

**Regulation of corticotropin-releasing factor and urocortin 2/3 mRNA by leptin in  
hypothalamic N39 cells**

(視床下部 N39 細胞におけるレプチンによる

CRF 及び urocortin 2/3 遺伝子発現の調節作用)

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## **Abstract**

Corticotropin-releasing factor (CRF) activates the pituitary-adrenal axis during stress, and shows anorectic effects via CRF type 1 receptors in the hypothalamus. Both urocortin (Ucn) 2 and Ucn3 also act as anorectic neuropeptides via CRF type 2 receptors. Leptin, a product of the obesity gene secreted mainly from adipose tissue, reduces food intake and increases energy expenditure. A possible interaction between leptin and CRF/Ucns has been suggested, as leptin can regulate expression and activation of CRF and Ucns in the hypothalamus. This study aimed to explore the possible function of leptin in the hypothalamus, and its effects in regulating CRF and Ucns. The study identified mRNA expression of the leptin receptor (Ob-R) and its subtypes, CRF, and Ucn2/3 in mouse hypothalamic N39 cells. Leptin stimulated signal transducer and activators of transcription type 3 (STAT3) phosphorylation, directly increased the mRNA levels of both CRF and Ucn2/3 in hypothalamic cells, and increased Ob-Rb mRNA levels. A Janus kinase inhibitor inhibited the leptin-mediated increase in STAT3 phosphorylation, and then the increases in CRF and Ucn2/3 mRNA levels. Leptin may contribute to a stress response or anorectic effect via the regulation

of CRF and Ucn2/3 in the hypothalamus.

## **Introduction**

Corticotropin-releasing factor (CRF), a major regulatory peptide in the hypothalamic-pituitary-adrenal (HPA) axis [61, 62], mediates a variety of physiological functions including the regulation of HPA axis activity during periods of stress [15]. A related compound is Urocortin (Ucn) 1, a 40-amino acid peptide originally cloned from the Edinger-Westphal nucleus, and a member of the CRF family of peptides [63]. Both CRF and Ucn1 contribute to stress responses via G protein-coupled receptors [29, 58, 62]. The CRF type 1 receptor (CRF<sub>1</sub> receptor) [6, 11, 65], for which both CRF and Ucn1 have high affinity [62], is predominately expressed in the brain and pituitary gland.

Ucn2 and Ucn3 prohormones were identified in the human genome database and in mouse genomic DNA, respectively [27, 35, 50], which led to predictions regarding the existence and identity of endogenous peptides [20]. Ucn2 and Ucn3 are more similar to each other than they are to CRF, and both have very high affinity for the CRF type 2 receptor (CRF<sub>2</sub> receptor) but little to none for the CRF<sub>1</sub> receptor [33, 38, 48, 57]. In the mouse brain, Ucn2 is expressed in restricted areas, including the magnocellular division

of the paraventricular nucleus (PVN), the arcuate nucleus (ARC), and the locus coeruleus [50]. Ucn3 is expressed in the median preoptic nucleus and ventromedial nucleus (VMH) of the hypothalamus [35].

CRF and Ucn1 show anorectic effects in rodents [25, 44, 51, 56]. Both Ucn2 and Ucn3 also act as anorectic neuropeptides via the CRF<sub>2</sub> receptor [7, 19], and Ucn3 additionally regulates glucose-stimulated insulin secretion and energy homeostasis [36]. Ucn3 that innervates the VMH is also a critical molecular mediator for regulating feeding and peripheral energy metabolism [7].

Leptin, a product of the obesity (ob) gene secreted mainly from adipose tissue, regulates various reproductive, neuroendocrine, immune, and metabolic functions [39]. Leptin also regulates energy homeostasis, reduces food intake [4, 23, 34, 46], and increases energy expenditure [34]. The mRNA coding for the long form of the leptin receptor (Ob-Rb) has been found in various brain structures, including nuclei involved in the regulation of energy balance such as the VMH, the PVN, and the ARC [26, 41]. Ob-Rb is well-known to mediate the effects of leptin through the Janus kinase (JAK)-signal transducer and activators of transcription (STAT) pathway [22], although

in the rodent hypothalamus, leptin activates only STAT type 3 (STAT3) [60].

Leptin has been reported to increase CRF secretion from the rat hypothalamus in *in intro* and *ex vivo* [49, 12]. CRF neurons in the PVN and VMH are considered to be an important mediator for leptin that contribute to regulation of feeding and adiposity [40].

Leptin can also influence the activity of Ucn1 neurons in the mouse Edinger-Westphal nucleus [66]; however, little is known about the direct regulation of CRF and Ucn1 by leptin in the hypothalamus. Belsham et al. have managed to develop cell lines that are representative of the enormous range of hypothalamic cell types [2, 14]. N39, developed from primary mouse fetal hypothalamic cultures, is one such homologous neuronal cell line. As they express both CRF<sub>1</sub> and CRF<sub>2</sub> receptors and Ob-R [2, 32] these hypothalamic N39 cells have been used to further understand endogenous receptor signal transduction, the possible function of leptin, and the regulation of CRF and Ucn1 by leptin in the hypothalamus. Cui et al. used these cells to demonstrate that leptin signaling involves the JAK-STAT3 pathway [13]. In the present study, we first examined mRNA expression of Ob-R subtypes as well as the leptin-induced phosphorylation of STAT3. To further elucidate the possible function of leptin and the

regulation of CRF and Ucn3 in the hypothalamus, we then examined the direct effects of leptin on mRNA levels of CRF, Ucn3, and Ob-Rb, in hypothalamic N39 cells.

## **Materials and Methods**

### *Materials*

Recombinant mouse leptin was purchased from R&D Systems (Minneapolis, MN). JAK inhibitor I was purchased from Calbiochem (San Diego, CA).

### *Cell cultures*

N39 cells were obtained from CELLutions Biosystems Inc. (Burlington, Ontario, Canada), and incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, and 100 U/mL penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Cells were plated at 10<sup>4</sup> cells/cm<sup>2</sup> for 4 days before each experiment, and the medium was changed every 48 h. On day 5, to remove the effect of factors contained in FBS, the cells were washed and then starved overnight using DMEM supplemented with 0.2% bovine serum albumin prior to each experiment. At the end of each experiment, total cellular RNA or protein was collected and stored at -80°C until the assay was performed. All treatments were performed in triplicate.



### *RNA extraction*

Cells were incubated with medium alone (control) or with medium containing leptin for the times indicated in Fig. 2. To examine the dose-dependent effects of leptin, cells were incubated for the indicated times with medium alone (control) or with medium containing increasing concentrations of leptin (10 pM-1 nM). At the end of each experiment, total cellular RNA was extracted with an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. cDNA was synthesized from total RNA (0.5 µg) using random hexamers as primers with the SuperScript First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions.

### *Reverse transcriptase-polymerase chain reaction*

RT-PCR was carried out in a programmable thermal controller (Bio-Rad, Hercules, CA) with the following oligonucleotide primers [26]: Ob-R-forward (F)

(5'-CAGATTCGATATGGCTTAATGGG-3'), Ob-R-reverse (R)

(5'-GTAAAATTCACAAGGGAAGCG-3'), Ob-Ra-F

(5'-ACACTGTTAATTTACACCAGAG-3'), Ob-Ra-R

(5'-AGTCATTCAAACCATTAGTTTAGG-3'), Ob-Rb-F

(5'-GTGTGAGCATCTCTCCTGGAG-3'), and Ob-Rb-R

(5'-ACCACACCAGACCCTGAAAG-3'). Conditions for the Ob-R and Ob-Rb were 1

× (94°C, 4 min), 40 × (94°C, 1 min; 55°C, 1 min; 72°C, 1 min), and 1 × (72°C, 10 min)

and conditions for the Ob-Ra were 1 × (94°C, 4 min), 40 × (94°C, 1 min; 54°C, 1 min;

72°C, 1 min), and 1 × (72°C, 10 min). Products were separated by electrophoresis on a

1.2% agarose gel containing ethidium bromide. The expected sizes of PCR products for

the Ob-R and Ob-Ra were 473 bp and 237 bp, respectively. The expected size of PCR

products for the Ob-Rb was 533 bp.

#### *Real-time RT-PCR*

Total cellular RNA extraction and cDNA synthesis were performed as described earlier [30, 31]. The resulting cDNA was then subjected to real-time PCR as follows.

The expression levels of mouse CRF, Ucn3, and Ob-Rb mRNA were evaluated with quantitative real-time PCR with the following specific sets of primers and probes (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA).  $\beta$ 2-microglobulin (B2MG) was used as a housekeeping gene to standardize values, as B2MG mRNA levels were not changed during any treatments in this study. Each reaction consisted of 1  $\times$  TaqMan universal PCR Master Mix (Applied Biosystems), 1  $\times$  Assays-on-Demand Gene Expression Products (Mm01293920\_s1 for mouse CRF, Mm00445261\_m1 for mouse Ucn1, Mm01227928\_s1 for mouse Ucn2, Mm00453206\_s1 for mouse Ucn3, Mm00440181\_m1 for mouse Ob-Rb, and Mm00437762\_m1 for mouse B2MG) and 1  $\mu$ L of cDNA in a total volume of 25  $\mu$ L with the following parameters on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

The above assays involved specific sets of primers and a TaqMan probe spanning the exon/exon junction and should not, therefore, have been influenced by DNA contamination. Data were collected and recorded with ABI PRISM 7000 SDS software (Applied Biosystems) and expressed as a function of the threshold cycle ( $C_T$ ). The

amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical when analyzed with diluted samples.

#### *Western blot analysis*

Western blot analysis was performed to examine protein expression of the phosphorylated (p) STAT3/STAT3. Cells were washed twice with phosphate-buffered saline (PBS) and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation, and the supernatant was recovered. Samples were boiled and subjected to electrophoresis on a 4-20% gradient polyacrylamide gel, and proteins were transferred to a polyvinylidene fluoride membrane (Daiichi Kagaku, Tokyo, Japan). After blocking with Detector Block<sup>®</sup> buffer (Kirkegaard & Perry Laboratories, Gaithersburg, MD), the membrane was incubated for 1 h with anti-STAT3 antibody (dilution 1/1000) (Cell Signaling Technology, Beverly, MA), or anti-pSTAT3 antibody (dilution 1/1000) (Cell Signaling Technology), washed with PBS containing 0.05% Tween 20, and incubated with horseradish peroxidase-labeled anti-rabbit immunoglobulin G (Daiichi Kagaku). The chemiluminescent substrate SuperSignal

West Pico (Pierce Chemical Co., Rockford, IL) was used for detection, and the membrane was exposed to BioMax film (Eastman Kodak Co., Rochester, NY).

### *Statistical analysis*

Each experiment was performed at least three times. Samples were provided in triplicate for each group of experiments. Each value is expressed as the mean  $\pm$  standard error of the mean (SEM). Statistical analysis was performed with analysis of variance (ANOVA), followed by Fisher's protected least-significant difference post-hoc test. The level of statistical significance was set at  $P < 0.05$ .

## Results

### *Expression of Ob-R mRNA*

N39 cell mRNA expression of Ob-R and its subtypes Ob-Ra and Ob-Rb was examined by RT-PCR. N39 cells were found to express Ob-R, Ob-Ra, and Ob-Rb mRNA (Fig. 1A).

N39 cells were incubated with 1 nM leptin to determine its effects on STAT3 phosphorylation. A time-course study showed that leptin stimulated STAT3 phosphorylation from 15 min to 1 h in hypothalamic N39 cells (Fig. 1B-1). To determine whether the JAK pathway was involved in leptin-induced phosphorylation of STAT3, N39 cells were pre-incubated for 30 min with JAK I inhibitor, prior to the addition of leptin. JAK I inhibitor inhibited a leptin-mediated increase in STAT3 phosphorylation (Fig. 1B-2).

### *Effects of leptin on CRF, Ucn2/3, and Ob-Rb mRNA levels*

Expression of CRF, Ucn2, and Ucn3 but not Ucn1 mRNA was detected in N39 cells by real-time RT-PCR. We then examined the effects of leptin on time- and

dose-dependent changes in CRF and Ucn2/3 mRNA levels. A time-course study showed that 1 nM leptin significantly increased CRF mRNA levels (ANOVA;  $P < 0.005$ ; Fig. 2A). The maximum effect of leptin was observed at 2 h, with approximately 2.0-fold increases in CRF mRNA levels compared with the basal level. Leptin significantly stimulated CRF mRNA levels in a dose-dependent manner (ANOVA;  $P < 0.0005$ ) with significant effects observed at 1 nM (Fig. 2A).

Leptin also significantly increased Ucn2/3 mRNA levels (ANOVA;  $P < 0.0005$  and  $P < 0.0001$ , respectively; Fig. 2B). The maximum effect of leptin was observed at 2 h, with approximately 1.5- and 1.4-fold increases in their mRNA levels, respectively, compared with basal levels. Significant effects of leptin on both levels were observed at 1 nM (Fig. 2B).

We then investigated the effects of leptin on the time- and dose-dependent changes in Ob-Rb mRNA levels. Leptin significantly increased mRNA levels of its receptor, Ob-Rb (ANOVA;  $P < 0.05$ ; Fig. 2C). The maximum effect of leptin was observed at 6 h, with approximately 1.4-fold increases in Ob-Rb mRNA levels

compared with the basal level. Significant effects of leptin were observed at 1 nM (Fig. 2C).

*Effects of JAK inhibitor I on leptin-induced increases in CRF and Ucn2/3 mRNA levels*

We examined whether the JAK-STAT pathway was involved in leptin-induced increases in CRF and Ucn2/3 mRNA levels. N39 cells were pre-incubated for 30 min with JAK I inhibitor, prior to the addition of leptin. JAK I inhibitor significantly inhibited leptin-mediated increases in CRF and Ucn2/3 mRNA levels (Fig. 3).



## Discussion

In this study, mRNA expression of both Ob-Ra and Ob-Rb subtypes was detected in hypothalamic N39 cells. The short-form Ob-Rs, Ob-Ra and Ob-Rc, contribute to transport of leptin into the brain [3]. The long-form Ob-R, Ob-Rb, is abundantly expressed in the ARC, PVN, VMH, and lateral hypothalamic area [16, 17, 41]. Leptin activates STAT3 with Jak2. Leptin similarly stimulates STAT3 phosphorylation in the rodent hypothalamus [60] and hypothalamic N39 cells. A JAK inhibitor inhibits the leptin-mediated increase in STAT3 phosphorylation. The present results in hypothalamic N39 cells are consistent with those of earlier studies [13]. Through the activation Ob-Rb and subsequent activation of the Jak2-STAT3 signaling pathway in the hypothalamus, leptin reduces food intake and boosts energy expenditure [53, 60].

Possible interaction between leptin and CRF/Ucns has been suggested, as leptin can regulate expression or activation of CRF and Ucns in the hypothalamus. In the present study, we found that leptin directly increased the mRNA levels of both CRF and Ucn2/3 in hypothalamic N39 cells, indicating that leptin acts directly on CRF and

Ucn2/3 neurons via the Ob-Rb in the hypothalamus. Both *in vivo* and *in vitro* studies have shown that leptin increases expression of CRF in the hypothalamus [12, 28, 43, 54]. In fact, simultaneous injection of leptin and a non-specific CRF receptor antagonist confirmed that a ligand for CRF receptor contributes to leptin's anorectic effect [21, 59]. Furthermore, this anorectic effect was substantially attenuated in rats pretreated with intracerebroventricular (icv) injection of an anti-CRF antibody, but not with an anti-Ucn1 antibody. These results suggest that the anorectic effect of leptin is at least partially mediated by CRF, but not by Ucn1, in the hypothalamus [45]. In fact, it is possible that leptin acts directly on the PVN, as leptin is generally assumed to be transported across the blood-brain barrier via Ob-Rs [52]. Even if leptin does not act directly, there is evidence for its indirect control of the PVN via the Ob-R-rich ARC [52]. Therefore, at the least, CRF itself may be responsible for the central actions of leptin that lead to suppression of food intake.

Central administration and direct injection of Ucn2 or Ucn3 into the VMH decrease food intake [18, 44, 47, 64]. Both Ucn2 and Ucn3 act as anorectic neuropeptides via the CRF<sub>2</sub> receptor [7, 19]. Though both CRF<sub>1</sub> and CRF<sub>2</sub> receptors are

involved in stress-induced inhibition of food intake, CRF<sub>1</sub> receptor mediates an immediate or transient inhibition of intake, whereas CRF<sub>2</sub> receptor is associated with a delayed or late inhibition of food intake [55, 58]. Therefore, Ucn2 and Ucn3 may contribute to the delayed or late inhibition of food intake via the CRF<sub>2</sub> receptor.

Although Ucn2 mRNA is expressed in the rat hypothalamus [1, 67], the innervation is undetermined. The VMH, which is densely innervated by Ucn3-positive fibers [8, 37], expresses CRF<sub>2</sub> receptor mRNA [5]. The CRF<sub>2</sub> receptor-expressing glutamatergic neurons of the VMH project to the ARC, and stimulate production of an anorectic neuropeptide, proopiomelanocortin [10]. Huang et al. [28] reported that chronic icv infusion of leptin increased VMH expression of the CRF<sub>2</sub> receptor. Nishiyama et al. [43] reported that expression of CRF<sub>2</sub> receptor mRNA in the VMH was dependent on plasma leptin levels, and a low dose of leptin, representative of physiological levels, increased CRF<sub>2</sub> but not CRF<sub>1</sub> receptor mRNA levels in the VMH. Therefore, leptin can modulate the effects of Ucn3 via the regulation of the CRF<sub>2</sub> receptor. A recent study demonstrated that leptin remains effective on food intake in mice deficient in CRF<sub>2</sub> receptor [24]. As global CRF<sub>2</sub> receptor knockout mice often result in functional

compensation such as exaggerated CRF<sub>1</sub> receptor activity, specific brain area deletions of CRF<sub>2</sub> may be necessary to further evaluate the interaction between leptin and the CRF system [9].

In the present study, leptin increased Ob-Rb mRNA levels in hypothalamic cells. Ob-Rb gene expression in the ARC and VMH is increased after fasting and decreased by refeeding [42]. Leptin challenge also increases Ob-Rb expression in the ARC, although the regulation of Ob-Rb by leptin may be dependent on cell type or nucleus [42]. Although it is unclear whether the changes in mRNA levels directly correlate with the protein expression levels, it is possible that leptin-induced increases in Ob-Rb expression levels may enhance responsiveness to the ligand. Together, the leptin system would regulate its effects in cooperation with CRF or Ucn2/3 in the hypothalamus (Fig. 4). Further study is required to determine the effects of leptin-induced CRF/Ucn regulation on stress *in vivo*.

In summary, this study identified mRNA expression of both Ob-Ra and Ob-Rb in hypothalamic N39 cells. Leptin stimulated STAT3 phosphorylation, and directly increased the mRNA levels of both CRF and Ucn2/3 in hypothalamic cells.

Leptin also increased Ob-Rb mRNA levels. A JAK inhibitor inhibited the leptin-mediated increase in STAT3 phosphorylation, and then the increases in CRF and Ucn2/3 mRNA levels. Leptin may contribute to a stress response or anorectic effect via the regulation of CRF and Ucn2/3 in the hypothalamus.

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### Legend to Figures

**Fig. 1** Expression of Ob-R in N39 cells. (A) Expression of Ob-R, Ob-Ra, and Ob-Rb mRNA in N39 cells. M: marker, N39: reverse transcriptase-PCR shown in triplicate, PC: positive control (mouse hypothalamus) for reverse transcriptase-PCR, NC: negative control for reverse transcriptase-PCR. (B) Effects of leptin on STAT3 phosphorylation in N39 cells. Independent experiments were repeated in triplicate, and a representative blot is shown. (B-1) Time-dependent changes in leptin-induced STAT3 phosphorylation. Cells were incubated with medium containing 1 nM leptin for the durations shown. (B-2) Effects of JAK inhibitor I on leptin-induced STAT3 phosphorylation. Cells were pre-incubated for 30 min with medium containing 100 nM JAK inhibitor I (JAK I) or vehicle, and then incubated for 30 min with medium containing 1 nM leptin or vehicle.

**Fig. 2** Effects of leptin on CRF, Ucn2/3, and Ob-Rb mRNA levels in N39 cells.

Control cells were treated with medium alone. Cells were treated in triplicate and the average of at least three independent experiments is shown. Statistical analysis was performed using one-way ANOVA, followed by post-hoc tests. \*:  $P < 0.05$  (compared with control). Time-dependent effects of leptin on CRF (A), Ucn2/3 (B), and Ob-Rb (C)



mRNA levels (left panel): cells were incubated with medium containing 1 nM leptin.

Dose-dependent effects of leptin on CRF (A), Ucn2/3 (B), and Ob-Rb (C) mRNA levels (right panel): cells were incubated for 2 h with medium containing from 10 pM to 1 nM leptin.

**Fig. 3** Effects of JAK inhibitor I on leptin-induced increases in CRF and Ucn2/3 mRNA levels in N39 cells. Cells were treated in triplicate and the average of at least three independent experiments is shown. Statistical analysis was performed using one-way ANOVA, followed by post-hoc tests. \*  $P < 0.05$  (compared with control). <sup>+</sup>  $P < 0.05$  (compared with leptin). Cells were pre-incubated for 30 min with medium containing 100 nM JAK inhibitor I (JAK I) or vehicle, and then incubated for 2 h with medium containing 1 nM leptin or vehicle.

**Fig. 4** Schematic representation of the role of leptin in the regulation of CRF and Ucn2/3 gene expression in N39 cells. Solid lines indicate the main route of CRF and

Ucn regulation by leptin as suggested in this study. Dashed lines indicate a hypothesized role.