

ORIGINAL ARTICLE

Nesfatin-1 exerts long-term effect on food intake and body temperature

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OBJECTIVE: To determine whether the anorexigenic peptide, nesfatin-1 affects energy expenditure, and to follow the time course of its effects.

DESIGN: Food intake duration, core body temperature, locomotor activity and heart rate of rats were measured by telemetry for 48 h after a single intracerebroventricular injection of 25 or 100 pmol nesfatin-1 applied in the dark or the light phase of the day. Body weight, food and water intake changes were measured daily. Furthermore, cold-responsive nesfatin-1/NUCB2 neurons were mapped in the brain.

RESULTS: Nesfatin-1 reduced duration of nocturnal food intake for 2 days independently of circadian time injected, and raised body temperature immediately, or with little delay depending on the dose and circadian time applied. The body temperature remained higher during the next light phases of the 48 h observation period, and the circadian curve of temperature flattened. After light phase application, the heart rate was elevated transiently. Locomotion did not change. Daily food and water intake, as well as body weight measurements point to a potential decrease in all parameters on the first day and some degree of compensation on the second day. Cold-activated (Fos positive) nesfatin-1/NUCB2 neurones have been revealed in several brain nuclei involved in cold adaptation. Nesfatin-1 co-localised with prepro-thyrotropin-releasing hormone in cold responsive neurones of the hypothalamic paraventricular nucleus, and in neurones of the nucleus raphe pallidus and obscurus that are premotor neurones regulating brown adipose tissue thermogenesis and skin blood flow.

CONCLUSION: Nesfatin-1 has a remarkably prolonged effect on food intake and body temperature. Time course of nesfatin-1's effects may be varied depending on the time applied. Many of the nesfatin-1/NUCB2 neurones are cold sensitive, and are positioned in key centres of thermoregulation. Nesfatin-1 regulates energy expenditure a far more potent way than it was recognised before making it a preferable candidate anti-obesity drug.

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INTRODUCTION

Obesity and its complications cause death of thousands of people every year worldwide, therefore research for possible therapeutic targets is intensively continued. Nesfatin-1 was discovered a few years ago as a new agent in the regulation of the food intake.¹ It is the N-terminal fragment of the nucleobindin 2 protein (NUCB2) named after nucleobindin2-encoded satiety- and fat-influencing protein. Other fragments like nesfatin-2 and nesfatin-3 have DNA-binding domains and unknown function.¹ Intracerebroventricular (icv) administration of nesfatin-1 decreases nocturnal food intake in a dose-dependent manner.¹ Injected into fasted rats, nesfatin-1 activates food intake regulatory autonomic centres in the brain, such as the neurons of the hypothalamic paraventricular nucleus (PVN), and the nucleus of the solitary tract (NTS).² Moreover, fasting results in depletion of nesfatin-1 /NUCB2 mRNA and peptide in the supraoptic nucleus and in the PVN, whereas icv administration of an antisense oligonucleotide prepared against nesfatin-1 /NUCB2 induces an increase in food intake and body weight gain.^{1,3}

Localisation and wide distribution of nesfatin-1-producing neurons in the brain predispose its involvement in many other

functions. As it has been already shown, nesfatin-1 decreases water intake, increases the mean arterial pressure, evokes anxiety-related behaviour, and participates in the stress reaction.^{4–6} Recently, a relationship between nesfatin-1 level and occurrence of epileptic seizures has also been established in human patients.⁷

Long-term energy balance is determined both by the regulation of the food intake and by the energy expenditure. This is supported by the fact that many anorexigenic and orexigenic neuropeptides are also involved in the central control of thermogenesis, like corticotropin-releasing hormone, thyrotropin-releasing hormone (TRH) and oxytocin in the PVN, proopiomelanocortin and cocaine- and amphetamine-regulated transcript in the arcuate nucleus (ARC), prolactin-releasing peptide in the NTS and in the caudal ventrolateral medulla and melanin-concentrating hormone in the tuberohypothalamic area.^{6,8–11} Nesfatin-1 is co-expressed with all of the above-mentioned neuropeptides suggesting a possible association with thermoregulation that had not been investigated, yet.^{11–17} Besides, both body temperature and food intake have a diurnal rhythm, and there are no data in the literature about longer than 24 h observations on nesfatin-1's effect. Considering these, the present

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work aimed to investigate (1) whether any prolonged visceral consequences of a single icv nesfatin-1 injection exist, (2) whether actions of nesfatin-1 interact with the diurnal cycle of the animals, and (3) whether certain nesfatin-1/NUCB2 neurons in the central nervous system are functionally involved in thermoregulation. To determine this, we injected nesfatin-1 or vehicle icv into the lateral ventricle of rats either at the beginning of the light phase, or at the beginning of the dark phase, and used a telemetric device to follow the core body temperature, heart rate and locomotion for 48 h. Additionally, in another experiment, we subjected rats to cold and activation of subsets of nesfatin-1/NUCB2 immunopositive neurons in the brain was examined.

MATERIALS AND METHODS

Animals

Male Wistar rats (TOXI-COOP Ltd., Budapest, Hungary) weighing 250–300 g were used for the studies ($n=37$). Animals were kept with light–dark cycle of 12:12 h at room temperature ($21 \pm 1^\circ\text{C}$), and had free access to standard rodent chow and tap water except otherwise indicated. Experiments were performed according to the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were supervised by the Animal Welfare Committee of the Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, Hungary.

Biotelemetry

The biotelemetric recordings were made by means of a 12-channel VitalView system (Minimitters Co., Bend, OR, USA) in every minute for 48 h.¹⁸

Feeding duration was determined by infrared feeding monitor belonging to the Vital View system designed for use with the Nalgene food chambers. When the subject places its head in the feeding chamber, a beam of infrared light is broken. At the same time, a clock starts and continues to run as long as the beam is interrupted. This equipment allows ad libitum feeding without restriction, but it is narrow enough not to be convenient to be in there, unless feeding.

In case of light phase experiments, lights were on at 06:00 h and off at 18:00 h. In case of dark phase injections, rats were housed in opposite diurnal cycle (light on at 22:00 h and off at 10:00 h) for three weeks before the injections. A polyethylene guide cannula was inserted into the right lateral ventricle (stereotaxic coordinates: 0.8 mm caudal to the Bregma, 2 mm from the midline, 4 mm ventrally) under deep anaesthesia with ketamine (50 mg kg^{-1} , Richter Gedeon Nyrt, Budapest, Hungary) and xylazine (15 mg kg^{-1} , CP-Pharma, Bönsensell, Germany). The cannula was fixed to the skull with acrylic dental cement. At the same time, VitalView biotelemetry emitters were implanted into the abdominal cavity. The negative and positive heart rate leads were attached to the anterior right side of the chest (near the clavicle) and to the posterior chest wall (left to the sternum and anterior to the last rib), respectively. Animals were allowed to recover for one week, whereas handled daily to reduce future experimental stress. Once recovered, they have received either 5 μl of nesfatin-1 dissolved in physiological saline, (25 pmol, $n=10–12$, light phase injections and $n=5–6$, dark phase injections, or 100 pmol, $n=7$, light phase injections) or 5 μl of saline icv. The icv injections were always made between 10:00 and 10:30 h applying it through a 26-gauge needle, which was connected by polyethylene tubing to a 10 μl Hamilton syringe. Telemetric data were calculated as sum \pm s.e.m. (food intake duration and locomotion) or mean \pm s.e.m. (core temperature and heart rate) in each 4 h (2 days measurement), or 10-min (short time observations) time bin. Temperature data were normalised to the average temperature between 6 h and 7 h on the day of the injection (2 days measurements), or to the average temperature of 10 min before the injection (short-term observations). Heart rate data for short-term observations were expressed in percentage of average values measured 10 min before the injections. Body weight, food and water intake were measured daily, data were calculated as differences between the consecutive days for each individual, and expressed as mean \pm s.e.m. Evaluation of the data was performed by using

two ways repeated measure ANOVA with the help of the STATISTICA 9.0 software (StatSoft, Tulsa, OK, USA).

Cold exposure

Rats were kept one per cage for 3 days before the experiment. On the experimental day, animals were placed to cages without bedding, and were exposed to 4°C in a cold room ($n=3$), or were kept at room temperature ($n=3$). After 2 h, the animals were anaesthetised (see above) and transcardially perfused with 4% paraformaldehyde.

Immunohistochemistry (IHC)

Brains were cryoprotected in 20% sucrose overnight, and cut into 50 μm thick serial coronal sections on a frigomobil (Frigomobil, Reichter-Jung, Vienna, Austria). Immunostainings always started with blocking the endogenous peroxidase activity, using a 3% H_2O_2 solution for 15 min. Then, the sections were blocked in 1% BSA and 0.5% TritonX-100/PBS for 1 h. The same solution was used to dilute the antibodies. Incubations were made for 2 days at 4°C in the primary antibodies and for 1 h at room temperature, when secondary or tertiary antibodies were used. The sections were washed three times for 5 min in PBS following each incubation step. As multiple labellings were performed with primary antibodies raised in the same hosts, sections were microwave-treated in 0.1 M citric-acid (pH 6.0) for 5 min after each immunostaining to block the peroxidase enzyme used for visualisation in the previous step, and to prevent crossreactions.¹⁹

Fos and nesfatin-1/NUCB2 double and Fos, nesfatin-1/NUCB2 and prepro-TRH triple immunostainings

Sections of cold exposed and control animals were incubated first in rabbit anti-Fos (1:30 000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), in biotinylated anti rabbit IgG (1:1000, Vector Laboratories, Inc., Burlingame, CA, USA), and in extravidine-peroxidase (1:1000, Sigma, Budapest, Hungary). The immunostaining was visualised by FITC-conjugated tyramide (Invitrogen, Budapest, Hungary). After this, the sections were incubated in rabbit anti-nesfatin-1/NUCB2 (1:12 000, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) and in anti-rabbit IgG polymer-HRP (Millipore, Budapest, Hungary). The second immunostaining was developed by tyramide-conjugated Alexa Fluor 568 (Invitrogen).

In case of triple labelling, the Fos IHC was performed first, as described. Sections were then incubated in rabbit anti-prepro-TRH (1:5000, kindly provided by Eva Redei), and in anti-rabbit IgG polymer-HRP (Millipore). The prepro-TRH antigen was visualised by tyramide-conjugated Alexa Fluor 568 (Invitrogen). The third IHC was performed using a rabbit anti-nesfatin-1/NUCB2 (1:12 000), a goat anti-rabbit IgG polymer-HRP (Millipore), and Alexa Fluor 405-tyramide (Invitrogen).

Colocalisation of prepro-TRH and nesfatin-1/NUCB2 in the brainstem

As previous report suggested that TRH immunoreactivity in brainstem raphe neurons can be revealed only after colchicine treatment,²⁰ sections from control and from colchicine-treated animals were also used. Colchicine ($200 \mu\text{g}/20 \mu\text{l}$) was injected under deep anaesthesia and stereotaxic control to the right lateral ventricle of rats by a Hamilton syringe. After 2 days, the animals were anaesthetised again, and transcardially perfused with 4% paraformaldehyde. Nesfatin-1/NUCB2 immunostaining was performed first, using anti-rabbit IgG polymer-HRP (Millipore) and tyramide-conjugated Alexa Fluor 568 (Invitrogen). The rabbit anti-prepro-TRH was applied next, followed by anti rabbit IgG polymer-HRP secondary antibody (Millipore), and FITC-conjugated tyramide (Invitrogen) for visualisation. Sections were counterstained with DAPI (Invitrogen).

Sections were mounted on non-coated slides, air dried and coverslipped with DPX (Sigma). Images were captured by a Nikon Eclipse E800 microscope attached to a Bio-Rad Radiance 2100 Rainbow confocal scanning system (Bio-Rad Microscience Ltd, Hemel Hempstead, England, UK).

RESULTS

Effects of icv nesfatin-1 on physiological parameters

Nesfatin-1 (25 pmol), added at the onset of the dark phase, diminished the duration of nocturnal food intake in the first 6 h and also decreased it at the beginning of the next dark phase (Figure 1a) (effect of treatment: $F_{(1,9)} = 4.63, P = 0.05$). Additionally, nesfatin-1 blunted the duration of nocturnal food intake for 48 h, when it was applied during the beginning of the light phase, too (Figure 1b) (effect of treatment: $F_{(1,11)} = 11.9, P < 0.01$). A circadian rhythm of food intake was observable in both cases (effect of time: $F_{(11,99)} = 10.5, P < 0.01$ for dark and $F_{(11,121)} = 5.83, P < 0.01$ for light phase application), but with much smaller amplitude than that of the controls (Figure 1) (treatment \times time interaction: $F_{(11,99)} = 3.06, P < 0.01$ for dark and $F_{(11,121)} = 4.41, P < 0.01$ for light phase application). Light phase injections had no significant effect on the daily amount of food consumed, indicating that animals either may have eaten more during the shortened durations at night, or made up for food intake during the day. However, dark phase application showed a clear reduction in food consumption on the first day and a compensation on the second day (effect of time: $F_{(1,21)} = 4.66, P < 0.05$, treatment \times time interaction: $F_{(1,21)} = 6.09, P < 0.05$, (Figure 1c). Nesfatin-1 treatment reduced water intake on the first day, that was followed by a compensatory increase on the second day in both cases (effect of treatment: $F_{(1,9)} = 6.1, P < 0.05$, effect of time: $F_{(1,21)} = 5.2, P < 0.05$, tendency for treatment \times time interaction: $F_{(1,21)} = 4.8, P = 0.056$ for dark phase, and treatment within the first day $P < 0.05$ (post hoc comparison Holm-Sidak method), effect of time: $F_{(1,23)} = 7.02, P < 0.05$, tendency for treatment \times time interaction: $F_{(1,23)} = 4.89, P = 0.05$ for light phase injections) (Figure 1c). Body weight changes reflected the tendencies observed with food and water intake, but they did not reach the level of significance in any cases (Figure 1c).

Nesfatin-1 (25 pmol) also affected the core body temperature of rats. When it was applied at the onset of the dark phase, the temperature started to elevate immediately (Figures 2a and b)

(effect of treatment: $F_{(1,9)} = 7.85, P < 0.05$). The difference between the groups was not so pronounced during the dark phases, but it was markedly significant during the light phases of the 48 h observation period (Figure 2a). In case of light phase injections, core body temperature started to elevate slightly approximately 1.5 h after the injection (Figures 2c and d) (effect of treatment: $F_{(1,14)} = 14.2, P < 0.01$). Body temperature of control and treated rats converged during the dark phase, but next day the temperature of the treated animals remained higher resulting in a maximal difference between the groups. The temperature difference between the groups existed also the second night (Figure 2c). Both dark phase and light phase injections resulted in a flattened circadian curve (effect of time: $F_{(11,99)} = 10.4, P < 0.01$ for dark and $F_{(11,154)} = 8.25, P < 0.01$ for light phase application), mainly as a consequence of the higher daytime temperature.

There was no notable influence of 25 pmol of nesfatin-1 on the heart rate, except a short interim elevation approximately 1.5 h after the light phase injection (Figures 3a-d) (effect of time: $F_{(11,88)} = 19.7, P < 0.01$ for dark and $F_{(11,198)} = 16.7, P < 0.01$ for light phase application). To elucidate whether changes in the locomotion may be responsible for the alterations in the heart rate and the core body temperature, locomotion data were evaluated through the 48 h observation period and also in more detail regarding a 2.5 h long period immediately after the injections. Significant changes were found in neither case (Figures 3e and f, Supplementary Figures 1A and B).

Effects of higher dose of nesfatin-1 (100 pmol) administered during the light phase showed the same tendencies observed with that of 25 pmol (food intake duration: effect of treatment: $F_{(1,154)} = 9.07, P < 0.01$, effect of time: $F_{(11,154)} = 4.24, P < 0.01$, treatment \times time interaction: $F_{(11,154)} = 3.12, P < 0.01$; body temperature: effect of treatment: $F_{(1,132)} = 6.45, P < 0.05$, effect of time: $F_{(11,132)} = 9.98, P < 0.01$, treatment \times time interaction: $F_{(11,132)} = 5.75, P < 0.01$; heart rate: effect of time: $F_{(11,380)} = 4.74, P < 0.01$, (Supplementary Figure 2). Temperature and heart rate changes were enhanced immediately after the injection, compared with

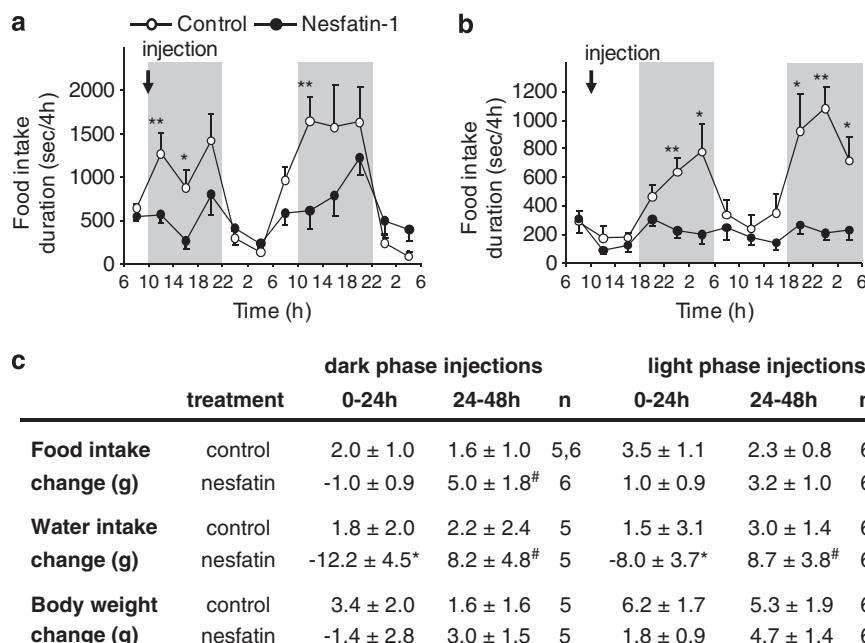


Figure 1. Food intake and related parameters in rats after icv administration of 25 pmol of nesfatin-1. (a, b) Time spent with food intake in case of dark ($n = 5-6$) and light phase ($n = 10-12$) injections (arrows) of nesfatin-1 or saline, respectively. Data were recorded continuously, summarised at 4 h intervals and plotted over a 48 h period. Dark phases are labelled by grey shadows. Sum \pm s.e.m., * $P < 0.05$, ** $P < 0.01$. (c) Daily changes in food and water intake, as well as in the body weight in case of dark ($n = 5-6$) and light phase ($n = 6$) injections of nesfatin-1, or saline. Mean \pm s.e.m., * $P < 0.05$ for treatment, [#] $P < 0.05$ for time effect. See text for details of statistical analysis.

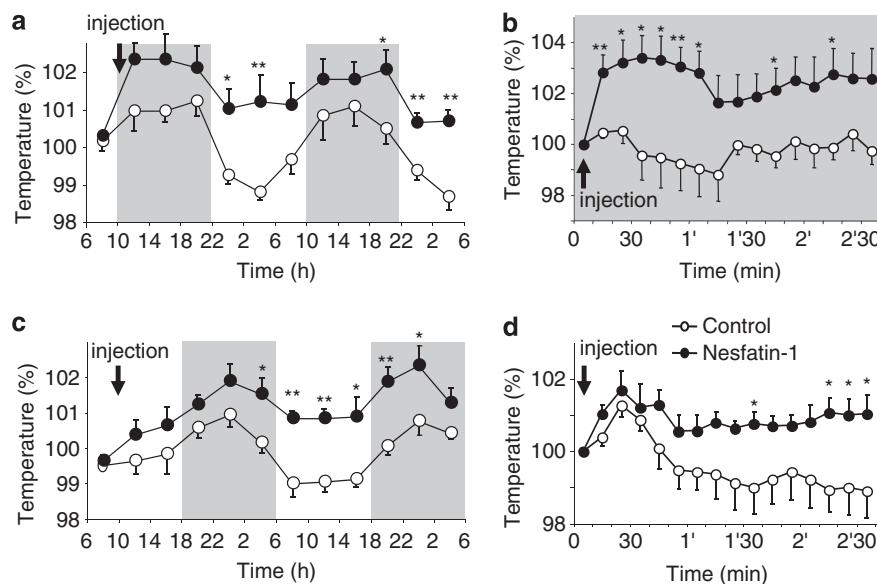


Figure 2. Effect of nesfatin-1 (25 pmol) on core body temperature of rats. Nesfatin-1 or saline were injected intracerebroventricularly (arrows) and core body temperature was recorded in every minute by remote radio telemetry. (a, c) Results after dark and light phase injections, respectively, averaged at 4 h intervals and plotted over a 48 h period. Data of each animal were normalised to the mean temperature value of the same animal measured between 6–7 h, at the beginning of the recording (100%). (b, d) Detailed analysis of (a, c), respectively, showing the mean values calculated at 10 min intervals over a 2.5 h period immediately after the injection. Data were normalised to the average temperature of 10 min before the injection. Dark phases are labelled by grey shadows. Mean \pm s.e.m., $n=5-6$ (a, c) or 8 (b, d) per group, $*P<0.05$, $**P<0.01$. See text for details of statistical analysis.

the lower dose applied (Supplementary Figures 2B and C). Again, there was no change in locomotion either immediately after the injections (Supplementary Figure 1C), or all along the observation period (not shown). A definite reduction in the amount of consumed food was observed on the first day after the injections, followed by compensatory eating next day, that probably happened mainly during the second light phase (effect of treatment: $F_{(1,12)}=8.55$, $P<0.05$, tendency for time effect: $F_{(1,27)}=4.33$, $P=0.06$, treatment \times time interaction: $F_{(1,27)}=9.88$, $P<0.01$), (Supplementary Table 1 and Supplementary Figure 2A). Changes in water intake were comparable in groups treated with 25 or 100 pmol nesfatin-1 (tendency for treatment effect: $F_{(1,12)}=3.89$, $P=0.07$, treatment within first day (post hoc comparison Holm-Sidak method): $P<0.05$, time: $F_{(1,27)}=5.29$, $P<0.05$, treatment \times time interaction: $F_{(1,27)}=4.83$, $P<0.05$) (Supplementary Table 1). Higher dose of nesfatin-1 enhanced tendencies observed previously on body weight (effect of time: $F_{(1,27)}=5.15$, $P<0.05$, tendency for treatment \times time effect: $F_{(1,27)}=4.33$, $P=0.06$), (Supplementary Table 1).

Effect of cold stress on activation of nesfatin-1/NUCB2 expressing neurons

Exposure of rats to cold induced strong Fos activation in neurons at several brain nuclei, as previously described.²¹ Control rats showed only minimal labelling (Figures 4a, c, e, g; 5a and 6a-c). In the preoptic region, most of the Fos-positive cells were located in the medial preoptic area lacking nesfatin-1/NUCB2 labelling (Figures 4a and b). Cold activated only few nesfatin-1/NUCB2 neurons in the periventricular preoptic nucleus (Figures 4a-d). Here, a group of double-labelled cells was seen, in the close vicinity of the third ventricle (Figure 4d, arrow). Nesfatin-1/NUCB2 and Fos double IHC revealed heavy labelling of the magnocellular cells in the supraoptic nucleus (Figures 4e and f), and less

extensive activation in the medial and lateral parts of the ARC (Figures 4g and h). Activated nesfatin-1/NUCB2 immunopositive neurons were present in high number also in the magnocellular and parvocellular subdivisions of the PVN (Figures 5a and b). Within the parvocellular PVN, Fos and nesfatin-1/NUCB2 double positive neurons were observed both in the hypophysiotropic (Figures 5a and b, mp and p) and in the autonomic projecting subdivisions (Figures 5a and b, arrow and asterisk). Triple IHC demonstrated that many cold-activated nesfatin-1/NUCB2 cells in the parvocellular PVN were prepro-TRH positive (Figures 5c-f).

In the lower brainstem, cold exposure induced a very strong Fos labelling in all of the nesfatin-1/NUCB2 expressing neurons in the nucleus raphe pallidus (Figures 6a and d) and raphe obscurus (Figures 6b and e), scattered double-labelled cells were found in the NTS (Figures 6c and f). Almost all of the nesfatin-1/NUCB2 immunoreactive cells in the nucleus raphe pallidus and obscurus contained prepro-TRH, too (Figures 6g-i).

DISCUSSION

Although nesfatin-1 is a potent anorexigen that may have therapeutic significance, long-term effects of a single injection have not been investigated yet. In the present paper, we report for the first time that icv-administered nesfatin-1 influences nocturnal food intake, as well as core body temperature over a 48 h period, modifies the circadian amplitude of these two parameters, and its effects interact with the circadian rhythm. We also provide new data on functional relevance of certain nesfatin-1/NUCB2 neurons located at thermoregulatory key centres in response to cold, further confirming its involvement in regulation of energy expenditure.

The 25 pmol dose of icv nesfatin-1 for rats was chosen according to Oh-I *et al.*¹, who applied this dose as a maximal one, establishing that 5 pmol is already effective. Most of the

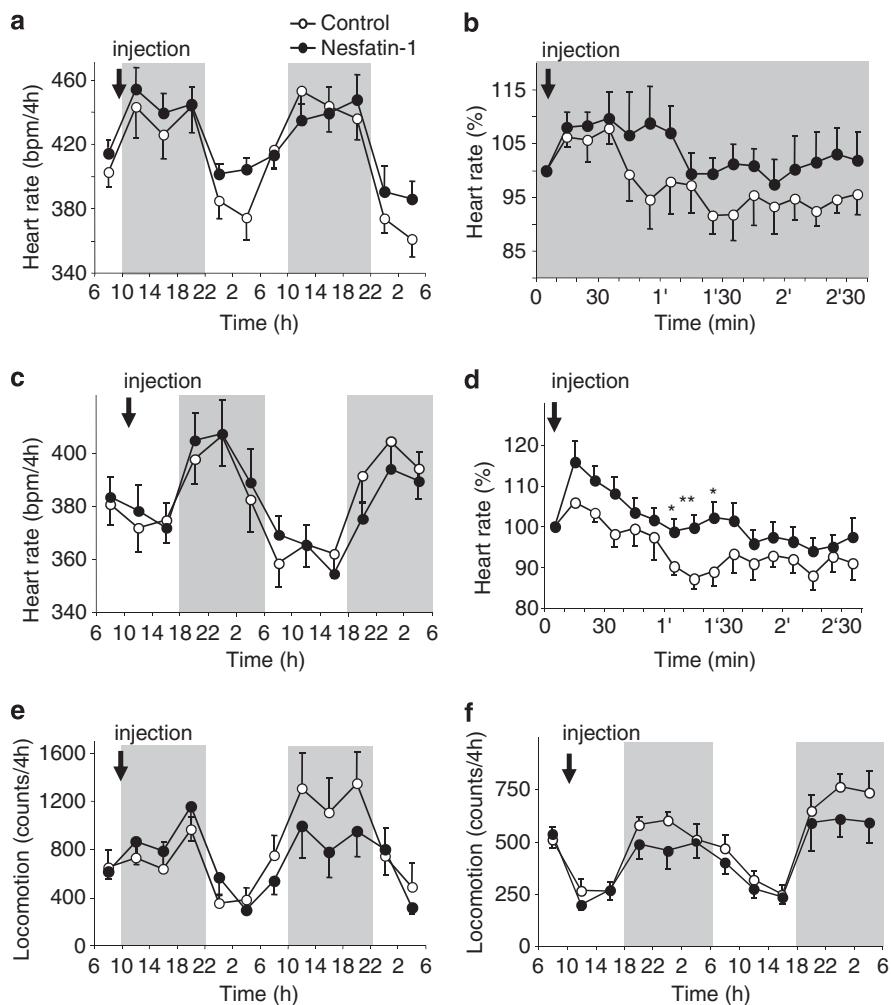


Figure 3. Effect of nesfatin-1 (25 pmol) on heart rate (**a–d**) and locomotion (**e, f**) of rats. Nesfatin-1 or saline were injected intracerebroventricularly (arrows) and parameters were recorded in every minute by remote radio telemetry. (**a, c**) Heart rate results after dark and light phase injections, respectively, averaged at 4 h intervals and plotted over a 48 h period. (**b, d**) Detailed analysis of (**a, c**) respectively, showing the mean values calculated at 10 min intervals over a 2.5 h period immediately after the drug applications, and expressed in percentage of average values measured 10 min before the injections. (**e, f**) Locomotion after dark and light phase injections, respectively, recorded in every minute, summarised at 4 h intervals and plotted over a 48 h period. Dark phases are labelled by grey shadows. Dark phase injections ($n=5-6$), light phase injections ($n=10$). Mean \pm s.e.m. (heart rate) or sum \pm s.e.m. (locomotion). $^*P<0.05$. See text for details of statistical analysis.

authors working on rats and using icv injections investigating different aspects of nesfatin-1's actions have also found the 5–20 pmol dose range effective, higher doses only caused earlier timing of the effect, and/or enhanced the tendencies observed with lower dose.^{5,22,23} We also found the 25 pmol dose as an adequate one, increasing the dose to 100 pmol did not lead to any substantially new observations.

Long-lasting effects and interference with the diurnal rhythm are not exclusive among the peptides regulating energy balance. Orexin initiates daytime food intake only, and NPY's action on body temperature is maintained for at least 24 h.^{24,25} Earlier data showed that nesfatin-1 reduced nocturnal food intake in the first 6 h after icv injections or given intranasally at the beginning of the dark phase.^{1,3} Report about longer than 24 h observations is not published so far. In our experiments, duration of food intake was also decreased in the first 6 h after injection of the drug at the beginning of the dark phase. In addition, nesfatin-1 decreased nocturnal food intake duration at the beginning of the second night, too. Nesfatin-1's long-time nocturnal food intake-reducing capability was evident even when it was given during the light

phase of the day, supposing a possible interaction with the circadian rhythm. So far, food intake duration after nesfatin-1 treatment was measured in mice only, where it also had a tendency to reduce total mealtime and time spent with food consumption.²⁶ Daily food and water intake, as well as body weight measurements point to a potential decrease in all parameters on the first day and some degree of compensation on the second day. Therefore, as it seems, chronic treatment is necessary for developing a permanent body weight-reducing effect.¹

Anorexigenic compounds, like prolactin-releasing peptide, corticotropin-releasing hormone, cocaine- and amphetamine-regulated transcript and TRH often affect not only the food intake, but-through the energy expenditure-also the thermoregulation.^{12,27–29} To reveal whether nesfatin-1 may alter the body temperature, we injected it during the light phase when the body temperature of the nocturnal animals is naturally lower. Lower dose of nesfatin-1 caused only a transient 1% elevation in body temperature within the first 3 h. Nonetheless, the main effect was seen during the next light phase when the body temperature of

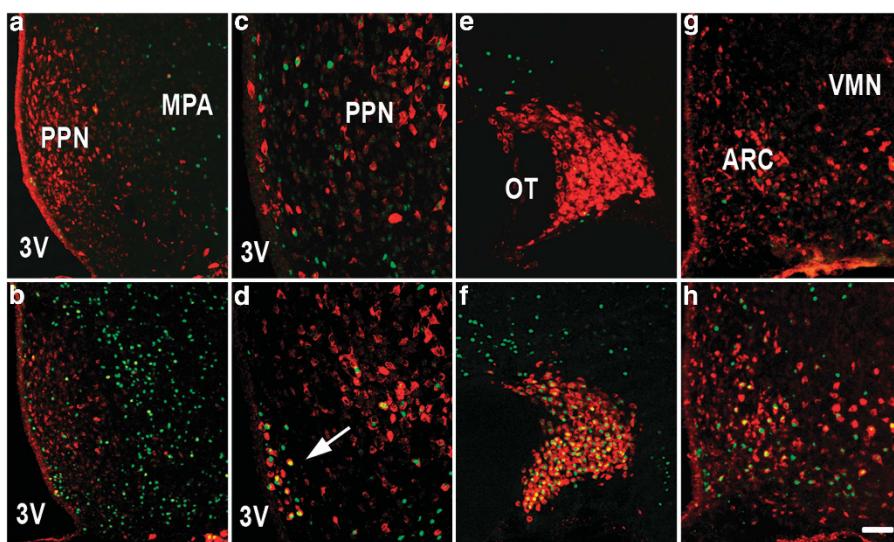


Figure 4. Cold stress activated nesfatin-1/NUCB2 immunopositive neurons in the hypothalamus. Activated, Fos-positive neuronal cell nuclei are green, nesfatin-1/NUCB2 labelled neurons are red, double labelled cells are yellow. Top (a, c, e, g); control, bottom (b, d, f, h); cold exposed animals. (a, b) Strong Fos activation without nesfatin-1/NUCB2 labelling in the medial preoptic area. (c, d) Scattered double-labelled cells in the periventricular preoptic nucleus. A group of neurons located at the vicinity of the 3rd ventricle is strongly activated by cold (d, arrow). (e, f) Heavy Fos activation in the supraoptic nucleus. (g, h) Double-labelled neurons in the ARC. MPA, medial preoptic area; OT, optic tract; PPN, periventricular preoptic nucleus; 3V, third ventricle; VMN, ventromedial nucleus. Scale bar: 100 μ m for a, b, e, f, and 50 μ m for c, d, g, h.

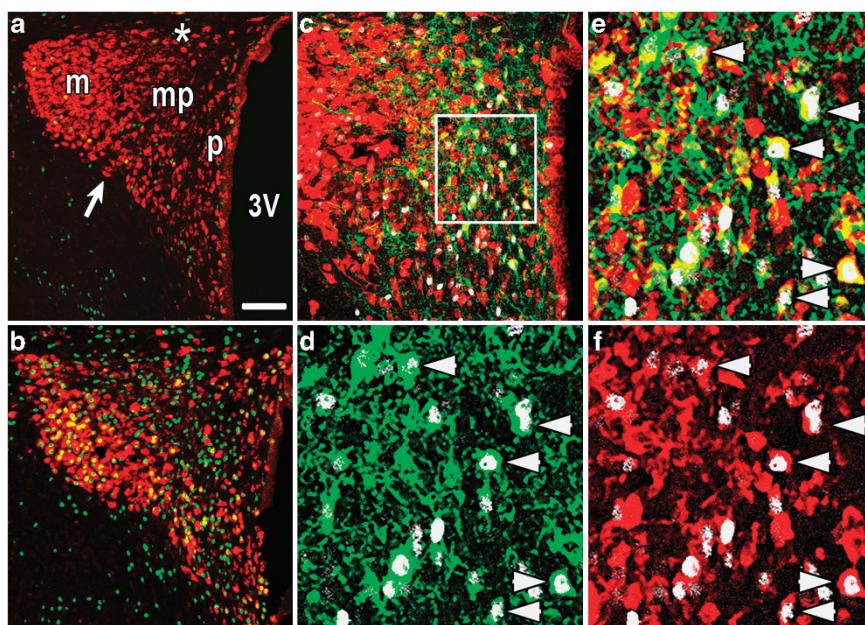


Figure 5. Cold stress activated nesfatin-1/NUCB2 immunopositive neurons in the hypothalamic PVN. (a, b) Control and cold-exposed animals, respectively. Activated, Fos-positive neuronal cell nuclei are green, nesfatin-1/NUCB2-labelled neurons are red, double-labelled cells are yellow. Double-labelled cells are seen in all subdivisions. (c–f) Cold stress activated prepro-TRH / nesfatin1/NUCB2-positive neurons in the medial parvocellular PVN. Fos is shown in white, prepro-TRH in green, and nesfatin1/NUCB2 in red. Triple-labelled cells are yellow with white nucleus. (c) Triple labelling, smaller magnification. (e) Enlarged picture of the framed area in (c). (d) The same area as in (e) showing the Fos and prepro-TRH labelling separately. (f) The same area as in (e) showing the Fos and nesfatin-1/NUCB2 labelling separately. The arrows on (d, e) and (f) point to identical cells immunopositive for all the three peptides. Hypophyseotrophic subdivisions: m, magnocellular; mp, medial parvocellular; p, periventricular parvocellular. Autonom projecting subdivisions: asterisk and arrow; dorsal and ventral parvocellular components, respectively. 3V, third ventricle. Scale bar: 100 μ m for a, b, 250 μ m for c, 50 μ m for e, f.

the rats failed to decrease to the level of the controls and remained higher during the second night too. In contrast, nesfatin-1 injected at the beginning of the dark phase elevated body temperature immediately for 1 h, indicating that there may be a potentiating action on the increased sympathetic activity

characteristic to the animals at this time of the day.^{30–32} The temperature of the treated rats practically did not differ at nights, but again it failed to fall during the next two light phases. Higher dose of nesfatin-1 one applied during the light phase caused somehow similar effect then the lower dose applied at dark,

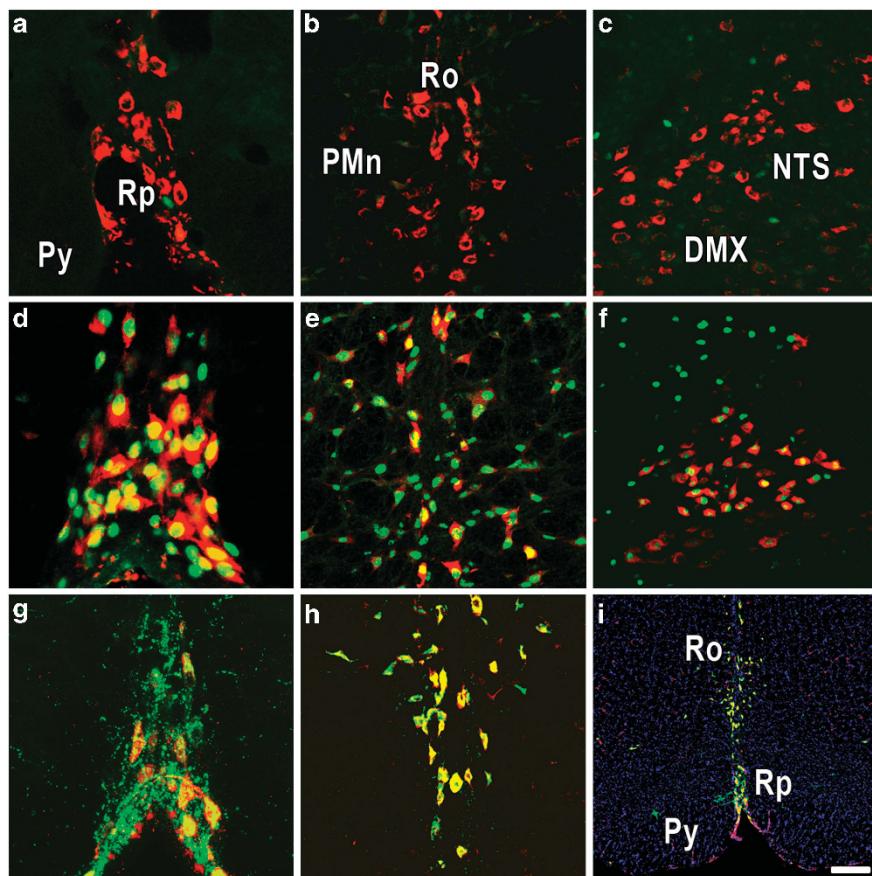


Figure 6. Cold sensitivity and characterisation of nesfatin-1/NUCB2 immunopositive neurons in the brainstem. (a-f) Effect of cold stress. Activated, Fos-positive neuronal cell nuclei are green, nesfatin-1/NUCB2-labelled neurons are red, double-labelled cells are yellow. Pictures of control (a-c) and cold exposed (d-f) animals are under each other, respectively. All nesfatin-1/NUCB2 neurons are activated in the nucleus raphe pallidus (a, d) and in the nucleus raphe obscurus (b, e). Many double-labelled cells are observed in the NTS, commissural part (c, f). (g-i) Colocalisation of nesfatin-1/NUCB2 and prepro-TRH in the nucleus raphe pallidus and obscurus. Prepro-TRH-positivity is seen as green, nesfatin-1/NUCB2 neurons are red, double-labelled cells are yellow. DAPI counterstaining on *i* is blue. Colchicine treatment (h, i) eliminates immunostaining of the fibres that partly mask the cells (g). DMX, dorsal vagal nucleus; PMn, paramedian reticular nucleus; Py, pyramidal tract; Ro, nucleus raphe obscurus; Rp, nucleus raphe pallidus. Scale bar: 30 μ m for a, d, g; 50 μ m for b, c, e, f, h and 220 μ m for i.

indicating that the elevated concentration may compensate for the reduced daytime sensitivity. There is a general consensus in the literature that nesfatin-1 itself is able to increase sympathetic activity, therefore such interaction is not surprising.^{4,23} It is interesting, however, that there was no effect of nesfatin-1 on the heart rate applied at night, meanwhile a limited and belated (starting more than 1 h after injection) heart rate elevation was observed, when it was given during the light phase. This became more definite using higher dose of nesfatin-1. Yosten *et al.*⁴ did not find any change in heart rate after nesfatin-1 treatment during the light phase, but they measured it only for 1 h after injection.

The mechanism through nesfatin-1 is able to modify the core temperature of the animals is not yet clear. Participation of nesfatin-1/NUCB2 in several autonomic functions is proposed, as nesfatin-1/NUCB2 neurons are present in high number in the hypothalamus and the lower brainstem autonomic centres.^{9,33} Many of these neurons were activated by cold, suggesting that nesfatin-1/NUCB2 may mediate thermoregulatory, as well as other responses to cold. Regulation of heat loss and thermogenesis are the two main effectors to maintain body temperature.³⁴ Setting heat loss includes adjusting thermoregulatory behaviour and skin blood flow, whereas controlling thermogenesis is realized through regulation of the skeletal muscle (shivering) and/or the brown adipose tissue (BAT) heat production (non shivering).³⁴ Although importance of BAT was earlier recognised only in mammals, it had

recently been demonstrated that humans also possess essential amounts of functioning BAT.³⁵ Retrograde, transsynaptic virus labelling of the BAT is a good tool to reveal areas in the brain connected directly or indirectly to the BAT. By this way, it has been shown that the PVN, the ARC, the nucleus raphe pallidus and raphe obscurus, and the NTS, where cold stress activated a number of nesfatin1/NUCB2-positive cells, are all among the elements of the polysynaptic pathways toward the BAT.^{36,37} Moreover, many effects of nesfatin-1 are mediated through melanocortin-3/4 receptors, and the melanocortin-3/4 receptor antagonist SHU9119 significantly decreases BAT temperature.^{4,23,38} In addition, prepro-TRH, precursor of TRH, a key factor regulating basal metabolic rate and thermogenesis, co-localised in the cold responsive nesfatin-1/NUCB2 cells in the PVN and in the brainstem raphe nuclei.¹² Although Johnson *et al.*²⁰ suggested colchicine treatment for TRH immunostaining, the prepro-TRH antibody we used worked without it. Colchicine treatment was still helpful, as it reduced the number of immunopositive fibres that might otherwise mask the cells (see Figures 6g and h for comparison). Nesfatin-1/NUCB2 has previously been colocalised with serotonin in the caudal raphe nuclei,⁹ and serotonin-containing neurons here are TRH positive.²⁰ Therefore, TRH, serotonin and nesfatin-1/NUCB2 are probably co-expressed in these nuclei. Serotonin expressing raphe pallidus and obscurus neurons exert their actions as preganglionic premotor neurons

regulating skin blood flow.³⁴ Thus, nesfatin-1 expressed in these cells is in a position to modify also heat dissipation.

In conclusion, our observations provide a new insight on nesfatin-1 function, and on neurocircuitry that may mediate its actions. As nesfatin-1 is expressed in human, acts as anorexigen, and its role has recently been suggested in many other functions such as, epilepsy and puberty onset, our data may be relevant regarding nesfatin-1, as a potential pharmacotherapeutic target.^{7,39,40}

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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