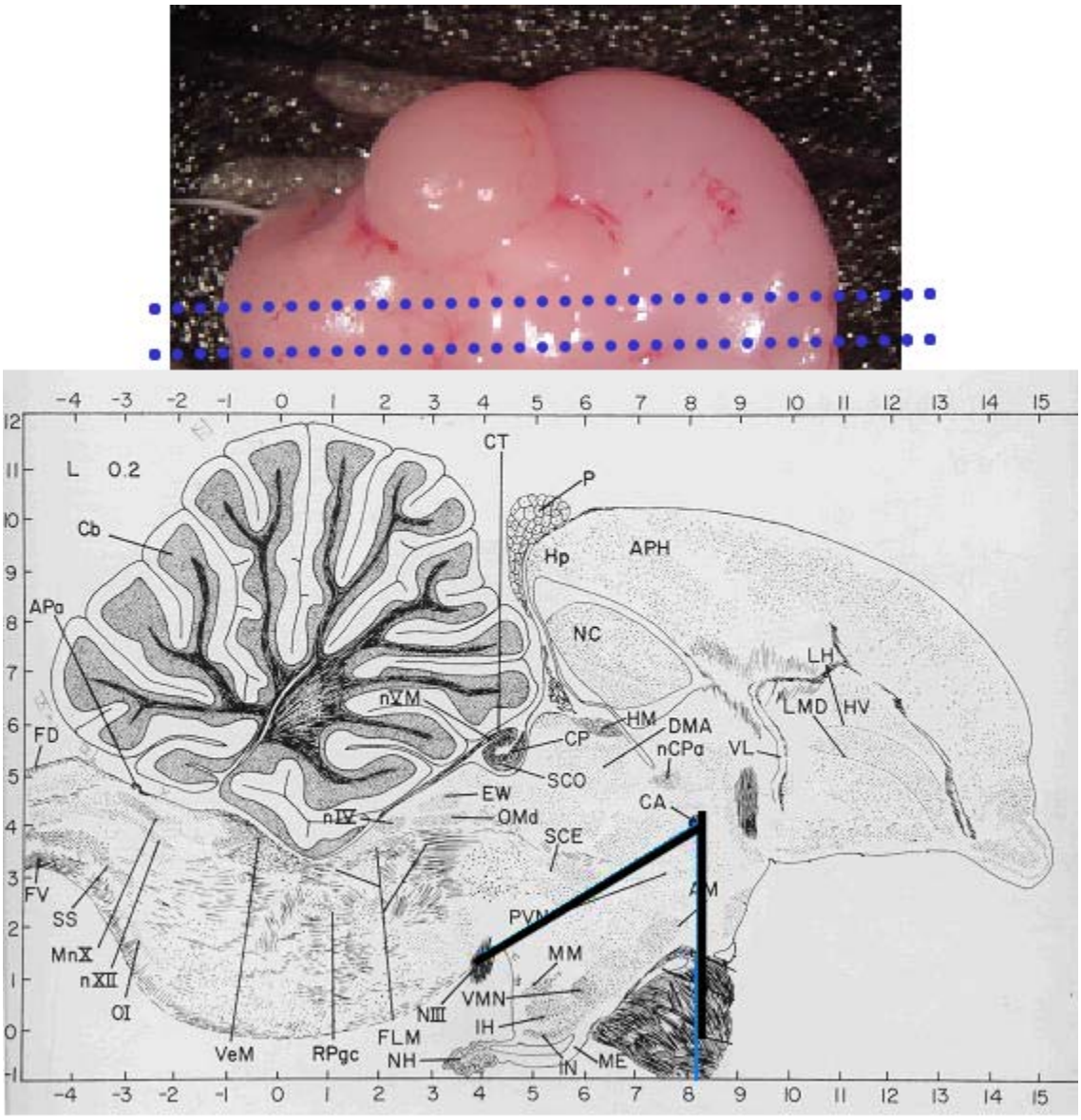
Hypothalami were dissected in a saggital plane using the anterior commisure and the oculomotor nerve as neuroanatomical markers from day 20 chicken embryos, since they are primarily independent of myelination, which begins around embryonic day 14 but occurs primarily after hatch (5% myelinated in 3 day old chicks[132]). The brains were pinned to a dissecting tray and two sagittal were made, 1mm from the left and right side of the midline using a scalpel. (Fig 1 and 2). Primary hypothalamic neurons were cultured for 4 days, since neurons grown 4 days *in vitro* have not formed synaptic connections with other neurons, implying that data reflect mRNA levels independent of stimulation received from synaptic interactions with other





Atlas of the Brain: Kuenzel and Masson (1988)

Fig 1. **Hypothalamic dissection step 1.** The black arrows indicate the location of the hypothalamus on a chicken brain. The brain was pinned to the dissecting tray in location distal to the arrows. The dashed lines indicated the initial scalpel cuts made on each side of the midline as represented[133].



Atlas of the Brain: Kuenzel and Masson (1988)

Fig. 2. **Step 2 in the dissection of the hypothalamus.** After the sagittal cuts on each side of the midline were made the slice was pinned on its side, as seen in the atlas above. A cut was made from the anterior commisure and the oculomotor nerve (diagonal line) and another cut made straight down from the anterior commisure[133].

or 10 ng BDNF (Invitrogen) added 0, 1, 3, 6 or 24 hr prior to harvesting. Cell viability was determined by staining with trypan blue. Cells were cultured for 4 days, since BDNF mRNA levels stabilized by day 3 *in vitro* (data not shown). Cells were harvested with 0.05% trypsin, centrifuged and snap frozen in liquid nitrogen.

RNA extraction and qPCR

Cell culture RNA was extracted using RNeasy Mini kits (Qiagen, Valencia, CA). RNA was quantified using UV absorbance (260/280 nm) and with a bioanalyzer (Agilent Technologies, Palo Alto, CA), and Superscript III reverse transcriptase (Invitrogen) was used to create cDNA from 400 ng of RNA. Levels of mRNA for specific genes were quantified with Sybr green realtime qPCR master mix containing the following: 1unit of Taq, 20X SYBR green I, 400 nM fluoroscein, 10 mM dNTPs, 25 mM $MgCl_2$, 10X PCR buffer and water. Primers were designed with Primer Express (v2.0 Applied Biosystems) and the primer sequences previously described were used (Chapter 3).

The qPCR output provided a Ct value for the threshold cycle. Data were first transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to two housekeeping genes, β-actin and GAPDH, using the geNorm software and methods [88].

Methimazole and Thyroid Hormone treatment

Chicken embryos were injected every 24 hours on embryonic day (e) 18 and e19 with 0.1 ml of 10^{-7} M T_3, 0.075g/mL methimazole (a thyroid hormone synthesis

inhibitor) or a similar volume of water, and then hypothalami were collected on e20. To inject the eggs, a whole was gently drilled through the shell and the the whole was then covered with wax until the injection on the next day, with the wax being replaced each time. Hypothalami were dissected using a scalpel and neuroanatomical markers, the anterior commisure and the ventral portion of the oculomotor nerve.

Statistical analysis and graphical display of data

Differences in gene expression for all experiments were tested using a one-way (cell culture treatment) analysis of variance (ANOVA) with a PDIFF post-hoc analysis to identify differences between the groups (Statistical Analysis System, v.8.02, SAS Institute, Cary, NC). Values reported are means ± SEM, and $P < 0.05$ was required for statistical significance. All graphs were constructed using Graphpad (Graphpad Prism v.3.0, Graphpad Software).

Results

BDNF, TrkB and TRH gene expression

Hypothalamic neuronal cultures were treated with 10^{-9}M T_3 or 10 ng/mL BDNF for 0, 1, 3, 6 and 24-hr and assayed with quantitative real-time PCR (qPCR) to determine BDNF, TRH and TrkB mRNA levels. Treating hypothalamic neurons with T_3 suppressed expression of BDNF, TrkB and TRH mRNA relative to untreated controls (0-hr) (Fig. 3a, c and e, respectively). Strikingly, treatment of neurons with BDNF modulated expression of the same genes in the opposite direction, relative to T_3 treatment (Fig. 3b, d and f, respectively). β-actin was not different after either treatment (Fig. 3m, n). This indicated that T_3 and BDNF act reciprocally to regulate

genes involved with their own signaling processes. Since LEPR is one gene known to modulate many other genes influencing body composition and food intake [8], we determined whether a similar pattern of gene expression would be seen. In fact, T_3 treatment suppressed LEPR gene expression after 1, 3, 6 and 24-hr (Fig. 3g), and BDNF treatment increased expression of the LEPR gene after 6-hr (Fig. 3h). This suggests that BDNF and T_3 reciprocally modulate expression of a key gene in the obesity gene circuit. These data suggest that there is an interaction between BDNF and T_3, which paralleled the data observed in the Fat and Lean lines (Chapter 3). Thus, we selected candidate genes differentially expressed between the lines in our previous studies to determine if mRNA levels are modulated by BDNF or T_3 *in vitro*.

Anabolic and Catabolic gene expression after T_3 or BDNF treatment

Given that T_3 and BDNF reciprocally modulated catabolic gene expression for BDNF, TrkB, TRH and LEPR, we proposed that T_3 would suppress the catabolic genes in our candidate gene list, POMC and CRH, and increase the anabolic genes, AGRP and NPY, whereas BDNF would have the opposite affect. Indeed, T_3 suppressed POMC mRNA at 1 and 3-hours (Fig. 3i) and increased AGRP expression at 1 and 24-hours (Fig. 3k). However, BDNF had no effect on these genes (Fig. 3j and l, respectively). BDNF and T_3 had no effect on NPY or CRH (Fig. 4a-d). This

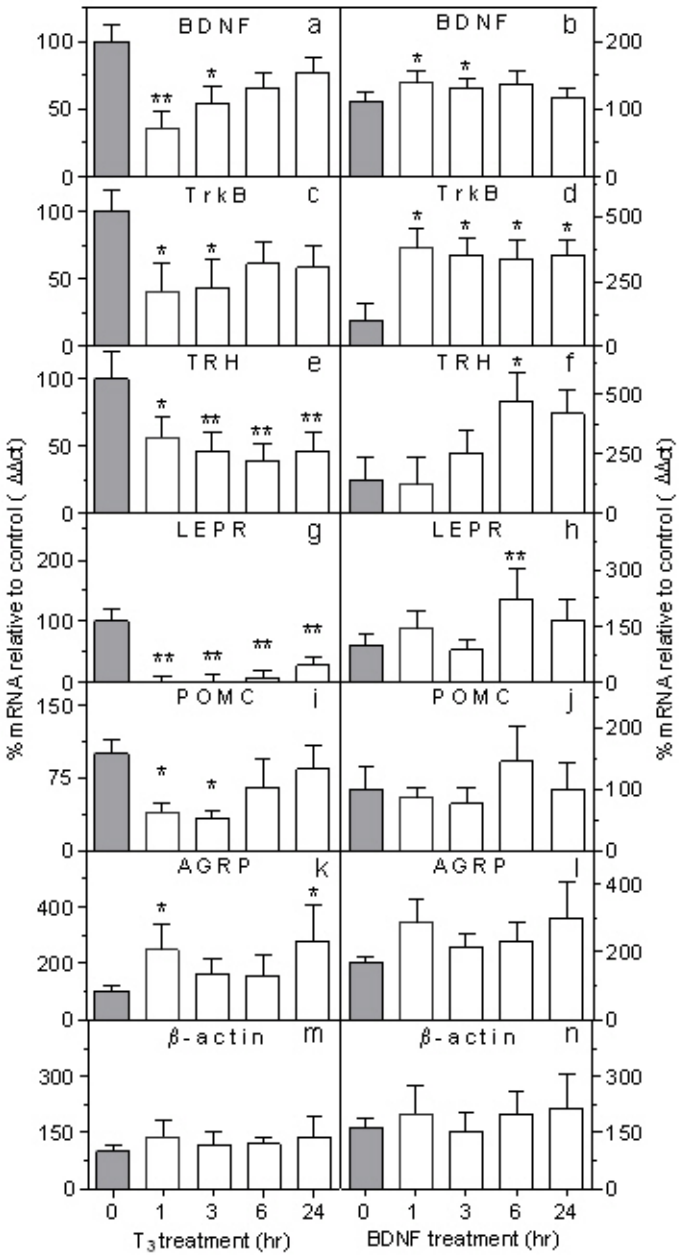

BDNF a
BDNF b
TrkB c
TrkB d
TRH e
TRH f
LEPR g
LEPR h
POMC i
POMC j
AGRP k
AGRP l
β-actin m
β-actin n
% mRNA relative to control (ΔΔct)
% mRNA relative to control (ΔΔct)
0 1 3 6 24
T3 treatment (hr)
0 1 3 6 24
BDNF treatment (hr)

Figure 3. **T_3 and BDNF have opposite effects on hypothalamic genes known to regulate energy homeostasis *in vitro*. a,c,e,g,i,k,m.** T_3 treatment decreased BDNF (**a**), TrkB (**c**), TRH (**e**), LEPR (**g**), and POMC (**i**) gene expression, increased expression of AGRP (**k**), but not β-actin (**m**) mRNA levels. (n=4-10). **b,d,f,h,j,l,n.** BDNF treatment increased gene expression of BDNF (**b**), TrkB (**d**), TRH (**f**), and LEPR (**h**) relative to control, but not POMC (j), AGRP (**l**) and β-actin mRNA levels (**n**) (n=4-6). Expression levels are normalized to a control value of 100%. * $p<.05$, **$p<.01$, treatment versus 0-hour control.

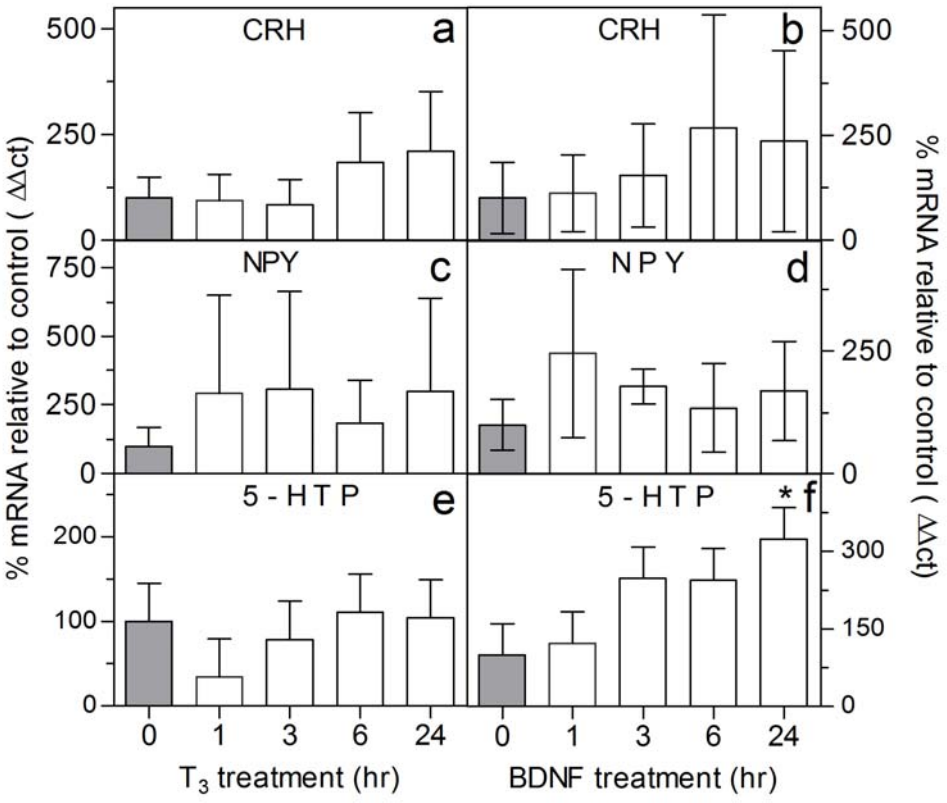

CRH
a
CRH
b
NPY
c
NPY
d
5 - HTP
e
5 - HTP
* f
500
250
0
750
200
100
150
300
0
1
3
6
24
% mRNA relative to control (ΔΔct)
% mRNA relative to control (ΔΔct)
T3 treatment (hr)
BDNF treatment (hr)

Figure 4. **Effects of T_3 and BDNF on hypothalamic genes known to regulate energy homeostasis. a,c,e.** T_3 treatment does not change CRH (**a**), NPY (**c**), and 5-HTP (**e**) (n=4-10). **b,d,f.** BDNF treatment did not alter expression of CRH (**b**) or NPY (**d**), but did increase expression of 5-HTP(**f**) mRNA levels (n=4-6). Expression levels are normalized to a control value of 100%. *p<.05, **p<.01, treatment versus 0-hour control.

suggests that T_3 directly suppresses expression of the catabolic gene, POMC, and increases expression of the anabolic gene, AGRP. BDNF was able to increase expression of most catabolic genes directly (BDNF, TrkB, TRH and LEPR), but indirect modulation of other genes, POMC and AGRP, may occur through BDNF's ability to increase LEPR gene expression or TRH's ability to modulate thyroid hormone levels and, thus, BDNF gene expression.

The rate-limiting enzyme for neurotransmitter synthesis (5-tryptophan hydroxylase, 5-HTP) was differentially expressed between the Fat and Lean lines at week 5 of age. Interestingly, this profile correlated with TrkB expression at week 5 of age, which suggests that an interaction between the genes may be biologically relevant. As predicted, treating hypothalamic neuronal cultures with BDNF modulated 5-HTP gene expression. 5-HTP gene expression increased after treating cultures for 24-hr with BDNF (Fig. 4f), but no differences were observed after T_3 treatment (Fig. 4e). Thus, the *in vitro* data indicate that BDNF modulates expression of 5-HTP.

T_3 and Methimazole treatment on anorexigenic and orexigenic neuropeptide genes in vivo

We injected T_3 or Methimazole (MMI), a thyroid hormone synthesis inhibitor, into the developing embryo and collected hypothalami on embryonic day 20. Interestingly, we observed similar patterns of expression for BDNF, TrkB and TRH (Fig. 5a-c, respectively) relative to cell culture data (Fig 3a, c, and e), but surprisingly both T_3 and MMI decreased LEPR, increased POMC mRNA levels (Fig. 5d and e,

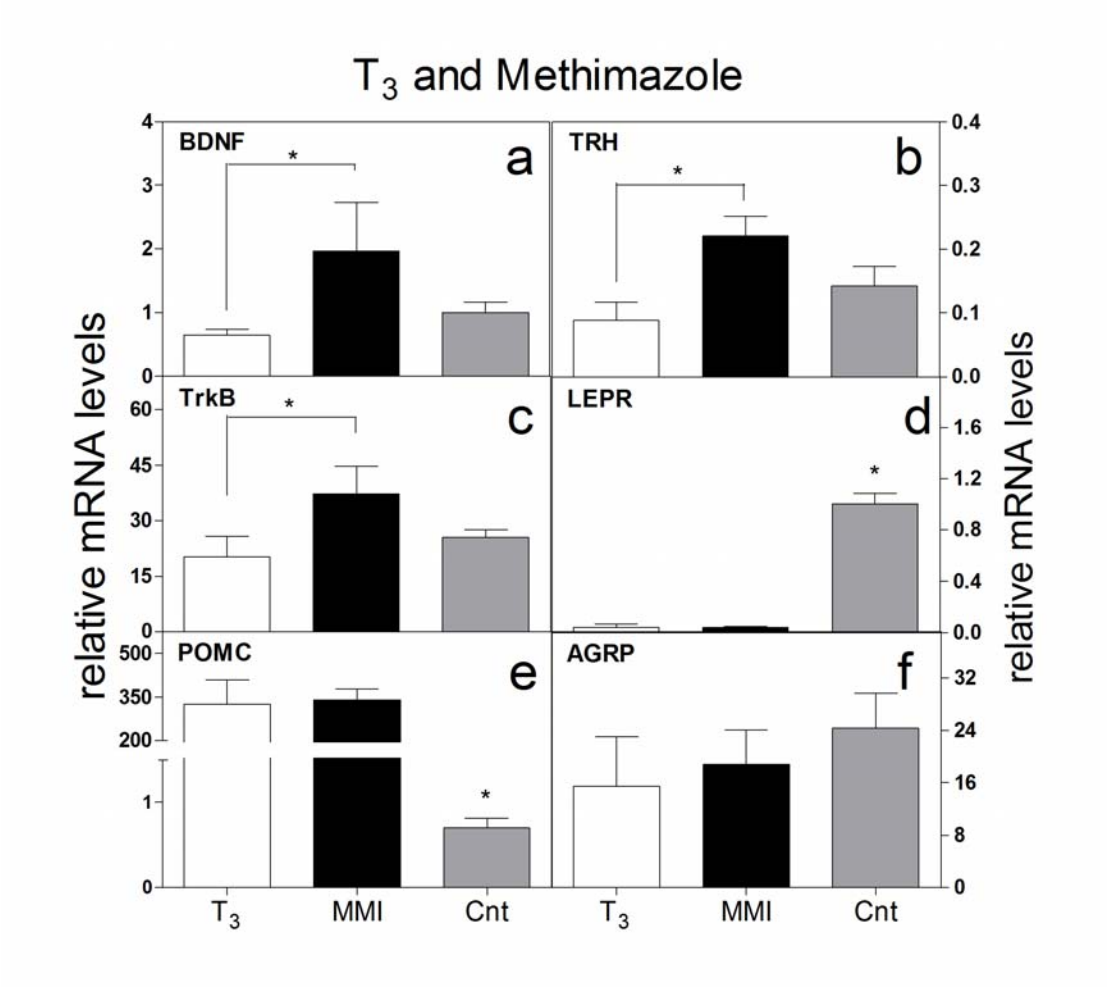

T3 and Methimazole
BDNF
a
TRH
b
TrkB
c
LEPR
d
POMC
e
AGRP
f
relative mRNA levels
relative mRNA levels
T3
MMI
Cnt
T3
MMI
Cnt

Figure 5. **T_3 reciprocally modulates catabolic and anabolic genes. a-f.** T_3 treatment at embryonic day 18 and 19 had different effects on gene expression. T_3 suppressed BDNF (**a**), TrkB (**b**), TRH (**c**), and LEPR (**d**) gene expression, whereas methimazole increased BDNF (**a**), TrkB (**b**), and TRH (**c**), but not LEPR (**d**). T_3 and methimazole both increased POMC (**e**) and had no affect on AGRP (**f**) mRNA. (n = 6). * $p<.05$, ** $p<.01$. T_3, triiodothyronine, MMI, methimazole, CNT, control (water).

respectively) and had no effect on AGRP (Fig. 5f). This suggested that T_3's ability to regulate anabolic and catabolic gene expression may be attributable to developmental or experimental parameters.

Discussion

Our data show that T_3 decreased hypothalamic BDNF, TrkB, TRH and LEPR gene expression (Fig. 3a,c,e,g) and that BDNF reciprocally increased expression of the same genes (Fig 3b,d,f,h). Additionally, we identified differential 5-HTP expression as another downstream factor modulated by BDNF that could also be involved in regulating the development of increased or decreased adipose state (Fig. 4f). T_3 or Methimazole (MMI) injection resulted in a similar pattern of expression for BDNF, TrkB and TRH (Fig. 5h-i, respectively) as cell culture (Fig 3a, c, e), but surprisingly both T_3 and MMI decreased LEPR, increased POMC mRNA levels (Fig. 5j-k, respectively) and had no effect on AGRP (Fig. 5i). This suggested that T_3's ability to regulate anabolic and catabolic gene expression may be attributable to developmental, embryonic day 20 versus post-hatch day 29 (Chapter 5) or experimental parameters, intermittent delivery every 24 hours versus chronic delivery via an osmotic minipump (Chapter 5). Thus, we present a model summarizing our *in vivo* and *in vitro* data showing reciprocal regulation of gene expression by T_3 and BDNF (Fig. 6).

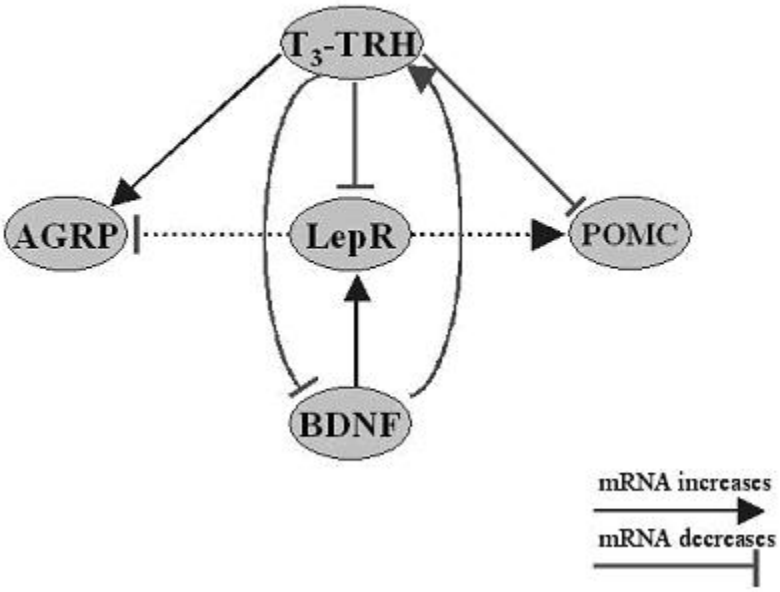

Gene Expression
T3-TRH
AGRP
LepR
POMC
BDNF
mRNA increases
mRNA decreases

Figure 6. **Model for BDNF and T_3 interactions modulating body composition and metabolism.** *In vitro* T_3 inhibits BDNF, TrkB, TRH and LepR mRNA levels, whereas BDNF increases BDNF, TrkB, TRH, 5-HTP and LepR gene expression. Fat relative to Lean expression patterns *in vivo* support interactions presented in this model. In the Fat line, increased T_3 levels may inhibit BDNF expression at wk 1 and 3 and TRH at wk 3. In the Lean line increased BDNF expression may increase expression of TRH at wk 3, likely in conjunction with decreased T_3, and TrkB, LepR and 5-HTP at wk 5.

T_3 was also able to increase anabolic gene expression (AGRP, Fig. 3k and 5f) and decrease catabolic gene expression for the following genes *in vivo* and *in vitro*: BDNF, TrkB, TRH, LEPR and POMC (Fig. 3a, c, e, g and Fig. 5a-e). BDNF was able to directly modulate all catabolic genes in the opposite direction as T_3, except POMC and AGRP (Fig. 3b, d, f, h, j). Furthermore, it has been previously shown that LEPR signaling can suppress AGRP and increase POMC gene expression [134], and since T_3 and BDNF reciprocally modulate LEPR gene expression, they may indirectly modulate expression of both AGRP and POMC via LEPR. T_3 was able to modulate expression of AGRP, but not NPY, although they are co-localized to the same cell type in the arcuate nucleus, suggesting that T_3 is able to directly modulate expression via the promoter region of the AGRP gene. This mechanisms for gene expression is most likely not present in the promoter region of the NPY gene.

To summarize our findings, we present a model summarizing our *in vivo* and *in vitro* data showing reciprocal regulation of the obesity gene circuit by T_3 and BDNF (Fig. 6), suggesting that BDNF and T_3 levels may explain how some individuals are more susceptible to develop an obese phenotype. The primary locations in the hypothalamus modulating the effects presented in the model are the arcuate nucleus (ARC) and the paraventricular nucleus (PVN). AGRP is primarily expressed in the ARC and POMC is expressed in both the ARC and PVN. TrkB is colocalized with TRH in PVN neurons, with BDNF modulated TRH expression in this area via the TrkB receptor[54]. Although we utilized whole hypothalami in order to determine the effects of BDNF and T3, the specialized expression of these genes

suggests that the predominating effects presented in the model (Fig. 6) occur in the ARC and PVN.

During the development of adiposity, it is plausible that interactions between T_3 and BDNF serve to program an initial set point in order to establish long-term levels of body fat stores, possibly by regulating the maturation of homeostatic signaling circuitry (e.g. neural, hormonal or metabolic). For example, in the Fat and Lean animals, one mechanism altered during the development of adiposity could be initially signaled by T_3 or BDNF (Chapter 3) and secondarily by a metabolic parameter, such as glucose utilization [90,117,135], lipolysis [136] or fuel partitioning [137]. Currently, the rate of neuromodulator release in response to T_3 or BDNF is unknown and may modulate neural circuitry more than protein or mRNA production.

It is quite possible that interactions between BDNF and LEPR signaling may also modulate fat deposition and metabolism. LEPR is a key component in plasma leptin's ability to modulate body composition. Furthermore, it has been demonstrated that LEPR gene expression in the brain is the primary signal that modulates body composition and not LEPR gene expression in the periphery[138]. In the mouse, leptin increases BDNF mRNA levels in the hypothalamus [139], and here we show for the first time that BDNF increases LEPR mRNA in the hypothalamus. In the Fat relative to Lean line, liver leptin levels are increased 1.7 fold [122], but a controversy about the validity of this identified chicken leptin sequence exists [123,140]. Regardless, the avian LEPR has been accurately sequenced [124]. Thus, it is probable that BDNF and LEPR levels are modulating energy expenditure and energy balance to influence adipose deposition by modulating anorexigenic and orexigenic signaling networks in the

hypothalamus (Fig. 4). Therefore, it is not surprising that T_3 acts in a reciprocal manner to inhibit BDNF and LEPR gene expression, potentially to compensate for changes in energy expenditure and ultimately establish a feedback loop. We propose that altered feedback may be an underlying mechanism regulating body fat stores.

In conclusion, the balance between BDNF and T_3 could modulate expression of genes that establish neuropeptide and hormonal signaling processes, which regulate long-term body fat stores. BDNF and T_3 reciprocally modulate expression of the genes presented here, suggesting that expression levels may act as a fine tipping point for determining whether hypothalamic gene expression and adiposity increase or decrease over development. Thus, we propose that obesity therapeutics should simultaneously target BDNF and T_3 to inhibit or reverse further fat deposition.

Chapter 5: Differential effects of Glucocorticoids and Thyroid Hormone on Hypothalamic gene expression linked to a fat phenotype.

Abstract

Glucocorticoids and thyroid hormones modulate gene expression in the hypothalamus to regulate food intake and body composition [43]. Under conditions of fasting, corticosterone (Cort) levels increase in order to increase blood glucose levels, and thyroid hormone levels decrease in order to preserve energy and decrease basal metabolic rate. Since the hypothalamus is the primary area of the brain known to modulate energy homeostasis [4,5], we sought to determine how triiodothyronine (T_3) and/or corticosterone regulate anabolic and catabolic gene expression in the hypothalamus. Juvenile chickens were surgically implanted at 29 days of age with osmotic minipumps that delivered T_3, Cort, T_3 plus Cort, or a control vehicle for 72 hours (n=4), since the chicken genome has more synteny with the human genome than a rodent species, such as the mouse [108,109]. We show that Cort and T_3 have overlapping roles in regulating many of the anabolic and catabolic genes, TRH, LEPR, and POMC, and that Cort and T_3 have opposite effects on BDNF, NPY and AGRP. Putative transcription factor binding sites were identified that respond to both glucocorticoid and thyroid hormone located upstream of exon 1 in five of the anabolic and catabolic genes investigated. This suggests a plausible explanation for the complex interactions observed when hormone levels are elevated simultaneously or individually. We also identified for the first time a novel gene regulated by T_3, a gene induced by tumor necrosis factor alpha (TNF AP), and a novel gene regulated by simultaneously elevated levels of Cort plus T_3, zinc finger protein 36 (ZFP 36).

Collectively, these findings suggest that differentially expressed genes in the hypothalamus that contribute to increased food intake or obesity could be regulated by levels of T_3 and/or Cort, which may serve to develop the neural circuitry to respond appropriately to reset or maintain energy homeostasis in the body. These interactions explain a potential homeostatic mechanism that links hypothalamic energy regulation with food intake and body weight.

Introduction

Thyroid hormone and glucocorticoids regulate energy homeostasis and often act in opposition to one another. Increased levels of thyroid hormone decrease adipose tissue mass and increase food intake, whereas glucocorticoids have been shown to increase adipose tissue mass [43]. These hormones have receptors in nuclei of the hypothalamus and modulate food intake and body weight by regulating transcriptional processes for anabolic and catabolic neuropeptides [77,141,142]. Although it is known that thyroid hormone and corticosterone act in opposition to one another, with corticosterone (Cort) being considered an anabolic hormone and T_3 a catabolic hormone, they have been shown to have synergistic effects when used in combination. For example, T_3 treatment alone slightly enhanced protein synthesis in muscle, whereas Cort treatment increased protein breakdown and Cort in conjunction with thyroid hormone enhanced protein breakdown in muscle 6-fold [143]. Under states of chronic stress, glucocorticoids have been shown to increase food intake which may be due to a dysregulation of normal mechanisms that modulate food intake [144]. When glucocorticoid signalling is no longer effective, increased food intake mimics the food intake phenotype observed when thyroid hormone levels are elevated, however adiposity is still regulated in opposite directions. Therefore we wanted to determine which hypothalamic genes known to regulate both food intake and body weight are modulated in the same or different directions after chronic 72-hour treatment with Cort, T_3, or Cort plus T_3.

Previously, we demonstrated that thyroid hormone, T_3, has been shown to modulate neuropeptide levels in the hypothalamus by increasing TRH, BDNF, TrkB,

and POMC gene expression and decreasing levels of AGRP mRNA in cell culture (Chapter 4). Cort levels are also able to modulate expression of these genes in the opposite direction by decreasing AGRP and NPY expression and increasing POMC and BDNF expression [55,69]. Here we tested whether Cort, T_3 or Cort plus T_3 treatments *in vivo* were able to reciprocally modulate 8 known anabolic or catabolic genes, as well as 6 candidate genes differentially expressed in Fat and Lean chicken lines (Chapter 2). The simultaneous delivery of Cort plus T_3 will allow us to begin to determine which hormone has the predominant effect on regulating expression of each gene.

Our results show that many of the predicted effects were present when Cort or T_3 levels were increased individually, but surprisingly when levels of both hormones were elevated simultaneously the predicted effect was no longer present. This suggests that transcriptional mechanisms are altered by elevated T_3 or Cort levels. Thus, we identified thyroid and glucocorticoid response elements in anabolic and catabolic genes and found that response elements for both hormones exist in the same region, suggesting an explanation for the complex gene expression patterns. Lastly, we identified one novel gene regulated by thyroid hormone, one novel gene regulated by T_3 plus Cort only and verified *in vivo* one gene previously known to be regulated by Cort.

Materials and Methods

Animal and tissue preparation

At 29 days of age, broiler chickens were given a subcutaneous implant of an osmotic minipump (Alzet® , model 2001; ALZA, Mountain View, CA) to

continuously release hormone for seven days at a rate of 1.0 µl/hour. Corticosterone and L-tri-idothyronine (T_3) hormones (Sigma Chemicals, St. Louis, MO) were dissolved in solutions of 50% DMSO and 50% propylene glycol, so as to deliver 600 µg Cort/kg/day and 192 µg T_3/kg/day, respectively. Animals were given *ad libitum* access to food and water at all times. The light/dark cycle was: 24 hr of light for the first two days and then 14 hr of light and 10 hr of dark thereafter.

Animals were sacrificed after 72 hours of hormone or vehicle treatment, with the hypothalamus being immediately dissected and snap frozen in liquid nitrogen (n=4 for group) and stored at –80 ºC until further processing. All procedures were handled in accordance with the Institutional Animal Care and Use Committee at the University of Maryland and the University of Delaware.

Primer design, validation and identification of GRE or TRE

Primers were 18-30 nucleotides in length with a melting temp between 58-64 ºC or 69-72 ºC and were designed using Primer Express (v 2.0, Applied Biosystems, Foster City, CA). The primer product was between 100 and 150. Forward and reverse primer sequences are listed in Table 1.

Glucocorticoid and thyroid hormone response elements were identified using TESS software, Transcriptional Element Search System (University of Pennsylvania, 1997, URL: http://www.cbil.upenn.edu/tess). Sequences were first identified in NCBI having the following annotations: AGRP: AB029443.1, BDNF: XM_419645.1, CRH: AJ621492.3, LEPR: NM_204323 , NPY: NM_205473.1, POMC: NM_001031098,

GENE	Sense primer	Antisense primer
AGRP	5'AGAGCGGACCGTGAGGACACTT3'	5'GTTGGCATTTCCTCCCAAAGGA3'
BDNF	5'TGGGTAACAGCAGCGGAGAA3'	5'TATTGCTTCAGTTGGCCTTTAG3'
CRH	5'CACAGCAACAGGAAACTGATGGAAA3'	5'AAAGAGGTGACATCAGAGCAGCACTATG3'
LEPR	5'AAAACCCAGAGCGTAGCGTCCAA3'	5'TTGCTTACGCGATCGTTCACAAG3'
NPY	5'GGGAAAGCACAGAAAACATTCC3'	5'AAATCCCATCACCACATCGAA3'
POMC	5'AGGGACCTCAGGGATCATCAA3'	5'TGTTCAAGGGCAGGTTGGA3'
TRH	5'AGCATCTTTTGGAGACATTCAG3'	5'CAGCTCCAGGTAGTTGACAAGGT3'
TrkB	5'GTCCTGGGTGCTCACTAACCTT3'	5'TTATGGTTAACGAGGCAGGATTC3'
ZFP 36	5'CGACATCGACTTCTTGTGCAA3'	5'TGCTGGTGCGGATCGAGAACT3'
Induced by TNF	5'ACAACGCCGTCACCCTATTC3'	5'GTTCCCCAGGAAGAGGACGAA3'
GHRHR	5'CCTTGGCATTCGGCTTTATTTAG3'	5'TCAGGAAACAGTAGAGGAGTGCTACA3'
B-actin	5'CCCAAAGCCAACAGAGAGAAG3'	5'ACCATCACCAGAGTCCATCAC3'

Tabel 1. Primers for qPCR.

TrkB: NM_205231, and TRH: XM_414458.1. The sequence was blasted to the chicken genome using Ensembl (URL: http://www.ensembl.org) and 1900 bp upstream of exon 1 for each gene was identified and searched for a GRE or TRE sequence.

RNA isolation and cDNA preparation

Individual tissue samples were homogenized and total RNA extracted according to manufacturer's protocol with Rneasy Midi kit (Qiagen, Valencia, CA). RNA was quantified using UV absorbance (260nm) and 1µg total RNA was used to create cDNA using the stated protocol for Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo(dT) primer. In order to have a negative control which measures genomic DNA contamination, cDNA was made that had no reverse transcriptase added. cDNA was diluted (1:20) and mRNA levels quantified using 2µl of cDNA in a 20µl quantitative real time PCR (qPCR) reaction using a master mix that consisted of recombinant Taq, 2X PCR buffer containing 100 mM KCl, 20 mM Tris-HCl (pH 9.0), 0.2% triton-X 100, 3.8 mM MgCl2, 0.12 U/µl Taq Polymerase, 400 nM dNTPs, 40 nM fluorescein, and SYBR Green I Nucleic Acid Gel Stain diluted 1:10,000 (all Invitrogen, Carlsbad, CA) and analyzed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). A three-step PCR cycle protocol was used: initial denaturation at 95 ^{o}C for 3 min, followed by 40 cycles of 94 ^{o}C for 45s, 66 ^{o}C for 45s and 72 ^{o}C for 45s, followed by melting temperature analysis at 94 ^{o}C to verify that one PCR product was amplified.

Statistical analysis and graphical display of data

PCR products were verified by dissociation curve analysis and gel electrophoresis for the appropriate size. The qPCR output data provided a Ct value for the threshold cycle. The ΔΔCt value was generated using geNorm software and methods [88]. Briefly, the data were first transformed to a ΔCt value by subtracting the sample Ct value from the sample with the highest expression level in order to control for amplification efficiency. The ΔΔCt value was then calculated by normalizing gene expression to β-actin.

Differences in gene expression for all experiments were tested using a one-way analysis of variance (ANOVA), and a fisher post-hoc analysis performed in order to determine interactions (Statistica, v6.0, StatSoft, Tulsa, OK, USA). Values reported are means ± SEM, and $P < 0.05$ was required for statistical significance.

Results

72-hour Thyroid Hormone treatment

In order to verify prior results from primary hypothalamic cell cultures (Chapter 4), T_3 was delivered via osmotic minipump for 72-hours. We demonstrated that T_3 was able to inhibit expression of catabolic neuropeptides TRH, BDNF, TrkB, LEPR and POMC (Fig 1a-e). We also found that T_3 was able to increase expression of AGRP, an anabolic neuropeptide (Fig. 1f). Delivering T_3 via osmotic minipump had no effect on NPY or CRH (Fig. 1g and h), as previously demonstrated in cell culture. This suggests that *in vivo* T_3 treatment alone is able to directly suppress expression of

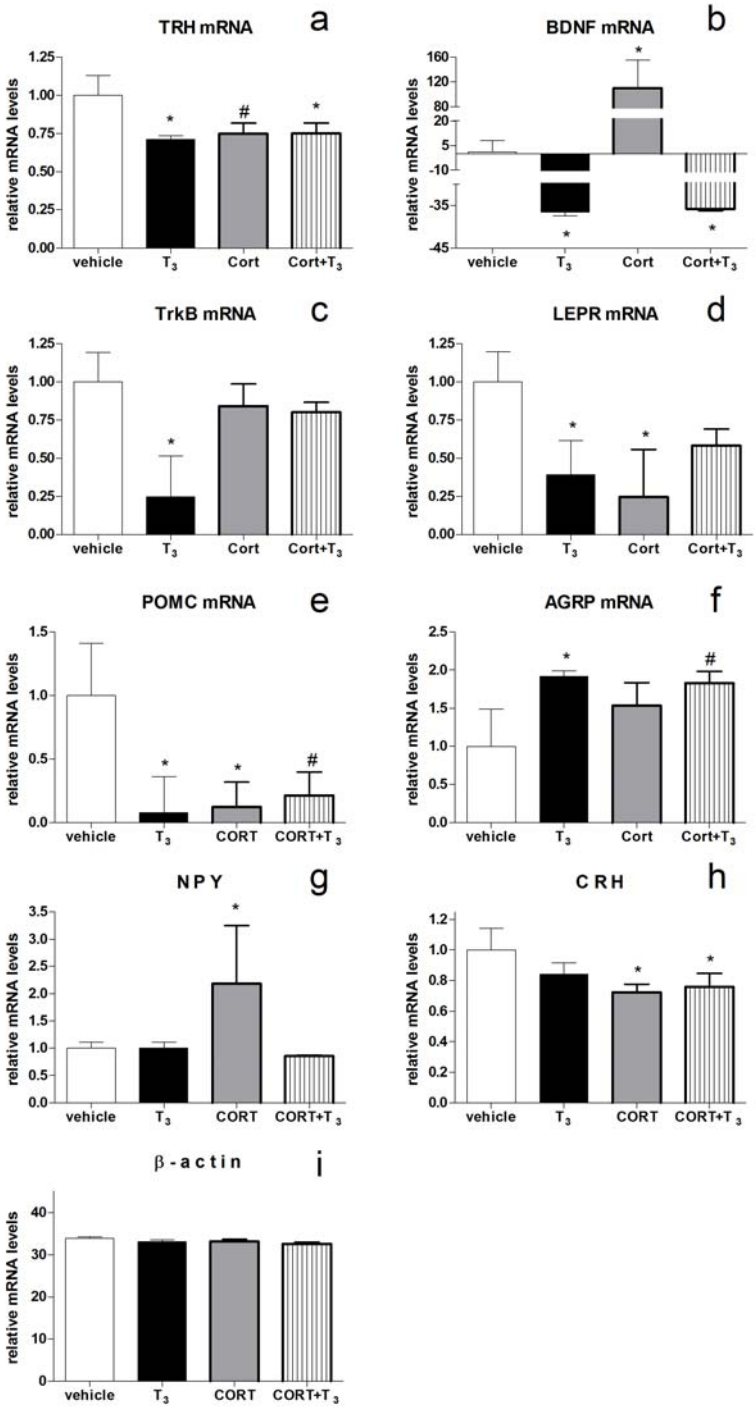

TRH mRNA
a
relative mRNA levels
1.25
1.00
0.75
0.50
0.25
0.00
vehicle
T3
Cort
Cort+T3
BDNF mRNA
b
160
120
80
20
5
-10
-35
-45
TrkB mRNA
c
LEPR mRNA
d
POMC mRNA
e
CORT
CORT+T3
AGRP mRNA
f
NPY
g
CRH
h
β-actin
i
40
30
20
10
0

Figure 1. **Anabolic and catabolic gene expression in the hypothalamus after 72-hour hormone treatment. a-h,** Quantification of hypothalamic BDNF (**a**), TrkB (**b**), TRH (**c**), LEPR (**d**), POMC (**e**), AGRP (**f**), NPY (**g**), CRH (**h**) and β-actin (**i**) mRNA levels (n = 4) by qPCR analysis. * $p<0.05$, hormone treatment relative to vehicle control.

catabolic genes and increases expression of an anabolic gene. There was no change in β-actin gene expression after any of the treatments (Fig. 1i).

72-hour Corticosterone treatment

After treating chickens for 72-hours with corticosterone, we expected to observe the opposite effects as that seen with T_3. Surprisingly, for many of the genes we observed a similar expression pattern as seen after T_3 treatment. For example, we observed that Cort also inhibited expression of the catabolic neuropeptides TRH, LEPR and POMC (Fig 1a, d, and e). On the other hand, opposite to that observed with T_3, Cort treatment increased expression of BDNF (Fig. 1b). Cort had no effect on TrkB or AGRP (Fig. 1c and f, respectively), and unlike T_3, Cort increased expression of the anabolic neuropeptide NPY and decrease expression of the catabolic neuropeptide CRH (Fig. 1g and h).

72-hour simultaneous delivery of Corticosterone and Thyroid Hormone

Delivering Cort in conjunction with T_3 had surprising effects on anabolic and catabolic gene expression in the hypothalamus. Similar to T_3 treatment alone, T_3 plus Cort decreased expression of TRH and BDNF mRNA (Fig 1a and b). Decreased POMC and increased AGRP expression had only a trend toward significance, which is surprising since T_3 or Cort alone were able to decrease POMC mRNA levels and T_3 alone was able to increase AGRP mRNA levels (Fig. 1e and f). Surprisingly, simultaneous treatment with Cort and T_3 had no effect on TrkB, LepR and NPY (Fig 1c, d and g, respectively). As predicted, Cort treatment, even in conjunction with T_3 delivery, suppressed expression of CRH (Fig. 1h).

GRE and TRE identification in anabolic and catabolic genes

Glucocorticoid and thyroid hormone response elements (GRE and TRE, respectively) were identified in the upstream region 5' of the start site for anabolic

and catabolic genes differentially regulated by Cort and/or T_3 treatment. Putative GREs and TREs were identified in the 2000 bp region upstream of exon 1 and were present in the following genes: AGRP, BDNF, CRH, LEPR, NPY, POMC, TRH and TrkB (Table 2). POMC was the only gene that did not have a TRE site in this region. BDNF contained the most TRE sites, and AGRP contained the most GRE sites, but regardless Cort treatment over 72-hours did not alter AGRP expression, suggesting that regulation of gene expression may occur prior to 72-hours or that the GREs sites in the AGRP gene simply are not functional. POMC was the only gene that did not contain a TRE, yet T_3 treatment was able to suppress gene expression, suggesting that inhibition of POMC expression does not occur via direct interactions with T_3, but more likely via another protein which is regulated by T_3 expression or via a thyroid hormone receptor in the membrane [145]. Five out of the eight genes (AGRP, BDNF, NPY, LEPR and TRH) had potential transcription factor binding sites for both the glucocorticoid and thyroid hormone receptors, perhaps providing an explanation for the gene expression pattern observed when the level of both hormones are elevated simultaneously.

Candidate Genes

The following six candidate genes, differentially regulated in the hypothalamus of Fat and Lean lines (Chapter 2), were selected to determine if expression could be modulated by thyroid hormone, Cort or thyroid hormone plus Cort treatment: a gene induced by tumor necrosis factor alpha (TNFα AP), growth hormone releasing hormone receptor (GHRH-Rec), zinc finger protein 36 (ZFP 36), CCAAT/enhancer-binding protein (CEBP), and Metalloprotease 1 (ADAM12). TNFα AP decreased mRNA levels after T_3 and Cort plus T_3 treatment relative to vehicle (Fig. 2a). GHRHR mRNA levels were suppressed after Cort treatment,

GENE	TRE	#	bp from 5' start	GRE	#	bp from 5' start
AGRP	Yes	2	**-1594**, -1662	Yes	29	-147, -199, -251, -355, -381, -459, -537, -563, -589, -641, -756, -895, -951, -1035, -1051, -1147, -1156, -1230, -1267, -1360, -1499, **-1594**, -1606, -1630, -1659, -1778, -1800, -1833, -1892
BDNF	Yes	4	**-26**, -246, **-1593**, -1726	Yes	24	**-26**, -139, -429, -468, -741, -898, -974, -1031, -1066, -1100, -1126, -1251, -1295, -1319, -1368, -1405, -1446, -1500, -1518, **-1593**, -1664, -1682, -1742, -1812
CRH	Yes	1	-1585	Yes	9	-22, -33, -61, -84, -163, -166, -1694, -1852, -1905
LEPR	Yes	3	**-308**, -997, **-1420**	Yes	19	-67, -118, **-308**, -467, -495, -513, -530, -831, -839, -872, -895, -945, -955, -995, -1270, -1336, -1391, **-1420**, -1546
NPY	Yes	2	-981, **-1133**	Yes	14	-90, -151, -211, -277, -278, -370, -617, -659, -873, -1020, -1053, -1096, -1105, **-1133**
POMC	Yes	0	---	Yes	13	-314, -603, -636, -710, -781, -867, -976, -1017, -1088, -1167, -1522, -1740, -1914
TRH	Yes	3	-716, **-921**, -1652	Yes	21	-85, -161, -239, -284, -319, -336, -469, -541, -802, -823, **-921**, -1032, -1066, -1241, -1290, -1299, -1350, -1615, -1684, -1696, -1885
TrkB	Yes	1	-26,	Yes	4	-13, -307, -584, -820,

Table 1. **Potential Glucocorticoid (GRE) and thyroid hormone response elements (TRE) located upstream of the 5' start site for each gene.** # indicates the number of potential GREs or TREs identified and the base pair number from the 5' region of exon 1 for each gene. Items in bold indicate sites identified as both a GRE and TRE.

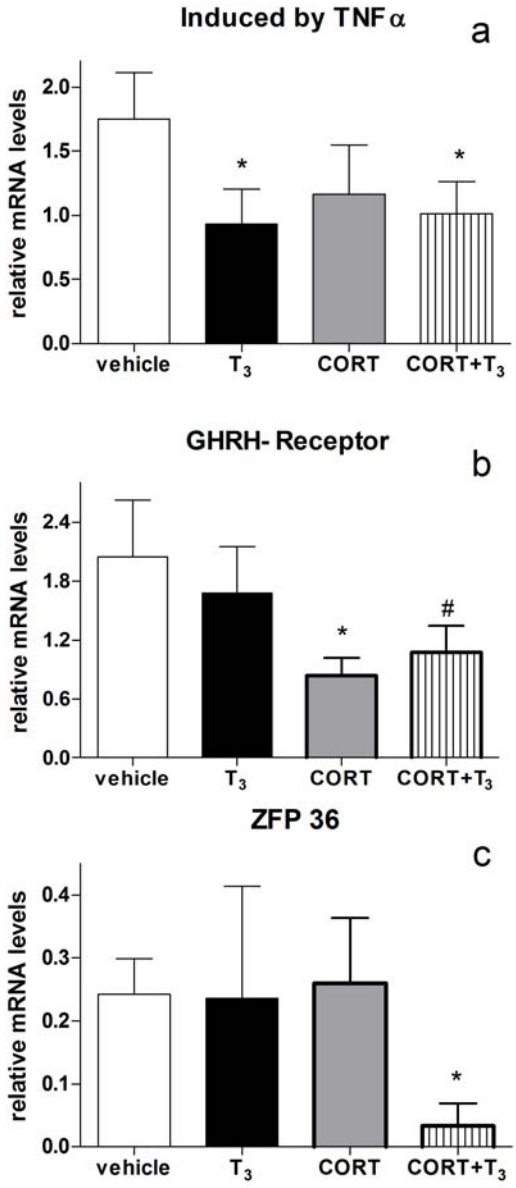

Induced by TNFα
a
relative mRNA levels
2.0
1.5
1.0
0.5
0.0
*
*
vehicle
T3
CORT
CORT+T3
GHRH- Receptor
b
relative mRNA levels
2.4
1.8
1.2
0.6
0.0
*
#
vehicle
T3
CORT
CORT+T3
ZFP 36
c
relative mRNA levels
0.4
0.3
0.2
0.1
0.0
*
vehicle
T3
CORT
CORT+T3

Fig 2. **Candidate gene expression in the hypothalamus after Corticosteone or Thyroid Hormone treatment. a-c,** Quantification of hypothalamic expression for TNF AP (**a**), GHRH receptor (**b**) and ZFP 36 (n = 4) by qPCR analysis. * $p<0.05$, hormone treatment relative to vehicle control.

with a trend toward suppression after Cort plus T_3, but no change in mRNA levels after T_3 treatment alone (Fig. 2b). ZFP 36 decreased expression in response to Cort plus T_3 treatment, with unaltered mRNA levels after treatment with either T_3 or Cort alone (Fig. 2c). There were no alterations in gene expression for CEBP, ADAM12, and TNF-ra2 after hormone treatments.

Discussion

Our data suggest that Cort and T_3 play overlapping roles in regulating mRNA levels in the hypothalamus for genes known to modulate food intake and body weight, specifically TRH, LEPR, and POMC. We also demonstrate that Cort and T_3 regulate gene expression in opposite directions, Cort treatment was able to increase BDNF expression, which was decreased by T_3 treatment. Some genes are specifically regulated by one hormone only; NPY mRNA levels were increased by Cort whereas T_3 had not effect on NPY, T_3 decreased TrkB mRNA levels whereas Cort had no effect on TrkB expression, and T_3 increased AGRP gene expression whereas Cort had no effect. Cort and T_3 were able to modulate expression of AGRP and NPY, but in opposite directions. These effects correspond with increased Cort levels resulting in anabolic activity by increasing expression of the anabolic gene NPY. On the other hand, increased T_3 levels generally increase catabolic activity, which would explain the downregulation of the anabolic gene AGRP.

Glucocorticoids are known to modulate adipose mass and food intake. Increased glucocorticoid levels decrease insulin and leptin, increase AGRP/NPY mRNA and decrease POMC gene expression in the hypothalamus [60]. We show here that after 72 hours glucocorticoid treatment increased NPY levels, as predicted, but

no alteration in AGRP mRNA levels was observed, which suggests that AGRP might be sensitive to the duration of the treatment or that this is a species specific phenomenon. In concordance with prior findings, we also demonstrated that POMC gene expression decreased in response to elevated Cort levels.

Thyroid hormones have been shown to decrease adipose tissue, and to compensate they also increase food intake. Elevated levels of thyroid hormone increase leptin levels as well as NPY mRNA and decrease Cocaine and Amphetamine regulated transcript (CART) and proopiomelanocortin (POMC) mRNA in the hypothalamus [55]. We demonstrated that POMC gene expression was decreased after T_3 treatment, but we show that there was no change in NPY mRNA levels, which was unexpected based on prior literature[55]. This suggests that our results might again demonstrate a species specific phenomenon or that, similar to AGRP after Cort treatment, alterations in NPY gene expression might be sensitive to the duration of the T_3 treatment.

Thyroid hormone and Cort modulate adiposity in the opposite direction but have been shown to increase food intake. Thus, we wanted to determine if treating with both Cort and T_3 simultaneously would modulate gene expression patterns in a fashion that resembles the increase in food intake, or in the opposite direction similar to body weight. We have shown that BDNF gene expression is regulated in the opposite direction by thyroid hormone or Cort levels, but in conjunction T_3 appears to have the predominating ability to decrease BDNF gene expression (Fig 1b). NPY mRNA levels were increased after Cort treatment, and no change was present after T_3 alone. However, when Cort and T_3 were delivered together the presence of T_3 was

able to ablate the increased gene expression observed with Cort treatment alone, suggesting again that T_3 is the primary regulator for gene expression.

Interestingly, identification of transcription factor binding sites revealed that BDNF has two sites and NPY has one site that potentially functions as both a TRE and GRE. If the GRE site induces transcription, it would be possible for T_3 to inhibit transcription by competitively binding to the site, or perhaps binding with a higher affinity. TRH gene expression decreased in the presence of T_3 or T_3 and Cort, but there was only a trend toward decreased expression with Cort treatment alone, regardless of TRH having 1 out of the 2 TRE sites also identified as a GRE. However, it is quite plausible that this is not an active GRE or TRE site. LEPR was another gene having a putative transcription factor site that potentially serves as both a GRE and TRE. LEPR gene expression decreased in the presence of Cort or T_3 alone, but the combination of the two delivered together only partially decreased expression. This could be due to the duration of hormone delivery, the possibility that expression patterns vary over time due to compensatory mechanisms, or the 2 out of 3 TRE sites that also function as a GRE. It is unknown whether these are excitatory or inhibitory response elements or functional in regards to transcription, but they do provide possibilities that deserve further investigation.

Paradoxically, T_3 inhibited POMC gene expression despite the lack of TRE sites upstream of exon 1. It has been shown that thyroid hormone receptors are located in the cell membrane and activate a MAPK signalling cascade [145], which could explain the decreased POMC mRNA levels observed. It is also possible that T_3 could increase expression of POMC mRNA by increasing expression of another,

unknown, protein. However, we observed that POMC mRNA levels increase after treating hypothalamic neurons in cell culture for 1 and 3 hours (Chapter 4), suggesting that the activation of thyroid receptors in the membrane is the more plausible explanation for T_3's rapid ability to modulate POMC gene expression.

The results for TNFα AP and ZFP 36 identified them as novel gene targets for Cort and T_3. Previous work in the pituitary has shown that GHRH-receptor mRNA levels decrease in response to elevated levels of glucocorticoids [146,147], thus this was not surprising. The ability for thyroid hormone to modulate TNF AP has not been previously demonstrated, but interactions between thyroid hormone and TNFα signalling have been suggested. For example in humans with thyroid hormone resistance, it has been proposed that the increased circulating levels of thyroid stimulating hormone (TSH) might stimulate lymphocytes to secrete more TNFα which may cause TNFα to bind receptors on thyrocytes [148]. Here we show that T_3 is able to inhibit a gene induced by TNFα, perhaps as a feedback to prevent or limit TNFα signalling processes. The results that Cort plus T_3 treatment modulated ZFP36 expression was quite surprising given that nothing has been previously reported about this interaction. Here we report for the first time that Cort and T_3 suppress ZFP 36 mRNA levels, but Cort or T_3 treatment alone has no effect on ZFP 36 gene expression (Fig. 2c).

Our findings demonstrate the finely orchestrated interactions between hormone levels and gene expression. Elevated levels of one hormone may not necessarily modulate gene expression in the same manner as it would if another hormone is simultaneously elevated. Here we begin to elucidate the underpinnings for

how hormone interactions may modulate anabolic and catabolic gene expression in the hypothalamus, with future work testing the functionality of the transcription factor binding sites identified that include both a GRE and TRE being much deserved.

Chapter 6: General discussion

In this dissertation, novel mechanisms which may be linked to obesity have been identified. Fat and Lean lines that develop a 250% difference in visceral fat show differential genetic expression patterns in the hypothalamus. The Fat and Lean lines are a unique model because they dissociate the development of an obese phenotype from alterations in food intake, which often occur simultaneously. These lines also show alterations in thyroid hormone as well as anabolic and catabolic gene expression. Thus, we elucidate how hormone interactions regulate the hypothalamic obesity circuit. The specific aims were the following: 1) identify genes contributing to the fat or lean phenotype via microarray, 2) identify alterations in anabolic and catabolic gene expression in Fat and Lean lines, 3) verify direct interactions in cell culture and *in vivo* and 4) further characterize *in vivo* interactions by chronically delivering thyroid hormone and corticosterone (Cort) to determine whether anabolic, catabolic or candidate gene expression in the hypothalamus is modulated.

Three hypotheses were developed describing potential underlying mechanisms in the hypothalamus that modulate body composition. The following two key processes were identified via microarray technology in the Fat and Lean chicken lines: genes involved with glycolysis have been associated with Klotho, ADAM12 and DRP2 (Table 1c and Fig. 6b, Chapter 2) and a gene induced by TNFα, TNF AP, has been identified to be associated with other genes previously known to regulate the obese phenotype, for example CEBPζ (Table 3 and Fig 6a, Chapter 2). We were also able to identify potential biological links between many other genes known to regulate obesity. From this we were able to identify for the first time which catabolic

and anabolic genes are associated with the development of adiposity independent of food intake alterations (Chapter 3). This identification is quite important because it begins to untangle the polygenic nature of diseases associated with body composition.

Another hypothesis developed and tested demonstrated that that Brain-derived Neurotrophic Factor (BDNF) and T_3 are able to reciprocally modulate the obesity gene circuit in the hypothalamus by regulating expression of many anabolic and catabolic genes. Using the Fat and Lean line data, we selected candidate genes that were differentially expressed between lines. Since the Fat line has increased T_3 levels relative to the Lean line at weeks 1 and 3 of age and since BDNF mRNA levels are decreased in the Fat relative to Lean at week 1, 3 and 5 of age, we tested whether BDNF or T_3 were directly able to modulate expression of candidate genes (Chapter 4 and 5). We tested these interactions in primary hypothalamic cell culture (in vitro) and by directly treating embryos over 48-hours with T_3 or methimazole (MMI), a thyroid hormone synthesis inhibitor, or by treating animals 29 days of age with an osmotic minipump that delivers T_3, Cort or T_3 plus Cort for 72-hours.

We observed that BDNF and T_3 reciprocally modulate some of the anabolic and catabolic genes differentially expressed in the Fat and Lean lines, suggesting that this may be a mechanism that regulates the divergence of adiposity between the lines. Our data show that T_3 decreased hypothalamic BDNF, TrkB, TRH and LEPR gene expression and that BDNF reciprocally increased expression of the same genes (Fig 1, Chapter 4). We present a model summarizing our *in vivo* and *in vitro* data showing reciprocal regulation of gene expression by T_3 and BDNF (Fig. 4, Chapter 4). Fat and Lean line data demonstrated that BDNF or T_3 levels could serve as the primary signalling factor to initiate hypothalamic gene expression during the different stages of adiposity development. Corresponding with predictions presented in our model

(Fig. 4, Chapter 4), before and at the onset of adiposity divergence (week 1 and 3), plasma T_3 was increased and hypothalamic BDNF expression was decreased in the Fat relative to lean line (Fig 1, 2 and 3, Chapter 3). An interaction between BDNF and T_3 was also demonstrated *in vitro* (Chapter 4). Additionally, at week 3 hypothalamic TRH mRNA levels were lower in the Fat relative to the Lean line, which also corresponds to *in vitro* experiments, suggesting that either T_3 could inhibit expression of TRH in the Fat line or increased BDNF expression in the Lean line could increase expression of TRH (Fig. 1, Chapter 4). Finally, at week 5 of age, hypothalamic BDNF levels continued to increase in the Lean relative to Fat line, which corresponds to an increase in TrkB, LEPR and 5-HTP levels that were also shown *in vitro* (Chapter 3 and 4). It is still unknown whether BDNF or T_3 initiates the differential gene expression for TRH, TrkB, LEPR or 5-HTP. In summary, our data support a novel pathway that contributes to differences in visceral fat independent of environmental alterations.

Utilizing the microarray data from Fat and Lean lines, candidate genes were selected (Chapter 2) and for the first time we show that expression of these genes is modulated by T_3 and/or glucocorticoids (Chapter 5). We found that delivery of glucocorticoids for 72-hours via osmotic minipump suppresses GHRH receptor gene expression in the hypothalamus, which has been previously demonstrated in the pituitary. We have also shown that T_3 delivery for 72-hours via minipump suppresses a gene induced by TNFα (TNF AP), a gene previously not known to be modulated by thyroid hormone. Lastly, we have shown that the Cort and T_3 together suppress ZFP 36, with either hormone alone having no effect on mRNA levels. The interaction between ZFP 36 and these hormones has never been shown or suggested. This is an important association since it is possible that ZFP 36 could regulate expression of genes involved in glycolysis (Chapter 2). This implies a possible mechanism for how

increased levels of glucocorticoids and thyroid hormone may modulate glucose metabolism, at least within the hypothalamus.

In summary, we have demonstrated that BDNF and T_3 reciprocally modulate expression of many genes, suggesting that expression levels may act as a fine tipping point for determining whether hypothalamic gene expression and adiposity increase or decrease over development. The implications of this are huge, since thyroid hormone supplements are commonly given to treat thyroid hormone disorders, which may disrupt the balance between BDNF and T_3 signalling and ultimately disrupt expression of many genes in the hypothalamus that serve to maintain energy regulation within a healthy range. Thus, we propose that obesity therapeutics should simultaneously target BDNF and T_3 to inhibit or reverse further fat deposition.

We have also identified a possible link between many genes known to regulate body composition, suggesting that new drug developments should be designed to modulate expression of all these genes simultaneously, to prevent dysregulation of the system or negative compensation. Here we show that it is not BDNF or T_3 alone causing the divergence of adiposity between the Fat and Lean lines, but it may also be genes involved with glycolysis as well as many other genes not modulated by T_3 levels. Thus, we present here multiple hypothalamic gene networks that may simultaneously be modulating the phenotype, potentially through alterations in hormone levels. For example, elevated levels of both Cort and T_3 are able to decrease expression of many genes identified by the microarray (Chapter 2), specifically a gene induced by TNFα, ZFP 36 and these hormones had a trend toward decreasing levels of GHRH receptor. We also have shown that elevated levels of both Cort and T_3 decrease expression of TRH, BDNF and CRH, with a trend toward suppression for POMC and a trend to elevated levels for AGRP gene expression. Please note that

these “trends” toward decreased or increased expression may be priming the system to respond more quickly when one hormone becomes the dominantly expressed hormone relative to the other one, since the genes were responsive to elevations in just one hormone. In conclusion, the finely orchestrated expression between hormone levels and hypothalamic gene expression modulate physiological processes to express either a fat or lean phenotype. We have shown that these hormones alter more than one hypothalamic gene network simultaneously, thus demonstrating the complexity of the hypothalamic obesity circuit.

Abbreviations

α-melanocyte-stimulating hormone (αMSH)

Agouti-related protein (AGRP)

AMP-activated protein kinase (AMPK)

Brain-Derived Neurotrophic Factor (BDNF)

CCAAT/enhancer-binding protein (CEBP)

Cholecystokinin-B receptor (CCKr)

Cocaine- and amphetamine-regulated transcript (CART)

Corticosterone (Cort)

Corticotropin releasing hormone (CRH)

Cytochrome b (Cyto b)

Dihydropyrimidase protein-2 (DRP2)

Elongation factor 1 α (EF1α)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Growth hormone releasing hormone receptor (GHRHR)

Induced by TNF alpha (TNF AP)

Leptin Receptor (LEPR)

Melanin concentrating hormone, MCH

Melanocortin-4 receptor (MC4R)

Metalloprotease 1 (ADAM12)

Neural Enolase (Neuro Eno)

Neuropeptide Y (NPY)

No description (ND)

Orexin (ORX)

Phosphoglucose isomerase (Phosg isomerase)

Phoshpoglycerate mutase (Phosg mut)

Pro-opiomelanocortin (POMC)

Soluble NSF-attachment protein (SNAP α)

Sparc/osteonectin (SPOCK2)

Thyroid stimulating hormone (TSH)

Thyrotropin releasing hormone (TRH)

Triiodothyronine (T_3)

Thyroxine (T_4)

TNF receptor associated factor 1 (TRAF-1)

TrkB tyrosine protein kinase receptor (TrkB)

Tumor necrosis factor α (TNFα)

5-tryptophan hydroxylase (5-HTP)

Bibliography

1. WHO, W.H.O. Obesity and Overweight, Fact sheet. (2003).

2. WHO, W.H.O. Global strategy on diet, physical activity and health. **Publication WHA57.17**(2005).

3. Zigman, J.M. & Elmquist, J.K. Minireview: From anorexia to obesity--the yin and yang of body weight control. *Endocrinology* **144**, 3749-56 (2003).

4. Anand, B.K. & Brobeck, J.R. Nutrition classics. The Yale Journal of Biology and Medicine. Volume XXIV 1951-1952. Hypothalamic control of food intake in rats and cats. *Nutrition Reviews* **42**, 354-6 (1984).

5. Hetherinton, A. & Ranson, S. Nutrition Classics. The Anatomical Record, Volume 78, 1940: Hypothalamic lesions and adiposity in the rat. *Nutrition Reviews* **41**, 124-7 (1983).

6. Kaye, W.H., Frank, G.K., Bailer, U.F. & Henry, S.E. Neurobiology of anorexia nervosa: clinical implications of alterations of the function of serotonin and other neuronal systems. *Int J Eat Disord* **37 Suppl**, S15-9; discussion S20-1 (2005).

7. Tataranni, P.A. & DelParigi, A. Functional neuroimaging: a new generation of human brain studies in obesity research. *Obes Rev* **4**, 229-38 (2003).

8. Schwartz, M.W., Woods, S.C., Porte, D., Jr., Seeley, R.J. & Baskin, D.G. Central nervous system control of food intake. *Nature* **404**, 661-71 (2000).

9. Chagnon, Y.C., Perusse, L. & Bouchard, C. Familial aggregation of obesity, candidate genes and quantitative trait loci. *Curr Opin Lipidol* **8**, 205-11 (1997).

10. Chagnon, Y.C., Perusse, L. & Bouchard, C. The human obesity gene map: the 1997 update. *Obes Res* **6**, 76-92 (1998).

11. Chagnon, Y.C., Perusse, L., Weisnagel, S.J., Rankinen, T. & Bouchard, C. The human obesity gene map: the 1999 update. *Obes Res* **8**, 89-117 (2000).

12. Chagnon, Y.C. et al. The human obesity gene map: the 2002 update. *Obes Res* **11**, 313-67 (2003).

13. Perusse, L., Chagnon, Y.C., Dionne, F.T. & Bouchard, C. The human obesity gene map: the 1996 update. *Obes Res* **5**, 49-61 (1997).

14. Perusse, L., Chagnon, Y.C., Weisnagel, J. & Bouchard, C. The human obesity gene map: the 1998 update. *Obes Res* **7**, 111-29 (1999).

15. Perusse, L. et al. The human obesity gene map: the 2000 update. *Obes Res* **9**, 135-69 (2001).

16. Perusse, L. et al. The human obesity gene map: the 2004 update. *Obes Res* **13**, 381-490 (2005).

17. Rankinen, T. et al. The human obesity gene map: the 2001 update. *Obes Res* **10**, 196-243 (2002).

18. Rankinen, T. et al. The human obesity gene map: the 2005 update. *Obesity (Silver Spring)* **14**, 529-644 (2006).

19. Leclercq, B., Blum, J.C. & Boyer, J.P. Selecting broilers for low or high abdominal fat - initial observations. *British Poultry Science* **21**, 107-113 (1980).

20. Abasht, B. et al. Fatness QTL on chicken chromosome 5 and interaction with sex. *Genet Sel Evol* **38**, 297-311 (2006).

21. Levin, B.E., Dunn-Meynell, A.A., Balkan, B. & Keesey, R.E. Selective breeding for diet-induced obesity and resistance in Sprague-Dawley rats. *Am J Physiol* **273**, R725-30 (1997).

22. Hinton, E.C. et al. Neural contributions to the motivational control of appetite in humans. *Eur J Neurosci* **20**, 1411-8 (2004).

23. Del Parigi, A. et al. Neuroimaging and obesity: mapping the brain responses to hunger and satiation in humans using positron emission tomography. *Ann N Y Acad Sci* **967**, 389-97 (2002).

24. Comings, D.E. & Blum, K. Reward deficiency syndrome: genetic aspects of behavioral disorders. *Prog Brain Res* **126**, 325-41 (2000).

25. Kelley, A.E. Ventral striatal control of appetitive motivation: role in ingestive behavior and reward-related learning. *Neurosci Biobehav Rev* **27**, 765-76 (2004).

26. Barbarich, N.C., Kaye, W.H. & Jimerson, D. Neurotransmitter and imaging studies in anorexia nervosa: new targets for treatment. *Curr Drug Targets CNS Neurol Disord* **2**, 61-72 (2003).

27. Jaccoby, S., Arnon, E., Snapir, N. & Robinzon, B. Effects of bilateral basomedial hypothalamic lesions on feeding, fattiness, and reproductive functions in the White Leghorn hen. *Physiol Behav* **56**, 1081-9 (1994).

28. Pritchard, L.E., Turnbull, A.V. & White, A. Pro-opiomelanocortin processing in the hypothalamus: impact on melanocortin signalling and obesity. *J Endocrinol* **172**, 411-21 (2002).

29. Nijenhuis, W.A., Oosterom, J. & Adan, R.A. AgRP(83-132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Mol Endocrinol* **15**, 164-71 (2001).

30. Fox, E.A. & Byerly, M.S. A mechanism underlying mature-onset obesity: evidence from the hyperphagic phenotype of brain-derived neurotrophic factor mutants. *Am J Physiol Regul Integr Comp Physiol* **286**, R994-1004 (2004).

31. Friedel, S. et al. Mutation screen of the brain derived neurotrophic factor gene (BDNF): identification of several genetic variants and association studies in patients with obesity, eating disorders, and attention-deficit/hyperactivity disorder. *Am J Med Genet B Neuropsychiatr Genet* **132**, 96-9 (2005).

32. Kernie, S.G., Liebl, D.J. & Parada, L.F. BDNF regulates eating behavior and locomotor activity in mice. *Embo J* **19**, 1290-300 (2000).

33. Ribases, M. et al. Association of BDNF with anorexia, bulimia and age of onset of weight loss in six European populations. *Hum Mol Genet* **13**, 1205-12 (2004).

34. Lyons, W.E. et al. Brain-derived neurotrophic factor-deficient mice develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities. *Proc Natl Acad Sci U S A* **96**, 15239-44 (1999).

35. Rios, M. et al. Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol Endocrinol* **15**, 1748-57 (2001).

36. Ernfors, P., Van De Water, T., Loring, J. & Jaenisch, R. Complementary roles of BDNF and NT-3 in vestibular and auditory development. *Neuron* **14**, 1153-64 (1995).

37. Monteleone, P. et al. Opposite changes in the serum brain-derived neurotrophic factor in anorexia nervosa and obesity. *Psychosom Med* **66**, 744-8 (2004).

38. McGough, N.N. et al. RACK1 and brain-derived neurotrophic factor: a homeostatic pathway that regulates alcohol addiction. *J Neurosci* **24**, 10542-52 (2004).

39. Pickar, D. et al. Endorphins in the cerebrospinal fluid of psychiatric patients. *Ann N Y Acad Sci* **398**, 399-412 (1982).

40. Torregrossa, M.M., Folk, J.E., Rice, K.C., Watson, S.J. & Woods, J.H. Chronic administration of the delta opioid receptor agonist (+)BW373U86 and antidepressants on behavior in the forced swim test and BDNF mRNA expression in rats. *Psychopharmacology (Berl)* **183**, 31-40 (2005).

41. Guillin, O. et al. BDNF controls dopamine D3 receptor expression and triggers behavioural sensitization. *Nature* **411**, 86-9 (2001).

42. Xu, B. et al. Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. *Nat Neurosci* **6**, 736-42 (2003).

43. Griffin, J.E. & Ojeda, S.R. Textbook of Edocrine Physiology, 4th Edition. (2000).

44. Michalaki, M.A. et al. Thyroid function in humans with morbid obesity. *Thyroid* **16**, 73-8 (2006).

45. Lechan, R.M. & Fekete, C. Role of thyroid hormone deiodination in the hypothalamus. *Thyroid* **15**, 883-97 (2005).

46. Kohrle, J. Local activation and inactivation of thyroid hormones: the deiodinase family. *Mol Cell Endocrinol* **151**, 103-19 (1999).

47. Kakucska, I., Rand, W. & Lechan, R.M. Thyrotropin-releasing hormone gene expression in the hypothalamic paraventricular nucleus is dependent upon feedback regulation by both triiodothyronine and thyroxine. *Endocrinology* **130**, 2845-50 (1992).

48. Rodriguez, E.M. et al. Hypothalamic tanycytes: a key component of brain-endocrine interaction. *Int Rev Cytol* **247**, 89-164 (2005).

49. Peruzzo, B., Pastor, F.E., Blazquez, J.L., Amat, P. & Rodriguez, E.M. Polarized endocytosis and transcytosis in the hypothalamic tanycytes of the rat. *Cell Tissue Res* **317**, 147-64 (2004).

50. Blazquez, J.L. et al. Antibodies obtained by xenotransplantation of organ-cultured median eminence specifically recognize hypothalamic tanycytes. *Cell Tissue Res* **308**, 241-53 (2002).

51. Riskind, P.N., Kolodny, J.M. & Larsen, P.R. The regional hypothalamic distribution of type II 5'-monodeiodinase in euthyroid and hypothyroid rats. *Brain Res* **420**, 194-8 (1987).

52. Guerra-Crespo, M., Ubieta, R., Joseph-Bravo, P., Charli, J.L. & Perez-Martinez, L. BDNF increases the early expression of TRH mRNA in fetal TrkB+ hypothalamic neurons in primary culture. *Eur J Neurosci* **14**, 483-94 (2001).

53. Camboni, D., Roskoden, T. & Schwegler, H. Effect of early thyroxine treatment on brain-derived neurotrophic factor mRNA expression and protein amount in the rat medial septum/diagonal band of Broca. *Neurosci Lett* **350**, 141-4 (2003).

54. Ubieta, R. et al. BDNF up-regulates pre-pro-TRH mRNA expression in the fetal/neonatal paraventricular nucleus of the hypothalamus. Properties of the transduction pathway. *Brain Res* **1174**, 28-38 (2007).

55. Ishii, S. et al. Hypothalamic neuropeptide Y/Y1 receptor pathway activated by a reduction in circulating leptin, but not by an increase in circulating ghrelin, contributes to hyperphagia associated with triiodothyronine-induced thyrotoxicosis. *Neuroendocrinology* **78**, 321-30 (2003).

56. Wirth, M.J., Patz, S. & Wahle, P. Transcellular induction of neuropeptide Y expression by NT4 and BDNF. *Proc Natl Acad Sci U S A* **102**, 3064-9 (2005).

57. Woods, S.C., Lotter, E.C., McKay, L.D. & Porte, D., Jr. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* **282**, 503-5 (1979).

58. McMinn, J.E. et al. NPY-induced overfeeding suppresses hypothalamic NPY mRNA expression: potential roles of plasma insulin and leptin. *Regul Pept* **75-76**, 425-31 (1998).

59. Schwartz, M.W. et al. Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* **46**, 2119-23 (1997).

60. Makimura, H. et al. Role of glucocorticoids in mediating effects of fasting and diabetes on hypothalamic gene expression. *BMC Physiol* **3**, 5 (2003).

61. Roberge, C. et al. Adrenocortical dysregulation as a major player in insulin resistance and onset of obesity. *Am J Physiol Endocrinol Metab* (2007).

62. Krabbe, K.S. et al. Brain-derived neurotrophic factor (BDNF) and type 2 diabetes. *Diabetologia* **50**, 431-8 (2007).

63. Ribases, M. et al. Met66 in the brain-derived neurotrophic factor (BDNF) precursor is associated with anorexia nervosa restrictive type. *Mol Psychiatry* **8**, 745-51 (2003).

64. Schule, C. et al. Brain-derived neurotrophic factor Val66Met polymorphism and dexamethasone/CRH test results in depressed patients. *Psychoneuroendocrinology* **31**, 1019-25 (2006).

65. Falkenberg, T. et al. Increased expression of brain-derived neurotrophic factor mRNA in rat hippocampus is associated with improved spatial memory and enriched environment. *Neurosci Lett* **138**, 153-6 (1992).

66. Chao, H.M. & McEwen, B.S. Glucocorticoids and the expression of mRNAs for neurotrophins, their receptors and GAP-43 in the rat hippocampus. *Brain Res Mol Brain Res* **26**, 271-6 (1994).

67. Chao, H.M., Sakai, R.R., Ma, L.Y. & McEwen, B.S. Adrenal steroid regulation of neurotrophic factor expression in the rat hippocampus. *Endocrinology* **139**, 3112-8 (1998).

68. Rage, F., Givalois, L., Marmigere, F., Tapia-Arancibia, L. & Arancibia, S. Immobilization stress rapidly modulates BDNF mRNA expression in the hypothalamus of adult male rats. *Neuroscience* **112**, 309-18 (2002).

69. Marmigere, F., Givalois, L., Rage, F., Arancibia, S. & Tapia-Arancibia, L. Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus* **13**, 646-55 (2003).

70. Givalois, L. et al. Immobilization stress rapidly and differentially modulates BDNF and TrkB mRNA expression in the pituitary gland of adult male rats. *Neuroendocrinology* **74**, 148-59 (2001).

71. Pelchat, M.L. Of human bondage: food craving, obsession, compulsion, and addiction. *Physiol Behav* **76**, 347-52 (2002).

72. Berthoud, H.R. Brain, appetite and obesity. *Physiol Behav* **85**, 1-2 (2005).

73. Grilo, C.M. & Masheb, R.M. Childhood psychological, physical, and sexual maltreatment in outpatients with binge eating disorder: frequency and associations with gender, obesity, and eating-related psychopathology. *Obes Res* **9**, 320-5 (2001).

74. Cannon, C.M. & Palmiter, R.D. Reward without dopamine. *J Neurosci* **23**, 10827-31 (2003).

75. Blum, K. et al. Increased prevalence of the Taq I A1 allele of the dopamine receptor gene (DRD2) in obesity with comorbid substance use disorder: a preliminary report. *Pharmacogenetics* **6**, 297-305 (1996).

76. Bowirrat, A. & Oscar-Berman, M. Relationship between dopaminergic neurotransmission, alcoholism, and Reward Deficiency syndrome. *Am J Med Genet B Neuropsychiatr Genet* **132**, 29-37 (2005).

77. Bailer, U.F. & Kaye, W.H. A review of neuropeptide and neuroendocrine dysregulation in anorexia and bulimia nervosa. *Curr Drug Targets CNS Neurol Disord* **2**, 53-9 (2003).

78. DelParigi, A. et al. Persistence of abnormal neural responses to a meal in postobese individuals. *Int J Obes Relat Metab Disord* **28**, 370-7 (2004).

79. Spangler, R. et al. Opiate-like effects of sugar on gene expression in reward areas of the rat brain. *Brain Res Mol Brain Res* **124**, 134-42 (2004).

80. Mercer, M.E. & Holder, M.D. Antinociceptive effects of palatable sweet ingesta on human responsivity to pressure pain. *Physiol Behav* **61**, 311-8 (1997).

81. Fedoroff, I.C., Polivy, J. & Herman, C.P. The effect of pre-exposure to food cues on the eating behavior of restrained and unrestrained eaters. *Appetite* **28**, 33-47 (1997).

82. Goldberg, J.R., Ehrmann, B. & Katzeff, H.L. Altered triiodothyronine metabolism in Zucker fatty rats. *Endocrinology* **122**, 689-93 (1988).

83. Leclercq, B., Guy, G. & Rudeaux, F. Thyroid hormones in genetically lean or fat chickens: effects of age and triiodothyronine supplementation. *Reprod Nutr Dev* **28**, 931-7 (1988).

84. Touchburn, S., Simon, J. & Leclercq, B. Evidence of a glucose-insulin imbalance and effect of dietary protein and energy level in chickens selected for high abdominal fat content. *J Nutr* **111**, 325-35 (1981).

85. Geraert, P.A., MacLeod, M.G., Larbier, M. & Leclercq, B. Nitrogen metabolism in genetically fat and lean chickens. *Poult Sci* **69**, 1911-21 (1990).

86. Cahaner, A. & Leenstra, F. Effects of high temperature on growth and efficiency of male and female broilers from lines selected for high weight gain, favorable feed conversion, and high or low fat content. *Poult Sci* **71**, 1237-50 (1992).

87. Hood, R.L. The cellular basis for growth of the abdominal fat pad in broiler-type chickens. *Poult Sci* **61**, 117-21 (1982).

88. Vandesompele, J. et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* **3**, RESEARCH0034 (2002).

89. Tamayo, P. et al. Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci U S A* **96**, 2907-12 (1999).

90. Leclercq, B., Kouassi-Kouakou, J. & Simon, J. Laying performances, egg composition, and glucose tolerance of genetically lean or fat meat-type breeders. *Poult Sci* **64**, 1609-16 (1985).

91. Wu, C. et al. Enhancing hepatic glycolysis reduces obesity: differential effects on lipogenesis depend on site of glycolytic modulation. *Cell Metab* **2**, 131-40 (2005).

92. Mori, K. et al. Disruption of klotho gene causes an abnormal energy homeostasis in mice. *Biochem Biophys Res Commun* **278**, 665-70 (2000).

93. Rhee, E.J. et al. Relationship between polymorphisms G395A in promoter and C1818T in exon 4 of the KLOTHO gene with glucose metabolism and cardiovascular risk factors in Korean women. *J Endocrinol Invest* **29**, 613-8 (2006).

94. Do, M.S. et al. Inflammatory gene expression patterns revealed by DNA microarray analysis in TNF-alpha-treated SGBS human adipocytes. *Yonsei Med J* **47**, 729-36 (2006).

95. Shindo, T. et al. ADAMTS-1: a metalloproteinase-disintegrin essential for normal growth, fertility, and organ morphology and function. *J Clin Invest* **105**, 1345-52 (2000).

96. Boyd, R.S. et al. Proteomic analysis of the cell-surface membrane in chronic lymphocytic leukemia: identification of two novel proteins, BCNP1 and MIG2B. *Leukemia* **17**, 1605-12 (2003).

97. Choi, J. et al. Proteomic identification of specific oxidized proteins in ApoE-knockout mice: relevance to Alzheimer's disease. *Free Radic Biol Med* **36**, 1155-62 (2004).

98. Charrier, E. et al. Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. *Mol Neurobiol* **28**, 51-64 (2003).

99. Bodnar, R.J. et al. Mediation of anorexia by human recombinant tumor necrosis factor through a peripheral action in the rat. *Cancer Res* **49**, 6280-4 (1989).

100. Mace, O.J., Affleck, J., Patel, N. & Kellett, G.L. Sweet taste receptors in rat small intestine stimulate glucose absorption through apical GLUT2. *J Physiol* **582**, 379-92 (2007).

101. Abribat, T., Finkelstein, J.A. & Gaudreau, P. Alteration of somatostatin but not growth hormone-releasing factor pituitary binding sites in obese Zucker rats. *Regul Pept* **36**, 263-70 (1991).

102. Ceci, F. et al. The effects of oral 5-hydroxytryptophan administration on feeding behavior in obese adult female subjects. *J Neural Transm* **76**, 109-17 (1989).

103. Manda, T., Nishigaki, F., Mori, J. & Shimomura, K. Important role of serotonin in the antitumor effects of recombinant human tumor necrosis factor-alpha in mice. *Cancer Res* **48**, 4250-5 (1988).

104. Alt, J.A. et al. Influenza virus-induced glucocorticoid and hypothalamic and lung cytokine mRNA responses in dwarf lit/lit mice. *Brain Behav Immun* **21**, 60-7 (2007).

105. Chiu, C.H., Lin, W.D., Huang, S.Y. & Lee, Y.H. Effect of a C/EBP gene replacement on mitochondrial biogenesis in fat cells. *Genes Dev* **18**, 1970-5 (2004).

106. Glowinska-Olszewska, B. & Urban, M. Elevated matrix metalloproteinase 9 and tissue inhibitor of metalloproteinase 1 in obese children and adolescents. *Metabolism* **56**, 799-805 (2007).

107. Bouret, S.G., Draper, S.J. & Simerly, R.B. Formation of projection pathways from the arcuate nucleus of the hypothalamus to hypothalamic regions implicated in the neural control of feeding behavior in mice. *J Neurosci* **24**, 2797-805 (2004).

108. Burt, D.W. et al. The dynamics of chromosome evolution in birds and mammals. *Nature* **402**, 411-3 (1999).

109. Hedges, S.B. & Kumar, S. Genomics. Vertebrate genomes compared. *Science* **297**, 1283-5 (2002).

110. Kopelman, P.G. Obesity as a medical problem. *Nature* **404**, 635-43 (2000).

111. Bray, G.A. & York, D.A. The MONA LISA hypothesis in the time of leptin. *Recent Prog Horm Res* **53**, 95-117; discussion 117-8 (1998).

112. Herbert, A. et al. A common genetic variant is associated with adult and childhood obesity. *Science* **312**, 279-83 (2006).

113. Jeanrenaud, B. & Rohner-Jeanrenaud, F. Effects of neuropeptides and leptin on nutrient partitioning: dysregulations in obesity. *Annu Rev Med* **52**, 339-51 (2001).

114. Elmquist, J.K. Hypothalamic pathways underlying the endocrine, autonomic, and behavioral effects of leptin. *Physiol Behav* **74**, 703-8 (2001).

115. Morton, G.J. & Schwartz, M.W. The NPY/AgRP neuron and energy homeostasis. *Int J Obes Relat Metab Disord* **25 Suppl 5**, S56-62 (2001).

116. Kuenzel, W.J., Beck, M.M. & Teruyama, R. Neural sites and pathways regulating food intake in birds: a comparative analysis to mammalian systems. *J Exp Zool* **283**, 348-64 (1999).

117. Simon, J. & Leclercq, B. Longitudinal study of adiposity in chickens selected for high or low abdominal fat content: further evidence of a glucose-insulin imbalance in the fat line. *J Nutr* **112**, 1961-73 (1982).

118. Saadoun, A. & Leclercq, B. In vivo lipogenesis of genetically lean and fat chickens: effects of nutritional state and dietary fat. *J Nutr* **117**, 428-35 (1987).

119. Saadoun, A., Simon, J., Williams, J. & Leclercq, B. Levels of insulin, corticosterone, T3, T4 and insulin sensitivity in fat and lean chickens. *Diabete Metab* **14**, 97-103 (1988).

120. Ur, E. & Wilkinson, M. Endocrine and neuroendocrine signals of energy stores: view from the chair. *Int J Obes Relat Metab Disord* **25 Suppl 5**, S30-4 (2001).

121. Baskin, D.G., Breininger, J.F. & Schwartz, M.W. Leptin receptor mRNA identifies a subpopulation of neuropeptide Y neurons activated by fasting in rat hypothalamus. *Diabetes* **48**, 828-33 (1999).

122. Dridi, S., Buyse, J., Decuypere, E. & Taouis, M. Potential role of leptin in increase of fatty acid synthase gene expression in chicken liver. *Domest Anim Endocrinol* **29**, 646-60 (2005).

123. Friedman-Einat, M. et al. The chicken leptin gene: has it been cloned? *Gen Comp Endocrinol* **115**, 354-63 (1999).

124. Richards, M.P. & Poch, S.M. Molecular cloning and expression of the turkey leptin receptor gene. *Comp Biochem Physiol B Biochem Mol Biol* **136**, 833-47 (2003).

125. Mistry, A.M., Swick, A. & Romsos, D.R. Leptin alters metabolic rates before acquisition of its anorectic effect in developing neonatal mice. *Am J Physiol* **277**, R742-7 (1999).

126. Tohei, A., Akai, M., Tomabechi, T., Mamada, M. & Taya, K. Adrenal and gonadal function in hypothyroid adult male rats. *J Endocrinol* **152**, 147-54 (1997).

127. Dupont J et al. Metabolic differences between genetically lean and fat chickens are partly attributed to the alteration of insulin signaling in liver. *J Nutr.* **129**, 1937-44 (1999).

128. Leclercq, B. & Saadoun, A. Selecting broilers for low or high abdominal fat - comparison of energy-metabolism of the lean and fat lines. *Poult Sci* **61**, 1799-1803 (1982).

129. Molteni, R., Barnard, R.J., Ying, Z., Roberts, C.K. & Gomez-Pinilla, F. A high-fat, refined sugar diet reduces hippocampal brain-derived neurotrophic factor, neuronal plasticity, and learning. *Neuroscience* **112**, 803-14 (2002).

130. Molteni, R. et al. Exercise reverses the harmful effects of consumption of a high-fat diet on synaptic and behavioral plasticity associated to the action of brain-derived neurotrophic factor. *Neuroscience* **123**, 429-40 (2004).

131. Wu, A., Ying, Z. & Gomez-Pinilla, F. The interplay between oxidative stress and brain-derived neurotrophic factor modulates the outcome of a saturated fat diet on synaptic plasticity and cognition. *Eur J Neurosci* **19**, 1699-707 (2004).

132. Saxod, R. & Bouvet, J. Quantitative analysis of growth and myelination of cutaneous nerve fibers in the chick. *Dev Neurosci* **5**, 143-55 (1982).

133. Kuenzel, W.J. & Masson, M. A Stereotaxic Atlas of the Brain of the Chick (Gallus domesticus). (1988).

134. Korner, J. et al. Effects of leptin receptor mutation on Agrp gene expression in fed and fasted lean and obese (LA/N-faf) rats. *Endocrinology* **141**, 2465-71 (2000).

135. Dupont, J. et al. Metabolic differences between genetically lean and fat chickens are partly attributed to the alteration of insulin signaling in liver. *J Nutr* **129**, 1937-44 (1999).

136. Saadoun, A., Simon, J. & Leclercq, B. Effect of exogenous corticosterone in genetically fat and lean chickens. *Br Poult Sci* **28**, 519-28 (1987).

137. Saadoun, A.a.L.B. Selecting broilers for low or high abdominal fat - comparison of energy-metabolism of the lean and fat lines. *Poultry Science* **61**, 1799-1803 (1982).

138. Guo, K. et al. Disruption of peripheral leptin signaling in mice results in hyperleptinemia without associated metabolic abnormalities. *Endocrinology* **148**, 3987-97 (2007).

139. Komori, T., Morikawa, Y., Nanjo, K. & Senba, E. Induction of brain-derived neurotrophic factor by leptin in the ventromedial hypothalamus. *Neuroscience* **139**, 1107-15 (2006).

140. Pitel, F., Monbrun, C., Gellin, J. & Vignal, A. Mapping of the LEP (OB) gene to a chicken microchromosome. *Anim Genet* **30**, 73-4 (1999).

141. Dallman, M.F. et al. Feast and famine: critical role of glucocorticoids with insulin in daily energy flow. *Front Neuroendocrinol* **14**, 303-47 (1993).

142. Dhillo, W.S. Appetite regulation: an overview. *Thyroid* **17**, 433-45 (2007).

143. Hayashi, K., Kayali, A.G. & Young, V.R. Synergism of triiodothyronine and corticosterone on muscle protein breakdown. *Biochim Biophys Acta* **883**, 106-11 (1986).

144. Adam, T.C. & Epel, E.S. Stress, eating and the reward system. *Physiol Behav* **91**, 449-58 (2007).

145. Davis, P.J., Davis, F.B. & Cody, V. Membrane receptors mediating thyroid hormone action. *Trends Endocrinol Metab* **16**, 429-35 (2005).

146. Miller, T.L. & Mayo, K.E. Glucocorticoids regulate pituitary growth hormone-releasing hormone receptor messenger ribonucleic acid expression. *Endocrinology* **138**, 2458-65 (1997).

147. Porter, T.E., Ellestad, L.E., Fay, A., Stewart, J.L. & Bossis, I. Identification of the chicken growth hormone-releasing hormone receptor (GHRH-R) mRNA and gene: regulation of anterior pituitary GHRH-R mRNA levels by homologous and heterologous hormones. *Endocrinology* **147**, 2535-43 (2006).

148. Gavin, C., Meggison, H. & Ooi, T.C. Proposing a causal link between thyroid hormone resistance and primary autoimmune hypothyroidism. *Med Hypotheses* (2007).