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## Leptin But Not Ciliary Neurotrophic Factor (CNTF) Induces Phosphotyrosine Phosphatase-1B Expression in Human Neuronal Cells (SH-SY5Y): Putative Explanation of CNTF Efficacy in Leptin-Resistant State

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Growing evidences suggest that obesity is associated with hypothalamic leptin resistance, leading to the alteration of food intake control. Alternative treatment using ciliary neurotrophic factor (CNTF) has been suggested because CNTF exerts a leptin-like effect, even in leptin-resistant states, but the mechanisms by which CNTF maintains this effect are not yet understood. Both leptin and CNTF act in the hypothalamus through similar signaling pathways including janus kinase-2/signal transducer and activator of transcription (STAT)-3 pathway. To explore the differences and interactions between leptin and CNTF signaling pathways, differentiated human neuroblastoma cells (SH-SY5Y) were exposed to either leptin or CNTF and then challenged for each cytokine. Leptin pretreatment completely abolished leptin-dependent STAT-3 and ERK 1/2 phosphorylations without affecting CNTF action. The lack of cross-desensitization between leptin and CNTF signaling pathways occurred despite the induction of suppressor of cytokine signaling-3 in response to both cytokines. Interestingly, leptin as well as insulin induced the expression of phosphotyrosine phosphatase (PTP)-1B, whereas CNTF treatment did not affect its expression. In addition, acute leptin treatment but not CNTF induced PTP-1B expression in mouse hypothalamic arcuate nucleus. Furthermore, the overexpression of human PTP-1B in SH-SY5Y cells completely abolished leptin- and insulin-dependent janus kinase-2, STAT-3, and ERK 1/2 phosphorylations, but CNTF action was not altered. Collectively, our results suggest that PTP-1B constitutes a key divergent element between leptin/insulin and CNTF signaling pathways at the neuronal level, which may constitute a possible mechanism that explains the efficacy of CNTF in leptin-resistant states. (*Endocrinology* 150: 1182–1191, 2009)

**L**eptin, an adipocyte-derived satiety factor, has a primary role as an antiobesity hormone by both increasing energy expenditure and reducing food intake (1). Changes in circulating leptin levels inform the central nervous system, mainly the hypothalamus, on the body energy status to control body energy homeostasis and weight (2–4). In the hypothalamus, leptin binding to long isoform leptin receptor (ObRb) acti-

vates janus kinase (JAK)-2 tyrosine kinase to mediate several signaling pathways and mainly the phosphorylation of the transcription factor signal transducer and activator of transcription (STAT)-3 that controls the expression of anorexigenic and orexigenic neuropeptides (1, 2). Thus, the attenuation of leptin action as observed in obese rodents and humans results in increased appetite and reduced energy expenditure despite

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Abbreviations: CNTF, Ciliary neurotrophic factor; ECL, enhanced chemiluminescence; JAK, janus kinase; ObRb, long isoform leptin receptor; PTP, phosphotyrosine phosphatase; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription.

high leptin plasma levels, suggesting a leptin resistance state (1, 5, 6). Furthermore, leptin resistance is frequently associated with other metabolic diseases such as type 2 diabetes (7, 8).

The mechanisms that cause the failure of elevated leptin levels to induce weight loss in obese humans or rodents have not yet been established and may involve multiple mechanisms such as an alteration of leptin signaling by the increased expression of suppressor of cytokine signaling (SOCS)-3 (7, 9) or as a defect in leptin transport to the central nervous system (10–12). Circulating leptin levels are positively correlated to the degree of adiposity; thus, whether the elevated leptin associated with obesity contributes to leptin resistance or is the consequence of obesity is, however, still a matter of speculation. Hence, overcoming hypothalamic leptin resistance may constitute a major step of reestablishing a normal control of food intake and energy expenditure in leptin-resistant obese.

The ciliary neurotrophic factor (CNTF), a cytokine from the IL-6 family, has shown leptin-like effects even in the leptin resistance state or animal models of obesity (13–15). CNTF clearly inhibits food intake, reduces body weight, and improves insulin sensitivity in normal and leptin-resistant mice (13, 15–17). CNTF acts on arcuate nucleus of the hypothalamus that expresses a tripartite complex receptor consisting of CNTF receptor- $\alpha$ , leukemia inhibitory receptor, and Glycoprotein 130 (16, 18). Similarly to leptin, CNTF binding to its receptor activates JAK-2/STAT-3 signaling pathway, which controls the expression of both anorexigenic and orexigenic neuropeptides. Despite the similarity between CNTF and leptin signaling pathways, it has been reported that the two cytokines initiate differential patterns of gene expression in the hypothalamus, suggesting distinct mechanisms of action (19, 20). Nevertheless, the divergence in early neuronal intracellular signaling steps between leptin and CNTF is still unknown, and its characterization is necessary for the understanding of the leptin resistance phenomenon and may identify new therapeutic targets. In the present work, we used a human differentiated neuroblastoma cell line (SH-SY5Y) that express both leptin and CNTF receptors in addition to insulin receptor (21–25). In this neuronal cell model, we show that in contrast to the cross-down-regulation between ObRb and insulin receptor (21, 26), the overexposure to leptin does not affect the CNTF-dependent STAT-3 or ERK 1/2 phosphorylation. This demonstrates the efficiency of CNTF in a hyperleptinemic environment such that observed in obese or leptin-resistant state.

For the first time, we demonstrate that the divergence between CNTF and leptin signaling pathways involves phosphotyrosine phosphatase (PTP)-1B. We show that leptin but not CNTF is able to induce the expression of PTP-1B in SH-SY5Y cells and mouse hypothalamic arcuate nucleus. Furthermore, overexpression of PTP-1B cDNA in human neuronal cells dramatically affects leptin and insulin but not CNTF signaling pathways. These data reveal the critical role of PTP-1B in the onset of

neuronal leptin resistance and demonstrate that PTP-1B is unable to inhibit CNTF-dependent signaling pathways in leptin-resistant states.

## Materials and Methods

### Materials and chemicals

The human neuroblastoma cell line (SH-SY5Y) was obtained from Dr. B. Dufy (Unit Mixte de Recherche 5543, Centre National de la Recherche Scientifique, Bordeaux-II University, Bordeaux, France). All cell culture reagents were purchased from Invitrogen (Cergy-Pontoise, France). Antibodies directed toward STAT-3, phospho-STAT-3, ERK 1/2, phospho-ERK 1/2, JAK-2, and phospho-JAK-2 were from Cell Signaling (Ozyme, Saint Quentin-en-Yvelines, France). Antibodies against PTP-1B (sc-14021) were from Santa Cruz Biotechnology (Santa Cruz, CA). Insulin, all *trans*-retinoic acid, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membranes were from Euromedex (Mundolsheim, France), enhanced chemiluminescence (ECL) detection reagents were from Amersham Biosciences (Buckinghamshire, UK). Recombinant ovine leptin was produced in *Escherichia coli* as previously described (27). Human CNTF (C17S) and optimized for expression in *E. coli* that was purchased from Entelchon GmbH (Regensburg, Germany) and subcloned into pMon3401 expression vector using *Nde*I and *Hind*III restriction sites. Similar steps were used for the production of human CNTF as those previously described for the production of ovine leptin (27). cDNA encoding human PTP-1B and mutant human PTP-1B (nonfunctional) were a generous gift from Dr. T. C. Meng (University of Taiwan).

### Cell stimulation and immunoblotting

SH-SY5Y cells were seeded in six-well dishes and differentiated for 15 d with retinoic acid as previously described (21). Serum-starved cells were incubated in serum-free DMEM in presence or absence of insulin (100 nM), leptin (15 nM), or CNTF (1 nM) for 2, 4, or 16 h. Cells were then rinsed and stimulated for 10 min with insulin, leptin, or CNTF. After hormonal treatment, protein lysates were prepared and immunoblotting was performed. Briefly, proteins from whole-cell lysates (50–100  $\mu$ g) were resolved by SDS-PAGE and electrotransferred to nitrocellulose. Membranes were then incubated with the appropriate primary and secondary antibodies, and targeted proteins were visualized by enhanced chemiluminescence reagents (ECL detection kit) followed by autoradiography.

### Quantification of SOCS-3 and PTP-1B mRNA expression by quantitative RT-PCR

Total RNA from differentiated SH-SY5Y cells was extracted using RNA Insta Pure kit (Eurogentec, Angers, France) according to the manufacturer's recommendations. One microgram of total denatured RNA was reverse transcribed, and the resulting cDNAs were submitted to quantitative PCR analysis as previously described (26). The PCR primer sequences used were as follows: SOCS-3 forward, 5'-GTCACCCACAGCAAGTTT-3', SOCS-3 reverse, 5'-CTGAGCGTGAAGAAGTGG-3'; PTP-1B forward, 5'-CGAGCAGATCGACAAGTCCGG-3', PTP-1B reverse, 5'-AACTCCTTTGGGCTTCTTCCA-3'; 18S forward, 5'-GGA-CAGGATTGACAGATTGATAG-3', 18S reverse, 5'-CTCGTTCCGTTATCGGAATTAAC-3'.

Real-time PCR was carried out using the Roche LightCycler apparatus and the Fast Start DNA Master SYBER Green I kit (Roche Diagnostics, Mannheim, Germany). PCR amplification was performed in triplicate using the following conditions: initial activation of the hot start DNA polymerase for 15 min at 94 C followed by denaturation for 10 sec at 94 C, annealing for 10 sec at 48 C for SOCS-3 and at 57 C for PTP-1B, and extension for 10 sec at 72 C.

Forty cycles of PCR were programmed to ensure that the threshold crossing point (cycle number) was attained. Fluorescence emission was monitored continuously during cycling. At the completion of cycling, melting curve analysis was carried out to establish the specificity of the amplified product. The expression level of each mRNA and their estimated crossing point in each sample were determined relative to the standard preparation using the LightCycler computer software (Roche). A ratio of specific mRNA to 18S amplification was then calculated to correct for any difference in efficiency at reverse transcription.

### Transient transfection

SH-SY5Y cells were seeded in 6-well plates and differentiated as previously described (21). Seventy percent confluent cells were then transfected using Exgen 500 (Euromedex) according to the manufacturer's recommendations. Each well received 20  $\mu$ l of Exgen 500 and 4  $\mu$ g of plasmid DNA in 2ml of serum-free optiMEM medium (Invitrogen, Cergy-Pontoise, France). Cells were either not transfected or transfected with an expression vector encoding wild-type or mutant PTP-1B. After 3 h, cell medium was supplemented with 10% fetal calf serum. Twenty-four hours after transfection, SH-SY5Y cells were serum deprived for 16 h before stimulation with insulin (100 nM), leptin (15 nM), or CNTF (1 nM) for 10 min. Cells were then harvested and protein lysates were prepared for immunoblotting.

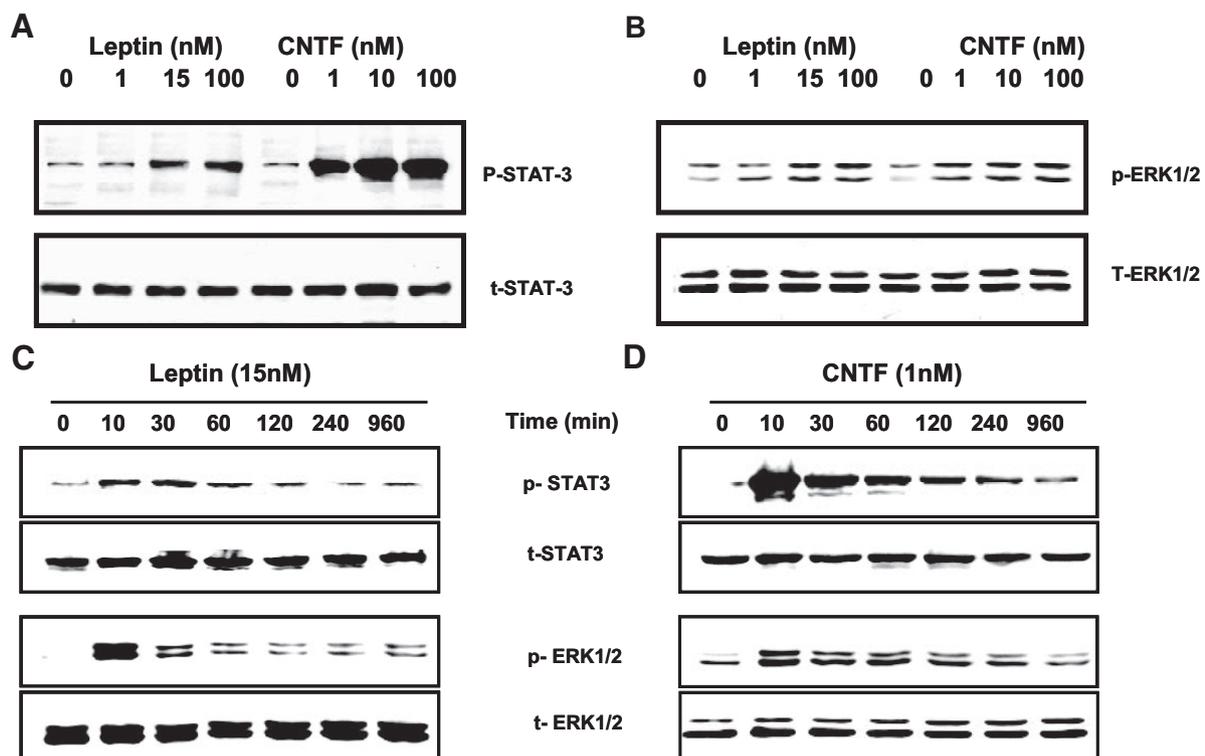
### Animal treatment

All experiments were performed in agreement with European legal requirements (Decree 86/609/EEC) and approved by the local com-

mittee on animal welfare. Fifteen 3-month-old male C57/BL6 mice were used in this study. Animals were housed under 12-h light, 12-h dark cycles (lights on at 0700 h) with access to food and water *ad libitum*. They were injected ip once daily on 4 successive days with either CNTF (0.3 mg/kg · d) or leptin (1 mg/kg · d). Control mice received a similar volume (depending on the weight) of saline solution only, according to the same schedule. Mice were killed 5 h after the last injection.

### Fluorescent immunohistochemistry

The immunohistochemical protocol used in this study was modified from Vacher *et al.* (28). Mice were anesthetized by ip injection of pentobarbital (60 mg/kg of body weight) and transcardially perfused with PBS followed by 4% paraformaldehyde. Brains were removed and conserved in the same fixative solution at 4 C for 4 h and cryoprotected in 20% sucrose. Serial coronal 16- $\mu$ m-thick sections were cut on a cryostat at -21 C (Microm; Microtech, Francheville, France). Sections were incubated in 50 mM NH<sub>4</sub>Cl for 20 min before blocking nonspecific immunoglobulin binding sites by a 2-h incubation in PBS containing 0.2% fish gelatin, 2% normal donkey serum, and 0.2% Triton X-100. They were then incubated with rabbit antiserum raised against PTP-1B (1:100; Santa Cruz Biotechnology) or SOCS-3 (1:100; Santa Cruz Biotechnology) for 48 h at 4 C. Their specificity was tested by omission of the antibodies. Primary antibodies were visualized by using Alexa-488 (Invitrogen-Molecular Probes, Paisley, UK) conjugated donkey antirabbit (1:400) for 1 h at room temperature. Sections were mounted with antifading mounting media (Vectashield; Vector Laboratories, Peterborough, UK).



**FIG. 1.** CNTF and leptin induce phosphorylation of STAT-3 and ERK in dose- and time-dependent manners. Differentiated SH-SY5Y cells were either subjected to increased concentrations of leptin (A and B) or CNTF (A and B) for 10 min or treated with leptin (15 nM) (C) or CNTF (1 nM) (D) at various times (from 10 to 960 min). Cell lysates were then collected and subjected to Western blot analysis. Membranes were probed sequentially with antiphospho-STAT-3 (p-STAT3) followed by anti-STAT-3 (t-STAT3) antibodies or antiphospho-ERK 1/2 (p-ERK 1/2) followed by anti-ERK 1/2 (t-ERK 1/2). The proteins on the blots were revealed by ECL. This is a representative experiment independently performed three times.

## Confocal microscopy

### Acquisition

Immunofluorescence was examined by confocal laser-scanning microscopy with a Zeiss LSM 510 system (Zeiss Microscopy, Jena, Germany). The argon laser was used to excite the fluorochrome at 488 nm. Optical sections were taken through the hypothalamus sample in the Z axis at 0.4- $\mu$ m intervals. Each optical section was obtained by a sequential scanning and averaged three times.

### Quantification

All quantification steps were performed with ImageJ 1.36b software (National Institutes of Health, Bethesda, MD). The differences in level expression of PTP-1B or SOCS-3 in the arcuate nucleus of control and treated mice were assessed by comparing the fluorescence intensities emitted by Alexa-488. The integrated fluorescence density level quantifies the sum of the values of the pixels within the reproductive contours of a whole arcuate nucleus. All measurements were performed in defined-size outline drawn and reused for every condition examined and averaged for each animal from 15 different focal planes homogeneously distributed through three hypothalamus sections.

### Statistical analysis

Statistical analysis was performed using ANOVA (Statview Software program, version 5; ASAP Software, St. Ouen, France) to detect significant intergroup differences. Data are presented as means  $\pm$  SEM, and  $P < 0.05$  was considered statistically significant.

## Results

### Leptin and CNTF increase STAT-3 and ERK phosphorylations in dose- and time-dependent manners

To examine the effects of leptin and CNTF on STAT-3 and ERK 1/2 phosphorylations, SH-SY5Y cells were treated for 10 min with increasing concentrations of leptin or CNTF. Cell lysates were subjected to Western blot analysis using adequate antibodies. Leptin and CNTF induced a dose-dependent increase in STAT-3 (Fig. 1A) and ERK 1/2 (Fig. 1B) phosphorylation levels. These phosphorylations were detectable already with 15 or 1 nM of leptin or CNTF, respectively (Fig. 1, A and B).

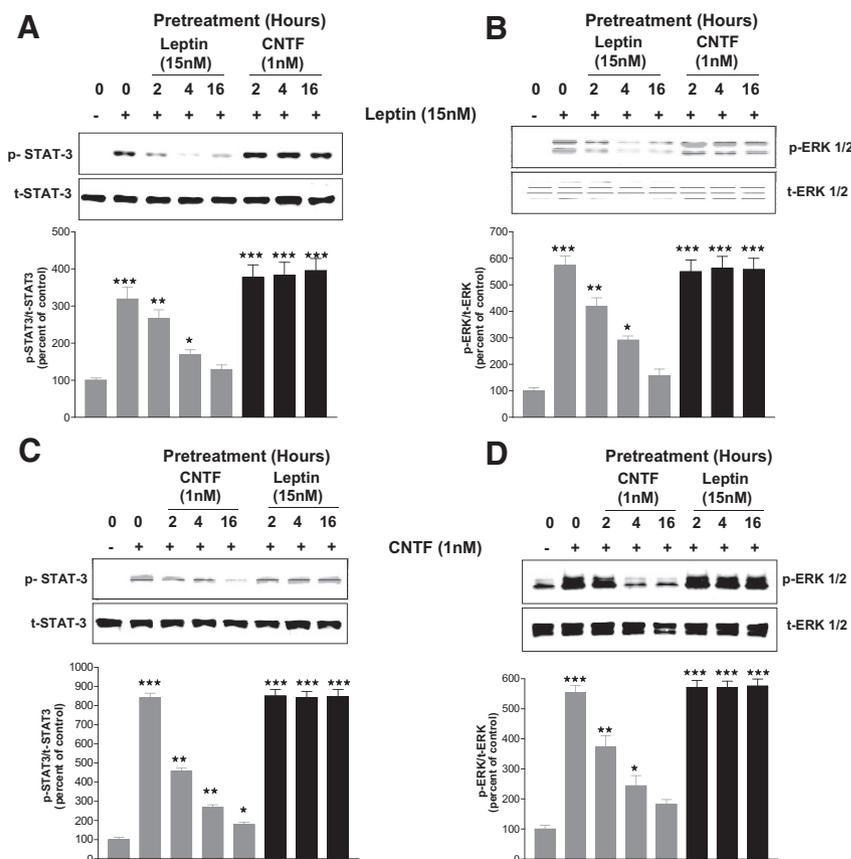
To establish the kinetics of STAT-3 and ERK 1/2 phosphorylations in response to leptin or CNTF, SH-SY5Y cells were treated with leptin (15 nM) or CNTF (1 nM) for various periods of times. STAT-3 and ERK 1/2 phosphorylations were estimated by Western blot. Leptin (Fig. 1C) and CNTF (Fig. 1D) induced the phosphorylation of STAT-3 from 10 min of incubation, which was maintained until 30 min and then gradually declined. The

same pattern of phosphorylation was observed for ERK 1/2 with a maximum phosphorylation level at 10 min (Fig. 1, C and D) in response to both cytokines.

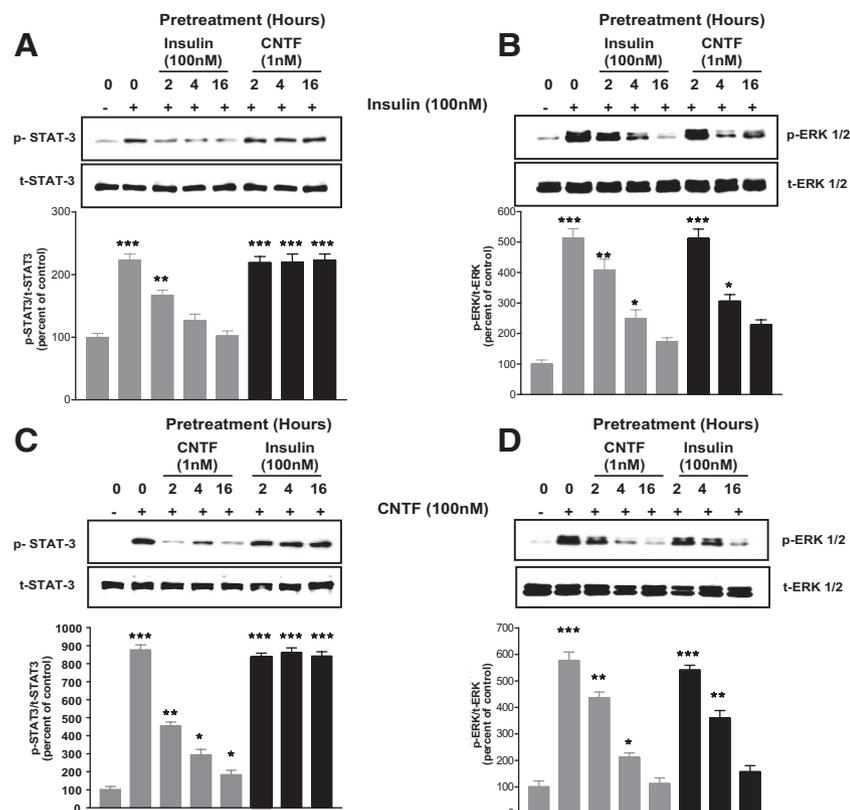
### Chronic leptin treatment affects leptin-induced phosphorylation of STAT-3 and ERK but not CNTF action and vice versa

To investigate the cross-regulation between leptin and CNTF signaling pathways in SH-SY5Y cells, we examined the impact of chronic leptin or CNTF treatment on STAT-3 and ERK 1/2 phosphorylations in response to both cytokines. Cells were pretreated with or without leptin (15 nM) or CNTF (1 nM) for various periods of time (2, 4, and 16 h) and then acutely stimulated for 10 min with 15 nM leptin or 1 nM CNTF. Phosphorylation of STAT-3 and ERK 1/2 was estimated by Western blot using adequate antibodies. The results were normalized to either total STAT-3 or total ERK 1/2.

Cells overexposed to leptin had significantly decreased leptin-dependent phosphorylation of STAT-3 (Fig. 2A) and ERK 1/2



**FIG. 2.** Chronic effect of leptin or CNTF on leptin or CNTF-induced STAT-3 and ERK phosphorylations in SH-SY5Y neuronal cells. Serum-deprived SH-SY5Y cells were incubated with 15 nM leptin or 1 nM CNTF for a various period of time (2, 4, and 16 h) and then stimulated for 10 min without or with 15 nM leptin (A and B) or 1 nM CNTF (C and D). Cell lysates were then subjected to Western blot analysis. Membranes were probed sequentially with antiphospho-STAT-3 (p-STAT3) followed by anti-STAT-3 (t-STAT3) antibodies or antiphospho-ERK 1/2 (p-ERK 1/2) followed by anti-ERK 1/2 (t-ERK 1/2). The proteins on the blots were revealed by ECL and bands quantified by densitometry (using Bio-1D software, Viber Lourma, France). The results were expressed as the ratio of p-STAT3 to t-STAT3 or p-ERK to t-ERK and presented as the means  $\pm$  SEM (n = 3). \*, \*\*, and \*\*\*,  $P < 0.05$ ,  $P < 0.001$ , and  $P < 0.0001$ , respectively, compared with the control.



**FIG. 3.** Chronic effect of insulin or CNTF on CNTF- or insulin-induced STAT-3 and ERK phosphorylation in SH-SY5Y neuronal cells. Serum-deprived SH-SY5Y cells were incubated with 100 nM insulin or 1 nM CNTF for a various period of time (2, 4, and 16 h) and then stimulated for 10 min without or with 100 nM insulin (A and B) or 1 nM CNTF (C and D). Cell lysates were then subjected to Western blot analysis. Membranes were probed sequentially with antiphospho-STAT-3 (p-STAT3) followed by anti-STAT-3 (t-STAT3) antibodies or antiphospho-ERK 1/2 (p-ERK 1/2) followed by anti-ERK 1/2 (t-ERK 1/2). The proteins on the blots were revealed by ECL and bands quantified by densitometry (using Bio-1D software). The results were expressed as the ratio of p-STAT3 to t-STAT3 or p-ERK to t-ERK and presented as the means  $\pm$  SEM ( $n = 3$ ). \*, \*\*, and \*\*\*,  $P < 0.05$ ,  $P < 0.001$ , and  $P < 0.0001$ , respectively, compared with the control.

(Fig. 2B) in a time-dependent manner. In contrast, CNTF-dependent phosphorylation of STAT-3 and ERK 1/2 was not affected (Fig. 2, C and D). When cells were overexposed to CNTF, STAT-3 (Fig. 2C), and ERK 1/2 (Fig. 2D), phosphorylation in response to CNTF were diminished in a time-dependent manner but leptin effect was not altered (Fig. 2, A and B).

### Chronic insulin treatment affects CNTF-induced phosphorylation of ERK but not STAT-3 phosphorylation

Next we examined the possible cross talk between CNTF and insulin, another anorexic hormone, signaling in SH-SY5Y cells. Cells were pretreated with or without insulin (100 nM) or CNTF (1 nM) for a various period of time (2, 4, and 16 h) and then acutely stimulated for 10 min with 100 nM insulin or 1 nM CNTF. Then STAT-3 and ERK 1/2 phosphorylation were estimated as described above. Cell overexposed to insulin had significantly reduced insulin-dependent STAT-3 and ERK 1/2 phosphorylation in a time-dependent manner (Fig. 3, A and B). Insulin pretreatment did not alter CNTF-dependent STAT-3 phosphorylation (Fig. 3C) but reduced CNTF-

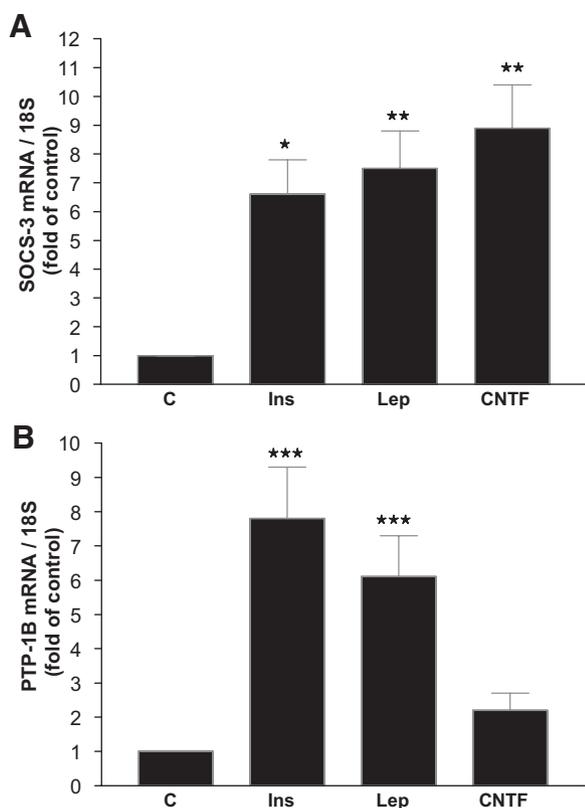
dependent ERK 1/2 phosphorylation (Fig. 3D). When cells were overexposed to CNTF, insulin-dependent phosphorylation of ERK 1/2 was significantly altered in a time-dependent manner (Fig. 3B) but not STAT-3 phosphorylation (Fig. 3A). In summary these results clearly indicate a cross talk between insulin and CNTF to desensitize their signaling pathways at the level of ERK 1/2 but not at the level of STAT-3.

### Chronic effect of leptin, insulin, or CNTF pretreatments on SOCS-3 and PTP-1B mRNA expression in SH-SY5Y cells

To understand the lack of cross-regulation between CNTF and leptin or insulin signaling pathways in SH-SY5Y cells, we analyzed the impact of insulin, leptin, or CNTF on the induction of the common negative regulators: SOCS-3 and PTP-1B. Cells were incubated with or without insulin (100 nM), leptin (15 nM), or CNTF (1 nM) for 4 h. Incubation of 4 h was chosen according to the results obtained in Figs. 2 and 3, because leptin, CNTF, or insulin led to dramatic down-regulation of their signals after 4 h of treatment. Then SOCS-3 and PTP-1B mRNA expressions were measured using quantitative RT-PCR. Insulin, leptin, or CNTF treatment significantly increased SOCS-3 expression by more than 5-fold (Fig. 4A). Insulin and leptin in contrast to CNTF had also significantly increased PTP-1B expression (Fig. 4B).

### Overexpression of PTP-1B differentially regulates insulin, leptin, and CNTF signaling pathways in SH-SY5Y cells

To investigate whether PTP-1B affects leptin, insulin, and CNTF signaling in SH-SY5Y cells, we analyzed the effects of PTP-1B overexpression on the activation of two major signaling pathways: JAK-2/STAT-3 and ERK 1/2. Cells were transiently transfected with an expression vector encoding human wild-type PTP-1B cDNA (wt-PTP-1B) or its nonactive mutant PTP-1B (mut-PTP-1B). The total cell lysates from untransfected or transfected cells were immunoblotted with PTP-1B antibodies to ensure the overexpression of wt-PTP-1B and mut-PTP-1B in SH-SY5Y cells (Fig. 5). Cells were then stimulated for 10 min with insulin, leptin, or CNTF and JAK-2, STAT-3, and ERK 1/2 phosphorylation estimated by Western blot. In untransfected cells or cells transfected with mut-PTP-1B cDNA, insulin, leptin, and CNTF significantly activated JAK-2, STAT-3, and ERK 1/2 phosphorylation (Fig. 6, A, C, D, F, G, and I). Furthermore, the effect of CNTF on JAK-2/STAT-3 signaling pathway was more predominant than those of leptin and insulin. In contrast, in cells transfected with wt-PTP-1B cDNA (Fig. 6, B, E, and H) both leptin and



**FIG. 4.** Leptin, insulin, and CNTF chronic effects on SOCS-3 and PTP-1B mRNA expression in SH-SY5Y neuronal cells. Serum-deprived cells were incubated for 4 h in the absence (C) or the presence of 100 nM insulin (Ins), 15 nM leptin (Lep), or 1 nM CNTF. Total RNAs were then extracted and subjected to reverse transcriptase. The resulting cDNAs were used to perform quantitative PCR, as described in *Materials and Methods*, using specific primers for SOCS-3 (A) or PTP-1B (B). Results were expressed as ratio of specific mRNA to 18S RNA and presented as the means  $\pm$  SEM ( $n = 3$ ). \*, \*\*, and \*\*\*,  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively, compared with the control.

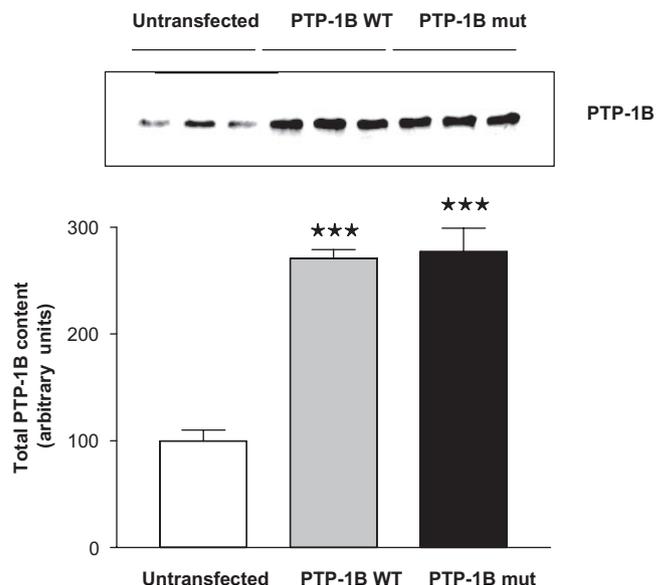
insulin effects on JAK-2, STAT-3 and ERK 1/2 phosphorylations were totally abolished, whereas CNTF action was fully preserved.

#### Effect of chronic leptin or CNTF treatment on PTP-1B and SOCS-3 expression on mouse hypothalamic arcuate nucleus

To investigate whether the differential effect of leptin and CNTF on PTP-1B expression could be extrapolated *in vivo*, mice were treated for 4 successive days by leptin or CNTF, and then PTP-1B and SOCS-3 expression was determined by immunohistochemistry in mice arcuate nucleus. Leptin clearly induced both SOCS-3 and PTP-1B expression (Fig. 7), whereas CNTF was able to induce SOCS-3 but not PTP-1B (Fig. 7). We even observed a reduced amount of PTP-1B in mice treated with CNTF compared with animals treated with saline (Fig. 7).

#### Discussion

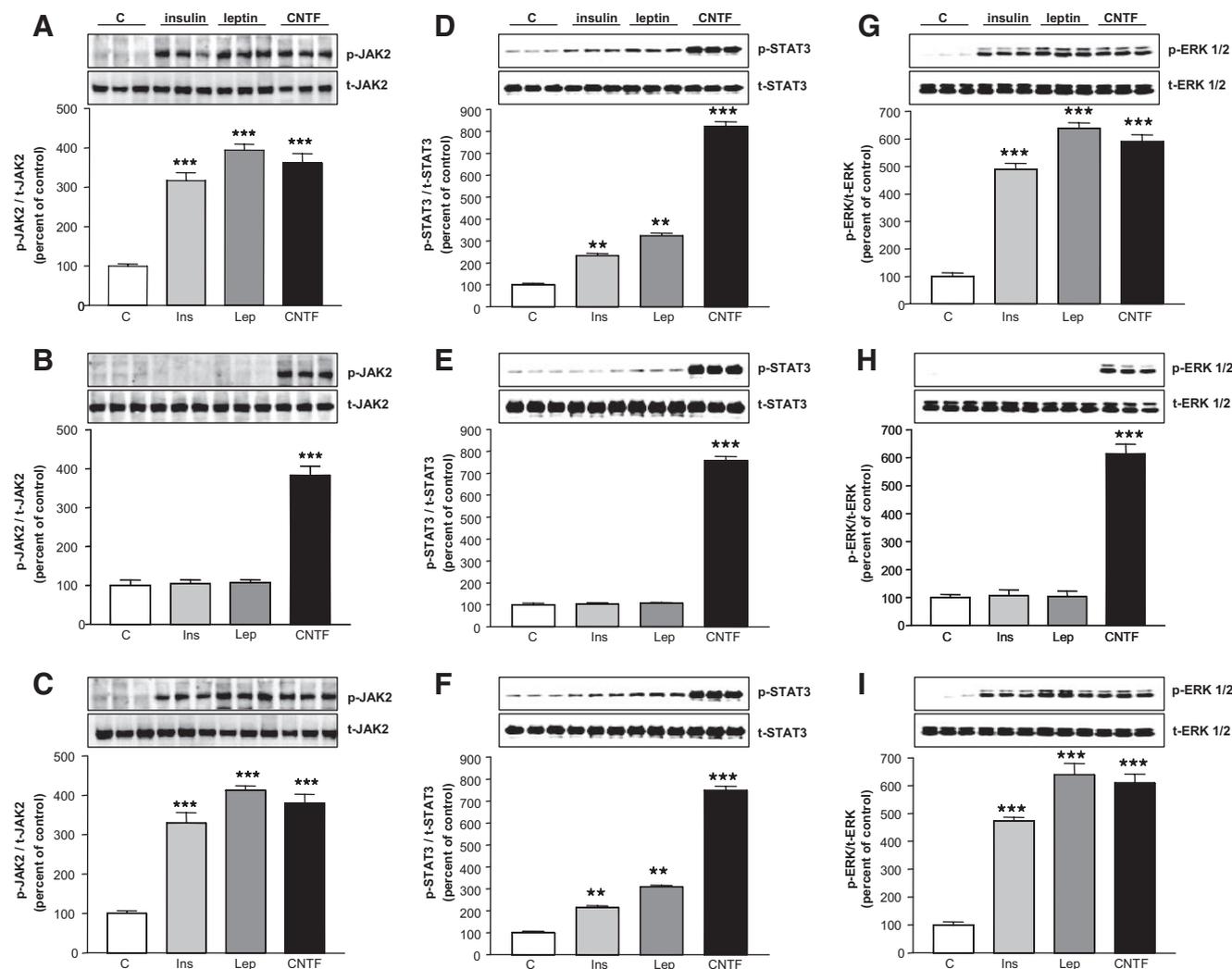
Despite the recruitment of signaling pathways similar to those of leptin, CNTF anorectic effect was maintained in leptin re-



**FIG. 5.** Overexpression of wild-type and mutant PTP-1B cDNA in SH-SY5Y human neuronal cells. Untransfected cells or transiently transfected cells with either wild-type (PTP-1B WT) or mutant type (PTP-1B mut) expression were solubilized and subjected to Western blot analysis. Membranes were probed with anti-PTP-1B antibodies. Blotted proteins were revealed by ECL and bands quantified by densitometry (using Bio-1D software). The results were expressed as the means  $\pm$  SEM ( $n = 3$ ), where \*\*\* indicates  $P < 0.001$ , compared with the control.

sistance state. As suggested by Anderson *et al.* (16), one possible explanation is that CNTF and leptin do not act on the same neuronal populations in the hypothalamus. Indeed CNTF and leptin mainly activate STAT-3 phosphorylation, respectively, in the ventromedial and the lateral parts of hypothalamic arcuate nucleus. Nevertheless, CNTF and leptin similarly target hypothalamic proopiomelanocortin neurons, indicating that both cytokines induce proopiomelanocortin expression through a STAT-3-dependent mechanism (17). We first showed that both leptin and CNTF activate STAT-3 and ERK 1/2 phosphorylation with a higher efficiency of CNTF compared with leptin in SH-SY5Y cells. The effect of leptin is significant from 15 nM, which is in good agreement with previous data obtained on the same cells, whereas the effect of leptin on STAT-3 phosphorylation, on cell proliferation or dephosphorylation of  $\tau$ -protein was observed only at 100 nM (23, 25). It is noteworthy that the common concentrations of leptin used in cell models or even *in vivo* for acute treatment are 50- to 100-fold higher than circulating leptin. This could be attributed to the sensitivity of Western blot technique used for measuring changes in protein phosphorylation levels. In contrast and as previously reported on SH-SY5Y cells, CNTF is efficient at 1 nM (29).

Despite the common signaling pathways shared by these cytokines, they probably mechanistically diverge on signaling components that are not yet identified. In the present study, we attempted to address this issue, and we show that the overexposure of SH-SY5Y human neuronal cells to leptin does not affect CNTF-dependent STAT-3 or ERK 1/2 phosphorylation and vice versa. Similar results were obtained when

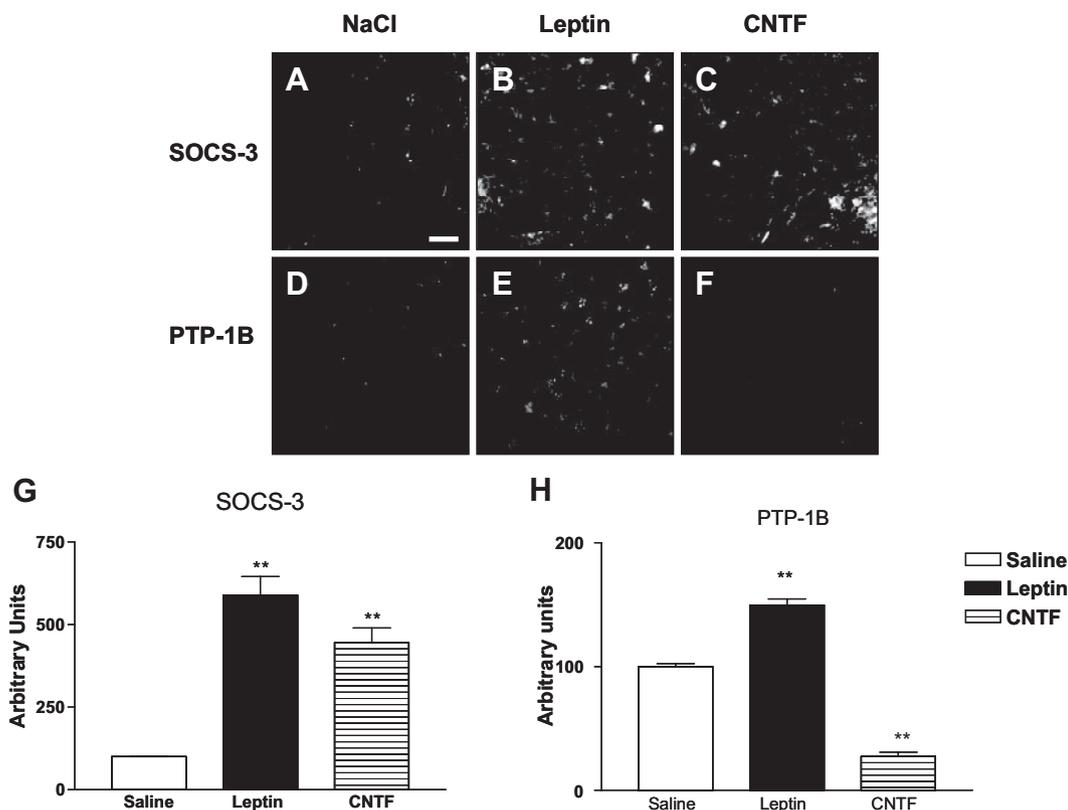


**FIG. 6.** Effect of PTP-1B overexpression on leptin-, insulin-, or CNTF-dependent JAK-2, STAT-3, and ERK phosphorylations in SH-SY5Y human neuronal cells. Untransfected cells (A, D, and G) or transiently transfected cells with either wild-type (B, E, and H) or mutant type PTP-1B (C, F, and I) expression vector were stimulated with 100 nM insulin (Ins), 15 nM leptin (Lep), or 1 nM CNTF for 10 min. Cell lysates were then subjected to Western blot analysis. Membranes were probed sequentially with antiphospho-JAK-2 (p-JAK2) followed by anti-JAK-2 (t-JAK2) antibodies (A–C), antiphospho-STAT-3 (p-STAT3) followed by anti-STAT-3 (t-STAT3) antibodies (D–F) or antiphospho-ERK 1/2 (p-ERK 1/2) followed by anti-ERK 1/2 (t-ERK 1/2) antibodies (G, H, and I). Blotted proteins were revealed by ECL and bands quantified by densitometry (using Bio-1D software). The results were expressed as the ratio of p-JAK2/t-JAK2, p-STAT3/t-STAT3, or p-ERK/t-ERK and presented as the means  $\pm$  SEM ( $n = 3$ ), where \*\* and \*\*\* indicate  $P < 0.01$  and  $P < 0.001$ , respectively, compared with the control.

cells were overexposed to insulin, another anorectic hormone, except that CNTF-dependent ERK 1/2 phosphorylation was attenuated, indicating a partial cross-desensitization of ERK 1/2 signaling pathway in response to insulin or CNTF. However, the mechanism underlying this phenomenon is still unknown.

In cells overexposed to leptin, SOCS-3 expression levels were significantly increased and accompanied with altered leptin-dependent STAT-3 phosphorylation but maintained CNTF action. The induction of SOCS-3 expression has been reported in several leptin-resistant models such as in diet-induced obesity (30), whereas overexpression of SOCS-3 inhibits leptin signaling through SH2 binding to tyrosine residues of ObRb leading to the abolition of STAT-3 activation (31–33). Furthermore, the conditional neuronal-specific SOCS-3 knockout conferred resistance to diet-induced obesity with elevated leptin sensitivity (34). Here we show that

cell overexposure to CNTF induced SOCS-3 expression without altering leptin or insulin-dependent STAT-3 phosphorylation. Thus, SOCS-3 expression does not lead to a cross-desensitization phenomenon between CNTF and leptin signaling pathways in SH-SY5Y human neuronal cells. This is in good agreement with previous reports, indicating that GH pretreatment of adipocytes did not affect STAT-3 phosphorylation induced by cardiotropin-1a or CNTF but completely abolished GH-dependent STAT-5 phosphorylation (35). Our finding could, at least partially, explain the anorectic effect of CNTF in a leptin-resistant environment with leptin-induced SOCS-3 overexpression. It is noteworthy that overexposure to CNTF completely abolished CNTF-dependent STAT-3 and ERK 1/2 phosphorylations suggesting that SOCS-3 may only inhibit signaling pathways of the inducer cytokine. This is in accordance with our previous studies in which SOCS-3 induction in response to prolactin overexposure did not affect



**FIG. 7.** Effect of leptin or CNTF chronic treatment on PTP-1B and SOCS-3 expression in mouse hypothalamic arcuate nucleus. Mice were treated for 4 d with saline, leptin (1 mg/kg · d), or CNTF (0.3 mg/kg · d) and using adequate antibodies SOCS-3 (A–C) and PTP-1B (D–F) were detected and signal quantified using ImageJ software 1.36b (National Institutes of Health). The results of quantification are reported in G for SOCS-3 and H for PTP-1B. The results in G and H are expressed as mean  $\pm$  SEM where \*\* indicates  $P < 0.005$  ( $n = 5$ ).

leptin signaling in CHO cells expressing both OBRb and prolactin receptors (36). It has been also reported that SOCS-3 blocks insulin signaling by ubiquitin-mediated degradation of insulin receptor substrate-1 and insulin receptor substrate-2 (37), and SOCS-3 inhibits IL-1 signaling by targeting the TNF receptor-associated factor-6/TGF $\beta$ -activated kinase complex through the ubiquitination of TNF receptor-associated factor-6. We may suggest that similar mechanisms could affect leptin and CNTF signaling pathways.

SOCS-3 expression is similarly induced by leptin and CNTF and because it is not involved in a cross-desensitization process between these two cytokines, we focused on PTP-1B, the other major negative regulator involved in leptin resistance (38, 39). Indeed, previous studies have evidenced a resistance to diet-induced obesity and increased leptin signaling in PTP-1B-deficient mice (40). Likewise more recent studies reported hypersensitivity to leptin in neuron-specific PTP-1B knockout mice (41). In addition, age-dependent leptin resistance has been reported to be associated with an increased hypothalamic PTP-1B expression (42). Here the overexposure of SH-SY5Y human neuronal cells to leptin or insulin, but not CNTF, induced the expression of PTP-1B at the mRNA level. Interestingly, these observations were confirmed *in vivo* because a chronic treatment with leptin induces PTP-1B expression in mice hypothalamic arcuate nucleus but not CNTF. To our knowledge, this is the first evidence demonstrating that

leptin and insulin are able to induce the expression of PTP-1B in a neuronal cell model. Leptin has been reported to induce PTP-1B protein expression in liver and HepG2 cells, which may possibly maintain insulin signaling sensitivity (43). Thus, PTP-1B may constitute a potential signaling component involved in the divergence between leptin and CNTF signaling pathways at the neuronal level. This hypothesis was corroborated by the fact that the transient overexpression of PTP-1B completely abolished leptin and insulin signaling pathways without altering CNTF action in SH-SY5Y human neuronal cells. Thus, we may not suggest, as for SOCS-3, that PTP-1B inhibits only the stimulating cytokine because when PTP-1B is overexpressed, it selectively affects leptin/insulin signaling but not CNTF. The mechanisms underlying the selective effect of PTP-1B are still unclear.

Taken together, these data indicate that overexposure to leptin induces both SOCS-3 and PTP-1B, whereas CNTF induces only SOCS-3, and overexpression of PTP-1B in SH-SY5Y human neuronal cells alters leptin and insulin but not CNTF signaling pathways. This may constitute a major step in the understanding of the mechanisms involved in the efficacy of CNTF to reduce body weight in a variety of rodent models of obesity/type 2 diabetes and even in humans exhibiting leptin resistance. In conclusion, PTP-1B is clearly a divergent component between leptin and insulin *vs.* CNTF signaling, and understanding the mechanism involved in its inefficacy to inhibit CNTF signaling may

reveal new therapeutic targets for the treatment of leptin resistance.

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