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Paraventricular NUCB2/nesfatin-1 is directly targeted by leptin and mediates its anorexigenic effect

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ABSTRACT

An adipokine leptin plays a central role in the regulation of feeding and energy homeostasis via acting on the hypothalamus. However, its downstream neuronal mechanism is not thoroughly understood. The neurons expressing nucleobindin-2 (NUCB2)-derived nesfatin-1 in the hypothalamic paraventricular nucleus (PVN) have been implicated in feeding and energy homeostasis. The present study aimed to explore the role of PVN NUCB2/nesfatin-1 in the leptin action, by using adeno-associated virus (AAV) vectors encoding shRNA targeting NUCB2 (AAV-NUCB2-shRNA). Leptin directly interacted and increased cytosolic Ca^{2+} in single neurons isolated from the PVN, predominantly in NUCB2/nesftin-1-immunoreactive neurons. Treatment with leptin *in vivo* and *in vitro* markedly increased NUCB2 mRNA expression in the PVN. Peripheral and central injections of leptin failed to significantly inhibit food intake in mice receiving AAV-NUCB2. These results indicate that PVN NUCB2/nesfatin-1 is directly targeted by leptin, and mediates its anorexigenic effect.

1. Introduction

The discovery of leptin, the ob (obese) gene product, in 1994 was remarkable since it established an endocrine status of the adipocytes and a molecular basis for the link between the adipocytes and brain [1]. Leptin is secreted primarily from the white adipose tissue and transported to the brain across the blood–brain barrier (BBB), thereby reducing food intake and increasing energy expenditure [2,3]. It has long been thought that leptin exerts these metabolic effects via interacting with the first-order neurons in the arcuate nucleus (ARC), particularly the pro-opiomelanocortin (POMC) neurons, via the long form leptin receptor, Ob-Rb [4,5]. However, recent studies have shown that deletion of Ob-Rb selectively in ARC POMC neurons only partially account for the massive obesity seen with global deletion of Ob-Rb, indicating that the ARC POMC neuron is not the sole but one of several key neurons through which leptin regulates body weight (BW) [6]. In fact, selective deletion of Ob-Rb in the neurons of ventromedial hypothalamus (VMH) also results in an increase in BW, but to a moderate extent [7,8]. These studies suggest a yet unidentified "non-ARC POMC, non VMH" additional neuronal center(s) for sensing leptin to execute its metabolic effects [8].

The paraventricular nucleus (PVN) has been recognized as the integrative center for feeding and energy metabolism [9]. Accumulating evidences support that the PVN could also serve as a target for circulating leptin. First, leptin receptor is expressed abundantly in the PVN neurons as revealed by in situ hybridization and immunohistochemistry [10]. Second, leptin injected intravenously and into the third ventricle elevates c-Fos expression in the PVN [11]. Third, whole cell patch-clamp experiments demonstrated that leptin depolarizes the neurons in the PVN slices [12]. Forth, single microinjection of recombinant AAV vector-encoding leptin into the PVN reverses diet-induced obesity [13]. These reports suggest that leptin might act directly on the PVN neurons to modulate feeding and/or energy expenditure. However, the molecule and neuron in the PVN that mediate the anorexigenic effect of leptin remain unknown.

Abbreviations: NUCB2, nucleobindin2; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; PVN, paraventricular nucleus; ARC, arcuate nucleus; NTS, nucleus tractus solitarius; POMC, pro-opiomelanocortin.

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In the PVN, although several neurons have been shown to be related to feeding behavior, the neuron species or neuropeptide responsible for the prominent role of this nucleus in controlling feeding is less defined. Nesfatin-1 is an 82 amino acids peptide processed from its precursor nucleobindin-2 (NUCB2) [14]. Central and peripheral injections of nesfatin-1 decrease food intake in rats and mice [15,16]. Conversely, immunoneutralization of endogenous NUCB2/nesfatin-1 in the brain significantly enhances food intake [14,17]. These findings have supported that nesfatin-1 is an anorexigenic neuropeptide. Moreover, accumulating evidences suggest that the NUCB2/nesfatin-1 localized in the PVN is an emerging new player in regulation of food intake and energy metabolism [14,18]. First, the NUCB2/nesfatin-1 protein and its mRNA expression are detected most abundantly in the hypothalamic nuclei including the PVN and brainstem areas [19,20]. Second, the NUCB2/nesfatin-1 mRNA and protein levels in the PVN are selectively altered with energy status, being down-regulated after short or chronic under-nutrition and up-regulated after refeeding in rats [14,20]. Third, refeeding induces c-Fos expression preferentially in the PVN, primarily in NUCB2/nesfatin-1 neurons [20]. Forth, systemic metabolic signals, glucose and insulin, directly activate nesfatin-1 neurons in the PVN [21]. These findings prompted us to hypothesize that the PVN NUCB2/nesfatin-1 could be directly targeted by leptin and serves as a physiological regulator of feeding.

The present study aimed to clarify whether leptin acts through the PVN NUCB2/nesfatin-1 for its anorexigenic and/or metabolic effects. For this, we used AAV vector-mediated RNA interference to suppress NUCB2 expression specifically in the PVN.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice were single-housed for *in vivo* experiments and group-housed for Ca^{2+} imaging under a 12-h light/dark cycle condition (7:30 lights on). Water and food were available ad libitum except that food was withdrawn in particular experiments. All animal procedures were conducted in compliance with protocols approved by Jichi Medical University Animal Care and Use Committee.

2.2. Measurement of $[Ca^{2+}]_i$ and NUCB2/nesfatin-1-immunocytochemistry in single PVN neurons

Single neurons were prepared from mice aged 5–6 weeks according to procedures reported previously [21] with slight modifications. $[Ca^{2+}]_i$ was measured by ratiometric fura-2 fluorescence imaging as previously reported [21]. Briefly, single neurons on coverslips were incubated with 2 μmol/l fura-2/AM (Dojin chemical, Kumamoto, Japan) for 40 min at room temperature, mounted in chamber and superfused with HKRB composed of (in mmol/l) 129 NaCl, 5.0 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.8 $CaCl_2$, 1.2 $MgSO_4$, and 10 HEPES at pH 7.4. Fluorescence images due to excitation at 340 and 380 nm were captured and ratio (F340/F380) images produced by Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). After $[Ca^{2+}]_i$ measurement, NUCB2/nesfatin-1 neurons were detected by immunocytochemistry using anti-NUCB2 antibody (Sigma).

2.3. PVN slice cultures

Transverse brain slices were prepared from 5 week-old mice. A 200 μm coronal brain slice containing rostrocaudally middle part of the PVN was prepared using a vibratome. Bilateral portions of PVN were dissected and explanted onto culture membrane (Millicell CM; pore size, 0.4 μm; Millipore) in a 35 mm culture dish containing 1 mL of DMEM (Gibco) supplemented with 2.7 mM $NaHCO_3$, 10 mM HEPES, 20 mg/L kanamycin, 100 mg/mL apo-transferin, 5 μg/mL insulin, 16 μg/mL putrescine, 7.6 μM progesterone and 20 nM sodium selenite. Following incubation with leptin, total RNAs were extracted.

2.4. Intracerebroventricular (icv) injection

In 10 week-old mice, a guide cannula was inserted into 3rd cerebral ventricular with the tip located at 1.5 mm caudal, 0.15 mm lateral to the bregma and 5.0 mm below the skull. Mice were allowed to recover from surgery for at least one week before being subjected to tests. For mRNA expression experiments, test substances were dissolved in sterile saline and were injected intracerebroventricularly in a volume of 50 pmole/3 μl. Injections were performed within 1 h before the onset of dark cycle and PVN was isolated at 2 h after the injection.

2.5. RT-PCR

RT-PCR was performed as previously reported [15]. Briefly, at 2 h after leptin or α-MSH injection, bilateral portions of PVN were immediately collected from hypothalamic slices.

Quantitative RT-PCR assay was performed using SYBR Premix Ex Taq II polymerase in Thermal Cycler Dice (Takara bio). Expression levels of mRNAs were calculated by the ΔΔCT method of relative quantification, and normalized to products glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were as follows:

NUCB2 5′-GTCACAAAGTGAGGACGAGACTG-3′, 5′-TGGTTCAGGT GTTCAAACTGCTTC-3′,

GAPDH 5′-GGCACAGTCAAGGCTGAGAATG-3′, 5′-ATGGTGGTGA AGACGCCAGTA-3′.

2.6. Construction of short hairpin RNAs (shRNAs) and viral vector production

Target sequence was chosen to design shRNAs for NUCB2 gene (Accession No. NM 016773: 5′-GGATCATCCAAGTACAGTA-3′). In addition, scrambled oligonucleotide sequence was used for specificity control (5′-CAACACTAGTTGACATGTA-3′). To construct shRNAs, forward oligonucleotides were designed to contain the sense and antisense sequences connected with a hairpin loop (TAGTGCTCCTGGTTG) followed by a poly(dT) termination signal. The annealed forward and reverse oligonucleotides were ligated into the pBAsi-mU6 vector (Takara Bio, Japan) of the production of shRNAs downstream of mouse U6 promoter. Then the mU6 promoter, hairpin sequence and terminator sequences were subcloned into pAAV plasmid. The shRNA constructs were then cloned into an AAV serotype 9 (AAV9) for stable shRNA delivery. Briefly, AAV9-NUCB2-shRNA and AAV9-Scr-shRNA viruses were produced following triple-transfection of HEK293 cells with pAAV-shRNA, an adenoviral helper plasmid pAdeno, and a chimeric helper plasmid encoding AAV2 rep/AAV9 cap genes (pAAV2rep/AAV9cap). [22,23].

2.7. Microinjection of virus vector into the PVN of hypothalamus and intracerebroventricular (icv) cannula implantation

rAAV-NUCB2-shRNA or control rAAV-Scrambled-shRNA at 1×10^8 vg/0.2 μl volume in saline were injected stereotaxically into bilateral PVN. The coordinates for the injections into the PVN were determined as anteroposterior (AP) = −0.82 mm, mediolateral (ML) = ±0.15 mm and dorsoventral (DV) = −5.0 mm with respect to bregma. Then, 3rd cerebral ventricular cannulations

for icv injection were performed. The stereotaxic coordinates used were AP = −1.5 mm and DM = −5 mm. For feeding experiments, tests substances were dissolved in sterile saline and icv injected in a volume of 50 pmole/3 μl at 3 weeks after the operation. Injections were performed within 1 h before the onset of dark cycle and food intake was measured after the injection. Intraperitoneal (ip) injection of leptin was performed at 4 weeks after the AAV injection; mice received sterile saline or leptin (600 pmole/g BW) dissolved in saline in 10 μl/g BW volume.

2.8. Immunohistochemistry

Coronal sections (40 μm) of the hypothalamus were cut using a freezing microtome and collected at 120 μm intervals. After blocking of endogenous peroxidase, sections were incubated with rabbit anti-NUCB2 primary antibody (1:1000) (Sigma) overnight at 4 °C. Primary antibodies were detected using a VECTASTAIN ABC kit (Vector lab.). The reaction product was visualized by incubation in 0.02% diaminobenzidine (DAB) in 0.05 M Tris buffer with 0.005% hydrogen peroxide for 5–7 min.

2.9. Western blot analysis

PVN lysate proteins were subjected to 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. NUCB2 proteins were detected with the anti-NUCB2 IgG (Sigma) and ECL system. Immunoreactive (IR) signal was quantified by using FAS-1000 (Fujifilm, Japan) and expression levels of proteins were normalized to β-actin (Santa Cruz).

2.10. Statistical analysis

Data are expressed as means ± s.e.m. Data were analyzed for statistical significance by one-way ANOVAs for two groups or two-way ANOVAs for knockdown experiments followed by a Bonferroni's multiple comparison test. $p < 0.05$ was considered significant.

3. Results

3.1. Leptin increases $[Ca^{2+}]_i$ in PVN NUCB2/nesfatin-1 neurons

To determine the direct effect of leptin on PVN NUCB2/nesfatin-1 neurons, we used a method of $[Ca^{2+}]_i$ imaging in single neurons isolated from the PVN followed by immunocytochemical identification of NUCB2/nesfatin-1 neurons [21]. Administration of 10^{-11} M leptin increased $[Ca^{2+}]_i$ in the PVN neuron (Fig. 1A, left panel) that was subsequently proved to be IR to NUCB2/nesfatin-1 by immunocytochemistry (Fig. 1A, right panel). Among 208 PVN neurons examined, 44 neurons (21.2%) responded to leptin with $[Ca^{2+}]_i$ increases (Fig. 1B). Furthermore, 30 of 44 leptin-responsive PVN neurons (68.2%) were IR to NUCB2/nesfatin-1 (Fig. 1C), indicating that NUCB2/nesfatin-1 neuron is the direct and major target of leptin in the PVN.

3.2. Leptin stimulates NUCB2 mRNA expression in the PVN

The result that leptin directly activated NUCB2/nesfatin-1 neurons suggested that leptin could serve as an up-stream regulator of NUCB2. The *in vitro* treatment of PVN slices with 10^{-8} M leptin overnight in culture significantly increased NUCB2 mRNA expression (Fig. 2A). Moreover, in *in vivo* experiments, icv injection of leptin (50 pmol) increased NUCB2 mRNA expression in the PVN by 15 fold at 2 h after injection (Fig. 2A). These results indicate that leptin directly interacts with the PVN to up-regulate NUCB2 mRNA expression.

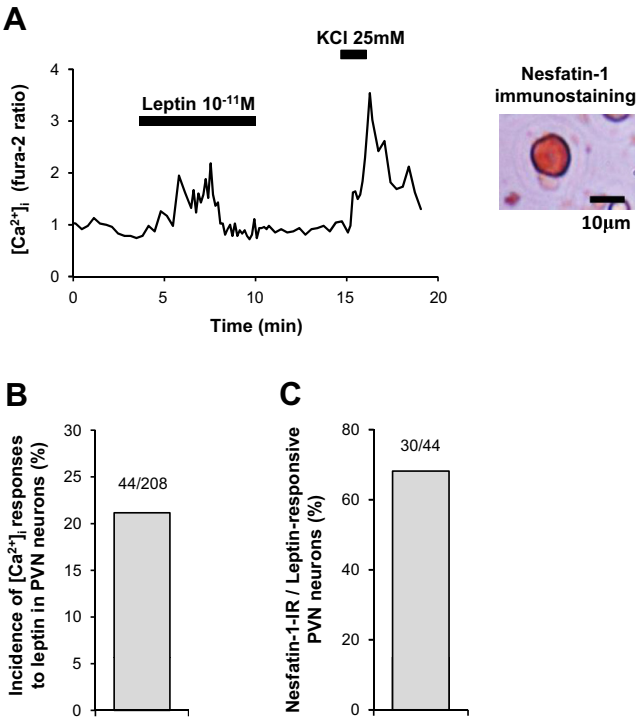

Fig. 1. Leptin increases $[Ca^{2+}]_i$ in PVN NUCB2/nesfatin-1 neurons. (A) Leptin at 10^{-11} M increased $[Ca^{2+}]_i$ (left panel) in a PVN neuron that was subsequently shown to be immunoreactive (IR) to nesfatin-1 (right panel). (B) Incidence of $[Ca^{2+}]_i$ responses to leptin in PVN neurons, expressed by percentage. (C) Thirty of 44 leptin-responsive neurons (68.2%) were IR to NUCB2/nesfatin-1.

3.3. Efficiency of AAV-mediated knockdown of PVN NUCB2

To investigate whether PVN NUCB2 mediates anorexigenic effect of leptin, we prepared PVN specific NUCB2 knockdown mice using AAV vector. To confirm the efficacy of AAV-NUCB2-shRNAs, brain sections containing the PVN were processed for NUCB2/nesfatin-1 immunohistochemistry. AAV-NUCB2-shRNA injection decreased NUCB2/nesfatin-1 staining in the PVN (Fig. 3A). To quantify the knockdown of NUCB2, the PVN samples were collected and analyzed with western blotting. At 4 weeks after injection, AAV-NUCB2-shRNA injection significantly reduced the NUCB2 protein level in the PVN by 56% compared to the control injected with AAV-Scr-shRNA (Fig. 3B).

3.4. Anorexigenic effect of leptin is impaired in PVN NUCB2 knockdown mice

To investigate whether PVN NUCB2 mediates anorexigenic effect of leptin, leptin was injected ip and food intake was measured. In control mice injected with AAV-Scr-shRNA in the PVN, ip injection of leptin, compared to vehicle, significantly reduced food intake at 3 h after injection (Fig. 2A). In contrast, ip leptin injection failed to inhibit food intake in mice injected with AAV-NUCB2-shRNA (Fig. 3C). Likewise, icv injection of 50 pmol leptin markedly reduced food intake approximately by 60% at 3 h after injection. In contrast, in mice treated with AAV-NUCB2-shRNA, although icv leptin tended to suppress food intake, the change was not statistically significant (Fig. 3D). These data suggest that the neural pathway involving the PVN NUCB2/nesfatin-1 substantially mediates the anorexigenic effect of both peripherally and centrally administered leptin.

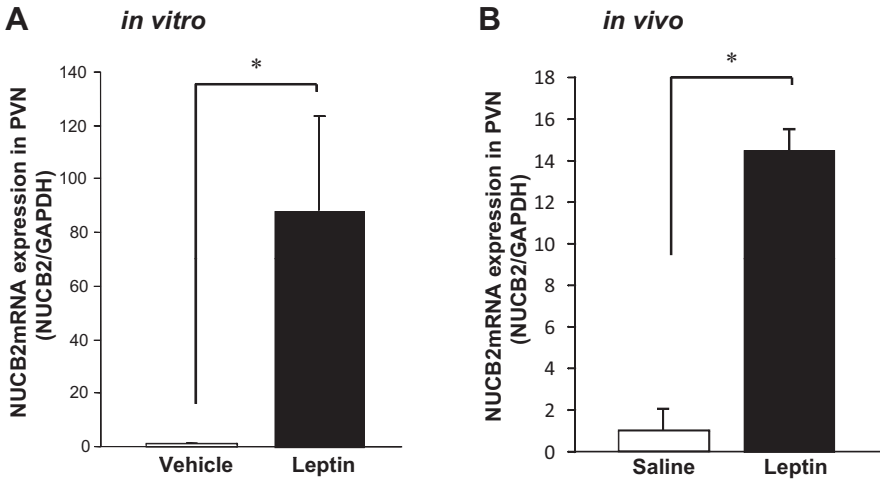

Fig. 2. Treatment with leptin *in vitro* and *in vivo* induces NUCB2 mRNA expression in the PVN. (A) NUCB2 mRNA expression in hypothalamic PVN explants cultured with or without 10^{-8} M leptin for 18 h. $n = 7$. (B) NUCB2 mRNA expression in the hypothalamic PVN of mice after icv injection of saline (control, open bars) or 50 pmol leptin (filled bars). Samples were collected 2 h after icv injection. $n = 4$. Bars represent the mean ± s.e.m. $^{*}p < 0.05$.

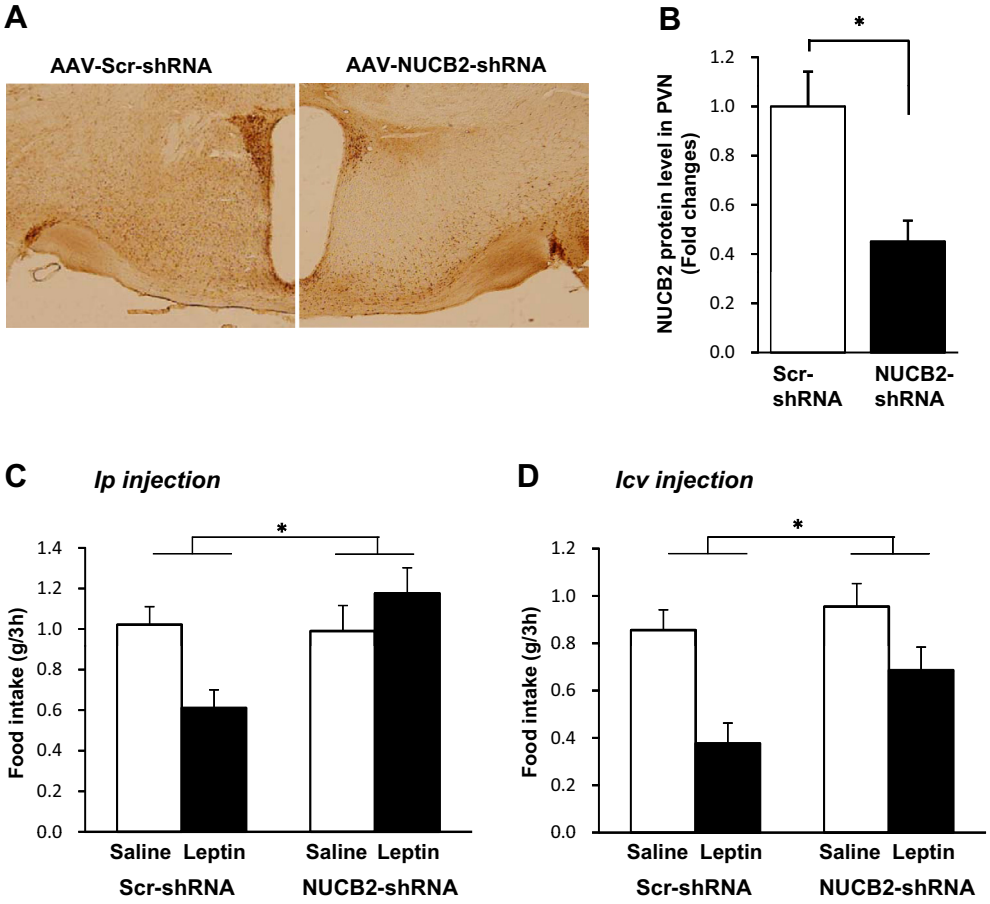

Fig. 3. Hypothalamic PVN-specific knockdown of NUCB2 by AAV-NUCB2-shRNA on feeding and energy homeostasis. (A) Immunohistochemistry for NUCB2/nesfatin-1 in PVN. AAV-Scr-shRNA was injected in left side and AAV-NUCB2-shRNA (NUCB2-shRNA) in right side. (B) Western blot analysis of NUCB2 protein in the PVN of control AAV-Scr-shRNA and NUCB2-shRNA mice at 4 weeks after injection. Protein levels of NUCB2, relative to β-actin, in the PVN are expressed. $n = 4–5$ for each group. (C) Cumulative food intake for 3 h after ip injection of leptin (600 pmol/g BW) or saline in AAV-Scr-shRNA (control, open bar) and AAV-NUCB2-shRNA (filled bar) injected mice ($n = 6$ each group). (D) Cumulative food intake for 3 h after icv injection of leptin (50 pmol) or saline in control and AAV-NUCB2-shRNA injected mice ($n = 5–6$). All data are presented as means ± s.e.m. $^{*}p < 0.05$ by two-ways ANOVAs with Bonferroni's multiple comparison test.

4. Discussion

In this study, we found that leptin directly interacts with the PVN NUCB2/nesfatin-1 neuron to up-regulate its neuronal activity and NUCB2 mRNA expression. Moreover, anorexigenic effect of leptin, administered peripherally and centrally, is substantially mediated by PVN NUCB2/nesfatin-1. Previous studies with global vs. region-specific leptin receptor deficiency have suggested yet-unidentified target neuron(s) for anorexigenic leptin action, in addition to ARC POMC and VMH neurons [7,8]. The present study

has identified the PVN NUCB2/nesfatin-1 (neuron) as a novel target for the anorexigenic effect of leptin.

In the present study, a substantial fraction (21.2%) of isolated PVN neurons responded to leptin with $[Ca^{2+}]_i$ increases, and majority (68.2%) of the PVN neurons that responded to leptin were NUCB2/nesfatin-1 neurons. The result is consistent with previous histological report that the OB-Rb is expressed abundantly in several hypothalamic regions including the PVN [23,24]. The $[Ca^{2+}]_i$ responses were evoked by leptin at 10^{-11} M, the estimated physiological concentration of this hormone in the CSF of rats [25,26], and the $[Ca^{2+}]_i$ increase reflects the activation of neurons [25]. Taken together, the present results demonstrate that leptin directly interacts with and activates NUCB2/nesfatin-1 neurons in the PVN, which may function under physiological conditions.

Icv injection of leptin significantly increased NUCB2 mRNA expression in the PVN at 2 h after injection (Fig. 2B). In isolated PVN explants in culture, leptin treatment also remarkably stimulated NUCB2 mRNA expression, indicating that leptin up-regulates NUCB2 mRNA expression via its direct effect on the PVN. We previously reported that the NUCB2 mRNA expression in the PVN was raised specifically during light phase in normal rats, and that the light phase-associated rise in NUCB2 mRNA was blunted in Zucker-fatty rats with mutated leptin receptor [17]. These current and previous results taken together support that leptin up-regulates PVN NUCB2 mRNA expression. Alternatively, it is well known that leptin activates ARC POMC neurons, which project to PVN neurons [26]. We have recently reported that α-MSH, the POMC-derived neuropeptide, increases $[Ca^{2+}]_i$ in NUCB2/nestatin-1 neurons in PVN [27]. The present and previous results taken together demonstrate that leptin potently activates PVN NUCB2/nesfatin-1 via both its direct action on the PVN and indirect pathway through the ARC POMC to PVN.

We showed that the anorexigenic effect of ip leptin injection was blunted and that of icv leptin injection was markedly attenuated in PVN-specific NUCB2 knockdown mice. The results suggest that NUCB2/nesfatin-1 in the PVN substantially mediates anorexigenic effect of leptin. Alternatively or additionally, it is possible that the reduced leptin action results from the increased BW and consequent leptin resistance in some feeding-regulating neurons other than PVN NUCB2/nesfatin-1 neurons in the knockdown mice. In our study, however, the obesity-associated leptin resistance appears not to substantially contribute to the leptin ineffectiveness detected in the early period of 3 weeks after viral injection, since the BW was not elevated in this period. Hence, our data indicate that PVN NUCB2/nesfatin-1 plays a pivotal role in mediating anorexigenic effect of leptin administered both centrally and peripherally.

The present study is significant for several reasons. First, this is the first to demonstrate that leptin directly activates NUCB2/nesfatin-1 neurons in the PVN. Second, this study successfully used RNAi technology and revealed that the PVN-specific NUCB2 knockdown results in the inability of leptin to inhibit food intake. The interaction of leptin with PVN NUCB2/nesfatin-1 controls feeding behavior and possibly BW. These findings provide important implications regarding the neural signaling pathway by which leptin regulates energy homeostasis.

Author contributions

D.G., M.N. and T.Y designed the experiments. D.G., M.N., L.W. E.L., A.S. and M.M. performed the *in vivo* and slice experiments. T.O. and M.N contributed to preparation of AAV vector. D.G. contributed to $[Ca^{2+}]_i$ measurements. M.M. participated in discussions. D.G., M.N. and T.Y. prepared the manuscript.

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