Hypothalamic Nesfatin-1/NUCB2 Knockdown Augments Hepatic Gluconeogenesis that is Correlated with Inhibition of mTOR-STAT3 Signaling Pathway in Rats

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Abstract

Nesfatin-1, an 82 amino acid neuropeptide, has recently been characterized as a potent metabolic regulator. However, the metabolic mechanisms and signaling steps directly associated with the action of nesfatin-1 have not been well delineated. We established a loss-of-function model of hypothalamic nesfatin-1/NUCB2 signaling in rats using an adenoviral-mediated RNAi. Using this model, we found that inhibition of central nesfatin-1/NUCB2 activity markedly increased food intake and hepatic glucose flux, and decreased glucose uptake in peripheral tissue in both normal chow diet (NCD)- and high fat diet (HFD)-fed rats. The change of hepatic glucose fluxes in the hypothalamic nesfatin-1/NUCB2 knockdown rats was accompanied by increased hepatic levels of G-6-Pase and PEPCK and decreased insulin receptor (InsR), insulin receptor substrate 1 (IRS-1), and AKT kinase (AKT) phosphorylation. Furthermore, knockdown of hypothalamic nesfatin-1 led to decreased phosphorylation of mammalian target of rapamycin (mTOR) and signal transducer and activator of transcription 3 (STAT3), and the subsequent suppressor of cytokine signaling 3 (SOCS3) levels. These results demonstrated that hypothalamic nesfatin-1/NUCB2 plays an important role in glucose homeostasis and hepatic insulin sensitivity, which at least in part, is associated with the activation of the mTOR-STAT3 signaling pathway.

Key words: hypothalamus / Hepatic Gluconeogenesis / nesfatin-1/NUCB2 knockdown /mTOR-STAT3 Signaling Pathway
INTRODUCTION

The hypothalamus, where numerous neuropeptides and transmitters are released to participate in the control of essential body functions, has been considered to be a key integrator involved in the central control of food intake and energy balance (1, 2). Nesfatin-1, an 82 amino acid polypeptide, was originally discovered in hypothalamus by Oh et al (3) in the search for new appetite-controlling signals. Nesfatin-1 is the cleavage product of the calcium and DNA-binding protein, nucleobindin2 (NUCB2), a 396 amino acid peptide which is highly conserved in rat, mouse and humans. NUCB2 mRNA was detected in both brain nuclei, including the arcuate nucleus (ARC), paraventricular (PVN), and supraoptic nucleus (SON), the nucleus of the solitary tract (3), and in peripheral tissues, such as rat stomach, pancreas, pituitary gland, and testis (4).

Nesfatin-1 was identified as an anorectic molecule. Thus, central injection of nesfatin-1 in rats decreased food intake and body weight in a dose-dependent manner (3) and several other studies have shown that nesfatin-1, when injected into the lateral (5, 6) or the 4th brain ventricle (5), the PVN (7), or into the abdominal cavity (8), induced a sustained suppression of dark-phase feeding in rodents. Importantly, increasing evidence suggest that nesfatin-1 may also play an import role in the regulation of glucose metabolism as elevated plasma nesfatin-1 concentrations have been found in subjects with impaired glucose tolerance (IGT), with type 2 diabetes mellitus (T2DM) and to be associated with insulin resistance (9).

Signal transducer and activator of transcription 3 (STAT3), a member of the STAT family of cytoplasmic transcription factors, was found to be activated by
glycoprotein (gp) 130-coupled cytokines, such as IL-6 (10, 11). Recent studies have reported that liver-specific STAT3 knockout mice had increased expression of gluconeogenic genes (12). Further, STAT3 has been found to directly target the regulatory regions of glucose-6-phosphatase (G-6-Pase) and phosphoenolpyruvate carboxykinase (PEPCK), and to regulate their expression (11) and has been shown to be involved in the pathogenesis of IL-6-induced insulin resistance in the liver (13).

We recently reported that hypothalamic nesfatin-1 was involved in the regulation of hepatic glucose production (HGP) via the hepatic AKT kinase/AMP-dependent protein kinase/ mammalian target of rapamycin (AKT/AMPK/mTOR) pathway (14), suggesting a connection between hypothalamic nesfatin-1 and the STAT signaling pathway. To our knowledge, such a connection has not been reported and it has been suggested that it should be confirmed by a loss-of-function study (15). In the current study, we have therefore determined whether attenuation of the central nesfatin-1/NUCB2 signal will lead to a change in peripheral glucose metabolism. To this end, we have created a loss-of-function animal model by knocking down the hypothalamic nesfatin-1/NUCB2. This has allowed us to determine molecular mechanisms, by which the central nesfatin-1/NUCB2 knockdown can lead to the alteration in insulin signaling.
RESEARCH DESIGN AND METHODS

*Generation of nesfatin-1/NUCB2 knockdown vector*

To construct vectors expressing shRNA against nesfatin-1/NUCB2, we designed several oligonucleotides and complementary strands to target specifically rat nesfatin-1/NUCB2. A recombinant vector Ad-\textit{shNUCB2} was generated by transfection in 293 cells with the AdEasy system. The Ad-\textit{shNUCB2} (sequence 5'-GATCCCGGTGAAAGTGCAAGGATATTCAAGAGATATCCTTGCACTTTCCACCTTTTTGGAAA-3') was the most effective one and was therefore selected for subsequent experiments. A control recombinant vector Ad-\textit{shGFP} encoding enhanced green fluorescence protein (GFP; Clonetech Mountain View, CA, USA) was used as a control for viral infection. Large-scale amplification and purification of recombinant adenoviruses were performed using the ViraBind Adenovirus Purification Kit according to the manufacturer’s instructions (Cell Biolabs Inc., San Diego, CA, USA).

*Animals*

One hundred and two 14-week-old male Sprague-Dawley rats (the Experimental Animal Center of Chongqing Medical University, Chongqing, China) were studied. Rats were housed in a controlled environment, subjected to a standard light (6:00am-18:00pm) and randomly divided into two groups fed with either a normal-chow diet (NCD) or a high-fat diet (HFD) for 10 weeks. The NCD (3.49kcal/g) provided 60% calories from carbohydrates, 21% from protein, and 19% from fat. The HFD (4.72kcal/g) contained 33% of calories from carbohydrates, 13% from protein, and 54% from fat (lard) (Medicience Ltd, Jiangsu, China). All experimental
procedures were approved by the Animal Experimentation Ethics Committee (Chongqing Medical University) and were in accordance with the National Health and Medical Research Council of China Guidelines on Animal Experimentation.

**Cannulation and injection**

14 days before the *in vivo* studies, rats were equipped with chronic catheters in the third cerebral ventricle. After being anesthetized with intraperitoneal ketamine (87mg/kg), rats were fixed in a stereotaxic apparatus with ear bars and a nosepiece set at + 5.00 mm. A stainless steel guide cannula was implanted into the third ventricle. After full recovery (7 days), rats were equipped with indwelling catheters placed in the right internal jugular vein and the left carotid artery (Fig. 3a), and randomly divided into six groups: 1) NCD- artificial cerebrospinal fluid (aCSF) (NCA, n=10); 2) NCD- Ad-shGFP (NCG, n=10); 3) NCD- Ad-shNUCB2 (NCN, n=10); 4) HFD- aCSF (HFA, n=10); 5) HFD- Ad-shGFP (HFG, n=10); 6) HFD- Ad-shNUCB2 (HFN, n=10).

Three days before the clamp studies, rats received an injection of aCSF (10µl/rat), Ad-shGFP (10⁹PFU/rat) or Ad-shNUCB2 (10⁹PFU/rat). Only rats displaying a complete recovery from the surgery were studied.

**Seven day-phase food intake in conscious rats**

In different experiments, NCD and HFD-fed rats infused with aCSF (10 µl), Ad-shGFP (10⁹PFU/rat) or Ad-shNUCB2 (10⁹PFU/rat) into the third ventricle were individually housed. The infusion was initiated at 17:00 clock and continued for one hour at a rate of 10µl/h. Food intake and body weight were monitored every 24 hours for 7 days. All injections were performed once in ad libitum fed rats maintained in
their familiar housing cages. At the end of the seventh day, rats were anesthetized, and liver samples were freeze-clamped in situ and stored at -80°C for glycogen analysis.

**Hyperinsulinemic-euglycemic clamp studies**

Twelve hours before the clamp studies, food was removed from every cage. The clamps were performed as previously described (14). Briefly, a primed-continuous infusion of high-performance liquid chromatography-purified [3-H\textsuperscript{3}] glucose (Amersham, Los Angeles, CA, USA; 6 µCi bolus, 0.2 µCi/min) was initiated at 0 min and maintained throughout the study to assess glucose kinetics. A hyperinsulinemic-euglycemic clamp was performed during the final 2 hours (120-240 min) of the study. Insulin (6 mU·kg\textsuperscript{-1}·min\textsuperscript{-1}) was continuously infused, and a variable infusion of 25% glucose was started and adjusted every 5 min to maintain the plasma glucose concentration at approximately 6 mmol. Blood samples (100 µl) were obtained from the jugular vein catheter at 0, 120, 200, 220, 230, and 240 min for determination of insulin, NEFA, and glucose-specific activity. In another study cohort (n=4 for each group), 2-deoxy-D-[H\textsuperscript{3}]glucose (2-DG; Amersham; 30 µCi bolus) was administered forty-five minutes before the end of the clamp studies to determine insulin-mediated glucose uptake in individual tissues. Extra blood samples (50 µl) were taken at 2, 5, 10, 15, 20, 30, and 45 min after the injection to determine tracer disappearance. At the end of the clamp, the rats were anesthetized and tissue samples were freeze-clamped in situ with aluminum tongs precooled in liquid nitrogen and stored at -80°C for subsequent analysis.
**Analytical procedures**

Plasma glucose was measured with the glucose oxidase method. Plasma insulin was measured using a commercial insulin enzyme-linked immunosorbent assay kit (Diagnostic Products, Los Angeles, CA, USA). NEFA was determined spectrophotometrically using an acyl-CoA oxidase-based colorimetric kit (Wako Pure Chemical Industries, Osaka, Japan). Triacylglycerol (TG), total cholesterol (TC), HDL cholesterol (HDL-C), and LDL cholesterol (LDL-C) concentrations were measured using enzymatic colorimetric kits. Hepatic glycogen levels were measured using glycogen assay kits (BioVision, Mountain View, CA, USA) (14). Plasma [3-H\(^3\)] glucose specific activity was measured in duplicates in the supernatants of Ba(OH)\(_2\) and ZnSO\(_4\) precipitates (Somogy procedure) of plasma samples after evaporation to dryness to eliminated tritiated water. Under steady-state conditions, the rate of glucose disappearance (Rd) equals the rate of glucose appearance (Ra). The latter was calculated as the ratio of the rate of [3-H\(^3\)] glucose infusion (dpm/min) and the steady-state plasma [H\(^3\)] glucose-specific activity (dpm/mg). When exogenous glucose was given, the rate of hepatic glucose production (HGP) was calculated as the difference between Ra and glucose infusion rate (GIR) (16). The rate of 2-DG uptake was calculated as described by Pascal et al (17) with the equation:

\[
R_i = \frac{[2-deoxyglucose-6-phosphate]}{LC\int_0^\infty \left( \frac{C_s}{C_{bs}} \right) dt}
\]

The lumped constant (LC), which is a correction factor for the discrimination against
2-DG in glucose transport and phosphorylation pathway, is determined in vitro by comparing glucose and 2-DG fractional extraction by the different tissues. \( C^*_B \) is the blood 2-DG expressed in term of radioactivity and \( C_B \) is the blood glucose concentration.

**RNA extraction and quantitative real-time RT-PCR**

Total RNA was isolated from frozen tissue with TRIzol reagent (Invitrogen, NY, USA) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed with a SYBR Green PCR kit (Takara Bio, Otsu, Japan), and a Corbett Rotor Gene6000 real-time PCR system (Corbett Research, Sydney, Australia) according to the manufacturer’s instructions. Gene expressions were analyzed using the comparative threshold cycle method and normalized with \( \beta \)-actin. The following sequences of the primers were used: 5’-CCCTGAACCCTAAGGCAACCGTGAA AA-3’ and 5’-TCTCCGGAGTCCATCACAATTGTCTTG-3’ for \( \beta \)-actin; 5’-CACCTTGACACTACACCCTT-3’ and 5’-GTGGCTGTGAACACCTCT-3’ for G-6-Pase; and 5’-AGTCACCATCACTTCCTGGAAGA-3’ and 5’-GGTGCGAGAATCGCGAGTT-3’ for PEPCK.

**Nesfatin-1/NUCB2 immunohistochemistry**

After being injected with Ad-shNUCB2 or aCSF for 48 hours, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (1 mg/kg) and perfused first with saline containing heparin (20 U/ml) for 3 min and then with 4% paraformaldehyde in 0.1 M PBS for 20 min. The brains were excised, transferred to 4% paraformaldehyde, and then fixed at 4°C for 24 h. After being dehydrated with
ethanol-xylene, the specimens were embedded in paraffin and coronal sections of the hypothalamus were obtained. Immunohistochemistry for nesfatin-1/NUCB2 protein (rabbit anti-c-NUCB2, 1:200; Abcam, Cambridge, MA, USA) was performed as described previously (18).

**Western blot analyses**

For analysis of nesfatin-1/NUCB2 protein expression in the brain, the hypothalamus was dissected out by a horizontal cut 2 mm in depth with following the limits: 1 mm anteriorly from the optic chiasm, the posterior border of mammillary bodies, and the hypothalamic fissures (19). Hypothalamus, liver and muscle tissues were homogenized, and protein concentration was measured with a BCA quantification kit (Pierce Biotechnology, Rockford, IL, USA). Protein lysates were subjected to 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were probed at 4 °C in the presence of 1:1000 dilutions of primary antibodies against the insulin receptor (InsR), phospho-InsR, IRS-1, phospho-IRS-1, AKT, phospho-AKT, mTOR, phospho-mTOR, STAT3, phospho-STAT3, SOCS3 (all from Cell Signaling Technology, Boston, MA, USA), G-6-Pase and PEPCK (from Santa Cruz Biotechnology, Texas, USA) and β-actin (Research Diagnostics, Santa Clara, CA, USA). After being washed 3 times with TBST (Tris-buffered saline containing 0.1% Tween-20), the membranes were incubated with horseradish peroxidase-labeled sheep anti-rabbit antibody for 1h. Then, the blots were visualized with enhanced chemiluminescence (ECL). The intensity of the bands was quantified with a Bio Imaging System Densitometer (Bio-Rad, Hercules, CA, USA) and quantification of
antigen-antibody complexes was performed using Quantity One analysis software (Bio-Rad).

Statistical analyses

All results are presented as means ± SEM. A Two-way ANOVA with a least significant difference post hoc test was used to compare mean values between multiple groups and a two-sample, unpaired Student t-test was used for two-group comparisons. $p<0.05$ was considered significant.
RESULTS

Molecular approaches to nesfatin-1/NUCB2 inhibition

To inhibit hypothalamic nesfatin-1/NUCB2 signaling, we constructed a shRNA expression vector Ad-shNUCB2 specific to rat nesfatin-1/NUCB2. Three days after injection of Ad-shNUCB2 into the third ventricle, hypothalamic nesfatin-1/NUCB2 protein levels were decreased by 66% (p<0.05) compared with nesfatin-1/NUCB2 protein levels of rats who received aCSF or Ad-shGFP; whereas there was no reductions of nesfatin-1/NUCB2 protein abundance in adipose, liver or muscle tissues (Fig. 1a).

Effect of intracerebroventricular (ICV) Ad-shNUCB2 injection on nesfatin-1/NUCB2 expressing neurons

To determine the effect of ICV Ad-shNUCB2 on nesfatin-1/NUCB2 expressing neurons, we performed immunohistochemistry in rat hypothalamic. As shown in Fig. 1b-c, ICV Ad-shNUCB2 led to a significant decrease in the number of nesfatin-1/NUCB2 immunoreactive cells in the ARC, PVN, SON and supraoptic retrochiasmatic nucleus (SOR) (p<0.01, Fig. 1b and c), which have been implicated in glucose homeostasis.

Effect of hypothalamic nesfatin-1/NUCB2 inhibition on food intake

ICV injection of Ad-shNUCB2 (10⁹PFU/rat) caused a significant increase in food intake in NCD and HFD-fed rats between days 2-7 (Fig. 2a-c). Since the greatest increase in food intake induced by ICV Ad-shNUCB2 was on day 3, all subsequent experiments were performed at the third day after ICV Ad-shNUCB2 injection. We
also examined the change in body weight (BW) on day 7, and no significant
differences were found among the rats received Ad-shNUCB2, Ad-shGFP, or aCSF injection (Fig. 2d). Although HFD-fed rats had significantly decreased hepatic
glycogen stores compared with NCD-fed rats (57.04±2.05 vs. 73.36±2.64 mg/g, p<0.01), hypothalamic nesfatin-1/NUCB2 inhibition failed to change glycogen levels in both NCD- and HFD-fed rats (Fig. 2e).

**Effects of hypothalamic nesfatin-1/NUCB2 inhibition on glucose kinetics**

To examine the effect of ICV administration of Ad-shNUCB2 on glucose kinetics, both NCD- and HFD-fed rats received an injection of Ad-shNUCB2, Ad-shGFP, or aCSF 3 days before the hyperinsulinemic-euglycemic clamp studies. After 12 hours of fasting, the plasma insulin, glucose, TC, TG, LDL-C, HDL-C, NEFA levels were similar in NCD-fed rats who had received aCSF, Ad-shNUCB2, or Ad-shGFP injections. However, HFD-fed rats had significantly increased plasma insulin, TC, TG, LDL-C, NEFA, and decreased HDL-C levels compared with NCD-fed rats (p<0.01, Supplemental Table). During the clamp, NEFA and triglyceride levels were significantly suppressed in all of groups, although they were higher in HFD-fed rats than that in NCD-fed rats (p<0.05, Table 1).

GIR, GRd and HGP were determined during the final 30 minutes of the clamp studies when steady state conditions were achieved for plasma glucose and insulin concentrations and GIR. As expected, HFD-feeding decreased GIR (Fig. 3c), GRd (Fig. 3d) and suppression of endogenous glucose production by insulin (Fig. 3e), and increased HGP (Fig. 3f). Hypothalamic nesfatin-1/NUCB2 inhibition significantly
decreased GIR in both NCD-fed (p<0.01) and in HFD-fed groups (p<0.01, Fig. 3c). In addition, ICV Ad-shNUCB2 administration significantly increased HGP in both NCD- and HFD-fed rats compared with the aCSF controls (p<0.05 or p<0.01, Fig. 3f). Finally, Ad-shNUCB2 injection significantly decreased insulin induced suppression of endogenous glucose production (Fig. 3e). Taken together, our data showed that hypothalamic nesfatin-1/NUCB2 knockdown significantly increased peripheral and hepatic insulin resistance.

**Effect of hypothalamic nesfatin-1/NUCB2 inhibition on peripheral glucose intake**

To further determine whether central nesfatin-1/NUCB2 knockdown decreases insulin-induced peripheral glucose utilization, 2-DG was injected through the intracarotid catheter during the last 45 minutes of the hyperinsulinemic-euglycemic clamp studies (Fig. 4a). We found that 2-DG utilization rate was significantly decreased in gastrocnemius soleus muscle (Fig. 4b), white adipose tissue (WAT) (Fig. 4d), and interscapular brown adipose tissue (BAT) (Fig. 4c) by the central nesfatin-1/NUCB2 knockdown in both NCD and HFD groups. The results indicated that central nesfatin-1/NUCB2 knockdown attenuated peripheral insulin action.

**Effect of central nesfatin-1/NUCB2 knockdown on hepatic expression of PEPCK and G-6-Pase**

Since central nesfatin-1/NUCB2 knockdown significantly attenuated hepatic insulin action (Fig. 3), we next examined whether expressions of PEPCK and G-6-Pase, two gluconeogenic enzymes, were altered by this knockdown. HFD feeding for 10 weeks increased hepatic PEPCK and G-6-Pase protein levels (p<0.05). Intriguingly, both
NCD- and HFD-fed rats that received ICV Ad-shNUCB2 further increased expression of PEPCK and G-6-Pase proteins and mRNAs (Fig. 5) compared with the aCSF controls. These results indicated that central nesfatin-1/NUCB2 knockdown blocked, at least in part, the inhibitory effects of insulin on PEPCK and G-6-Pase in the liver, and led to increased HGP.

**Effects of central nesfatin-1/NUCB2 knockdown on insulin signaling in liver and muscle**

To determine the underlying mechanisms, by which central nesfatin-1/NUCB2 knockdown inhibited insulin signaling, we examined hepatic phosphorylation levels of InsR, IRS-1, and AKT by Western blots in liver and muscle. As showed in Fig. 6, 10 weeks HFD feeding induced a marked decrease in InsR, IRS-1, and AKT phosphorylation in both liver and muscle tissue (p<0.05). Upon central Ad-shNUCB2 treatment, the phosphorylated InsR (Tyr 1105) (Fig. 6a, b), IRS-1 (Tyr612) (Fig. 6c, d), and Akt (Ser473) (Fig. 6e, f) (p<0.05) were dramatically reduced in liver and muscle tissues of both NCD and HFD rats. These data confirmed that insulin signaling was attenuated in liver and muscle by the central Ad-shNUCB2 knockdown.

**Effects of central nesfatin-1/NUCB2 knockdown on the mTOR-STAT3 pathway in the liver**

We previously reported that hypothalamic nesfatin-1 is involved in regulation of HGP via hepatic AKT/AMPK/mTOR signaling (14). mTOR has been shown to activate STAT3 by phosphorylation of Ser727 (10, 20–22). To further characterize signaling mechanism associated with the action of nesfatin-1/NUCB2, we examined the effects
of central nesfatin-1/NUCB2 knockdown on STAT3 signaling in the liver. As shown in Fig. 7a and b, total STAT3 protein levels were similar in all groups. However, in both NCD- and HFD-fed rats, ICV nesfatin-1/ NUCB2 knockdown induced a dramatic decrease in STAT3 tyrosine phosphorylation in positions 705 (Tyr705) (Fig. 7a), and serine phosphorylation in position 727 (Ser727) (Fig. 7b) (p<0.05). Consistent with our previous reports, 10 weeks HFD inhibited phosphorylation of mTOR (Ser2448) (Fig. 7c). Furthermore, ICV nesfatin-1/NUCB2 knockdown led to a significant decrease in SOCS3 (Fig. 7d), a transcriptional target of STAT3.
DISCUSSION

Nesfatin-1 is a hypothalamic signal peptide involved in feeding behaviors and body weight control (3). Although several lines of evidence have suggested that nesfatin-1 may function as a regulator for energy homeostasis (14), the mechanisms associated with the action of nesfatin-1 remain to be characterized. In this study, we have established an in vivo hypothalamic nesfatin-1 knockdown model using RNAi of nesfatin-1/NUCB2 expression in the hypothalamus of rats. Using this model, we observed that central nesfatin-1/NUCB2 knockdown did not significantly change the body weight of the mice although food intake was increased about 10% with a maximal effect on day 3. No significant changes in body weight may be related to that treatment time is not long enough (7 days) (3) or the relative small increase in food intake (~10%). There are several possibilities for such small effect on food intake: 1) one injection of Ad-shNUCB2 may not be enough for significantly knocking down central nesfatin-1/NUCB2. Because adenovirus-mediated gene expression was considered to be dose and time dependent in a linear fashion (23, 24), multiple injections to increase vector dose may be required for a large effect on gene expression and consequently on the food intake; 2) there was insufficient blockade (50%) of NUCB2 translation at key hypothalamic nuclei related to food intake; and 3) central nesfatin-1 knockdown may lead to an increase of an anorectic hormone, such as leptin (as nesfatin-1 and leptin expression are not parallel) (25). Interestingly, a recent study reported that knockdown of hypothalamic NUCB2 had no effects on food intake or body weight in pubertal female rats (26), suggesting a sex-difference in nesfatin-1
signaling (i.e., a more prominent role in the control of feeding in male rodents).

ICV nesfatin-1/NUCB2 knockdown decreased GIR and glucose uptake in peripheral tissues, and increased HGP during euglycemic-hyperinsulinemia clamp. These changes were accompanied by a significant increase in the hepatic expression of the enzymes G-6-Pase and PEPCK. In addition, hypothalamic nesfatin-1/NUCB2 knockdown markedly decreased InsR, IRS-1 and Akt phosphorylation in both liver and muscle. As the insulin signaling pathway is generally thought to proceed through receptor-mediated tyrosine phosphorylation of IRS-1, which leads to activation of phosphoinositide 3-kinase (PI3K) and Akt. In blocking gluconeogenesis, insulin reduces transcription of several crucial genes in glucose production, including PEPCK and G-6-Pase (27). Consistent with our previous report(14), our results further confirmed that nesfatin-1 plays an important role in hepatic insulin signaling through regulating hepatic glucose fluxes, G-6-Pase and PEPCK expression.

How does central nesfatin-1/NUCB2 signaling regulate hepatic G-6-Pase and PEPCK expression? It is well known that STAT3 signaling is involved in hypothalamic regulation of food intake, and hepatic glucose fluxes (28). STAT3 is activated through phosphorylation by a wide range of cytokines and growth factors in response to various stimuli (29~32). We found that central nesfatin-1/NUCB2 knockdown suppressed phosphorylation of STAT3 at serine in position 727 (Ser727) and tyrosine in position 705 (Tyr705) in the liver. It is known that Tyr705 phosphorylation is required for activation, whereas phosphorylation at Ser727 is essential for maximal activation of STAT3 (33). After Tyr705 phosphorylation,
STAT3 can bind to the upstream regions of the G-6-Pase and PEPCK promoters to suppress their expression (11). Decreased Tyr705 phosphorylation of STAT3 by knocking down nesfatin-1/NUCB2 signaling may release this suppression resulting in increased G-6-Pase and PEPCK expression. Therefore, our findings provided evidence that nesfatin-1 regulated hepatic glucose production in association with STAT3 induced suppression of gluconeogenic genes.

SOCS3 is one of the transcriptional targets of STAT3 and plays a role in cytokine-induced insulin resistance by reducing IRS levels (34-36). Our result showed decreased SOCS3 protein abundance in the liver in hypothalamic nesfatin-1/NUCB2 knockdown rats, further supporting decreased STAT3 activity. Interestingly, although SOCS3 was down regulated in liver and total IRS levels were slightly increased (Fig. 6b), phosphorylation of both IRS and AKT was decreased, which suggested that decreased phosphorylation of IRS and AKT plays a major role in the increased hepatic insulin resistance induced by hypothalamic nesfatin-1/NUCB2 knockdown.

We have previously reported that hypothalamic nesfatin-1 is involved in regulation of hepatic glucose production via hepatic AKT/AMPK/mTOR signaling (14). As a member of the phosphatidylinositol kinase-like Ser/Thr kinase family, mTOR appears to activate STAT3 by phosphorylation of Ser727 (10, 20-22). We found that central nesfatin-1/NUCB2 inhibition decreased the activity of mTOR, particularly in HFD-fed rats, leading to decreased phosphorylation of STAT3. Recently, it has been proposed that over-activation of the mTOR/S6K1 pathway
contributes to defective activation of phosphatidylinositol 3-kinase (PI3K) by directly phosphorylating IRS on serine residues (37, 38). Ser2448 in mTOR has been identified as a phosphorylation site by AKT in vivo and this phosphorylation depends on PI3K (39). Therefore, it is likely that hypothalamic nesfatin-1/ NUCB2 knockdown decreased mTOR Ser2448 phosphorylation through inhibition of insulin signaling. Although it is not clear whether these signal transduction changes are direct effects of hypothalamic nesfatin-1 knockdown or indirect effects associated with increased food intake, our previous study demonstrated that central nesfatin-1 infusion could independently and immediately affect hepatic insulin action supporting a direct effect (14). However, some limitations of this study need to be considered such as lack of a pair-feeding controls, lack of access to insulin levels in the portal vein and an experimental approach to induce a selective inhibition in these signaling pathways. Therefore, further studies are needed to address these issues.

The neural pathways involved in the effects of nesfatin-1/ NUCB2 on glucose homeostasis remain to be defined. A recent study has reported that nesfatin-1 has either hyperpolarizing or depolarizing effects on most neurons in the PVN (40). In addition, glucose and insulin may directly activate nesfatin-1 neurons in PVN (41). Our previous study found that ICV nesfatin-1 activated c-fos-positive neurons in the PVN (14). It is known that the hypothalamus, especially the PVN, via the modulation of the sympathetic parasympathetic balance, takes part in the control of whole body glucose metabolism (42–47). Therefore, it is possible that, like leptin, nesfatin-1/NUCB2 signaling was relayed to the liver, skeletal muscle, or adipocytes
via the autonomic nervous system.

In summary, we demonstrated here that an adenoviral-mediated RNAi can induce a loss of function in hypothalamic nesfatin-1/NUCB2. Using this tool, we detected an important role of hypothalamic nesfatin-1 knockdown in the regulation of liver glucose fluxes and insulin signaling, which was associated with inhibition of the mTOR-STAT3 pathway. Further studies are required to validate the cross talk between mTOR-STAT3 pathway and nesfatin-1/NUCB2 signaling.
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G. Y. and L.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Author contribution

D.W. researched data/ wrote manuscript. M.Y. researched data. Y.C. researched data. Y.J. researched data. Z.A.M reviewed/edited manuscript. G.B. reviewed/edited manuscript. L.L. contributed to discussion, reviewed/edited manuscript. G.Y. contributed to discussion, reviewed/edited manuscript. All the authors gave final approval to the submission of the manuscript.

Conflict of interest

There is not any duality of interest.
Reference


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### TABLE 1 General characteristics during the hyperinsulinemic clamp studies

<table>
<thead>
<tr>
<th>Diet</th>
<th>Treatment</th>
<th>Weight (g)</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (mU/L)</th>
<th>TG (mmol/l)</th>
<th>TC (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
<th>NEFA (mmol/l)</th>
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<tr>
<td>NCD</td>
<td>aCSF</td>
<td>357.7±4.4</td>
<td>5.27±0.94</td>
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**Two-way ANOVA P value**

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NCD, normal chow-diet; HFD, high fat-diet; aCSF, artificial cerebrospinal fluid; TG, Triglyceride; TC, Total cholesterol; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol. n=10. Data are means ± SEM. Two-way ANOVA analysis indicated that only 10 weeks of HFD feeding altered weight, serum insulin, TC, TG, LDL-C, HDL-C, NEFA in these rats.
Figure legends

**Figure 1** Effects of central ad-shNUCB2 injection on nesfatin-1/NUCB2 immunoreactivity (ir) in hypothalamic regions and nesfatin-1/NUCB2 protein expression. (a) Representative Western blots analysis. (b) Photomicrographs of coronal brain sections showing nesfatin-1/NUCB2 immunostaining in the arcuate (ARC), paraventricular (PVN) nucleus, supraoptic nucleus (SON) and supraoptic retrochiasmatic nucleus (SOR) after ICV aCSF (NCA) or Ad-shNUCB2 treatment (NCN). (c) Number of nesfatin-1/NUCB2-ir cells per section in hypothalamic regions. Data are means ± SEM; n = 3. *P < 0.01 vs. NCA groups.

**Figure 2** Changes in food intake and hepatic glycogen after ICV infusion of Ad-shNUCB2. Daily food intake in (a) NCD-fed and (b) HFD-fed rats, (c) total food intake, (d) weight changes, and (e) hepatic glycogen content (mg/g wet tissue) were determined (n=6). Data are means ± SEM, *p< 0.05, **p< 0.01 vs. NCA group, ▲ p< 0.05, ▲▲ p< 0.01 vs. HFA group.

**Figure 3** Aggravation of insulin resistance by central Ad-shNUCB2 treatment. (a) Experimental protocol. (b) Hyperinsulinemic-euglycemic-clamp procedure. (c) The rate of glucose infusion (GIR). (d) The glucose disappearance rate (GRd). (e) Percentage of the suppression of endogenous glucose production-induced by insulin infusion. (f) Hepatic glucose production (HGP). n=6, *p< 0.05, **p< 0.01 vs. NCA group, ▲ p< 0.05, ▲▲ p< 0.01 vs. HFA group.

**Figure 4** Central nesfatin-1/NUCB2 knockdown decreases glucose utilization in tissues. (a) Hyperinsulinemic-euglycemic clamp procedure. Glucose utilization was
assessed in (b) gastrocnemius and soleus, (c) interscapular brown adipose tissue (BAT), and (d) white adipose tissue (WAT). n=4. *p< 0.05, **p< 0.01 vs. NCA group; ▲p< 0.05, ▲▲p< 0.01 vs. HFA group.

**Figure 5** Effects of central nesfatin-1/NUCB2 knockdown on protein and mRNA levels of PEPCK and G-6-Pase in the liver. (a) PEPCK protein abundance. (b) G-6-Pase protein abundance. (c) PEPCK mRNA expression. (d) G-6-Pase mRNA expression. n=4. *p< 0.05, **p< 0.01 vs. NCA group, ▲p< 0.05, ▲▲p< 0.01 vs. HFA group.

**Figure 6** Effects of central nesfatin-1/NUCB2 knockdown on molecules of insulin signaling pathway in liver and muscle tissues. (a) Phospho-tyrosine of InsR in the liver. (b) Phospho-tyrosine of InsR in muscle. (c) Phospho-tyrosine of IRS-1 in the liver. (d) Phospho-tyrosine of IRS-1 in muscle. (e) Phospho-serine of Akt in the liver. (f) Phospho-serine of Akt in muscle. n=4. *p< 0.05, **p< 0.01 vs. NCA group, ▲p< 0.05, ▲▲p< 0.01 vs. HFA group.

**Figure 7** Effects of central nesfatin-1/NUCB2 knockdown on liver STAT3, mTOR and SOCS3 phosphorylation in liver from NCD- and HFD-fed rats. (a) Western blot analysis of phosphorylated STAT3 (p^{Tyr705}STAT3) and total STAT3. (b) Western blot analysis of phosphorylated STAT3 (p^{Ser727}STAT3) and total STAT3. (c) Western blot analysis of phosphorylated mTOR (p^{Ser2448}mTOR) and total mTOR. (d) Western blot analysis of SOCS3 and β-actin. *p< 0.05, **p< 0.01 vs. NCA group, ▲p< 0.05, ▲▲p< 0.01 vs. HFA group.
Figure 1

a

<table>
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<tr>
<td>Nestin-1/NUCB2 protein levels</td>
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<tr>
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</table>

b

![Image of tissue sections from ARC, PVN, SON, SOR regions with aCSF and ad-shNUCB2 treatments]
C

![Bar graph showing immunoreactive cells (number/section) for ARC, PVN, SON, and SOR.](chart)

- **ARC**
- **PVN**
- **SON**
- **SOR**

Legend:
- **NCA**
- **NCN**

* *
Figure 2

(a) Food intake (kcal) over time after injection (day).

(b) Food intake (kcal) over time after injection (day).

(c) Total food intake (kcal) and weight changes (g).

(d) Hepatic glycogen (mg/g) levels.
Figure 3

a

Day-14 → ICV Cannulae → Vascular Catheters → Euglycemic Clamps → Recovery

b

0 min → [3-H+] Glucose (0.2 µCi/min) → Insulin (6 mU/kg/min) → 25% Glucose (as needed) → 120 min → 240 min

NCA  NCG  NCN          HFA  HFG  HFN

GIR (mmol/kg/min)

0.00  0.02  0.04  0.06  0.08  0.10  0.12

NCA  NCG  NCN          HFA  HFG  HFN

GRd (mmol/kg/min)

0.00  0.05  0.10  0.15  0.20

NCA  NCG  NCN          HFA  HFG  HFN

% suppression

0  10  20  30  40  50

NCA  NCG  NCN          HFA  HFG  HFN

HGP (mmol/kg/min)

0.00  0.02  0.04  0.06  0.08  0.10  0.12

NCA  NCG  NCN          HFA  HFG  HFN
Figure 4

a

![Diagram of experiment timeline including Insulin (6mU/kg/min) administration, 25% Glucose (as needed), and 2-DG (30μCi) injection.]

b

![Bar chart showing Muscle Glucose utilization (µmol/100g/min) for Gastrocnemius and Soleus muscles.]

Gastrocnemius

Soleus

Muscle Glucose utilization (µmol/100g/min)

0

1

2

3

4

5

NCA  NCG  NCN  HFA  HFG  HFN

0.0

0.1

0.2

0.3

0.4

0.5

10

8

6

4

2

0

NCA  NCG  NCN  HFA  HFG  HFN

0.0

0.1

0.2

0.3

0.4

0.5

WAT Glucose utilization (µmol/100g/min)
Figure 5

(a) PEPCK protein levels
(b) G-6-Pase protein levels
(c) PEPCK mRNA expression
(d) G-6-Pase mRNA expression
Figure 6

(a) 

(b) 

(c) 

(d)
Figure 7

(a) p-STAT3 (Tyr705) / total STAT3

(b) p-STAT3 (Ser727) / total STAT3

(c) p-m TO R / total m TO R

(d) SOCS3

Diabetes
### Supplemental Table  Baseline characteristics of the experimental groups

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<th>Diet</th>
<th>Treatment</th>
<th>Glucose (mmol/l)</th>
<th>Insulin (pmol/l)</th>
<th>TG (mmol/l)</th>
<th>TC (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>LDL-C (mmol/l)</th>
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<td>5.56±0.16</td>
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<td>Ad-shGFP</td>
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<td>Ad-shNUCB2</td>
<td>5.21±0.16</td>
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**Two-way ANOVA P value**

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aCSF, artificial cerebrospinal fluid; TG, Triglyceride; TC, Total cholesterol; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol. n=4. Data are means ± SE. * p<0.05, **p<0.01 vs. normal-chow rats treated with aCSF. Two-way ANOVA analysis indicated that 10 weeks of high-fat diet feeding but not central Ad-shNUCB2 administration altered serum glucose, insulin, TC, TG, LDL-C, HDL-C, NEFA in these rats.
Supplemental Figure  Experimental protocol

- Total rats (n=102)
- Western blot and immunohistochemistry analyses to testify NUCB2 knockdown (n=3/group)
- Clamp study using [3-H2] glucose (n=6/group)
- Clamp study using 2-deoxy-D-[H2]glucose (n=4/group)
- Food intake experiments (n=6/group)

Tissue samples were taken at the end of the clamp.

Western blot analyses and RT-PCR (n=4/group)