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LEPTIN INDUCES NITRIC OXIDE SYNTHASE TYPE II IN C6 GLIOMA CELLS

and

ROLE OF DEIODINASE TYPE II-PRODUCING T3 AND UNCOUPLING PROTEIN 2 IN THE REGULATION OF ENERGY BALANCE

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PART I

LEPTIN INDUCES NITRIC OXIDE SYNTHASE TYPE II IN C6 GLIOMA CELLS

1. INTRODUCTION

In the late 1950s, a genetic defect that caused a severely obese phenotype due to overeating and decreased energy expenditure was identified in mice (Hausberger et al, 1959). The gene was named *ob* and the obese mice carrying the mutation were called *ob*/*ob* (Coleman et al, 1978). In 1994, the molecular defect responsible for the obesity syndrome in *ob*/*ob* mice was identified by Friedman and colleagues (Zhang et al, 1994). The protein encoded by the *ob* gene was named leptin, from the Greek *leptos*, meaning thin. Because a defect in leptin led to overeating and obesity, leptin was proposed to be a satiety factor.

Parabiotic animal experiments suggested that the *ob/ob* animals were unable to make a factor that results in overeating, but *ob/ob* mice could respond to such a factor from a parabiotic mate. Similar experiments suggested that *db/db* mice, which have a mutation in the *db* gene and display a phenotype

identical to *ob/ob* mice, make the factor that is missing in *ob/ob* mice, but *db/db* mice cannot respond to it (Fantuzzi and Faggioni, 2000). Therefore, it was hypothesized that the *db* gene would encode for the *ob* receptor. After the cloning of leptin, the leptin receptor (Ob-R) was cloned shortly thereafter by virtue of its high affinity to leptin through an expression cloning strategy (Tartaglia et al, 1995). The OB-R is indeed the product of the *db* gene and is abundant in the hypothalamus, an area in the brain involved in the control of food intake (Lee et al, 1996).

Thus, both leptin-deficient and leptin receptor-deficient mice display identical severe hereditary obesity phenotype, characterized by increased food intake and body weight plus decreased energy expenditure (Zhang et al, 1995; Montague et al, 1997; Chen et al, 1996). Furthermore, administration of leptin reverses the obese phenotype in *ob/ob* mice and decreases food intake in normal mice. In contrast, *db/db* mice are resistant to the weight-reducing effects of leptin (Campfield et al, 1995; Halaas et al, 1995; Pelleymounter et al, 1995).

1.1. Leptin

Leptin is a 16 kDa protein synthesized mainly by adipose tissue. The structure of leptin and its receptor suggest that leptin is a member of the cytokine family. Despite the absence of sequence similarity between leptin and other long-chain helical cytokines, there is a striking similarity in their tertiary structures. Leptin has a four-helix bundle similar to that of the long-chain helical cytokine family, which also includes IL-6, IL-11, IL-12, leukemia inhibitory factor (LIF), G-CSF, CNTF, and oncostatin M (Zhang et al, 1997).

Circulating leptin levels are directly related to adipose tissue mass (Frederich et al, 1995). High leptin levels signal the presence of sufficient energy stores to sites in the central nervous system, which respond by reducing appetite and increasing energy expenditure, preventing severe obesity (Maffei et al, 1995). Therefore, leptin signals the nutritional status from the periphery to the area of the brain involved in the homeostasis of energy balance (Faggioni, 2001).

Leptin, initially discovered as a regulator of food intake and energy expenditure, is emerging as a pleiotropic molecule involved in a variety of physiological and pathological conditions. A pleiotropic role for leptin in mammalian physiology is clearly suggested by the complex syndrome

exhibited by leptin-deficient *ob/ob* mice and leptin receptor-deficient *db/db* mice. Those mice are not only obese, but have abnormalities in reproductive function, hormone levels, wound repair, bone structure, and immune function (Flier, 1998).

1.2. Leptin receptor

The Ob-R is a cytokine receptor and belongs to the class I cytokine receptor family that includes gp-130, the common signal-transducing component for the IL-6-related family of cytokines (Tartaglia et al, 1995). The Ob-R is alternately spliced into at least five transcripts from the single *db* gene. These transcripts encode proteins called the long (Ob-Rb), short (Ob-Ra, -c, and -d), and soluble (Ob-Re) forms of the leptin receptor with varying length of their cytoplasmic tail (Lee et al, 1996). The complexity of the Ob-R system is typical of cytokine biology, where the signal triggered by the binding of a cytokine to its receptor on target cells might be modulated by soluble receptors, decoy receptors, receptor antagonists, and binding proteins.

The long form is essential in mediating most of the biological effects of leptin (Lee et al, 1996; Chen et al, 1996). Ob-Rb is highly expressed in the

hypothalamus (Vaisse et al, 1996), but is also present in peripheral tissues, including those of the immune system such as spleen, thymus, lung, and leukocytes (Tsuchiya et al, 1999; Lord et al, 1998; Cioffi et al, 1996).

The short isoform (Ob-Ra) is the most abundant isoform found in most tissues (Tartaglia et al, 1995). It is highly expressed in the choroid plexus where it is likely to function as a transporter across the blood–brain barrier (Kastin et al, 1999). Although evidence of signaling capabilities has been reported for Ob-Ra (Bjorbaek et al, 1997), the complete role of the signal it triggers is still unclear; it remains to be determined whether it might also function as a decoy receptor or have a role as a ligand-passing receptor.

Leptin circulates both in free form and bound to its soluble receptor Ob-Re (Sinha et al, 1996). Ob-Re appears to act as a carrier, since the binding of leptin to it delays its clearance from circulation and potentiates its effect on food intake (Huang et al, 2001). Other cytokine receptors, such as tumor necrosis factor (TNF), also use their soluble receptors as binding proteins (Engelmann et al, 1990).

1.2.1. Leptin receptor signaling

Ob-Rb contains an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic signaling domain (Tartaglia, 1997). Ob-Rb shares the same signal transduction pathway with gp-130-like cytokines (Baumann et al, 1996). It activates Janus kinase/signal transducers and activators of transcription (STAT) as well as mitogen-activated protein kinase signal transduction pathways (Vaisse et al, 1996). In common with cytokine-transducing pathways, leptin induces expression of SOCS (suppressor of cytokine signaling)-3 mRNA in the hypothalamus. SOCS proteins are thought to function as inducible intracellular negative regulators of cytokine signal transduction. Accordingly, transfection data suggest that SOCS-3 is an inhibitor of leptin signaling (Bjorbaek et al, 1999).

Unliganded Ob-Rb exists as a preformed homodimer; leptin binding alters the conformation of the Ob-Rb dimer, enabling transphosphorylation and activation of the intracellular Ob-Rb-associated Janus associated kinases-2 (Jak2) molecules (Devos et al, 1997). The activated Jak2 molecule then phosphorylates other tyrosine residues within the Ob-Rb/Jak2 complex to mediate downstream signaling (White et al, 1997).

All the four membrane-bound leptin receptors contain in the cytoplasmic tail a box 1 motif, strongly conserved within most members of this receptor family, whereas box 2 and 3 motifs are found only in the long isoform. Domains 1 and 2 are involved in the interaction and activation of JAK2 tyrosine kinase that phosphorylates and activates members of the STAT family of transcription factors, such as STAT1, STAT3 and STAT5. The activation of STAT3 has been observed *in vivo* in the hypothalamus and requires a box 3 motif in the receptor intracellular domain (Vaisse et al, 1996). These transcription factors, after tyrosine-phosphorylation in response to JAK activation, translocate to the nucleus, where they activate gene transcription.

1.3. Leptin and inflammation

In addition to its well-defined role as regulator of energy balance, leptin appears to be involved in the host response to infection and inflammation (Fantuzzi and Faggioni, 2000). In fact, leptin levels are up-regulated by many acute phase factors, such as TNF, IL-1 and IL-6, and during bacterial infection, or lipopolysaccharide challenge (Faggioni et al, 2001). Leptin, per

se, acts on monocytes/macrophages by inducing eicosanoids synthesis, nitric oxide and several pro-inflammatory cytokines (Mancuso et al, 2004). In addition, this hormone potentiates IFN-γ-induced expression of nitric oxide synthase and cyclo-oxygenase-2 in murine macrophage J774A.1 (Raso et al, 2002). Moreover, leptin induces chemotaxis of neutrophils, the release of oxygen radicals (Caldefie-Chezet et al, 2001), and also affects natural killer (NK)-cell development and activation both *in vitro* and *in vivo* (Tian et al, 2002).

Leptin has been demonstrated also to modulate adaptive immune response. This is divided classically into T helper 1 and 2 immune responses on the basis of the cytokine pattern produced. T helper 1 lymphocytes produce mainly pro-inflammatory cytokines that are necessary for macrophage activation and cell-mediated response, whereas T helper 2 lymphocytes secrete modulatory and anti-inflammatory peptides that are important factors for the activation of B cells and basophils. It has been found that leptin deficiency is responsible for the immunosuppression and thymic atrophy observed during acute starvation and reduced caloric intake. The impairment of the immune response is associated with a dramatic fall in serum leptin and in rodents is reverted by exogenous leptin. In mice leptin is necessary for an effective cell-mediated immunity and the $CD4^+$ T lymphocytes function is

suboptimal in the absence of leptin as observed in ob/ob mice (Lord et al, 1998). In addition, there are several evidences that leptin participates in the maintenance of thymic maturation of double positive $CD4^+/CD8^+$, cells and prevents glucocorticoids-induced apoptosis in thymocytes (Howard et al, 1999).

1.4. Inducible nitric oxide synthase

In mammals, three distinct isoform of nitric oxide synthase (NOS) exist: neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and endothelian (eNOS or NOS3). nNOS and eNOS are primarily expressed in neurons and endothelian cells, respectively. iNOS expression is induced in a wide range of cells and tissues by cytokines and other agents. After induction, iNOS produces continuosly NO until the enzyme is degraded (MacMicking et al, 1997).

Although, NO production by iNOS has been described to have benefical immunological effects (MacMicking et al, 1997), aberrant iNOS induction seems to be involved in the pathophysiology of human diseases such as

asthma, arthritis, multiple sclerosis, neurodegenerative diseases, or septic shock (Bogdan, 2001; Kroncke et al, 1998).

No data report a direct iNOS induction by leptin, although we have previously demonstrated that in a macrophage cell line J774A.1 leptin synergized with IFN-γ in NO and PGE2 release, increasing iNOS and COX-2 expression (Raso et al, 2002). Moreover in human primary chondrocytes and ATDC5 cells IFN-γ-induced iNOS expression was increased by leptin and occurred via activation of JAK-2 (Otero et al, 2003).

1.4.1. Regulation of iNOS expression

The pathways regulating iNOS expression seem to vary in different cells or species. Activation of the transcription nuclear factor (NF)-kB and signal transducer and activator of transcription (STAT)-1α seems to be an essential step in the regulation of iNOS expression in most cells (Kleinert et al, 2004). In particular, the transcriptor factor NF-kB seems to be a central target for activators or inhibitors of iNOS expression. It has been shown that tyrosine phosphorylation of JAK-2 and the activation of NF-kB by several cytokines were required for iNOS induction in rat glial cells, C6 (Nomura, 2001).

NF-kB activation is regulated by a related inhibitory protein called IkB (Blackwell and Christman, 1997; Ghosh et al, 1998; Karin and Delhase, 2000). The IkBα isoform retains NF-kB in the cytoplasm by masking its nuclear translocation sequences under basal conditions. In response to a proinflammatory signal, IkB-α is rapidly phosphorylated by IKB kinase (IKK), thus targeting IkBα for polyubiquitination and degradation by the 26S proteasome. Proteolytic processing of IkB-α by the proteasome complex (26S) unmasks NF-kB nuclear translocation sequences, thus allowing NF-kB to enter the nucleus for direct transcription of specific pro-inflammatory genes.

1.5. Astrocytes and inflammation

There are two groups of glial cells in the central nervous sistem: microglia and macroglia, the latter of which includes astrocytes, oligodentrocytes, and ependymal cells. Astrocytes are the major glial cells and have a number of important physiological properties related to CNS homeostasis. Astrocytes affect neuronal function, guide neuronal development and play a significant role during infections and autoimmuno diseases of the CNS.

Astrocytes, as part of the blood–brain barrier (BBB), are in close proximity to endothelial cells; they also secrete cytokines, and present antigens to T-cells (Dong and Benviste, 2001). In particular, expression of inflammatory cytokines by astrocytes is an important regulator of brain response to injury and infection.

In the CNS, inducible or immunological NOS is mainly expressed in activated astrocytes and microglia after ischemic, traumatic, neurotoxic or inflammatory damage in rodents and humans (Lee et al, 2003).

High levels of NO induced by these stimuli and different mediators, such as cytokines locally released, are implicated in several diseases: NO production contributes to oligodendrocyte degeneration (Merrill et al, 1993), neuronal injury during trauma (Uehara et al, 1999a) and cerebral ischemia (del Zoppo et al, 2000).

Evidence suggests that leptin may be a risk indicator for ischemic and hemorrhagic stroke in patients of both sexes (Soderberg et al, 1999; 2003). Moreover, cytokines seem to be important modulators of neuroendocrine disturbances, including diurnal rhythmicity of cortisol and leptin, which are common and often profound soon after strokes (Johansson et al, 2000).

1.6. Aim of the study

The long form of the receptor, Ob-Rb, has a cytoplasmatic region with consensus aminoacid sequences involved in receptor binding to Jak-STAT tyrosine kinases (Vaisse et al, 1996; McCowen et al, 1998). It has been shown that tyrosine phosphorylation of Janus associated kinases-2 and NF-kB activation by IFN-γ are required for the induction of iNOS in C6 astroglioma cells (Nishiya et al, 1995).

High levels of NO induced by different stimuli, such as cytokines, can be cytotoxic and play a critical role in the brain. NO derived from iNOS activation in glial cells is involved in neuronal cell death (Uehara et al, 1999). Since a close relationship appears to exist between inflammation, cytokines, and gliosis, the purpose of this study was to analyze the modulation by leptin of iNOS expression induced by inflammatory cytokines in C6 cells. Moreover, considering the role of NF-kB in the regulation of iNOS, we investigated the effect of pyrrolidine dithiocarbamate (PDTC) and N-α-tosyl-L-lysine chloromethyl ketone (TLCK), two NF-kB pathway inhibitors, on leptin-induced iNOS expression.

The antioxidant PDTC is thought to inhibit NF-kB activation by depleting the cells of oxygen radicals. TLCK is a serine protease inhibitor and its site of

action appears to be at the proteasome complex (26S), although TLCK may have other sites of action. Conversely, MG132 is a potent and specific inhibitor of the proteasome complex, and hence of IkB-α degradation (Sherman et al, 1993; Kim et al, 1995; Epinat and Gilmore, 1999). We therefore evaluated the degradation of IkB-α, which is necessary for NF-kB activation, after hormonal stimulation with or without MG132.

2. MATERIALS AND METHODS

2.1. Cell culture and treatments

C6 glioma cell line was cultured in 75-cm^2 flasks in Dulbecco's modified essential medium (DMEM) with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C under 5% CO2 humidified air. Cells were passaged at confluence using a solution of 0.025% trypsin and 0.01% EDTA. Cells were cultured for 3 days to confluence in complete medium. After 12 h of starvation in serum free 5 DMEM/F12, cells were stimulated with leptin (filtered with Detoxi-gelTM columns in order to remove pyrogens). Our protocol utilized hormone concentrations that produced detectable NO release. The hormone (NIDDK's National Hormone & Peptide Program; 1, 3, or 10 μg/ml) was used alone or in combination with IFN-γ (Calbiochem; La Jolla, CA, USA; 200 U/ml), or TNF-α (Sigma, St. Louis, MO, USA; 500 U/ml), or IFN-γ (100 U/ml) plus IL-1β (Calbiochem; 10 ng/ml). After 22 h of incubation the supernatants were analyzed for NOx assay. The effect of PDTC (Sigma, St. Louis, MO, USA; 10, 50, and 100 μM), TLCK (Sigma; 10, 30 and 50 μM), and MG132 (Sigma; 1, 5, and 10 μM) on iNOS induction was assessed by adding them 60 min before leptin stimulation. To evaluate the temporal effect of leptin on IkB-α degradation, C6 cells were stimulated with leptin (10 μg/ml) for the indicated time periods (0-5-15-30-45-60-90 and 120 min) in the absence or presence of MG132 (10μM).

2.2. Measurement of nitrite and nitrate (NOx) on the supernatants

NOx production was measured in the culture medium. Briefly, the nitrate in the samples was first reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 µM) at room temperature for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction, by adding 100 µl of Griess reagent (0.1% (w/v) naphtylethylenediamine-HCl and 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid (vol.1:1) to 100 µl samples. The optical density at 550 nm was measured using a microplate reader Titertek. Nitrite concentrations were calculated from a sodium nitrite standard curve freshly prepared in culture medium and expressed as micromolar unit.

2.3. Semi-quantitative RT-PCR

After 22 h of cell stimulation total RNA was extracted by a modified method of Chomczynski and Sacchi (1987), using TRIzol Reagent (Life Technologies, Milan, Italy) according to the manufacturer's instructions. Reverse transcription was performed by a standard procedure (Brenner et al., 1989) using 2 µg of total RNA. After reverse transcription, 2 µl of RT products were diluted 6 in 48 µl of PCR mix, to give a final concentration of 50 U/ml of Taq DNA polymerase (Promega, Madison, WI, USA) 4 µM of 5' and 3' primers, 50 µM of each dNTP, 1.5 mM MgCl2, and 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). The following oligonucleotides were used:

iNOS; 5'-TGCCCCTTCAATGGTTGGTA-3'

5’-ACCGGAGGGACCAGCCAAAT-3’

cyclophilin: 5’-CGACATCACGGCTGATGGCGAGCCC -3’

5’-TTACAGGGTATTGCGAGCAGATGGGG-3’.

iNOS and cyclophilin cDNAs underwent 28 cycles, each one performed at 94°C for 1 min, Tm 60°C for 1 min, and 72°C for 1 min. After this treatment 10µl of RT-PCR products were separated by 1.5% agarose gel electrophoresis in TBE 1X (Tris-base 0.089 M, boric acid 0.089 M) containing 0.2 µg/ml of ethidium bromide. Fragments of DNA were seen under UV light. Cyclophilin was used as an internal reference and no bands were observed in absence of cDNA.

2.4. Western blot analysis

For protein detection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and resuspended in Tris-HCl 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulphonylfluoride, 1 mM Na3VO4, leupeptin (10 µg/ml), and trypsin inhibitor (10 µg/ml). After 40 min, cell lysates were obtained by centrifugation at 100,000 g for 15 min at 4°C.

Protein concentrations of cell lysates were estimated by the Bio-Rad protein assay using bovine serum albumin as standard.

Equal amounts of protein of the cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% and 12 % polyacrylamide for iNOS and IkB-α detection, respectively). Western blotting was performed by transferring proteins from slab gel to a sheet of nitrocellulose membrane at 240 mA for 40 min at room temperature. The filters were then blocked with 1X PBS and 5% non-fat dried milk for 40 min at room temperature and incubated with the

specific monoclonal antibody IgG1 anti-iNOS (Transduction Laboratories, Lexington, KY, USA; 1:10,000) or anti-IκB-α (SC-371; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1,500) antibody in 1X PBS, 5% non-fat dried milk and 0.1% Tween 20 overnight at 4°C. Thereafter, the filters were incubated with the appropriate secondary antibody (anti-mouse or anti-rabbit IgG horseradish peroxidase conjugates; Jackson, West Grove, PA, USA) for 1 h at room temperature.

The antibody-reactive bands were revealed by chemiluminescence (ECL). To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against the β-actin protein. The protein bands on X-ray film were scanned and densitometrically analyzed by Model GS-700 Imaging Densitometer.

2.5. Statistical analysis

Data are presented as mean ± SEM values of 3 independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed by two-way ANOVA test and multiple comparisons were made by Bonferroni's test. A value of $P<0.05$ was considered significant.

3. RESULTS

3.1. Effect of leptin alone or in cytokine combination on NOx production

Leptin (1-3-10 μg/ml) induced NO accumulation in C6 supernatant (control was 1.11±0.53, leptin was 2.92±0.23, 3.41±0.24, and 4.78±0.02 μM at 1, 3, 10 μg/ml, $p<0.05$, $p<0.01$ and $p<0.001$ vs control, respectively). Moreover leptin potentates IFN-γ (200 U/ml)-induced NOx release in a concentration-dependent manner and this synergistic effect was significant at 3 and 10 μg/ml of leptin (Fig.1, $p<0.001$, vs IFN-γ alone). As shown in Fig.2, leptin also in combination with TNF-α potentiates cytokine-induced NO release. This effect was concentration-dependent and significant at 3 and 10 μg/ml of leptin ($p<0.05$ and $p<0.001$ vs TNF-α alone, respectively). IL-1β did not induce NO release (data not shown), but when it was associated with leptin (3 or 10 μg/ml) a significant increase of NO production was evidenced (7.80±0.10 and 12.50±0.08 $p<0.05$ and $p<0.01$ vs leptin alone). Moreover, when a lower concentration of IFN-γ (100 U/ml) was added to IL-1β (10 ng/ml) a synergistic effect was evident using 3 and 10 μg/ml leptin (Fig. 3, #$p<0.05$ vs cytokine combination).

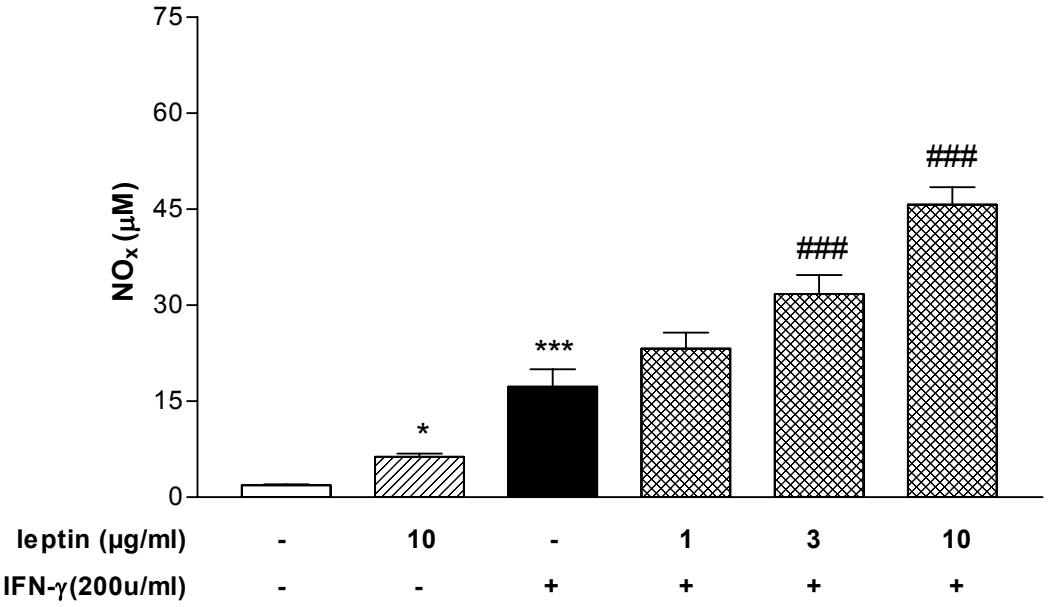

Fig. 1 Evaluation of NO production by C6 cells stimulated for 22 h with leptin (1, 3, and 10 μg/ml) in combination with IFN-γ (200 U/ml).

The values expressed as NOx (μM) are means of at least 3 determinations ± SEM. *$p<0.05$ and ***$p<0.001$ vs control; ### $p<0.001$ vs IFN-γ alone.

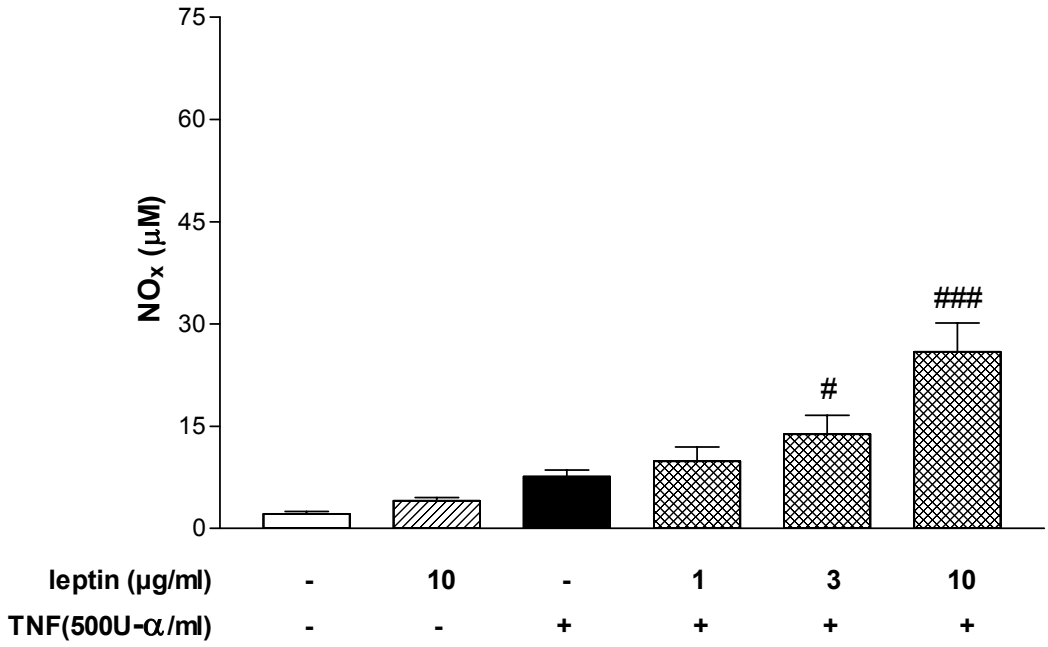

Fig. 2 Evaluation of NO production by C6 cells stimulated for 22 h with leptin (1, 3, and 10 µg/ml) in combination TNF-α (500 U/ml).

The values expressed as NOx (µM) are means of at least 3 determinations ± SEM. # $p<0.05$ and ### $p<0.001$ vs TNF-α alone.

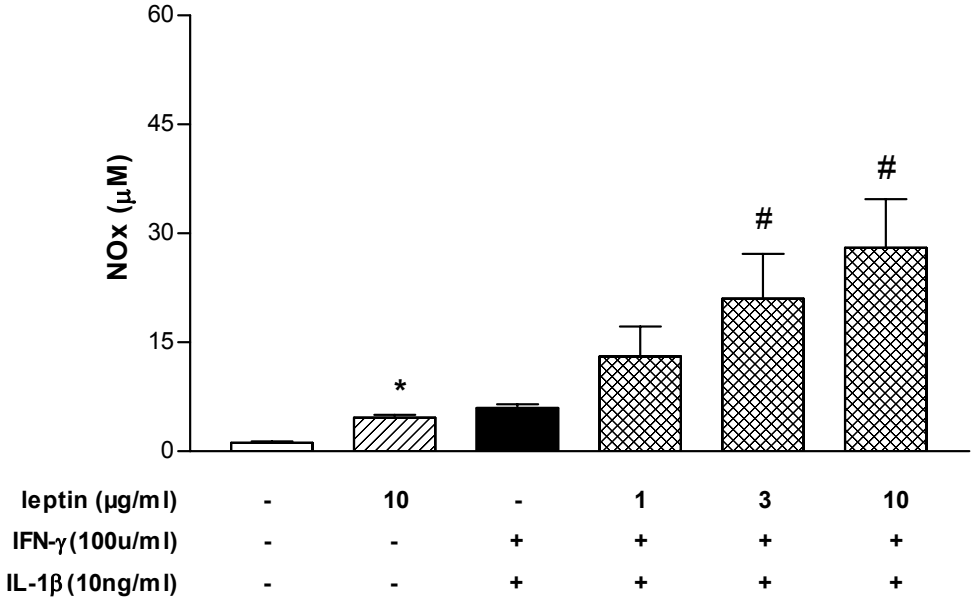

Fig. 3 Evaluation of NO production by C6 cells stimulated for 22 h with leptin (1, 3, and 10 μg/ml) in combination with or IFN-γ (100 U/ml) plus IL-1β (10 ng/ml).

The values expressed as NOx (μM) are means of 3 determinations ± SEM. *$p<0.05$ vs control; # $p<0.05$ vs IFN-γ and IL-1β combination.

3.2. Leptin induced iNOS and potentated cytokine-induced protein expression

To determine whether leptin effect on NO release was related to enhanced iNOS expression, RT-PCR and Western blot analysis were carried out. Representative RT-PCR and blot of RNA extracts and lysates obtained from cells stimulated with leptin alone or in combination with different cytokines are shown in Fig. 4.

In unstimulated control cells iNOS expression was not detectable. Using semi-quantitative RT-PCR and Western blot analysis, we showed a concentration-dependent increase of iNOS (305 bp or 130 kDa) expression after leptin stimulation. IFN-γ and TNF-α by themselves, as well as leptin induced iNOS in mRNA and protein. The cytokine effect was up-regulated by increasing concentration of leptin (Fig.4 panel A and B). A synergistic effect was observed when leptin was used in combination with IL-1β and, interestingly, it was increased by a lower concentration of IFN-γ (Fig.4 panel C).

Cyclophilin was used as internal reference for RT-PCR analysis, while to ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated in the presence of the antibody against the β-actin protein (data not shown).

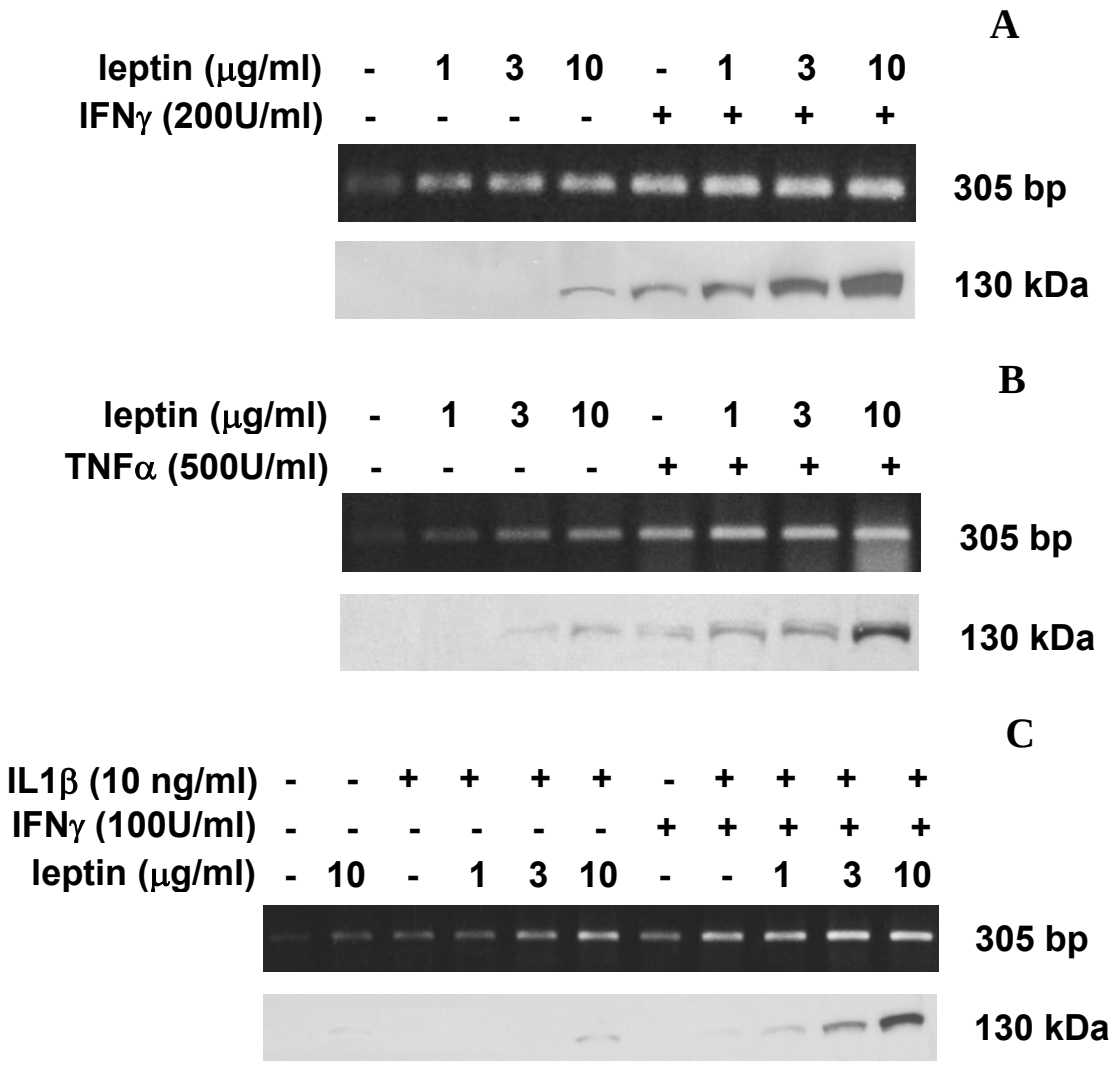

Fig. 4 Leptin induction of iNOS expression and its potentation by cytokines. Representative semiquantitative RT-PCR (A,B,C upper panels) and Western blot analysis (A,B,C lower panels) of extracts and lysates prepared by unstimulated and stimulated cells with the hormone alone and in combination with different cytokines are shown. iNOS mRNA band was found at 305 bp and protein band corresponded to 130 kDa. Similar results were obtained in three additional separate experiments.

3.3. Inhibition of leptin induced iNOS by NF-kB inhibitors

The possible role of NF-kB pathway in the regulation of iNOS expression by leptin was examined using two well known inhibitors: PDTC and TLCK (Fig.5 and Fig.6). Leptin effect was markedly reduced by both inhibitors. In particular TLCK was more active than PDTC as evidenced by the concentration-dependent inhibition of the enzyme induction.

To confirm NF-kB role in leptin-induced enzyme expression, we used the more potent and specific membrane-permeable proteasome inhibitor MG132 (Fig. 7). The pre-treatment of cells with this drug (1-10 μM) inhibited concentration-dependent leptin-induced iNOS. Lower panels of figure 5, 6 and 7 showed densitometric analysis of protein bands obtained from three separate experiments.

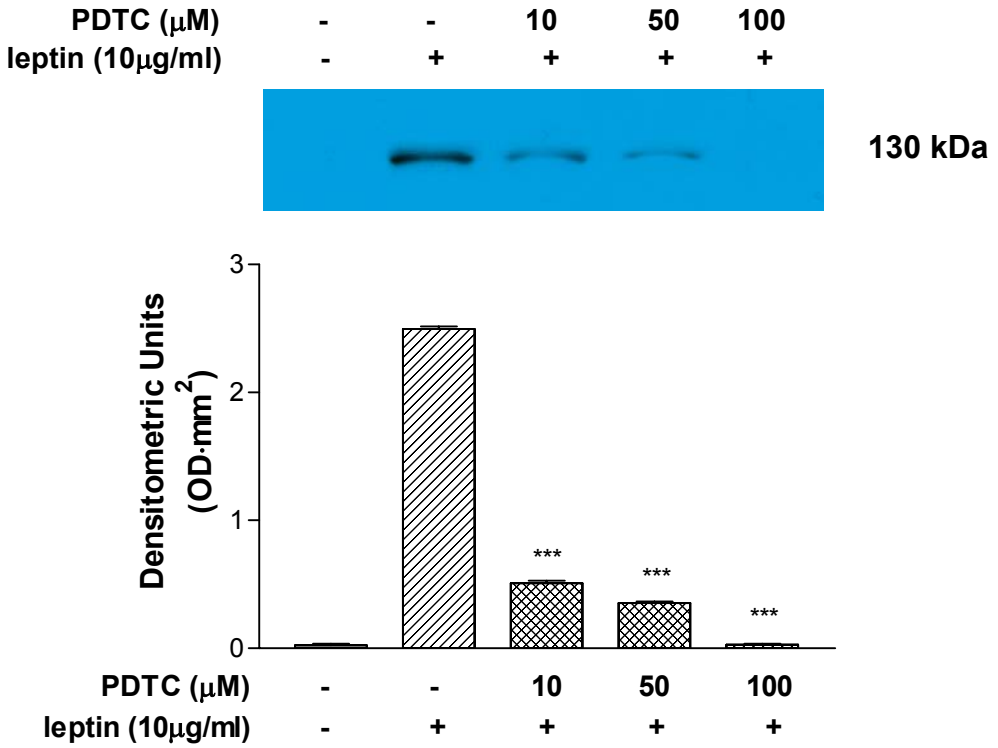

Fig. 5 Effect of PDTC on leptin-induced iNOS expression. Representative Western blot analysis is shown. PDTC (10-50-100 μM) was added 1 h before leptin stimulation (10 μg/ml). Respective densitometric analysis of protein bands is reported in lower panels and obtained from three separated experiments (***$p<0.001$ vs leptin).

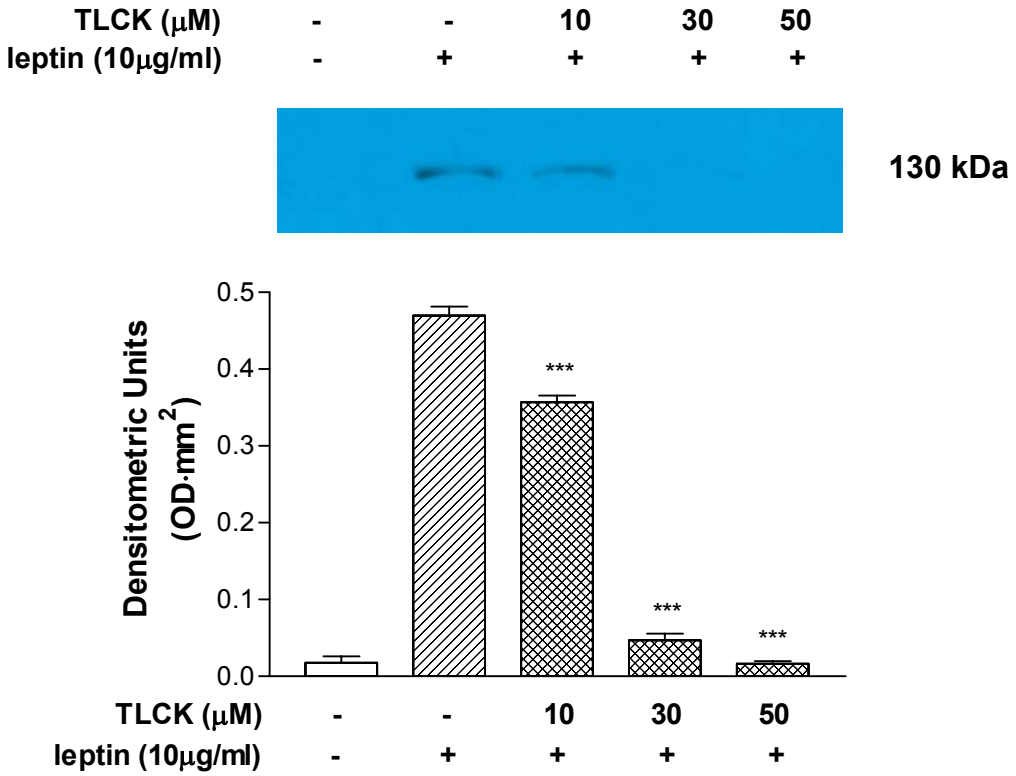

Fig. 6 Effect of TLCK on leptin-induced iNOS expression. Representative Western blot analysis is shown. TLCK (10-30-50 μM) was added 1 h before leptin stimulation (10 μg/ml). Respective densitometric analysis of protein bands is reported in lower panels and obtained from three separated experiments (***$p<0.001$ vs leptin).

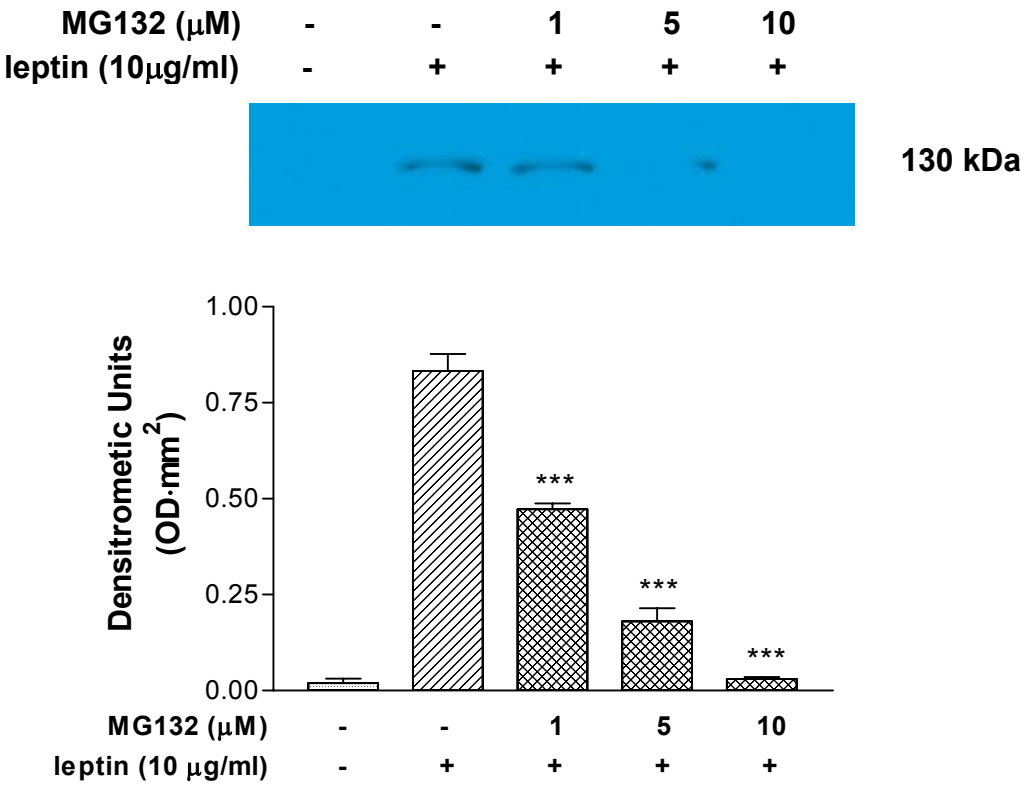

Fig. 7 Effect of MG132 on leptin-induced iNOS expression. A representative Western blot analysis is shown (upper panel). MG132 (1-5-10 μM) was added 1 h before leptin (10 μg/ml).

Respective densitometric analysis of protein bands is reported in lower panel and obtained from three separated experiments (***$p<0.001$ vs leptin).

3.4. Time-course of the effects of leptin on the IκB-α degradation

IkB-α degradation was evaluated after hormonal stimulation by Western blot analysis (band at 37 kDa) at the indicated sampling times (0-120 min). The levels of IkB-α were decreased after 5-15 min in response to leptin and restored at 30 min, returning to basal level (Fig. 8). Next we examined the effect of MG132 on leptin-induced IkB-α degradation. Pre-treatment of C6 cells with this inhibitor reverted the protein degradation of the protein induced by leptin both at 5 and 15 min (Fig. 9).

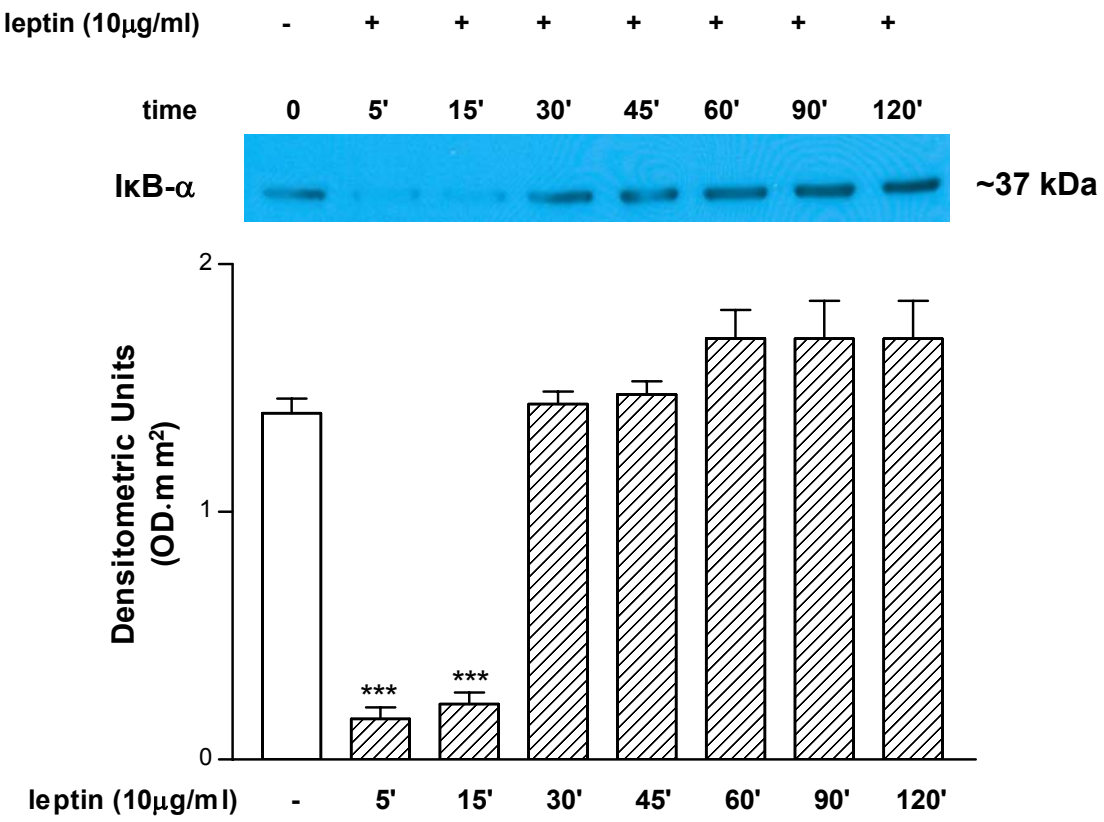

Fig. 8 Effect of leptin (10 μg/ml) on IkB-α degradation at the indicated sampling times (0-120 min). A representative immunoblot analysis of three independent experiments is shown (***p<0.001 vs control lane).

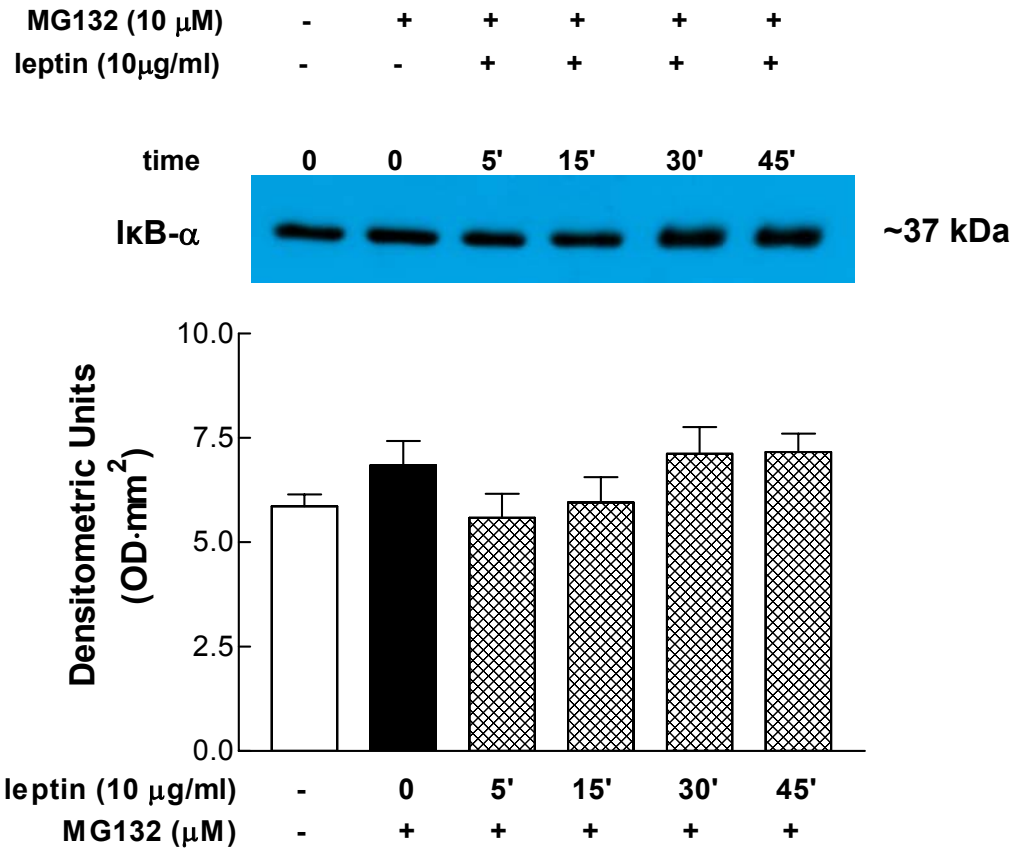

Fig. 9 MG132 (10 μM) reverses IkB-α degradation induced by leptin (10 μg/ml). A representative immunoblot analysis of three independent experiments is shown.

4. DISCUSSION

In our study, we report that *in vitro* leptin stimulation increases NO production and potentiates the effects induced by different Th1 cytokines, modulating the inflammatory properties of astrocytes, which represent the most abundant glial cell type in the CNS.

Using C6 cells we found that leptin increases iNOS derived NO production and enzyme expression both alone and in combination with IFN-γ, TNF-α, or IFN-γ/IL-1β. Since NO production is proportional to cell number, we excluded the possible interference of a proliferative effect by leptin on NO release using MTT assay (Raso et al, 2002): no change in cell proliferation was evident when C6 were treated with the used concentrations of leptin alone or in combination with all tested cytokines (data not shown). The regulation of iNOS gene expression is complex; several data indicate that it is primary under a transcriptional control mechanism and that transcription factors, such as NF-kB, AP-1, and STAT1α may function synergistically (Taylor and Geller, 2000; Nomura, 2001).

In C6 cells several studies showed that iNOS induction by LPS in presence of cytokines required more than one signal pathway. In particular TNF-α and

LPS stimulation involves NF-kB and AP-1 (Lee et al, 2003), while LPS and IFN-γ activate NF-kB and JAK-2/STAT1α, respectively.

We have demonstrated for the first time that leptin-induced iNOS expression was dependent from NF-kB activation. In fact, both TLCK and PDTC inhibited enzyme expression induced by the hormone, suggesting a role for this transcriptional factor. Although different mechanisms underlined the activity of these inhibitors (Xie et al, 1994; Schreck et al, 1992), both compounds have been shown to inhibit iNOS expression induced by leptin in a concentration-dependent manner. In particular TLCK, that blocks early events of iNOS induction preventing IkB degradation, appears to be more active than PDTC, which acts by anti-oxidant properties.

Since proteasome inhibition is known to inhibit NF-kB via blocking IkB degradation (Haas et al, 1998), the cells were also treated with MG132, a specific proteasome inhibitor. MG132 reduced leptin-induced iNOS expression in a concentration-dependent manner.

After the rapid translocation of NF-kB, this factor binds to kB DNA motifs present in a variety of promoter regions including that of iNOS (Xie et al, 1994), as well as of IkBα (Simeonidis et al, 1999). The *de novo* transcription of IkB-α results in its rapid protein expression, which can reassociate with active cytoplasmatic and nuclear NF-kB, reducing the ongoing NF-kB activity.

Thus we investigated the effect of leptin on IkB-α degradation: the hormone processes this protein, that is rapidly resynthesized at 30 min, most likely through the positive regulation of its transcription by NF-kB. In addition the pretreatment with MG132 caused an accumulation of IkB-α

IkB-α, completely preventing hormonal-induced protein degradation at 5 and 15 min. All cytokines used potentiated the induction of iNOS by leptin and this effect was more evident when the hormone was in presence of IFN-γ. As reported, it was previously shown in primary condrocytes and ATDC5 cells that IFN-γ induced iNOS expression was increased by leptin (Otero et al, 2003). Since both the functional isoform of Ob-R and the INF receptor signal via JAK/STAT pathway, the authors argued that leptin potentiation occurs by this same pathway, without excluding the involvement of other transcription factors. However, in their experimental conditions leptin alone failed in iNOS induction.

Our data demonstrate a role for NF-kB in hormone activity and we suggest that leptin-activated NF-kB can cooperate with the JAK-2/STAT1α pathway induced by IFN-γ to promote the transcription of iNOS. In fact, when MG132 was added to cells stimulated with leptin and IFN-γ we observed a significant but partial reduction of iNOS expression (data not shown).

The hormone synergized iNOS expression also in combination with other pro-inflammatory cytokines such as TNF-α or IL-1β. Although these

cytokines are known to signal through NF-kB by proteosome activation (Hu, 2003; Uehara et al, 1999b), the mechanism of the potentiating effect of leptin on iNOS induction needs further studies. Interestingly the hormone is able to induce an evident increase of NO production when added to IL-1β, which was inactive itself.

Hence leptin, in addition to its well defined role as an adipostat hormone, may be considered a paracrine and/or autocrine mediator of neuroendocrine physiopathological functions and may cooperate in turn with cytokines to amplify and/or sustain CNS inflammation.

PART II

ROLE OF DEIODINASE TYPE II-PRODUCING T3 AND UNCOUPLING PROTEIN 2 IN THE REGULATION OF ENERGY BALANCE

5. INTRODUCTION

For more than a century, increasingly sophisticated methods have been applied to identify and isolate the brain's role in the regulation of energy metabolism.

In the 1940s and 1950s, the hypothalamus emerged as one of the critical sites for energy homeostasis during degeneration studies. Destruction of the hypothalamic ventromedial, paraventricular, and dorsomedial nuclei induced abnormal increase in appetite and food intake (hyperphagia; Hetherington, 1942). By contrast, lesions of the lateral hypothalamus reduced food intake (hypophagia; Anand, 1951).

The idea that the CNS, and the hypothalamus in particular, is key in metabolism regulation was reinforced by the discovery of leptin. Since the identification of leptin, there has been a considerable effort in research to

identify the neural circuits that mediate the effects of adiposity signals in the CNS to limit food intake and elevate energy expenditure. Dozens of neurotransmitter systems with actions in the hypothalamus and other brain regions have been identified that influence food intake and body weight. An important challenge is to organize all of them into functional circuits to identify opportunities to intervene and produce significant and sustained weight loss.

5.1. Hypothalamic circuits

The hypothalamus consists of several nuclei involved in food intake. In the arcuate nucleus (ARC), neurons are located at the bottom of the hypothalamus around the third ventricle in direct contact with peripheral satiety factors like leptin and insulin. In the median eminence (ME), which overlies the ARC, the blood brain barrier (BBB) is not present, and, while the neuronal cell bodies in the ARC are protected by the BBB, the axon terminals are in direct contact with the bloodstream (Peruzzo et al, 2000). For this reason, they are called ‘first order neurons’.

The ARC contains at least two distinct groups of neurons controlling energy balance, one as neurons that contain the orexigenic neuropeptides agouti-gene-related protein (AgRP) and neuropeptide Y (NPY), and two as neurons that contain the anorexigenic neuropeptides pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). From the ARC, neurons project to 'second order neurons' in the paraventricular nucleus (PVN), the ventromedial nucleus (VMH), the dorsomedial nucleus (DMH), and in the lateral hypothalamic area (LHA; Schwartz, 2000).

NPY/AgRP and melanocortinergic neurons are also found to connect with neurons that synthesize other regulators of energy homeostasis, such as melanocortin-concentrating hormone (MCH) and orexin neurons in the LH, which is also named the 'feeding center' (Elias et al, 1998). 'Second order neurons' project to, among others, the nucleus of the solitary tract (NTS) in the brainstem and the dorsomotor nucleus of the vagus (DMV). This communication between hypothalamic pathways and the caudal brainstem, responding to meal-related satiety signals, is essential for long-term regulation of energy homeostasis (Hillebrand et al, 2002).

These hypothalamic regions contain the highest concentration of leptin receptors in the brain, numerous insulin receptors, and also binding sites for glucocorticoids, ghrelin and other peripheral signals, suggesting an important role of this cell group in converting information about metabolic balance

from the periphery to the brain. Peripheral hormones influence energy homeostasis either by activating or inhibiting the activity of the orexigenic or anorexigenic peptides within the hypothalamus (Sainsbury et al, 2002).

5.2. The CNS melanocortin system

The melanocortin system was first linked to the control of skin and hair pigmentation. Several important melanocortin receptor ligands come from the precursor peptide proopiomelanocortin (POMC). In particular, alpha-melanocyte-stimulating hormone (α-MSH) acts on the first of five identified melanocortin receptor subtypes (MC1) located primarily in the skin and hair to produce dark pigmentation. A second melanocortin ligand produced from POMC is adrenocorticotrophic hormone (ACTH). ACTH is produced in the pituitary as an essential part of the stress response. ACTH circulates in the blood and acts upon MC2 receptors found primarily in the adrenal gland to stimulate glucocorticoid secretion (Grill et al, 1998). POMC and α-MSH appear ideally situated to be critical regulators of both food intake and energy expenditure. α-MSH is a potent for agonist not only MC1 receptors but also for MC3 and MC4 receptors that are found primarily within the CNS.

Although MC3 receptors have a rather narrow distribution within specific subregions of the hypothalamus, MC4 receptors are broadly distributed in the CNS, including several regions of the hypothalamus, and are also found in the nucleus accumbens and the dorsal motor nucleus of the vagus (Kishi et al, 2003; Liu et al, 2003).

AGRP is a competitive antagonist/inverse agonist of MC4 receptors (MC4R) in the CNS, so its actions oppose those of α-MSH. Consistent with its important role as an anabolic effector, AGRP expression is increased in fasting and leptin-deficient mice (Haskell-Luevano et al, 2001). Both the POMC and the AGRP/NPY neurons in the arcuate are the main regulators of appetite, satiety, and the regulation of energy expenditure. The POMC cells maintain an anorexigenic tone, whereas the NPY/AGRP neurons maintain an orexigenic tone (Sommer et al, 1967). In this model, activation of POMC neurons by leptin triggers the release of α-MSH from POMC axon terminals, which in turn activates MC4R, leading to suppressed food intake and increased energy expenditure. Simultaneously, leptin suppresses the activaty of arcuate nucleus NPY/AgRP neurons (Cowley et al, 2001), which otherwise would antagonize the effect of α-MSH on MC4Rs through the release of AgRP (Ollmann et al, 1997).

Other peripheral peptides have been hypothesized to act directly upon melanocortinergic neurons in the arcuate. For example, the stomach makes a peptide called ghrelin that, when given either peripherally or directly into the CNS, stimulates food intake. Consistent with this action of ghrelin, circulating levels of ghrelin are increased after periods of fasting and decreased during feeding, the opposite of leptin (Horvath et al, 2001; Inui, 2001). Moreover, ghrelin reduces activity in POMC neurons via actions on ghrelin receptors, also termed growth hormone secretagogue receptors (Cowley et al, 2003). Additionally, circulating levels of peptide YY(3-36) [PYY(3-36)] are increased after meals, and peripheral or interarcuate injections of PYY(3-36) have been reported to reduce food intake. PYY(3-36) also results in increased activity of POMC neurons (Batterham et al, 2002). These data support the hypothesis that a variety of circulating peripheral factors may act upon melanocortin neurons in the arcuate in order to mediate control of energy balance.

5.3. Thyroid hormones

Thyroxine (T_4) and triiodothyronine (T_3) are essential for normal development, differentiation, and metabolic balance. Normally, thyrotropin-

releasing hormone (TRH), a product of the hypothalamus, stimulates the pituitary gland to secrete thyroid-stimulating hormone (TSH). TSH activates the thyroid gland to secrete T_3 and T_4. Quantitatively, T_4 is the largest hormone secreted, but T_3 is the active hormone. The major source of circulating T_3 is the peripheral conversion of T_4 to T_3 by iodothyronine deiodinases. Thyroid hormones travel in the bloodstream linked to the carrier proteins, T_4-binding globulin (TBG), transthyretin, and T_4-binding prealbumin. Free T_3, the unbound hormone, mediates the cellular action of the thyroid gland. In target cells, thyroid hormone binds to nuclear receptors that alter the expression of specific genes (Zhang, 2000).

5.3.1. Thyroid hormone receptors

Thyroid Receptors (TRs) belong to the nuclear receptor superfamily that also includes the receptors for retinoids, vitamin D, fatty acids, and prostaglandins, as well as "orphan receptors" with no identified ligands (Evans, 1988; Mangelsdorf et al, 1995; Ribeiro et al, 1995). TR is encoded by two separate genes, designated TRα and TRβ, located in different chromosomes (17 and 3, respectively, in humans). Alternative splicing from

each gene generates multiple TR isoforms, including TR α1, TR α2, and TR α3 from the TRα gene, and TRβ1 and TRβ2 from the TRβ gene (Lazar, 1993).

TR forms a heterodimer with the retinoid X receptor (RXR) and binds to T3 responsive elements (TREs) in the promoter region of thyroid hormone-responsive genes. Binding of T3 to its receptor effects a change in gene expression. In case of positive gene regulation, the T3-TR interaction induces the release of corepressors and the recruitment of coactivator, resulting in the stimulation of transcription (Jansen et al, 2005).

5.4. Deiodinase

The iodothyronine deiodinases constitute a highly specialized family of enzymes located in several tissues that metabolize thyroid hormone by removal of an iodine atom from either the outer (5′ position) or inner (5 position) ring. Depending on the compound undergoing the reaction, deiodination can result in either the activation or inactivation of a thyroid hormone. Thus, the principal secreted product of the thyroid gland, the

prohormone T4 is converted to the biologically active T3 by 5′-deiodination. In contrast, 5-deiodination of T4 or T3 results in the formation of 3,3′,5′-triiodothyronine (reverse T3) and 3,3′-diiodothyronine, compounds with little or no functional activity (Hernandez, 2003).

Most vertebrate species express three deiodinase enzymes, called types 1, 2, and 3 (DI, DII, DIII), which are encoded from three separate genes. All the deiodinases are membrane-bound proteins, approximately of 30 kDa in size, and contain the uncommon amino acid selenocysteine at the active catalytic site (St. Germain, 2001; Berry, 2002; Bianco, 2002; Kohrle, 2002).

DI is most highly expressed in the adult liver, kidney, and thyroid gland. Although it has both outer and inner ring deiodinase activities, its major physiological function is the generation of plasma T3 (and clearance of plasma rT3). DII is expressed predominantly in the brain, pituitary gland, thyroid, skeletal muscule, and has exclusively a 5′-deiodinase. Although DII may contribute to circulating T3, it appears especially important for the local formation of T3 in these tissues, in particular in the brain. DIII functions as a pure 5-deiodinase are strongly expressed in the pregnant uterus, placenta, and fetal tissues as well as in adult brain and skin (Jansen et al, 2005).

Thyroid hormone metabolism in rat CNS is subject to a highly specific regulatory mechanism that differs substantially from that described in other tissues such as the liver or kidney. In these latter organs, most of the active iodothyronine compound T_3 is taken up directly from the blood, whereas the T_3 supply of the brain depends mainly on cellular uptake and intracellular deiodination of T_4 (Crantz et al, 1982).

5.4.1. Deiodinase type II

Centrally, DII has been found to be highly expressed in the hypothalamus (Riskind et al, 1987; Tu et al, 1997; Diano et al, 1998a), specifically in the arcuate nucleus and the region surrounding the third ventricle. Most recently, it has been demonstarted that the cell types that produce DII are glial cells (Tu et al, 1997; Diano et al, 2003a), astrocytes, and tanycytes. In particular, tanycytes express one of the highest concentrations of DII activity and DII mRNA in the brain (Tu et al, 1997). Astrocytes are homogeneously distributed within the arcuate nucleus, contributing to the establishment of the blood brain barrier, while the cell bodies of tanycytes are predominately located on the floor and ventrolateral walls of the third ventricle, between the

rostral and caudal limits of the hypothalamic median eminence (Bruni, 1974; Krisch et al, 1978). Characteristic of tanycytes are apical microvilli and stereocilia that extend into the cerebrospinal fluid (CSF), and a basal process that ramifies into the underlying neuropil, encircling blood vessels in the adjacent arcuate nucleus, and terminating in numerous endfeet processes on or near fenestrated capillaries of the primary portal plexus in the external zone of the median eminence (Bruni, 1974). Thus, tanycytes may provide a bidirectional, cytoplasmic conduit between the CSF in the third ventricle and the blood in the vascular elements of the arcuate nucleus and/or median eminence, allowing the movement of substances from one compartment to the other. Because DII activity and DII mRNA are not present in the hypothalamic PVN (Tu et al, 1997), it has been hypothesized that conversion of T_4 to T_3 by DII-expressing tanycytes may be part of the feedback regulation of the hypothalamic-pituitary-thyroid axis after uptake of T_4 by tanycytes from CSF or vascular compartments and release of T_3 back into the CSF, bloodstream, or median eminence (Tu et al, 1997).

5.4.2. Deiodinase type II regulation

Deiodinases are regulated by circulating thyroid hormone levels (Bianco et al, 2002). In particular, DII mRNA and activity are increased when circulating T4 levels are decreased, and the opposite occurs when T4 levels are increased. It has been suggested that the major role of the DII is to maintain T_3 homeostasis, producing adequate intracellular levels of T_3 to ensure all T_3-dependent cellular functions in the tissue (Calvo et al, 1990; Leonard et al, 1981). In fact, in hypothyroid adult rats, the increased activity of DII is able to normalize T_3 levels in the brain and other tissues after infusion of T_4 in doses insufficient to adjust plasma T_4 and T_3 levels (Escobar-Morreale et al, 1995). In parallel with altered DII activity, failure of the thyroid gland [thyroidectomy or chemical inhibition of thyroid hormone production through 6-*n*-propyl-2-thiouracil (PTU), methimazole, or methimazole plus iopanoic acid] induces a modest elevation in hypothalamic TRH levels (Rondeel et al, 1992). In contrast, hypothyroid conditions induced by fasting coincide with suppressed production and release of TRH in the hypothalamic paraventricular nucleus and median eminence (Van

Haasteren et al, 1995). The central mechanism that underlies the emergence of this apparent paradox in thyroid feedback is ill defined. A different expression of type II deiodinase during fasting-induced hypothyroidism and hypothyroidism due to the failure of the thyroid gland may contribute to the development of this apparent paradox in thyroid feedback. During food deprivation, DII mRNA and activity are elevated in the hypothalamus. As expected, this increase coincides with a decrease in the plasma levels of T4 as well as T3. T4 replacement in these fasted animals, surprisingly, does not restore DII mRNA and activity to lower levels (Diano et al, 1998b). Thus, it would appear that, during fasting, the signal for the elevation of DII does not originate in the thyroid gland and, therefore, the lower circulating thyroid hormone levels under this condition are not the cause but, rather, the result of suppressed TRH production and release.

5.5. Uncoupling proteins

Uncoupling proteins (UCPs) are homologous proteins constituting a subfamily of mitochondrial anion carriers that are evolutionarily related and possibly derived from an ancestral protein that acted as a proton/anion carrier

(Lanni et al, 2003). The first of these proteins, UCP1, was discovered in brown adipose tissue, where it has a well-described role in thermogenesis. UCPs are located in the inner membrane of the mitochondria, and it is thought that their primary function is to leak hydrogen protons from the intermembrane space to the matrix of the mitochondria (Bouillaud et al, 1985; Fleury et al, 1997). In the individual mitochondrion, these proteins may deprive the driving force of ATP synthase from catalyzing ATP synthesis, dissipate energy in the form of heat, diminish the production of superoxides, and decrease the likelihood of calcium entry to the mitochondrial matrix (Negre-Salvayre et al, 1997). However, despite decreased ATP production by an individual mitochondrion, the neurons overall will have more ATP available because uncoupling supports mitochondrial proliferation in the brain (Diano et al, 2003b) as well as in the adipose tissue (Rossmeisl, 2002).

UCP1, the most well characterized UCP, is expressed solely in brown adipose tissue and is responsible for thermogenesis in small rodents (Nicholls et al, 1984). In the last few years, however, several other members of the UCP family have been discovered and found to promote partial uncoupling of oxidation from phosphorylation *in vitro*. These proteins include UCP2, UCP3, UCP4, and BMCP1/UCP5 (Fleury et al, 1997; Boss et al, 1997; Mao et al, 1999; Sanchis et al, 1998). The five putative UCPs differ greatly among

themselves, in tissue distribution and regulation, and may have distinct physiological roles. While UCP1 and UCP3 are expressed only in peripheral tissues (UCP1 in brown adipose tissue and UCP3 in skeletal muscle and the heart in humans), UCP4 and BMCP1 are predominantly expressed in the central nervous system (Mao et al, 1999; Sanchis et al, 1998), and UCP2 is expressed in muscle, adipose tissue, spleen, and the central nervous system (Fleury et al, 1997; Richard et al, 1998; Horvath et al, 1999; Diano et al, 2000).

5.5.1. Uncoupling protein 2 in the brain

In the brain, in both rodents and primates, UCP2 is expressed predominantly in neuronal populations of subcortical regions that are involved in the central regulation of autonomic, endocrine, and metabolic processes. These regions include key brain stem and hypothalamic nuclei (Richard et al, 1998; Horvath et al, 1999; Diano et al, 2000). In rat extracts from hypothalamic regions with abundant UCP2 expression, the mitochondrial respiratory control ratio (RCR: ratio between state 3 and state 4) was found significantly lower than that measured in regions that lack UCP2 expression (the striatum-lateral thalamus

region; Horvath et al, 1999). On the one hand, the reduced RCR in the hypothalamus was attributable exclusively to an enhanced state 4 mitochondrial respiration rate. State 4 respiration is caused only by the proton leak across the inner membrane, and mitochondria respiring in the absence of ADP or nonphosphorylating mitochondria. On the other hand, state 3 mitochondrial respiration rate (phosphorylating mitochondria in which the respiration is not controlled by proton leak) measured in the two regions did not differ. These results demonstrate regional variations in uncoupling activity in which higher mitochondrial uncoupling activity is positively correlated with UCP2 expression. The presence of decreased mitochondrial energy coupling efficiency (increased proton leak) in UCP2-containing brain regions supports the hypothesis that a thermogenic mechanism is intrinsic to distinct neuronal pathways (Horvath et al, 1999). Besides, brain tissue temperature in UCP2-containing brain regions has a significantly higher local level when compared to that in other sites or to the core body temperature. Brain sites other than those containing UCP2, such as the striatum and thalamus, exhibit a significant mitochondrial proton leak, which is consistent with the subsequent discovery of other putative brain uncouplers, such as UCP4 and BMCP1 (Mao et al, 1999; Sanchis et al, 1998).

UCP2 expression has been found in mitochondria of neurons, particularly in axon terminals, suggesting a direct role in interneuronal communication for mitochondrial uncoupling/thermogenic mechanism in circuits involved in the central regulation of homeostasis. Acute heat production in axon terminals could immediately accelerate synaptic transmission by affecting synaptic vesicle formation and traffic, neurotransmitter release and reuptake, and the tertiary structure of neuromodulators, as well as directly influence the postsynaptic membrane potential (Horvath et al, 1999). UCP2 and NPY have been found an abundant coexpression in the arcuate nucleus neurons (Horvath et al, 1999). In further support of the involvement of UCP2 in central metabolic pathways, UCP2 cells and processes were closely associated with leptin receptors, and after fasting, all of the c-fos-expressing neurons (marker for neuronal activity) were targeted by numerous UCP2-containing boutons. In accordance with the projection of arcuate nucleus neurons, UCP2-containing axons were found to innervate other hypothalamic peptidergic systems that participate in metabolic regulation, including MCH- and orexin-producing cells.

5.6. Aim of the study

In the CNS, the active form of the thyroid hormone, T3, derives from the cellular uptake and intracellular 5'-monodeiodination of T4 by DII. In the adult rat brain, high DII activity is present in the arcuate nucleus/median eminence region, and DII-producing cells are both astrocityes and tanicityes (Guadano-Ferraz et al, 1997; Diano et al, 1998a). Arcuate nucleus DII-producing glial cells may actively participate in metabolism regulation because they are in close proximity to T3-targeted neurons, including those producing NPY, AgRP and POMC, all of which project onto the paraventricular TRH cells (Legradi and Lechan 1998; Fekete et al, 2000). In support of this theory, it has been found that arcuate nucleus DII activity is elevated during fasting (Diano et al, 1998b), a situation during which negative feedback of circulating thyroid hormones is diminished in the hypothalamus.

The aforementioned observations give impetus to our hypothesis that it is the activation of arcuate nucleus DII by diminishing leptin and rising corticosterone levels that disrupts normal thyroid feedback during short-term food deprivation. On the one hand, we propose that during fasting, elevated

arcuate nucleus DII activity promotes both activation of the inhibitory NPY/AgRP and inhibition of the excitatory α-MSH projections onto paraventricular TRH neurons, thereby explaining the paradox that suppressed levels of circulating T4 and T3 parallel the inactivation of TRH.

On the other hand, we propose that the interest in the interplay between DII and UCP2 in the regulation of the melanocortin system emerges when the proximity of these mechanisms in the arcuate nucleus is revealed.

NPY cells express UCP2 and its expression and activity are regulated by thyroid hormones, T3 in particular. Furthermore, the activation of UCP2 may lead to elevated local temperature (Horvath et al, 1999), mitochondrial proliferation and ATP levels (Diano et al, 2003b), all of which enhance neuronal activity. We propose that, taken together, elevated corticosterone levels during negative energy balance trigger T3-regulated mitochondrial uncoupling in NPY/AgRP neurons, a key mechanism that allows NPY neurons to learn to remember the depletion of the energy stores. This learning/memory process within the melanocortin system entails mitochondrial proliferation and increased available ATP levels in NPY/AgRP neurons. This, in turn, enables elevated NPY/AgRP and suppressed POMC neuronal activity despite increasing metabolic signals in the circulation following re-feeding.

6. MATERIALS AND METHODS

6.1. Experiment 1

6.1.1. Animal treatment

Seventy Sprague Dawley male rats from Taconic Farms Inc. (200-250gr) were used in this study.

Animals were divided into 14 experimental groups:
Group 1: Intact rats with food *ad libitum* (n=5)
Group 2: Sham-operated rats with food *ad libitum* (n=5)
Group 3: Adrenalectomized (ADX), *ad libitum*-fed rats (n=5)
Group 4: ADX, low corticosterone-treated *ad libitum*-fed rats (n=5)
Group 5: ADX, high corticosterone-treated, *ad libitum*-fed rats (n=5)
Group 6: Leptin-treated, *ad libitum*-fed rats (L; n=5)
Group 7: Intact rats fasted for 2 days (48 hours) (n=5)
Group 8: 2-day fasted sham-operated rats (n=5)
Group 9: 2-day fasted and ADX rats (n=5)
Group 10: 2-day fasted, ADX and low corticosterone-treated rats (ADX+C; n=5)
Group 11: 2-day fasted, ADX and high corticosterone-treated rats (ADX+C(h); n=5)
Group 12: 2-day fasted and leptin-treated rats (L; n=5)

Group 13: 2-day fasted, adrenalectomized and high corticosterone and leptin-treated rats (ADX+L+C(h); n=5)
Group 14: 2-day fasted, ADX and leptin-treated rats (n=5)

Bilateral adrenalectomy or a sham operation was performed under general anesthesia 1 week before starting the experiments. Immediately after surgery, adrenalectomized rats received drinking water containing 0.9% (w/v) NaCl.

6.1.2. Corticosterone treatment

Corticosterone treatment was initiated at the time of surgery. Corticosterone (0.2 mg/ml, low dose, or 0.6 mg/ml, high dose; Sigma St. Louis, MO) was dissolved in ethanol and this solution was added to the water yielding a final concentration of 2% ethanol. All the other groups non-treated with corticosterone were given similar drinking water without the hormone. To confirm that the daily water, and thereby, corticosterone intake reached the estimated amount and to ensure that the corticosterone supplementation did not alter drinking, we measured water consumption over 24 hrs.

6.1.3. Leptin treatment

At the time of the initiation of food deprivation (1 week after adrenalectomy), all groups were implanted subcutaneously with Alzet micro-osmotic pumps (Model 1003D, 1 μl per hour, 3 days; Alza, Palo Alto, CA) which were filled

with either PBS (as control in groups 1-5 and 7-11) or 500 ng/µl leptin (groups 6, 12-14; recombinant rat leptin, PeProTech Inc., Rocky Hill, NJ).

6.1.4. DII enzymatic activity

The animals were sacrificed and the mediobasal hypothalamus (defined rostrally by the optic chiasma, caudally by the mamillary bodies, laterally by the optic tract and superiorly by the apex of the third ventricle) was dissected and stored at -80ºC prior to DII activity measurements. DII activity was measured based on the release of radioiodide from the ^{125}I-labeled substrate. Using 100,000 cpm of L[^{125}I] T4 (PerkinElmer Life Sciences, Boston MA) for each sample, an incubation mixture containing 100 µg tissue homogenate in 0.1 M potassium phosphate buffer (pH=7.0), 1 mM EDTA in the presence of 5 nM T4, 30 mM DTT, 1 mM PTU and 1 µM T3 (to inhibit the inner ring deiodination of T4 due to the presence of D3 activity) were incubated in duplicate at 37°C for 1 h. The reactions were stopped by addition of 50 µl ice-cold 5% BSA followed by 200 µl 20 % ice-cold trichloroacetic acid and centrifuged at 4000 g for 20 min. The supernatant was further purified by cation exchange chromatography on 1.6 ml Dowex 50 W-X2 (100-200 mesh; Sigma Chemical Co., St. Louis, MO). The iodide was then eluted with 2x1 ml 10% glacial acetic acid and counted in a *gamma* counter. Enzymatic deiodination was corrected for non-enzymatic ^{125}I production as determined in blank incubations without homogenates and multiplied by 2 to account for the random labeling and deiodination for the 3' and 5' positions in labeled T4. Enzymatic activity is expressed in femtomoles of ^{125}I- released per hour per milligram of protein.

For the determination of proteins, the bicinchonic acid assay (BCA Protein Assay, Pierce Rockford, IL) was employed.

6.1.5. Hormone measurements

Plasma total T4 (TT4), free T4 (FT4), T3 and thyroxine binding capacity (TBC) were measured by the Microparticle Enzyme Immunoassay (MEIA), using AxSym System in the Clinical Chemistry Laboratory of the Yale-New Haven Hospital. Plasma leptin, corticosterone was measured using RIA kits from Linco Research Inc. (St. Charles, MO) according to the manufactures protocols. Due to the high number of samples measured in duplicates, assays conducted in our laboratory were run at different time. However, to ensure the consistency of the results, samples from fed and fasted intact rats were always included in every assay as control.

6.1.6. Statistical analysis

For each experiment, means were compared between experimental groups using one-way analysis of variance (ANOVA) with mean comparisons by the Student-Newman-Keuls Method. A level of confidence of $p< 0.05$ was used to determine significant differences.

6.2. Experiment 2

6.2.1. Hypothalamic TRH mRNA levels

Twenty Sprague-Dawley male rats (200-250g BW; Taconic Farms, Inc.) were implanted with a cannula into the third ventricle (Bregma -0.8 mm). Animals were divided into 2 experimental groups: a control group (n=10) was infused with 0.9% saline, while the second group (n=10) was infused with 10^{-7}M IOP diluted in 0.9% saline (Alzet micro-osmotic pump 1µl/hour, 3 days). All the animals were infused for the duration of the experiment. Twenty-four hours after the implantation of the pump, the animals were further divided in the following groups: n=5 infused with 0.9% saline and fed *ad libitum* ; n=5 infused with 0.9% saline and fasted for 48 hours; n=5 infused with 10^{-7}M IOP and fed *ad libitum*; n=5 infused with 10^{-7}M IOP and 48 hour fasted. The animals were then perfused and processed for semi-quantitative *in situ* hybridization histochemistry.

An 826 bp fragment of complementary DNA (cDNA) of TRH was amplified based on the RT-PCR reaction, using specific oligonucleotide primers derived from the coding region of the rat TRH sequence. Total RNA was extracted from the hypothalamus by guanidium thiocyanate-phenol-chloroform method using TriZol reagent (Life Tecnologies, Grand Island, NY) and transcribed using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Piscataway, NJ). Linearized DNA was transcribed using T7 polymerase (antisense cRNA probe) and T3 polymerase (sense cRNA probe; Riboprobe Combination System T3/T7, Promega Corporation, Madison, WI) and labeled with ^{35}S-UTP (Amersham; 10 mCi/ml).

The purified cRNA probes was heated at 80°C for 2 minutes with 500 μg/ml yeast tRNA and 50 μM DTT in water before being diluted to an activity of 5.0 x 10^7 dpm/ml with hybridization buffer containing 50% formamide, 0.25 M sodium chloride, 1x Denhardt's solution and 10% dextran sulfate. Sections with this hybridization solution (150 μl/slide) were incubated overnight at 50°C. Following hybridization, the slides were washed in 4xSSC prior to RNase digestion (20 μg/ml for 30 min at 37°C), rinsed at room temperature in decreasing concentrations of SSC that contained 1 mM DTT (2x, 1x, 0.5x; 10 minutes each) to a final stringency of 0.1xSSC at 65°C for 30 minutes.

After dehydration in increasing alcohols, the sections were exposed to β-max hyperfilm (Amersham) for 5 days before being dipped in Kodak NTB liquid emulsion diluted 1:1 with distilled water. The dipped autoradiograms was developed 21 days later with Kodak D-19 developer, fixed, and the sections counterstained through the emulsion with hematoxylin. Sections were examined under brightfield and darkfield illumination.

The density of the hybridization product was assessed in the different experimental groups. In order to digitally analyze, quantitate and compare the amount of TRH mRNA, an Image-1/AT image processor (Universal Imaging Corporation, West Chester, PA) using an Olympus IMT-2 inverted microscope with dark field optics (Olympus Corporation, Lake Success, New York) and a Hamamatsu CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) was employed. Six sections per animal were selected from the same area to assess the intensity of the hybridization product. The total surface covered by the hybridization product was assessed within a test region measuring 2x10^5 mm^2 that contains the paraventricular nucleus. The threshold for measurement was assessed for each slide by determining the background labeling in the nearby ventromedial nucleus.

6.2.2. Hypothalamic T3 measurements

Twenty male Sprague-Dawley rats (200-250 gr. BW; Taconic Farm, Inc) were used in this study. Each animal was implanted with one cannula into the third ventricle (Bregma -0.8 mm) connected to a micro-osmotic pump (Alzet Corp., Palo Alto, CA; 1.0 μl/hr for 3 days). Animals were divided into 4 experimental groups: group 1 (n=4) and group 2 (n=4) infused with 0.9% saline, group 3 (n=4) and group 4 infused with 10^{-7}M IOP diluted in 0.9% saline. Rats were infused for the duration of the experiment. Twenty-four hours after implantation, group 2 and 4 were food-deprived for 48 hrs. All animals were then sacrificed and the hypothalamus was collected and immediately frozen and stored at –80 ˚C.

Triiodothyronine (T3) was extracted from the hypothalamus by adding methanol 95% containing PTU 10^{-4} M. Tissues were homogenized and centrifuged at 13000 rpm and the pellets re-suspended twice using methanol solution. The supernatants were evaporated to dryness and resuspended in GAB buffer (0.2 M Glycine/0.13 M Acetate with 0.02% BSA). T3 was determined by radioimmunoassay system (RIA). Samples and standard curve were incubated at 4^{o}C with polyclonal antibody against T3 (Fitzgerald Industries International, Concord MA) in RIA buffer GAB. Three days later, 10,000 cpm of radiolabeled [^{125}I]-T3 (Specific activity 2200Ci/mmol; PerkinElmer Life Sciences, Boston MA) was added to each tube. After two days of incubation, a rabbit gamma globulin (Jackson ImmunoResearch Laboratories) diluted in 0.1 M EDTA and 16% polyethylene glycol with goat

antirabbit IgG (Antibodies Incorporated, Davis CA) in GA buffer (0.2 M Glycine/0.13 M Acetate) was added to precipitate the antibody- T3 complex. After centrifugation the precipitates were counted in a γ-counter.
T3 is expressed in pg per mg of weight tissue.

6.2.3. Double immunocytochemistry

Adult C57Bl6 mice carrying GFP in NPY/AgRP neurons (Pinto et al., 2004) were used in this study. Animals were perfused transcardially with 4% paraformaldehyde, 15% picric acid and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB; pH 7.35). Brain sections were pre-incubated in 1% H2O2 and 1% sodium borohydride, before overnight incubation with rabbit anti-DII (Diano et al., 2003b). After the secondary antibody (biotinylated goat antirabbit IgG; Vector Laboratories, Inc., Burlingame, CA) the sections were incubated with either avidin-biotin-peroxidase (ABC Elite Kit, Vector Laboratories, Inc.) or avidin-Texas Red for immunofluorescent microscopy.
For electron microscopy, the immunolabeling for DII was visualized with a modified version of the nickel-diaminobenzidine (Ni-DAB) reaction (15 mg DAB, 0.12 mg glucose oxidase, 12 mg ammonium chloride, 600 µl 0.05 M nickel ammonium sulfate, and 600 µl 10% ß-D-glucose in 40 ml PB) and were further incubated with a mouse anti-GFP antibody (Molecular Probes, Eugene OR). The immunoreactivity for GFP was visualized with DAB resulting in a brown reaction product. Sections were then postosmicated (1% OsO4), dehydrated through increasing ethanol concentrations and embedded in Durcupan (Electron Microscopy Science, Fort Washinghton, PA, USA). Ribbons of ultrathin sections (Reichert-Jung Ultramicrotome) were collected

on Formvar-coated single slot grids, and examined using a Philips CM-10 electron microscope.

6.2.4. Brain mitochondria preparation and uncoupling activity measurements

Twenty-four C57B6 mice were used in this study and divided in the following groups: 8 mice were injected i.p. with T3 (5 ug/30g BW); 8 mice were injected i.p. with saline; 8 mice were fasted for 24 hours and 8 rats were fed *ad libitum*. Mice injected with saline or T3 were sacrificed 6 hours after the injection. The hypothalamus was dissected and homogenized in the isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% fatty acid-free BSA, 20 mM HEPES, 1 mM EGTA, pH adjusted to 7.2 with KOH). Protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL). Mitochondrial respirations were assessed using a Clark-type oxygen electrode (Hansatech Instruments, Norfolk, UK) at 37°C with pyruvate and malate (5 and 2.5 mM) as oxidative substrates in respiration buffer (215 mM mannitol, 75 mM sucrose, 0.1% fatty acid-free BSA, 20 mM HEPES, 2 mM MgCl, 2.5 mM KH_2PO_4, pH adjusted to 7.2 with KOH). After the addition of ADP and oligomycin, an inhibitor of H^+-transporting ATP synthase, UCP-mediated proton conductance was measured as increased fatty acid-induced respiration which was then compared with state 4 respiration induced by oligomycin.

6.2.5. Fasting and refeeding

Five male DII knockout, 5 wild type, 5 UCP2 knockout, and 5 wild type mice were fasted for 24 hours. Food was replaced after 24 h at which time body weight and core body temperature were recorded and food intake was measured in a 60 minute period following re-feeding.

6.2.6. Semi-quantitative RT-PCR

RT-PCR was used to assess mRNA levels of UCP2 in DII KO animals (n=5) compared to those in their wild type controls (n=5), and in T3-treated rats compared to those in saline treated rats. Animals were sacrificed and hypothalami were dissected. Total RNA was isolated using Trizol. cDNA was synthetized by a First-Strand cDNA Kit (Amersham Biosciences, Piscataway, NJ).
Primers to amplify UCP2 were:
forward 5'-CTACAAGACCATTGCACGAGAGG-3' and
reverse 5'-AGCTGCTCATAGGTGACAAACAT-3' (396 bp).
As internal control a 201 bp fragment of GAPDH
5'-CTCATGACCACAGTCCATGC-3' and reverse
5'-CACATTGGGGGTAGGAACAC-3' was amplified.
Reactions were analyzed by gel electrophoresis and intensity of the bands was analysed by Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD).

6.2.7. Quantification of mitochondria in GFP-NPY/AgRP cells of the arcuate nucleus

C57Bl6 adult mice carrying GFP in NPY/AgRP neurons and GFP-NPY ob/ob mice were perfused as described above and their brains were processed for GFP immunolabeling for electron microscopic examination. The unbiased stereological methods for mitochondria number assessment that were used are based upon our published paper (Pinto et al, 2004). A series of sections was defined with equal spacing of a known distance, and the volume of the structure of interest was estimated using the Cavalieri method (Gundersen and Jensen, 1987). The counts obtained represent the number of mitochondria per unit volume of the structure of interest. These counts were then corrected for the total volume of the structure obtained with the Cavalieri method, yielding a value for total mitochondrial number for the cell class and structure sampled.

6.2.8. Statistical analysis

For each experiment, means were compared between experimental groups using one-way analysis of variance (ANOVA) with mean comparisons by the Student-Newman-Keuls Method. A level of confidence of $p< 0.05$ was used to determine significant differences.

7. RESULTS

7.1. Experiment 1

7.1.1. Hormone measurements

Plasma levels of corticosterone, leptin, TT4, FT4, T3 and TBC are shown in Table 1 for fasted animals and in Table 2 for *ad libitum* fed rats.

The forty-eight hour fasting increased corticosterone levels in the intact rats (Table 1) compared to fed controls (Table 2). Adrenalectomy with or without leptin replacement in fasted rats resulted in undetectable levels of corticosterone in their plasma (Table 1). Corticosterone administration at low doses (ADX+C) in fasted rats significantly increased circulating corticosterone to physiological levels (value not significantly different from intact fed rats in Table 2). The levels of corticosterone in fasted adrenalectomized rats with high dose of corticosterone (ADX+C(h)) and in fasted rats treated with leptin and high corticosterone (L+C(h)) were comparable to the level of the intact fasted rats. Leptin administration to fasted animals reduced corticosterone to the levels of intact fed rats.

In *ad libitum* fed animals (Table 2), adrenalectomy was successfully performed as shown by the undetectable levels of corticosterone. Corticosterone replacement to physiological levels was conducted successfully as was corticosterone replacement to fasted levels.

Fasting induced a significant decrease in plasma leptin levels (Table 1) compared to *ad libitum* fed rats (Table 2). Adrenalectomy of fed animals significantly decreased leptin levels (Table 2) although they were statistically higher than the leptin levels of fasted rats. Corticosterone administration at low and high doses increased leptin levels in fed ADX animals (Table 2). Only high doses of corticosterone significantly increased plasma leptin (Table 1).

Leptin levels in fasted ADX+L, and L groups (Table 1) were comparable to leptin level in the intact fed group (Table 2).

Total T4 as well as free T4 in fasted intact rats was significantly lower compared to fed intact animals. Moreover, none of the treatments in any of the fasted groups could elevate TT4 and FT4 levels to those of fed intact rats.

Fasted rats with high levels of circulating corticosterone (intact, ADX+C(h) and L+C(h)) showed significantly lower levels of TT4 compared to animals with no detectable or just physiological levels of corticosterone (Table 1). In contrast, no statistically significant values were observed in the circulating levels of free T4 between any of the fasted rats.

In fed animals, TT4 as well as FT4 showed a significant decrease in the group treated with the high dose of corticosterone compared to all other groups with undetectable, low or physiological levels of corticosterone (Table 2).

T3 levels in fasted animals also showed significant decreases compared to those in fed rats. In fasted animals, plasma T3 in ADX+C(h) and leptin treated rats showed values similar to those in intact fed rats. In addition, ADX rats treated with corticosterone to physiological levels showed an increase in circulating T3 compared to intact fasted rats. This increase was significantly lower than that observed in leptin treated and ADX+C(h) groups (Table 1).

Fasted rats showed a thyroxine binding capacity (TBC) that, although slightly higher, was not significantly different from that observed in fed animals. Moreover, undetectable levels of corticosterone resulted in a significant increase in TBC both in fasted and fed animals, although ADX fed rats with physiological levels of corticosterone showed an elevated TBC compared to that in the intact fed group.

FASTED RATS	CORT µg/dl	Leptin ng/ml	TT4 µg/dl	FT4 ng/dl	T3 ng/dl	TBC µg/dl
Intact	**38.1±6.8**[b,c,d,f]	**0.3±0.2**[d,e,f,g]	**3.1±0.14**[b,c,d,f]	**2.9±0.1**	**33±3.0**[b,c,d,e,f]	**5.1±0.4**[b,d]
ADX	**ND**[a,c,e,f,g]	**0.2±0.1**[c,d,e,f,g]	**4.6±0.12d**[a,e,g]	**2.6±0.1**	**49.8±2.1**[a,c,e,f]	**7.9±0.5**[a,c,e,f,g]
ADX+C	**11.2±2.2**[a,b,d,e,g]	**0.6±0.2**[b,d,e,f,g]	**4.3±0.13**[a,e,g]	**2.9±0.2**	**59.5±1.1**[a,b,d,e,f,g]	**5.5±0.6**[b,d]
ADX+L	**ND**[a,c,e,f,g]	**2.9±0.1**[a,b,c,e,g]	**4.9±0.32**[a,e,g]	**2.7±0.3**	**49.3±2.5**[a,c,e,f]	**8.6±1.1**[a,c,e,f,g]
ADX+C(h)	**31.8±2.8**[b,c,d,f]	**1.7±0.4**[a,b,c,d,g]	**3.6±0.1**[b,c,d,f]	**3.1±0.5**	**70.3±4.5**[a,b,c,d,g]	**5.6±0.7**[b,d]
L	**11.6±1.1**[a,b,d,e,g]	**2.1±0.3**[a,b,c,d,g]	**4.5±0.2**[a,e,g]	**3.2±0.2**	**70.0±4.4**[a,b,c,d,g]	**5.07±0.3**[b,d]
L+C(h)	**30.4±4.7**[b,c,d,f]	**6.2±0.1**[a,b,c,d,e,f]	**3.5±0.8**[b,c,d,f]	**1.6±1.0**	**39±13.3**[c,e,f]	**5.7±1.0**[b,d]

Table 1 Effects of ADX, corticosterone replacement at low or high doses, and /or leptin administration on hormone levels of food deprived animals (n=5 for each group).
Results are expressed as mean±SEM.

[a]P<0.05 compared to Intact.

[b]P<0.05 compared to ADX.

[c]P<0.05 compared to ADX+C.

[d]P<0.05 compared to ADX+L.

[e]P<0.05 compared to ADX+C(h).

[f]P<0.05 compared to L.

[g]P<0.05 compared to L+C(h) CORT=Corticosterone; TT4= Total T4; FT4=Free T4; T3= 3,5,3' triiodothyronine; TBC= Thyroxine binding capacity; ND= non detectable

FED RATS	**CORT** μg/dl	**Leptin** ng/ml	**TT4** μg/dl	**FT4** ng/dl	**T3** ng/dl	**TBC** μg/dl
Intact	**11.6±5.0**[b,d,e]	**2.9±0.3** [b,c,d,e]	**5.5±0.8** [d]	**3.6±0.4**[d]	**76.0±6** [d]	**4.5±0.7**[b,c]
ADX	**ND**[a,c,d,e]	**1.2±0.03** [a,c,d,e]	**5.2±0.2** [d]	**3.2±0.1**[d]	**70.33±4.1** [d]	**6.1±0.3**[a,e]
ADX+C	**11.9±3.5**[b,d,e]	**4.2±0.7**[a,b]	**5.1±0.6** [d]	**4.4±0.1** [b,d,e]	**79.7±8.9** [d]	**6.4±0.4**[a,e]
ADX+C(h)	**24.2±1.4**[a,b,c,e]	**5.6±0.7**[a,b]	**2.7±0.5** [a,b,c,e]	**2.2±0.3**[a,b,c,e]	**60.3±6.4**[a,b,c,e]	**5.2±0.6**[b,c]
L	**3.3±0.2**[a,b,c,d]	**5.8±2.1**[a,b]	**4.1±0.5** [d]	**3.1±0.1**[c,d]	**71.3±3.4**[d]	**4.8±0.3**[b,c]

Table 2 Effects of ADX, ADX plus corticosterone replacement at low or high doses, and leptin administration on hormone levels of *ad libitum* fed animals (n=5 for each group).

Results are expressed as mean±SEM.

[a]P<0.05 compared to Intact.

[b]P<0.05 compared to ADX.

[c]P<0.05 compared to ADX+C.

[d]P<0.05 compared to ADX+C(h).

[e]P<0.05 compared to L.

CORT= Corticosterone; TT4= Total T4; FT4=Free T4; T3=3,5,3' triiodothyronine; TBC= Thyroxine binding capacity; ND= non detectable.

7.1.2. DII enzymatic activity

Fasting in intact animals (94.33±2.69 fmol/h/mg protein) increased DII enzymatic activity compared to that in *ad libitum* fed rats (80.5±4.95 fmol/h/mg protein; Fig. 1).

In *ad libitum* fed rats, DII activity (Fig. 2A) of intact and sham-operated animals (80.5±4.95 and 78.76±1.23 fmol/h/mg protein, respectively) did not show any significant changes compared to those in ADX (86.39 ±0.99 fmol/h/mg protein) rats or ADX rats treated with corticosterone at either low (85.45±1.87 fmol/h/mg protein) or high doses (85.63±3.08 fmol/h/mg protein).

On the other hand, in fasted animals (Fig 2B), ADX significantly reduced DII activity (78.67±3.35 fmol/h/mg protein) compared to that in intact and sham-operated rats (94.33±2.69 and 98.33±2.87 fmol/h/mg protein, respectively). When corticosterone was replaced in ADX fasted rats, DII activity showed a dose-dependent increase, with the greater elevation at the high dose of corticosterone, that mimics the serum level of the intact fasted rats (88.25±0.87 fmol/h/mg protein and 96.27±1.46 fmol/h/mg protein, in low dose and high dose, respectively). Indeed, at

the high dose of corticosterone, DII activity did not significantly differ from that in intact fasted animals (96.27±1.46 fmol/h/mg protein in corticosterone treated compared to 94.33±2.69 fmol/h/mg protein in the intact rats).

This difference in the effect of corticosterone on DII activity in fed compared to fasted rats, led us to analyze the consequences of leptin administration in the same paradigm (Fig. 3). When leptin was administered to fed animals, no changes were observed between this group and their control fed rats (80.5±4.95 and 78.42± 4.21 fmol/h/mg protein). On the other hand, leptin administration to fasted animals decreased DII activity (76.52±1.61 fmol/h/mg protein) compared to that in their control intact fasted rats (94.33±2.69 fmol/h/mg; Fig.4) and reduced DII activity to the levels of fed control rats (80.5±4.95 fmol/h/mg protein; Fig. 3). Leptin treatment to fasted rats also reduced corticosterone plasma levels further supporting the fact that, during negative energy balance, the decrease in plasma leptin and the simultaneous increase in corticosterone levels are necessary for the elevation of DII activity.

We examined the effect of the administration of leptin, corticosterone or both on DII activity of fasted rats (Fig. 4) and compared the following groups: intact fasted, intact fasted plus leptin, ADX fasted, ADX fasted plus leptin and ADX fasted plus high corticosterone levels. As described above, leptin administration to fasted animals restored DII activity to the level of intact fed rats. ADX rats fasted for 48 hrs and ADX fasted rats treated with leptin did not show any differences in DII activity compared to that in the intact rats given leptin. When we administered high doses of corticosterone to fasted ADX rats, DII activity was statistically elevated to the levels of intact fasted rats and when these animals (ADX+C at high doses) were treated with leptin to physiological levels, we did not observe the increase in DII activity that was seen without leptin treatment (Fig. 4).

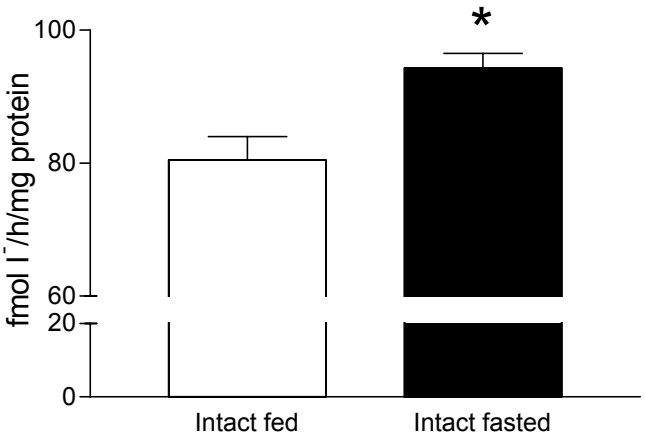

Fig.1 Hypothalamic DII activity expressed in femtomoles of I^- per hour per mg of protein in intact *ad libitum* fed rats (n=5) and intact fasted rats (n=5). Using one-way ANOVA, results are expressed as the mean ± SEM. * $P<0.05$ compared to fed intact.

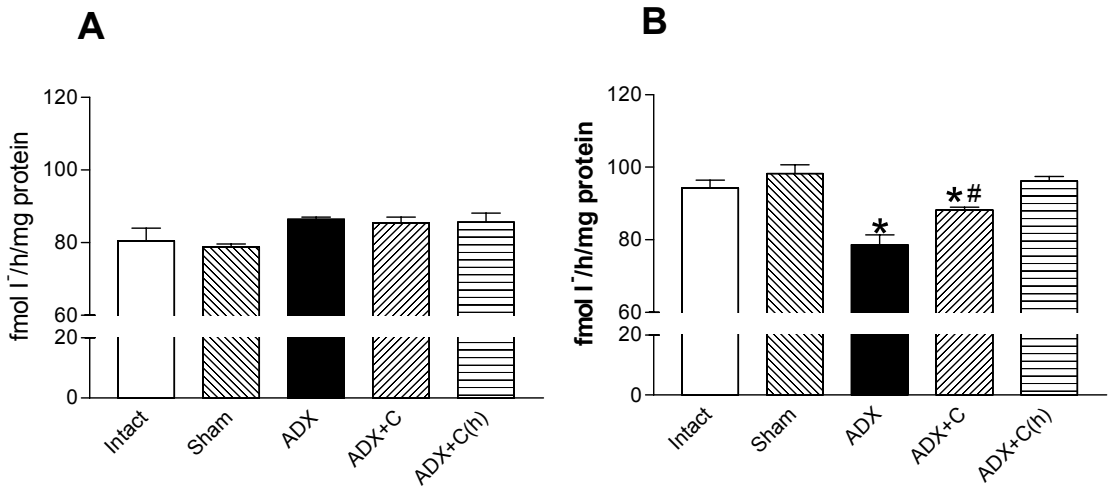

Fig. 2 A. Hypothalamic DII activity measurements in all *ad libitum* fed rats (n=5 for each group) either left intact or sham operated or adrenalectomized (ADX) or ADX plus corticosterone treatment at low doses (ADX+C) or at high doses (C(h)). No significant differences were observed between these groups. **B.** Bar graph showing hypothalamic DII activity measurements in all fasted animals. Results are expressed as mean± SEM.* $P<0.05$ compared to Intact, Sham and ADX+C(h).

# $P<0.05$ compared to ADX.

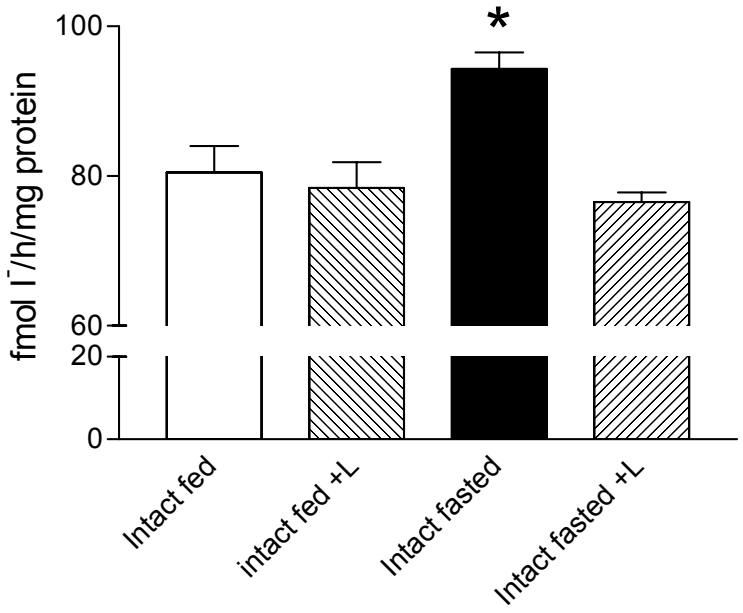

Fig. 3 Hypothalamic DII activity measurements in intact *ad libitum* fed rats (n=5), intact *ad libitum* fed rats plus leptin treatment (Intact fed+L; n=5), intact fasted animals (n=5) and intact fasted rats plus leptin treatment (Intact fasted +L; n=5). While leptin treatment to fed animals does not affect hypothalamic DII activity, leptin replacement to fasted rats induces a significant decrease as compared to intact fasted rats in DII measurement. Results are expressed as mean± SEM.* $P<0.05$ compared to Intact fed, Intact fed+L and Intact fasted +L animals.

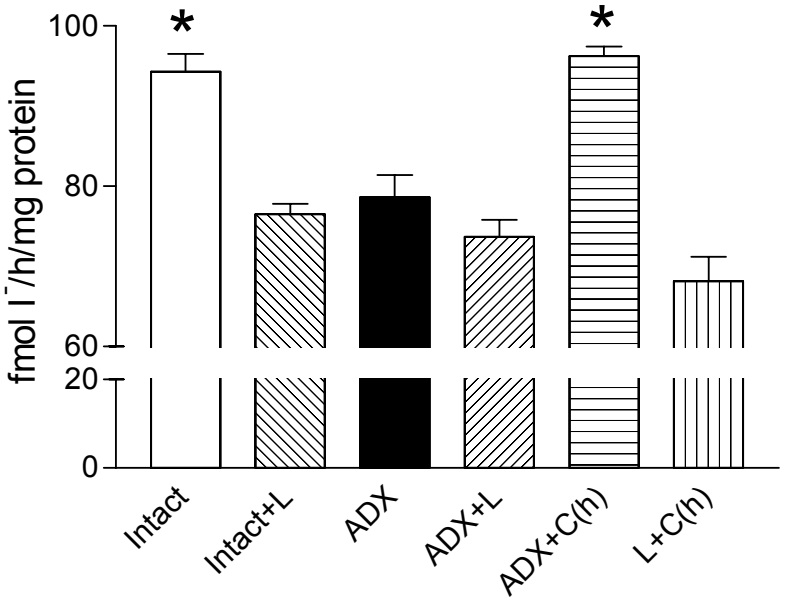

Fig. 4 Hypothalamic DII activity measurements in all fasted animals. Intact and adrenalectomized plus corticosterone treatment at high dose (ADX+C(h)) show a statistically significant elevation of DII activity (*P<0.05) compared to the intact plus leptin treatment (Intact+L), adrenalectomized (ADX), ADX plus leptin treatment (ADX+L) and leptin plus corticosterone treatment at high dose (L+C(h)). Results are expressed as mean± SEM.

7.2. Experiment 2

7.2.1. Hypothalamic TRH mRNA levels

In situ hybridization for TRH mRNA in the paraventricular nucleus of the hypothalamus of saline infused rats showed that fasting induces a decrease in TRH mRNA levels compared to those in fed control animals. The TRH mRNA levels in saline treated fasted rats was 68% ± 4.7 of that seen in saline treated fed animals ($P<0.05$; Fig. 5). When the animals were treated with IOP, fasting blunted this decrease in TRH mRNA levels being 89% ± 7.8 of those seen in IOP treated fed animals ($P>0.05$; Fig. 5).

7.2.2. Hypothalamic T3 measurements

We previously showed that during food deprivation, DII activity levels are increased in the hypothalamus. To assess whether this elevation in activity also induces increased tissue levels of T3, we performed T3 measurements in the hypothalamic tissue of fasted and fed animals. Moreover, to determine whether the effect of IOP in blunting TRH mRNA decline during fasting is

due to a change in the tissue T3, we assessed hypothalamic T3 levels in IOP infused rats that were either fasted or fed *ad libitum*. Hypothalamic T3 levels were significantly higher in saline-treated fasted rats (2.32±0.13 pg/mg wet tissue) compared to those in the saline-treated *ad libitum* fed animals (1.71±0.03 pg/mg wet tissue; Fig.6). On the other hand, in IOP-infused rats, food deprivation did not affect hypothalamic T3 levels (1.81±0.03 pg/mg wet tissue) that were not significantly different from the levels of the fed animals (1.95±0.04 pg/mg wet tissue).

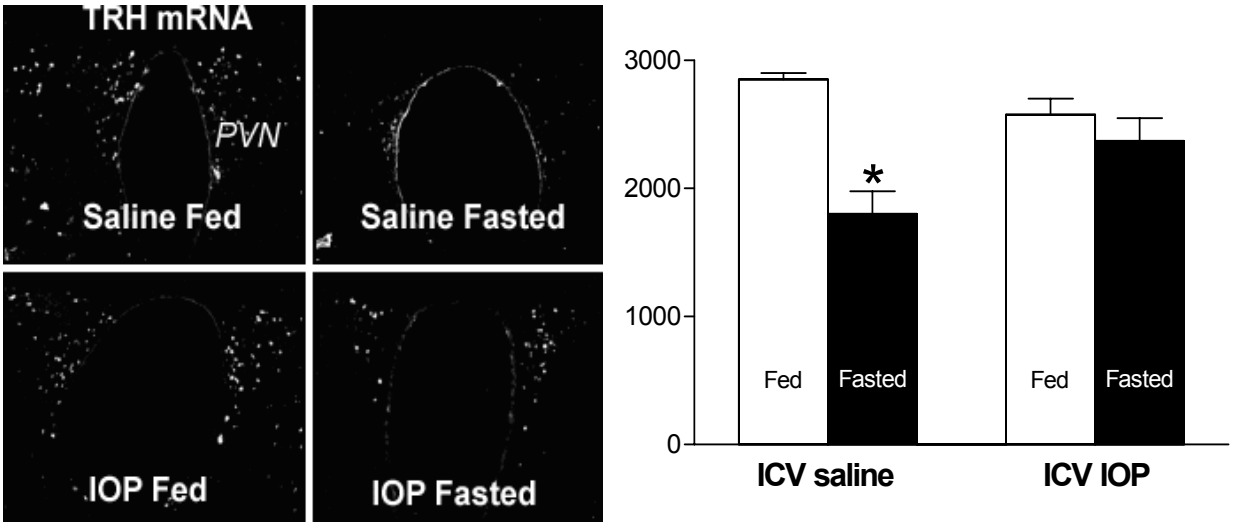

Fig. 5 *In situ* hybridization for TRH mRNA in the PVN revealed that while fasting suppressed TRH mRNA levels in the saline-treated animals ($P<0.05$), ICV IOP (dissolved in saline) injections blunted the fasting-induced decline in TRH mRNA levels of the PVN. Results are expressed as mean± SEM. *$p<0.05$ compared to saline-treated fed, IOP-treated fed and fasted rats.

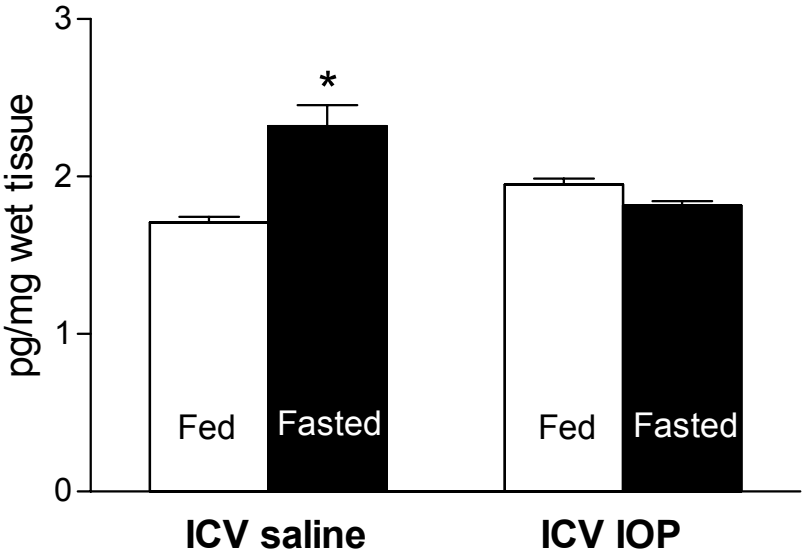

Fig. 6: Graph showing hypothalamic T3 levels in fed or fasted rats after ICV injection of saline or IOP (n=4 for each group). Results are expressed as mean± SEM. *p<0.05.

7.2.3. Double immunocytochemistry for DII and GFP

This study was performed to show the existence of a direct apposition between DII expressing glial cells and perikarya of GFP-NPY neurons in the arcuate nucleus. Immunostaining for DII in GFP-NPY transgenic mice revealed a close association between DII-producing glial processes and NPY/AgRP neurons (Fig. 7). Electron microscopic examination showed (Fig. 8 panel B) tanycytes immunostained for DII in contact with a GFP-NPY immunoreactive cell. Thus, if DII is present in astrocytic processes, then it is reasonable to assume that arcuate nucleus cells, including neurons, may be exposed to locally formed T3. This observation reveals that locally formed T3 by glial cells may have direct access to the melanocortin system, particularly the NPY/AgRP neurons. This provides a basis for neuronal glial interaction in metabolism regulation as DII activity is upregulated during fasting.

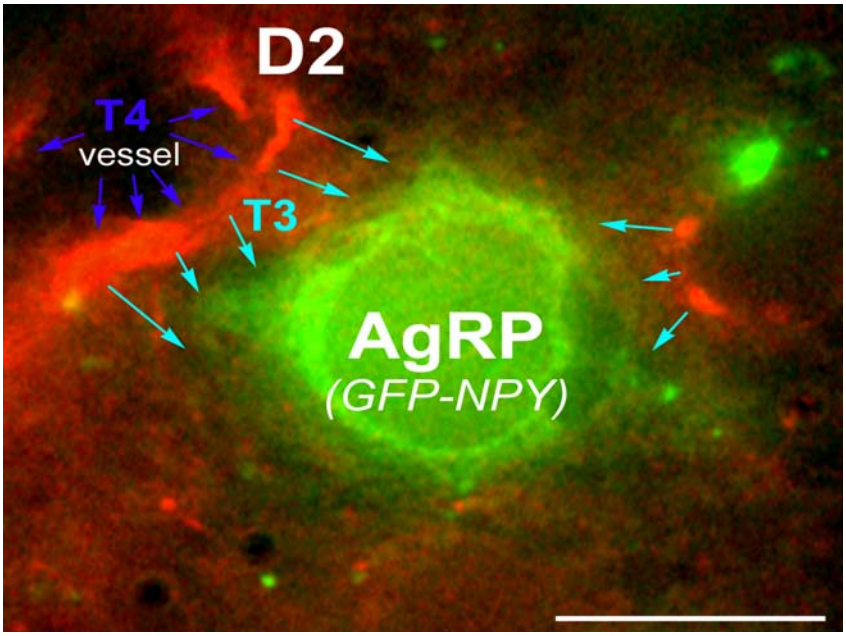

Fig. 7 Immunostaining for DII in GFP-NPY transgenic mice revealed close associations between DII-producing glial processes and NPY/AgRP neurons. This observation revealed that locally formed T3 by glial cells may have direct access to the melanocortin system, particularly the NPY/AgRP neurons.

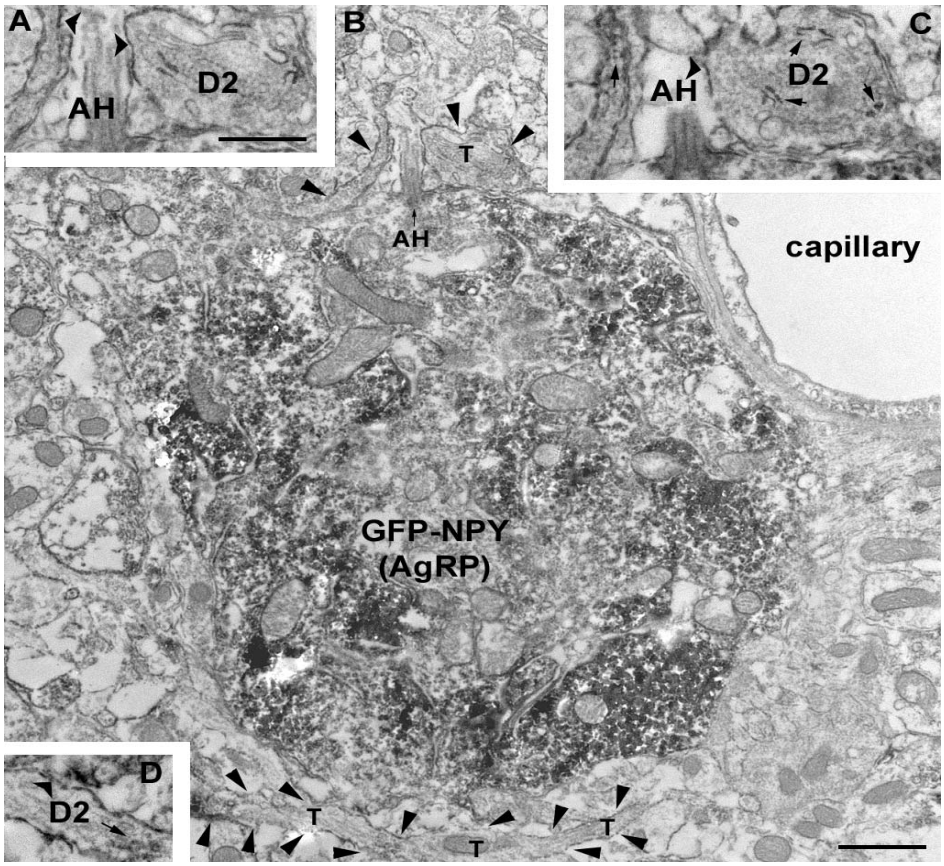

Fig. 8 Electron photomicrograph showing (panel B) tanycytes immunostained with DII (arrow heads) in contact with a GFP-NPY immunoreactive cell. A and C are high power magnification photomicrographs showing 2 tanycytes immunopositive for DII in contact (arrow heads) with an axon hillock (AH) of a GFP-NPY cell in the arcuate nucleus of the hypothalamus. Panel D shows another tanycyte immunostained for DII in contact (arrow head) with the same GFP-NPY cell of the arcuate nucleus.

7.2.4. Measurements of UCP2 uncoupling activity and RNA messenger

It has been shown that in peripheral tissue, T3 regulates mitochondrial uncoupling activity (Lanni et al, 1997; Masaki et al, 1997). To investigate whether T3 triggers mitochondrial uncoupling activity also in the brain, we measured the uncoupling activity in the hypothalamic mitochondria of T3-treated and fasted mice compared to that in saline-treated and fed control mice, respectively. UCP2 is a highly active H^+ transporter in the presence of CoQ_{10} and free fatty acids. Whereas CoQ_{10}, the electron acceptor for complexes I and II of the electron transfer chain, is constitutively expressed in mitochondria, endogenous free fatty acids are sequestered by BSA. Thus, isolated mitochondria were incubated with palmitic acid (300 μM), and the increase in respiration was expressed relative to state 4 respiration induced by the ATP synthase inhibitor oligomycin to give an index of uncoupling activity. T3-treated and fasted mice showed a significant increase in uncoupling activity compared to that in their controls (+50% and +30%, respectively; Fig. 9). Moreover, T3 treatment induced also an elevation of hypothalamic UCP2 mRNA (Fig. 10).

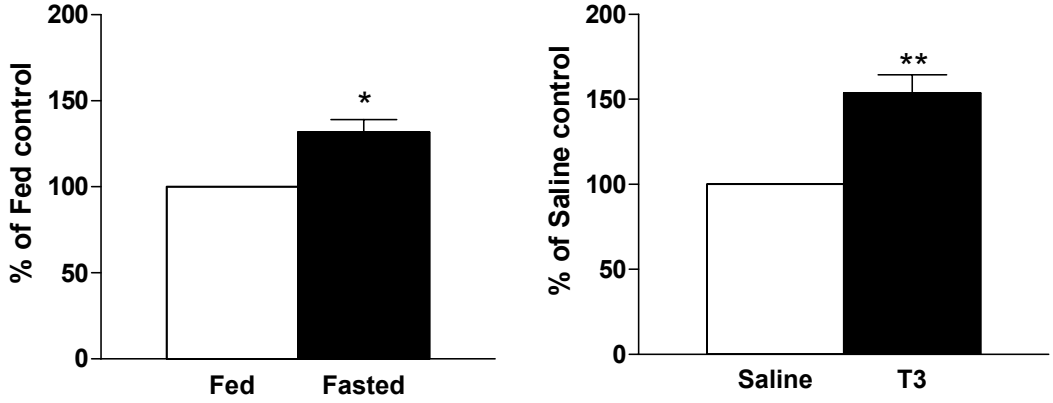

Fig. 9 Fatty acid-induced mitochondrial uncoupling. Exposure to the free fatty acid palmitate induces increased uncoupling activity in fasted and T3-treated mice compared to fed and saline-treated mice, respectively. *$p<0.05$ compared to fed, and **$p<0.001$ compared to saline-treated mice.

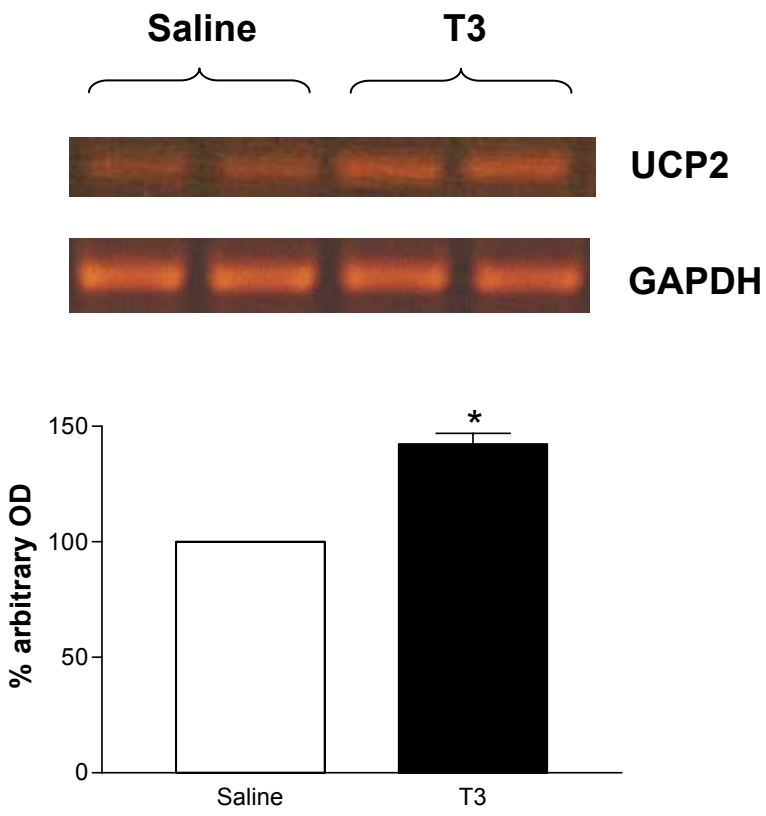

Fig. 10 RT-PCR from representative samples of saline- and T3-treated mice shows an elevation of hypothalamic UCP2 mRNA. ***** $p<0.05$ compared to saline-treated mice.

7.2.5. Quantification of mitochondria in GFP-NPY/AgRP cells of the arcuate nucleus

To study whether elevated mitochondrial uncoupling triggers mitochondria proliferation in the hypothalamus, we assessed the mitochondria number in two models of negative energy balance, fasted and ob/ob mice. We found that in both fasted and ob/ob animals the number of mitochondria in NPY/AgRP neuron was elevated compared to that in their fed controls or wild type, respectively (Fig. 11). In particular, in NPY-AgRP neurons of fasted mice the mitochondrial number was 91% greater compared to that in their fed controls, while in the ob/ob mice the increase was about 113% compared to that in their wild type controls.

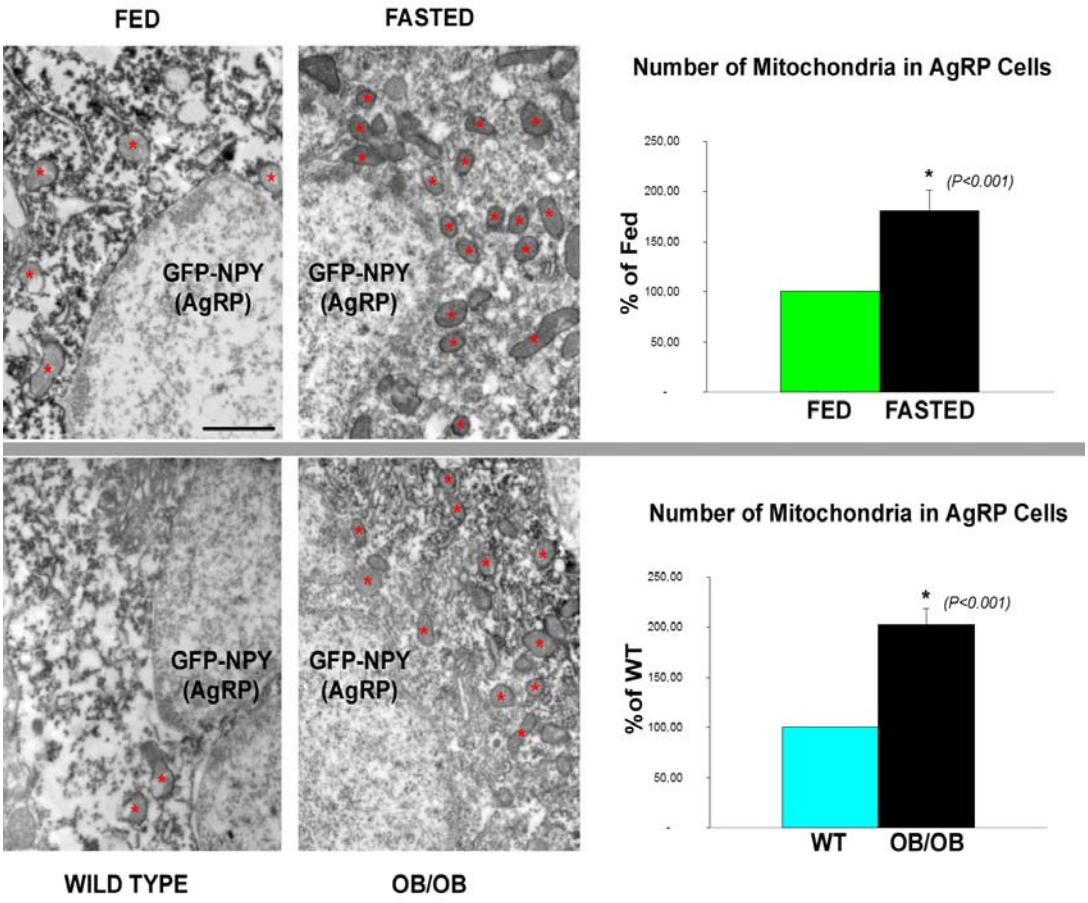

Fig. 11 GFP-NPY neurons in the hypothalamic arcuate nucleus of a fed mouse and a fasted mouse (upper micrographs) and a wild type and an ob/ob mouse (lower micrographs). Red points indicate some of the mitochondria which appear to be more numerous in fasted and ob/ob NPY/AgRP neurons compared to the values of their respective controls.

7.2.6. Fasting and refeeding in DII KO animals

We analyzed the acute responses of DII knockout animals to a 24 hour fasting regimen. Five male DII knockout and 5 male wild type animals were fasted. Food was replaced after 24 h at which time body weight and core body temperature were recorded and food intake was measured in a 60-minute period following re-feeding. Twenty-four hour fasting reduced the body weight of both wild type and DII KO animals. However, the weight reduction in DII KO animals unmasked a significant difference in body weight between KO and wild type animals in which DII KO animals weighed less (24.11±0.66 g) than their wild type littermates (27.98±0.52 g; Fig. 12). The core body temperature of both KO and wild type animals declined during the fasting, and here again, fasting unmasked a significant difference in core body temperature between groups, in which DII KO animals had significantly lower core body temperature after fasting (34.9±0.35 ºC) than their wild type littermates (35.78±0.18 ºC). Based on the differences in these parameters, it is reasonable to indicate that lack of DII increases the depth of negative energy balance following food deprivation. More pronounced negative energy balance should trigger a more robust rebound effect following re-feeding. Strikingly, however, food intake at the

end of 24 h fasting, was significantly lower in DII KO animals compared to that in wild type littermates both in the first (0.39±0.06 g in DII KO; 0.56±0.014 in the wild type) and second 30-minute period after re-feeding (0.065±0.005 g in D2KO; 0.14±0.015 g in wild type; Fig. 12).

7.2.7. UCP2 messenger RNA in the hypothalamus of DII KO mice

RT-PCR showed (Fig.13) that in the DII KO mice the levels of UCP2 mRNA (0.9±0.1 arbitrary density) are significantly lower than those in their wild type controls (1.3±0.09 arbitrary density). This evidence further supports our central hypothesis that an interplay between glial DII and neuronal UCP2 in the hypothalamus is responsible for the rebound feeding.

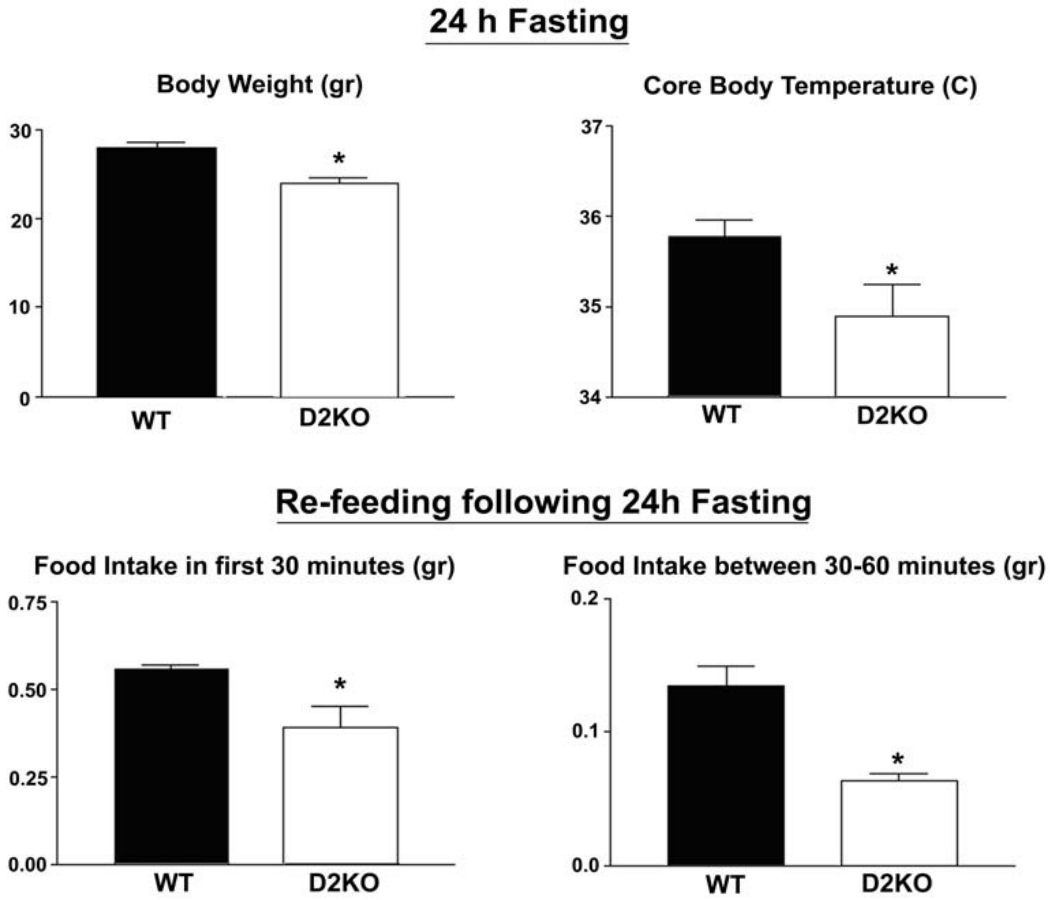

Fig. 12 Acute responses of DII knockout mice to a fasting regimen.
After 24 h, DII KO mice weighed less than their wild type littermates, and DII KO animals had significantly lower core body temperature than their wild type littermates. Food intake at the end of 24 h fasting, was significantly lower in DII KO animals compared to wild type littermates both in the first and second 30-minute period after re-feeding.

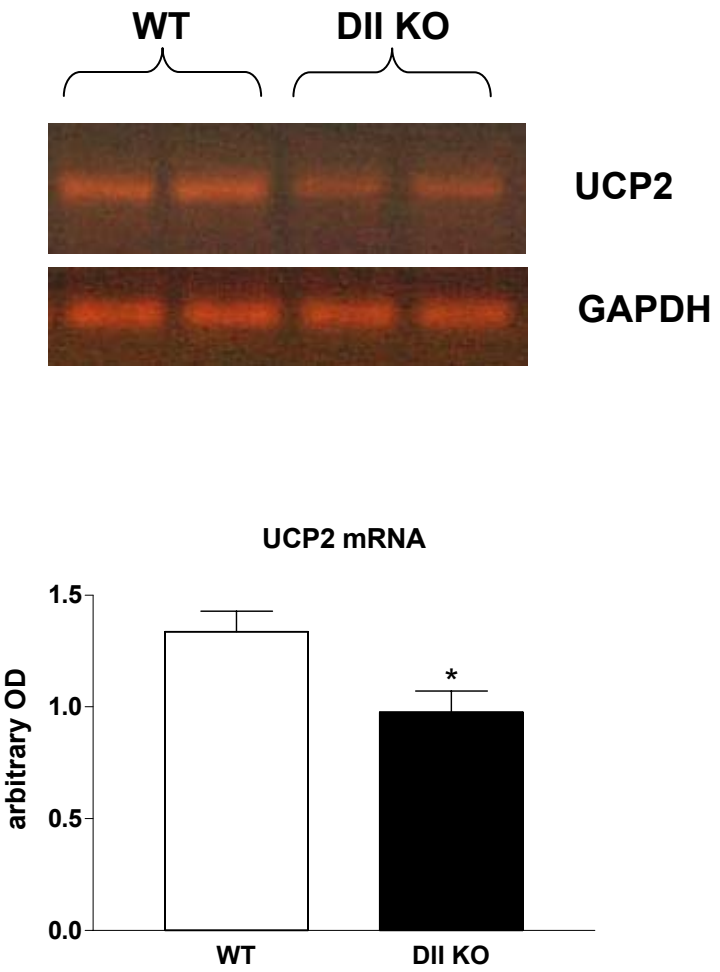

Fig. 13 RT-PCR shows that in the DII KO mice the levels of UCP2 mRNA are significantly lower than their wild type controls.

7.2.8. Fasting and refeeding in UCP2 KO mice

We analyzed the acute response of UCP2 KO animals to 24 h fasting. The initial feeding response of UCP2 KO mice after food deprivation was diminished (0.52±0.13 g; 0.35±0.03 g) compared to that in the fasted wild type littermates (0.86±0.09 g; 0.54±0.05 g; Fig. 14). Note that the normal feeding behavior of these animals is not affected nor do these animals show visible signs of gross metabolic alterations (data not shown).

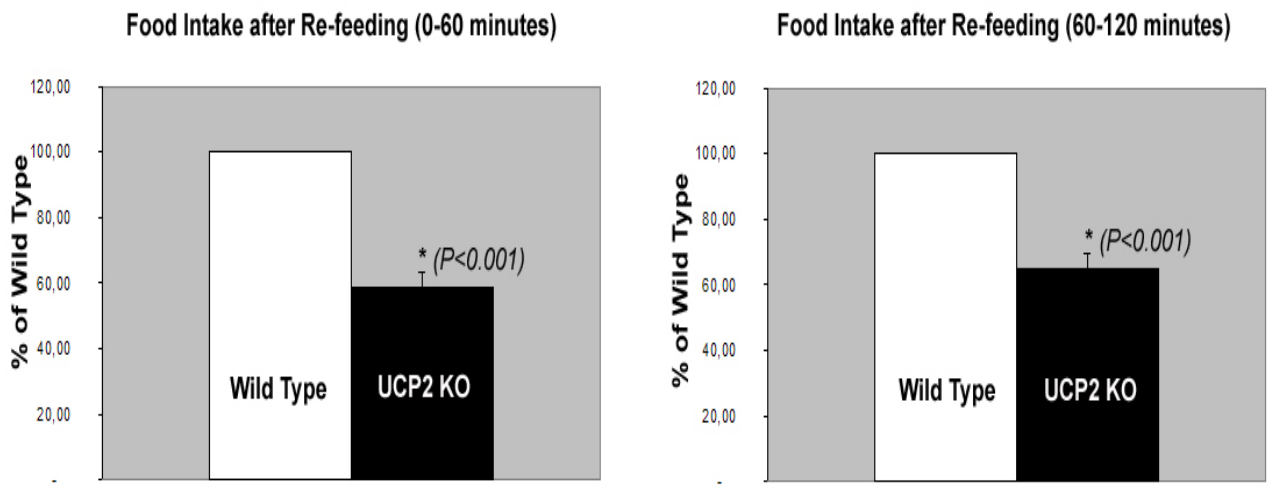

Fig. 14 Diminished rebound feeding in UCP2 KO mice.

After 24 h fasting, food intake of UCP2 KO mice was lower compared to the values of wild type littermates.

8. DISCUSSION

The results of our study demonstrate that inverse changes in circulating levels of the stress hormone, corticosterone, and the metabolic signal, leptin, are critical triggers for increased DII activity in the hypothalamus during food deprivation. In fasted rats, adrenalectomy significantly reduces hypothalamic DII activity to the level of the animals given food *ad libitum*. Conversely, corticosterone administration to ADX fasted rats increases DII activity in a dose-dependent manner. When leptin was administered to fasted rats, DII activity was reduced to the level of intact fed animals. A comparison of plasma leptin and corticosterone levels showed, in fact, that the leptin-treated fasted group had hormone values similar to those in intact fed animals. Moreover, leptin administration to ADX fasted rats with or without corticosterone replacement showed no changes in the levels of DII compared to those in fasted ADX animals (Fig. 4), indicating that the decrease in plasma leptin allows a corticosterone-dependent increase in DII activity during fasting.

Our results show that in fasted ADX rats, when corticosterone is replaced to physiological levels, DII activity is significantly lower than that found in

fasted intact, fasted sham-operated, as well as ADX fasted rats with high dose corticosterone replacement (Fig. 2B). Although an indirect effect of corticosterone on TRH levels via DII activation might be hypothesized, there is direct evidence that glucocorticoids affect TRH synthesis through the presence of glucocorticoid receptors on TRH-producing cells (Ceccatelli et al, 1989), and the existence of the glucocorticoid-responsive element in the promoter region of the pro-TRH gene (Lee et al, 1988).

The current results clearly indicate that circulating leptin and corticosterone levels play a key regulatory role in DII activity in fasted animals. It is also clear, however, that neither of these hormones is critical for baseline expression of DII activity since neither of them appeared to affect hypothalamic DII levels in fed animals (Fig.2A and 3).

When leptin is administered to fasted animals, it induces an increase of TRH mRNA to the level of that in intact animals (Legradi et al, 1997). Therefore, it has been suggested that leptin may directly act on TRH neurons since the long form of leptin receptors is widely distributed in the hypothalamus and has been found to be expressed in TRH cells (Fekete et al, 2002). We propose, however, that leptin-restored TRH mRNA levels in the hypothalamic paraventricular nucleus might be due, at least in part, through their effect in lowering corticosterone levels (see Table 1) and, consequently, DII activity in the arcuate nucleus/median eminence region (Fig. 3).

The results of this study suggest that activation of hypothalamic DII during fasting contributes to elevated local T3 production, which, in turn, could trigger suppression of TRH mRNA levels in the paraventricular nucleus.

To block DII activity, we employed IOP, a potent competitive and irreversible inhibitor of DII *in vivo* as well as *in vitro* (Larsen et al, 1979; Obregon et al, 1980; Courtin et al, 1985; Goswami, and Rosenberg, 1986).

In our study, we observed that IOP had a different effect in all the parts of the brain examined. The intracerebroventricular infusion of IOP in fasted rats showed that IOP at a concentration of 10^{-7}M can selectively inhibit the activity of DII in the hypothalamus without interfering with its activity in other brain regions or in the pituitary gland (data not shown). We propose that this differential effect is the consequence of the accessibility of IOP. Because the cannulae in the third ventricle were positioned in the anterior portion of the hypothalamus (Bregma -0.8), it is reasonable to hypothesize that the strongest effect of IOP at the lower dose (10^{-7} M) was found in the hypothalamus, compared to that in the hippocampus, cerebellum, and pituitary gland.

Although IOP treatment did not reduce hypothalamic T3 levels in fed animals, it did prevent T3 elevation induced by fasting. We found that in fed animals treated with IOP hypothalamic, T3 levels were slightly elevated compared to those in the saline-treated fed animals and this could be the

reason for the slight suppression of TRH levels in the IOP-treated fed group. While it may appear to be a paradox, we suggest that the elevated hypothalamic T3 levels in the IOP-treated fed group could be due to increased transport of circulating T3 to the hypothalamus (rather than local formation). However, fasting-induced DII increase was blocked by IOP, and hence the diminished suppression of TRH.

Thus, we propose that the previously unsuspected existence of hypothalamic hyperthyroidism in the face of systemic hypothyroidism during fasting may be responsible both for the decline of TRH mRNA levels and for the reversal of negative feedback of the thyroid axis.

The arcuate nucleus has been found to contain an abundant number of thyroid receptor-producing neuronal nuclei (Lechan et al, 1993), as well as populations producing various regulatory peptides and neurotransmitters, such as neuropeptide Y, opioid peptides, growth hormone releasing hormone, and dopamine, all of which are known to influence the production and release of TRH (Judd and Hedge, 1982; Liao et al, 1991; Fekete et al, 2000). Also demonstrated has been the existence of a monosynaptic pathway between the arcuate nucleus that contains DII-producing glial cells, and the paraventricular TRH neurons that project to the median eminence with direct access to fenestrated capillaries (Diano et al, 1998b). In addition, it has been shown (Diano et al, 1998c; Legradi and Lechan, 1998) that the arcuate

nucleus NPY/AgRP neurons provide a massive inhibitory input on TRH cell bodies and proximal dendrites via symmetric synapses. Another study (Legradi and Lechan, 1999) has also reported that TRH cells are symmetrically contacted by nerve terminals containing AgRP, which is co-produced in the NPY arcuate neurons. In the present study, we show that activation of DII induces alterations in arcuate nucleus T3 levels and may be responsible for the decrease in TRH mRNA levels during fasting. Thus we hypothesize that the increase in T3 levels during food deprivation could affect and alter neuropeptide expression in leptin-responsive arcuate neurons that strongly project to paraventricular TRH neurons. Studies are underway to delineate this signaling modality in the hypothalamic regulation of the thyroid axis.

By light and electron microscopic examination, we showed a direct relationship between DII-containing, and hence triiodothyronine (T3)-producing, glial elements and components of the melanocortin system, including the NPY/AgRP-expressing arcuate nucleus neurons. On the one hand, DII-produced T3 can have the immediate effect of increasing mitochondrial ATP by enhancing electron transport in the cytochrome chain that could contribute to the acute activation of NPY cells during fasting (Takahashi and Cone, 2005). On the other hand, we have shown that NPY cells express UCP2, the expression level and activity of which are enhanced

by thyroid hormones, T3 in particular. Activation of UCP2 may lead to elevated local temperature, mitochondrial proliferation and increased ATP levels, which together could enhance neuronal activity in a sustained manner. This sustained elevation of ATP due to mitochondrial proliferations is the mechanism that would enable AgRP neurons to have increased firing after refeeding.

In support of this theory, we have shown that DII KO, as well as UCP2 KO mice, have an impaired response to negative energy balance. After 24 hours of food deprivation, both DII KO and UCP2 KO mice consumed less food than their wild type controls did. In addition, we also showed that DII KO mice have reduced levels of UCP2 mRNA in their hypothalamus.

Under normal conditions (satiety), peripheral anorexigenic hormones, such as leptin, control the melanocortin system (Elmquist, 2001). In fasting, orexigenic hormones, such as ghrelin, will reverse the tone of the melanocortin system in which NPY/AgRP neuronal activity will dominate (Zigman and Elmquist, 2003). At the same time, elevating corticosteroid levels will trigger DII activity (Diano et al, 1998b) and local T3 production, which will trigger UCP2 production and activity in mitochondria of NPY/AgRP neurons. The elevated activity of NPY/AgRP neurons will suppress POMC tone. That this cascade is triggered by corticosteroids is supported by the study of Drazen et al (2003) in which adrenalectomy was

observed to alter the sensitivity of the melanocortin system. On the basis our study, we suggest that by the time of re-feeding, activated UCP2 will proliferate mitochondria in NPY/AgRP neurons which, in turn, will be a critical factor in maintaining increased firing of these orexigenic cells so that food intake will remain elevated and POMC neuronal firing suppressed subsequent to refeeding, at a time when neither orexigenic nor anorexigenic circulating signals dominate.

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