REAL TIME RT-PCR ANALYSIS OF HYPOTHALAMI FROM RODENTS TREATED CENTRALLY AND PERIPHERALLY WITH PEPTIDE HORMONES

by

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(Under the Direction of Dr. Clifton A. Baile)

ABSTRACT

The hypothalamus is a primary organ for the regulation of feeding, energy metabolism, and a variety of other physiological functions. It has numerous receptors to peripheral signals and has widespread interactions with other brain areas. To investigate how peptide hormones modulate the hypothalamic gene profile in rodents, we employed real time reverse transcription-polymerase chain reaction (RT-PCR) to measure the mRNA levels of selected genes. The hormones used were leptin, ciliary neurotrophic factor (CNTF), and glucose-dependent insulinotropic peptide (GIP). The genes were divided into clusters according to their biological functions, and mRNA levels in the whole hypothalamus or in an individual hypothalamic nucleus were compared between the control and the peptide-treated rodents.

In the first study, we compared and contrasted leptin and CNTF on rat hypothalamic gene expression. Our results showed that both intracerebroventricular (ICV) leptin and CNTF for 4 successive days increased mRNA levels of CART, POMC, STAT3, and SOCS3 in the rat hypothalamic arcuate nucleus (ARC). Leptin increased ARC mRNA level of GHRH, while CNTF increased JAK2 mRNA and reduced mRNA levels of AVP, GNRH1, MAPK1, OXT, and TH. Our findings show that leptin and

CNTF engaged both convergent and divergent pathways involved in feeding, cellular signaling, inflammation, and other biological regulatory systems.

The second study showed that *ob/ob* and lean mice have different hypothalamic gene profiles. The results indicated that the major hypothalamic mRNA expression difference between *ob/ob* and lean control mice was the divergent gene profile for those biomarkers involved in feeding regulation. While leptin dramatically modified hypothalamic mRNAs of these feeding-related genes in both genotypes, leptin altered hypothalamic expression of only some of the other genes assayed.

In the third experiment, we showed that unilateral leptin exposure with VMH injections, as well as the bilateral leptin exposure with ICV injections, induced divergent physiological behaviors and hypothalamic ARC gene profiles, and that tyrosine hydroxylase (TH) may play an important role in the crosstalk between the two VMH sides.

The fourth experiment was conducted to study how the absence of GIP receptor (GIPR) affects the hypothalamic gene expression profile in mice. Our results suggest that the hypothalamic mRNAs are down-regulated on AVP, CART, OXT, PTGES, STAT3, TH, and UCN3, while up-regulated on MAPK1 and NPY in GIPR knockout (KO) mice. In the fifth study, GIP was ICV injected in rats and the results indicated that GIP changed the mRNA levels of biomarkers related to stress and anxiety behaviors.

In general, our results suggest that real time RT-PCR, when combined with microfluidic technology provides a convenient, sensitive, and reliable way to profile multiple hypothalamic mRNA changes. The mRNA changes of these selected genes in

the hypothalamus provide useful information for understanding the complexity of integrated neural circuits and the action mechanisms of such hormones.

INDEX WORDS: Hypothalamus, Real time RT-PCR, Leptin, GIP, CNTF, *ob/ob*, ARC, VMH, ICV, Subcutaneous, Gene expression

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Doctor of Philosophy

ATHENS, GEORGIA

2006

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December 2006

DEDICATION

I dedicate my thesis to my parents, Yangchun Duan and Zhiying Kuang, and my wife, Hongliang Hu, my son, Richard Duan, whose love and support have made all my accomplishments possible.

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my major professor, Dr. Clifton A. Baile, for his invaluable guidance, support and encouragement during the past three and a half years. I greatly appreciate his patience, advice, and inspiration motivating me to achieve my goal.

I would like to thank my supportive and patient committee, Drs. Roger Dean, Gaylen Edwards, Silvia Giraudo, and Gary Hausman, for their valuable suggestions, expert advice and continual encouragement.

Thanks to Diane Hartzell for providing me tremendous help for which I am always very appreciative. Thanks to Dr. MaryAnne Della-Fera for her great patience on reading and correcting my papers.

I also would like to thank a wonderful group of people to which I am grateful for their support: Kara Huff and Roger Nilsen for the excellent sample processing; Kimberly Freeman and Drs. Romdhane Rekaya, Wensheng Zhang, Dan Hall, Mark Hamrick, Ji Lin, and Yang-Ho Choi for their help on my project; Rhonda Wheeler and Gily Raz for correcting my thesis; and the rest of the Baile lab.

Abbreviations:

ACSF, artificial cerebrospinal fluid;

AGRP, agouti related protein;

ANOVA, analysis of variance;

ARC, arcuate nucleus;

AVP, arginine vasopressin;

BBB, blood-brain-barrier;

BMC, body mineral content;

BMD, body mineral density;

CALM2, calmodulin 2;

CART, cocaine and amphetamine regulated transcript;

CCK, cholecystokinin;

CNTF, ciliary neurotrophic factor;

CNS, central nervous system;

COX-2, cyclooxygenase-2;

CREB1, cAMP responsive element binding protein 1;

CRH, corticotropin-releasing hormone;

Ct, threshold cycle;

DA, dopamine;

DMH, dorsomedial hypothalamus;

FOS, FBJ osteosarcoma oncogene;

FRET, fluorescence resonance energy transfer;

FSH, follicle-stimulating hormone;

GABA, γ-aminobutyric acid;

GABRD, gamma-aminobutyric acid A receptor;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase;

GIP, glucose-dependent insulinotropic peptide;

GIPR, glucose-dependent insulinotropic peptide receptor;

GH, growth hormone;

GHRH, growth hormone releasing hormone;

GNRH1, gonadotropin-releasing hormone 1;

HCRT, hypocretin;

HKG, housekeeping gene;

HPA axis, hypothalamus-pituitary-adrenal axis;

HPG axis, hypothalamus-pituitary-gonadal;

HPT axis, hypothalamo-pituitary-thyroid axis;

ICV, intracerebroventricular;

IL-6, interleukin 6;

JAK2, Janus kinase 2;

KO, knockout;

LIF, leukemia inhibitory factor;

LH, lateral hypothalamus;

*l*VMH, left side of VMH;

MAPK1, mitogen activated protein kinase 1;

MCH, melanin-concentrating hormone;

ME, medial eminence;

MSF, melanocyte-stimulating hormone releasing factor;

MSH, melanin - stimulating hormone;

NA, nucleus accumbens;

NE, norepinephrine;

NPY, neuropeptide Y;

NTS, nucleus of solitary tract;

Ob-Rb, long form of leptin receptor;

OVLT, organum vasculosum laminae terminalis;

OXT, oxytocin;

PBN, parabrachial nucleus;

PCR, ploymerase chain reaction;

PO-LH, preoptic lateral hypothalamus;

POMC2, proopiomelanocortin, beta;

PPIA, peptidylprolyl isomerase A;

PRF, prolactin-releasing factor;

PTGES, prostaglandin E synthase;

PVN, paraventricular nucleus;

*r*VMH, right side of VMH;

RQ, relative quantification;

RT, reverse transcription;

SC, subcutaneous;

SCN, suprachiasmatic nucleus;

SCT, secretin;

SDS, sequence detection system;

SNS, sympathetic nervous system;

SOCS3, suppressor of cytokine signaling 3;

SON, supraoptic nucleus;

STAT3, signal transducer and activator of transcription 3;

TH, tyrosine hydroxylase;

TNF, tumor necrosis factor;

TRH, thyrotropin-releasing hormone;

VIP, vasoactive intestinal polypeptide;

VMH, ventromedial hypothalamus;

UCN3, urocortin 3;

UCP, uncoupling protein;

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

The hypothalamus is a brain region located below the thalamus [1]. It forms the main portion of the ventral diencephalon and is a major organ in the limbic system [2]. The hypothalamus is involved in a variety of biological and physiological functions such as feeding, energy homeostasis, thermogenesis, emotion, and circadian rhythms [3]. It responds to various external and internal signals and is widely connected with many parts of the central nervous system (CNS) such as the forebrain and hindbrain [4]. In addition to the neural connections, the rich localization of vascularized capillaries provides easy access for peripheral humoral information, such as leptin, insulin, ghrelin, cytokines, and glucose [5-7]. These peripheral signals travel through the blood-brain-barrier (BBB) and bind to their corresponding receptors in different regions of the hypothalamus [8, 9]. Thus, the hypothalamus is an important integrator organ that can receive and relay information to and from a variety of neuronal and humoral systems, and it plays a critical role in neuroendocrine and homeostatic regulation. The wide projections and interactions, together with numerous neuropeptides and neurotransmitters in the hypothalamus, form the complexity of neuronal circuits [10].

1.1 Hypothalamus

1.1.1 Subdivisions and boundaries

The hypothalamus consists of three regions: the periventricular, medial, and lateral hypothalamus [11]. The anatomical boundaries of the hypothalamus are: rostral, the lamina terminalis; caudal, the mamillary bodies; dorsal, the hypothalamic sulcus; medial, the 3rd ventricle; lateral, the subthalamus; ventral, the optic chiasm and posterior pituitary [4]. Numerous nuclei are located within each regional area of the hypothalamus (Fig. 1) [12]. The medial preoptic nucleus is in the medial area; the supraoptic nucleus (SON), suprachiasmatic nucleus (SCN), and paraventricular nucleus (PVN) can be found in the anterior; the dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and arcuate nucleus (ARC) are in the tuberal; whereas the lateral preoptic nucleus and lateral hypothalamus (LH) can be found in the lateral area [4, 13]. The medial hypothalamus has extensive connections with the amygdala and hippocampus [4, 14]. The lateral preoptic lateral hypothalamic (PO-LH) area has a variety of reciprocal connections with the forebrain, brainstem, and spinal cord [15]. The hypothalamus projects to both caudal and rostral areas through the medial forebrain bundle, mammillotegmental tract, fornix, and stria terminalis [15, 16].

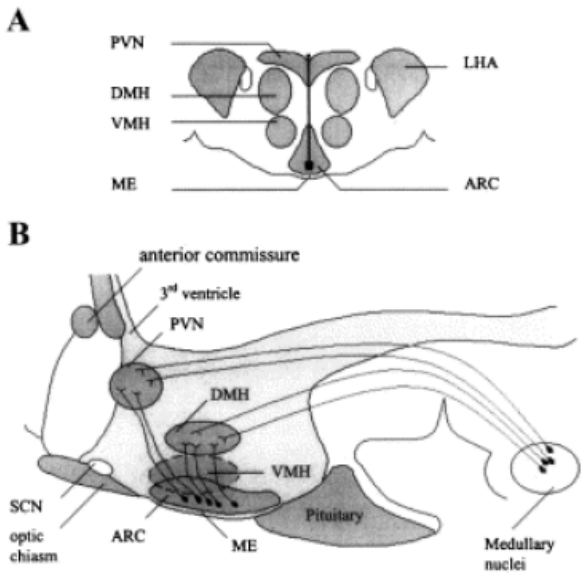


Fig. 1. Anatomy of the rat hypothalamus. (A) Coronal view; (B) Sagittal section [12].

1.1.2 Hypothalamic nuclei and their interactions

In the hypothalamus, there are a number of nuclei that consist of specific cell types with various neuroendocrine functions [4]. The ARC of the hypothalamus, located at the bottom of the 3rd ventricle and adjacent to the medial eminence (ME), is an extremely important organ in that it receives various peripheral stimuli [4]. Peripheral signals can get through the highly vascularized ME region into the ARC [17]. The ARC interacts not only with other hypothalamic nuclei such as LH and PVN, but also with multiple regions in both the forebrain and hindbrain [18]. Moreover, ARC produces numerous receptors and neuropeptides that are related to various endocrine functions. The orexigenic neuropeptide system neuropeptide Y/agouti related protein (NPY/AGRP) and the anorectic neuropeptide system proopiomelanocortin/cocaine and amphetamine

regulated transcript (POMC/CART) are two such examples of mutually antagonized systems related to feeding [19, 20].

The PVN on the top of the 3rd ventricle is involved in behaviors including feeding, thirst, and autonomic and endocrine responses to stress [21]. PVN produces corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH) [22]. The CRF-producing neurons are inhibited by the NPY/AGRP from the ARC, while TRH-producing neurons are stimulated by the POMC/CART terminals projected from the ARC [23, 24]. Not only does PVN have wide neuronal connections with ARC, LH, and DMH in the hypothalamus, but it also interacts with numerous areas such as nucleus of solitary tract (NTS) in the brainstem, as well as amygdala and hippocampus in the limbic system [15].

The LH has the most intensive projections and connections with both the intrahypothalamic nuclei (such as ARC, PVN, DMH, and VMH) and a variety of extrahypothalamic areas including the forebrain and hindbrain [4]. In both LH and the perifornical area, there are neurons that produce orexin and melanin-concentrating hormone (MCH), which have orexigenic effects on feeding and body weight [25]. The VMH is the largest nucleus in the hypothalamus and has been thought of as a satiety center [26]. It is involved in regulation of feeding and metabolism, as lesions of the VMH and surrounding fibers result in hyperphagia and obesity [27]. VMH is also critical in linking nutritional status to circadian rhythmicity [28]. In the hypothalamus, VMH has connections with LH and DMH, which locates close to VMH and is involved in regulation of energy deprivation [4]. DMH has connections with PVN, VMH, and LH in

the hypothalamus [15]. There are also other nuclei such as SON and SCN which are associated with circadian rhythm [29].

1.1.3 Hypothalamic biomarkers

A variety of neuropeptides and neurotransmitters have been produced in the hypothalamus [30]. The hypothalamic neurons communicate information by multiple biochemical signals, including neurotransmitters, peptides, cannabinoids, and gases such as nitric oxide [10]. While neurotransmitters locate at presynaptic ends and alter the excitability of other neurons, the neuropeptides are stored in large vesicles in the neurons and have diverse and prolonged effects [31]. The hypothalamic parvocellular neurons produce a number of releasing and inhibiting hormones including TRH, CRH, gonadotropin-releasing hormone (GNRH), growth hormone-releasing hormone (GHRH), prolactin-releasing factor (PRF), and melanocyte-stimulating hormone releasing factor (MSF) [30]. The peripheral signals, once bound to their corresponding receptors in the hypothalamic neurons, can activate the immediate early genes such as c-fos and c-jun, as well as signaling molecules such as Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3), and mitogen activated protein kinase (MAPK) [32-34]. The hypothalamic biomarkers are related to various physiological activities. For example, the POMC/CART and NPY/AGRP produced in the hypothalamic ARC are involved in regulation of feeding and energy metabolism [19, 20, 35]. Some other biomarkers, such as arginine vasopressin (AVP), oxytocin (OXT), and cholecystokinin (CCK), are involved in learning and memory behavior [36, 37].

1.1.4 Hypothalamic functions

HPG and HPA axis

The hypothalamus plays an integrative role in the hypothalamus-pituitary-gonadal (HPG) axis, which controls maternal and reproductive behavior [38]. GNRH producing neurons in the hypothalamus are sensitive to gonadal steroids, which exert negative feedback regulation on GNRH release [39]. The pulsatile stimulation of the anterior pituitary by GNRH initiates the pulsatile release of follicle-stimulating hormone (FSH) and luteinizing hormone at the pituitary [40]. It has been found that spikes of electrical activity in the medial basal hypothalamus correspond to pulses of luteinizing hormone release [41]. The critical brain area involved in sexual behavior has been found at the medial preoptic area in males and VMH in females [42]. The hypothalamus-pituitary-adrenal (HPA) axis is a key player in an animal's response to stress [43]. CRF, which is produced in the medial part of PVN and regulated by the humoral factors such as glucocorticoids, has a protective role during both physical and emotional stress [44].

Sleep and circadian timing

The hypothalamus plays an important role in sleep-wake regulation [45]. The medial hypothalamus is related to circadian functions, while the SCN is considered as an endogenous circadian clock [46]. This nucleus receives afferents from the retina in order to synchronize the day-night cycle [46]. Lesions of the preoptic region produce insomnia, while lesions of the caudal hypothalamus produce somnolence, suggesting that sleep and arousal are regulated by different regions [47, 48]. It has been found that

circadian timing is controlled and maintained by many clock genes including orexin, γ-aminobutyric acid (GABA), NPY, serotonin, and melatonin [49-52].

Fluid homeostasis

Fluid homeostasis is regulated by OXT and AVP in the SON and PVN of the hypothalamus [53]. The inputs from osmoreceptors in the organum vasculosum laminae terminalis (OVLT) and mechanoreceptors in the pulmonary artery reach the hypothalamic integration centers to initiate drinking [54]. In addition, angiotensin II, which activates the neural circuit for drinking through its action on the subfornical organ, can activate the vasopressin system to reduce the loss of water through the kidney [55].

Thermogenesis and temperature control

Thermosensitive neurons in the PO-LH area have been found to be involved in regulation of body temperature and fever production [56]. These hypothalamic neurons modulate the thermogenesis through increasing or decreasing peripheral metabolism [57]. Studies have shown that cold exposure as well as ICV leptin injection in rats increase the body temperature by altering the VMH's output to brown adipose tissue (BAT) through the sympathetic nervous system (SNS) [58]. It has been demonstrated that the melanocortin system interacts with the hypothalamo-pituitary-thyroid (HPT) axis, and this interaction allows controlled energy balance by the regulation of both energy input and energy expenditure [59, 60].

1.1.5 Crosstalk with other CNS areas

The hypothalamus has intensive interactions with a variety of brain areas. For example, the cortical inputs innervate the LH [61]. The hypothalamus also receives projections from limbic regions such as hippocampus and amygdala [62]. Signals from the gastrointestinal (GI) tracts can get through the highly vascularized area postrema (AP) and bind to the receptors in NTS, which then relay the information to the hypothalamus via the parabrachial nucleus (PBN) [63, 64].

The main output of hypothalamic nuclei are directed to ME, posterior pituitary, brainstem, and spinal cord to influence autonomic functions [16]. Several hypothalamic cell groups also project to the amygdala, bed nucleus of the stria terminalis, and brainstem to regulate various neuroendocrine and behavioral responses [62].
The amygdala interacts with hypothalamic nuclei such as LH, and has been thought to be an important organ of feeding regulation [65]. The nucleus accumbens (NA) and the adjacent ventral pallidum is related to appetitive instrumental learning and has functional relation with LH [66, 67].

1.1.6 Feeding regulation: a hypothalamic focus

Feeding is a very complex process and involves integration of central and peripheral nervous system, the GI tract, and adipose tissue, as well as numerous hormonal and neurochemical signals. Among this integrative system, the hypothalamus plays a critical part [68]. Firstly, several hypothalamic nuclei are involved in feeding. For example, lesions of the LH can cause decreased food intake, while lesions of VMH increase feeding [69, 70]. Secondly, the hypothalamus contains receptors for the energy

status-related peripheral signals and specialized glucose-sensitive neurons in the ARC and VMH [71, 72]. Furthermore, a huge body of neuropeptides and neurotransmitters in the hypothalamus are related to feeding, for example, the POMC/CART, NPY/AGRP, GABA, MCH, TRH, dopamine, noradrenaline, and cannabinoid receptors [73, 74]. These biomarkers interact with each other, for example, there is functional relationship between the melanocortin and opioid systems which facilitate feeding regulation [75]. Also the first-order neuropeptides in ARC influence the second-order neuropeptides in other hypothalamic nuclei such as LH and PVN. For example, NPY from the ARC binds to the Y1 and Y5 receptors in the PVN and down-regulates the CRH secretion, while POMC binds to MC-3R and MC-4R and up-regulates the TRH expression in the PVN [23, 24]. In the LH, NPY increases the expression of both orexin and MCH [76].

While it is widely accepted that adiposity signals such as leptin and insulin act on the hypothalamus and exert a long-term regulatory effects on feeding, the gut peptides are involved in the short-term regulation of feeding via NTS [77]. The gut peptides secreted from the GI tract include CCK, apolipoprotein, amylin, bombesin-like peptides, ghrelin, *etc* [78, 79]. These peptides, through vagal afferent terminal of splanchnic afferent nerve terminal, project to the NTS where they are integrated with descending hypothalamic input [64]. Thus, the short-term and long-term regulatory systems are functionally integrated. It is this delicate integrating system that allows an animal to maintain a relatively constant body weight over a long period of time.

1.2 CNTF and GIP

Ciliary neurotrophic factor (CNTF) was originally considered as a trophic factor for motor neurons in the ciliary ganglion and spinal cord [80, 81]. It is a cytokine that shares structural and functional properties with interleukin 6 (IL-6) and leukemia inhibitory factor (LIF). The observation that implantation of CNTF-secreting cells results in rapid wasting syndrome raises the possibility that it might be a powerful anorectic cytokine and might induce cachectic-like effects [81]. Like leptin, CNTF triggers similar intracellular JAK-STAT pathways to produce anorexia [82].

Glucose-dependent insulinotropic peptide (GIP), a 42 amino acid peptide, is released from endocrine cells in the small intestine [83]. It was initially named as gastric inhibitory polypeptide and is now considered to be a major incretin factor of the enteroinsular axis [84]. GIP receptors (GIPRs) belong to the seven transmembrane G protein-coupled receptor family and are distributed both in peripheral organs and in the CNS [85]. GIP regulates lipid metabolism and storage [86]. It has an anabolic effect on bone, and GIP treatment to ovariectomized mice prevents bone loss [87]. GIP now is considered to be a potential pharmaceutical target for the treatment of diabetes, obesity, and other metabolic syndromes [88, 89]. The widespread distribution of GIPR in the CNS and high binding affinity of GIP to the brain [85, 90, 91], together with the observation that GIP can regulate the release of hormones from pituitary and adrenal glands, imply that GIP might regulate the HPA axis through binding to the GIPR in the hypothalamus [92, 93]. Also recently, GIP was found expressed in the adult brain [94]. Although the role of GIPRs in the brain is unknown, given that many of the GIPR cell populations and regions are part of the limbic system, this receptor and its potential

endogenous ligand may be involved with memory, emotion, and vegetative functions [85].

1.3 Real time RT-PCR

1.3.1 Introduction

Polymerase chain reaction (PCR) is a DNA amplification method that has wide applications in biological sciences [95]. In PCR, the DNA polymerase and a pair of primers complementary to the DNA sequence enable logarithmic amplification of the DNA template [96]. Real time RT-PCR is a PCR method that combines with reverse transcription (RT) to convert mRNA into complementary cDNA, enabling quantitation of gene expression in the tissues [97]. The method is called the real-time version of quantitative PCR because the DNA is quantified after each round of amplification [98].

Traditional mRNA detection methods, such as northern blotting, RNase protection assay, and *in situ* hybridization, measure genes one by one with high sensitivity [99, 100]. Meanwhile, high throughput methods such as SAGE, differential display, and microarray, measure thousands of genes simultaneously with compromised sensitivity and resolution [101-104]. Recent development of novel chemistries and instrumentation platforms has led to the wide application of real-time RT-PCR for quantitating mRNA changes [105]. Real-time RT-PCR has become the confirmatory method for validating results obtained from microarray analyses and other techniques that measure gene expression changes on a global scale [106].

In the general procedures of the real-time RT-PCR, RNA is first extracted from the particular tissues and used to synthesize cDNA by a RT reaction. The target gene is

then amplified and detected by the real-time PCR [107]. The relative quantity values, which have been normalized to a housekeeping gene (HKG), are used for statistical analysis to study the fold changes of genes under the given treatment. Real time RT-PCR can detect multiple mRNAs in small tissue or even a single cell in a very short period of time [107]. This method is very precise, accurate, and reproducible as it collects quantitative data at the constant, exponential phase of amplification [108, 109].

1.3.2 Principle of real time RT-PCR

There are four different real time RT-PCR chemistries: TaqMan® (Applied Biosystems), Molecular Beacons, Scorpions®, and SYBR® Green (Molecular Probes) [110]. All of these methods depend on fluorescence resonance energy transfer (FRET) to generate the fluorescence signal, and allow detection of multiple DNA species because fluorescent dyes are attached to the different probes [111]. The TaqMan fluorogenic detection system can monitor PCR using a dual-labeled fluorogenic hybridization probe [111]. The Molecular Beacons and Scorpions form a stem-loop structure, while SYBR Green will bind to any double-stranded DNA in the reaction [112].

The TaqMan assay is simple and sensitive for quantitative analysis of mRNA abundance [113]. TaqMan probes are designed to have a fluorescent reporter dye attached to the 5' end and a quencher dye to the 3' end. These probes can hybridize to an internal region of a PCR product. TaqMan probes depend on the 5'- nuclease activity of the DNA polymerase used for PCR to hydrolyze a hybridized sequence [112]. Primers are designed as spanning the exon-exon junction, thus preventing detection of genomic DNA and providing specificity [114]. If there is no DNA template in the sample, the

probe will keep intact. Since the proximity of the fluorescence and the quench dyes prevents the detection of fluorescent signals, there will be FRET and no fluorescence emission will be observed. If the DNA template exists, the polymerase will cleave the probe during PCR. Thus, the two fluorescent dyes will be separated and FRET no longer occurs [115, 116]. The fluorescence increases in each cycle and is in direct proportion to the amount of cDNA amplified. Thus, the more cDNA input, the less PCR cycles would be needed to reach the fluorescence detection threshold.

1.3.3 Quantitation and validation

Two methods are used to quantify the real time PCR results: the standard curve method and the relative threshold method. The standard curve method can measure the absolute amount of mRNA by constructing a standard curve from RNA of known concentration [117]. This curve is then used as a reference standard to obtain quantitative data for mRNA target in the sample. The relative threshold method uses relative quantification (RQ) values to measure mRNA abundance across different samples, using an HKG for sample normalization [118]. Results are expressed as ratios of mRNA abundance of the target gene to mRNA abundance of the HKG. The C_t values of both the control and the samples of interest are normalized to an appropriate endogenous HKG. The relative C_t method is also known as the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$. Here, $\Delta C_{t,sample}$ is the C_t value for any sample normalized to the endogenous HKG and $\Delta C_{t,\,reference}$ is the C_t value for the control normalized to the endogenous HKG [118].

To investigate the effects of peripheral hormones on CNS gene expression, the relative threshold method but not absolute standard curve method is preferred, because only the relative fold changes of individual genes but not the absolute mRNA copy itself will be of interest. The sequence detection system (SDS) software program allows production of threshold cycle (Ct) and RQ values showing gene expression levels compared between samples in different treatments, expressed as a fold difference between samples. To validate the real time PCR results, the system needs to be optimized to minimize the intra- and inter-assay variation [109]. It is very important to have an appropriate HKG, since the expression of these HKGs can be varied with different tissue, animal species, treatment, and age [119, 120]. With appropriate HKGs such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, cyclophilin, and 18S (ribosomal RNAs) as internal controls, differentiation of mRNA expression in tissues with different treatments can be compared [120, 121].

1.3.4 Applications

Real time RT-PCR has wide applications in molecular biological laboratories. In the brain, numerous investigations have been performed with real time RT-PCR to study the mRNA levels of interested biomarkers in specific brain regions with a given treatment or in a particular pathological condition [122, 123]. This method can be applied to model systems to measure responses to experimental stimuli and to gain insight into potential changes in protein level and function, thus better understanding biological processes. The peripheral hormones induce complicated biological events by

up-regulating or down-regulating the expression of some protein biomarkers (the products of messenger RNA) in the CNS. Since the amount of mRNA produced usually correlates with the amount of protein synthesized and the relative ease for mRNA detection, the hypothalamic mRNA profile can be measured to dissect the mechanism involved in the hormone-induced activity in the hypothalamus. The Taqman method, when integrated with the ABI Microfluidic cards with 384-well format, cansimultaneously measure gene expression of multiple biomarkers in a small piece of tissue in a very short period of time. Thus it provides a very sensitive, reliable, and high throughput way to investigate the hypothalamic involvement in the neuroendocrine circuits.

Chapter 2

THE BIOLOGY OF LEPTIN

[1] Duan, J. and C.A. Baile. To be submitted to *Peptides.*

2.1 Structure, expression, and functions of leptin

Leptin is a non-glycosylated protein with a molecular mass of 16 KD [124]. It is a cytokine-like hormone which is expressed primarily by adipose tissue, with low levels also detected in the skeletal muscles, placenta, and the brain [125]. Its molecular structure shows similarities with members of the IL-6 cytokine family, including IL-11, IL-12, CNTF, IL-6, and LIF [126]. The observations that normal rats lose weight when parabiosed with obese rats with VMH lesions, while the VMH-lesioned rats gain weight when parabiosed with lean rats suggested that the existence of a circulating satiety factor, which was later characterized as leptin [124]. In 1994, the mouse *ob* gene and its human homologue were cloned and sequenced [127]. The expression of leptin is up-regulated by a variety of factors such as glucocorticoids, acute infection, and pro-inflammatory cytokines, and is down-regulated by cold exposure, adrenergic stimulation, and GH [125].

Although many researchers view leptin as an anti-obesity hormone, it is suggested that leptin may function as an adaptive mechanism in an environment where food availability is limited [128]. Moreover, leptin plays a role in regulation of cardiovascular function, bone formation, anorexia, and apoptotic induction by mediation of the CNS [129-131]. Furthermore, leptin has a concomitant fluctuation with seasonal changes and is probably involved in seasonal control of body fat [128]. Taken together, leptin is not so much a body weight controlling peptide as a hormone with multiple functions on most of the body systems [132]. The profound behavioral and neuroendocrine effects of leptin on multiple systems illustrate the complex circuitry involved in leptin biology.

2.2 Leptin receptors and leptin resistance

Leptin receptors belong to the class I cytokine receptor superfamily [133]. There are full length, long form receptors (known as Ob-Rb) and several short forms (Ob-Rs, including Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Re), with the difference between the subtypes being the length of the intracellular domain [133]. All leptin receptor variants possess an identical 816 amino acid extracellular domain and a transmembrane domain of 23 amino acids [32]. The Ob-Re is a soluble receptor and lacks both transmembrane and intracellular domain [134]. Leptin receptors are widely expressed throughout the brain in areas such as the cortex, cerebellum, brainstem, basal ganglia, and hippocampus [124]. It is generally accepted that the hypothalamus is the critical action site of leptin [135-137]. The anatomic studies indicate that ob-Rb localizes to several hypothalamic nuclei such as ARC, DMN, and VMN where leptin receptors are co-expressed with several neuropeptides [138]. Extrahypothalamic leptin receptors have been described in the rodent and human brain including receptors within the cerebellum, thalamus, PBN, and NTS [139]. Although the role of these extrahypothalamic leptin receptors is unknown, the presence of leptin receptor mRNA in the meninges and the microcirculation implies that leptin receptors at one or all these sites are responsible for transporting leptin into or out of the CNS [139].

The *ob/ob* and *db/db* mice have genetically mutated forms of leptin and leptin receptors, respectively. In *ob/ob* mice, a missense leptin mutation (C^{105} to T^{105}) results in production of an abnormal protein, which is functionally inactive [140]. In *db/db* mice, a stop codon is inserted in the 3'-end of the Ob-Rb mRNA transcript and leads to the synthesis of Ob-Ra, which is insensitive to leptin [141]. In *fa/fa* rats, a Q^{269} to P^{269}

substitution in leptin receptors results in multiple functional abnormalities including a defective JAK/STAT signaling pathway [142, 143]. The fact that leptin levels are very high in obese animals suggests the presence of leptin resistance, which is thought to result from a number of potential mechanisms including transport saturation across the blood-brain barrier or the presence of SOCS3 [144, 145].

2.3 Feeding-related neuropeptides

Both peripheral and central leptin can regulate food intake and body weight by modulating the expressions of feeding-related neuropeptides in the hypothalamus. In the hypothalamic ARC, two important neuron groups are regulated by leptin: the neurons producing orexigenic NPY/AGRP and those producing anorectic POMC/CART [12, 146]. Leptin-induced inhibition of food intake results from both suppression of orexigenic and induction of anorexigenic neuropeptides [147]. For example, expression of NPY mRNA in the ARC is increased in *ob/ob* and *db/db* mice, and this increase is suppressed with exogenous leptin treatment [12, 146]. It has been found that leptin-induced NPY transactivation is mediated through the 221-bp region of the NPY gene promoter, which has two STAT3 binding sites [148].

2.4 Leptin and the reproductive axis

It has been found that leptin functions as an important link between nutritional state and reproductive capacity [149]. Leptin receptors have been identified within the ovary and testis [150-152]. Leptin plays a permissive role on pubertal maturation and later reproduction through action on the hypothalamus [153]. While *ob/ob* mice fail to

undergo puberty, leptin administration in normal mice at the time of weaning accelerates the onset of puberty [154]. Leptin stimulates GNRH release from hypothalamic explants along with FSH and luteinizing hormone release from the anterior pituitary of adult male rats in vitro [155]. ICV administration of leptin antiserum decreases pulsatility of luteinizing hormone and suppresses ovarian cyclic activity in female rats [156]. Furthermore, leptin therapy restores gonadotropin secretion, luteinizing hormone, and thyroid-stimulating hormone pulsatility [157, 158].

2.5 Interaction with other hormones

Leptin has widespread interactions with a variety of hormones. Leptin regulates GH secretion in rats by regulating hypothalamic GHRH gene expression [159]. GH has a positive effect on leptin secretion, while leptin has a direct negative effect on GH [160]. Leptin regulates the thyroid axis in that the TRH neurons in the PVH express leptin receptors, and the TRH promoter is probably a direct site of leptin action [161]. Leptin modulates adipocyte insulin signaling indirectly through neuroendocrine pathways and normalizes insulin-stimulated PI-3-K pathways [162, 163].

2.6 Energy metabolism

The SNS and adipose tissue form a feedback loop and leptin plays an important part in this regulation process [164]. It has been suggested that leptin affects the CNS to increase sympathetic outflow and affect fat cell energy utilization [165, 166]. Short-term administration of leptin into the cerebral ventricles increases renal sympathetic activity

through activation of hypothalamic melanocortin receptors [167, 168]. It has been found that leptin action is required for normal basal peroxisome proliferators-activated receptor (PPAR) expression in BAT in rodents [169]. The feedback regulation of leptin expression in BAT is primarily on SNS activity [170].

Leptin up-regulates expression of glucose transporter 4 (GLUT4) mRNA in white adipose tissue (WAT) [171]. ICV injection of leptin has extended effects on sustained energy expenditure [58]. Leptin induced β-adrenergic stimulation results in an increase in uncoupling protein-2 (UCP2) expression and adipose tissue thermogenesis [172]. UCP-2 mRNA expression in subcutaneous adipose tissue is linked to local plasma leptin level and may contribute to the pathogenesis of obesity [173], while UCP-3 is a mediator of thermogenesis which can be regulated by leptin [174].

2.7 Bone metabolism

Leptin modulates the reciprocal differentiation between osteoblastic and adipocyte pathways and inhibits adipogenesis in a negative feedback loop [175]. Leptin increases osteoblastic activity by inhibiting apoptosis in primary human osteoblast culture [130]. Leptin exerts its bone regulatory effects through receptor binding on VMH neurons, the autonomous nervous system, and β-adrenergic receptors on osteoblasts [176, 177]. The SNS is found to be the peripheral mediator of leptin's antiosteogenic function [129]. It has been found that leptin-triggered CART pathways were involved in this bone regulatory process [178].

2.8 Leptin signaling in the hypothalamus

The long form leptin receptor activates the JAK-STAT pathway and regulates expression of many hypothalamic neuropeptides, while the short receptor isoform may mediate leptin clearance in the kidney and transport leptin from the blood into the brain capillary endothelium [179]. While the short isoforms of the leptin receptor are not able to trigger signal transduction, Ob-Rb activates some immediate early genes like c-fos and c-jun in the hypothalamus [32]. Ob-Rb contains a highly conserved proline-rich motif which is thought to recruit JAKs and provide docking sites for downstream molecules [180]. The binding of leptin to its receptors activates the tyrosine phosphorylation of JAK2 and the subsequent phosphorylation of Tyr^{985} and Tyr^{1138} of leptin receptors. Phosphorylated Tyr^{1138} recruits the STAT3, allowing its dimerization and subsequent translocation into the nucleus, where it modulates expression of target genes [181]. In the nucleus, STAT3 also mediates gene transcription of SOCS3, which in turn suppresses leptin signaling by binding to the activated receptor complex and thus triggering degradation [180]. Phosphorylated Tyr^{985} recruits the phosphatase SHP-2 that mediates dephosphorylation and attenuation of leptin receptors [180, 182]. In addition to the JAK/STAT pathway, the MAPK pathway is also involved in leptin signaling via ob-Rb [34].

2.9 Leptin-induced adipocyte apoptosis

WAT is a major endocrine organ involved in a complex network influencing energy homeostasis, glucose metabolism, lipid metabolism, and immune response [125, 183]. Leptin is produced from WAT and in turn modulates adiposity [184]. The

observations that leptin induces a reduction of fat-pad weight in either systemic or central administration, together with the adipocyte apoptosis found in ICV leptin-treated rats, suggest that leptin injections not only reduce the cell size of the adipocytes, but also triggers some signaling pathways to induce adipocyte apoptosis [131]. This induced adipocyte apoptosis is responsible for the unexpected delay in return to initial energy status following leptin treatment [131]. It has been found that PPARγ may be directly involved in the leptin-induced adipocyte apoptotic signaling pathway, whereas UCP and TNFα may function in the leptin induced lipolytic process [185].

2.10 Leptin-induced inflammation and cachexia

The cytokine-CNS interactions are involved in many physiological and pathological processes [186]. Leptin is a sample of the overlapping molecules within the neuroendocrine system and the immune system [187]. Leptin deficiency increases susceptibility to infectious and inflammatory stimuli and is associated with dysregulation of cytokine production [188]. The administration of leptin to rats increases the level of IL-1β in the hypothalamus, while the leptin-induced effects on food intake and body temperature are abolished by administration of the IL-1 receptor antagonist (IL-1Ra) and are absent in IL-1 receptor-deficient mice [188]. The abnormalities in macrophages from *ob/ob* mice, which include increased expression of cyclooxygenase-2 (COX-2) and prostaglandin E synthase (PTGES), have suggested that these macrophage changes may be involved in obesity related pathophysiology [189]. Both COX-2 and PTGES are capable of reducing food intake as well as inducing the cancer anorexia-cachexia syndrome [190].

2.11 Leptin *vs*. CNTF: regulation of food intake

CNTF was originally considered as a trophic factor for motor neurons in the ciliary ganglion and spinal cord [80, 81]. It is a cytokine that shares structural and functional properties with IL-6 and LIF [191]. The observation that implantation of CNTF-secreting cells results in rapid wasting syndrome raises the possibility that CNTF might be a powerful anorectic cytokine [81]. CNTF shares many similarities with leptin on the regulation of food intake and induction of cachexia [192]. Both leptin and CNTF bind to their corresponding receptors at the medial ARC, involve suppression of NPYergic signaling in the hypothalamus, and trigger similar intracellular JAK-STAT signal transduction pathways [193]. Like leptin, CNTF also induces SOCS-3 mRNA expression [82]. It has been found that the Ob-Rb and CNTF receptors regulate cholinergic neurotransmitter and neuropeptide synthesis in a similar manner [194]. Although there is an overlap of the mechanisms by which leptin and CNTF regulate food intake, the action of these two hormones on the hypothalamus are likely to be divergent [195]. For example, CNTF has inflammatory properties distinct from leptin [86, 196, 197]. Moreover, CNTF reduces obesity-related phenotypes in *db/db* mice and diet-induced obese mice, which suggests that its effects are mediated by pathways different from those of leptin [86].

Although clinical trials have indicated that the potential application of leptin on the prevention of human obesity is not as promising as we previously thought, the studies on leptin have opened the door for understanding the mechanisms of food intake and energy metabolism, the interrelationship between nutrition and physiology, and the

molecular interactions among adipose tissue, immune system, and the CNS. A detailed understanding of the complexity of integrated neuronal circuits, particularly the hypothalamic pathways mediated by leptin actions, will help decipher the pathogenesis of obesity and other related disorders.

Chapter 3

COMPARATIVE EXPRESSION ANALYSIS OF MRNAS IN RATS BY REAL TIME RT-PCR IN THE HYPOTHALAMIC ARCUATE NUCLEUS FOLLOWING ICV INJECTION OF LEPTIN OR CNTF

[1] Duan, J., Duff, E., Choi, Y.H., Hartzell, D.L., Della-fera, M.A. and C.A. Baile. Submitted to *Peptides*, 09/30/2006.

Abstract

Leptin and CNTF mediate neuroendocrine responses and regulate food intake and energy balance. Both are cytokine-like hormones and act on their corresponding receptors in the hypothalamic ARC. However, it is unclear if these two cytokines engage similar CNS pathways to exert their effects. To assess the effects of ICV injection of leptin and CNTF on hypothalamic gene expression, real-time RT-PCR was used to quantitatively compare mRNA levels of selected genes in micropunched hypothalamic ARC samples from rats. Treatments included ICV injections of 10 μg/d leptin or 5 μg/d CNTF for four days. Leptin and CNTF significantly increased ARC mRNA levels of STAT3, SOCS3, CART, and POMC2. Leptin increased ARC mRNA level of GHRH, while CNTF increased JAK2 mRNA and reduced mRNA levels of AVP, GNRH1, MAPK1, OXT and TH. Our findings show that leptin and CNTF engaged both convergent and divergent pathways involved in feeding, cellular signaling, inflammation, and other biological regulatory systems. The gene expression profile in ARC following ICV leptin or CNTF administration helps to understand the pathogenesis of obesity.

Introduction

Leptin, a cytokine-like hormone produced primarily in adipocytes, functions as an afferent signal in a negative feedback loop for the regulation of food intake and body weight [198-201]. Leptin regulates directly and indirectly a series of complicated downstream pathways, both by down-regulating the orexigenic neuropeptides and by up-regulating the anorexigenic neuropeptides in the hypothalamus and extrahypothalamic regions [202-205]. Leptin is also involved in the mobilization of stored lipids, initiation

of energy expenditure, regulation of reproduction and inflammation, as well as mediation of adipocyte apoptosis [185, 206-212]. However, how these downstream factors interact with leptin and how they act in leptin-mediated processes in both physiological and pathological states are yet to be established.

Although a large number of neuropeptides have been found to be involved in leptin regulation, several lines of evidence indicate that NPY/AGRP and POMC/CART neurons in the ARC of the hypothalamus are the most important targets of leptin [20, 213-215]. ICV administration of leptin can suppress NPY/AGRP expression and increase POMC/CART expression in the hypothalamic ARC [195, 214, 215]. Leptin receptors and the neurons producing these neuropeptides are coexpressed in the ARC [216]. There is experimental evidence that leptin can activate the JAK-STAT signaling pathway, especially activating STAT3 via the long form of the leptin receptor in the hypothalamus [33, 217]. The SOCS3 is also likely to play a critical role in the negative feedback regulation of the hypothalamic leptin signaling system [180, 218].

CNTF, a member of the cytokine family that is structurally related to LIF, can down-regulate NPY expression and has leptin-like effects on food intake and body weight regulation [193, 219]. It has been suggested that the anorectic effects of CNTF may not be mediated by leptin because CNTF treatment rapidly reduced leptin secretion [220]. Thus, we hypothesized that leptin and CNTF might initiate both overlapping and divergent downstream pathways in the hypothalamic ARC. In this study, we employed real time RT-PCR with microfluidic cards on micropunched ARC samples to compare the effects of ICV injection of leptin and CNTF in rats on mRNA profiling patterns. We measured several potential downstream hypothalamic biomarkers that are involved in

food intake regulation, cellular signaling, inflammation, and a variety of other biological functions.

Materials and Methods

Animals

Male Sprague Dawley rats (250-274 g initial body weight) were purchased from Harlan, Inc. (Indianapolis, IN). They were individually housed in hanging plastic shoebox cages under constant temperature (22 ± 1ºC) and a 0600 h/ 1800 h light/dark cycle. They had free access to standard rat chow and tap water. All surgical and experimental procedures proposed were conducted in accordance with the NIH Guidelines and were approved by the Animal Care and Use Committee of The University of Georgia.

Treatment

All of the rats were surgically prepared with lateral ventricular cannulas for ICV administration of control and test agents as described previously [196]. Following recovery from cannulation surgery and angiotensin confirmation of proper placement of cannulas, twenty-four male Sprague Dawley rats were randomly divided into three groups. CNTF (Serological Inc., MA) and recombinant rat leptin (R&D Systems, Minneapolis, MN) were dissolved in an artificial cerebrospinal fluid (aCSF) which consisted of (in g/l): NaCl, 8.66; KCl, 0.224; $CaCl_2.2H_2O$, 0.206; $MgCl_2.6\ H_2O$, 0.163; $Na_2HPO_4.7\ H_2O$, 0.214; $NaH_2PO_4\ .H_2O$, 0.027. The CNTF and leptin solutions were divided into aliquots for daily injections and stored at -80ºC. Three treatments (Control:

aCSF 10 µl; Leptin: 10 µg/10 µl; CNTF: 5 µg /10 µl) were administered once daily for 4 successive days as ICV injections. The experiment was terminated approximately 24 h after the last treatment. Rats were then killed by CO_2 asphyxiation and decapitation [196].

RNA extraction

After decapitation, the brains were removed rapidly and immediately frozen by placing them on plastic cassettes on top of powdered dry ice. Once completely frozen, they were stored at -80ºC. The brains were thawed to about -5ºC before the sectioning. A micropunch technique was performed to dissect the hypothalamic ARC using blunted needles with a diameter of 0.75 mm (Fig. 1) [221, 222]. Tissue homogenization and total RNA isolation were performed according to the protocol from Invitrogen. Briefly, the dissected ARC tissues were homogenized in 1 ml of Trizol reagent using the power homogenizer. Then the homogenized samples were incubated for 5 min at room temperature and 0.2 ml of chloroform was added. The samples were centrifuged at 12000 × *g* for 15 min at 4ºC and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at 12000 × g for 10 min at 4ºC, the pellet was washed with 1 ml of 75% ethanol and then dissolved in RNase-free water. The integrity of the RNA produced from all samples used was verified and quantified using a RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA).

RT and real-time PCR

One hundred ng of total RNA in a 20 µl reaction was reverse transcribed using the cDNA Archive Kit (Applied Biosystems Inc., part #4322171, CA) according to the manufacturer's protocols using the MultiScribe™ Reverse Transcriptase. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (Taqman™) assays were chosen for the transcripts to be evaluated from Assays-On-Demand™ (ABI, CA), a pre-validated library of QPCR assays, and incorporated into 384-well MicroFluidic cards™. All of the oligonucleotide primer and fluorogenic probe sets for Taqman™ real time PCR were from ABI (Table 1). Two µl of the cDNA samples, along with 50 µl of 2× PCR master mix were loaded into respective channels on the microfluidic card followed by a brief centrifugation (3000 rpm for 3 min). The card was then sealed and real-time PCR and RQ was carried out on the ABI PRISM 7900 SDS system. The cycle conditions were: 94.5ºC for 15 min, followed by 40 cycles of 97ºC for 30 s, 59.7ºC for 1 min. Data were analyzed using SDS software and the RQ, which presents the fold difference of mRNA level in treatment groups relative to the aCSF control group. mRNA expression was normalized by using PPIA as an endogenous control to correct the differences in the amount of total RNA added to each reaction. The ΔC_T values were first calculated by using C_T for a specific gene mRNA minus C_T for PPIA mRNA in the sample. Then the mean mRNA expressions from the treatment groups were compared with the aCSF control group using the formula: $RQ = 2^{-\Delta\Delta CT}$ ($\Delta\Delta C_T$ is the average aCSF control group ΔC_T values minus the average experimental group ΔC_T values and $\Delta\Delta C_T$ of 1 equates to a twofold difference in cDNA added into the PCR reaction).

Statistical Analysis

Data are means ± SEM of RQ values from the SDS file for all the genes. Statistical significance was assessed by general linear model analysis of variance (ANOVA) for multiple comparisons between the means for the different treatment groups.

Results

mRNA expression of ARC signaling molecules (Figure 3)

The mRNA level of 18 S did not change with either leptin or CNTF treatment when compared to the aCSF control group. ICV administration of leptin resulted in a 259% increase in SOCS3 mRNA expression ($P < 0.001$), while ICV CNTF induced an approximate 10-fold increase (1064%) in SOCS3 ($P < 0.001$). There was a significant difference between leptin and CNTF groups in SOCS3 mRNA expression ($P < 0.001$). Thus, although both leptin and CNTF induce hypothalamic SOCS3 mRNA expression, CNTF appeared to be much more potent in this respect. Both ICV leptin and CNTF treated rats showed a significant increase in STAT3 mRNA expression by 65% and 125%, respectively (both $P < 0.001$). The mRNA expression of TH was decreased by 75% in CNTF treated rats ($P < 0.05$), but the decrease in leptin treated rats was not significant. JAK2 mRNA was increased by 32% ($P < 0.01$), while MAPK1 was down-regulated by 19% in CNTF treated rats ($P < 0.05$). There were no significant changes in mRNA of JAK2 and MAPK1 following leptin treatment, and CREB1 mRNA level did not change with either leptin or CNTF treatment.

mRNA expression of ARC molecules regulating food intake (Figure. 4)

Hypothalamic POMC2 mRNA expression was significantly increased in leptin and CNTF treated groups by 374 and 264%, respectively (both $P < 0.001$). There were also significant increases in another anorexigenic neuropeptide, CART, in both leptin and CNTF treated groups by 103 and 123%, respectively (both $P < 0.001$). NPY was up-regulated with leptin treatment by 115% ($P < 0.001$) and was unchanged with CNTF treatment. No significant differences in mRNA levels of CCK and GABRD were observed in hypothalamic ARC with leptin or CNTF treatment.

mRNA expression of ARC molecules involved in cachexia (Figure. 5)

CNTF increased the mRNA level of TNF by 43% and leptin treatment increased the mRNA levels of both PTGES and TNF by 56% and 40% respectively, but none of these changes were significantly different.

mRNA expression of ARC molecules involved in other functions (Figure 6)

ICV CNTF treatment down-regulated the mRNA level of AVP by 73% ($P < 0.05$), OXT by 76% ($P < 0.01$), and GNRH1 by 60% ($P < 0.01$). Leptin treatment increased GHRH mRNA (310%, $P < 0.001$) and there was a trend for an increase in the CNTF treatment groups (112%, $P = 0.06$). There were no significant changes in mRNA expression of CALM2, HCRT, SCT, TRH, and UCN3 after ICV treatment of either leptin or CNTF.

Discussion

The hypothalamus is a primary CNS site that receives central and peripheral stimuli and integrates the information for the regulation of feeding, energy homeostasis, and a variety of other biological and physiological functions [223, 224]. In this study, we focused on ARC, which has been recognized as a key target site for leptin and CNTF's actions in the hypothalamus [80, 225, 226]. Other physiological responses to the leptin and CNTF treatments of the rats used for this study have been previously reported by Duff, *et al.* (Fig. 2) [196]. Briefly, it was shown that while CNTF had a stronger effect than leptin on the reduction of cumulative food intake, both leptin and CNTF reduced body weight and adipose tissue mass in rats [196]. In this study, we used real time RT-PCR with microfluidic cards to investigate how ICV administration of leptin and CNTF regulate gene expression of a series of biomarkers in hypothalamic ARC. We found that leptin and CNTF induced both convergent and distinct patterns of gene expression in the ARC of the rat hypothalamus.

Binding of leptin to Ob-Rb activates the JAK-STAT pathway, which in turn can be inhibited by SOCS3, a crucial negative regulator of cytokine signaling and a candidate for involvement in leptin resistance [180, 227]. The ObRb exhibits structural similarities with the CNTF receptor [195, 228]. In agreement with the previous investigations, our results show that mRNA levels of SOCS3 and STAT3 in rat hypothalamic ARC are significantly up-regulated with ICV treatment of leptin or CNTF. The significant reduction of mRNAs of TH and MAPK1 as well as increase of JAK2 with CNTF treatment but not with leptin treatment suggests that, although partially overlapping, CNTF induced signaling transduction pathways differ from those of leptin.

There is considerable evidence that the effects of leptin on feeding and energy metabolism are mediated by inhibition of the orexigenic peptides and stimulation of the anorexigenic peptides [20, 229]. Consistent with these findings, our study indicated that the mRNA expressions of CART and POMC (the precursor molecule of anorexigenic peptide α-MSH) in hypothalamic ARC were up-regulated by either leptin or CNTF treatment. It was surprising that NPY was up-regulated at the mRNA level in our study. We think that the different dosage and treatment duration might be responsible for this apparent disparity between our results and the previous findings on mRNA level of NPY induced by leptin or CNTF. We speculate that, in our study, the energy depletion by continuous ICV injection of high dosage of leptin or CNTF might initiate a potentiated food intake response to compensate for the energy loss. Thus, the hypothalamus might employ a compensatory mechanism to resist the over reduction of body weight. Since NPY is the most powerful anabolic factor, it might be the first candidate to be employed in this compensatory mechanism, although we do not know why POMC does not go down as part of this compensatory mechanism. It is possible that following the continuous ICV injection, the hypothalamic ARC, in which a series of first order neuropeptide producing neurons are located, might receive negative feedback information from the other hypothalamic nuclei such as LH or PVN, given the extensive synaptic pathways and projections between these regions. Taken together, the above observations strongly suggest that leptin and CNTF exerted similar influences on hypothalamic neurons involved in regulation of food intake and body weight. The redundant pathways and compensatory feeding mechanism imply that simultaneous blocking of actions of

multiple hormones and pathways are needed to obtain desirable effects for the prevention and therapy of obesity.

There is much evidence that shows strong relationships between leptin and the immune system [230, 231]. PTGES is capable of reducing food intake and inducing the cancer anorexia-cachexia syndrome, and has been known as the major target for antipyretic therapy [232]. The increased expression of PTGES in macrophages from *ob/ob* mice have suggested that it might be involved in obesity related pathophysiology [232]. TNF has been found to be involved in leptin induced adipose tissue apoptosis and inhibition of adipose cell differentiation in vitro [233, 234]. CNTF administration has been shown to induce the expression of COX-2, which is related to PTGES in the febrile process [197, 235, 236]. In this study, there was a trend for mRNA of PTGES to be up-regulated in the leptin treated group, but the increase was not statistically significant.

Both AVP and OXT are implicated in the central control of lipolysis through the sympathetic nervous system outflow from brain to WATs, and the release of both hormones is under direct regulation of the hypothalamic ARC [237]. An *in vitro* study on rat SON has shown that long-term treatment of CNTF significantly reduced the apoptosis and increased the survival of both OXT and AVP producing neurons [238]. In our study, ICV CNTF treatment significantly reduced the expression of AVP and OXT in the rat hypothalamic ARC. The *in vitro* studies suggested that both leptin and CNTF stimulate GNRH release from hypothalamic explant harvested from proestrous female rats [239]. Long-term food restriction in the male rats reduced secretion of luteinizing hormone without altering the expression of GNRH [240]. In this study, ICV CNTF treatment decreased GNRH1 mRNA levels to nearly one fourth of the controls. It has been found

that ICV injection of NPY suppressed the pulsatile release of luteinizing hormone in the rhesus monkey [241]. This raises the possibility that the up-regulated NPY following ICV leptin or CNTF administration may have blunted the up-regulation of GNRH1 in leptin treated groups or even down-regulated the mRNA of GNRH in ICV CNTF groups.

Previous studies indicated that leptin up-regulates the GH responses at the pituitary level [242]. The increased mRNA level of GHRH in hypothalamic ARC with leptin treatment in this study suggested that this up-regulatory effect might be mediated by changing the gene expression of hypothalamic GHRH. HCRT is known as a weak orexigenic peptide and interacts with hypothalamic NPY and POMC [243, 244]. The fact that its expression was unchanged in both treatments suggests that HCRT responds differently from NPY to leptin or CNTF stimulus.

When studying the mRNA expression in brain, the animal species, genotype, experimental conditions, dosage of agents, duration of treatment, method of administration (peripherally or centrally), and *in vivo* vs *in vitro* methods are all possible confounding factors that might contribute to the discrepancies of the results from different studies. All these factors have to be taken into consideration when comparing results from different investigations. Also, we have to be very cautious to interpret the physiological relevance with our real time PCR results, because we cannot extrapolate our mRNA findings to protein expression levels for the complicated post-translational modifications.

In summary, our study showed that real time RT-PCR, when combined with microfluidic cards, provides a very convenient and reliable way to profile the multiple mRNA changes in rat hypothalamic ARC in a quantitative manner. Our results

demonstrated that ICV treatment of leptin or CNTF induced both overlapping and divergent pathways in the hypothalamic ARC in rats. The hypothalamic mRNA profile obtained in this study helps decipher the pathogenesis of obesity.

Table 1. Probes used in real time PCR

Gene Symbol	Gene Name	Probe Sequence
18 S	18 S	CCATTGGAGGGCAAGTCTGGTGCCA
AVP	arginine vasopressin	CATGGAGCTGAGACAGTGTCTCCCC
CALM2	calmodulin 2	CTACGAAGAGTTTGTACAAATGATG
CART	cocaine and amphetamine regulated transcript	AGAAGGAGCTGCCAAGGCGGCAACT
CCK	cholecystokinin	AGGTCCGCAAAGCTCCCTCTGGCCG
CREB1	cAMP responsive element binding protein 1	GTCTAATGAAGAACAGGGAAGCAGC
GABRD	gamma-aminobutyric acid A receptor, delta	ACCATGGCGCCAGAGCAATGAATGA
GHRH	growth hormone releasing hormone	GGAGAGCATCTTGCAGGGATTCCCA
GNRH1	gonadotropin-releasing hormone 1	TTTCCAAGAGATGGGCAAGGAGGAG
HCRT	hypocretin	TCTACAAAGGTTCCCTGGGCCGCCG
JAK2	janus kinase 2	CTCCCGGGAAGGCCGATGTTCTGAA
MAPK1	mitogen activated protein kinase 1	TTAAATTGGTCAGGACAAGGGCTCA
NPY	neuropeptide Y	CAGCCCGCCCGCCATGATGCTAGGT
OXT	oxytocin	GCTGTAGCCCGGATGGCTGCCGCAC
POMC2	proopiomelanocortin, beta (endorphin, beta)	GCAACCTGCTGGCTTGCATCCGGGC
PPIA	peptidylprolyl isomerase A	AGGATTCATGTGCCAGGGTGGTGAC
PTGES	prostaglandin E synthase	GCTGCCTCAGAGCCCACCGCAACGA
SCT	secretin	GGGGAAGCGCAGCGAGGAGGACACA
SOCS3	suppressor of cytokine signaling 3	CCGGAGCACGCAGCCAGTGCCCCGC
STAT3	signal transducer and activator of transcription 3	AGCTGACCCAGGTAGTGCTGCCCCT
TH	tyrosine hydroxylase	AAGGACAAGCTCAGGAACTATGCCT
TNF	tumor necrosis factor	CGTAGCCCACGTCGTAGCAAACCAC
TRH	thyrotropin releasing hormone	ACCTCGGTGCTGCCTTAGACTCCTG
UCN3	urocortin 3	GGCCTCCTGCGGATCTTACTGGAAC

Fig. 1. Dissection of hypothalamic arcuate nucleus.[221]

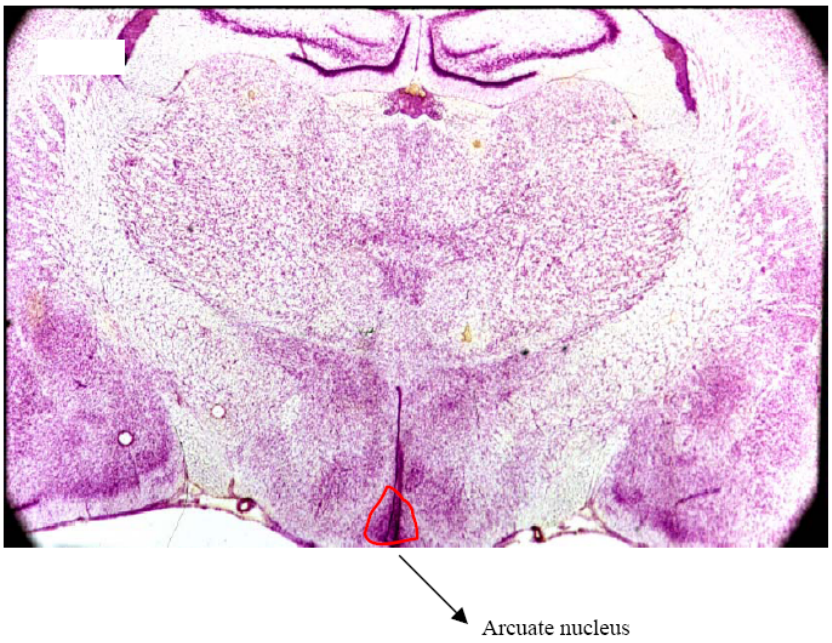


Fig. 2. Physiological changes in rats after 4 daily ICV injections of aCSF, leptin or CNTF. Within each parameter, means that don't share a letter in common are different. a, b—$p < 0.05$.

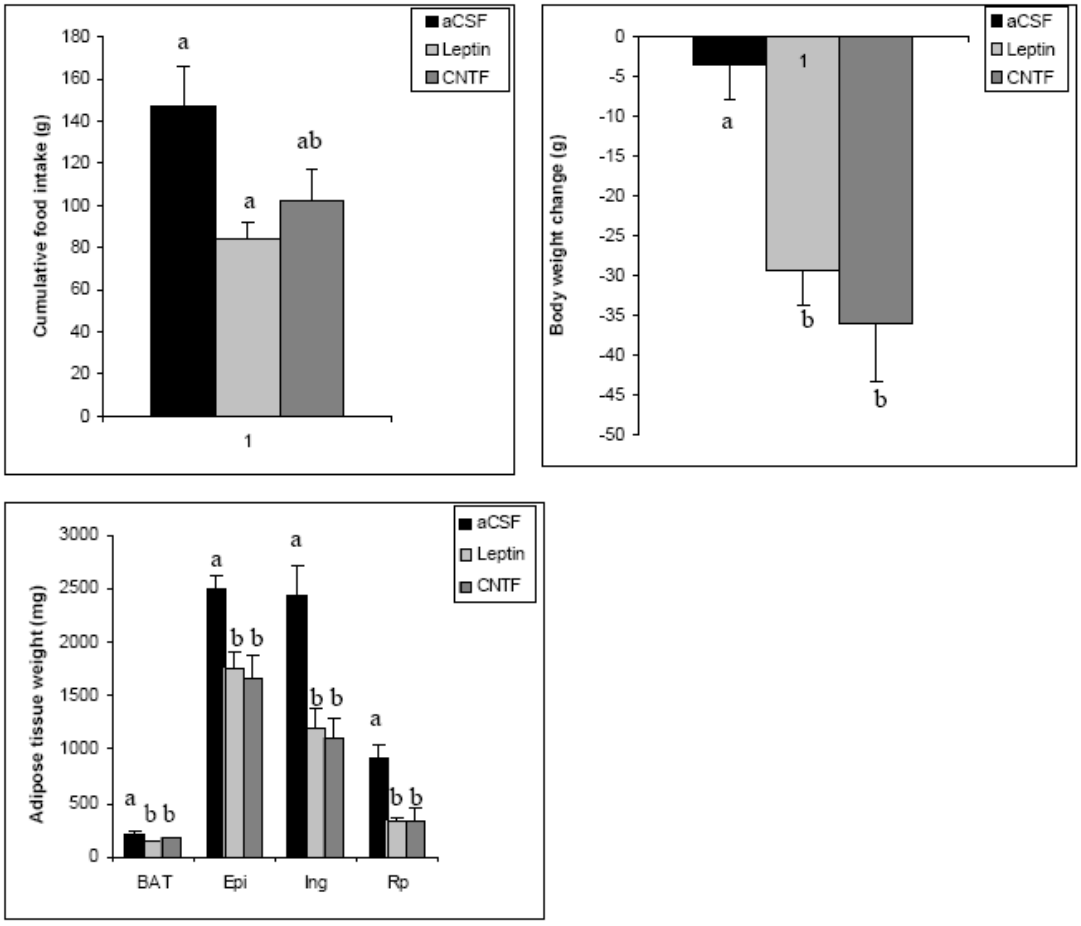


Fig. 3. mRNA expression in hypothalamic arcuate nucleus of rats after 4 daily ICV injections of aCSF, leptin or CNTF. Data are expressed as means ± SEM for each group normalized to the PPIA values and then expressed as a % change from the aCSF-treated group. Within each gene, means that don't share a letter in common are different. a, b, c—$p < 0.05$; x, y, z—$p < 0.01$.

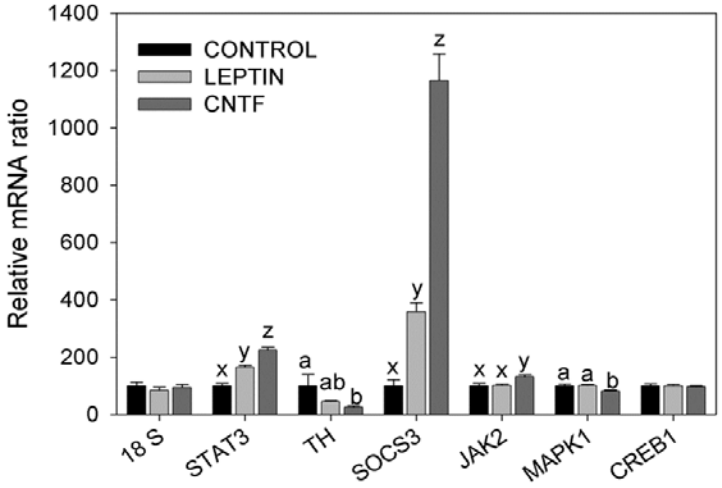


Fig. 4. mRNA expression in hypothalamic arcuate nucleus of rats after 4 daily ICV injections of aCSF, leptin or CNTF. Data are expressed as means ± SEM for each group normalized to the PPIA values and then expressed as a % change from the aCSF-treated group. Within each gene, means that don't share a letter in common are different. a, b, c—$p < 0.05$; x, y, z—$p < 0.01$.

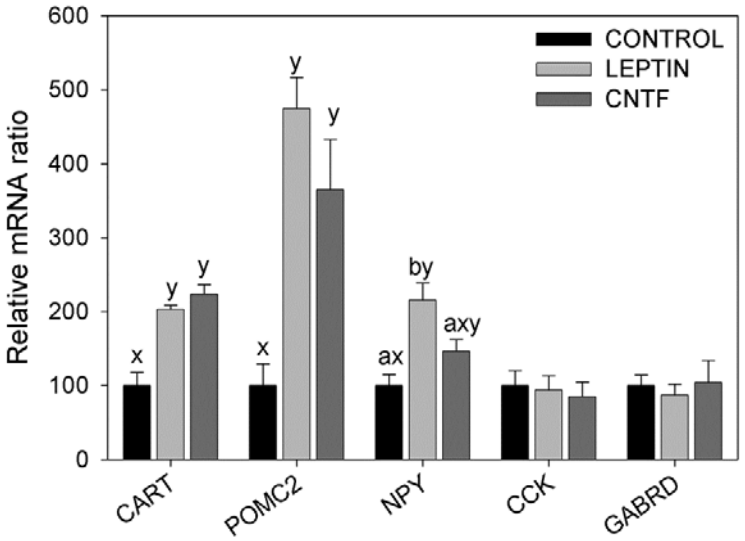


Fig. 5. mRNA expression in hypothalamic arcuate nucleus of rats after 4 daily ICV injection of aCSF, leptin or CNTF. Data are expressed as means ± SEM for each group normalized to the PPIA values and then expressed as a % change from the aCSF-treated group. Within each gene, means that don't share a letter in common are different. a, b—$p < 0.05$.

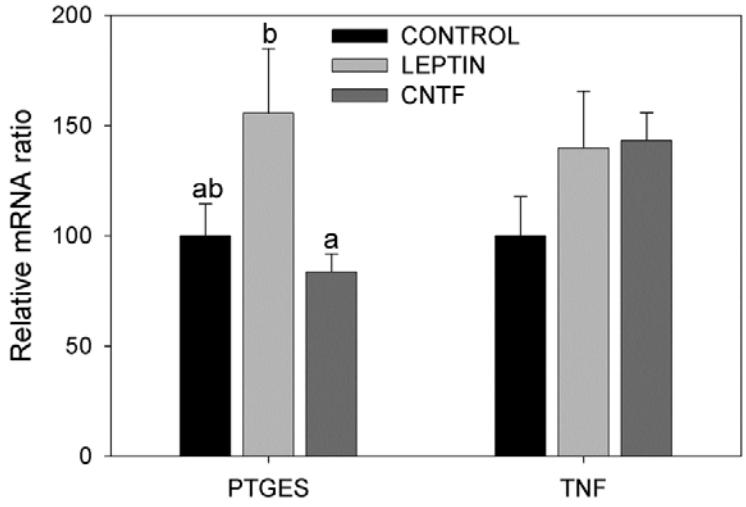
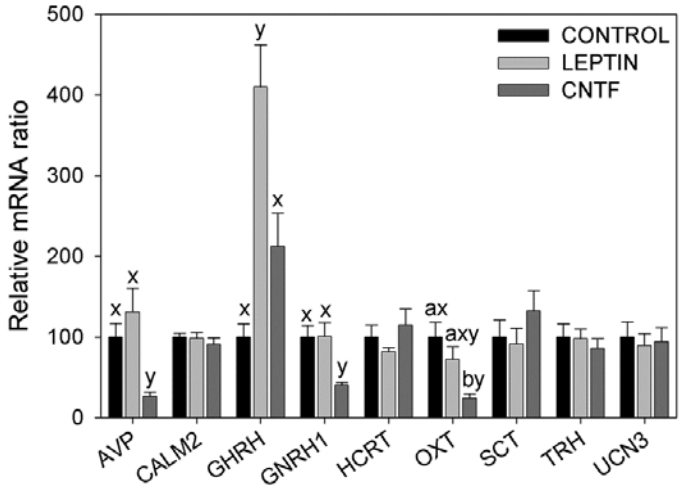


Fig. 6. mRNA expression in hypothalamic arcuate nucleus of rats after 4 daily ICV injections of aCSF, leptin or CNTF. Data are expressed as means ± SEM for each group normalized to the PPIA values and then expressed as a % change from the aCSF-treated group. Within each gene, means that don't share a letter in common are different. a, b, c—$p < 0.05$; x, y, z—$p < 0.01$.



Chapter 4

SUBCUTANEOUS LEPTIN INJECTIONS ON HYPOTHALAMIC GENE PROFILES IN LEAN AND *OB/OB* MICE

[1] Duan J., Choi Y.H., Hartzell D.L., Della-Fera M.A., Hamrick M.W., C.A. Baile. Submitted to *Obesity Research,* 10/13/2006.

Abstract

Leptin-deficient *ob/ob* mice are more sensitive to exogenous leptin than lean mice and leptin treatment normalizes many of the phenotypic characteristics of *ob/ob* mice. The primary objective of this experiment was to investigate whether this altered leptin sensitivity in *ob/ob* mice was reflected in the hypothalamic mRNA profile. Fifteen-week-old female *ob/ob* and lean mice were treated with 14 days of subcutaneous (sc) infusions of PBS or leptin (10 μg/day) using osmotic pumps. Real-time Taqman™ RT-PCR (ABI Microfluidic cards) was used to quantitatively compare the mRNA levels of selected hypothalamic genes in these groups. Hypothalamic mRNA levels for *ob/ob* control mice were higher for AGRP, NPY and AVP, and lower for CART, CREB1, POMC1, and UCN3 compared to lean controls. In leptin-treated *ob/ob* mice, hypothalamic mRNA levels were reduced for NPY, AGRP, AVP, and increased for SOCS3 compared to *ob/ob* controls. Leptin treatment dramatically up-regulated hypothalamic mRNA levels of POMC1 in both lean and *ob/ob* mice. Leptin did not change levels of hypothalamic PTGES, TNF, OXT, SCT, VIP, GABRD, TH, MAPK1, or STAT3 mRNA in either genotype. Strong correlations were observed between hypothalamic JAK2 and CREB1, STAT3 and CREB1, JAK2 and STAT3, NPY and AVP in all samples. *ob/ob* and lean mice have different hypothalamic mRNA expression patterns (particularly those of feeding-related genes), and selected genes in *ob/ob* mice are more sensitive to exogenous leptin stimulation compared to lean mice.

Introduction

Leptin, a cytokine-like hormone produced primarily in adipose tissue, plays a very important role in the regulation of feeding and energy homeostasis [199, 201]. Leptin modulates feeding primarily by down-regulating the orexigenic neuropeptides, NPY/AGRP, and by up-regulating the anorexigenic neuropeptides POMC/CART in the ARC of the hypothalamus and hindbrain regions [203-205]. Leptin initiates a series of complicated signaling pathways, primarily the JAK-STAT pathway [245]. The SOCS3 is likely to play a critical role in the negative feedback regulation of the hypothalamic leptin signaling system [218]. Leptin is also involved in mobilization of stored lipid and mediation of adipocyte apoptosis, and all of these functions are directly or indirectly mediated by the hypothalamus [185, 212].

The *ob/ob* mice, with a point mutation of the leptin gene, are genetically leptin deficient. It is hypothesized that the lack of functional circulating leptin in *ob/ob* mice might up-regulate hypothalamic leptin receptors [246]. Moreover, *ob/ob* mice are more responsive to leptin than lean mice in reducing feeding, body weight, serum insulin, and glucose levels [247]. Furthermore, prolonged leptin treatment reduces leptin uptake in the central nervous system and induces the development of leptin resistance, in which SOCS3 might be involved [218, 247]. Thus, we hypothesized that, given widespread distribution of leptin receptors in the hypothalamus, lack of leptin signaling in *ob/ob* mice might be associated with an alteration of hypothalamic gene profile patterns, and *ob/ob* mice might respond differently to leptin than lean mice. In this study, we treated both

lean and *ob/ob* mice with 14 days of sc leptin injection. We dissected the mouse hypothalami and extracted the total RNA. Then we employed real time RT-PCR with microfluidic cards to investigate the effects of sc administration of leptin in lean or *ob/ob* mice on a series of potential biomarkers, whose mRNA expressions in the hypothalamus are critical in regulation of feeding, cellular signaling, inflammation, and several other biological functions.

Methods and procedures

Animals and leptin administration

Fifteen-week old female obese *ob/ob* (strain B6.V-Lep^{ob}, n = 16) and wild type lean mice (n = 16) were purchased from Jackson Laboratories, Inc. Mice were initially housed individually in suspended cages in a room with a 12h/12h light/dark cycle, 22 ± 1ºC ambient temperature, and 50% humidity. They were provided with food and water *ad libitum* throughout the study. All mice were surgically implanted with osmotic minipumps (Alzet Corp., Cupertino, CA Model 1002, 0.25 µl/hr) for sc infusion of treatment solutions. Mice were anesthetized briefly with 0.5% oxygen/isofluorane and the prefilled and primed pumps were inserted into a subcutaneous pocket as described previously [248]. Then the 16 mice within each genotype were randomly divided into two groups treated with either PBS solution or recombinant mouse leptin (R&D Systems, Minneapolis, MN) at a dose of 10 µg/day by sc infusion for 14 continuous days. Thus, there were 8 mice in each group (control/lean: lean mice sc treated with PBS; control/obob: *ob/ob* mice sc treated with PBS; leptin/lean: lean mice sc treated with leptin; leptin/obob: *ob/ob* mice sc treated with leptin). Mice were killed by decapitation

after sedation in a CO_2 chamber at the end of the 14th day of injection [248]. All of the animals and surgical procedures in this study were approved by the Animal Care and Use Committee of The University of Georgia.

RNA extraction

The brains were removed rapidly after decapitation and immediately frozen by placing them on plastic cassettes on top of powdered dry ice. Once completely frozen, they were stored at -80ºC. The brains were thawed to -5ºC before the hypothalamic dissection (Fig. 1). Tissue homogenization and total RNA extraction were performed according to the protocol from Invitrogen. Briefly, each dissected hypothalamic block was homogenized in 1 ml of Trizol reagent using the power homogenizer. Then the homogenized samples were incubated for 5 min at room temperature and 0.2 ml of chloroform was added. The samples were centrifuged at 12,000 × *g* for 15 min at 4ºC and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at 12,000 × *g* for 10 min at 4ºC, the pellet was washed with 1 ml of 75% ethanol and then dissolved in RNase-free water. The integrity of the RNA produced from all samples was verified and quantified using a RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA).

RT and real-time PCR

One hundred ng of total RNA in a 20 µl reaction was reverse transcribed using the cDNA Archive Kit (Applied Biosystems Inc., part #4322171, CA) according to the

manufacturer's protocols. Reactions were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (Taqman™) assays were performed using 384-well MicroFluidic cards™ on the ABI PRISM 7900 Sequence Detection System. All of the oligonucleotide primer and fluorogenic probe sets for Taqman™ real time PCR were made by ABI (Table 1). The cycle conditions were: 94.5ºC for 15 min, followed by 40 cycles of 97ºC for 30 s, 59.7ºC for 1 min. mRNA expressions were normalized by using β-ACT as an endogenous control to correct the differences in the amount of total RNA added to each reaction. The RQ values from each gene were used to compare the hypothalamic gene expression of all the groups.

Statistical Analysis

Data were expressed as means ± SEM of RQ values from each SDS file for all the genes. Statistical significance was assessed by a general linear model two-way ANOVA to determine the possible effects of genotype and leptin treatment. The one-way ANOVA with posthoc statistical procedures were also performed even when the two-way ANOVA did not show an interaction between genotype and leptin treatment, as the large biological variation observed in some genes distorted significant treatment effects. Multivariate analyses between genes were also performed with Pearson correlation.

Results

The genes we detected fell into different categories according to their reported physiological and cellular functions in the hypothalamus (Table 3). These candidate genes have been reported as being involved in feeding and body weight regulation,

cellular signal transduction, and also various other activities such as temperature and fluid balance. Overall, our results indicated that the major hypothalamic mRNA expression difference between *ob/ob* and lean control mice was the divergent gene profile for those biomarkers involved in feeding regulation. While leptin dramatically modified hypothalamic mRNAs of these feeding-related genes in both genotypes, leptin altered hypothalamic expression of only some of the other genes assayed (Table 3).

mRNA expression of hypothalamic signaling molecules involved in leptin pathways in lean and ob/ob mice (Fig. 2)

The results showed similar gene expression patterns of the selected signaling molecules between the *ob/ob* and lean control mice except for CREB1. Leptin injections increased only the hypothalamic mRNA level of FOS by 27% in lean mice ($P < 0.01$) and mRNA level of SOCS3 by 32% in *ob/ob* mice ($P < 0.05$). In *ob/ob* control mice, the hypothalamic mRNA level of CREB1 was lower by 24% than that of lean control mice ($P < 0.01$). There was no effect of leptin treatment on mRNA levels of JAK2, CREB1, TH, STAT3, and MAPK1.

Strong positive correlations were observed between CREB1, JAK2 and STAT3 in all of the treatment combinations. The correlation coefficient was 0.92 between JAK2 and CREB1 mRNA levels; 0.75 between STAT3 and CREB1 mRNA levels; and 0.84 between JAK2 and STAT3 mRNA levels (Fig. 6 a-c).

mRNA expression of hypothalamic molecules regulating food intake and body weight in lean and ob/ob mice (Fig. 3)

The data indicate that for *ob/ob* control mice, the hypothalamic mRNA levels of anorectic neuropeptides were lower and mRNA levels of orexigenic neuropeptides were higher when compared to those of lean controls. Leptin treatment increased the hypothalamic mRNA levels of anorectic neuropeptides and reduced mRNA levels of orexigenic neuropeptides in *ob/ob* mice, but it modified only some of these markers in lean mice. For hypothalamic POMC1, there were effects of treatment ($P < 0.01$) and genotype ($P < 0.05$), and an interaction between these two factors ($P < 0.05$). The hypothalamic POMC1 mRNA level was lower in *ob/ob* control mice by 54% compared to lean control mice ($P < 0.01$). It was dramatically up-regulated by leptin treatment in both *ob/ob* and lean mice (about 8 and 135 fold increase in lean and *ob/ob* mice, respectively, both $P < 0.01$). The hypothalamic CART mRNA level in *ob/ob* control mice was lower by 16% than in lean control mice ($P < 0.05$); sc leptin treatment increased hypothalamic CART mRNA in *ob/ob* ($P < 0.05$) but not in lean mice. For hypothalamic NPY, there were effects of treatment ($P < 0.01$) and genotype ($P < 0.01$) as well as an interaction between the two factors ($P < 0.01$). The hypothalamic NPY mRNA level in *ob/ob* control mice was higher by 65% than in lean control mice. While NPY was not changed in lean mice with sc leptin administration, its mRNA level was down-regulated by 26% in leptin-treated *ob/ob* mice ($P < 0.01$). For hypothalamic AGRP, there were effects of treatment ($P < 0.01$) and genotype ($P < 0.01$) as well as an interaction ($P < 0.01$). The hypothalamic AGRP mRNA level in *ob/ob* control mice was higher (by 387%) than in lean control mice. The hypothalamic AGRP mRNA level was down-regulated by 45% in leptin-treated *ob/ob* mice ($P < 0.01$), but was up-regulated by 28% in

leptin-treated lean mice ($P < 0.05$). No effects of treatment and genotype were observed for hypothalamic mRNA level of GABRD.

mRNA expression of hypothalamic molecules regulating inflammation and cachexia in lean and ob/ob mice (Fig. 4)

Leptin had no effect on the hypothalamic mRNA levels of two cachectic factors, TNF and PTGES. For both TNF and PTGES, there were no differences in the hypothalamic mRNA levels between lean and *ob/ob* control mice, and leptin did not alter the hypothalamic mRNA levels of these two cytokines in either mouse genotype.

mRNA expression of hypothalamic molecules regulating other biological functions in lean and ob/ob mice (Fig. 5)

The results showed that *ob/ob* control mice have a higher hypothalamic mRNA level of AVP and lower level of UCN3 when compared to their lean counterparts. Of all the five genes in this category, leptin-induced hypothalamic mRNA change was only observed with AVP in *ob/ob* mice. The data showed that there were genotype effects on the hypothalamic mRNA level of AVP ($P < 0.01$), and a trend for a treatment effect ($P = 0.07$) as well as a genotype x treatment interaction ($P < 0.01$). While the hypothalamic AVP mRNA level was higher in *ob/ob* control mice than in lean control mice by 96% ($P < 0.01$), sc leptin treatment dramatically reduced its mRNA level by 32% in *ob/ob* mice ($P < 0.01$) but not in lean mice. Strong positive correlation ($r = 0.83$) was observed between NPY and AVP mRNA levels (Fig. 6 d). Although the hypothalamic mRNA level of UCN3 was lower in *ob/ob* control mice than in lean control mice by 23% ($P <$

0.01), no leptin treatment effect was observed. There were no changes in expression of OXT, SCT, and VIP between the two genotypes, and sc leptin treatment did not change the mRNA expression of these genes.

Discussion

The widespread presence of leptin receptor in the hypothalamus, a center for the regulation of energy metabolism and various other biological activities, suggests the hypothalamic involvement in the leptin-induced physiological functions [224]. Therefore, *ob/ob* mice, with a mutated *ob* gene and the resulting lack of leptin signaling, might have an altered hypothalamic gene profile, and respond differently than lean mice when subjected to exogenous leptin stimulation. Our previous studies have shown that leptin decreased serum insulin level and dramatically increased serum leptin level in both *ob/ob* and lean mice, and *ob/ob* mice had higher overall sensitivity to leptin than lean mice on feeding and body weight (Table 2) [248]. In this study, we speculate that similar phenomena might also occur in the hypothalamic gene expression. Our data not only confirm the previous reports showing a clear difference between *ob/ob* and lean mice on mRNA levels of some hypothalamic neuropeptides such as NPY and CART, but show some novel findings as well. We found that there were differences between two genotypes on mRNA levels of hypothalamic AVP, CREB1 and UCN3. We found that not only do *ob/ob* mice have different hypothalamic gene patterns, but also that specific genes (e.g., CART, POMC, NPY and AVP) were more sensitive to the effects of leptin stimulation in *ob/ob* mice (Table 2). Also with leptin treatment, the up-regulated mRNA level of AGRP in lean mice and the extraordinary increase of mRNA level of POMC1 in

ob/ob mice suggest a delicate hypothalamic regulation in response to various energy conditions.

Leptin-induced hypothalamic JAK-STAT signaling has been intensively studied. STAT3 and Ob-Rb have been shown to be co-localized in many hypothalamic ARC neurons, and leptin increased hypothalamic STAT3 phosphorylation and up-regulated SOCS3 expression [245, 249]. In our study, hypothalamic mRNA levels of SOCS3 were significantly up-regulated with sc leptin treatment in *ob/ob* mice, but not in their lean counterparts. STAT3 mRNA levels in the ARC in *ob/ob* mice have been shown to be lower than in lean mice [250]. This difference, however, was not observed in our study, although our use of whole hypothalamus may have prevented us from detecting site-specific changes in STAT3 expression. The mRNAs of TH and MAPK1 were unchanged with leptin treatment in both lean and *ob/ob* mice. This is not unexpected since these signaling molecules might be involved in leptin signaling by phosphorylation of the protein rather than up-regulation of the gene. We cannot eliminate the possibility, however, that their expression changes could be blunted by leptin-induced negative feedback signals such as SOCS3 during the prolonged sc leptin treatment.

The cAMP response element binding protein (CREB) is a transcription factor and usually considered as a marker for neuron activation [251]. In our study, hypothalamic mRNA level of CREB1 in *ob/ob* control mice was lower than that in lean control mice, but its mRNA level was unaltered with sc leptin treatment in either genotype. The up-regulated hypothalamic mRNA level of another neuronal activation marker, c-FOS, in lean but not *ob/ob* mice indicated that neuronal activity was different between the two genotypes in response to leptin treatment. It has been found that there were regional

differences in c-FOS expression in the hypothalamus in response to leptin treatment [252]. Thus, the c-FOS difference observed in the whole hypothalamus in our study reflects only an average value that prevents us from measuring region-specific changes. Even though our results showed that leptin failed to alter the hypothalamic mRNA levels of JAK2, STAT3 and CREB1 in either genotype, the strong positive correlation between these molecules in all of the groups suggests that their expression might be closely interrelated under our experimental conditions. It must be stated that a high correlation may not necessarily mean definite causation, since the development of leptin resistance in *ob/ob* mice could distort any normal relationship between one biomarker and the other.

Leptin has been shown to induce febrile and inflammatory responses, and there are strong relationships between leptin and the immune system, although the mechanisms of leptin's involvement in inflammation and cachexia are still poorly understood [253]. In our study, sc leptin treatment had no effect on hypothalamic mRNA levels of two inflammatory factors, TNF and PTGES. Considering the redundant inflammatory pathways, it is quite possible that TNF and PTGES are not the only cytokines induced by leptin, and the lack of leptin signaling could induce alterations in other inflammatory molecules that might in turn modulate the expression of both cachectic molecules.

Both AVP and OXT in the hypothalamic ARC have been shown to be involved in the communication between brain and WAT [237]. In our study, the mRNA level of AVP in *ob/ob* control mice was significantly higher than that in lean control mice, and leptin dramatically decreased its mRNA level in *ob/ob* but not in lean mice. It has been shown that food-restriction in rats alter the circadian rhythms and the HPA axis, thus reducing AVP level in the blood or in the suprachiasmatic nucleus [254, 255]. Although

decreased plasma leptin levels and increased NPY levels in the hypothalamus have been reported in both food-restricted rats and *ob/ob* mice, there is an apparent disparity on AVP expression. We speculate that, in *ob/ob* mice, the intrinsic mutation of functional leptin might be followed by early disruption of a series of metabolic and endocrine factors, e.g. the increased hypothalamic AVP and NPY; while during food restriction, the change of functional leptin level and associated hypothalamic neuropeptides regulated by leptin might be temporary and dynamic. Other studies have demonstrated the anatomical relationship between nerve terminals containing NPY and AVP in the hypothalamus [256]. The strong correlation between hypothalamic NPY and AVP in this study supports these observations.

UCN has been found to regulate gastric emptying and reduce feeding as well as body weight in *ob/ob* mice [257]. In this study, although the hypothalamic mRNA level of UCN3 was higher in *ob/ob* control mice than lean control mice, its mRNA level was unchanged with leptin treatment in either genotype. Thus, UCN3, a weak anorectic peptide, may not be as important as POMC in the compensatory mechanism following prolonged leptin stimulation. There were no significant treatment or genotype effects on expression of SCT and VIP. Whether the use of whole hypothalamic tissue in our study resulted in dilution of site-specific differential expression changes of these molecules is unknown.

It is accepted that leptin regulates feeding by modulating the expression of orexigenic and anorectic peptides in the hypothalamus [201]. POMC is the precursor molecule of α-MSH and the latter is a primary hypothalamic anorectic neuropeptide [258]. In our study, leptin treatment drastically increased its level in both genotypes,

although the increase in *ob/ob* mice was more pronounced. In contrast, the mRNA level of another hypothalamic anorectic neuropeptide, CART, was increased only in *ob/ob,* but not in lean mice, with sc leptin treatment. Thus, although results from anatomical studies suggested that α-MSH and CART-producing neurons are morphologically co-localized [23], they may differ in sensitivity to leptin signaling. Previous studies have shown that CART was involved in leptin's regulation of bone resorption [178]. Our phenotypic data have shown that *ob/ob* mice have lower whole body mineral content (BMC) and body mineral density (BMD) and leptin increased BMC and BMD in *ob/ob* mice but provided no change on lean mice [259]. In this study, the decreased hypothalamic CART mRNA in *ob/ob* mice, together with the increased CART level with leptin treatment in *ob/ob* mice but not in lean mice supports its involvement in bone metabolism. It has been suggested that the reduction of GABA might be involved indirectly in leptin's effect on POMC expression [260]. In this study we measured the mRNA level of hypothalamic GABA receptor and found it was unchanged in either genotype with leptin treatment. Whether the dramatic up-regulation of POMC1 might in turn blunt the hypothalamic GABA receptor mRNA expression is unknown.

NPY and AGRP are the primary orexigenic neuropeptides in the hypothalamic arcuate nucleus, and it has been found that fasting increases the mRNA levels of both peptides in the hypothalamus [213]. In our study, the mRNA levels of hypothalamic NPY and AGRP were higher in *ob/ob* control mice than lean control mice. The mRNA level of NPY was significantly reduced in *ob/ob* mice with leptin treatment. This suggests that NPY neurons of *ob/ob* mice have increased sensitivity to leptin, and is in

agreement with the previous literature, which suggested an enhanced interaction between leptin and NPY signaling in *ob/ob* mice [261].

It has been hypothesized that in lean mice, prolonged leptin treatment might suppress endogenous leptin secretion and induce leptin resistance [262]. Also in lean mice, reduction in feeding and body weight that occurs with continuous leptin injection might stimulate hypothalamic orexigenic mechanisms to compensate for the energy loss. In our study, the increased hypothalamic mRNA level of orexigenic AGRP provides explanation for the unchanged body weight in lean mice with leptin treatment. In *ob/ob* mice the lack of leptin signaling results in increased expression of orexigenic and decreased expression of anorectic neuropeptides, and leptin treatment appears to correct this abnormality, resulting in reversal of obesity [263]. Since POMC is the most powerful catabolic factor, it would be the first candidate neuropeptide to be employed in the hypothalamus that helps normalize the food intake and body weight of *ob/ob* mice undergoing leptin treatment. In this study, we observed a strong positive correlation ($r = 0.68$, $P < 0.01$) between body weight loss and mRNA level of POMC (but not other feeding-related neuropeptides) in all the samples.

Previous studies have demonstrated that in *ob/ob* mice, the lack of leptin up-regulates expression of the leptin receptor and increases its binding affinity for leptin [246, 264]. It is possible that in lean mice leptin receptors in the hypothalamus are down-regulated in response to continuous leptin treatment, while in *ob/ob* mice the inherent absence of leptin and the corresponding up-regulation of leptin receptors may cause them to be very sensitive to exogenous leptin stimulation [246, 264]. Up-regulation of the hypothalamic leptin receptor has also been found during fasting [265]. Considering the

widespread localization of leptin receptors in the hypothalamus and various extra-hypothalamic regions, as well as the possible diversified regulation of gene expression in these different areas, further investigation, such as injections of leptin receptor inhibitors in specific areas like hypothalamic ARC, may help decipher the role of leptin receptors in depth.

In summary, our data demonstrate that *ob/ob* and lean mice have different hypothalamic mRNA expression patterns (particularly for those feeding-related genes), and selected genes in *ob/ob* mice are more sensitive to exogenous leptin stimulation compared to lean mice. Our results both confirm previous reports and establish a number of candidate hypothalamic biomarkers (e.g., AVP) whose changes in expression may warrant further study. The hypothalamic gene expression profiles we obtained may be useful in deciphering the pathogenesis of obesity and thus be of potential clinical importance for prevention or treatment of obesity.

Table 1. Probes used in real time PCR

Gene Symbol	Gene Name	Context Sequence
β-ACT	actin, beta, cytoplasmic	TACTGAGCTGCGTTTTACACCCTTT
AGRP	agouti related protein	CCCAGAGTTCCCAGGTCTAAGTCTG
AVP	arginine vasopressin	TGCAGCGACGAGAGCTGCGTGGCCG
CART	cocaine and amphetamine regulated transcript	CCACGAGAAGGAGCTGCCAAGGCGG
CREB1	cAMP responsive element binding protein 1	GGCCTTCCTACAGGAAAATTTTGAA
FOS	FBJ osteosarcoma oncogene	AACACACAGGACTTTTGCGCAGATC
GABRD	gamma-aminobutyric acid A receptor, delta	CCGCACCATGGCGCCAGGGCAATGA
JAK2	Janus kinase 2	TCCCTCCCGCGAAGGCCAATGTTCT
MAPK1	mitogen activated protein kinase 1	GCATGGTTTGCTCTGCTTATGATAA
NPY	neuropeptide Y	TCTCATCACCAGACAGAGATATGGC
OXT	oxytocin	CGCTGAGCCCACTTTCTGGGAATAC
POMC1	pro-opiomelanocortin-alpha	GCAACCTGCTGGCTTGCATCCGGGC
PTGES	prostaglandin E synthase	GAGCGCTGCCTCAGAGCCCACCGCA
SCT	secretin	CTACAGGACTGGCTTCTGCCCAGGC
SOCS3	suppressor of cytokine signaling 3	CCAGCGCCACTTCTTCACGTTGAGC
STAT3	signal transducer and activator of transcription 3	CACGGCAGCCCAGCAAGGGGGCCAG
TH	tyrosine hydroxylase	CTGTCACGTCCCCAAGGTTCATTGG
TNF	tumor necrosis factor	AAAGGGATGAGAAGTTCCCAAATGG
UCN3	urocortin 3	ACAAGCTGGAAGATGTGCCCTTGCT
VIP	vasoactive intestinal polypeptide	AAAGAGGAGCAGTGAGGGAGATTCT

Table 2. Physiological changes of lean and *ob/ob* mice treated with subcutaneous leptin

	Leptin treated *ob/ob* mice	Leptin treated lean mice
Body weight	↓↓ 34%	→
Physical activity	↑↑ 400%	↓ 13%
Total body fat	↓↓ 43%	→
Food intake	↓↓ 71%	↓ 34%

Table 3. Hypothalamic mRNA expressions of lean and *ob/ob* mice treated with subcutaneous leptin

	Gene	*ob/ob* vs. lean mice	Leptin treated lean mice	Leptin treated *ob/ob* mice
Signaling	CREB1	↓↓	→	→
	FOS	→	↑↑	→
	JAK2	→	→	→
	MAPK1	→	→	→
	SOCS3	→	→	↑
	STAT3	→	→	→
	TH	→	→	→
Feeding	AGRP	↑↑	↑	↓↓
	CART	↓	→	↑
	GABRD	→	→	→
	NPY	↑↑	→	↓↓
	POMC1	↓↓	↑↑	↑↑
Inflammation	PTGES	→	→	→
	TNF	→	→	→
Others functions	AVP	↑↑	→	↓↓
	OXT	→	→	→
	SCT	→	→	→
	UCN3	↓↓	→	→
	VIP	→	→	→

Fig. 1. Dissection of hypothalamus in mouse [221]

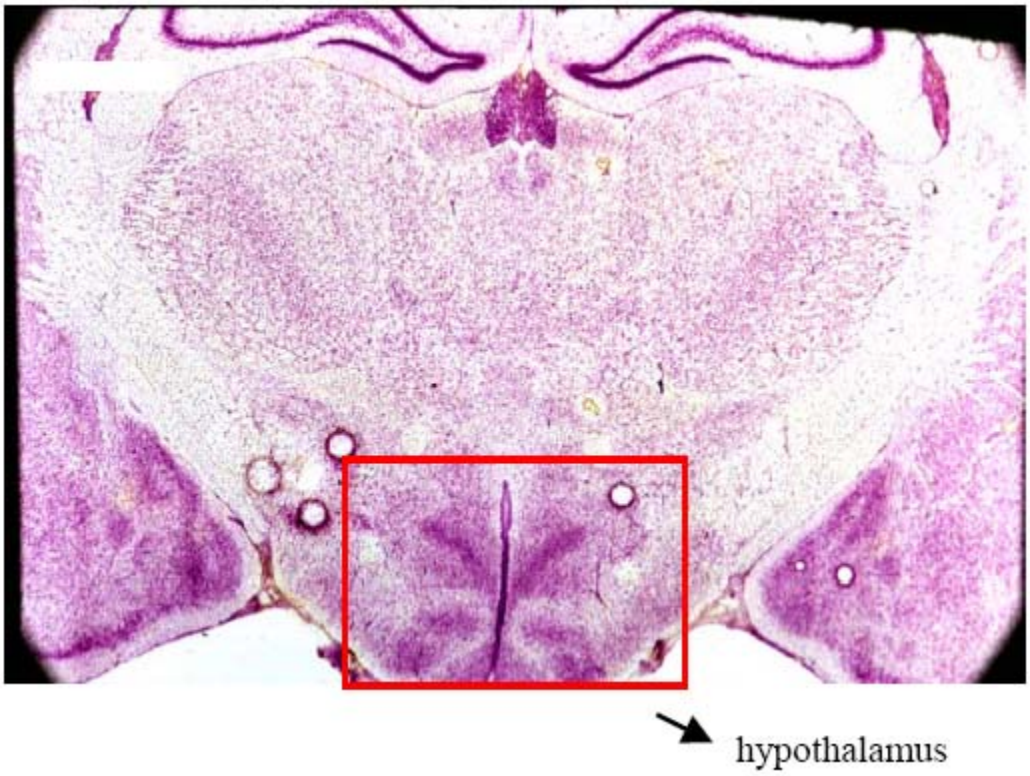


Fig. 2. Hypothalamic mRNA expression of *ob/ob* and lean mice after 14 days of subcutaneous (sc) leptin treatment (10 μg/day) as determined by real time RT-PCR. Data are expressed as means ± SEM for each group normalized to the β-ACT values and then expressed as RQ. Within each gene, bars denoted with a common letter are not different. a, b: $P < 0.05$; w, x: $P < 0.01$.

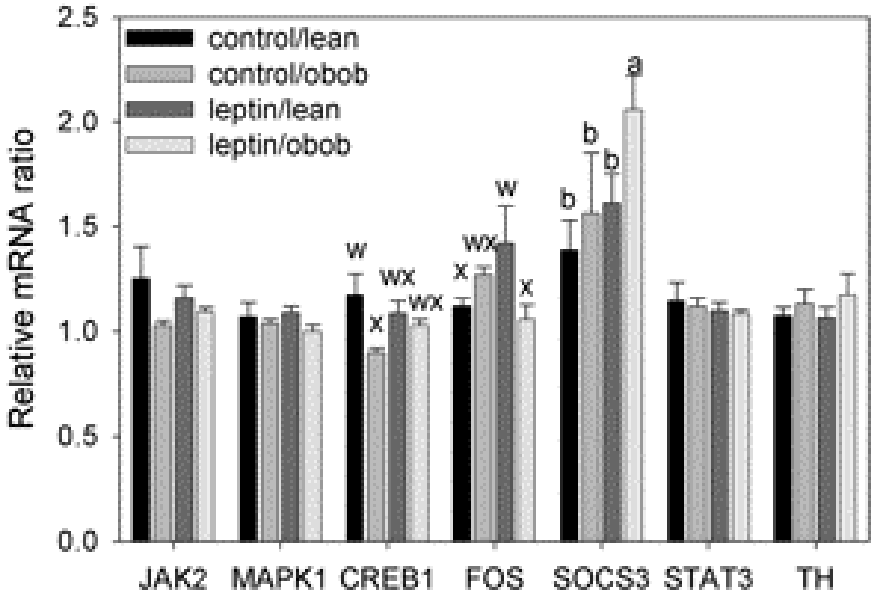


Fig. 3. Hypothalamic mRNA expression of *ob/ob* and lean mice after 14 days of subcutaneous (sc) leptin treatment (10 μg/day) as determined by real time RT-PCR. Data are expressed as means ± SEM for each group normalized to the β-ACT values and then expressed as RQ. Within each gene, bars denoted with a common letter are not different. a, b: $P < 0.05$; w, x, y, z: $P < 0.01$.

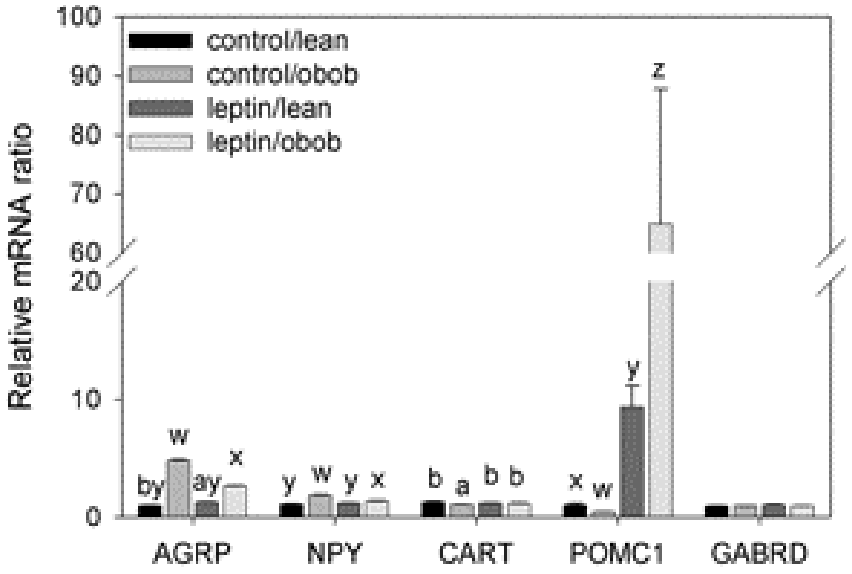


Fig. 4. Hypothalamic mRNA expression of *ob/ob* and lean mice after 14 days of subcutaneous (sc) leptin treatment (10 μg/day) as determined by real time RT-PCR. Data are expressed as means ± SEM for each group normalized to the β-ACT values and then expressed as RQ.

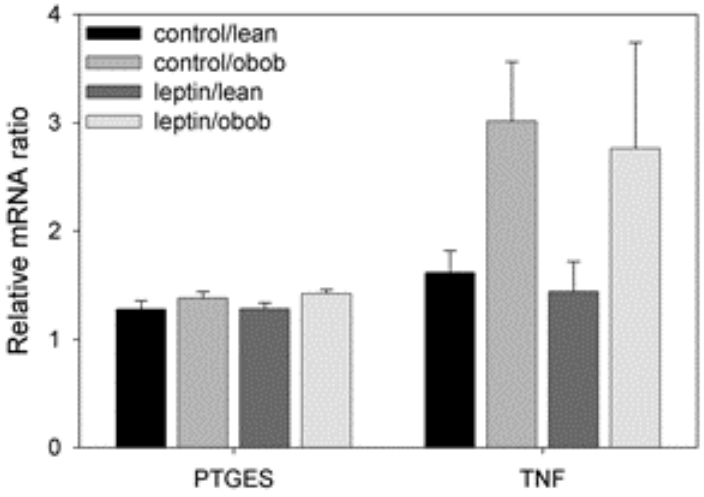


Fig. 5. Hypothalamic mRNA expression of *ob/ob* and lean mice after 14 days of subcutaneous (sc) leptin treatment (10 μg/day) as determined by real time RT-PCR. Data are expressed as means ± SEM for each group normalized to the β-ACT values and then expressed as RQ. Within each gene, bars denoted with a common letter are not different. w, x: $P < 0.01$.

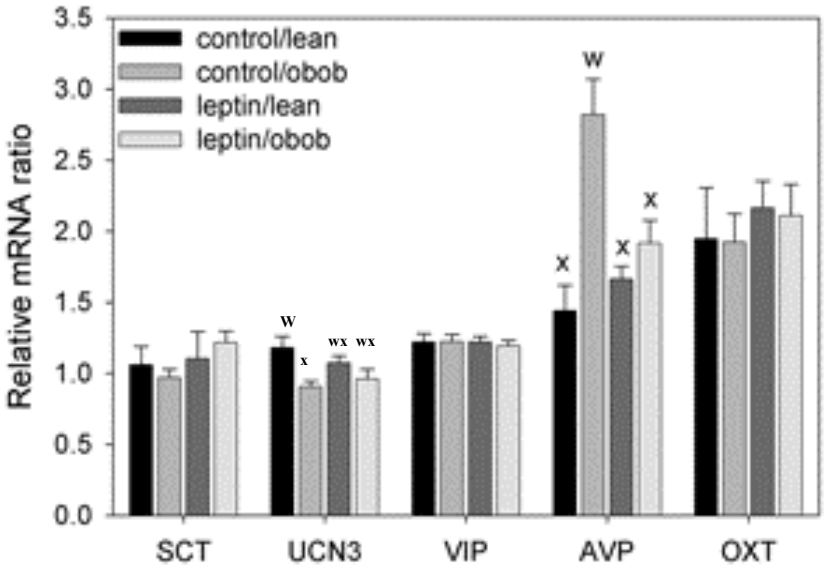
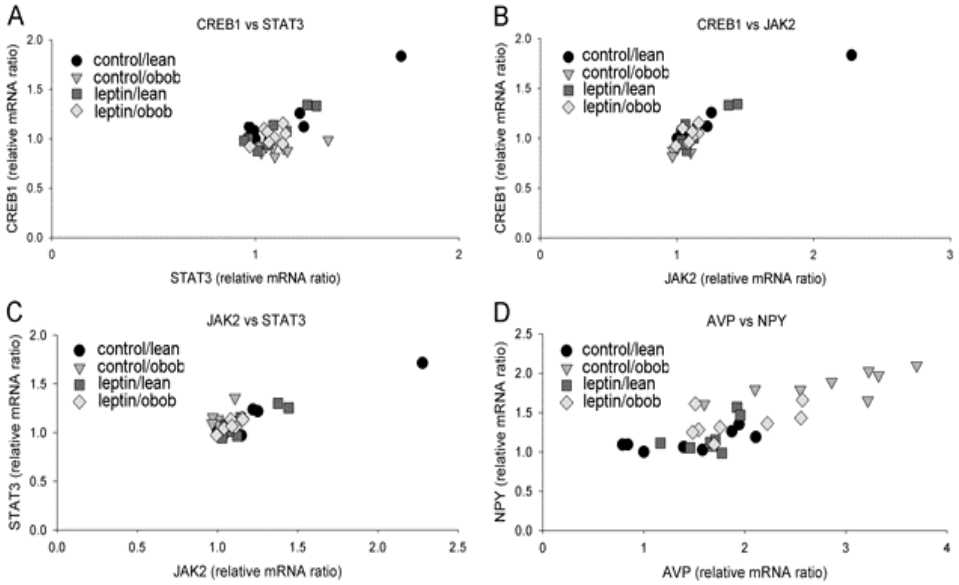


Fig. 6. Relationship between hypothalamic mRNA levels of (a): CREB1 and STAT3; (b): CREB1 and JAK2; (c): STAT3 and JAK2 and (d): NPY and AVP in lean and *ob/ob* mice with 14 days of subcutaneous leptin treatment.



Chapter 5

COMPARISON OF VMH AND ICV INJECTIONS OF LEPTIN ON GENE EXPRESSION IN VARIOUS HYPOTHALAMIC NUCLEI IN RATS

[1] Duan J., Hartzell D.L., Choi Y.H., Hamrick M.W., Della-Fera M.A. and C. A. Baile. To be submitted to *Peptides.*

Abstract

Leptin mediates neuroendocrine responses and contributes to the control of feeding and energy homeostasis. Leptin receptors are localized in specific areas of the hypothalamus, including the ARC and VMH. Both VMH and ICV leptin injections decrease food intake and body weight in rats. To compare the effects of VMH and ICV (lateral ventricle) injections of leptin on hypothalamic ARC gene expression, real-time Taqman RT-PCR (ABI Microfluidic cards) was used to quantitatively compare mRNA levels of selected genes in ARC samples from rats. Rats were implanted with unilateral right side VMH or ICV cannulas. Both VMH and ICV rats were randomly assigned to treatments of control (aCSF, 0.5 μl) or leptin (0.05 μg/injections) twice/day for 4 days. Total RNA was extracted from micropunched ARC, the right side of VMH (*r*VMH) and the left side of VMH (*l*VMH) and was used for real time RT-PCR to determine the mRNA levels of selected genes. RT-PCR results showed that VMH injections of leptin up-regulated the mRNA levels of TRH, and down-regulated the mRNA levels of JAK2, PTGES, SOCS3, STAT3, TH and TNF at the hypothalamic ARC. None of these mRNA changes were observed in the hypothalamic ARC of rats with ICV leptin injections. VMH injections of leptin induced similar reductions in mRNA levels of AVP, CART, SOCS3, OXT and PTGES in both sides of VMH, with the only exception being that TH was down-regulated in *r*VMH and up-regulated in *l*VMH. We conclude that the unilateral leptin exposure with VMH injections and the bilateral leptin exposure with ICV injections induce divergent physiological behaviors and hypothalamic ARC gene profiles, and TH may play an important role in the crosstalk between the two VMH sides.

Introduction

Leptin receptors are shown to be localized to specific areas of the hypothalamus, including the ARC and VMH [266]. Leptin administration induces nuclear STAT3 expression in the DMH, ARC and VMH, indicating that these areas have specific functional activity to leptin [249]. A number of studies involving intrahypothalamic injections of leptin or site-specific leptin gene therapy have been carried out to define the areas involved in leptin's effects on food intake, body weight and metabolism. Satoh *et al* showed that injections of leptin at 0.125 - 0.5 μg/site into the VMH, ARC or LH decreased food intake and body weight gain in a dose-dependent manner [267]. Another study showed that injections of leptin directly into the VMH of rats for 3 days (0.05 μg/day) reduced food intake and body weight, whereas the same dose administered either into the dorsal raphe nucleus or ICV had no effect [268].

Although the majority of recent studies investigating leptin action have focused on the ARC, leptin administration directly into VMH provides evidence supporting a role for this area in leptin-mediated suppression of food intake and stimulation of sympathetic nervous system. And recently it was found that leptin could directly activate steroidogenic factor-1 (SF1) neurons in the VMH and this action is required for normal body-weight homeostasis [269]. We hypothesized that a low dose of leptin that is effective in reducing food intake and body weight while injected into the VMH might have different effects when ICV administered leptin diffuses into the surrounding brain areas and accesses more than one action site. We also hypothesized that the unilateral leptin exposure with VMH injections and the bilateral leptin exposure with ICV

injections might induce divergent physiological behaviors and gene profiles in different hypothalamic nuclei. In this study, we investigated the effects of VMH and ICV administration of leptin on gene profiles in the hypothalamic ARC. We also compared the unilateral leptin VMH injections on the gene expression pattern of the bilateral VMH sides.

Material and Methods

Animals and treatments

Thirty-two male Sprague-Dawley rats (250 - 274 g) were purchased from Harlan, Inc. (Indianapolis, IN). They were individually housed in suspended polycarbonate cages under constant temperature (22 ± 1ºC) and a 0900 h/ 2100 h light/dark cycle. They had access to standard rat chow and tap water ad libitum throughout the study. Rats were implanted with ICV cannulas or unilaterally, right side VMH cannulas. After recovery from surgery, Both ICV and VMH rats were randomly assigned to treatments of control (aCSF, 0.5 µl/injections) or 0.05 µg/10 µl injections of leptin. Treatments were administered twice daily for 4 successive days. Recombinant rat leptin (R&D Systems, Minneapolis, MN) was dissolved in an artificial cerebrospinal fluid (aCSF) which consisted of (in g/l): NaCl, 8.66; KCl, 0.224; $CaCl_2.2H_2O$, 0.206; $MgCl_2.6\ H_2O$, 0.163; $Na_2HPO_4.7\ H_2O$, 0.214; $NaH_2PO_4\ .H_2O$, 0.027. All surgical and experimental procedures proposed were conducted in accordance with the NIH Guidelines and were approved by the Animal Care and Use Committee of The University of Georgia. The experiment was terminated approximately 24 h after the last treatments. On the day 5, rats were killed by CO_2 asphyxiation and decapitation [196].

RNA extraction

After decapitation, the brains were removed rapidly and immediately frozen by placing them on plastic cassettes on top of powdered dry ice. Once completely frozen, they were stored at -80ºC. The brains were thawed to -5ºC before the sectioning. A micropunch technique was performed to dissect the hypothalamic ARC and both sides of VMH using blunted needles with a diameter of 0.75 mm (Fig. 1) [222]. Tissue homogenization and total RNA isolation were performed according to the protocol from Invitrogen. The dissected hypothalamic nucleus tissues (weigh about 1 mg) were homogenized in 1 ml of Trizol reagent using a power homogenizer. The homogenized samples were incubated for 5 min at room temperature and 0.2 ml of chloroform was added. The samples were centrifuged at 12000 × g for 15 min at 4ºC and the aqueous phase was removed and mixed with 0.5 ml of isopropyl alcohol. After centrifugation at 12000 × g for 10 min at 4ºC, the pellet was washed with 1 ml of 75% ethanol and then dissolved in RNase-free water. The integrity of the RNA produced from all samples used was verified and quantified using a RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA).

RT and real-time PCR

One hundred ng of total RNA in a 20 µl reaction was reverse transcribed using the cDNA Archive Kit (Applied Biosystems Inc., part #4322171, CA) according to manufacturer's protocols using the MultiScribe^{TM} Reverse Transcriptase. Reactions were

incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (Taqman™) assays were chosen for the transcripts to be evaluated from Assays-On-Demand ™ (ABI, CA), a pre-validated library of QPCR assays and incorporated into 384-well MicroFluidic cards™. All of the oligonucleotide primer and fluorogenic probe sets for Taqman™ real time PCR were from ABI (Table 1). Two µl of the cDNA samples, along with 50 µl of 2× PCR master mix were loaded into respective channels on the microfluidic card followed by a brief centrifugation (3000 rpm for 3 min). The card was then sealed and real-time PCR and RQ was carried out on the ABI PRISM 7900 Sequence Detection System. The cycle conditions were: 94.5ºC for 15 min, followed by 40 cycles of 97ºC for 30 s, 59.7ºC for 1 min. Data were analyzed using SDS software (Applied Biosystems, CA) and the RQ, which represents the fold difference of mRNA level in treatment groups relative to the aCSF control group. mRNA expressions were normalized by using 18 S as an endogenous control to correct the differences in the amount of total RNA added to each reaction. The ΔC_T values were first calculated by using C_T for a specific gene mRNA minus C_T for 18 S mRNA in the sample. Then the mean mRNA expressions from the treatment groups were compared with the aCSF control group using the formula: $RQ = 2^{-\Delta\Delta CT}$ ($\Delta\Delta C_T$ is the average aCSF control group ΔC_T values minus the average experimental group ΔC_T values and $\Delta\Delta C_T$ of 1 equates to a twofold difference in starting amount of cDNA).

Statistical Analysis

Data are means ± SEM of RQ values from SDS file for all the genes. Statistical significance was assessed by ANOVA with post hoc tests for multiple comparisons between the different treatment groups.

Results

The real time RT-PCR results are shown in Table 2. In ARC regions, RT-PCR results showed that VMH injections of leptin up-regulated the hypothalamic ARC mRNA level of TRH by 58% ($p < 0.05$), and down-regulated the mRNA levels of JAK2 by 45% ($p < 0.05$), PTGES by 96% ($p < 0.05$), SOCS3 by 86% ($p < 0.05$), STAT3 by 47% ($p < 0.05$), TH by 51% ($p < 0.05$) and TNF by 46% ($p < 0.05$). But none of these mRNA changes were observed in rats with ICV leptin injections at the same dosage.

In the contralateral VMH regions, VMH injections of leptin induced similar reductions in mRNA levels of AVP, CART, SOCS3, OXT, PTGES in both sides of VMH, with the only exception being TH, which was down-regulated in *r*VMH but up-regulated in *l*VMH.

Discussion

Leptin plays a significant role in feeding and energy metabolism by acting as a signaling molecule to the brain, and by doing this it can cause region-specific changes in biomarker mRNA levels in the hypothalamic nuclei and affect several central and neuroendocrine functions. In this study, we injected low dosages of leptin ICV or VMH

to compare the effects of the two administration methods and the collateral effects of VMH injections on gene expression of hypothalamic biomarkers in some important hypothalamic nuclei such as ARC and VMH. Our physiological data showed that VMH but not ICV leptin injections at the 0.05 μg dose reduced epididymal fat mass by 21% ($p < 0.01$) and retroperitoneal fat mass by 41% ($p < 0.01$). VMH injected rats had much lower blood leptin (by 77%, $p < 0.05$) and insulin levels (by 49%, $p < 0.05$) compared to their ICV injected counterparts.

The present study demonstrates that microinjections of low dosages of leptin into the VMH but not ICV in rats dramatically alter the gene expression profiles in various hypothalamic regions. For ICV injections, leptin was likely distributed quickly throughout the ventricular space, and, thus, was exposed to the walls of the 3rd and 4th ventricle. There the leptin was either degraded or subjected to dispersion and removal by the flow of the CSF and then reabsorbed into bloodstream. Considering the dynamics of the CSF flow and the low leptin dosage being used, the possible effects induced by the penetration of leptin into the periventricular organs in the forebrain or hindbrain, and the peripheral leptin effects from blood reabsorption might be negligible because systemic administration of the same amount of leptin had no effect on in these peripheral tissues [270]. For the VMH injections, it is possible that leptin might diffuse beyond the nucleus and stimulate the other hypothalamic nuclei which also express the functional leptin receptor and thus are likely be responsive to leptin. In this study, we observed that VMH leptin injections induced drastic mRNA changes in a variety of biomarkers especially SOCS3 and PTGES. But the implication of these changes is still yet to be investigated.

Hypothalamic monoamines such as norepinephrine (NE) and dopamine (DA) play a critical role in many of leptin's central and neuroendocrine effects such as regulation of feeding behavior, sympathetic outflow, and alterations in the HPA axis. It also has been found that leptin administration decreases dopamine in PVN and NE concentration in the ARC, PVN [271]. Hay-Schmidt *et al* found that in many of the hypothalamic dopaminergic nuclei, most TH-positive cells co-localized with leptin receptors [272]. In this study, although we did not measure NE and dopamine directly, the reduction of TH (a marker for dopamine neurons) mRNA level might change the DA and NE, since TH is common for the synthesis of both NE and DA [271].

In this study, the differences of the mRNA changes between the ARC and VMH induced by VMH leptin injections suggest that discrete hypothalamic targets of leptin are differentially regulated. Both hormones in the hypothalamus have been shown to be involved in the communication between brain and adipose tissue through the SNS system [237]. In this study with VMH leptin injections, both hormones are unchanged in ARC but are down-regulated in both sides of VMH. These results support the previous findings which suggest that leptin's effects on adipose tissue are mediated by the VMH and are dependent on SNS output [273]. Both AVP and OXT-producing neurons are also co-localized with TH immunoactive neurons. It is interesting that reduction of mRNA level of TH in *r*VMH and its increase in *l*VMH suggested that TH might be very important in leptin's action. This divergent mRNA change of TH in the opposite VMH sides might also suggest its importance in the crosstalk between the two sides. Furthermore, although we did not measure leptin receptors in both sides of the VMH, it is possible that while the continuous VMH injections could cause the development of leptin

resistance and thus the down-regulation of leptin receptors in the injected VMH side. A compensatory mechanism might have developed at the collateral VMH side. Except with TH, we found that the trends of the changes of other selected hypothalamic biomarkers are very similar on both VMH sides with VMH leptin treatment, although for some genes, the relatively large variation might have been the cause of the insignificant differences.

The changes of some genes were associated with region-specific changes in leptin responsiveness. For example, while AVP, OXT and CART are unchanged in ARC with either ICV or VMH leptin injections, their mRNA levels are decreased in both VMH sides with VMH leptin injections. While it was expected that the dosage of ICV leptin injections might be too low to change the mRNA levels of NPY and POMC in ARC, it was interesting to see that the mRNA levels of these two hormones were also unchanged with VMH leptin injections which were effective on feeding and body weight regulation. It suggests that VMH leptin treatment might induce reduction of food intake by different mechanisms from that of ICV injection. The decrease but not increase of some genes (such as STAT3 and SOCS3) in ARC with VMH leptin injections also suggests this possibility. In addition, we cannot rule out the possibility that a negative feedback crosstalk might exist between hypothalamic ARC and VMH.

In summary, we conclude that the unilateral leptin exposure with VMH injections and the bilateral leptin exposure with ICV injections induce divergent hypothalamic ARC gene profiles. The divergent expression of TH at the different VMH sides implies its importance in the leptin responsiveness.

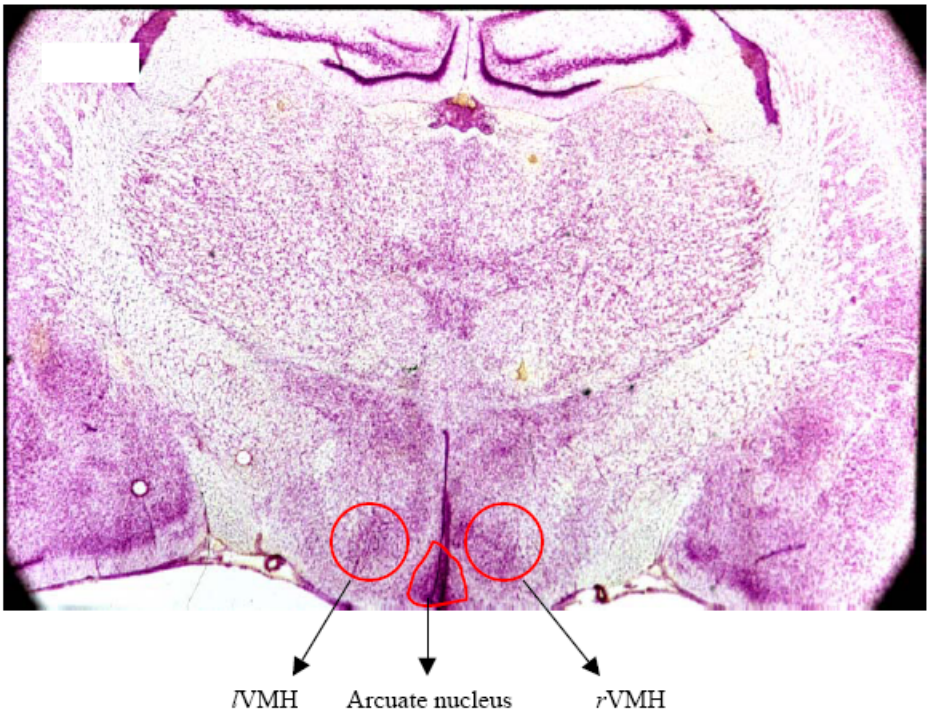
Table 1. Probes used in real time PCR

Gene Symbol	Gene Name	Probe Sequence
18 S	18 S	CCATTGGAGGGCAAGTCTGGTGCCA
AVP	arginine vasopressin	CATGGAGCTGAGACAGTGTCTCCCC
CALM2	calmodulin 2	CTACGAAGAGTTTGTACAAATGATG
CART	cocaine and amphetamine regulated transcript	AGAAGGAGCTGCCAAGGCGGCAACT
CCK	cholecystokinin	AGGTCCGCAAAGCTCCCTCTGGCCG
CREB1	cAMP responsive element binding protein 1	GTCTAATGAAGAACAGGGAAGCAGC
GABRD	gamma-aminobutyric acid A receptor, delta	ACCATGGCGCCAGAGCAATGAATGA
GHRH	growth hormone releasing hormone	GGAGAGCATCTTGCAGGGATTCCCA
GNRH1	gonadotropin-releasing hormone 1	TTTCCAAGAGATGGGCAAGGAGGAG
HCRT	hypocretin	TCTACAAAGGTTCCCTGGGCCGCCG
JAK2	janus kinase 2	CTCCCGGGAAGGCCGATGTTCTGAA
MAPK1	mitogen activated protein kinase 1	TTAAATTGGTCAGGACAAGGGCTCA
NPY	neuropeptide Y	CAGCCCGCCCGCCATGATGCTAGGT
OXT	oxytocin	GCTGTAGCCCGGATGGCTGCCGCAC
POMC2	proopiomelanocortin, beta (endorphin, beta)	GCAACCTGCTGGCTTGCATCCGGGC
PPIA	peptidylprolyl isomerase A	AGGATTCATGTGCCAGGGTGGTGAC
PTGES	prostaglandin E synthase	GCTGCCTCAGAGCCCACCGCAACGA
SCT	secretin	GGGGAAGCGCAGCGAGGAGGACACA
SOCS3	suppressor of cytokine signaling 3	CCGGAGCACGCAGCCAGTGCCCCGC
STAT3	signal transducer and activator of transcription 3	AGCTGACCCAGGTAGTGCTGCCCCT
TH	tyrosine hydroxylase	AAGGACAAGCTCAGGAACTATGCCT
TNF	tumor necrosis factor	CGTAGCCCACGTCGTAGCAAACCAC
TRH	thyrotropin releasing hormone	ACCTCGGTGCTGCCTTAGACTCCTG
UCN3	urocortin 3	GGCCTCCTGCGGATCTTACTGGAAC

Table 2. Data are expressed as means ± SEM of relative abundance for all of the groups normalized to the 18 S values and then expressed as a fold change from the control group. * P < 0.05 *vs* control group by ANOVA.

	ARC		**VMH0.05**	
	VMH0.05	**ICV0.05**	***l*VNH**	***r*VMH**
AVP	1.19	1.22	0.29*	0.16*
CALM2	1.29	2.36	0.91	1.30
CART	1.06	1.12	0.57*	0.36*
CCK	0.97	0.82	1.13	0.86
SOCS3	0.13*	0.88	0.44*	0.33*
CREB1	0.96	1.01	0.75	0.95
GABRD	1.57	0.73	1.14	1.16
GHRH	1.06	1.21	0.76	0.88
GNRH1	0.97	1.05	0.60	0.65
HCRT	1.16	0.83	0.62	0.14*
JAK2	0.55*	1.03	0.88	1.17
MAPK1	1.25	1.22	0.94	1.14
NPY	0.93	1.21	0.95	1.31
OXT	1.09	1.17	0.39*	0.36*
POMC2	1.26	1.12	1.27	1.06
PTGES	0.03*	0.93	0.56*	0.44*
SCT	0.45*	1.68	1.12	0.55
STAT3	0.53*	0.84	0.88	0.85
TH	0.48*	0.87	3.14*	0.22*
TNF	0.52*	0.64	0.53*	0.61
TRH	1.58*	1.03	0.84	1.05
UCN3	0.67	1.35	0.49*	0.81

Fig. 1. Dissection of rat hypothalamic ARC and VMH [221]



Chapter 6

HYPOTHALAMIC GENE PROFILES IN GIPR KNOCKOUT MICE

[1] Duan J., Della-Fera M.A., Isales C.M. and C. A. Baile. To be submitted to *Peptides.*

Abstract

GIP is a peptide synthesized and secreted from the small intestine. It is a major incretin factor of the enteroinsular axis and linked to dietary-induced obesity. In this study, real time RT-PCR is performed to determine the mRNA expression of a series of hypothalamic biomarkers in GIPR KO mice. Our results suggest that the hypothalamic mRNAs are down-regulated on AVP, CART, OXT, PTGES, STAT3, TH and UCN3, while up-regulated on MAPK1 and NPY in GIPR KO mice. There are significant positive correlations between hypothalamic OXT and AVP, OXT and AGRP, OXT and UCN3, TH and STAT3. This study provides a map of how the absence of GIPR affects the hypothalamic gene expression profile in mice and can be useful for further understanding the potential hypothalamic involvement in GIP induced activities.

Introduction

GIP is a peptide hormone synthesized and secreted from K-cells in the proximal small intestine [83]. It was initially named gastric inhibitory polypeptide but is now called glucose-dependent insulinotropic peptide for its stimulatory effect on insulin release and synthesis in the pancreas [274]. It is considered to be a major incretin factor in the enteroinsular axis [84]. GIP regulates lipid metabolism and storage [86]. It has an anabolic effect on bone, and GIP treatment of ovariectomized mice prevents bone loss [87]. GIPR KO mice are found to be resistant to diet-induced obesity and insulin resistance, and GIP now is considered to be a potential pharmaceutical agent for the treatment of diabetes, obesity and other metabolic syndromes [88, 89].

The GIPR is a glycoprotein which belongs to the secretin/VIP family of receptors,

a seven transmembrane G-protein-coupled receptor family [84]. GIPR mRNA is distributed both in peripheral organs and in the CNS [85]. The widespread distribution of GIPR in the CNS and high binding affinity of GIP in the brain [85, 90, 91], together with the observation that GIP can regulate the release of hormones from pituitary and adrenal glands [92, 93], raise the possibility that GIP might regulate the HPA axis by binding to the GIPR in the hypothalamus. Also recently, GIP was found in adult brain [94]. Furthermore, the wide localization of GIPRs in the limbic system implied its potential participation in some emotional behaviors such as memory and mood [85]. Given that the hypothalamus is a primary organ of the limbic system, we investigated the hypothalamic gene profile of the GIPR KO mice in this study. Considering the importance of choosing an appropriate (constantly expressed) HKG (housekeeping gene) in a given experimental condition in validating real time RT-PCR, initially we compared several HKGs for their hypothalamic mRNA levels and identified the one that was most stably expressed. Then we employed this particular HKG as the reference gene to investigate the effects of GIPR knockout on potential hypothalamic biomarkers that are involved in regulation of food intake, cellular signaling, and a series of other biological functions such as mood.

Material and Methods

The GIPR KO mice from the laboratory of Dr. Yuichiro Yamada (Japan) were bred and maintained on a C57Bl/6 genetic background in the Medical College of Georgia (Augusta, GA; the mice were very kindly provided for this work by Dr. Carolos Isales). Mice were group-housed and had ad libitum access to a pelleted standard lab chow

(Harlan TakLad Rodent Diet 8604) and water during the entire experiment. Mice were kept in cages of four animals/cage, at 25°C with a 12/12 h light/dark cycle. All experiments were approved by the Institutional Animal Care and Use Committee at Medical College of Georgia (Augusta, GA). Ten-week-old C57Bl/6 female mice (wild type, n = 20) and GIPR KO mice, n = 18) were used. The brains were removed rapidly after decapitation for hypothalamic dissection (Fig. 1) [221]. Hypothalamic blocks selected randomly from two mice of the same group were pooled together for RNA extraction. Tissue homogenization and total RNA extraction were performed according to the protocol from Invitrogen. The integrity of the RNA produced from all samples was verified and quantified using a RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA).

One hundred ng of total RNA in a 20 µl reaction was reverse transcribed using the cDNA Archive Kit (Applied Biosystems Inc., part #4322171, CA) according to manufacturers' protocols. Reactions were incubated at 25^{o}C for 10 min and subsequently at 37^{o}C for 120 min. Quantitative PCR (Taqman^{TM}) assays were performed using 384-well MicroFluidic cards^{TM} on the ABI PRISM 7900 SDS software. All of the oligonucleotide primer and fluorogenic probe sets for Taqman^{TM} real time PCR were made by ABI (Table 1). The cycle conditions were: 94.5ºC for 15 min, followed by 40 cycles of 97ºC for 30 s, 59.7ºC for 1 min.

β-ACT, 18 S, B2M and PPID have been established as endogenous controls for their comparatively constant expression in tissues and were used in real time RT-PCR to correct the differences in the amount of total RNA added to each reaction. To compare the expression stability of these HKGs in our experimental conditions, initially all of

these HKGs were normalized to each other and the RQ values were analyzed with repeated measure analysis of variance (ANOVA). Then the HKG with the lowest F value or high p value (which imply highest stability) was considered as the best reference gene in this study and was used later to normalize the mRNA expressions. Data are means ± SEM of RQ values from the SDS file for all the genes. Statistical significance was assessed by ANOVA to compare the hypothalamic gene expression of GIPR KO mice with that of control mice.

Results & Discussion

The HKG mRNA changes induced by GIPR knockout, using different HKGs as reference gene, are shown in Table 2. Both F and p values from the repeated measures ANOVA are presented. β-ACT was the most suitable as a reference gene due to its lowest F value (highest p value). Thus, it was then used to normalize the mRNA expression of the hypothalamic biomarkers in this study (Table 3). No significant differences were observed for hypothalamic mRNA of AGRP CREB1, FOS, GABRD JAK2, POMC, SCT, SOCS3, TNF and VIP. There were significantly positive correlations between OXT and AVP ($r = 0.92$, $p < 0.01$), OXT and AGRP ($r = 0.81$, $p < 0.01$), TH and STAT3 ($r = 0.84$, $p < 0.01$), and OXT and UCN3 ($r = 0.79$, $p < 0.01$) (Fig. 2).

Thus far, there has been no evidence to show GIP's effects on food intake and body weight. In this study, we did not observe mRNA changes of feeding-related peptides such as AGRP and POMC. Although there were changes in mRNA of hypothalamic NPY and CART, we think that these changes might be related not as much

to feeding regulation as to other physiological functions as mood and bone formation. For example, NPY has been reported to be an important neuromodulator in the regulation of emotional behaviors such as anxiety and depression [275]. Since CART was reported to participate in the regulation of bone resorption, the reduced hypothalamic CART mRNA in GIPR KO mice supports previous studies which showed the involvement of GIP in bone metabolism [178, 276]. Since both leptin and GIP are involved in food-dependent Cushing's syndrome in regulating cortisol secretion, we cannot rule out the possibility that leptin might interact with GIP in physiological conditions [277]. One study showed the compensation of the enteroinsular axis in GIPR KO mice [278]; thus, it is possible that hormones like leptin may also compensate in the absence of GIP action. UCN3 is a member of the corticotropin-releasing factor family and its reduced mRNA level in GIPR KO mice is in agreement with a previous report in which GIP was found to be involved in cortisol regulation in food-dependent Cushing's syndrome [277]. The decreased PTGES mRNA level in GIPR KO mice might imply a potential involvement of GIP in the febrile genesis.

Noradrenergic neurons innervate most parts of the limbic system and relate to moods such as depression and anxiety [279]. Both AVP and OXT (two noradrenergic hormones) are also implicated in the central control of lipolysis in WAT and the release of both hormones are under direct regulation of the hypothalamus [237]. The down-regulation of hypothalamic AVP and OXT with GIPR KO indicates that GIP might be involved in regulation of mood and helps explain why GIP does not change food intake but acts on adipose mobilization. Our results also showed that GIPR KO dramatically altered the cellular signaling pathways by down-regulating mRNA of STAT3 and TH and

up-regulating MAPK1. Although strong correlations were observed between several genes, the implication and significance of these interrelations are still yet to be elucidated.

Generally, the present results show that GIPR KO alters the gene expression of a series of selected hypothalamic biomarkers in mice. In addition, GIP might be involved in anxiety-related behaviors and bone metabolism by binding to the GIPRs in the limbic system.

Table 1. Probes used in real time PCR

Gene Symbol	Gene Name	Context Sequence
β-ACT	actin, beta, cytoplasmic	TACTGAGCTGCGTTTTACACCCTTT
AGRP	agouti related protein	CCCAGAGTTCCCAGGTCTAAGTCTG
AVP	arginine vasopressin	TGCAGCGACGAGAGCTGCGTGGCCG
CART	cocaine and amphetamine regulated transcript	CCACGAGAAGGAGCTGCCAAGGCGG
CREB1	cAMP responsive element binding protein 1	GGCCTTCCTACAGGAAAATTTTGAA
FOS	FBJ osteosarcoma oncogene	AACACACAGGACTTTTGCGCAGATC
GABRD	gamma-aminobutyric acid A receptor, delta	CCGCACCATGGCGCCAGGGCAATGA
JAK2	Janus kinase 2	TCCCTCCCGCGAAGGCCAATGTTCT
MAPK1	mitogen activated protein kinase 1	GCATGGTTTGCTCTGCTTATGATAA
NPY	neuropeptide Y	TCTCATCACCAGACAGAGATATGGC
OXT	oxytocin	CGCTGAGCCCACTTTCTGGGAATAC
POMC1	pro-opiomelanocortin-alpha	GCAACCTGCTGGCTTGCATCCGGGC
PTGES	prostaglandin E synthase	GAGCGCTGCCTCAGAGCCCACCGCA
SCT	secretin	CTACAGGACTGGCTTCTGCCCAGGC
SOCS3	suppressor of cytokine signaling 3	CCAGCGCCACTTCTTCACGTTGAGC
STAT3	signal transducer and activator of transcription 3	CACGGCAGCCCAGCAAGGGGGCCAG
TH	tyrosine hydroxylase	CTGTCACGTCCCCAAGGTTCATTGG
TNF	tumor necrosis factor	AAAGGGATGAGAAGTTCCCAAATGG
UCN3	urocortin 3	ACAAGCTGGAAGATGTGCCCTTGCT
VIP	vasoactive intestinal polypeptide	AAAGAGGAGCAGTGAGGGAGATTCT

Table 2. Change induced by GIPR knockout

Reference gene	**β-ACT**	**18 S**	**B2M**	**PPID**	**Statistics**
β-ACT	—	29%	–14%	–6%	F=0.04 p=0.8399
18 S	–22%	—	–33%	–28%	F=20.57 p=0.003
B2M	17%	9%	—	50%	F=11.20 p=0.0038
PPID	7%	39%	–7%	—	F=6.62 p=0.0198

Effects of GIPR knockout on the expression of HKGs normalized to different HKGs in the mouse hypothalamus. β-ACT: β-actin, 18 S: 18 S RNA, B2M: beta-2 microglobulin, PPID: peptidylprolyl isomerase D. The data are presented as % change when GIPR was knocked out. F and p values from repeated measures ANOVA were presented for each HKG used as reference. The gene with the lowest F value (or highest p value) was used as a valid reference gene in subsequent study.

Table 3. Effects of GIPR KO on mRNA expression of hypothalamic biomarkers in mice.

mRNA	**Control**	**GIPR KO**	**Change**
AVP	0.77±0.08	0.52±0.07	0.68*
CART	0.63±0.05	0.42±0.03	0.67**
OXT	0.72±0.06	0.50±0.05	0.69*
PTGES	1.04±0.06	0.73±0.04	0.70**
STAT3	1.02±0.03	0.86±0.03	0.84**
TH	0.76±0.06	0.59±0.04	0.78*
UCN3	0.69±0.06	0.49±0.03	0.71**
MAPK1	1.17±0.04	1.50±0.04	1.28**
NPY	1.15±0.05	1.39±0.06	1.21**

Data are expressed as means ± SEM for both groups normalized to the β-ACT values and then expressed as a fold change from the control group. * $P < 0.05$, ** $P < 0.01$, *vs* control group by ANOVA.

Fig. 1. Dissection of hypothalamus in mouse [221]

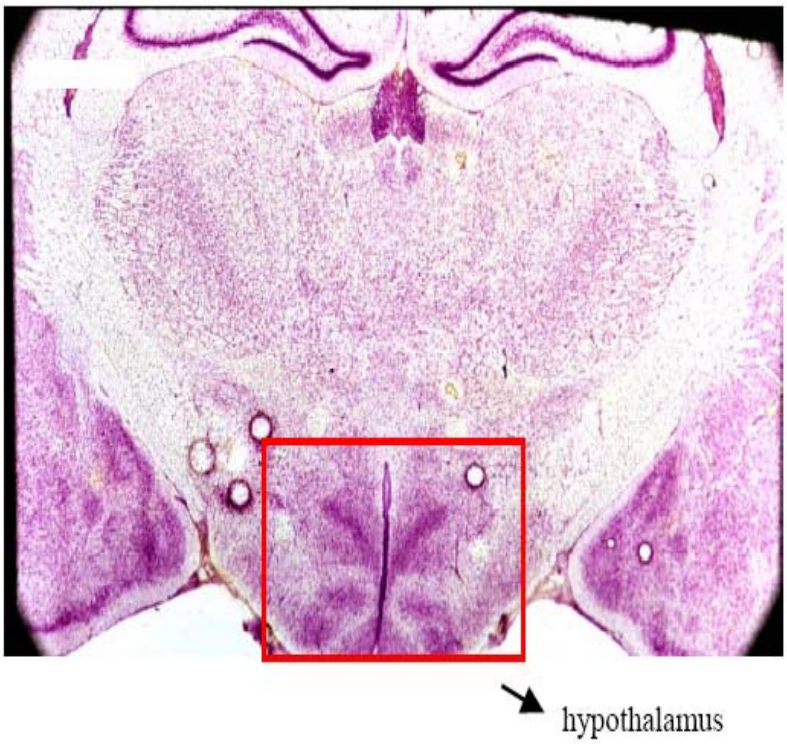
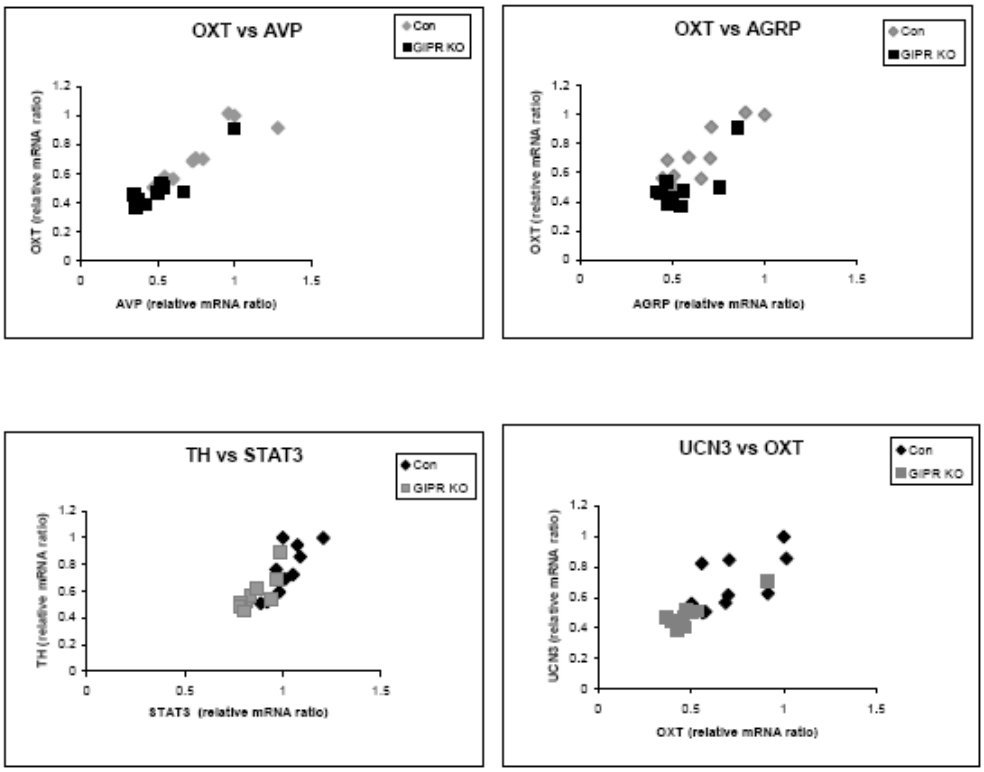


Fig. 2. Relationship between mRNA levels of (a) OXT and AVP; (b) OXT and AGRP; (c) TH and STAT3, and (d) UCN3 and OXT in GIPR KO and control mice.



Chapter 7

INTRACEREBROVENTRICULAR INJECTED GIP CHANGES HYPOTHALAMIC EXPRESSION OF ANXIETY-RELATED GENES IN RATS

[1] Duan J., Li Q., Choi Y.H., Hartzell D.L., Della-Fera M.A. and C. A. Baile. To be submitted to *Peptides*.

Abstract

GIP is a peptide synthesized and secreted from the small intestine. GIPRs are widespread in the limbic system. The objective of this study was to investigate the effects of central GIP administration on the hypothalamic gene expression profiles in rats. Brains were collected from male rats ICV injected with 10 μl of aCSF or 10 μg GIP in 10 μl daily for 4 successive days. Hypothalami were dissected and the total RNA was extracted and reverse transcribed, then real time RT-PCR was performed to determine the mRNA expression of a series of hypothalamic anxiety-related biomarkers. Central GIP treatment up-regulated the mRNA levels of hypothalamic AVP, CART, CCK, CREB1, HCRT, MAPK1, OXT, TH, TNF, and SCT. There were significant positive correlations between CREB1 and MAPK1 ($r = 0.87$, $p < 0.001$), CREB1 and CCK ($r = 0.89$, $p < 0.001$), and MAPK1 and CCK ($r = 0.78$, $p < 0.001$). These results demonstrate that GIP is involved in stress and anxiety-related behaviors and can be useful to further understanding the potential hypothalamic involvement in GIP induced activities.

Introduction

GIP is a 42-amino acid peptide hormone synthesized and secreted from K-cells in the proximal small intestine [83]. It was initially named gastric inhibitory polypeptide and is now called a glucose-dependent insulinotropic peptide for its stimulatory effect on insulin release and synthesis in the pancreas [274]. It is now considered to be a major incretin factor of the enteroinsular axis [84]. GIP regulates lipid metabolism and storage [86]. It has an anabolic effect on bone, and GIP treatment to ovariectomized mice prevents bone loss [87]. GIPR KO mice are found resistant to diet-induced obesity and

insulin resistance, and GIP now is considered to be a potential pharmaceutical target for the treatment of diabetes, obesity, and other metabolic syndromes [88, 89].

The GIPR is a glycoprotein which belongs to the secretin/VIP family of receptors, a seven transmembrane G-protein-coupled receptor family [84]. GIPR mRNA is distributed both in peripheral organs and in the CNS, but its possible central effects are yet to elucidated [85]. The widespread distribution of GIPR in the CNS and high binding affinity of GIP to the brain [85, 90, 91], together with the observation that GIP can regulate the release of hormones from pituitary and adrenal glands [92, 93], raise the possibility that GIP might regulate the HPA axis by binding to the GIPR in the hypothalamus. Also, recently GIP was found expressed in the adult brain [94]. Given that many of the GIPR cell populations and regions are part of the limbic system, this receptor and its potential endogenous ligand may be involved in emotional behaviors [85]. Thus, the following experiment was carried out to determine whether GIP acts centrally to affect anxiety, stress, and memory. In this study, we employed real time RT-PCR on rat hypothalamic samples to investigate the effects of ICV administration of GIP on mRNA levels of potential hypothalamic biomarkers that are involved in regulation of anxiety-related behaviors and other biological functions.

Materials and methods

Animals and Treatments

Sixteen male Sprague-Dawley rats (250 – 274 g) were purchased from Harlan, Inc. (Indianapolis, IN). Rats were housed in individual cages and had access to pelleted standard lab chow and water ad libitum throughout the study. Lights were on between

0900 h – 2100 h and off between 2100 – 0900 h, ambient temperature were set at 22 ± 1 ºC, and humidity 50 %. Rats were implanted with chronic lateral cerebroventricular cannulas, and after recovery from surgery rats were randomly assigned to 2 groups. Treatments were administered at 24-hour intervals for 4 days as 10 µl injections including 0 (aCSF) or 10 µg GIP in 10 µl. The injections were carried out using an injector cannula (C313I, Plastics One) that protruded 1.1 mm below the tip of the guide cannula, and were connected to a Gilmont microsyringe by way of polyethylene tubing (PE20 Intramedic, Cat. #427406, BD, Sparks, MD). All the animals and surgical procedures in this study were approved by the Animal Care and Use Committee of The University of Georgia.

Rats were anesthetized with CO_2 before decapitation on day 5. The brains were removed rapidly after decapitation and immediately frozen by placing them on plastic cassettes on top of powdered dry ice. Once completely frozen, they were stored at -80ºC. The brains were thawed to -20ºC before the hypothalamic dissection (Fig. 1) [221]. Tissue homogenization and total RNA extraction were performed according to the protocol from Invitrogen. The integrity of the RNA produced from all samples was verified and quantified using a RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA).

RT and real-time PCR

One hundred ng of total RNA in a 20 µl reaction was reverse transcribed using the cDNA Archive Kit (Applied Biosystems Inc., part #4322171, CA) according to manufacturer's protocols using the MultiScribeTM Reverse Transcriptase. Reactions

were incubated initially at 25°C for 10 min and subsequently at 37°C for 120 min. Quantitative PCR (TaqmanTM) assays were chosen for the transcripts to be evaluated from Assays-On-Demand TM (ABI, CA), a pre-validated library of QPCR assays and incorporated into 384-well MicroFluidic cards. All of the oligonucleotide primer and fluorogenic probe sets for TaqmanTM real time PCR were from ABI (Table 1). Two µl of the cDNA samples, along with 50 µl of 2× PCR master mix were loaded into respective channels on the microfluidic card followed by a brief centrifugation (3000 rpm for 3 min). The card was then sealed and real-time PCR and RQ was carried out on the ABI PRISM 7900 SDS software. The cycle conditions were: 94.5ºC for 15 min, followed by 40 cycles of 97ºC for 30 s, 59.7ºC for 1 min. Data were analyzed using sequence detection systems software (Applied Biosystems, CA) and the RQ, which presents the fold difference of mRNA level in treatment groups relative to the aCSF control group.

mRNA expressions were normalized by using 18 S as an endogenous control to correct the differences in the amount of total RNA added to each reaction. The ΔCT values were first calculated by using CT for a specific gene mRNA minus CT for 18 S mRNA in the sample. Then the mean mRNA expressions from the treatment groups were compared with the aCSF control group using the formula: $RQ = 2^{-\Delta\Delta CT}$ (ΔΔCT is the average aCSF control group ΔCT values minus the average experimental group ΔCT values and ΔΔCT of 1 equates to a twofold difference in starting amount of cDNA). Data are means ± SEM of RQ values from the SDS file for all the genes. Statistical significance was assessed by analysis of variance (ANOVA) to compare the hypothalamic gene expression between two groups.

Results

The results suggest that with GIP treatment, the hypothalamic mRNAs were up-regulated for all the anxiety-related biomarkers (Table 2). However, the hypothalamic mRNAs were unchanged in PPID, STAT3, SOCS3, and GHRH. There were significant positive correlations between CREB1 and MAPK1 ($r = 0.87$, $p < 0.001$), CCK and MAPK1 ($r = 0.78$, $p < 0.001$), and CREB1 and CCK ($r = 0.89$, $p < 0.001$) (Fig.2).

Discussion

The hypothalamus has a unique location at the interface between the limbic system and the endocrine and autonomic nervous systems, and is a primary organ for the regulation of a variety of other biological activities [223, 224]. The widespread localization of GIPRs in the brain, especially in the limbic system where the emotions are generated and the responses to emotional stimuli are coordinated, suggests its involvement in GIP-induced physiological functions. To our knowledge, there is no report on the central GIP effects on hypothalamic gene expressions and the significance of central GIP is still unknown. In this study, we used real time RT-PCR to investigate how ICV administration of GIP in rats regulates the gene expression of a series of selected hypothalamic biomarkers that are involved in anxiety-related behaviors. In addition, we found that central GIP can change the mRNA levels of these anxiety-related biomarkers in the hypothalamus.

Noradrenergic neurons innervate all parts of the limbic system and the cerebral cortex, where they play a major role in setting mood such as depression and anxiety

[280]. The up-regulation of AVP and OXT (two noradrenergic hormones) under GIP treatment indicated the potential GIP involvement in mood. CCK is one of the most abundant neuropeptides in the CNS which is implicated in the control and modulation of diverse centrally mediated physiological and behavioral processes, including satiety, stress and anxiety-related behaviors [281-283]. It regularly co-localized with other neurotransmitters such as dopamine and oxytocin, in central neurons [284, 285]. The role of HCRT in sleep/wakefulness and arousal and energy homeostasis has been widely recognized, and the pathophysiology of narcolepsy-cataplexy is linked to hypocretin ligand deficiency in the brain [286, 287]. GIP can induce mitogenic and anti-apoptotic signaling in insulin-producing β-cells by activating signaling modules such as CREB and MAPK [288-290]. Although a high correlation may not necessarily mean definite causation and we only measured mRNA instead of protein levels, the strong positive correlation between CCK, MAPK1, and CREB1 in this study confirms data from the previous studies from other tissues. These data add support to the notion that these molecules might be closely interrelated in GIP-induced signal transduction pathways.

Our results also confirm the previous findings about these molecules on functions other than anxiety-related behaviors. For example, both AVP and OXT are also implicated in the central control of lipolysis through the sympathetic nervous system outflow from brain to WAT and the release of both hormones are under direct regulation of the hypothalamus [237]. Thus, the up-regulation of both mRNAs in this study can provide explanation about the effects of GIP on adipose mobilization [86, 291]. Since CART was reported to participate in the regulation of bone resorption, the increased

hypothalamic CART mRNA in GIP treated rats supports previous studies which showed the involvement of GIP in bone metabolism [178, 276].

This study is the first to investigate how central GIP modulates the hypothalamic mRNA levels of those anxiety-related molecules. In conclusion, GIP may be involved in a variety of physiological activities as stress, and anxiety-related behaviors, and the MAPK signaling might be an important pathway in GIP-induced activities. This study furthers the understanding of the potential of hypothalamic involvement in GIP-induced activities.

Table 1. Probes used in real time PCR

Gene Symbol	Gene Name	Probe Sequence
18 S	18 S	CCATTGGAGGGCAAGTCTGGTGCCA
AVP	arginine vasopressin	CATGGAGCTGAGACAGTGTCTCCCC
CALM2	calmodulin 2	CTACGAAGAGTTTGTACAAATGATG
CART	cocaine and amphetamine regulated transcript	AGAAGGAGCTGCCAAGGCGGCAACT
CCK	cholecystokinin	AGGTCCGCAAAGCTCCCTCTGGCCG
CREB1	cAMP responsive element binding protein 1	GTCTAATGAAGAACAGGGAAGCAGC
GABRD	gamma-aminobutyric acid A receptor, delta	ACCATGGCGCCAGAGCAATGAATGA
GHRH	growth hormone releasing hormone	GGAGAGCATCTTGCAGGGATTCCCA
GNRH1	gonadotropin-releasing hormone 1	TTTCCAAGAGATGGGCAAGGAGGAG
HCRT	hypocretin	TCTACAAAGGTTCCCTGGGCCGCCG
JAK2	janus kinase 2	CTCCCGGGAAGGCCGATGTTCTGAA
MAPK1	mitogen activated protein kinase 1	TTAAATTGGTCAGGACAAGGGCTCA
NPY	neuropeptide Y	CAGCCCGCCCGCCATGATGCTAGGT
OXT	oxytocin	GCTGTAGCCCGGATGGCTGCCGCAC
POMC2	proopiomelanocortin, beta (endorphin, beta)	GCAACCTGCTGGCTTGCATCCGGGC
PPIA	peptidylprolyl isomerase A	AGGATTCATGTGCCAGGGTGGTGAC
PTGES	prostaglandin E synthase	GCTGCCTCAGAGCCCACCGCAACGA
SCT	secretin	GGGGAAGCGCAGCGAGGAGGACACA
SOCS3	suppressor of cytokine signaling 3	CCGGAGCACGCAGCCAGTGCCCCGC
STAT3	signal transducer and activator of transcription 3	AGCTGACCCAGGTAGTGCTGCCCCT
TH	tyrosine hydroxylase	AAGGACAAGCTCAGGAACTATGCCT
TNF	tumor necrosis factor	CGTAGCCCACGTCGTAGCAAACCAC
TRH	thyrotropin releasing hormone	ACCTCGGTGCTGCCTTAGACTCCTG
UCN3	urocortin 3	GGCCTCCTGCGGATCTTACTGGAAC

Table 2. Effects of ICV GIP on mRNA expression of hypothalamic biomarkers in rats

Gene Symbol	Control	GIP treatment	Change
AVP	0.78±0.06	1.14±0.03	46.2% **
CALM2	0.78±0.06	1.06±0.04	35.9%**
CART	0.97±0.07	1.22±0.02	25.8% *
CCK	0.98±0.03	1.49±0.07	52.0%**
CREB1	0.81±0.04	1.13±0.03	39.5%**
HCRT	0.80±0.06	1.1±0.05	37.5%**
MAPK1	0.85±0.06	1.14±0.06	34.1%**
OXT	0.85±0.09	1.27±0.05	49.4%**
SCT	0.85±0.08	1.14±0.15	34.1%**
TH	0.76±0.09	1.01±0.06	32.9% *
TNF	1.15±0.08	2.74±0.35	138.3%**

Data are expressed as means ± SEM for both groups normalized to the 18 S values and expressed as a fold change from the control group. * $P < 0.05$, ** $P < 0.01$, *vs* control group by ANOVA.

Fig. 1. Dissection of hypothalamus in mouse [221]

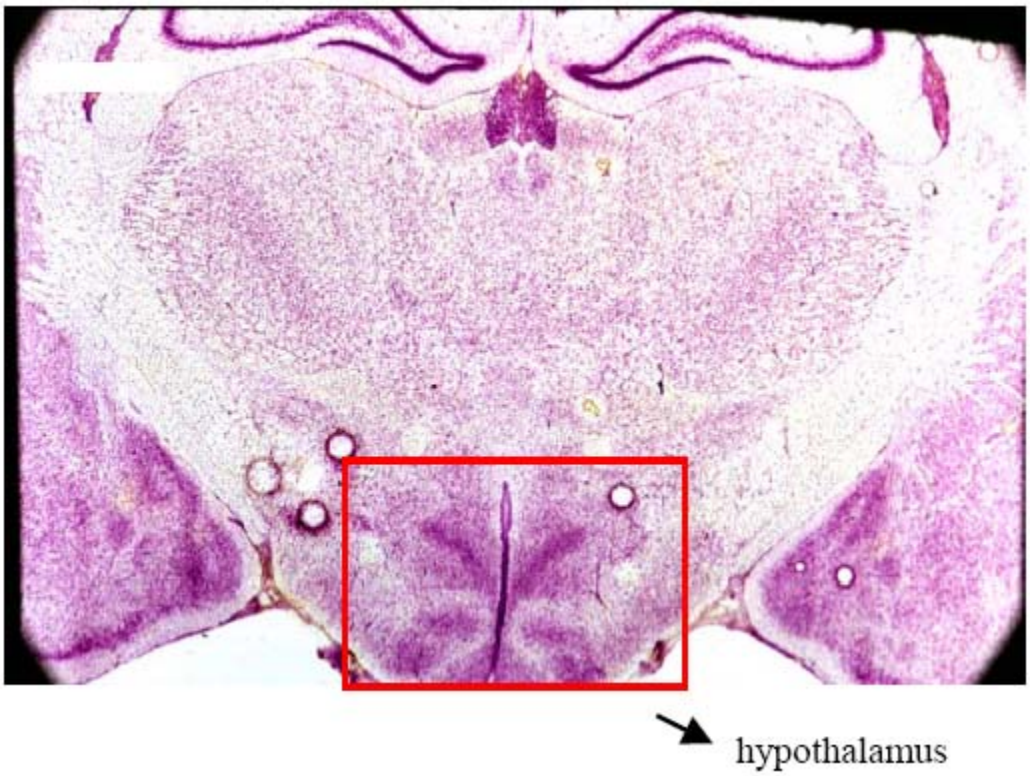
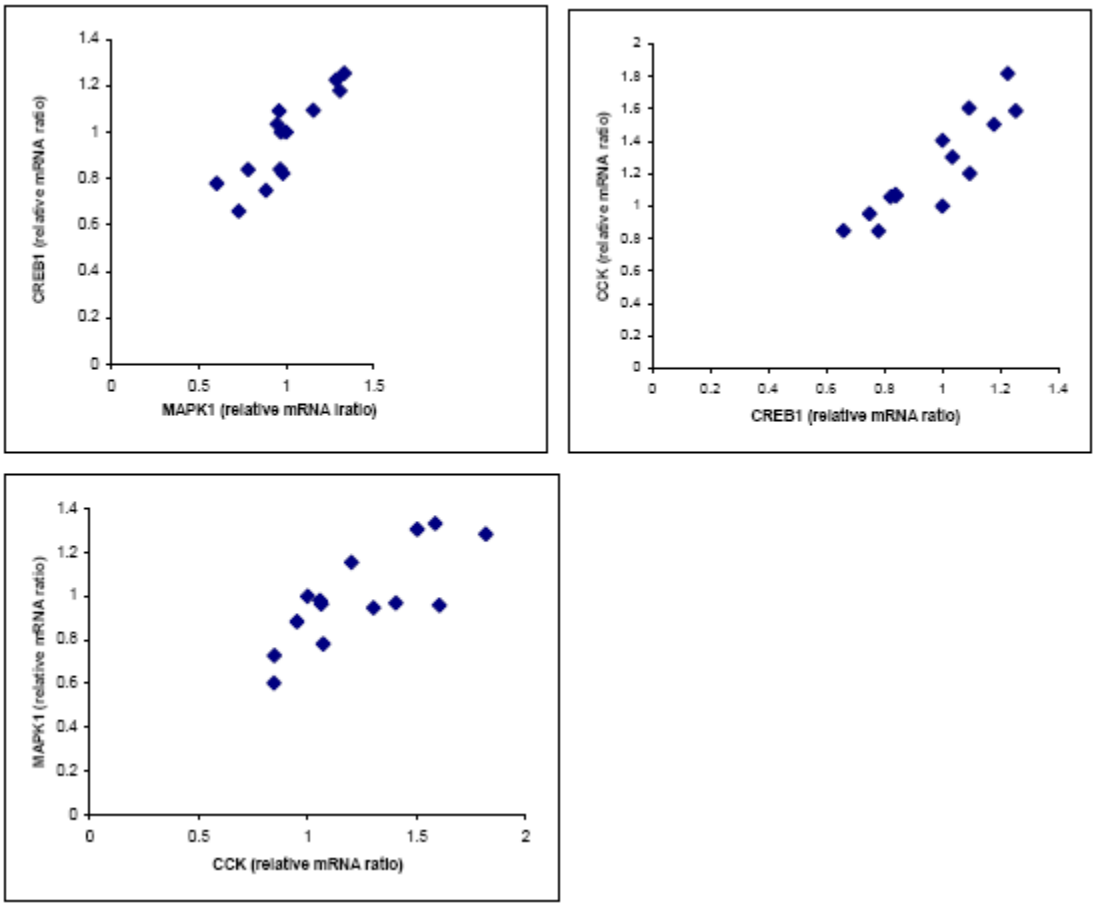


Fig. 2. Relationship between mRNA levels of MAPK1 and CCK; CREB1 and MAPK1; CCK and CREB1 in all samples



Chapter 8

SUMMARY AND CONCLUSION

Five studies were conducted to investigate the real time RT-PCR analysis of hypothalami from rodents treated centrally and peripherally with peptide hormones. In the first study, a comparison between two anti-obesity peptides - leptin and CNTF - was conducted to test their effects on hypothalamic gene expressions. Our results demonstrated that ICV treatment of leptin or CNTF induced both overlapping and divergent pathways in hypothalamic ARC in rats. The increased NPY expression with leptin and CNTF treatment indicated that the hypothalamus utilized a compensatory mechanism to resist the over reduction of body weight.

In the second study, we investigated the response of two different genotypes - *ob/ob* and lean mice - to subcutaneous leptin treatment. The data demonstrated that *ob/ob* and lean mice have different hypothalamic mRNA expression patterns (particularly in feeding-related genes), and selected genes in *ob/ob* mice were more sensitive to exogenous leptin stimulation as compared to lean mice. Multiple correlation analysis indicated a strong positive correlation between hypothalamic JAK2, STAT3, and CREB1. A strong correlation was also observed between body weight loss and the mRNA level of hypothalamic POMC. Our results establish a number of candidate hypothalamic biomarkers whose changes in expression may warrant further study. Our results also suggest that the hypothalamus exerts a very dynamic adaptive role by re-orchestrating the hypothalamic gene profile to a given condition or treatment.

The third study was undertaken to compare the effects of VMH and ICV (lateral ventricle) injections of leptin twice/day for 4 days on hypothalamic ARC gene expression. The results showed that VMH injections of leptin up-regulated mRNA levels of TRH, and down-regulated the mRNA levels of JAK2, PTGES, SOCS3, STAT3, TH, and TNF at the hypothalamic ARC. None of these mRNA changes were observed in the hypothalamic ARC of rats with ICV leptin injections. VMH injections of leptin induced similar reductions in mRNA levels of AVP, CART, SOCS3, OXT, and PTGES in both sides of VMH, with the only exception being that TH was down-regulated in *r*VMH and up-regulated in *l*VMH. We conclude that the unilateral leptin exposure with VMH injections and the bilateral leptin exposure with ICV injections induce divergent physiological behaviors and hypothalamic ARC gene profiles. Furthermore, TH may play an important role in the crosstalk between the two VMH sides.

In the fourth study, we investigated hypothalamic gene profiles in tissues from animals without GIPR. Our results indicate that the knockout of GIPR dramatically alters hypothalamic biomarkers involved in the regulation of food intake, cellular signaling, and a series of other biological functions such as anxiety, memory, sleep, and inflammation.

In the fifth study, we investigated the effects of central GIP administration on the hypothalamic gene expression profile in rats. Rats were ICV injected with 10 μg GIP daily for 4 successive days. Central GIP treatment up-regulated the mRNA levels of hypothalamic CART, AVP, CCK, CREB1, HCRT, MAPK1, OXT, TH, TNF, and SCT. There were significant positive correlations between CREB1 and MAPK1, CREB1 and CCK, as well as MAPK1 and CCK. These results demonstrate that GIP is involved in

stress and anxiety-related behaviors and can be useful in the further understanding hypothalamic involvement in GIP-induced activities.

In conclusion, our study indicates that real time RT-PCR, when combined with microfluidic cards, provides a convenient and reliable way to profile the multiple mRNA changes in the rodent whole hypothalamus or individual hypothalamic nuclei in a quantitative manner. The mRNA changes of these selected genes in the hypothalamus provide useful information for understanding the complexity of integrated neural circuits and the action mechanism of these hormones. The data also aids in understanding how these peptide hormones, central or peripheral, might act on the hypothalamus, and thus to modulate the gene expression profile which changes animal physiological behaviors such as feeding, signal transduction, psychological anxiety, and anorexia.

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