Acute but Not Chronic Central Administration of the Neuropeptide 26RFa (QRFP) Improves Glucose Homeostasis in Obese/Diabetic Mice

Marie-Anne Le Solliec a, Arnaud Arabo b, Saloua Takhlidjt a, Julie Maucotel b, Mélodie Devère a, Julien Riancho a, Hind Berrahmoune a, Jean-Luc do Rego c, Jean-Claude do Rego c, Alexandre Bénani d, Emmanuelle Nedelec d, Benjamin Lefranc a,e, Jérôme Leprince a,e, Youssef Anouar a, Marie Picot a, Nicolas Chartrel a, Gaëtan Prévost a,f

aInstitute for Research and Innovation in Biomedicine (IRIB), UNIROUEN, INSERM U1239, Laboratory of Neuronal and Neuroendocrine Differentiation and Communication (DC2N), Normandie University, Rouen, France; bInstitute for Research and Innovation in Biomedicine (IRIB), Department of Biological Resources (SRB), UNIROUEN, Normandie University, Rouen, France; cInstitute for Research and Innovation in Biomedicine (IRIB), UNIROUEN, Animal Behaviour Platform SCAC, Normandie University, Rouen, France; dCenter for Taste and Feeding Behaviour, CNRS (UMR6265), INRA (UMR1324), AgroSup Dijon, Université de Bourgogne-Franche Comté, Dijon, France; eCell Imaging Platform of Normandy, Normandie University, Rouen, France; fDepartment of Endocrinology, UNIROUEN, Rouen University Hospital, Diabetes and Metabolic Diseases, Normandie University, Rouen, France

Keywords
Brain · Glucose homeostasis · Insulin · Diabetes · Obesity

Abstract
Introduction: The aim of the study is to investigate whether acute or chronic central administration of the hypothalamic neuropeptide 26RFa may ameliorate the glycemic control of obese/diabetic mice. Methods: Mice were treated for 4 months with a high-fat (HF) diet and received a single i.c.v. injection of 26RFa (3 µg) or a chronic i.c.v. administration of the peptide during 28 days via osmotic minipumps (25 µg/day). i.p. and oral glucose (GLU) tolerance tests, insulin (INS) tolerance test, glucose-stimulated insulin secretion (GSIS), food/water intake, horizontal/vertical activity, energy expenditure, meal pattern, and whole-body composition were monitored. In addition, 26RFa and GPR103 mRNA expressions as well as plasma 26RFa levels were evaluated by RT-QPCR and radioimmunoassay. Results: Acute administration of 26RFa in HF mice induced a robust antihyperglycemic effect by enhancing INS secretion, whereas chronic administration of the neuropeptide is unable to improve glucose homeostasis in these obese/diabetogenic conditions. By contrast, chronic 26RFa treatment induced an increase of the body weight accompanied with an enhanced food intake and a decreased energy expenditure. Finally, we show that the HF diet does not alter the hypothalamic expression of the 26RFa/GPR103 neuropeptidergic system nor the levels of circulating 26RFa. Conclusion: Our data indicate that the central beneficial effect of 26RFa on glucose homeostasis, by potentiating GSIS, is preserved in HF mice. However, chronic administration of the neuropeptide is unable to balance glycaemia in these pathophysiological conditions, suggesting that the hypothalamic 26RFa/GPR103 neuropeptidergic system mainly affects short-term regulation of glucose metabolism.

© 2022 S. Karger AG, Basel
Introduction

Accumulated studies during the last decade provided the evidence that glucose (GLU) homeostasis is driven by a complex interplay between the pancreatic islets and the brain [1, 2]. This concept is illustrated, for instance, by the fact that the pancreatic islets are richly innervated by both sympathetic and parasympathetic fibers, with the former capable of powerfully inhibiting glucose-stimulated insulin secretion (GSIS) and the latter having the opposite effect [3, 4]. The crucial role of the brain in the control of glucose metabolism is supported by the observations that the nuclei of the hypothalamus, i.e., the arcuate nucleus (Arc), the paraventricular nucleus, the ventromedial hypothalamic nucleus (VMN), and the lateral hypothalamic area, all contain specific glucose-sensing neuronal populations that have a key role in initiating the glucose counter-regulatory response to hypo- or hyperglycemia [3, 5]. Indeed, photoactivation of VMN SF1 neurons induces a diabetogenic-like effect characterized by hyperglycemia and glucose intolerance partially mediated by inhibition of insulin (INS) secretion and enhanced glucagon release [6]. It was also found that chemogenetic activation of Arc AgRP neurons causes peripheral INS resistance and increased plasma INS [7]. These observations and many others raise the hypothesis that aberrant activity of the hypothalamic glucoregulatory circuits may be involved in the pathogenesis of type 2 diabetes (T2D) and suggest that correcting these underlying defects should normalize glycemia in diabetic animals. Supporting this notion, it was recently reported that leptin is able to normalize glycemia in mice with severe INS deficiency, and that a single i.c.v. injection of FGF1 can induce remission of hyperglycemia in rodent models of T2D for weeks or months [8–10]. However, our understanding of the hypothalamic glucoregulatory neurocircuitry and its contribution to T2D pathogenesis is still in its infancy and deserves the identification of novel peptidergic neuronal systems regulating glucose homeostasis within the hypothalamus.

In this context, the neuropeptide 26RFa (also referred to as QRFP) and its receptor GPR103, discovered by us and others, are of particular interest [11–16]. Indeed, 26RFa- and GPR103-expressing neurons are primarily localized in the VMN and the lateral hypothalamic, and i.c.v. administration of the neuropeptide stimulates food intake by modulating the NPY/POMC system in the Arc [11, 15, 17–20]. The 26RFa/GPR103 neuropeptidergic system is also involved in the control of glucose homeostasis at the periphery [21–24]. 26RFa and GPR103 are strongly expressed by the β cells of the pancreatic islets and in the gut [21–23]. 26RFa attenuates drastically glucose-induced hyperglycemia by acting as an incretin and by increasing INS sensitivity, and 26RFa mutant mice exhibit an impaired regulation of glucose homeostasis [22, 24]. In obese/diabetic mice, a loss of the incretin activity of 26RFa and a marked reduction of its INS-sensitive effect are observed [25]. Interestingly, this 26RFa resistance is associated with a downregulation of the 26RFa receptor in the pancreatic islets and INS target tissues [25].

Finally, we promoted, very recently, the evidence that the 26RFa neuronal system of the hypothalamus plays a key role in the central regulation of glucose homeostasis by lowering glucose-induced hyperglycemia and by mediating central INS-induced peripheral INS secretion [26]. With regard to all of these observations, the aim of the present study was to investigate whether acute or chronic central administration of 26RFa may ameliorate the glycemic control of obese/diabetic mice.

Materials and Methods

Animals

Male C57Bl/6 mice (Janvier Laboratory, Le Genest-Saint-Isle, France), weighing 22–25 g, were housed at 5 animals per cage, with free access to standard diet (U.A.R., Villemoisson-sur-Orge, France) and tap water. They were kept in a ventilated room at a temperature of 22 ± 1°C under a 12-h light/12-h dark cycle (light on between 7 h and 19 h). All the experiments were carried out between 09.00 h and 18.00 h in testing rooms adjacent to the animal rooms. Animal manipulations were performed according to the European Community Council Directive of November 24, 1986 (86/609: EEC) and were conducted by authorized investigators. Mice were submitted to a dietary challenge at 2 months of age by using a standard chow (U8200G10R; energy density: 2,830 kcal/kg; Scientific Animal Food & Engineering [SAFE], Augy, France) or a high-fat (HF) diet (U8978; energy density: 5,283 kcal/kg; SAFE). Experiments were performed after 3 months of dietary challenge. Our Institutional Animal Use and Care Committee (CENOMAX, Agreement of the Ministry of Research, No. 54) approved the experimental procedures (Agreement No.: #11752).

Surgery

Mice were anesthetized with isoflurane and placed in a stereotaxic frame. A stainless steel 26-gauge guide cannula (Phymep, Paris, France) was inserted into the right lateral ventricle (0.8 mm lateral to bregma and 2.2 mm ventral to dura mater). A stainless dummy small cap for 26-gauge cannula (Phymep) was inserted to prevent occlusion of the guide cannula and leakage of the cerebrospinal fluid. The cannula placement was secured with dental acrylic cement. At the end of the surgery, animals were treated with a nonsteroidal anti-inflammatory drug, buprenorphine, after guide cannula placement. Following the surgery, all animals were allowed to recover for 2 weeks and were handled 3 times in order to be habituated to handling by researchers. Correct cannula posi-
ioning was confirmed by histological examination after trypan blue injection.

For chronic i.c.v. infusion, mice were implanted with a cannu-
la to the right lateral ventricle using a brain infusion kit 3 (Al-
zet®, DURECT, Cupertino, CA, USA) at stereotaxic coordinates:
0.85 mm lateral, 0.22 mm posterior to bregma, and 2.5 mm below
the skull surface. The cannula was connected to a subcutaneous
28-day osmotic minipump (Alzet®) attached to polyethylene tube-
bearing an osmotic pump for 28 days (25 µg/day) and the other one that
received HEPES via the same procedure. After implantation of the
pumps, mice were placed in a combined indirect calorimetry sys-
tem for habituation (PhenoMaster, TSE Systems GmbH, Bad
Homburg, Germany). Ten days after pump implantation, food/
water intake, horizontal/vertical activity, and energy expenditure
were measured for 5 days. At the end of these calorimetric experi-
ments, mice were placed in individual BioDAQ cages (Research
Diets, Inc., New Brunswick, NJ, USA), acclimated for 3 days, and
metabolic biomarkers were measured on plasma samples using
IDEXX Catalyst One technology (Laboratories, Inc., Westbrook,
ME, USA).

Table 1. Sequence of the primers used for the Q-PCR experiments

| Name       | Forward | Reverse |
|------------|---------|---------|
| Mouse β-actin | AGATCAAGATCATGGTCTCCTCCTG | CCCAGCTCGTAACAGTTCGG |
| Mouse 26RFa | GAAGGGGACCACAGAGCATC | GTCTTGGCTTCCCTGAGCCAA |
| Mouse GPR103 | CACTGCTGTTGGACGGAAT | CCTTCCGGTGTAGTACTGCC |
| Mouse InsR | ATGGGCTTCCGGAGAGGAT | GGAATGTCATACCAAGGCGAC |
| Mouse IL6 | GGGACTGTGCTGGTGTACAA | AGACGTCTGTTGGAGTGGT |

InsR, insulin receptor.

Blood Glucose and INS Measurements in Mice

For chronic i.c.v. infusion, mice were placed in individual
BioDAQ cages (Research Diets, Inc., New Brunswick, NJ, USA), acclimated for 3 days, and
meal pattern was evaluated for 3 days. After this period, mice were
placed into standard individual cages until euthanasia. During the
4th and last week of treatment, mice were submitted to metabolic
assays. Throughout the treatment, the body weight was measured
each day and 6-h fasting glycemia and insulinemia were measured
each week. At the end of the treatment, whole-body composition
was assessed on vigil animals, using MiniSpec LF110 (Bruker Wis-
sembourg, France), a fast nuclear magnetic resonance method, and
mice were euthanized. Tissues and plasma samples were collected.
Metabolic biomarkers were measured on plasma samples using
IDEXX Catalyst One technology (Laboratories, Inc., Westbrook,
ME, USA).

26RFa Radioimmunoassay

Quantification of 26RFa in plasma samples was carried out us-
ing a specific radioimmunoassay setup in the laboratory [17]. For
the radioimmunoassay procedure, each plasma sample was diluted
(1:1) in a solution of water/trifluoroacetic acid (99.9:0.1; vol/vol).
Diluted plasmas were pumped at a flow rate of 1.5 mL/min through
one Sep Pak C18 cartridge. Bound material was eluted with aceto-
nitrile/water/trifluoroacetic acid (50:49.9:0.1; vol/vol/vol), and
acetonitrile was evaporated under reduced pressure. Finally, the
dried extracts were resuspended in 0.1 M PBS and assayed for
26RFa.
Statistical Analysis

Statistical analysis was performed with GraphPad Prism (version 6). A Kruskal-Wallis test with Dunn’s multiple comparison test or ordinary one-way ANOVA with Sidak’s multiple comparison was used for comparisons between different groups. ANOVA two ways were used for repeated measures. A post hoc comparison using a Bonferroni, Tukey, or Sidak test was applied according to ANOVA results. All data represent means ± SEM. Statistical significance was set up at \( p < 0.05 \).

Results

Effects of Acute i.c.v. Administration of 26RFa on the Glycemic Control of Obese/Diabetic Mice

The HF diet challenge induced a significant \( (p < 0.01) \) weight gain right from the first month of treatment (Fig. 1a). Plasma glucose was also significantly increased \( (p < 0.01) \) after 1 month of HF diet (Fig. 1b). After 4 months, the HF mice exhibited a fasting glycemia of 2.37 ± 0.17 g/L, whereas that of the SD mice at the same time was 1.65 ± 0.11 g/L \( (p < 0.01; \) Fig. 1b). By contrast, plasma 26RFa levels were not modified all along the HF treatment as compared to SD mice (Fig. 1c). However, it is noteworthy that, in the two groups of mice (SD and HF), plasma 26RFa increased gradually with the age of the animals to reach a significance at the end of the experimental protocol (4 months; \( p < 0.05 \) ) (Fig. 1c).

The impact of i.c.v. administration of 26RFa was investigated on basal glycemia in HF mice. As illustrated in Figure 1d, 26RFa did not affect significantly basal plasma glucose levels during the 90-min period of the test. By contrast, an IPGTT revealed that 26RFa significantly attenuated \( (p < 0.01) \) the hyperglycemia induced by an i.p. glucose challenge in HF mice (Fig. 1e). Concurrently, an acute GSIS (AGSIS) test indicated that i.c.v. injection of 26RFa allowed to maintain high level of INS production as compared to the mice that did not receive 26RFa and exhibited a progressively decreased INS production during the test (Fig. 1f). Consequently, the ratio glucose/INS (during the IPGTT) was significantly lower \( (p < 0.01) \) in 26RFa-injected mice as compared to HEPES-injected mice (Fig. 1g).

We investigated whether the expression of 26RFa and its receptor was impaired in a diabetogenic environment using in vitro and in vivo complementary approaches. First, we used the hypothalamic neuronal cell line m-HypoA-59 that expresses 26RFa and its receptor GPR103 as a model of 26RFa-producing neurons, and we treated these cells with palmitate to mimic conditions of lipotoxicity. Our data revealed that the palmitate treatment did not alter the expression of 26RFa and GPR103 in the m-HypoA-59 neuronal cells (Fig. 2a). The increased expression of IL6 in these cells indicates that palmitate-induced lipotoxicity was effective (Fig. 2a). The hypothalamic expression of 26RFa and GPR103 was also followed during the 4-month HF challenge of the mice. A significant increase of the expression of 26RFa \( (p < 0.05) \) and GPR103 \( (p < 0.05–0.01) \) was observed 1 and 2 months after the beginning of the protocol in the HF as well as in the SD mice (Fig. 2b, c). However, the HF treatment did not alter the expression of the neuropeptide and its receptor in the hypothalamus as compared to the SD treatment (Fig. 2b, c). Then, we compared the effects of 26RFa and INS (injected i.c.v.) during a glucose tolerance test in HF mice. 26RFa induced a robust antihyperglycemic effect, whereas the INS-induced attenuation of the glycemia was abolished in HF mice (Fig. 2d). Co-administration of 26RFa and INS induced an antihyperglycemic effect similar in terms of amplitude and kinetic to that of 26RFa alone (Fig. 2d). Concurrently, we evaluated the impact of i.c.v. injection of INS on the hypothalamic expression of 26RFa and that of its receptor (insulin receptor) in HF mice and compared it to that of SD mice. We found that INS slightly increased 26RFa expression in SD mice but not in HF animals (Fig. 2e). By contrast, the hormone does not impact the expression of its own receptor whatever the diet challenge given (Fig. 2e).

A 1 month in vivo protocol was set up to determine the impact of continuous administration of 26RFa on the metabolic and glycemic phenotype of the obese/diabetic mice (Fig. 3a). Chronic i.c.v. administration of 26RFa via Alzet pumps, 10 weeks after the beginning of the HF diet, induced a significant increase in body weight as compared to the mice receiving HEPES \( (p < 0.001; \) Fig. 3b). Concurrently, measurement of cumulative food intake for 5 days revealed that the mice treated with 26RFa ate more than the mice receiving the HEPES chronically \( (p < 0.001; \) Fig. 3c). This higher food consumption observed in 26RFa-treated mice was associated with a significant increase in the meal size (online suppl. Fig. S1a; see www.karger.com/doi/10.1159/000522287 for all online suppl. material), whereas the meal frequency, duration, and total mealtime were not impaired (online suppl. Fig. S1b–d). The increased food intake observed in 26RFa-treated mice was accompanied by a small increase of water intake (Fig. 3d) and a tendency to decreased energy expenditure (Fig. 3e). Concurrently, the locomotor activity (horizontal and vertical) was assessed, but no significant difference between the HF mice receiving 26RFa and those chronically injected with HEPES was observed (online suppl. Fig. S1e, f).
Fig. 2. Hypothalamic expression of the 26RFa/GPR103 peptidergic system in obese/diabetogenic condition: impact of insulin. a Expression levels of 26RFa, GPR103, and IL6 mRNA in m-HypoA-59 cells treated with palmitate (100 µM, red) for 12 h versus nontreated cells (black) (N = 4). b, c Expression levels of 26RFa and GPR103 mRNA in the hypothalamus of 4-month HF-treated mice (red) versus mice fed a standard chow (black) (n = 4–5/conditions). d Effect of an acute i.c.v. administration of insulin (10 mIU, red), 26RFa (3 µg, light blue), or insulin (10 mIU) + 26RFa (3 µg) (dark blue) on glycemia during a glucose tolerance test in mice submitted to a 4-month HF diet (n = 8–17/group). AUC represent the areas under the curves. e Expression levels of 26RFa and the InsR mRNA in the hypothalamus following the central administration of insulin (10 mIU, red) versus animals injected with the vehicle (black) (n = 7–8/group). Values represent the mean ± SEM. *, p < 0.05; **, p < 0.01 compared to mice fed a standard diet. InsR, insulin receptor.

Fig. 1. Effects of acute i.c.v. administration of 26RFa on the glycemic control of obese/diabetic mice. a–c Evolution of body weight (a), basal glycemia (b), and plasma 26RFa levels (c) during a 4-month standard diet (black) or HF diet (red) (n = 5 mice/condition). d–g Effect of central administration of 26RFa (3 µg) in HF mice on basal glycemia (d), during a glucose tolerance test (IPGTT) (e), an AGSIS (f), and the glucose/insulin ratio measured during an IPGTT (g) (n = 10–14/group). Values represent the mean ± SEM and AUC the areas under the curves. *, p < 0.05; **, p < 0.01 compared to mice injected with the vehicle.
Fig. 3. Effects of chronic i.c.v. administration of 26RFa on the metabolic and glycemic phenotype of the obese/diabetic mice. 

a. Schematic representation of the experimental protocol used to evaluate the impact of 26RFa, injected chronically (25 µg/day), on various metabolic and glycemic parameters during a 4-month HF diet. 

b. Evolution of the body weight of the HF mice for 28 days after the implantation of the Alzet pumps filled with 26RFa (red) or the vehicle (black) (n = 7–8/ groups).

c–e. Evolution of food intake (c), water intake (d), and energy expenditure (e) for 5 days in 26RFa-treated mice (red) versus HEPES-treated mice (black) (n = 7–8/group).

f, g. Evolution of basal glycemia (f) and insulinemia (g) in HF mice during the chronic treatment with 26RFa (red) or the vehicle (black) (n = 7–8).

h. Fat mass/body weight ratio measured after a 28-day chronic treatment with 26RFa (red) or the vehicle (black) (n = 7–8/group). Values represent the mean ± SEM. ***p < 0.001; ****p < 0.0001 compared to mice injected with the vehicle.
Evaluation of basal glycemia in HEPES- or 26RFa-treated mice did not reveal any significant difference between the two groups during the chronic treatment (Fig. 3f). In contrast, evaluation of insulinemia at the same time showed higher (although not significant) plasma INS levels in 26RFa-treated mice (Fig. 3g). Body fat mass also tended to be higher in 26RFa chronically injected mice (Fig. 3h), whereas the biochemical parameters were similar between the 26RFa-treated mice and the HEPES-treated animals (Table 2).

The impact of chronic i.c.v. administration of 26RFa on the "glycemic" phenotype of the HF mice was investigated using complementary in vivo tests performed during the last week of treatment. A IPGTT revealed a slight increase of glucose-induced hyperglycemia in the 26RFa-treated mice ($p = 0.07$) that was associated with slightly higher plasma INS levels during the first 15 min of the test as compared to HEPES-treated animals (Fig. 4a, b). However, calculation of the glucose/INS ratio during the first 30 min of the IPGTT did not reveal any difference between the two groups (Fig. 4c). ITT showed that INS tolerance was not significantly altered in 26RFa-injected mice (Fig. 4d). An OGTT revealed that the glycemic profile following the oral glucose load was not impaired in 26RFa-treated mice versus mice receiving HEPES (Fig. 4e). Finally, a Q-PCR experiment indicated that hypothalamic expression of the 26RFa receptor was not impaired by the chronic treatment with its ligand (Fig. 4f).

**Table 2. Measurement of metabolic parameters in HF mice chronically treated or not with 26RFa**

| Parameter | HEPES-treated mice | 26RFa-treated mice | $p$ value |
|-----------|---------------------|---------------------|-----------|
| GLU, g/L  | 2.44±0.10           | 2.54±0.10           | 0.51      |
| INS, µIU/mL | 21.54±2.79         | 29.08±4.81         | 0.22      |
| CHOL, g/L | 1.53±0.07           | 1.57±0.07           | 0.79      |
| TRIG, g/L | 0.87±0.05           | 0.93±0.04           | 0.46      |
| ALT, U/L  | 48±7                | 58±7                | 0.39      |
| AST, U/L  | 171±18              | 189±16              | 0.57      |
| ALKP, U/L | 40±1                | 40±3                | 0.90      |
| LIPA, U/L | 555±20              | 549±13              | 0.68      |

GLU, glucose; INS, insulin; CHOL, cholesterol; TRIG, triglycerides; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALKP, alkaline phosphatase; LIPA, lipase.

Discussion/Conclusion

During the last decade, a number of studies revealed that the 26RFa/GPR103 neuropeptidergic system is a key player in the regulation of energy and glucose metabolism. Indeed, 26RFa produced by neurons of the hypothalamus regulates feeding behavior, and at the periphery, the neuropeptide produced by the gut and the pancreatic islets regulates glucose homeostasis by acting as an incretin [14, 22, 27]. However in obese/diabetic mice, the antihyperglycemic effect of 26RFa is significantly blunted due, at least in part, to a decreased expression of its receptor (GPR103) both in beta cells and in INS target tissues such as the adipose tissue and the muscles [25]. More recently, we found that the 26RFa neuronal system of the hypothalamus was also able to promote GSIS and could be considered as a mediator for central INS effects on glucose tolerance [26]. With regard to these recent data, we thought important to evaluate the central effect of the 26RFa on glucose metabolism in a diabetogenic context.

In the present study, we show for the first time that an acute central administration of 26RFa in HF mice is still able to attenuate glucose-induced hyperglycemia by enhancing GSIS. This favorable central effect of 26RFa on INS secretion in this hyperglycemic model is therefore preserved and very similar in terms of amplitude and kinetic to that observed in euglycemic mice [26]. The dose we used for the acute i.c.v. administration of 26RFa (3 µg) is very low compared to that used for peripheral administration in our previous works and does not alter glucose tolerance when administered i.p. [22]. It is thus unlikely that the effects of 26RFa after an i.c.v. administration could be due to an infusion of the neuropeptide to the systemic circulation.

We also show that the obese/diabetogenic status of the mice does not impair the hypothalamic expression of 26RFa and GPR103, contrasting to what we observed at the periphery [25]. Supporting this observation, we also found that the expression of the 26RFa and GPR103 in the neuronal hypothalamic cell line m-HypoA-59 is not al-
tered under lipotoxic culture condition. It thus appears that the hypothalamic 26RFa/GPR103 system is protected from HF-induced defects and allows to maintain the antihyperglycemic effect of 26RFa, whereas the same peptidergic system is profoundly altered at the periphery, resulting in a loss of the incretin effect during of 26RFa.

Fig. 4. Effects of chronic i.c.v. administration of 26RFa on the glucose homeostasis of the obese/diabetic mice. a i.p. glucose tolerance test performed the last week of the chronic administration of 26RFa (red) or the vehicle (black) (n = 7–9/group). b AGSIS test performed the last week of the chronic administration of 26RFa (red) or the vehicle (black) (n = 7–9/group). c Glucose/insulin ratio measured during the first phase of the IPGTT in (a). d Insulin tolerance test performed the last week of the chronic administration of 26RFa (red) or the vehicle (black) (n = 7–9/group). e Oral glucose tolerance test performed the last week of the chronic administration of 26RFa (red) or the vehicle (black) (n = 7–9/group). f Hypothalamic expression of GPR103 mRNA measured the last week of the chronic administration of 26RFa (red) or the vehicle (black) (n = 7–9/group). Values represent the mean ± SEM.
However, we observed that, in these HF mice, central INS administration was not able to promote 26RFa hypothalamic expression, whereas we previously found that, in euglycemic mice, brain INS induced hypothalamic 26RFa expression and secretion during a glucose load [26]. In the same study, we also promoted the evidence that the 26RFa/GPR103 neuronal system mediates the central antihyperglycemic effect of INS [26]. Altogether, these observations support the idea that the inability of INS to trigger the 26RFa/GPR103 system in hyperglycemic condition is an additional consequence of the well-known central INS resistance observed in obesity/diabetes and probably participates to the glucose intolerance developed by the HF mice.

In addition, while the HFD does not impair the hypothalamic 26RFa/GPR103 system, our data reveal that the age influences the expression of this peptidergic system. Indeed, plasma 26RFa as well as hypothalamic expression of GPR103 and 26RFa is upregulated over time in our protocol in both normal and HFD mice. To our knowledge, it is the first time that evolution of the 26RFa peptidergic system is described over time. By contrast, previous studies reported stable or decreasing hypothalamic expression of other neuropeptides regulating energy metabolism such as NPY or AgRP over time, but the experimental conditions were different with extreme age analysis or long fasting period [28, 29]. Because of the limited length of our analysis, we speculate that the observed up-regulation of the 26RFa/GPR103 system may correspond to a maturation of this neuropeptidergic system rather than a sign of aging. However, further studies with extreme age analyses are warranted to confirm this hypothesis.

Given the beneficial effects of acute administration of 26RFa on glucose metabolism in HF mice, it was tempting to evaluate the impact of chronic administration of the peptide on metabolic parameters in an obese/hyperglycemic pathophysiological context. By using osmotic minipumps, 26RFa was chronically administered i.c.v. for 28 days and the stability of the peptide was checked all along the protocol by coupling HPLC analysis with mass spectrometry detection (data not shown). In 26RFa-treated mice, a significant increase of the body weight was observed during the experimental protocol that was accompanied with an enhanced food intake and a slight decrease of energy expenditure. Despite this increase of body weight, fasting blood glucose levels of the 26RFa-treated mice were not significantly different as compared to mice that did not receive the peptide. In addition, fasting insulinemia tended to be higher all along the protocol in the 26RFa-treated mice. However, the IPGTT and ITT tests revealed a slightly more pronounced glucose intolerance in 26RFa-treated mice, whereas GSIS was not improved, in contrast to what we observed after an acute administration of 26RFa. Considering the weight gain, our data are in agreement with previous results observed with the elongated form of 26RFa, 43RFa [19]. Indeed, this study reports that central chronic infusion of 43RFa for 13 days is associated with a significant increase of body weight and fat mass. Similarly to what we found, in this study, food intake was enhanced and energy expenditure was decreased because of a reduction of thermogenesis [19]. Considering the metabolic parameters, our results are not consistent with those of Moriya et al. [19], as these authors report higher fasting glucose levels as well as insulinemia in 43RFa-treated mice. However, no data concerning INS sensitivity or GSIS were available in this study. Supporting our findings, a previous study reports that acute administration of another incretin, GLP-1, enhances GSIS, whereas a 3-day i.c.v. infusion of the peptide does not affect INS secretion stimulation [30].

Altogether, these observations indicate that central chronic infusion of 26RFa or 43RFa stimulates appetite, leading to a significant increase of body weight and adiposity. In addition, the two peptides are able to promote fasting INS secretion that may also represent a major determinant for adiposity. Our results also suggest that fasting hyperinsulinemia results from a direct effect of the peptide on INS secretion rather than from an indirect effect simply due to the INS resistance caused by 26RFa-induced adiposity. Indeed, we did not find any significant difference in INS sensitivity during ITT between the 26RFa-treated mice and the control group. Nevertheless, it appears that, in the context of chronic central infusion of 26RFa in HF mice, the sustained orexigenic activity of the neuropeptide system leading to a significant weight gain exceeds the expected benefits of the peptide on glucose metabolism which is altered, especially considering the GSIS results. A decrease of GPR103 activity may be discarded as hypothalamic expression of the 26RFa receptor is not impaired in 26RFa-treated mice. However, further studies are warranted to understand whether the attenuation of the GSIS associated with chronic 26RFa treatment is linked to a central dysfunction of the 26RFa pathway rather than a beta cell failure. An alternative explanation, which has been suggested for GLP-1, would be that brain 26RFa might be specifically involved in the short-term regulation of glucose metabolism (especially postprandial), whereas its effects on the long-term control.
of glucose homeostasis would be mainly related to changes in body weight [30].

In conclusion, the present study provides the first evidence that in obese/diabetic condition, the 26RFa/GPR103 neuropeptidergic system is not altered in the hypothalamus, in contrast to the periphery. Indeed, the beneficial effect of 26RFa on glucose homeostasis, by potentiating GSIS, is preserved in HF mice. However, chronic central administration of the neuropeptide is unable to balance glycemia in these pathophysiological conditions, suggesting that the hypothalamic 26RFa/GPR103 neuropeptidergic system mainly affects short-term regulation of glucose metabolism.

**Statement of Ethics**

Animal manipulations were performed according to the European Community Council Directive of November 24, 1986 (86:609: EEC), and were conducted by authorized investigators. Our Institutional Animal Use and Care Committee (CENOMAX, Agreement of the Ministry of Research No. 54) approved the experimental procedures (Agreement No.: #11752).

**Conflict of Interest Statement**

The authors have no competing interests to declare.

**References**

1. Schwartz MW, Seeley RJ, Tschöp MH, Woods SC, Morton GJ, Myers MG, et al. Cooperation between brain and islet in glucose homeostasis and diabetes. *Nature*. 2013;503(7474):59–66.
2. Pozo M, Claret M. Hypothalamic control of systemic glucose homeostasis: the pancreas connection. *Trends Endocrinol Metab*. 2018; 29(8):581–94.
3. Rosario W, Singh I, Wautelet A, Patterson C, Flak J, Becker TC, et al. The brain-to-pancreatic islet neuronal map reveals differential glucose regulation from distinct hypothalamic regions. *Diabetes*. 2016;65(9):2711–23.
4. Thorens B. Central control of glucose homeostasis: the brain-endocrine pancreas axis. *Diabetes Metab*. 2010;36(Suppl 3):S45–9.
5. Shimazu T, Fukuda A, Ban T. Reciprocal influences of the ventromedial and lateral hypothalamic nuclei on blood glucose level and liver glycogen content. *Nature*. 1966; 210(5041):1178–9.
6. Meek TH, Nelson JT, Matsen ME, Dorfman MD, Guyenet SJ, Damian Y, et al. Functional identification of a neocircuit regulating blood glucose. *Proc Natl Acad Sci USA*. 2016; 113(14):E2073–82.
7. Steculorum SM, Ruud J, Karakashiliot I, Backes H, Enström Ruud L, Timper K, et al. AgRP neurons control systemic insulin sensitivity via myostatin expression in brown adipose tissue. *Cell*. 2016;165(1):125–38.
8. Fujikawa T, Berglund ED, Patel VR, Ramadori G, Vianna CR, Yong L, et al. Leptin engages a hypothalamic neurocircuitry to permit survival in the absence of insulin. *Cell Metab*. 2013;18(3):431–44.
9. Scarlett JM, Rojas JM, Matsen ME, Kaiyali K, Stefanovski D, Bergman RN, et al. Central injection of fibroblast growth factor 1 induces sustained remission of diabetic hyperglycemia in rodents. *Nat Med*. 2016;22(7):800–6.
10. Scarlett JM, Kota K, Brown JM, Rojas JM, Matsen ME, Acharya NK, et al. Peripheral mechanisms mediating the sustained antidiabetic action of FGF1 in the brain. *Diabetes*. 2019;68(3):654–64.
11. Chartrel N, Dujardin C, Amoury Y, Leprince J, Decker A, Clerens S, et al. Identification of a neuropeptide of the RFamide peptide family with orexigenic activity. *Proc Natl Acad Sci USA*. 2003;100(25):15247–52.
12. Fukusumi S, Yoshida H, Fujii R, Maruyama M, Komatsu H, Habata Y, et al. A new peptidic ligand and its receptor regulating adrenal function in rats. *J Biol Chem*. 2003; 278(47):46387–95.
13. Jiang Y, Luo L, Gustafson EL, Yadav D, Laverty M, Murgolo N, et al. Identification and characterization of a novel RF-amide peptide ligand for orphan G-protein-coupled receptor SP91155. *J Biol Chem*. 2003;278(30):27652–7.
14. Chartrel N, Alonzeau J, Alexandre D, Jeandel L, Alvear-Perez R, Leprince J, et al. The RFamide neuropeptide 26RFa and its role in the control of neuroendocrine functions. *Front Neuroendocrinol*. 2011;32(4):387–97.
15. Takayasu S, Sakurai T, Iwasaki S, Teranishi H, Yamanaka A, Williams SC, et al. A neuropeptide ligand of the G protein-coupled receptor GPR103 regulates feeding, behavioral arousal, and blood pressure in mice. *Proc Natl Acad Sci USA*. 2006;103(19):7438–43.
16. Leprince J, Bagnoù D, Bureau R, Fukusumi S, Granata R, Hinuma S, et al. The Arg-Phe-amide peptide 26RFa/glutamine RF-amide peptide and its receptor: IUPHAR review 24. *Br J Pharmacol*. 2017;174(20):3573–607.

**Funding Sources**

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) (Grant No.: U1239), the University of Rouen, the Institute for Research and Innovation in Bio-medicine (IRIB) (recurrent funding), the “Fondation pour la Recherche Médicale” (Grant No.: DEA 20140629966), the “Société Françophonie du Diabète” (Grant No.: R16038EE). The present study was also co-funded by European Union and Normandie Regional Council. Europe gets involved in Normandie with European Regional Development Fund (ERDF).

**Author Contributions**

M.A.L.S., N.C., A.A., and G.P. contributed to the study design and interpretation, and wrote the manuscript. M.A.L.S., S.T., A.A., J.M., M.D., J.L.D.O., and J.C.D.O. performed the in vivo experiments on mice. M.A.L.S., J.R., H.B., and M.D. contributed to the PCR experiments. A.B. and E.N. performed the INS assays. B.L. and J.L. produced synthetic 26RFa. J.L., M.P., and Y.A. revised and approved the final version of the manuscript. N.C. and G.P. are the guarantors of this work, as such, had full access to all the data in the study, and take responsibility for the integrity of the data and the accuracy of the data analysis.

**Data Availability Statement**

All data relevant to the study are included in the article or uploaded as online supplementary information.
17 Bruzzone F, Lectez B, Tollemer H, Leprince J, Dujardin C, Rachidi W, et al. Anatomical distribution and biochemical characterization of the novel RFamide peptide 26RFa in the human hypothalamus and spinal cord. J Neurochem. 2006;99(2):616–27.
18 Bruzzone F, Lectez B, Alexandre D, Jégou S, Mounien L, Tollemer H, et al. Distribution of 26RFa binding sites and GPR103 mRNA in the central nervous system of the rat. J Comp Neurol. 2007;503(4):573–91.
19 Moriya R, Sano H, Umeda T, Ito M, Taka-hashi Y, Matsuda M, et al. RFamide peptide QRFP43 causes obesity with hyperphagia and reduced thermogenesis in mice. Endocrinology. 2006;147(6):2916–22.
20 Lectez B, Jeandel L, El-Yamani FZ, Arthaud S, Alexandre D, Mardargent A, et al. The orexigenic activity of the hypothalamic neuropeptide 26RFa is mediated by the neuropeptide Y and proopiomelanocortin neurons of the arcuate nucleus. Endocrinology. 2009;150(5):2342–50.
21 Granata R, Settanni F, Trovato L, Gallo D, Gesundo I, Nano R, et al. RFamide peptides 43RFa and 26RFa both promote survival of pancreatic β-cells and human pancreatic islets but exert opposite effects on insulin secretion. Diabetes. 2014;63(7):2380–93.
22 Prévost G, Jeandel L, Arabo A, Coëffier M, El Ouahli M, Picot M, et al. Hypothalamic neuropeptide 26RFa acts as an incretin to regulate glucose homeostasis. Diabetes. 2015;64(8):2805–16.
23 Prévost G, Picot M, Le Solliec MA, Arabo A, Berrahmoune H, El Mehdi M, et al. The neuropeptide 26RFa in the human gut and pancreas: potential involvement in glucose homeostasis. Endocr Connect. 2019;8(7):941–51.
24 El-Mehdi M, Takhlidjt S, Khiar F, Prévost G, do Rego JL, do Rego JC, et al. Glucose homeostasis is impaired in mice deficient in the neuropeptide 26RFa (QRFP). BMJ Open Diabetes Res Care. 2020;8(1):e000942.
25 Prévost G, Arabo A, Le Solliec MA, Bons J, Picot M, Maucotel J, et al. Neuropeptide 26RFa (QRFP) is a key regulator of glucose homeostasis and its activity is markedly altered in obese/hyperglycemic mice. Am J Physiol Endocrinol Metab. 2019;317(1):E147–E157.
26 El Mehdi M, Takhlidjt S, Devêre M, Arabo A, Le Solliec MA, Maucotel, et al. Identification of a discrete neuronal circuit that relays insulin signaling into the brain to regulate glucose homeostasis. Medrxiv. 2021.
27 Chartrel N, Picot M, El Medhi M, Arabo A, Berrahmoune H, Alexandre D, et al. The neuropeptide 26RFa (QRFP) and its role in the regulation of energy homeostasis: a mini-review. Front Neurosci. 2016;10:549.
28 Gruenewald DA, Marck BT, Matsumoto AM. Fasting-induced increases in food intake and neuropeptide Y gene expression are attenuated in aging male brown Norway rats. Endocrinology. 1996;137(10):4660–7.
29 Kaneda T, Makino S, Nishiyama M, Asaba K, Hashimoto K. Differential neuropeptide responses to starvation with ageing. J Neuroendocrinol. 2001;13(12):1066–75.
30 Tudurí E, Beiroa D, Porteiro B, López M, Diéguez C, Nogueiras R. Acute but not chronic activation of brain glucagon-like peptide-1 receptors enhances glucose-stimulated insulin secretion in mice. Diabetes Obes Metab. 2015;17(8):789–99.