LEPTIN REGULATES PROTHYROTROPIN-RELEASING HORMONE (PROTRH) BIOSYNTHESIS: Evidence for direct and indirect pathways

Eduardo A. Nillni¹, Charles Vaslet¹, Mark Harris², Anthony Hollenberg², Christian Bjorbaek², Jeffrey S. Flier².

¹Division of Endocrinology, Brown University School of Medicine, Rhode Island Hospital, Providence RI 02903. ²Division of Endocrinology, Harvard Medical School. Beth Israel Deaconess Medical Center, Boston MA 02215

Address all correspondence and requests for reprints to:

Dr. Eduardo A. Nillni
Division of Endocrinology
Rhode Island Hospital
593 Eddy Street
Providence, RI 02903
Tel: (401) 444-5733
Fax: (401) 444-6964
E-mail: Eduardo_Nillni@Brown.edu

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ABSTRACT

The hypothalamic-pituitary-thyroid (HPT) axis is down-regulated during starvation, and falling levels of leptin are a critical signal for this adaptation, by acting to suppress preproThyrotropin releasing hormone (preproTRH) mRNA expression in the paraventricular nucleus of the hypothalamus. This study addresses the mechanism for this regulation, using primary cultures of fetal rat hypothalamic neurons as a model system. Leptin dose-dependently stimulated a 10-fold increase in proTRH biosynthesis, with a maximum response at 10 nM. TRH release was quantified using immunoprecipitation followed by isofocusing gel electrophoresis, and by specific TRH radioimmunoassay. Leptin stimulated TRH release by 7-fold. Immunocytochemistry revealed a substantial population of cells expresses TRH or leptin receptors and 8-13% of those expressing leptin receptors co-expressed TRH. Leptin produced a 5 fold induction of luciferase activity in CV-1 cells transfected with a TRH promoter and the long form of the leptin receptor cDNA.

Although the above data are consistent with a direct ability of leptin to promote TRH biosynthesis through actions on TRH neurons, addition of α-MSH produced a 3.5 fold increase in TRH biosynthesis and release, whereas NPY treatment suppressed ~3 fold proTRH biosynthesis. Furthermore, the MC4R antagonist, SHU9119 partially inhibited leptin stimulated TRH release from the neuronal culture. Consequently, our data suggest that leptin regulates the TRH neurons through both direct and indirect pathways.
INTRODUCTION

Food deprivation in animals and humans results in endocrine and metabolic changes that include decreases in circulating levels of thyroid hormones (1,2). Previous work in starved rats has shown that this is associated with a decrease in hypothalamic, but not thalamic reticular preproTRH mRNA, supporting the concept that the hypothyroidism of starvation is of hypothalamic origin (1). Leptin is a recently discovered peptide hormone that is synthesized and released by adipose tissue (3-6). Serum leptin levels decrease during starvation and it has been proposed to be a major regulator of the central nervous system mediated adaptation to starvation (2). Absence of leptin is responsible for the obese phenotype of ob/ob mice, and administration of this hormone to these animals reverses many of the endocrine defects (3-6).

It was recently suggested that leptin has an important role in the neuroendocrine regulation of the HPT axis (2,7,8). During prolonged fasting in rats, low levels of T3 and T4 are observed, and TSH is in the low to normal range. This is due in part to fasting-induced suppression of preproTRH gene expression in paraventricular nucleus of the hypothalamus (PVN) neurons. Since the decrease in thyroid hormone levels is blunted in fasted mice and rats by systemic administration of leptin (2,9), it has been proposed that the decrease in leptin during fasting alters the set point for feedback inhibition by thyroid hormones on preproTRH mRNA biosynthesis (8). The mechanism by which leptin regulates energy expenditure through the HPT axis is unknown. Leptin has direct actions on cell bodies in the arcuate nucleus, positively regulating pro-opiomelanocortin (POMC), and thus α-MSH, and negatively regulating the appetite-stimulating peptide, neuropeptide (NPY) and the Agouti-related peptide (AgRP) (10). NPY afferents on TRH neurons are proposed to be inhibitory. Thus the effect of leptin on TRH could be indirect, mediated by those arcuate neurons. In this study, using primary cultures of hypothalamic neurons that express high levels of endogenous proTRH (7,11), we examined the
hypothesis that leptin can regulate proTRH biosynthesis and TRH release by a
direct action on TRH neurons. To this end we determined the colocalization of
proTRH with the leptin receptor (ObR), investigated the ability of leptin to
activate the preproTRH promoter, and demonstrated that leptin induces
proTRH biosynthesis and release. To support an additional indirect regulatory
role of leptin on proTRH, we determined effects of α-MSH and NPY on the
biosynthesis of proTRH.
MATERIALS AND METHODS

Animals

The normal and timed-pregnant female Sprague-Dawley rats used in these studies were purchased from Charles River Laboratories (Wilmington, MA and Kingston, NY). The experimental protocols and euthanasia procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Rhode Island Hospital/ Brown University.

Tissue Culture

Primary cultures of hypothalamic neurons

Fetal rat hypothalamic cells (day 17) were prepared as described earlier by us (11). In brief, timed pregnant female rats on day 17 of gestation were anesthetized with pentobarbital (60 mg/kg). The diencephalon from fetuses was removed and dispersed enzymatically with neutral proteases (100 U/ml) for 2 hours. The dispersed cells were then plated (5 x 10^6 cells/ml) in tissue culture flasks for radiolabelling and release studies. All cells were plated in wells precoated with 20 µg/ml poly-D-lysine (Sigma). The cells were maintained in bicarbonate-buffered Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco BRL, Gaithersburg, MD), at 37 °C, 5% CO2 and 95% humidity. To induce differentiation of neuronal cells and preproTRH biosynthesis, the cells were cultured with 50 µM BrdU during the first four days as previously described (11). For immunocytochemistry (ICC) experiments, the cells were plated on 4-chamber glass LabTek (Nunc Inc., Naperville, IL) at low density (5 x 10^5 cells/ml).
CV-1 cells with the TRH promoter

The human TRH promoter and RSV180 were both cloned into the vector pA3 luc, as previously described (12). The ObRb and STAT3 constructs have been described previously (13). CV-1 cells were grown as described previously (14). The cells were seeded in 6 well plates and transfected in triplicate using the calcium-phosphate method. Each well received 1.7 ug of reporter, 0.8 ug of STAT3 and 0.8 ug of ObRb expression vectors. In addition, each well received 20 ng of a CMV β-galactosidase expression vector to control for transfection efficiency. Total amounts of DNA were held constant. Eighteen hours after transfection the cells were washed and placed in normal media. Thirty hours after transfection the cells were incubated with serum free media. Forty hours after transfection 100 nM leptin was added or not. The cells were assayed for luciferase and β-galactosidase enzyme activity.

CHO cells expressing leptin receptor

CHO cells stably expressing the short form of the leptin receptor, ObRa, were grown and generated as described earlier (13).

Mouse corticotropic AtT-20 cells

AtT-20 were grown in 75 cm² flasks at 37°C in an atmosphere of 5% CO₂, 95% air, and 90% humidity (15). Each flask was plated with 5 million cells and cultures were maintained for 6 days in Dulbecco's-Modified Eagle's Essential Medium, D-MEM (Gibco, MD) containing 10% Fetal Calf Serum (FCS) as previously described (15). Culture medium was removed every two days and replaced with fresh medium. Experiments were performed at a confluency corresponding to 20-30 x 10⁶ cells.
Quantitative $^{32}$P-RT-PCR of ObRb mRNA from fetal hypothalamic neurons.

ObRb mRNA was quantified as described earlier except for a few changes (16). Briefly, total RNA was isolated and 0.20 ug were subjected to cDNA synthesis (100 ul total), with or without reverse transcriptase. For amplification of rat β-actin cDNA, the following primers were used; upstream primer: 5'-TTGTAACCAACTGGGACGATATGG-3' and downstream primer: 5'-GATCTT-GATCTTCATGGTGCTAGG-3'. The following primers were used for specific PCR amplification of rat ObRb cDNA; upstream primer: 5'-TATGTCATTGTAACCGATAATTATT-3', and downstream primer: 5'-CCCCTTGGAATCTGGGAATGTT-3'. Each 50 ul PCR reaction was carried out with 5.0 ul of cDNA as template and 0.50 ul of α-$^{32}$P-dCTP (29.6 TBq/ mmol, 370 MBq/ ml)(NEN, Boston, MA). The samples were subjected to 18 cycles of amplification for β-actin and 30 cycles for ObRb. Five ul of the reaction were run on a denaturing urea-acrylamide gel. The gel was then dried and finally subjected to $^{32}$P PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA).

Radiolabeling experiments

After 12 days in culture 5 x 10$^6$ hypothalamic neurons (per flask) were stimulated with increasing concentrations of leptin for a period of 6 hours in low leucine, serum-deprived D-MEM medium. The cells were pulsed for the entire 6 hours with 0.3 mCi of (3,4,5, $^3$H)-leucine (156 Ci/ mmol) for proTRH and processing products radiolabelling and 0.3 mCi of (L-2,3,4,5$^3$H)-proline (100 Ci/ mmol) for TRH radiolabelling. For long term labelling we utilized 90% (9 volumes) leucine-free medium mixed with 1 volume of regular medium. After the incubations, the medium was removed and the cells washed three times.
After the last wash the cells were rapidly cooled on ice, and 2 ml of 2 N acetic acid containing 2 mM EDTA, 2 mM EGTA and enzyme inhibitors (phenylmethylsulphonylfluoride, aprotinin, bacitracin, bestatin, and pepstatin, each at 0.1%) were added. The cells were scraped and heated to 95°C for 10 minutes prior to sonication. One hundred µl of sample was removed for protein assay. The remainder of the cell extract was centrifuged at 15,000 rpm for 30 minutes. The supernatant was then lyophilized and held at -20°C until analyzed by polyacrylamide gel electrophoresis. For TRH analysis, media from these cultures was immunoprecipitated with anti-TRH antibodies followed by Isofocusing gel electrophoresis (IEF, see below).

Immunoprecipitation

An immunoprecipitation protocol was carried out as described previously (17). Briefly, lyophilized cell extracts were resuspended in 10 µl of 0.2% bovine serum albumin (BSA) and 200 µl of hypotonic buffer A (10 mM NaPO₄, pH 7.2/1 mM EDTA/0.1% Triton X-100). Following resuspension, cell extracts were incubated for 24 hours at 4°C with 1:500 dilution of protein G purified anti-proTRH₁₁₅₋₁₅₁ (15). Then, 1:1000 dilution of goat-anti-rabbit IgG was added along with 75 µl of buffer B (500 mM KCl/50 mM NaH₂PO₄, pH 7.4/5 mM NaEDTA/0.25% Triton X-100). Samples were further incubated for 4 hours at 4°C. Immunoprecipitates of cell extracts were washed once with buffer B and once with buffer C (10 mM NaH₂PO₄, pH 7.2/15 mM NaCl) which removes EDTA and Triton X-100. The immunoprecipitates were then resuspended in sample buffer (0.0625 M Tris, pH 6.8/1% SDS/15% glycerol/15 mM dithiothreitol), and boiled for 4 minutes prior to SDS-PAGE. Release media obtained from proline labelled cultures was immunoprecipitated with anti-TRH antibodies as described above and resuspended in sample buffer prior to IEF.
Radioactive or cold samples were fractionated by mobility by loading onto a discontinuous Tricine-polyacrylamide gel electrophoresis (SDS-PAGE) system for separation of low molecular weight peptides (18). A stacking gel was made to 3% crosslinking (acrylamide/bis solution) and the separating gel was made to 6% crosslinking (acrylamide/bis solution). Gels were run in the Protean 16 cm cell system (Bio-Rad, Richmond, CA). Following electrophoresis, gels were cut into 1mm slices in a gel slicer (Hoefer Scientific Instruments, San Francisco, CA), and prepared for either counting or radioimmunoassay. For tritium analysis, immunoprecipitated peptides were extracted from gel slices by incubation in 1ml of 1N acetic acid for 24 hours at 4°C. Scintillation fluid (Bio Safe II, RPI, IL) was added and samples were counted in a scintillation counter. Preparation for radioimmunoassay (RIA) included the same acetic acid extraction as described above, but, following incubation, gel slices were removed. Samples were then lyophilized and resuspended in the appropriate RIA buffer. Recovery of peptides from gel slices has been shown to be approximately 90% as determined by RIA prior to and following the electrophoresis. To identify the apparent molecular weight of fractionated peptides on SDS-PAGE, a series of molecular weight markers were used. Prestained bovine serum albumin, 80.0kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; lysozyme, 18.5 kDa (Bio-Rad, Richmond, CA); trypsin inhibitor, 20.4 kDa; myoglobin, 16.95 kDa; myoglobin fragment IV, 14.4 kDa; myoglobin fragment III, 8.16 kDa; myoglobin fragment II, 6.2 kDa; myoglobin fragment I, 2.5 kDa (Diversified Biotech, Newton, MA).

Isofocusing gel electrophoresis (IEF)
Immunoprecipitated $^3$H-proline-TRH samples with anti-TRH antibodies were fractionated according to an estimated isoelectric point for TRH (~7.00) based on theoretical calculations using MacVector software for protein analysis, and using SWISS-PROT annotated protein sequence database, TrEMBL Computer-annotated supplement to SWISS-PROT (~6.8-7.00). Samples dissolved in sample buffer (6 M urea, 1% chaps, 80 mM DTT, 1.6% Pharmalyte 5-8, 0.4% pharmalyte 2.5-5) were run onto 30% acrylamide/1% bis acrylamide gel tubes in a buffer containing 6 M urea, 1% chaps. To generate a 4.6 to 8.00 pH gradient, 1% Pharmalyte 2.5-5 and 4% Pharmalyte 5-8 (Pharmacia, LKB) was added to the solution. The pH gradient was pre-established by subjecting the gel tubes to 100 Volts for 15 minutes and 200 volts for another 15 minutes utilizing 100mM NaOH in the upper chamber and 10mM H$_3$PO$_4$ in the lower chamber. After the pre-run, samples were loaded and they were run for 2.5 hours at 400 volts. After the run each gel tube was sliced (0.5cm) and put in scintillation vials containing 2N acetic acid, keep at 4°C for three days and counted. The pH gradient was determined by measuring the pH in each slice. After the run a gel tube without sample was incubated in 2 ml of 50 mM KCl under vacuum overnight and the pH was measured with a pH meter, and using Bio-Rad’s IEF standards, a mixture of nine natural proteins with isoelectric points ranging from 4.45 to 9.0 (Bio-Rad, California)

TRH radioimmunoassays (RIA)

RIA for TRH was performed in triplicate samples removed from non radioactive neuronal cells previously exposed to increasing concentrations of leptin. The protocol for TRH RIA is standard in our laboratory and has been published elsewhere (19).

Double label immunocytochemistry (dICC)
Hypothalamic neurons (3x10⁵) from 12 day old cultures were fixed with 4% paraformaldehyde in PBS and subjected to an immunocytochemistry protocol as previously described (11,18). CHO cells transfected with an empty vector or expressing the short form of the leptin receptor were subjected to immunostaining using an antibody against the common extracellular domain of the leptin receptor. Generation of this polyclonal antibody was done as described earlier (20).

Immunoreaction of the primary anti-ObR antibody with the hypothalamic neurons was performed at 4°C for 24 hours. Goat anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate (FITC) was used as the fluorescence marker. A wide range of dilutions for the primary and secondary antibodies was tested. The optimal dilutions were found to be 1:1,000 for the primary antibody and 1:2,000 for the secondary antibody with an incubation time of 24 hours at 4°C for the primary antibody and 2 hours at room temperature for the secondary antibody. Control experiments including the incubation of cells without primary antibody or preimmune sera, and the blocking of the primary antibody with synthetic peptides against which the antibody was generated, were performed and did not show any positive staining. For colocalization experiments, cells previously stained with anti-ObR followed with FITC probe (green color) were then incubated with anti-pAV₃⁷-Texas Red or anti-pST₁₀-Texas Red for 24 hours at 4°C. Anti-pAV₃⁷ recognizes the TRH prohormone and anti-pST₁₀ recognizes the end product of processing preproTRH₁₆₀-₁₆₉ (11). The conjugation of Texas Red (red color) to these antibodies, as well as their ability to obtain successful colocalization with other proteins was previously described by us, in experiments demonstrating the colocalization of proTRH with the prohormone convertase 1 (18).

Statistics
RIA values were plotted against the gel slice number corresponding to a particular molecular mass peptide generated graphs. Protein assay results were used to correct for minor variations in total cell number. Data were displayed as ng/ml. Analysis of variance (ANOVA) followed by a multiple comparison (Tukey-Kramer test) was employed when appropriate.
RESULTS

Leptin stimulates proTRH biosynthesis in primary cultures of hypothalamic neurons.

We have previously used primary cultures of fetal rat hypothalamic neurons to study proTRH biosynthesis and processing (11,21). Using this primary cell system, we examined the effect of leptin on the biosynthesis of proTRH. After 12 days in culture, primary cultures of hypothalamic neurons were stimulated with leptin for 6 hours in the presence of radioactive leucine (leucine is present 21 times in the proTRH sequence). After treatment, radioactive peptides were acid extracted and subjected to double-immunoprecipitation with an antibody, previously characterized (18) by us, directed against the propreTRH116-151 (anti-pAV37), which recognizes the TRH precursor (26 kDa) and an intermediate form of its processing of about 16 kDa. The immunoprecipitates were then subjected to separation on a Tricine-SDS-PAGE system followed by slicing and radioactive counting. A typical SDS-PAGE profile of immunoprecipitated proTRH and the intermediate form of about 16 kDa during leptin treatment as compared with untreated controls is depicted in Fig. 1A.

The bar graph in Fig. 1B depicts the profile of proTRH (26 KDa) biosynthesis from three independent experiments similar to that shown in Fig. 1A. Since the radioactive incorporation into proteins, as well as the SDS-PAGE separation profile, vary from one experiment to the next, the values were normalized by calculating the ratio between the proTRH radioactive peak in cells treated with leptin vs the proTRH peak in untreated controls. Those values were also corrected against the total 3H-leucine incorporated by the cells for each experiment, as measured by TCA precipitation. Molecular masses of the identified peaks are indicated based on the migration of standards. The results
show an approximately 10-fold increase in proTRH biosynthesis in cells treated with 10nM as compared to untreated controls. These data indicate that leptin can stimulate the biosynthesis of proTRH.

**Leptin stimulates TRH release in primary cultures of hypothalamic neurons.**

Having determined that leptin stimulates the synthesis of proTRH, we wanted to determine whether leptin increased TRH release. We first evaluated the release of TRH synthesized de novo by subjecting the cells treated with leptin to a pulse with $^3$H-proline (which is present in the TRH molecule). Even though proline is a non-essential amino acid and may not have the advantages of an essential amino acid for radiolabeling, a dramatic increase in radiolabeling in proline-rich proteins has been reported in the rat parathyroid gland (22,23). More than 90% of the $^3$H-proline found in the parathyroid gland was incorporated into proline-rich proteins.

Radiolabeled TRH in the release medium was then immunoprecipitated with anti-TRH antibodies and subjected to isoelectrofocusing gel electrophoresis (IEF), pH gradient approximately 4.5-8. We have recently developed the conditions for the identification of TRH utilizing the isofocusing system. The approximate isoelectric point for TRH was about 7.00 based on theoretical calculations (see methods) and our experimental results are presented in Fig. 2A. The results depicted in Fig. 2A represent a typical IEF from media samples collected after treatment of hypothalamic cells with leptin for six hours in the presence of $^3$H-proline. The data show that treatment of hypothalamic neurons with leptin increased the release of TRH in a dose-dependent fashion from 1nM to 10 nM. Ten nM leptin showed an effect on TRH release of about 5 fold as compared with untreated controls. AtT-20 cells, which does not carry endogenous proTRH was used as control for the IEF system. As expected
immunoprecipitation of $^3$H-proline radio labelled release peptides in these cells showed background levels of radioactivity (Fig. 2A).

We next wanted to determine whether the increase in TRH release by leptin, in addition to induce a new biosynthesis of proTRH and TRH release (Fig. 2A), was also due to an action of leptin as a secretagogue. To this end, release media was collected from cultured cells exposed to increasing concentrations of leptin for six hours and compared with untreated control cells. The samples were analyzed by specific RIA against TRH as previously described (18). As seen in Fig. 2B the release of TRH increased in a dose-dependent manner with the maximum response being observed at 10 nM leptin (15 fold). The big difference in release measured by the two approaches indicates that leptin not only induce the activation of the preproTRH gene expression to ultimate translate more proTRH into protein, but also acts as a secretagogue. The ten fold difference observed between TRH measurement by radiolabeling vs. RIA indicates that leptin also induce the release of TRH stored in mature secretory granules near the plasma membrane of the cell, mechanisms that is independent of the gene activation. Since the samples were collected after 6 hours, the total amount of TRH measured by RIA may account for the newly synthesized plus TRH molecules accumulated in mature secretory granules. In our previous studies we showed that the newly formed proTRH need at least 120 minutes to be processed and deliver its end products of post-translational processing to mature secretory granules (24). Current studies by looking at short time leptin stimulation (less than 120 minutes) are underway to further quantify the amount of TRH release by leptin as secretagogue alone.

**ObRb mRNA expression and co-localization of leptin receptors with proTRH in primary cultures of fetal rat hypothalamus**
To further investigate whether leptin might have direct effects on TRH neurons, we first wanted to determine whether the long form of the leptin receptor, ObRb, is expressed in primary cultures of dissociated rat diencephalic neuronal culture by comparing it to the level in whole rat hypothalamic RNA. By applying semi-quantitative $^{32}$P-RT-PCR using a limiting number of PCR cycles of equal amounts of total RNA from the hypothalamic culture and from rat hypothalamus, we found that ObRb mRNA is highly expressed in the hypothalamic culture (Fig. 3). The levels were only ~4 fold less than that of the rat hypothalamus. As expected, we did not detect any ObRb mRNA in an RNA sample from the cultured neurons subjected to cDNA synthesis without the presence of reverse transcriptase (Lane 4). Neither did we detect any ObRb mRNA under these experimental conditions in the same amount of RNA isolated from rat lung tissue (not shown).

We then used primary cultures of dissociated rat diencephalic neurons to determine whether proTRH colocalizes with leptin receptors. We performed double-label ICC with antibodies directed against either proTRH-derived peptides or the extracellular domain of the leptin receptor to identify possible colocalization of the ObR and proTRH. Single immunostaining in non-permeabilized hypothalamic neurons with anti-ObR revealed, as expected, that a substantial proportion of these neurons express the ObR. As expected, the distribution of the ObR in the cells was mostly within the cell bodies and dendrites (Fig. 4A). Statistical analysis of more than 100 fields revealed that approximately 30-40% of the hypothalamic cells was positive for the leptin receptor. Of these, approximately 8-13% also expressed TRH (Fig. 4B). See also inserts in Fig. 4A and B for the colocalization of the leptin receptor and proTRH. The specificity of the ObR antibody was tested by using the same ICC conditions used in Fig. 5 on CHO cells transfected or not with leptin receptor cDNA. Parental CHO cells transfected with empty vector did not exhibit
specific fluorescence (Fig. 4A), whereas CHO cells expressing leptin receptors exhibited a population of cells with distinct staining (Fig. 5B).

**Leptin signaling positively regulates the human TRH promoter.**

Having established the presence of leptin receptors in the TRH neurons we next wanted determine whether the leptin-signaling pathway was capable of directly regulating proTRH at the transcriptional level. To pursue this we used the human TRH promoter (-900 to +55) fused to the luciferase reporter and assayed the effect of leptin receptor signaling. To determine the specificity of leptin signaling we employed a heterologous system whereby we cotransfected ObRb, the human TRH promoter and STAT3 in the presence or absence of leptin in the mammalian CV-1 cell line. As depicted in Fig. 6, cells stimulated with 100 nM leptin (similar results were seen with 10 nM leptin) for 6 hours showed almost a five fold increase in TRH promoter activity. Leptin had no effect in the absence of transfected leptin receptors. Furthermore, the leptin signaling system had no effect on a control luciferase reporter driven by the first 180 base pairs of the rous sarcoma virus (RSV) LTR promoter (data not shown). Thus, these results demonstrate that the leptin-signaling pathway directly activates the TRH promoter in a heterologous system.

**α-MSH stimulates the biosynthesis of proTRH and TRH release, while NPY has an opposite effect in primary cultures of hypothalamic neurons**

Although the forgoing data support the hypothesis that leptin can regulate TRH expression through direct effects on the TRH neuron, we recently show the first preliminary evidence in support of the hypothesis that the action of leptin on TRH neurons may occur also through an indirect pathway of signaling (7). This signaling may involve regulatory peptides produced in the arcuate nucleus of the hypothalamus (7,8,25,26). In addition, new data
supported a direct action of the hypothalamic melanocortin system in the fasting-induced suppression of the HPT axis (27). Two candidate neuropeptides for this role are NPY and α-MSH, which have been shown to be leptin responsive (28) and project nerve fibers to the PVN, where the hypophysiotropic neurons producing proTRH are located. Therefore, using our primary cultures of fetal rat hypothalamic neurons, we investigated whether these peptides affected the biosynthesis of proTRH in TRH neurons. Fig. 7A demonstrated that 10nM α-MSH for 4 hours resulted in a 3.5-fold increase in the biosynthesis of proTRH as determined by double immunoprecipitation of radiolabeled samples with anti-pAV37 followed by SDS-PAGE analysis. Concentrations ranging from 1 to 100 nM showed a dose dependent increase in proTRH biosynthesis (not shown).

Measurement of TRH release by RIA under the same experimental conditions described above showed that TRH release increased 4-fold (4.5pmol ±0.04 treated Vs 1.2pmol ±0.01) as compared with untreated controls. Using the same conditions we also evaluated the effect of NPY on the biosynthesis of proTRH. Fig. 7B shows that incubation of 5 nM NPY for 4 hours produced an almost 3-fold decrease in the biosynthesis of proTRH as measured by double-immunoprecipitation of radiolabeled ³H-leucine peptides followed by SDS-PAGE analysis. The release of ³H-proline TRH was also inhibited 2.8-fold as compared with untreated controls as measured by IEF (not shown). These results demonstrate that these two leptin responsive peptides regulate the biosynthesis of proTRH supporting the hypothesis that proTRH biosynthesis is also regulated by leptin through an indirect pathway.

**Direct stimulation of TRH neurons by Leptin**

In the experiments described above we showed that in primary cultures of hypothalamic neurons, under the same experimental conditions, leptin
increases 10 fold the biosynthesis of proTRH whereas the α-MSH peptide had only a 3 fold effect. This suggests that this difference in proTRH biosynthesis caused by leptin vs. α-MSH might be attributed to a direct action of leptin on TRH. To further clarify the direct involvement of leptin on the biosynthesis of proTRH, and the release of its end product, TRH from these cells, we stimulated the cells with leptin, the SHU9119 (an MC4 receptor antagonist, MC4A) compound or α-MSH peptide. Then, we monitored the release of TRH by double immunoprecipitation followed by IEF as shown before. Fig. 8A depicts a typical profile of three independent experiments. The data in this figure show that the SHU9119 compound inhibits leptin effect on TRH release by 25% (Fig. 8B). This is consistent with the hypothesis that leptin has a direct action on TRH neurons through its receptor and the 25% inhibition may represent the activation of TRH through the melanocortin-signaling pathway. Furthermore, data shows that stimulation of cells with the α-MSH peptide results in TRH release that is lower (43%, Fig. 8B) than the effect of leptin consistent with the results presented in Figs. 1 and 7.
DISCUSSION

Leptin is a hormone produced principally in adipose tissue, whose central physiologic role is to provide information on energy stores and energy balance to brain centers that regulate appetite, energy expenditure and neuroendocrine function (2-6). To effectively deliver this information, leptin must reach its central targets, and engage receptors on specific hypothalamic neurons (29-31). The output of these neurons is then integrated with other signals, ultimately engaging final effector pathways. When leptin signaling is deficient, due either to mutation of the leptin hormone or leptin receptor genes, severe obesity results in both rodents and humans (3,30,32-34), underscoring the fundamental role of leptin in physiology.

Thyroid hormone, the peripheral end product of the HPT axis, is an important regulator of energy expenditure, (35). This regulation involves release of hypophysiotropic TRH to stimulate pituitary TSH that in turn stimulates thyroid hormone production and release. TRH neurons in the medial and periventricular parvocellular subdivisions of the PVN are also subjected to negative feedback regulation by circulating levels of thyroid hormone (36). When plasma levels of thyroid hormone fall, the biosynthesis and secretion of TRH from these neurons increases, raising the threshold for feedback inhibition by thyroid hormone on anterior pituitary thyrotrophs, and thus increasing TSH secretion. Conversely, elevations in plasma concentrations of thyroid hormone suppress the biosynthesis and secretion of PVN TRH, causing a reduced threshold for feedback regulation by thyroid hormone on thyrotrophs and thus suppressed TSH secretion (36). During fasting, however, this regulatory system is altered, such that decreased circulating thyroid hormone levels are associated with a reduction in the biosynthesis of TRH and the secretion of TSH (1,37). By creating a transient state of central hypothyroidism, the resulting reduction of
thyroid thermogenesis may serve as an important energy conservation mechanism until re-feeding occurs.

Previous studies demonstrated that leptin can prevent the fall of thyroid hormone (T4 and T3) with starvation in rodents (2,9), and that this is accompanied by a maintained expression of TRH mRNA in the PVN (38). This underscores the importance of TRH neurons in the regulation of T3/T4 by leptin. Thus, hypophysiotrophic TRH neurons in the PVN are potential targets for leptin. The data presented in this study demonstrated the presence of leptin receptors in proTRH producing neurons. Since our hypothalamic cultures contains an array of different neurons including those producing TRH that do not belong to the HPT axis, we can not at this time demonstrate that the TRH neurons carrying the ObRb are those related to the HPT axis. However, since in vivo leptin treatment of starved rats only affects TRH mRNA expression within the PVN, and not extra PVN TRH neurons, it is likely that this is the case.

Consistent with a direct action of leptin on proTRH neurons, we demonstrated that transfected preproTRH promoter was activated by ObRb in a heterologous cell system. This effect of leptin signaling to activate the preproTRH promoter is consistent with the possibility that proTRH biosynthesis may be also positively affected. Utilizing our primary cultures of hypothalamic neurons, the data presented here clearly showed that leptin increased the biosynthesis of endogenous proTRH in a dose response fashion consistent with a receptor-ligand response. The greatest increase was observed at 10 nM leptin, ~10 fold increase over untreated control cells. Consistent with this increase in proTRH biosynthesis, we also showed that the amount of newly synthesized TRH released was ~5 times more in leptin treated than untreated cells, as monitored by a newly developed IEF assay for $^3$H-proline labelled TRH. In addition, TRH RIA analysis from non radiolabeled neuronal cultures that were treated with increasing concentrations of leptin revealed that leptin also induce
the release of TRH stored in mature secretory granules near the plasma membrane of the cell, mechanisms that is independent of the gene activation.. In summary, these combined results strongly support a direct regulation of proTRH biosynthesis at transcriptional, translational and peptide release levels.

Indirect regulation of proTRH through other peptide messengers has been also proposed. Using rats with arcuate nucleus lesions induced by monosodium glutamate (MSG), it has been suggested that this regulation may, at least in part, be indirect, requiring input from the arcuate nucleus (26). TRH neurons in the PVN are located in a region where they can be regulated by a large number of afferent neuroendocrine inputs. In addition to TRH neurons being densely innervated by NPY neurons, which originate in the arcuate nucleus (7), it was recently shown that α-MSH- and AgRP-expressing neurons have nerve fibers that project from the arcuate nucleus to the PVN. These projections are in close proximity to proTRH-expressing neurons (25,39). Since NPY, AgRP, POMC and CART expression in the arcuate nucleus are regulated by leptin in vivo (40-44), it is possible that these neuropeptides could play a role in modulating the regulation of TRH neurons by leptin. In the results presented here, we were able to demonstrate that α-MSH can positively regulate proTRH biosynthesis and TRH release in a dose-dependent manner, while NPY negatively regulated proTRH biosynthesis. The signaling mechanisms by which α-MSH and NPY regulate proTRH biosynthesis are unknown. We are currently pursuing these questions. In this study we were able to demonstrate that 75% of the leptin action on proTRH biosynthesis in hypothalamic neurons was through a direct action on the ObRb receptors in TRH neurons, whereas only 25% of the effect was through the melanocortin-signaling pathway (see Fig. 8).

We and others have shown that ObRb mRNA is highly expressed in specific hypothalamic neurons in the arcuate nucleus involved in regulation of
food intake (31,45). Among the neurons expressing ObRb mRNA include those expressing NPY, AgRP, POMC, and cocaine- and amphetamine-regulated transcript (CART) (40-44,46). A coherent view of the hypothalamic circuits regulated by leptin is beginning to emerge. The current paradigm envisions direct action by leptin on specific neural populations within the mediobasal hypothalamus, especially the arcuate nucleus. One population of arcuate neurons co-express NPY and AgRP, and expression of these mRNA’s are suppressed by leptin (Fig. 9). These neurons also express ObRb, and leptin acts directly on these neurons, as suggested by our finding of rapid induction of SOCS-3 (suppressor-of-cytokine-signaling) mRNA in these cells in response to leptin (20,45). Substantial evidence indicates that arcuate-derived NPY and AgRP promote feeding, and may induce obesity. A distinct subpopulation of arcuate neurons co-express POMC and CART, as well as ObRb. POMC and CART mRNA’s are positively regulated by leptin, once again through direct activation as suggested by leptin-induced SOCS-3 and c-Fos expression in these cells Elias, [1999 #4352]. Substantial physiologic and genetic evidence indicates that CART peptide and POMC-encoded α-MSH, mediate anorexigenic and very likely other actions of leptin (42-44). α-MSH exerts its effects primarily through melanocortin-4 receptors (MC4-R) (47-49), mutation of which causes obesity in mice and humans (50-52). AgRP antagonizes α-MSH binding and signaling via the MC4-R (53) (Fig. 9).

Taken together, the data presented here suggest that leptin may act at two levels to regulate proTRH biosynthesis and TRH release; a direct action through TRH neurons, and an indirect action through the arcuate nucleus via α-MSH, AgRP and NPY. This regulation may include a combination of positive and negative activation of the preproTRH gene as follows. A) an inhibitory action of leptin on NPY and AgRP release from the arcuate nucleus, leading to a reduced inhibitory effect of these peptides on TRH expression in the PVN; B) a stimulatory action of leptin on α-MSH release from the arcuate nucleus
resulting in stimulation of TRH release from the PVN, and C) a direct positive action of leptin on TRH neurons in the PVN. In support of the direct pathway of signaling for leptin to the PVN the following observations are worth noting.

Using in situ hybridization on rat brain sections, we previously showed that ObRb mRNA is expressed within the PVN (31). We have reported that peripheral administration of leptin to rodents activates SOCS-3 mRNA in neurons located in the PVN (20). In addition, in this study we have shown data that leptin can directly regulate proTRH promoter-activity via ObRb in a heterologous system. The signaling pathways and mechanisms by which leptin directly regulates proTRH mRNA and TRH biosynthesis are unknown and these are key issues we are currently investigating. Fig. 9 depicts our current model of leptin regulation of proTRH neurons from the HPT axis.

It is presently unknown how leptin may reach TRH neurons in the PVN from the circulation. We have earlier published data demonstrating high mRNA levels encoding the short form of the leptin receptor (ObRa) be present in rat brain microvessels, which comprise the blood-brain-barrier (BBB) (16). Consistent with this, we have demonstrated that this receptor has the capacity to mediate transport of intact leptin across a monolayer of polarized MDCK cells (54). This suggests that leptin may first enter the brain interstitial fluid via receptor-mediated transcellular transport at the BBB, followed by diffusion to finally reach ObRb receptors expressed on TRH neurons in the PVN. Further studies are clearly required to demonstrate this directly.

These studies provide the first direct evidence for leptin-mediated regulation of preproTRH mRNA expression and TRH prohormone biosynthesis. Although anatomical observations have implicated NPY, α-MSH and AgRP peptides in regulation of the HPT axis, the stimulation of TRH neurons by α-MSH is also shown for the first time in these studies. Taken together these results suggest that leptin may affect TRH neurons via both indirect pathways
(α-MSH and NPY), and direct pathways via leptin receptors expressed on TRH neurons in the PVN, leading to regulation of both TRH expression and processing.
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**Figure legends**

**Fig. 1.** Leptin stimulates proTRH biosynthesis in hypothalamic neurons. Panel A depicts the stimulation of proTRH biosynthesis by leptin from three independent experiments. In each experiment, $^{3}$H-radiolabelled peptides from primary cultures of hypothalamic neurons were immunoprecipitated with the anti-pAV$_{37}$ antibody. Immunoprecipitates were then resolved on a SDS-polyacrylamide gel and the radioactivity in individual gel-slices was counted. Panel B depicts a statistical representation of three independent experiments. The data presented in this figure was calculated based on three identical experimental conditions using the Tukey-Kramer test for multiple comparisons ($p<0.02$ for all conditions).

**Fig. 2.** Leptin stimulates TRH release from hypothalamic neurons. This figure depicts the stimulation of proTRH biosynthesis by leptin from three independent experiments in both A and B. Panel A, in each experiment, $^{3}$H-proline peptides from primary cultures of hypothalamic neurons were immunoprecipitated with the anti-TRH antibody. Immunoprecipitates were then resolved on an isoelectrofocusing-polyacrylamide gel and the radioactivity in individual gel-slices was counted. The pH gradient was determined by measuring the pH of each slice in a parallel gel and using pH standards (Bio-Rad). The formula depicted in panel A represent the calculation done on the data to bring it to a linear form. Panel B depicts specific RIA against TRH from media samples derived from hypothalamic cultures incubated for 6 hours with leptin. The data presented in this figure for TRH RIA was calculated based on six identical experimental conditions using the Tukey-Kramer test for multiple comparisons ($p<0.02$ for all conditions).
**Fig. 3.** ObRb mRNA is highly expressed in fetal hypothalamic neurons. Equal amounts of total RNA from fetal hypothalamic neurons and from a whole rat hypothalamus was subjected to quantitative $^{32}$P-RT-PCR under conditions of limiting number of PCR cycles (see Methods). Two independent samples of fetal hypothalamic neurons (lanes 1 and 2 from left), were amplified and run in parallel with a rat hypothalamic sample (lane 3 from left) and a RNA sample from fetal hypothalamic neurons that did not receive reverse transcriptase enzyme during cDNA synthesis (lane 4 from left). Shown is a Phospholmager image of the radioactive PCR products. The upper panel shows the ObRb results, while the lower shows the β-Actin results. This experiment was done twice. ObRb mRNA was not detected in the same amount of RNA from rat lung tissue (not shown).

**Fig. 4.** Co-localization of proTRH and ObR by double-ICC staining of primary cultures of hypothalamic neurons. Neuronal cells were cultured for up to 12 days and fixed with 4% paraformaldehyde. Fluorescein isothiocyanate conjugated to goat anti-rabbit globulin was used to visualize ObR immunostaining (green color). Anti-pAV$_{37}$ recognizes the TRH prohormone and anti-pST$_{10}$ recognizes the end product of processing preproTRH$_{160-169}$ (18). Anti-pAV$_{37}$ and anti-pST$_{10}$ antibodies were conjugated with Texas Red (red color) as described previously (18). Panel A shows non-permeabilized hypothalamic neurons positively stained for ObR. The insert in Panel A shows the co-localization of anti-ObR and anti-pST$_{10}$ antibodies; shown is a cell body with positive staining for ObR (green, arrow) and positive staining in axonal processes of the same cell (bottom) using anti-pST$_{10}$ (red, arrow heads). Panel B shows co-localization of ObR and proTRH (detected with anti-pAV$_{37}$), see inserts ObR (green) and proTRH (red) indicated by arrows. The combined figures of the same cells are shown above (yellow). This panel also shows a cell only positive for proTRH (red, arrowhead) and a cell only positive for ObR (long arrow, green).
Fig. 5. Specificity of ObR antibodies. Panel A shows ICC for ObR in CHO cells transfected with an empty vector. Panel B shows CHO cells expressing the short form of the leptin receptor. The leptin receptor was detected using an antibody against the common extracellular domain of the leptin receptor (13).

Fig. 6. Activation of the TRH promoter by leptin signaling in a heterologous transfection system. CV-1 cells were transiently transfected with the TRH promoter-luciferase construct with or without ObRb expression vectors. Forty-eight hours post transfection, including serum-starvation for 12 hours, cells were treated or not with 10nM of leptin for 6 hours. The figure shows measurements of luciferase activities. The data presented in this figure was calculated based on four identical experimental conditions using the Tukey-Kramer test for multiple comparisons (p<0.02 for all conditions).

Fig. 7. α-MSH stimulates the biosynthesis of proTRH and NPY inhibits it. This graph depicts an electrophoretic separation of immunoprecipitated ³H-leucine samples with anti-pAV₃₇ derived from hypothalamic neurons treated with α-MSH as compared with untreated controls. The data shows an approximately 3-fold increase in proTRH biosynthesis. The 26 kDa peak represents the proTRH polypeptide. The graphs shown in Panels A and B are a representative of three independent experiments.

Fig. 8. Direct stimulation of leptin on TRH hypothalamic neurons. Panel A depicts the stimulation of TRH release by leptin and a combination of treatments with SHU9119 (MC4RA) and α-MSH peptide. The graph is a representation of three independent experiments. In each experiment, ³H-proline peptides from primary cultures of hypothalamic neurons were immunoprecipitated with the anti-TRH antibody. Immunoprecipitates were then resolved on an isoelectrofocusing-polyacrylamide gel and the radioactivity in individual gel-
slices was counted. The pH gradient was determined by measuring the pH of each slice in a parallel gel and using pH standards (Bio-Rad, California). The formula depicted represents the calculation done on the data to bring it to a linear form. Panel B depicts the calculated percentage of TRH releases from Panel A. The hundred percent release value was arbitrarily set for leptin.

Fig. 9. Hypothetical model of leptin action on TRH neurons. This cartoon represents the proposed model for activation of the thyroid axis by leptin through actions on the proTRH neuron in the paraventricular nucleus (PVN) of the hypothalamus. Two mechanisms of leptin action on proTRH neurons are proposed in this study. In one, indirect regulation, leptin regulates arcuate (ARC) neurons expressing POMC, that give rise to α-MSH through post-translational processing (induced by leptin), and AgRP (suppressed by leptin), which then project their nerve fibers to a close contact with proTRH neurons. These peptides influence TRH expression by antagonistic actions of α-MSH (stimulatory) and AgRP (inhibitory) on melanocortin 4 receptors MC4R. Leptin inhibit the expression of the appetite-stimulating NPY, which then project its nerve fibers to a close contact with proTRH neurons producing an inhibitory action of proTRH neurons. We propose that leptin also acts directly on TRH neurons through leptin receptors on these cells (stimulatory). In the absence of leptin signaling, reduced negative feedback on the TRH neuron by low levels of T4/T3, acting on the thyroid receptor (TR), fails to produce compensatory increases in TRH expression.
REFERENCES


Fig. 1, Nilnii et al
Fig. 2, Nilini et al
Fig. 6, Nilini et al
Fig. 7, Nillni et al
Fig. 8, Nillni et al
Fig. 9, Nillni et al
LEPTIN REGULATES PROTHYROTROPIN-RELEASING HORMONE (PROTRH) BIOSYNTHESIS: Evidence for direct and indirect pathways
Eduardo A. Nillni, Charles Vaslet, Mark Harris, Anthony N. Hollenberg, Christian Bjorbaek and Jeffrey S. Flier

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