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Paraventricular NUCB2/nesfatin-1 rises in synchrony with feeding suppression during early light phase in rats

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ABSTRACT

Obesity often results from hyperphagia and involves rhythm disorder. Circadian feeding pattern is suggested to be implicated in energy homeostasis while its disorder in obesity. However, the mechanism underlying circadian feeding is little known. PVN is considered a regulatory center for feeding and circadian activities of hormone release and autonomic nerve. Nucleobindin2 (NUCB2) and its processing product nesfatin-1 (NUCB2/nesfatin-1) are localized in the hypothalamic paraventricular nucleus (PVN) and implicated in regulation of feeding. This study aimed to clarify whether the PVN NUCB2/nesfatin-1 expression exhibits diurnal rhythm and, if so, whether it is related to circadian feeding. Here we show that NUCB2 mRNA expression in the PVN rises during early light phase (LP) in parallel with suppression of food intake. Immunoneutralization of PVN NUCB2/nesfatin-1 with anti-nesfatin-1 IgG during LP, but not dark phase, increased food intake. PVN-selective shRNA-induced knockdown of NUCB2 mRNA expression elevated food intake. Furthermore, the rise of PVN NUCB2 mRNA during LP was blunted in Zucker-fatty obese rats which exhibited LP-preferential hyperphagia. The increases in food intake during LP and 24 h were significantly corrected by intracerebroventricular injection of nesfatin-1 during LP. These results reveal the diurnal rhythm of PVN NUCB2 mRNA expression characterized by early LP rise, which may serve as a factor to limit LP food intake, contributing to circadian feeding. Furthermore, impaired NUCB2/nesfatin-1 rhythm may be related to dysregulated feeding pattern and hyperphagia in Zucker-fatty rats.

1. Introduction

Nesfatin-1, a nucleobindin2 (NUCB2)-derived 82 amino acid peptide, is a satiety molecule in the hypothalamus [1]. Intracerebroventricular (icv) injection of nesfatin-1 suppresses feeding [1,2], while that of anti-nesfatin-1 antibody stimulates feeding [1]. Nesfatin-1 is expressed in the feeding related regions in the hypothalamus and brain stem [1]. Fasting down-regulates NUCB2 mRNA expression and NUCB2/nesfatin-1 protein content selectively in the PVN [1], while refeeding activates nesfatin-1 neurons in PVN [3], suggesting a regulatory role of PVN nesfatin-1 in feeding. PVN is not only an integrative center for feeding regulation [4,5] but also a center for circadian activities including hormone release and autonomic functions [6,7]. PVN is anatomically and functionally connected with the suprachiasmatic nucleus (SCN) [8,9], the master circadian clock [8,10]. Rhythm disorder has been implicated in obesity [11,12], and impaired diurnal feeding rhythm is suggested to induce obesity [13–15]. In the present study, we explored whether NUCB2 mRNA expression in PVN exhibits a diurnal rhythm and, if so, whether the diurnal NUCB2 rhythm is related to the maintenance of diurnal feeding rhythm in rats, and whether it is impaired and related to hyperphagia in an obese model of Zucker-fatty rats.

2. Materials and methods

2.1. Animals

Male Wistar (200–250 g), Zucker-fatty (270–350 g) and -lean (200–230 g) rats (SLC, Hamamatsu, Japan) were housed individually on 12 h light/dark cycle (7:30 lights on) and given standard food CE-2 (Clea, Osaka, Japan) and water *ad libitum*. Light phase (LP) and dark phase (DP) food consumptions were determined by manual measurement. Cumulative food intake for every 1 h in 8 week-old Wistar rats, for every 12 h in 9 week-old Zucker-fatty and -lean rats were measured. All experimental protocols were

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approved by the Jichi Medical University Institute of Animal Care and Use Committee.

2.2. Plasmid construction and preparation of transfection complex

Target sequences for shRNA of rat NUCB2 were chosen from Accession No. NM 021663: 5′-GGACTTAGTAAGTCACAAA-3′ (489–507). Briefly, single-stranded DNA oligonucleotides were chemically synthesized and annealed to form double-stranded DNA that consisted of shRNA sense sequence, the loop sequence TAGTGCTCCTGGTTG, shRNA antisense sequence, and a string of six thymidine residues Scramble sequence was chosen: 5′-CAACACTAGTTGACATGTA-3′ for the mock. The double-stranded DNA oligonucleotide was cloned in a pBAsi-mU6 vector (Takara Bio, Tokyo, Japan).

2.3. Intra-PVN NUCB2 shRNA transfection and icv cannulation

In Wistar rats, *in vivo*-jetPEI™/plasmid (Polyplus-transfection Inc., New York, NY) for NUCB2 or scramble (0.25 μg/0.5 μl) complexes in 5% glucose solution were injected stereotaxically into bilateral PVN 1.8 mm caudal to the bregma, 0.5 mm lateral, and 7.5 mm below the surface of the skull). The needle remained in PVN for 1 min after injection to prevent reflow of the solutions.

Guide cannula of 26-gage were inserted stereotaxically into 2.5 mm caudal to the bregma in the midline and 8.0 mm below the surface of the skull) or LV 0.5 mm caudal to the bregma, 1.5 mm lateral and 3.0 mm below the surface of the skull) and secured to the skull with screws and dental cement. Rats were allowed to recover from the operation for 10 days while they were habituated to handling.

2.4. Collection of PVN samples

Bilateral PVN were acutely collected from hypothalamic slice 0.8 mm - 2.12 mm caudal to bregma) by 6 h intervals in free-fed conditions for NUCB2 determination. In NUCB2 shRNA transfection experiment, bilateral PVN samples were collected at 9:00–11:00 at days 4 and 7 post-transfection.

2.5. Real-time RT-PCR

Total RNA was isolated from PVN samples using TRIzol (Invitrogen, Carlsbad, CA) and treated with RQ1-DNase (Promega, Madison, WI) to remove residual DNA contaminations. After conversion to cDNA, Real-time RT-PCR was performed with SYBR premix Ex taq II polymerase (Takara Bio, Tokyo, Japan) (95 °C for 5 s and 60 °C for 30 s × 40 cycles) in Thermal Cycler Dice Real Time System TP800 2.10B (Takara Bio). mRNA Expression levels were calculated by the ΔΔCT method of relative quantification, and normalized to housekeeping gene products glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Primers were as follows

NUCB2: 5′-GTCACAAAGTGAGGACGAGACTGG-3′ and 5′-TGGTTCAGGTGTTCAAACTGCTTC-3′, GAPDH: 5′-GGCACAGTCAAGGCTGAGAATG-3′ and 5′-ATGGTGGTGAAGACGCCAGTA-3′.

2.6. Western blot analysis of NUCB2 in PVN samples

PVN samples were lysed in lyses buffer (150 mM NaCl, 0.5% NP40, 1 mM EDTA, 10 mM Tris–HCl pH 7.5, 1 mM PMSF). Ten μg proteins were subjected to 10% SDS–PAGE and transferred to nitrocellulose filters. Nesfatin-1 proteins were detected with anti-nesfatin-1 IgG [1] and ECL system. Immunoreactive signal was quantified by using FAS-1000 and expression levels of proteins were normalized to β-actin.

2.7. Measurement of food intake

NUCB2 or scrambled shRNA were injected stereotaxically into bilateral PVN of Wistar rats and cumulative food intake at days 2–6 were measured. Substances were injected under the following conditions: nesfatin-1 (Yanaihara Institute Co., Shizuoka, Japan) 100 pmol/5 μl and NaCl 0.9% 5 μl (vehicle) into 3 V of Wistar, Zucker-lean and -fatty rats at 7:30 or 19:30; anti-nesfatin-1 IgG 8 μg/5 μl and anti-rabbit polyclonal IgG 8 μg/5 μl into LV of Wistar rats at 10:30 or 19:30. Cumulative food intakes at 12 and/or 24 h after injections were measured.

2.8. Statistical analysis

One-way ANOVA followed by Bonferroni's Multiple Range tests were used to compare multiple test groups and unpaired Student's *t* tests for two groups. $p < 0.05$ was considered significant.

3. Results

In Wistar rats, NUCB2 mRNA expression in PVN rose during early LP (9:00), declined in afternoon (15:00) and remained low during dark phase (DP) (21:00, 3:00), showing diurnal rhythm (Fig. 1A). The rise of NUCB2 mRNA expression at 9:00 was associated with rapid decline of food intake (Fig. 1A, and B). The reduced levels of NUCB2 mRNA expression at 15:00 and at 21:00–3:00 were associated with the onset and sustained elevation of food intake, respectively (Fig. 1A and B).

To assess the LP elevation of NUCB2 protein and its role, the anti-nesfatin-1 IgG, which was shown to immuneneutralize

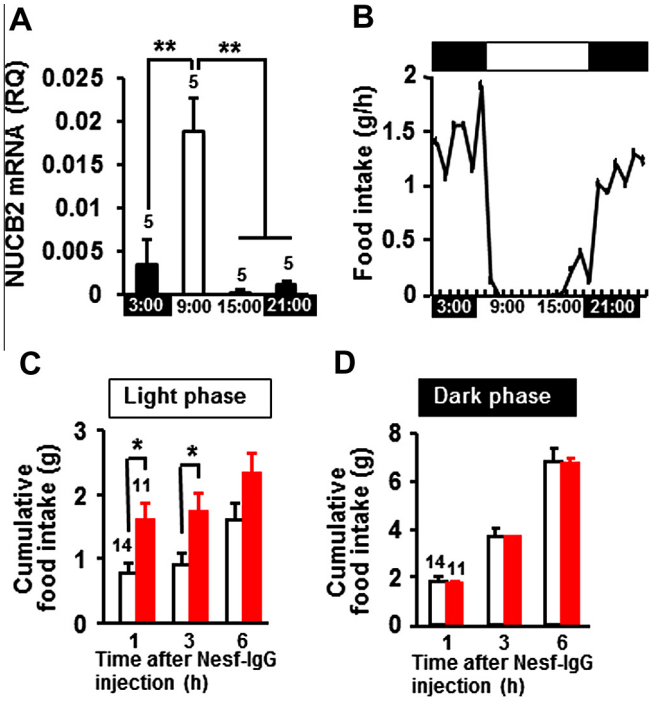

Fig. 1. Rise of PVN NUCB2 mRNA expression synchronizes with suppression of food intake during early light phase (LP) in Wistar rats. (A) NUCB2 mRNA expression in PVN was elevated during early LP (9:00) and declined during afternoon (15:00) and dark phase (DP) (21:00 and 3:00), exhibiting diurnal change. (B) Average food intake every 1 h during a day ($n = 20$). Filled and open squares above the trace indicate DP (19:30–07:30) and LP (07:30–19:30), respectively. (C and D) Cumulative food intake for 1, 3, and 6 h after anti-nesfatin-1 IgG (Nesf-IgG) injected icv at LP (10:30) (C) or DP (19:30) (D). Data represent means ± SEM. Numbers above the bars indicate the number of samples (A) or rats (C and D) examined. $^{*}p < 0.05$ and $^{**}p < 0.01$.

endogenous NUCB2 and nesfatin-1 [1], was icv injected. Injection of the anti-nesfatin-1 IgG during LP (10:30), when PVN NUCB2 mRNA level was elevated, markedly increased food consumption for the following 1 and 3 h (Fig. 1C), whereas during DP (19:30) when PVN NUCB2 mRNA level was suppressed, IgG injection had no effect on food intake (Fig. 1D).

To assess the physiological role of PVN NUCB2/nesfatin-1 in regulation of feeding in a longer term, NUCB2 shRNA was injected into bilateral PVN. This treatment significantly decreased PVN NUCB2 mRNA and protein levels at day 4 (Fig. 2A and C) and increased cumulative food intake at day 4 and 5 after transfection (Fig. 2D). The PVN NUCB2 level and food intake returned to the control levels at day 7 and 6 after transfection, respectively (Fig. 2B and D). Thus, PVN-selective NUCB2 knockdown increased food intake in a reversible manner in Wistar rats. The results strongly suggested that endogenous PVN NUCB2/nesfatin-1 regulates feeding.

We next examined diurnal rhythms of NUCB2 and feeding in an obese model of Zucker-fatty rats in which the leptin receptor is mutated [16]. In control Zucker-lean rats, similarly to Wistar rats, PVN NUCB2 mRNA expression and food intake exhibited normal diurnal rhythms (Fig. 3A and B). In Zucker-fatty rats, in contrast, LP rise of PVN NUCB2 mRNA expression was blunted (Fig. 3A), and food intake was elevated in which the hyperphagia was more pronounced during LP than DP (Fig. 3B), in consistent with previous report [17]. Accordingly, the ratio of food intake in Zucker-fatty over Zucker-lean rats (fatty/lean feeding ratio) was significantly greater during LP than DP (Fig. 3C). Thus, lack of PVN NUCB2 mRNA rise during LP paralleled with LP-preferential hyperphagia in obese Zucker-fatty rats.

To compensate for the impaired elevation of NUCB2/nesfatin-1, nesfatin-1 was icv injected at 7:30, the onset of LP. The nesfatin-1 treatment significantly ameliorated the hyperphagia for LP and for 24 h but not for DP in Zucker-fatty rats (Fig. 3D), while it had no effect on food intake in Zucker-lean rats. These changes resulted in significant correction of the elevated fatty/lean feeding ratio for LP and 24 h (Fig. 3E).

4. Discussion

This study for the first time revealed diurnal rhythm of NUCB2 mRNA expression in PVN in normal Wistar and Zucker-lean rats. Moreover, we found that the rise of PVN NUCB2 mRNA expressions during early LP parallels with suppression of food intake during LP. Furthermore, in an obese model of Zucker-fatty rats, the rise of PVN NUCB2 mRNA during LP is blunted in parallel with the LP-preferential hyperphagia, and LP nesfatin-1 administration partially corrects the feeding disorder. These results suggest that the LP rise of PVN NUCB2 mRNA expression serves as one of the factors to suppress food intake during LP, contributing to the diurnal feeding rhythm.

Although the mechanisms underlying the formation of PVN NUCB2/nesfatin-1 rhythm remain unknown, the SCN circadian center and/or meal/food-entrainable circadian pacemaker could serve as upstream regulators. The neurotransmitters of SCN neurons projecting to PVN including AVP [9,18] might be implicated. Alternatively, the factors evoked by meals might impact PVN NUCB2/nesfatin-1 rhythm. In fact, high glucose and insulin, meal-responsive factors, reportedly activate PVN nesfatin-1 neurons [19]. Hence, regular inputs of meal-responsive factors could underlie the maintenance of PVN NUCB2/nesfatin-1 rhythm.

The mechanism linking the PVN NUCB2/nesfatin-1 rise to feeding inhibition during LP remains unknown. However, it has been reported that anorexigenic effect of nesfatin-1 is substantially mediated by PVN oxytocin [2] and that the oxytocin release in PVN shows diurnal changes [11]. Hence, PVN oxytocin might be involved in the pathway linking the diurnal nesfatin-1 change to the feeding rhythm.

The results of this study suggest that the PVN NUCB2/nesfatin-1 elevation during early LP serves as one of the factors to restrict the LP feeding and thereby to maintain circadian feeding pattern. In an obese model of Zucker-fatty rats, lack of PVN NUCB2/nesfatin-1 elevation during LP underlies the insufficient suppression of feeding during LP, impaired diurnal feeding rhythm, and

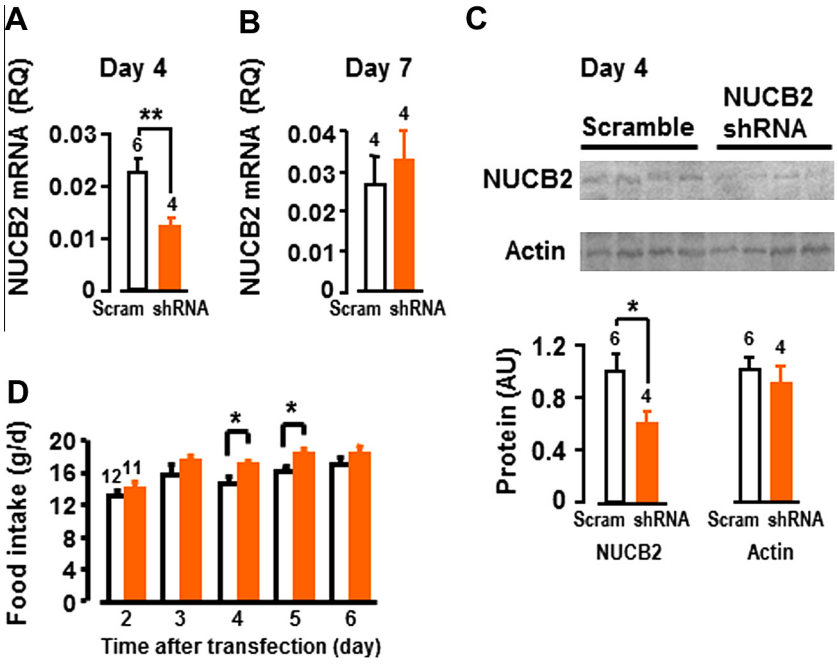

Fig. 2. NUCB2 shRNA transfection in PVN suppresses NUCB2 mRNA expressions and protein levels and increases food intake. Effects of transfection with NUCB2 shRNA or scramble RNA on NUCB2 mRNA expressions at day 4 (A) and 7 (B) post transfection, on protein levels of NUCB2 and β-actin in PVN at day 4 (C), and on cumulative food intake for 24 h from day 2 through day 6 (D). Bilateral PVN samples were collected at 9:00–11:00 at day 4 and 7 after transfection in (A–C). Data represent means ± SEM. Numbers above the bars indicate the number of samples (A–C) or rats (D). $^{*}p < 0.05$ and $^{**}p < 0.01$.

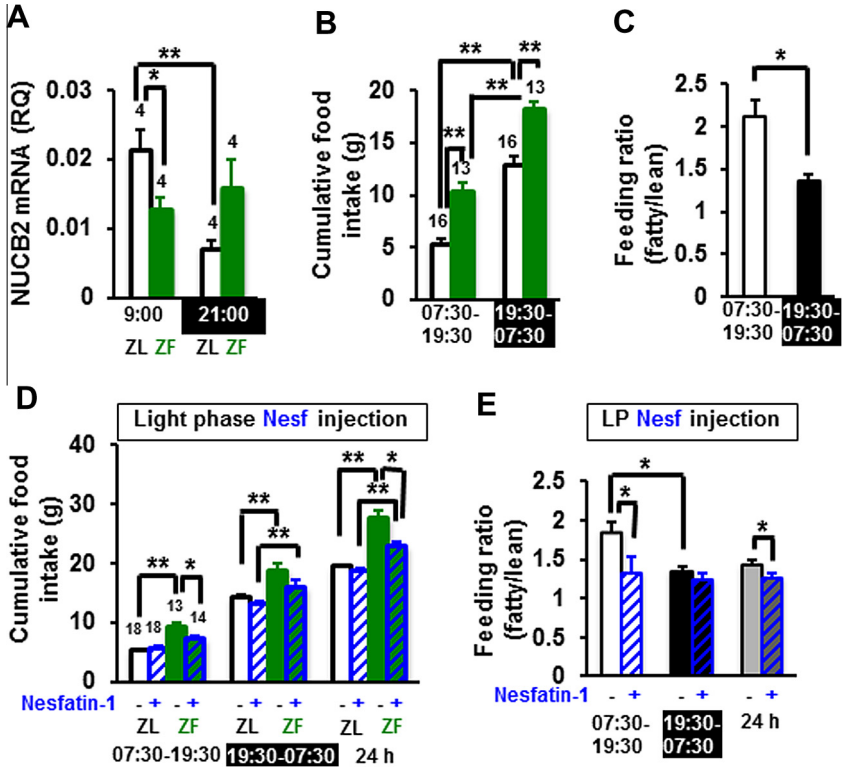

Fig. 3. Zucker-fatty rats show impaired rise of NUCB2 expression during LP and LP-preferential hyperphagia that was ameliorated by icv nesfatin-1 injection during LP. (A) Rise of PVN NUCB2 mRNA expression during LP observed in Zucker-lean (ZL) rats was impaired in Zucker-fatty (ZF) rats. (B) Larger amount of food intake in ZF than ZL rats during LP and DP. (C) The ratio of food intake in ZF rats to that in ZL rats, expressed by fatty/lean feeding ratio, was significantly greater during LP than DP. $n = 13$. (D) Icv injection of nesfatin-1 at LP (7:30) ameliorated elevated food intake during LP and 24 h significantly. Cumulative food intake during LP, DP and 24 h after icv injection of saline (solid bars) or nesfatin-1 (hatched bars) was measured in ZL (white bars) and ZF rats (green bars). (E) Elevated fatty/lean feeding ratios (solid bars) for LP and 24 h were ameliorated by icv nesfatin-1 injection (hatched bars). $n = 13–14$. Data represent means ± SEM. Numbers above the bars indicate the number of samples (A) and rats (B, D, E) examined. $^{*}p < 0.05$ and $^{**}p < 0.01$.

24 h-hyperphagia. Furthermore, these eating disorders are partially corrected by LP nesfatin-1 supplement. The results suggest that the diurnal PVN NUCB2/nesfatin-1 rhythm could be the potential causal site and novel therapeutic target for hyperphagic-obesity. Further study is definitely required to clarify the mechanism linking impaired LP rise of PVN NUCB2/nesfatin-1 and LP-preferential hyperphagia in obese Zucker-fatty rats.

Author contributions

U.S., Y.M., M.N. and T.Y. designed the study; U.S., Y.M. and M.N. performed experiments; U.S. collected and analyzed data; M.M. provided reagents; U.S. and T.Y. wrote the manuscript.

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