

# Neuroendocrine Gene Regulation in Hypothalamic Cell Lines

Sandeep S. Dhillon<sup>1</sup>, Ginah L. Kim<sup>1</sup> and Denise D. Belsham\*<sup>1,2</sup>

<sup>1</sup>Departments of Physiology, <sup>2</sup>Obstetrics and Gynaecology and <sup>2</sup>Medicine, University of Toronto and <sup>2</sup>Division of Cellular and Molecular Biology, Toronto General Hospital Research Institute, University Health Network, Toronto, ON, Canada M5S 1A8

**Abstract:** The physiological system implicated in the maintenance of energy homeostasis is situated predominantly in the hypothalamus. However, due to the inherent difficulty of studying individual neurons in the brain through *in vivo* analysis, cell models have been generated to investigate the direct action of hormones or other physiological compounds on metabolic effectors, such as neuropeptides, located in specific cell types from the hypothalamus. Immortalized, clonal cell lines represent an unlimited, homogeneous neuronal population that can be manipulated using a number of molecular techniques. In particular, cell lines have proven to be indispensable in the study of gene structure, gene expression and characterizing the molecular mechanisms responsible for regulating gene expression. In this review, we summarize recent studies that examine the direct transcriptional regulation of neuropeptide Y (NPY) by insulin and the leptin-mediated control of neurotensin (NT) gene expression. The use of these novel cell models has contributed profoundly to our understanding of how peripheral hormones, neuromodulators and neurotransmitters regulate transcriptional events that may ultimately contribute to the control of feeding behaviour.

**Keywords:** Hypothalamus, gene regulation, insulin, leptin, cell lines, neurotensin, neuropeptide Y.

## INTRODUCTION

### Hypothalamus

The hypothalamus is responsible for maintaining physiological homeostasis for some of our most vital and basic needs. To achieve this task, the hypothalamus receives input about the state of the body from numerous sources and is capable of initiating compensatory changes if any physiological variables stray from homeostatic levels. These inputs include: 1) retinal inputs that innervate the suprachiasmatic nucleus to regulate circadian rhythms; 2) limbic structures, including the amygdala, hippocampus and olfactory cortex that project to hypothalamic nuclei to regulate feeding, stress and reproductive behavior; 3) the nucleus of the solitary tract (NTS) collects information from the vagus nerve regarding blood pressure, gut motility and respiration and this information is relayed to numerous hypothalamic nuclei; and 4) finally, endocrine hormones derived from peripheral organs responsible for energy homeostasis, growth, stress and reproduction regulate the hypothalamus either directly through hypothalamic circumventricular structures that are deficient of the blood brain barrier (BBB) or are transported across the BBB *via* hormone specific transporters [1]. Disturbance to any hypothalamic inputs or to the delicate homeostatic mechanisms maintained by the hypothalamus can lead to a number of detrimental health concerns including obesity and diabetes [2].

This review focuses on the hypothalamic cell populations responsible for regulating feeding and energy homeostasis. Original experiments studying feeding and satiety in the hypothalamus included the stimulation or lesion of specific hypothalamic nuclei [3]. As a result, some of these nuclei were viewed as 'feeding' or 'satiety' centers. However, as our knowledge expanded, studies revealed that these 'feeding' and 'satiety' centers were comprised of a complex array of distinct neuronal populations, expressing a specific complement of neuropeptides, neurotransmitters and receptors that regulate energy homeostasis [4]. These neuronal populations are regulated by peripheral signals to maintain a balance in feeding and energy homeostasis [5]. The recognition of distinct neuronal populations responsible for feeding regulation calls for new methods and tools to study the molecular biology of these individual neurons. Developing a thorough understanding of the underlying cellular events and transcriptional regulation of unique peptidergic neurons from the hypothalamus is essential in order to understand how the brain achieves its diverse control of basic physiology. However, the cellular mechanisms involved in this process are poorly understood due to the complex circuitry of the hypothalamus and the lack of appropriate cell models available. Classical *in vivo* approaches have been instrumental in establishing synaptic connectivity between distinct hypothalamic nuclei and the functional purpose of numerous neuropeptides and neurotransmitters [6-9]. However, *in vivo* techniques cannot firmly establish the direct action of an agent on specific hypothalamic neurons and cellular events. Non-transformed primary hypothalamic cultures are difficult to maintain, have a short life span and represent a heterogeneous population of neurons and glial cells. For this reason, researchers have turned to immortalized, clonal cell lines to generate a

\*Address correspondence to this author at the Department of Physiology, University of Toronto, Medical Sciences Building 3247A, 1 Kings College Circle, Toronto, ON, Canada M5S 1A8; Tel: (416) 946-7646; Fax (416) 978-4940; E-mail: d.belsham@utoronto.ca

comprehensive picture of the molecular biology involved with the regulation of specific neuroendocrine hypothalamic neurons. Cell lines representative of the central nervous system (CNS) have previously been established from neuroblastomas (Neuro2A) and pheochromocytomas (PC12); however, these models are not truly representative of fully differentiated CNS neurons. As of 2004, few cell models existed that were representative of the hypothalamus and proved to be useful in understanding the molecular biology of specific peptidergic hypothalamic cells [10-12]. The paucity of cell models available to study the specific biology of neuroendocrine cells prompted our lab to generate an array of immortalized cell models from the hypothalamus [13]. Each clonal cell model has distinct cell morphology and genetic markers, suggesting they represent a unique cell type from the diverse neuronal population of the hypothalamus. These cell lines have proven to be indispensable in providing novel information on intracellular signaling cascades, transcriptional mechanisms and the secretory events involved with both feeding regulation and energy homeostasis.

### Generation and Characterization of Immortalized Hypothalamic Cell Models

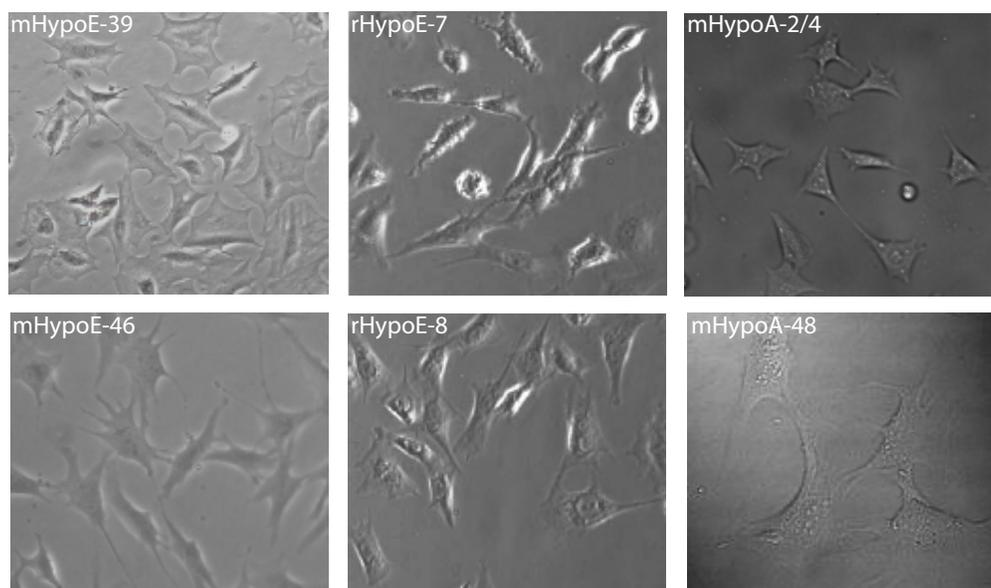
In order to study the cellular mechanisms and direct regulation of specific hypothalamic neurons, researchers have focused on immortalized cell models. One of the first fully differentiated immortalized hypothalamic cell lines is the gonadotropin-releasing hormone (GnRH)-secreting GT1 cells [12]. This cell line was created by directing tumorigenesis in GnRH-expressing neurons in transgenic mice using the 5' regulatory region of the GnRH gene to express the SV40 T-antigen oncogene. The GT1 cultures, which represent a heterogeneous population of hypothalamic cells, have been further subcloned into three homogeneous cell populations, labeled as GT1-1, GT1-3 and GT1-7. These cells have been extensively characterized [14] and have become the most accepted neuronal cell models for the study of GnRH and basic neuronal physiology. Other immortalized cell models from the hypothalamus have been generated in addition to the GT1 cells [10, 11, 15, 16]; however, the collection of these cell models represents only a small fraction of the distinct neuronal cell types found in the hypothalamus. In order to address this distorted representation, our group has generated an array of cell lines that are more illustrative of the entire hypothalamic cell population.

Taking a unique approach to immortalizing cells, our group transformed cells using retroviral gene transfer of the oncogene SV40 T-antigen into primary hypothalamic cell culture from mice at embryonic days 15, 17 and 18 [13]. The resulting mixed population of cells was further subcloned to obtain homogeneous cell lines. Altogether, we have established 38 embryonic, clonal, hypothalamic cell lines. These cell lines are designated as mHypoE-‘clone number’, although in previous studies they are referred to as N-‘clone number’. Each cell line possesses a neuronal phenotype with clearly defined perikarya and neurites; however, the precise cell morphology of each cell line appears to be distinct from one another, indicating that they represent unique cell types. Detailed characterization studies indicate that the cells express neuronal cell markers, including neuron-specific

enolase and neurofilament protein, but do not express glial fibrillary acidic protein. The cells also express markers of neurosecretory machinery, such as syntaxin, exhibit dense core granules that are indicative of secretory neurons, and demonstrate an intracellular calcium response after potassium chloride (KCl) depolarization. Thus, our cell lines exhibit the correct neuronal morphology, express mature neuronal markers and are capable of neurosecretion.

Further characterization studies were performed in our cell lines by examining the expression of various receptors and neuropeptides associated with energy homeostasis. Cell lines expressing peptides linked to energy homeostasis expressed peptide profiles consistent with those reported by *in vivo* studies. As an example, all neuropeptide Y (NPY)-expressing neurons, thought to have orexigenic properties in feeding behaviour, also expressed agouti-related peptide (AgRP), but not proopiomelanocortin (POMC), a precursor to the anorexigenic neuropeptide  $\alpha$ -melanocortin-stimulating hormone ( $\alpha$ -MSH) [17, 18]. In contrast, all POMC-expressing cell lines coexpressed cocaine- and amphetamine-regulated transcript (CART), consistent with findings from *in situ* colocalization studies [19]. Furthermore, the leptin receptor (ObR) and the insulin receptor (IR) were detected in many of the lines expressing peptides involved in energy homeostasis. In addition, cell lines were also generated expressing tropic hormones and enzymes involved in neurotransmitter production. Overall, our cell lines represent the multitude of neuronal phenotypes present in the hypothalamus, thereby providing suitable models for the study of energy regulation and feeding behavior at the molecular level.

In addition to the mouse cell lines, our group has generated 32 rat, embryonic hypothalamic cell lines using a similar technique [20]. This availability of hypothalamic cell lines from two highly utilized animal models will be greatly beneficial for studying the neuroendocrine regulation of the hypothalamus. However, it is important to note that these cell lines were, due to technical requirements, developed from the embryonic hypothalamus, and may or may not be an accurate representation of an adult hypothalamic neuron. In light of this, our group has recently developed a novel method to immortalize neurons from the adult mouse (D.D. Belsham *et al.*, submitted for publication). In order to infect cells with the SV40 T-antigen, cells must be dividing. Thus, we treated adult hypothalamic primary culture with ciliary neurotrophic factor (CNTF), a nerve growth factor that induces cell proliferation and neurogenesis. Cells were then retrovirally infected with SV40 T-antigen in a similar protocol used for the immortalization of embryonic neurons. We have established over 50 adult mouse cell lines labeled as mHypoA-‘clone number’. These adult cell lines express mature neuronal markers, exhibit neuronal morphology, and have been characterized for the expression of various neuropeptides and receptors. These lines will be key to understanding the physiology of the mature neuron, and can be used for the direct comparison with neurons of embryonic origin. Altogether, our repertoire includes models from the male and female adult mouse, embryonic mouse, embryonic rat and adult mouse pituitary (Fig. 1). This vast array of cell models will give rise to a comprehensive picture of the mechanisms involved in energy homeostasis and feeding behavior. Together, our cell lines present an optimal model



**Fig. (1).** Representative phase contrast micrographs of mouse embryonic (mHypoE-39 and -46), rat embryonic (rHypoE-7 and -8) and mouse adult (mHypoA-48 and -2/4) hypothalamic cell lines. The mouse embryonic mHypoE-39 and -46 cell lines were used in studies described in the leptin and insulin section, respectively.

for the study of neuroendocrine gene regulation that has not been possible in the whole brain.

### Gene Regulation

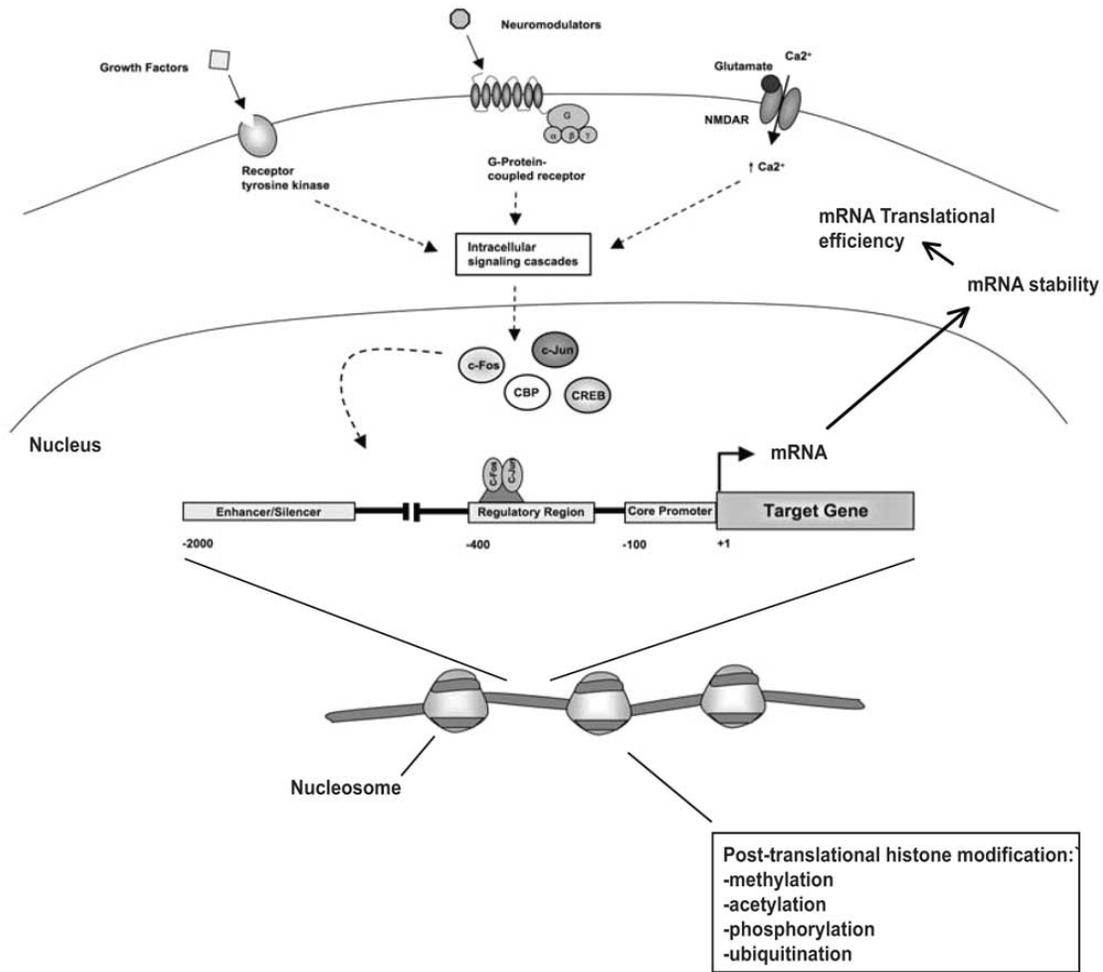
Knowledge of how transcriptional mechanisms regulate gene expression is fundamental to issues in physiology and medicine. Defects in transcription and gene regulation contribute to a wide range of diseases including cancers, cardiovascular hypertension, diabetes and many developmental abnormalities. Thus, understanding gene regulation in the hypothalamus is key to understanding the homeostatic mechanisms that maintain basic physiology. Gene expression is subject to a wide variety of regulatory controls that can be classified as transcriptional or post-transcriptional (Fig. 2). At the transcriptional level are promoters [21], enhancers [22] and silencers [22]. Promoters are comprised of a core promoter and a regulatory region. The core promoter is found immediately adjacent and upstream of the transcriptional start site and is believed to drive basal gene expression [21]. Regulatory regions are found upstream of the core promoter and contain one or more DNA sequences that transcription factors can bind to, resulting in an increase or decrease in transcriptional activity [22]. Enhancer and silencer regions are short DNA sequences that can be found thousands of base pairs upstream, downstream or even within the gene they control. These DNA motifs can increase (enhancer) and decrease (silencer) transcriptional activity. These fundamental *cis*-acting elements interact with a combination of transcription factors, co-factors, receptors and proteins to regulate transcriptional activity [21]. Additionally, the organization of chromatin can restrict physical access of nuclear proteins to underlying DNA elements. The restriction of nuclear factors is regulated by post-translational modifications to histone including phosphorylation, acetylation, methylation and ubiquitination [23]. Finally, post-transcriptional mechanisms including mRNA stability and mRNA degradation further contribute to gene levels and inherent translational

efficiencies [24]. Understanding the structure and functional activity of the transcriptional and post-transcriptional mechanisms is critical for understanding both basal gene regulation and neuron-directed gene expression. To decipher the mechanisms, we need to understand the wide range of processes involved in gene expression and develop strategic approaches to examine these processes. To date, a number of experimental methods (Table 1) have allowed for the detailed molecular analysis of the *cis*- and *trans*-acting elements involved in transcription. Combining these methods with immortalized hypothalamic cell lines, a number of groups have been able to define regions within endogenous neuronal genes critical for both basal gene regulation and neuron-directed expression. For example, the use of GT1 cells has allowed scientists to define the 173-bp promoter region proximal to the transcriptional start site, identify essential transcription factors necessary for basal GnRH expression, Oct1 and Otx2, and finally the characterization of the 300-bp enhancer located within the GnRH gene 5' flanking region between -1863 and -1571 [25-27]. Our hypothalamic cell models have provided a novel system to study the regulation of a number of endogenous neuronal genes and when combined with *in vivo* models, we can develop a comprehensive picture of the fundamental processes involved with the regulation of key genes in feeding behavior and energy homeostasis in the hypothalamus. In particular, our hypothalamic cell models have been key in elucidating the gene regulation of a number of neuropeptides including NPY, NT and AgRP by the feeding-related hormones leptin and insulin.

### IMMORTALIZED CELL LINES TO STUDY GENE REGULATION OF HYPOTHALAMIC FEEDING NEUROPEPTIDES

#### Feeding Regulation

The hypothalamus is a central regulator in feeding behaviour and energy homeostasis. Several distinct hypothalamic nuclei are involved in the regulation of



**Fig. (2).** Model depicting a typical gene and the components involved with activation and deactivation. Expression of genes can be regulated via transcriptional, post-transcriptional and post-translational mechanisms.

appetite and body weight including the lateral hypothalamic area (LH) [28, 29], the paraventricular (PVN) [7, 30], dorsomedial (DMH) [31], ventromedial (VMH) and arcuate (ARC) nuclei [29, 32]. It is now well known that these feeding nuclei are controlled by a neural circuitry comprised of orexigenic and anorectic neuropeptides (Table 2). Energy homeostasis is maintained through the regulation of these neuropeptides by peripheral signals [33]. Leptin and insulin are key peripheral hormones involved in regulating these peptidergic regulators of feeding by altering secretion and gene expression of these neuropeptides [34-39]. The following subsections will discuss how hypothalamic cell lines have advanced our knowledge in the leptin and insulin-mediated regulation of intracellular signaling cascades and neuropeptide gene expression.

**Leptin**

Leptin, an adipocyte-derived hormone and product of the obesity (*ob*) gene, acts on its receptors in the hypothalamus to reduce appetite and body weight [40-42]. Knockout mice without leptin (*ob/ob*) or the leptin receptor (*db/db*) are morbidly obese and infertile, and leptin administration is able to reverse this etiology in *ob/ob* mice [7]. Mice with a brain specific deletion of leptin receptors (ObR(Syn1)KO) are obese and infertile [43]. However, restoration of leptin

**Table 1. Experimental Techniques for Assessing Cellular Function**

Luciferase bioluminescence
Chromatin immunoprecipitation (ChIP)
RT-PCR
Radioactive immunoassays (RIA)
Real-time RT-PCR
Site-directed mutagenesis
RNA interference
Western blot analysis
Mass spectrometry
DNA microarray
Proteomics
mRNA stability assays
Immunocytochemistry (ICC)
DNA Footprinting

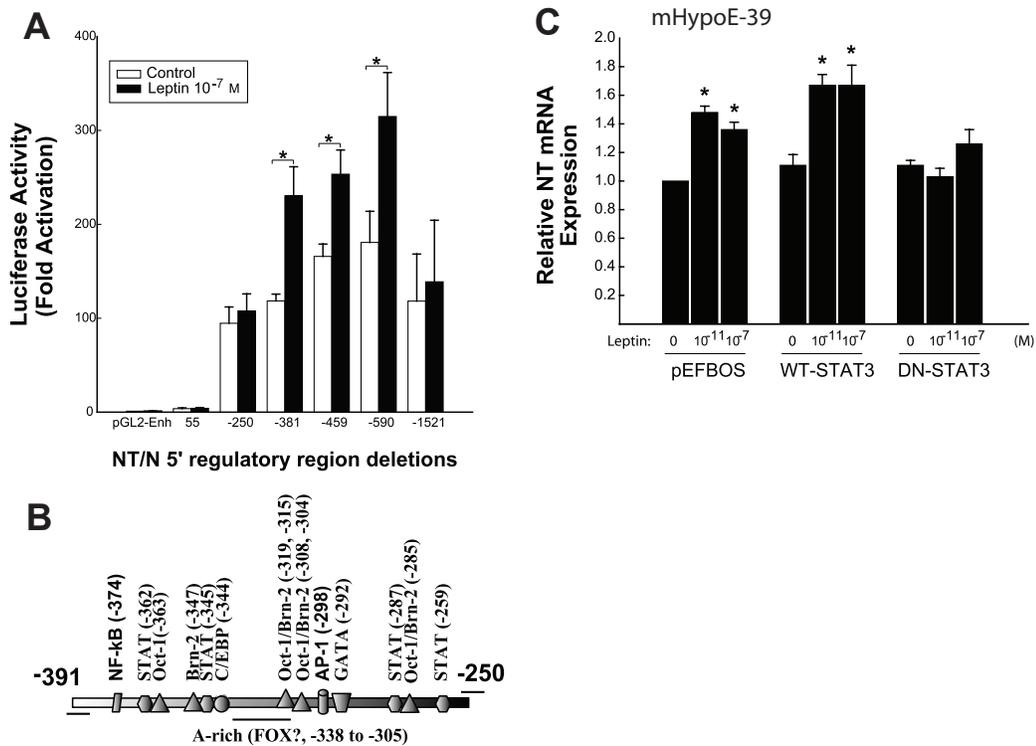
**Table 2. Hypothalamic Neuromodulators Involved in Feeding Behavior and Energy Homeostasis**

Increase Food Intake	Regulation by Insulin/Leptin
NPY	↓
MCH	↓
Galanin	↓
Orexin	?
Ghrelin	↓
Reduce Food Intake	
NT	↑
POMC/MSH	↑
CRH	↑
CCK	?
GLP-1	↑
CART	↑

receptor signaling exclusively in the brain is sufficient to reverse the obesity, infertility and diabetes of *db/db* mice. For that reason, major efforts are currently underway to identify key leptin-responsive hypothalamic cells for potential therapeutic treatments of obesity. The diverse actions of leptin on feeding and energy metabolism suggest differential regulation of neuronal circuitry in the hypothalamus. The recognition of the leptin receptor as a member of the class 1 cytokine receptor superfamily immediately implicated the JAK-STAT cascade as a major pathway in leptin signaling [41, 44]. Continuous leptin injections result in the activation of STAT3 in a number of feeding nuclei in the hypothalamus [44]. However, in addition to STAT3 activation, leptin can achieve highly divergent and apparently cell-specific activation of signal transduction pathways. Cell lines have been instrumental in determining the downstream effectors of leptin action in specific cell populations. Leptin has been shown to activate the phosphatidylinositol-3-kinase (PI3-K) [45], mitogen-activated protein kinase (MAPK) [46-48], AMP-activated kinase [49, 50], and classical janus kinase 2-signal transducer and activator of transcription (JAK-STAT) [44] pathways in a tissue specific manner, indicating leptin stimulation may activate a number of signaling pathways to carry out its diverse set of functions.

Thus far, leptin has been shown to regulate many key neuropeptides involved in appetite regulation (Table 2). NT neurons in particular appear to play an important role downstream of leptin. NT is a 13-amino acid peptide and is expressed throughout the CNS and digestive tract [51, 52]. The most notable functions of NT include feeding suppression, circadian regulation, and secretory stimulation of hypothalamic and anterior pituitary hormones [53-56]. NT neurons in the hypothalamus are responsive to leptin and may modulate the central effects of leptin on feeding behaviour [34-36, 57]. This was demonstrated in *ob/ob* mice

and leptin insensitive *fa/fa* rats, which have decreased NT expression in the hypothalamus. Conversely, central administration of leptin decreased body weight in association with an increase in NT gene expression [36, 57]. Additionally, Sahu *et al* 2001 demonstrated a reversal in leptin's satiety action by either NT immunoneutralization or NT-receptor antagonism [35]. Together, these studies suggest leptin-induced decreases in food intake and body weight may in part be mediated by the regulation of NT. Although *in vivo* studies have observed the overall response of NT to leptin treatments, it is difficult to determine whether the effect of leptin on NT-producing neurons is direct or through afferent connectivity *in vivo*. To study the molecular biology of leptin action on NT-expressing neurons, our lab utilized two hypothalamic cell lines that endogenously produce NT and also express the leptin receptor (ObR), N-39 and N-36/1 (mHypoE-39 and mHypoE-36/1) [58]. We found leptin treatments increased NT gene expression by approximately two-fold in both the mHypoE-39 and mHypoE-36/1 neurons. These results confirm the findings in previous *in vivo* studies, indicating leptin directly stimulates NT expression within the hypothalamus. This increase in NT mRNA was linked to specific signal transduction pathways and regulatory sites in the NT promoter. The leptin-responsive region of the NT gene was determined using a series of transiently transfected 5'-proximal sequential deletions of the 5' flanking region of the mouse NT gene, which was cloned onto a luciferase reporter gene, pGL2-enh. Luciferase activity observed allowed our lab to map the leptin *cis*-regulatory motif to within -381 bp of the NT gene 5' regulatory region (Fig. 3A). To determine whether the classical JAK-STAT leptin signal transduction pathway was important for the regulation of NT gene expression by leptin, we used wild-type and dominant-negative STAT3 protein. Transfection of the dominant negative STAT3 construct attenuated the leptin-mediated increase in NT mRNA compared to control (Fig. 3B). This result indicated STAT3 was essential in the increase of NT gene expression by leptin. Chromatin immunoprecipitation (ChIP) assays were used to confirm STAT3 did in fact bind to *cis*-elements within the -381 to -250 bp fragment of the NT promoter (Fig. 3C). However, additional studies using mHypoE-39 neurons found unique signaling events involved with NT gene expression [59]. Western blot analysis found leptin treatments induced an increase in the phosphorylational status of STAT3, ERK1/2, p38 and ATF-1 in the mHypoE-39 neurons. Pharmacological inhibitors directed against ERK1/2 (U0126) and p38 (SB203580, SB202190 and SB239063) signaling kinases confirmed that these MAP kinases are necessary for the up-regulation of NT transcription by leptin. A combination of studies using electrophoretic mobility shift assays (EMSA) and ChIP analysis then indicated enhanced binding of ATF-1 or c-Fos to the -252 to -36 bp region of the NT gene promoter. Intriguingly, the effects of leptin appear to differentially regulate the binding of ATF-1, c-Fos and STAT3 to the NT promoter depending upon the concentration of leptin used: physiologically relevant leptin concentrations ( $10^{-11}$  M leptin) resulted in a greater abundance of ATF-1 and STAT3 bound to the NT promoter, whereas supraphysiological leptin exposure ( $10^{-7}$  M leptin) resulted in increased c-Fos binding [59]. We propose leptin



**Fig. (3).** Leptin responsiveness can be mapped to a specific region of the mouse NT 5' regulatory region in mHypoE-39 neurons. **A)** The constructs containing indicated lengths of the mouse NT promoter cloned into the promoterless pGL2-enhancer luciferase vector were transiently cotransfected with pCMV {beta}-galactosidase (internal control) into N-39 cells. After transfection, cells were treated with or without 0.5 nM leptin for 48 h. Luciferase activity was normalized to {beta}-galactosidase expression. Each value is expressed as fold-increase relative to pGL2-enhancer vector alone. Values are mean  $\pm$  SEM from three experiments, each done in triplicate. \* $p < 0.05$  versus untreated. **B)** STAT3 is involved in the leptin-mediated induction of NT mRNA expression in mHypoE -39 neurons. Cells were transfected with pEFBOS, wild-type (WT) STAT3, or dominant-negative STAT3 (DN-STAT3) for 18 h and then serum-starved for 2 h and treated with leptin for 4 h at the indicated concentrations. The expression of NT mRNA was determined by real-time RT-PCR. Values for NT are expressed relative to {beta}-actin mRNA levels (mean  $\pm$  SEM;  $n = 4$ ). \* $p < 0.05$  compared with the untreated control by one-way ANOVA followed by Tukey's multiple-comparison test. M, Molecular mass marker size. **C)** Diagram of the NT/N promoter region from -381 to -250. Putative transcription factor binding elements are indicated (as analyzed by Match, BIOBASE Biological Databases), and sequences that were used as oligonucleotide primers in ChIP analysis are underlined. Originally published in [59].

may induce differential signaling and downstream effectors in response to physiological or supraphysiological leptin levels, perhaps providing a new mechanism by which high leptin levels result in aberrant leptin signaling. Overall, our studies offer further evidence that NT neurons may indeed be first-order neurons responsive to leptin. Considering NT is a potent anorexigenic compound, we suggest this may be a mechanism by which NT neurons from the hypothalamus contribute to feeding regulation and energy homeostasis.

### Insulin

Insulin is a peptide hormone that has metabolic effects in virtually all tissues. Disturbances in insulin levels are associated with a variety of metabolic conditions, highlighting the immense importance of insulin in energy homeostasis. Although this role of insulin in peripheral tissues has been extensively studied, the role of insulin in the brain has received significantly less attention. Central insulin action plays a crucial role in the maintenance of energy homeostasis [60]. Although it is still controversial whether insulin is produced in the brain, peripherally circulating insulin does cross the blood-brain-barrier in proportion to serum insulin levels [61]. Insulin receptors (IR) are also

found throughout the brain, with the highest expression in the hypothalamus, olfactory bulb, cerebral cortex, cerebellum and hippocampus [62, 63]. Like leptin, insulin affects both body weight and neuropeptide expression in the hypothalamus (Table 2) [64]. Studies in rats, baboons and humans have demonstrated that intracerebroventricular injection of insulin results in decreased body weight and/or food intake [65-67]. Insulin delivery to the brain has also been shown to suppress hepatic glucose production [68]. The generation of the neuron-specific IR knockout mouse (NIRKO) and neuron-specific IRS-2 knockout mouse, which have an obese and hyperinsulinemic phenotype, have further confirmed the critical role of central insulin action in the regulation of energy metabolism [69, 70]. In order to study the mechanisms involved in the centrally mediated actions of insulin, researchers have investigated the effects of insulin on orexigenic and anorexigenic neurons in the hypothalamus.

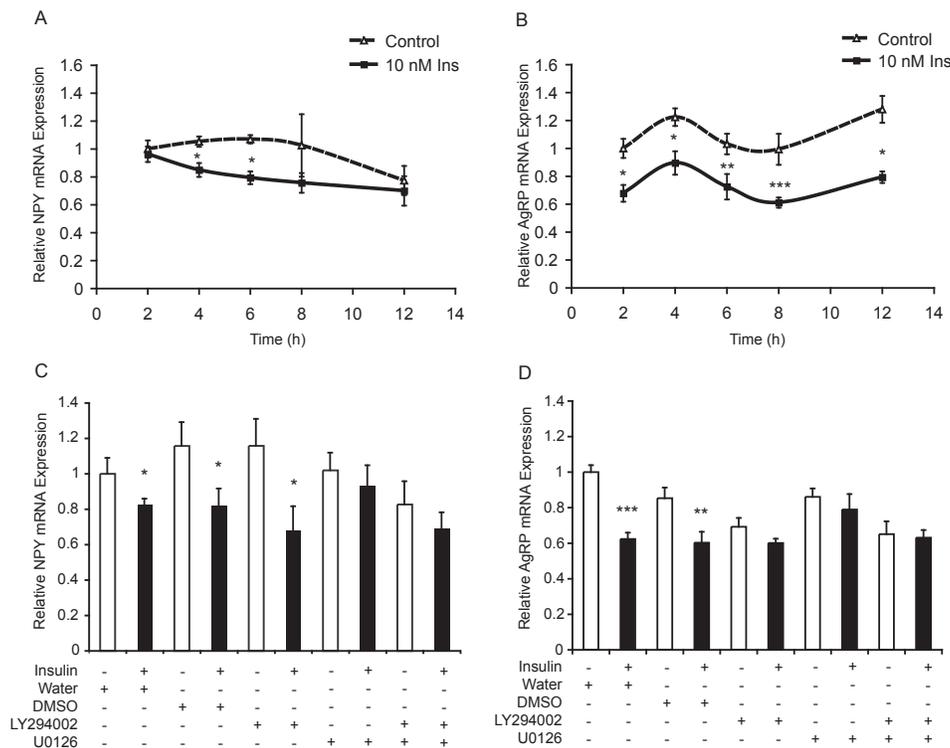
In the ARC of the hypothalamus, insulin has been shown to have actions on both orexigenic NPY/AgRP and anorexigenic POMC neurons [71-74]. In particular, the actions of insulin on NPY/AgRP neurons have been well studied. NPY and AgRP are both orexigenic neuropeptides,

causing increases in food intake and body weight when administered centrally [75, 76]. Antisense oligonucleotides directed against NPY or AgRP mRNA results in decreased food intake and body weight [77-79]. Insulin, which has anorexigenic effects in the CNS, has been found to downregulate NPY and AgRP gene expression and is thought to decrease the firing rate of NPY/AgRP neurons [71-73]. However, studies examining whether the effect of insulin on NPY/AgRP neurons is direct or indirect are conflicting. One group demonstrated that insulin directly activates NPY/AgRP neurons by creating a transgenic mouse that permits the measurement of PI3K in AgRP neurons [80]. By blocking synaptic transmission with either tetrodotoxin or low extracellular calcium, they found that insulin was able to directly activate PI3K. In support of this view, Konner *et al.* found that insulin-stimulated decreases in hepatic glucose output was abolished in mice in which the IR was knocked out in AgRP neurons [81]. In contrast, insulin has been demonstrated to indirectly decrease NPY gene expression via GABAergic neurons through the use of GABA agonists and antagonists in hypothalamic organotypic cultures [82]. Hypothalamic cell models of the NPY/AgRP neuron, which are devoid of afferent neuronal inputs, would be a valuable tool to help clarify the mode of action of insulin on NPY/AgRP neurons. Thus, our group utilized a mouse, hypothalamic, clonal cell model, labeled as mHypoE-46, to investigate this discrepancy. The mHypoE-

46 neurons express NPY, AgRP and the IR, and do not express POMC. We have demonstrated that treatment with insulin results in downregulation of both NPY and AgRP mRNA expression (Fig. 4A), and therefore provide new evidence that insulin can act directly on NPY/AgRP neurons to repress NPY and AgRP mRNA expression [83].

Two major signaling pathways activated by insulin in the periphery are the PI3K-Akt and MAPK MEK/ERK1/2 pathways. In NPY/AgRP neurons, several studies indicate that insulin acts specifically through PI3K [80, 84, 85], and it has been shown that the effects of insulin on food intake are blocked by PI3K inhibitors [85]. However, whether insulin activates either of these pathways to suppress NPY and AgRP gene expression has not yet been resolved. We were able to address this question using our hypothalamic cell lines. We have found that insulin activates the PI3K-Akt pathway in mHypoE-46 cells, and in contrast to previous studies, we also demonstrated that insulin activates the MEK-ERK pathway [83]. Treatment with inhibitors directed against ERK1/2 (U0126) and PI3K (LY294002) revealed that the MAPK MEK-ERK pathway, but not the PI3K pathway, is involved in the insulin-mediated attenuation of NPY and AgRP mRNA expression (Fig. 4B).

ERK1/2 can affect numerous transcription factors that regulate gene transcription. We analyzed the 5' regulatory regions of the human NPY and AgRP gene, since the NPY



**Fig. (4).** A, B: Insulin represses NPY and AgRP mRNA expression. mHypoE-46 cells were treated with 10 nM of insulin (solid line) or vehicle (dotted line). RNA was isolated at 2-12 h and analyzed using real-time RT-PCR with primers for NPY (A) and AgRP (B). C, D: U0126 attenuates insulin repression of NPY and AgRP mRNA expression. mHypoE-46 cells were pre-treated with either vehicle (DMSO), 25  $\mu$ M LY294002 or 10  $\mu$ M U0126 for 1 h and then treated with 10 nM of insulin (black bars) or vehicle (white bars). RNA was isolated after 4 h and analyzed using real-time RT-PCR with primers for NPY (C) or AgRP (D). Data was normalized to gamma-actin and shown with SEM (n = 3-7 independent experiments). \*P < 0.05 \*\*P < 0.01 \*\*\*P < 0.001 versus vehicle control or non-insulin treated paired sample. Originally published in [83].

and AgRP promoter regions of the mouse and rat have not yet been reported, and found binding sites for a variety of transcription factors, including SP-1, GATA, c-Myc, cAMP response element binding (CREB), STAT, c-Fos, c-Jun and Elk-1 [83]. By transiently transfecting luciferase reporter vectors containing the NPY and AgRP 5' regulatory sites in mHypoE-46 cells, we found that insulin treatment did not affect luciferase activity. These findings suggest that insulin does not regulate levels of NPY and AgRP mRNA through regulation of gene transcription, but through the regulation of mRNA stability. However, other explanations exist for this lack of effect on gene transcription. First, the 5' regulatory region used for the NPY and AgRP constructs may not have included potential *cis*-regulatory regions located upstream of the regions analyzed. Secondly, analysis of the human and mouse NPY and AgRP 5' regulatory regions were found to have low sequence similarity, and therefore the human 5' regulatory region may not contain the sequences required for the effects of insulin in mouse cells. Identification of the mouse NPY and AgRP 5' regulatory site will allow a more thorough analysis of potential insulin-responsive regulatory regions. Overall, the employment of hypothalamic cell models has been invaluable towards studying the gene regulation of two key orexigenic peptides, and there is great potential for further study of other components involved in both energy homeostasis and feeding behaviour.

## CONCLUSION

Recent studies have identified a number of hypothalamic neuropeptides important in the control of feeding and energy homeostasis. Despite our extensive knowledge concerning the overall importance of these peptides or receptors as demonstrated by knockout mice models, further investigations of the mechanisms of action of a specific neuropeptide, its gene regulation, and cellular signaling events must be carried out. Classical *in vivo* approaches fail to establish the direct action of an agent on specific hypothalamic neurons or on neuropeptide gene expression. The development of hypothalamic cell models has greatly expanded our understanding of these cellular characteristics. We have developed a novel technology to mass immortalize primary culture from the hypothalamus, generating hypothalamic cell lines from mouse and rat embryonic cultures and mouse adult cultures. A number of these neuronal cell lines express neuropeptides linked to the control of feeding behavior, including NPY, AgRP and NT. As discussed in this review, through careful experimentation, these neuronal cell lines can map the precise regulatory regions, cellular signaling cascades and transcriptional activity of specific neuropeptides. These novel cell models provide an innovative means to study the control of important feeding neuropeptides by hormones and neuromodulators involved with the regulation of energy homeostasis and the molecular events involved in the development of hormone resistance, a tremendous problem for obesity therapeutics.

## ACKNOWLEDGEMENT

We acknowledge funding from Canadian Institutes for Health Research, Natural Sciences and Engineering Research Council, Canadian Diabetes Association, Canada Foundation

for Innovation, and the Canada Research Chairs Program to DDB.

## REFERENCES

- [1] Faouzi M, Leshan R, Bjornholm M, *et al.* Differential accessibility of circulating leptin to individual hypothalamic sites. *Endocrinology* 2007; 148: 5414-23.
- [2] Kopelman PG. Obesity as a medical problem. *Nature* 2000; 404: 635-43.
- [3] Sahu A. Leptin signaling in the hypothalamus: emphasis on energy homeostasis and leptin resistance. *Front Neuroendocrinol* 2003; 24: 225-53.
- [4] Everitt BJ, Hokfelt T. Neuroendocrine anatomy of the hypothalamus. *Acta Neurochir Suppl (Wien)* 1990; 47: 1-15.
- [5] Friedman JM. A war on obesity, not the obese. *Science* 2003; 299: 856-8.
- [6] Smith MS, Grove KL. Integration of the regulation of reproductive function and energy balance: lactation as a model. *Front Neuroendocrinol* 2002; 23: 225-56.
- [7] Elmquist JK, Elias CF, Saper CB. From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron* 1999; 22: 221-32.
- [8] Dufourmy L, Warembourg M, Jolivet A. Quantitative studies of progesterone receptor and nitric oxide synthase colocalization with somatostatin, or neurotensin, or substance P in neurons of the guinea pig ventrolateral hypothalamic nucleus: an immunocytochemical triple-label analysis. *J Chem Neuroanat* 1999; 17: 33-43.
- [9] Hoffman GE, Smith MS, Verbalis JG. c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Front Neuroendocrinol* 1993; 14: 173-213.
- [10] Earnest DJ, Liang FQ, DiGiorgio S, *et al.* Establishment and characterization of adenoviral E1A immortalized cell lines derived from the rat suprachiasmatic nucleus. *J Neurobiol* 1999; 39: 1-13.
- [11] Zakaria M, Dunn IC, Zhen S, *et al.* Phorbol ester regulation of the gonadotropin-releasing hormone (GnRH) gene in GnRH-secreting cell lines: a molecular basis for species differences. *Mol Endocrinol* 1996; 10: 1282-91.
- [12] Mellon PL, Windle JJ, Goldsmith PC, *et al.* Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. *Neuron* 1990; 5: 1-10.
- [13] Belsham DD, Cai F, Cui H, *et al.* Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders. *Endocrinology* 2004; 145: 393-400.
- [14] Wetsel WC. Immortalized hypothalamic luteinizing hormone-releasing hormone (LHRH) neurons: a new tool for dissecting the molecular and cellular basis of LHRH physiology. *Cell Mol Neurobiol* 1995; 15: 43-78.
- [15] Radovick S, Wray S, Lee E, *et al.* Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice. *Proc Natl Acad Sci USA* 1991; 88: 3402-6.
- [16] Matsushita T, Amagai Y, Terai K, *et al.* A novel neuronal cell line derived from the ventrolateral region of the suprachiasmatic nucleus. *Neuroscience* 2006; 140: 849-56.
- [17] Elias CF, Saper CB, Maratos-Flier E, *et al.* Chemically defined projections linking the mediobasal hypothalamus and the lateral hypothalamic area. *J Comp Neurol.* 1998; 402:442-59.
- [18] Hahn TM, Breininger JF, Baskin DG, Schwartz MW. Coexpression of *Agrp* and *NPY* in fasting-activated hypothalamic neurons. *Nat Neurosci* 1998; 1: 271-2.
- [19] Elias CF, Lee C, Kelly J, *et al.* Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 1998; 21: 1375-85.
- [20] Gingerich S, Wang X, Lee PK, *et al.* The generation of an array of clonal, immortalized cell models from the rat hypothalamus: Analysis of melatonin effects on kisspeptin and gonadotropin-inhibitory hormone neurons. *Neuroscience* 2009; 162: 1134-40.
- [21] Maniatis T, Goodbourn S, Fischer JA. Regulation of inducible and tissue-specific gene expression. *Science* 1987; 236: 1237-45.
- [22] Goodbourn S, Burstein H, Maniatis T. The human beta-interferon gene enhancer is under negative control. *Cell* 1986; 45: 601-10.
- [23] Delcuve GP, Rastegar M, Davie JR. Epigenetic control. *J Cell Physiol* 2009; 219: 243-50.
- [24] Henkin TM. RNA-dependent RNA switches in bacteria. *Methods Mol Biol* 2009; 540: 207-14.

- [25] Eraly SA, Mellon PL. Regulation of gonadotropin-releasing hormone transcription by protein kinase C is mediated by evolutionarily conserved promoter-proximal elements. *Mol Endocrinol* 1995; 9: 848-59.
- [26] Kepa JK, Wang C, Neeley CI, *et al.* Structure of the rat gonadotropin releasing hormone (rGnRH) gene promoter and functional analysis in hypothalamic cells. *Nucleic Acids Res* 1992; 20: 1393-9.
- [27] Whyte DB, Lawson MA, Belsham DD, *et al.* A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. *Mol Endocrinol* 1995; 9: 467-77.
- [28] Horvath TL, Diano S, van den Pol AN. Synaptic interaction between hypocretin (orexin) and neuropeptide Y cells in the rodent and primate hypothalamus: a novel circuit implicated in metabolic and endocrine regulations. *J Neurosci* 1999; 19: 1072-87.
- [29] Broberger C, De Lecea L, Sutcliffe JG, Hokfelt T. Hypocretin/orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: relationship to the neuropeptide Y and agouti gene-related protein systems. *J Comp Neurol* 1998; 402: 460-74.
- [30] Elmquist JK, Bjorbaek C, Ahima RS, Flier JS, Saper CB. Distributions of leptin receptor mRNA isoforms in the rat brain. *J Comp Neurol* 1998; 395: 535-47.
- [31] Kalra SP, Dube MG, Pu S, *et al.* Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev* 1999; 20: 68-100.
- [32] Hahn TM, Breininger JF, Baskin DG, Schwartz MW. Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nat Neurosci* 1998; 1: 271-2.
- [33] Ahima RS, Osei SY. Molecular regulation of eating behavior: new insights and prospects for therapeutic strategies. *Trends Mol Med* 2001; 7: 205-13.
- [34] Richy S, Bulet A, Max J, Bulet C, Beck B. Effect of chronic intraperitoneal injections of leptin on hypothalamic neurotensin content and food intake. *Brain Res* 2000; 862: 276-9.
- [35] Sahu A, Carraway RE, Wang YP. Evidence that neurotensin mediates the central effect of leptin on food intake in rat. *Brain Res* 2001; 888: 343-7.
- [36] Sahu A. Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. *Endocrinology* 1998; 139: 795-8.
- [37] Watanobe H, Habu S. Leptin regulates growth hormone-releasing factor, somatostatin, and alpha-melanocyte-stimulating hormone but not neuropeptide Y release in rat hypothalamus *in vivo*: relation with growth hormone secretion. *J Neurosci* 2002; 22: 6265-71.
- [38] Cone RD. The central melanocortin system and energy homeostasis. *Trends Endocrinol Metab* 1999; 10: 211-6.
- [39] Elias CF, Aschkenasi C, Lee C, *et al.* Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 1999; 23: 775-86.
- [40] Elmquist JK, Maratos-Flier E, Saper CB, Flier JS. Unraveling the central nervous system pathways underlying responses to leptin. *Nat Neurosci* 1998; 1: 445-50.
- [41] Vaisse C, Halaas JL, Horvath CM, *et al.* Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice. *Nat Genet* 1996; 14: 95-7.
- [42] Zhang Y, Proenca R, Maffei M, *et al.* Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; 372: 425-32.
- [43] Cohen P, Zhao C, Cai X, *et al.* Selective deletion of leptin receptor in neurons leads to obesity. *J Clin Invest* 2001; 108: 1113-21.
- [44] Hubschle T, Thom E, Watson A, *et al.* Leptin-induced nuclear translocation of STAT3 immunoreactivity in hypothalamic nuclei involved in body weight regulation. *J Neurosci* 2001; 21: 2413-24.
- [45] Zhao AZ, Huan JN, Gupta S, Pal R, Sahu A. A phosphatidylinositol 3-kinase phosphodiesterase 3B-cyclic AMP pathway in hypothalamic action of leptin on feeding. *Nat Neurosci* 2002; 5: 727-8.
- [46] Catalano S, Mauro L, Marsico S, *et al.* Leptin induces, via ERK1/ERK2 signal, functional activation of estrogen receptor alpha in MCF-7 cells. *J Biol Chem* 2004; 279: 19908-15.
- [47] Bjorbaek C, Buchholz RM, Davis SM, *et al.* Divergent roles of SHP-2 in ERK activation by leptin receptors. *J Biol Chem* 2001; 276: 4747-55.
- [48] Banks AS, Davis SM, Bates SH, Myers MG, Jr. Activation of downstream signals by the long form of the leptin receptor. *J Biol Chem* 2000; 275: 14563-72.
- [49] Minokoshi Y, Alquier T, Furukawa N, *et al.* AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature* 2004; 428: 569-74.
- [50] Andersson U, Filipsson K, Abbott CR, *et al.* AMP-activated protein kinase plays a role in the control of food intake. *J Biol Chem* 2004; 279: 12005-8.
- [51] Carraway R, Leeman SE. The amino acid sequence of a hypothalamic peptide, neurotensin. *J Biol Chem* 1975; 250: 1907-11.
- [52] Carraway R, Leeman SE. The isolation of a new hypotensive peptide, neurotensin, from bovine hypothalamus. *J Biol Chem* 1973; 248: 6854-61.
- [53] Rostene WH, Alexander MJ. Neurotensin and neuroendocrine regulation. *Front Neuroendocrinol* 1997; 18: 115-73.
- [54] Dobner PR, Fadel J, Deitemeyer N, Carraway RE, Deutch AY. Neurotensin-deficient mice show altered responses to antipsychotic drugs. *Proc Natl Acad Sci USA* 2001; 98: 8048-53.
- [55] Meyer-Spasche A, Reed HE, Piggins HD. Neurotensin phase-shifts the firing rate rhythm of neurons in the rat suprachiasmatic nuclei *in vitro*. *Eur J Neurosci* 2002; 16: 339-44.
- [56] Remaury A, Vita N, Gendreau S, *et al.* Targeted inactivation of the neurotensin type 1 receptor reveals its role in body temperature control and feeding behavior but not in analgesia. *Brain Res* 2002; 953: 63-72.
- [57] Beck B, Stricker-Krongrad A, Richy S, Bulet C. Evidence that hypothalamic neurotensin signals leptin effects on feeding behavior in normal and fat-preferring rats. *Biochem Biophys Res Commun* 1998; 252: 634-8.
- [58] Cui H, Cai F, Belsham DD. Anorexigenic hormones leptin, insulin, and alpha-melanocyte-stimulating hormone directly induce neurotensin (NT) gene expression in novel NT-expressing cell models. *J Neurosci* 2005; 25: 9497-506.
- [59] Cui H, Cai F, Belsham DD. Leptin signaling in neurotensin neurons involves STAT, MAP kinases ERK1/2, and p38 through c-Fos and ATF1. *FASEB J* 2006; 20: 2654-6.
- [60] Plum L, Belgardt BF, Bruning JC. Central insulin action in energy and glucose homeostasis. *J Clin Invest* 2006; 116: 1761-6.
- [61] Baura GD, Foster DM, Porte D, Jr, *et al.* Saturable transport of insulin from plasma into the central nervous system of dogs *in vivo*: a mechanism for regulated insulin delivery to the brain. *J Clin Invest* 1993; 92: 1824-30.
- [62] van Houten M, Posner BI, Kopriwa BM, Brawer JR. Insulin-binding sites in the rat brain: *in vivo* localization to the circumventricular organs by quantitative radioautography. *Endocrinology* 1979; 105: 666-73.
- [63] Havrankova J, Roth J, Brownstein M. Insulin receptors are widely distributed in the central nervous system of the rat. *Nature* 1978; 272: 827-9.
- [64] Baskin DG, Figlewicz LD, Seeley RJ, *et al.* Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res* 1999; 848: 114-23.
- [65] Grossman AB, Rossmann WG, Kabigting EB, *et al.* The distribution of hypothalamic nitric oxide synthase mRNA in relation to gonadotrophin-releasing hormone neurons. *J Endocrinol* 1994; 140: 5-8.
- [66] Woods SC, Lotter EC, McKay LD, Porte D, Jr. Chronic intracerebroventricular infusion of insulin reduces food intake and body weight of baboons. *Nature* 1979; 282: 503-5.
- [67] Hallschmid M, Benedict C, Schultes B, *et al.* Intranasal insulin reduces body fat in men but not in women. *Diabetes* 2004; 53: 3024-9.
- [68] Obici S, Zhang BB, Karkanas G, Rossetti L. Hypothalamic insulin signaling is required for inhibition of glucose production. *Nat Med* 2002; 8: 1376-82.
- [69] Bruning JC, Gautam D, Burks DJ, *et al.* Role of brain insulin receptor in control of body weight and reproduction. *Science* 2000; 289: 2122-5.
- [70] Taguchi A, Wartschow LM, White MF. Brain IRS2 signaling coordinates life span and nutrient homeostasis. *Science* 2007; 317: 369-72.
- [71] Spanswick D, Smith MA, Mirshamsi S, Routh VH, Ashford ML. Insulin activates ATP-sensitive K<sup>+</sup> channels in hypothalamic neurons of lean, but not obese rats. *Nat Neurosci* 2000; 3: 757-8.

- [72] Schwartz MW, Marks JL, Sipols AJ, *et al.* Central insulin administration reduces neuropeptide Y mRNA expression in the arcuate nucleus of food-deprived lean (Fa/Fa) but not obese (fa/fa) Zucker rats. *Endocrinology* 1991; 128: 2645-7.
- [73] Wang J, Leibowitz KL. Central insulin inhibits hypothalamic galanin and neuropeptide Y gene expression and peptide release in intact rats. *Brain Res* 1997; 777: 231-6.
- [74] Benoit SC, Air EL, Coolen LM, *et al.* The catabolic action of insulin in the brain is mediated by melanocortins. *J Neurosci* 2002; 22: 9048-52.
- [75] Stanley BG, Kyrkouli SE, Lampert S, Leibowitz SF. Neuropeptide Y chronically injected into the hypothalamus: a powerful neurochemical inducer of hyperphagia and obesity. *Peptides* 1986; 7: 1189-92.
- [76] Rossi M, Kim MS, Morgan DG, *et al.* A C-terminal fragment of Agouti-related protein increases feeding and antagonizes the effect of alpha-melanocyte stimulating hormone *in vivo*. *Endocrinology* 1998; 139: 4428-31.
- [77] Marin-Bivens CL, Kalra SP, Olster DH. Intraventricular injection of neuropeptide Y antisera curbs weight gain and feeding, and increases the display of sexual behaviors in obese Zucker female rats. *Regul Pept* 1998; 75-76: 327-34.
- [78] Akabayashi A, Wahlestedt C, Alexander JT, Leibowitz SF. Specific inhibition of endogenous neuropeptide Y synthesis in arcuate nucleus by antisense oligonucleotides suppresses feeding behavior and insulin secretion. *Brain Res Mol Brain Res* 1994; 21: 55-61.
- [79] Makimura H, Mizuno TM, Mastaitis JW, Agami R, Mobbs CV. Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci* 2002; 3: 18.
- [80] Xu AW, Kaelin CB, Takeda K, *et al.* PI3K integrates the action of insulin and leptin on hypothalamic neurons. *J Clin Invest* 2005; 115: 951-8.
- [81] Konner AC, Janoschek R, Plum L, *et al.* Insulin action in AgRP-expressing neurons is required for suppression of hepatic glucose production. *Cell Metab* 2007; 5: 438-49.
- [82] Sato I, Arima H, Ozaki N, *et al.* Insulin inhibits neuropeptide Y gene expression in the arcuate nucleus through GABAergic systems. *J Neurosci* 2005; 25: 8657-64.
- [83] Mayer CM, Belsham DD. Insulin directly regulates NPY and AgRP gene expression via the MAPK MEK/ERK signal transduction pathway in mHypoE-46 hypothalamic neurons. *Mol Cell Endocrinol* 2009; 307: 99-108.
- [84] Mirshamsi S, Laidlaw HA, Ning K, *et al.* Leptin and insulin stimulation of signalling pathways in arcuate nucleus neurones: PI3K dependent actin reorganization and KATP channel activation. *BMC Neurosci* 2004; 5: 54.
- [85] Niswender KD, Morrison CD, Clegg DJ, *et al.* Insulin activation of phosphatidylinositol 3-kinase in the hypothalamic arcuate nucleus: a key mediator of insulin-induced anorexia. *Diabetes* 2003; 52: 227-31.

---

Received: May 29, 2009

Revised: August 05, 2009

Accepted: August 06, 2009

© Dhillon *et al.*; Licensee Bentham Open.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.