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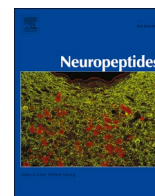
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## Interactions between the regulatory peptide 26RFa (QRFP) and insulin in the regulation of glucose homeostasis in two complementary models: The high fat 26RFa-deficient mice and the streptozotocin insulin-deficient mice

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### ABSTRACT

The regulatory peptide 26RFa (QRFP) is involved in the control of glucose homeostasis at the periphery by acting as an incretin, and in the brain by mediating the central antihyperglycemic effect of insulin, indicating the occurrence of a close relationship between 26RFa and insulin in the regulation of glucose metabolism. Here, we investigated the physiological interactions between 26RFa and insulin in two complementary models i.e. a model of obese/hyperglycemic mice deficient for 26RFa and a model of diabetic mice deficient for insulin. For this, transgenic 26RFa-deficient mice were made obese and chronically hyperglycemic by a 3-month high fat diet (HFD) and second group of mice was made diabetic by destruction of the  $\beta$  cells of the pancreatic islets using a single injection of streptozotocin. Our data reveal that 26RFa deficiency does not impact significantly the “glycemic” phenotype of the HFD mice. The pancreatic islets, liver, white adipose tissue masses are not altered by the lack of 26RFa production but the brown adipose tissue (BAT) weight is significantly increased in these animals. In diabetic insulin-deficient mice, the injection of 26RFa does not exhibit any beneficial effect on the impaired glucose homeostasis characterizing this model. Finally, we show that streptozotocin diabetic mice display lowered plasma 26RFa levels as compared to untreated mice, whereas the expression of the peptide in the duodenum is not affected.

Taken together, the present results indicate that dysregulation of glucose homeostasis in obese/hyperglycemic mice is not aggravated by the absence of 26RFa that may be compensated by the increase of BAT mass. In diabetic insulin-deficient mice, the antihyperglycemic effect of 26RFa is totally blunted probably as a result of the impaired insulin production characterizing this model, avoiding therefore the action of the peptide.

### 1. Introduction

26RFa (also referred to as QRFP) is a regulatory peptide initially discovered in the brain and by a bioinformatic approach (Chartrel et al., 2003; Fukusumi et al., 2003; Jiang et al., 2003). 26RFa is present in all vertebrate phyla from fish to human (Chartrel et al., 2011; Chartrel et al., 2016), and was identified as the cognate ligand of the human orphan G protein-coupled receptor, GPR103 (2-4 Takayasu et al., 2006;

Chartrel et al., 2011; Leprince et al., 2017). Neuroanatomical observations revealed that 26RFa- and GPR103-expressing neurons are primarily localized in hypothalamic nuclei involved in the control of feeding behaviour (Chartrel et al., 2003; Fukusumi et al., 2003; Takayasu et al., 2006; Bruzzone et al., 2006; Bruzzone et al., 2007). Indeed, i.c.v. administration of 26RFa stimulates food intake (Chartrel et al., 2003; Moriya et al., 2006; Takayasu et al., 2006; Lectez et al., 2009), and the neuropeptide exerts its orexigenic activity by modulating

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the NPY/POMC system in the arcuate nucleus (Arc) (Lectez et al., 2009). 26RFa also stimulates food intake in birds (Ukena et al., 2010) and fish (Liu et al., 2009), indicating that 26RFa plays a crucial role in the central regulation of energy metabolism in all vertebrates.

Later on, it was reported that 26RFa/GPR103 neuropeptidergic system is also present at the periphery. We, and others, found that 26RFa and GPR103 are strongly expressed by the  $\beta$  cells of the pancreatic islets and all along the gut (Granata et al., 2014; Prevost et al., 2015; Prévost et al., 2019b). This anatomical distribution suggested a possible involvement of the regulatory peptide and its receptor in the control of glucose homeostasis. Indeed, i.p. administration of 26RFa attenuates glucose-induced hyperglycemia by increasing plasma insulin via a direct insulinotropic effect on the pancreatic  $\beta$  cells, and by increasing insulin sensitivity (Prevost et al., 2015; Prévost et al., 2019b). In addition, an oral glucose challenge induces a massive secretion of 26RFa by the gut into the blood, strongly suggesting that this neuropeptide regulates glycemia by acting as an incretin (Prevost et al., 2015). This incretin effect of 26RFa was assessed by the observation that administration of a GPR103 antagonist reduces the global glucose-induced incretin effect, and also decreases insulin sensitivity (Prévost et al., 2019a).

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 insulin-induced peripheral insulin secretion (El Mehdi et al., 2022). More specifically, we observed that 26RFa-expressing neurons of the hypothalamus co-express the insulin receptor and that i.c.v. administration of insulin induces the expression of 26RFa in these neurons. Concurrently, we found that the central antihyperglycemic effect of insulin is abolished in presence of a 26RFa receptor (GPR103) antagonist as well as in 26RFa-deficient mice. Finally, our data indicate that the hypothalamic 26RFa neurons are not involved in the central inhibitory effect of insulin on hepatic glucose production, but mediate the central effects of the hormone on its own peripheral production (El Mehdi et al., 2022).

Altogether, these findings reveal the occurrence of a close relationship between 26RFa and insulin in the peripheral and central control of glucose homeostasis. Consequently, in the present study, we investigated the physiological interactions between 26RFa and insulin production in two complementary models i.e. a model of obese/hyperglycemic mice deficient for 26RFa and a model of diabetic mice deficient for insulin.

## 2. Materials and methods

### 2.1. Animals

8 weeks old male C57Bl/6 mice (Janvier laboratory, Le Genest-Saint-Isle, France) were housed at 3–5 animals per cage, with free access to food and tap water. Animals were kept in a ventilated room at a temperature of  $22 \pm 1$  °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 24th 1986 (86/609: EEC), and were conducted by authorized investigators. Our Institutional Animal Use and Care Committee (CENOMAX, Agreement of the Ministry of Research n°54) approved the experimental procedures (Agreement n°: # 201710131556206, 201,710,131,556,206 and 2,018,072,716,228, 990).

#### 2.1.1. 26RFa knock-out mice

26RFa<sup>-/-</sup> (iCre knock-in) mice were obtained from Professor T Sakurai (International Institute for Integrative Sleep Medicine, Tsukuba, Ibaraki, Japan). The entire coding region of exon 2 of prepro-26RFa sequence was replaced by iCre sequence and pgk-Neo cassette as previously described (El-Mehdi et al., 2020). Genotypes were determined

by PCR of DNA mouse tail. 8–9 weeks old 26RFa<sup>+/+</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>-/-</sup> mice were submitted to a 3 month high fat diet (HFD) (U8978; energy density: 5283 kcal/kg; SAFE) and all experiments were performed after three months of dietary challenge.

#### 2.1.2. Streptozotocin-treated mice

Mice were deprived of food 6 h before streptozotocin injection. Then, mice received one single ip-injection of 150 mg/kg of streptozotocin diluted in citrate buffer (pH 4.5). After injection, water was replaced by a glucose solution (30%) to avoid hypoglycemia and mice were monitored for 6 h after injection. Seven days after the injection, fasted glycemia was measured and all of the mice that did not display hyperglycemia (>3 g/l) were excluded from the experimental protocol.

### 2.2. Blood glucose and insulin measurements in mice

Intraperitoneal/oral glucose tolerance tests and glucose stimulated insulin secretion test were performed on 6 h-fasted mice that received either 2 g/kg of glucose or 1 g/kg for the streptozotocin-treated mice. For the insulin tolerance test, mice were also fasted for 6 h and then received an ip-injection of 0.75 UI/kg of insulin. Pyruvate tolerance tests were performed on 16 h-fasted mice that received a 2 g/kg dose of pyruvate via ip administration. The effect of 26RFa on the various glycemic parameters in streptozotocin-treated mice was investigated using a dose of 500  $\mu$ g/kg of the peptide via ip administration.

In all experiments, plasma glucose concentrations were measured from tail vein samplings at various times using an AccuChek Performa glucometer (Roche Diagnostic, Saint-Egreve, France) or Xpress 2 glucometer (Nova Biomedical, Les Ullis, France) when glycemia was above 6 g/l. Plasma insulin concentrations (from tail vein samplings) were determined using an ultrasensitive mouse insulin AlphaLisa detection kit (cat number AL204C) from Perkin Elmer.

### 2.3. Morphological analysis of mice pancreas

Deparaffinized sections (15 $\mu$ m thick) of 26RFa<sup>+/+</sup>, 26RFa<sup>+/-</sup>, 26RFa<sup>-/-</sup> mice pancreas were used for immunohistochemistry. To detect insulin and glucagon immunolabeling in pancreas sections, the following primary antibodies were used: guinea pig polyclonal anti-insulin (1:50, Gene Tex, Irvine, CA) and mouse monoclonal anti-glucagon (1:1000, Sigma Aldrich). Alexa-conjugated antibodies (Invitrogen Life Technologies) including goat anti-guinea pig-488 (1:300) and donkey anti-mouse-594 (1:300) were used as secondary antibodies. Sections were counterstained with 1  $\mu$ g/ml 4',6-diamino-2-phenylindole (Sigma-Aldrich) in PBS for 90 s. Images were captured using a confocal Leica TCS SP-8-X microscope equipped with a 40 $\times$  objective and a Leica Thunder Imager 3D (DM6 B). The specificity of the immunoreactions was controlled by (1) substitution of the primary antibodies with Tris buffer saline (TBS; pH 7.4) and (2) preincubation of the primary antisera with the corresponding synthetic hormones ( $10^{-6}$  M). In both cases, no immunoreaction was observed.

To study the morphological architecture, pancreas slices were stained with hematoxylin/eosin and examined under a Leica Z6 APO microscope and a Nikon E600 light microscope. Quantitative, qualitative and morphological analysis of the pancreas sections were performed using the Image J software (NIH, Washington, DC).

### 2.4. Quantitative RT-PCR (qRT-PCR)

Total RNA from duodenal fragments was isolated as previously described (Le Solliec et al., 2022). Relative expression of the 26RFa gene was quantified by real-time PCR using the following primers 5'-GAAGGGGACCCACAGACATC-3' and 5'-GTCTTGCCCTCCTAGACGGAA-3'.  $\beta$ -actin was used as internal control for normalization using the following primers 5'-AGATCAAGATCATTGCTCCTCTG-3' and 5'-CCCAGCTCAGTAACAGTCCG-3'. PCR was carried out using Gene

Expression Master Mix 2× assay (Applied Biosystems, Courtaboeuf, France) in an ABI Prism 7900 HT Fast Real-time PCR System (Applied Biosystems). The purity of the PCR products was assessed by dissociation curves. The amount of target cDNA was calculated by the comparative threshold (Ct) method and expressed by means of the  $2^{-\Delta\Delta Ct}$  method.

### 2.5. 26RFa radioimmunoassay

Quantification of 26RFa in plasma samples was carried out using a specific radioimmunoassay set up in the laboratory (Le Solliec et al., 2022). 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 acetonitrile/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.

### 2.6. 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 were 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. For experiments involving streptozotocin-treated mice, before and after treatment statistical analysis was performed using a paired student *t*-test. All data represent means  $\pm$  SEM. Statistical significance was set up at  $p < 0.05$ .

## 3. Results

### 3.1. Evolution of the carbohydrate metabolism of the 26RFa-deficient mice during a high fat diet

Evolution of the carbohydrate metabolism of the 26RFa<sup>+/-</sup> and 26RFa<sup>-/-</sup> during a 3-month HFD was deciphered and compared to that of their wild type littermates (Fig. 1). Measurement of body weight, plasma glucose, insulin levels and the ratio insulin/glucose all along the HFD did not reveal any significant difference between the wild type, heterozygous and 26RFa-KO mice (Fig. 1B-E).

### 3.2. Glycemic phenotype of high fat 26RFa-deficient mice

The “glycemic” phenotype of the HFD 26RFa-deficient and the heterozygous mice was investigated using complementary *in vivo* tests. An intraperitoneal glucose tolerance test (IPGTT) indicated that the glycemic profile induced by the glucose load was not affected by the lack of 26RFa (Fig. 2A). An oral glucose tolerance test (OGTT) revealed that the hyperglycemic peak induced by the glucose ingestion was not impaired in the 26RFa<sup>-/-</sup> mice nor in the 26RFa<sup>+/-</sup> mice as compared to the 26RFa<sup>+/+</sup> mice (Fig. 2B). However, the decrease of glycemia following the hyperglycemic peak tended to be attenuated from 30 to 75 mins in the 26RFa<sup>-/-</sup> and 26RFa<sup>+/-</sup> mice (Fig. 2B). Glucose-induced insulin secretion was not altered by the lack of 26RFa expression and production (Fig. 2C) as well as insulin sensitivity as assessed by an insulin tolerance test (ITT) (Fig. 2D). The impact of the 26RFa gene disruption on hepatic glucose production was also examined. A pyruvate tolerance test (PTT) showed that hepatic glucose production was not altered in the 26RFa<sup>-/-</sup> mice and the 26RFa<sup>+/-</sup> mice as compared to the wild type animals (Fig. 2E).

### 3.3. Metabolic phenotype of high fat 26RFa-deficient mice

Morphological features of liver, white adipose tissue, brown adipose tissue and pancreas of 26RFa<sup>+/+</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>-/-</sup> submitted to

a 3-month HFD were compared (Fig. 3). No difference in the weight of the liver and the perigonadal white adipose tissue was observed between the 3 groups of mice (Fig. 3A, B). By contrast, the 26RFa-KO mice showed a significant increase ( $p < 0.05$ ) of the interscapular brown adipose tissue weight as compared to their wild type littermates (Fig. 3C). Comparison of freshly dissected pancreas indicated that the number of pancreatic islets per pancreas tended to be higher in the 26RFa<sup>-/-</sup> mice, although statistically not significant (Fig. 3D). Conversely, the area and the perimeter of the pancreatic islets tended to be reduced in the 26RFa-deficient mice as compared to the wild type mice (Fig. 3E, F). Double labelling experiments of pancreas sections did not reveal any difference in the distribution and intensity of insulin- and glucagon-like immunoreactivity in the pancreatic islets between the wild type (Fig. 3G), the heterozygous (Fig. 3H) and the 26RFa-KO mice (Fig. 3I).

### 3.4. Effect of 26RFa on the glycemic phenotype of insulin-deficient mice

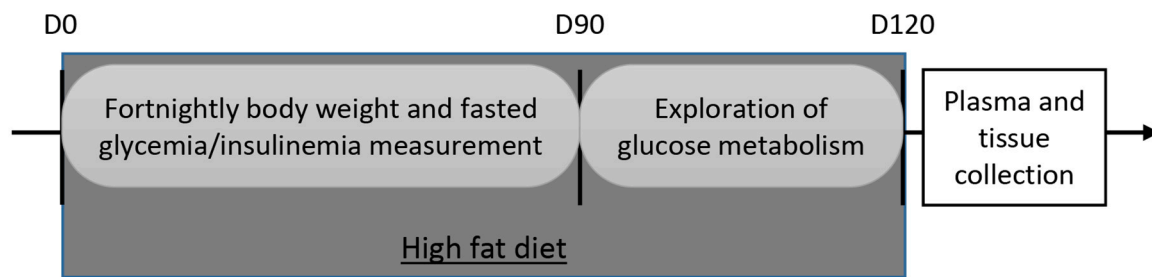
The effect of peripheral administration of 26RFa (500  $\mu$ g/kg) on glucose homeostasis was also investigated in another model of insulin-deficient mice (Fig. 4). Our data revealed that 26RFa did not modify basal glucose plasma levels of these mice (Fig. 4B). Similarly, ip administration of 26RFa did not alter the glycemic profile induced by an ip or oral glucose challenge (Fig. 4C, D). By contrast, an ITT revealed that 26RFa tended to reduce the insulin sensitivity of streptozotocin-treated mice, although this tendency was not statistically significant (Fig. 4E). Finally, we found that 26RFa did not impair glucose-induced insulin secretion of streptozotocin-treated mice (Fig. 4F). Phenotyping of the streptozotocin insulin-deficient mice revealed a significant ( $p < 0.001$ ) lower body weight of these mice (Fig. 5A). As expected, the streptozotocin-treated mice exhibited a highly significant ( $p < 0.001$ ) increase of their basal glycemia (Fig. 5B) associated with a dramatic decrease ( $p < 0.001$ ) of plasma insulin ( $p < 0.001$ ) (Fig. 5C). In addition, it appeared that plasma 26RFa levels were significantly ( $p < 0.05$ ) decreased in the insulin-deficient mice as compared to control animals (Fig. 5D) whereas the duodenal expression of the neuropeptide was not altered (Fig. 5E).

## 4. Discussion

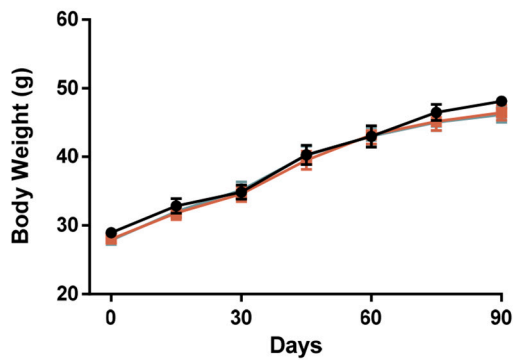
Previous studies performed in our team promoted the evidence of a bidirectional interaction between the 26RFa/GPR103 peptidergic system and insulin to regulate glucose homeostasis. Indeed, in the brain, 26RFa neurons express the insulin receptor and the hormone stimulates the expression of 26RFa within these neurons (El Mehdi et al., 2022). In addition, the 26RFa/GPR103 system in the hypothalamus relays the central stimulating effect of insulin on its own peripheral production (El Mehdi et al., 2022) whereas, at the periphery, 26RFa exerts an incretin-like activity by enhancing insulin secretion (Prevost et al., 2015). In 26RFa-deficient mice, insulin secretion is significantly impaired probably due to an important alteration of beta cell mass (El-Mehdi et al., 2020). In addition, the insulinotropic activity of 26RFa during a glucose challenge is completely blunted in a HFD mouse model (Prévost et al., 2019a). This dysfunction may be explained by a significant decrease of the 26RFa receptor expression in  $\beta$  cells and in insulin target tissues (Prévost et al., 2019a). Together, these observations led us to understand better the link between insulin and 26RFa in a pathophysiological context using two distinct models of diabetes i.e. the obese/hyperglycemic mice with insulin resistance and the insulinopenic streptozotocin-induced diabetic mice.

Firstly, we investigated the impact of 26RFa deficiency on the regulation of glucose homeostasis in HFD mice. Our data reveal that, after a 3-month HFD, the body weight of the 26RFa-mutant mice is similar to that of their wildtype littermates. It also appears that overall glucose tolerance during fasting or after an oral/intraperitoneal glucose load is not altered in 26RFa-deficient mice. Concurrently, we found that

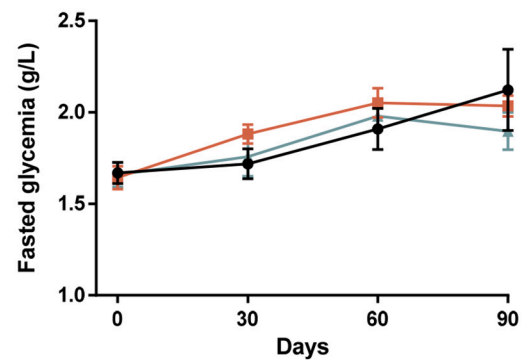
**A**



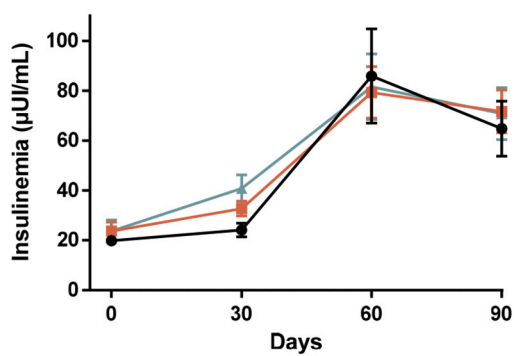
**B**



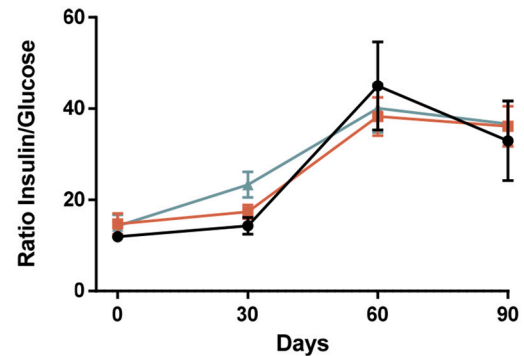
**C**



**D**



**E**



● Wild type mice  
 ■ 26RFa-heterozygous mice  
 ▲ 26RFa-KO mice

**Fig. 1.** Evolution of the carbohydrate metabolism of the 26RFa-deficient mice during a high fat diet. (A) Schematic representation of the experimental protocol used to evaluate various glycaemic parameters during a 3-month high fat diet (HFD) in 26RFa<sup>+/+</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>-/-</sup> mice. B-E: Evolution of body weight (B), glycemia (C), insulinemia (D) and the ratio insulin/glucose during the first 3 months of the HFD treatment in the 3 groups of animals (n = 8–10 per group). Data represent means ± SEM.

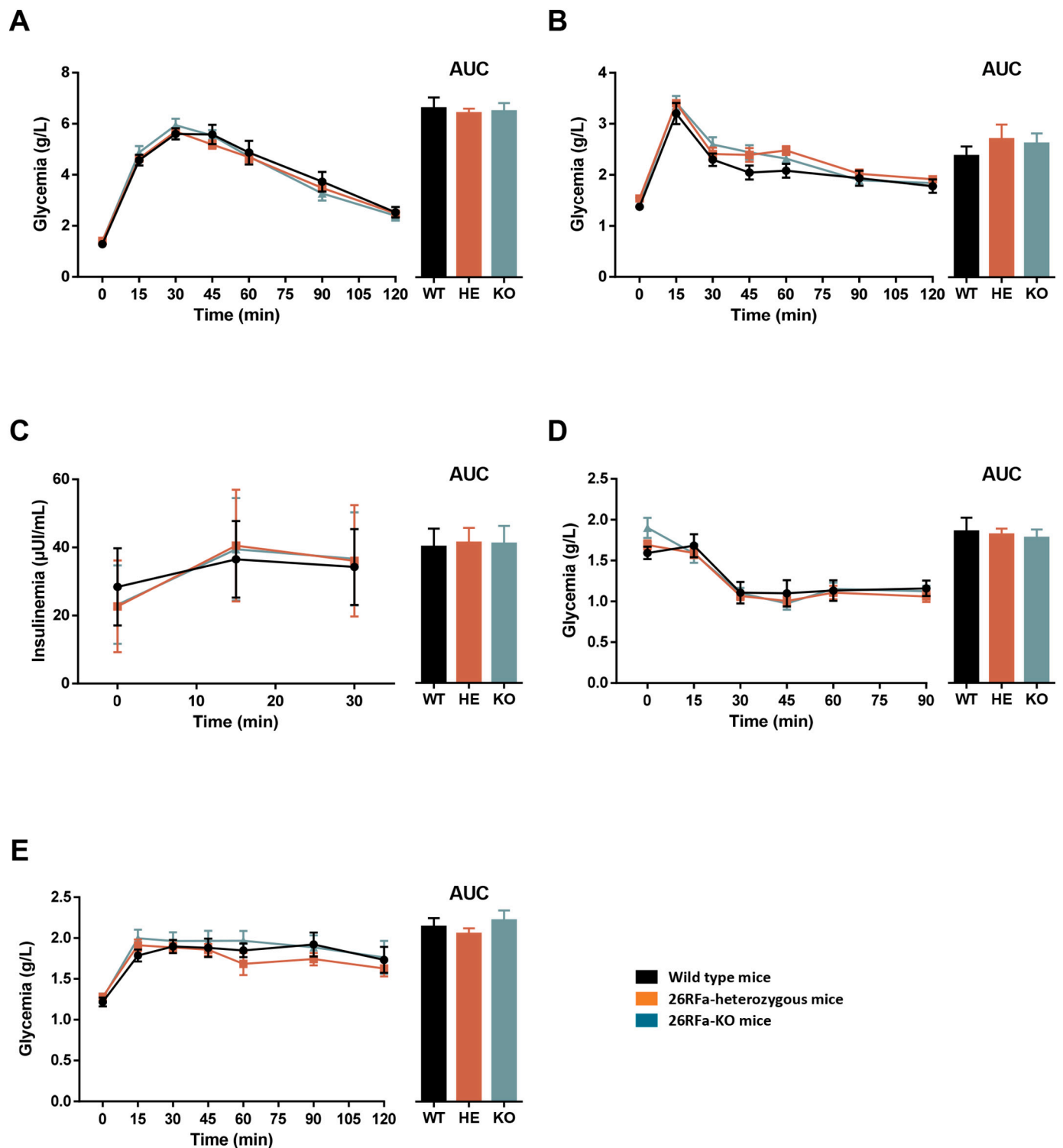
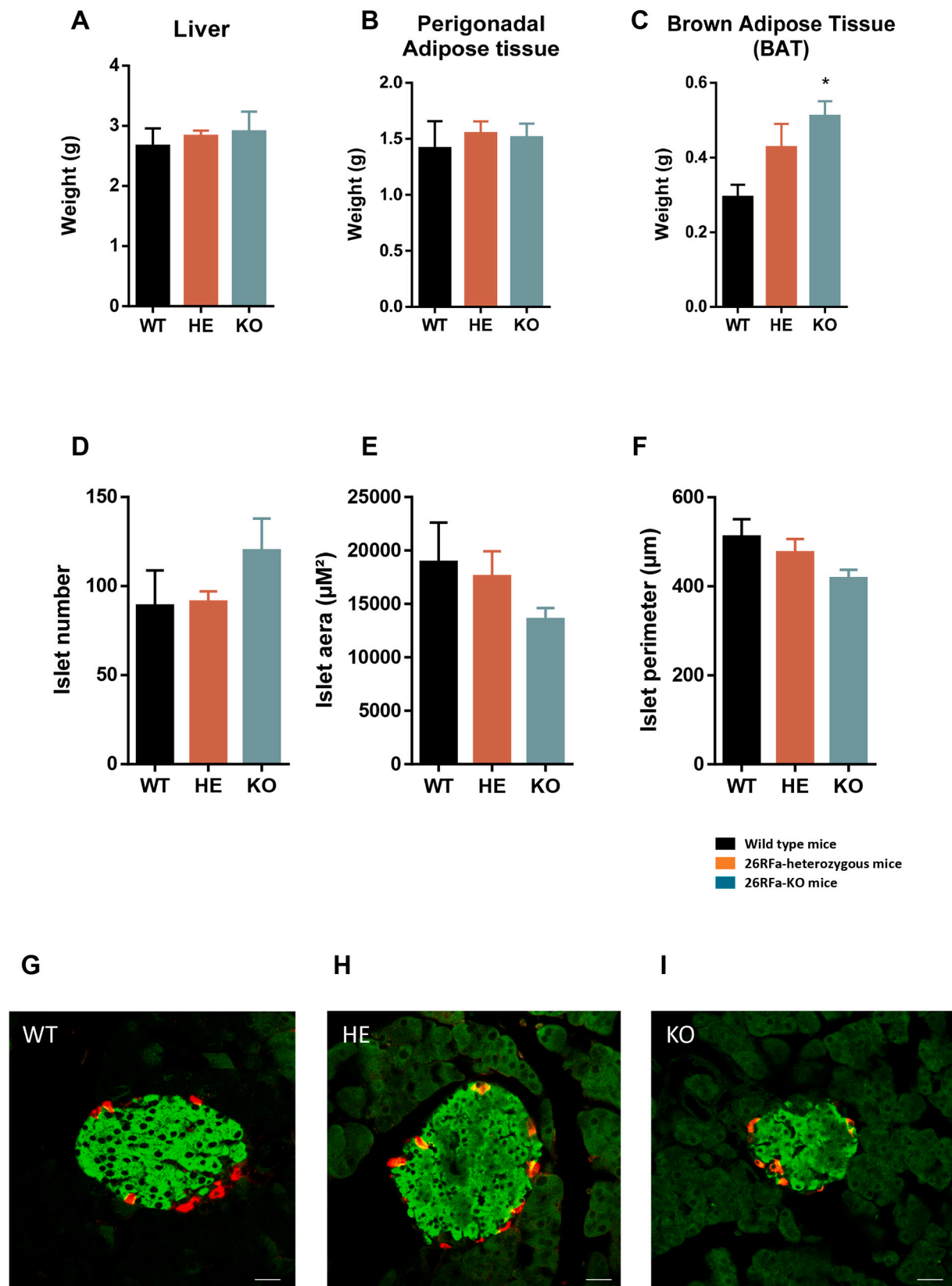


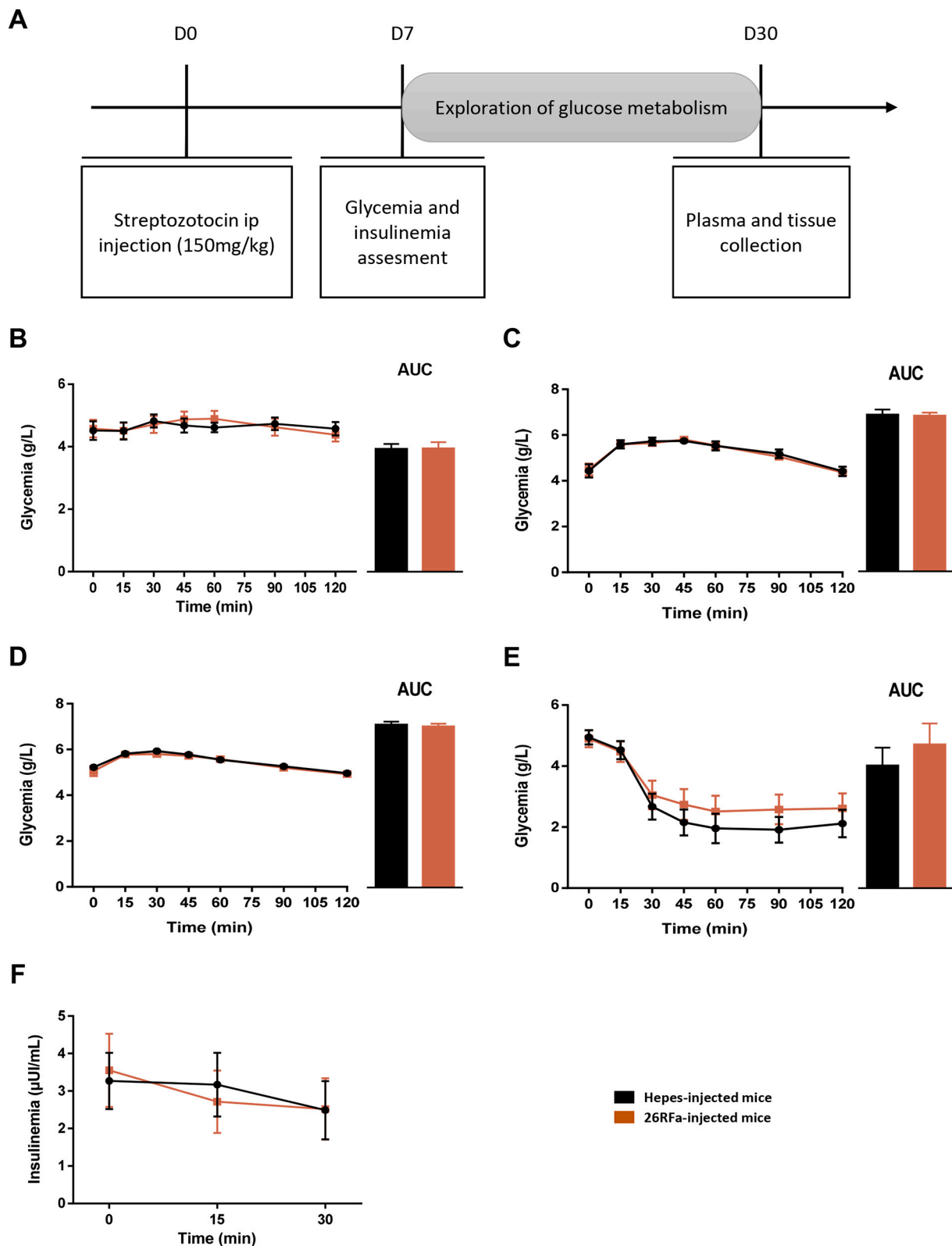
Fig. 2. Glycemic phenotype of high fat 26RFa-deficient mice. (A, B) Evaluation of plasma glucose in 26RFa<sup>-/-</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>+/+</sup> mice during an intra-peritoneal (A) and an oral glucose tolerance test (B) after a 3-month HFD treatment (n = 8–10 per group). (C) Evaluation of plasma insulin during an acute glucose-stimulating insulin secretion test in the 3 groups of animals after a 3-month HFD treatment (n = 8–10 per group). (D, E) Evaluation of plasma glucose in 26RFa<sup>-/-</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>+/+</sup> mice during an insulin tolerance test (D) and a pyruvate tolerance test (E) after a 3-month HFD treatment (n = 8–10 per group). Data represent means ± SEM and AUC the areas under the curves.

β cell mass is not affected by the lack of 26RFa although some anatomical changes were observed such as a smaller Langerhans islet size compensated with an increased number of these pancreatic islets. We hypothesize that the lack of glucose tolerance alteration observed in the obese/hyperglycemic mice deficient for 26RFa could be due to the

increase of brown adipose tissue (BAT) mass of the 26RFa<sup>-/-</sup> mice. More specifically, it is possible that the decrease of insulin secretion induced by 26RFa deficiency, previously reported (El-Mehdi et al., 2020), could be compensated by an increase of insulin sensitivity linked to the increase of BAT mass, resulting in a preserved glucose tolerance. Indeed,

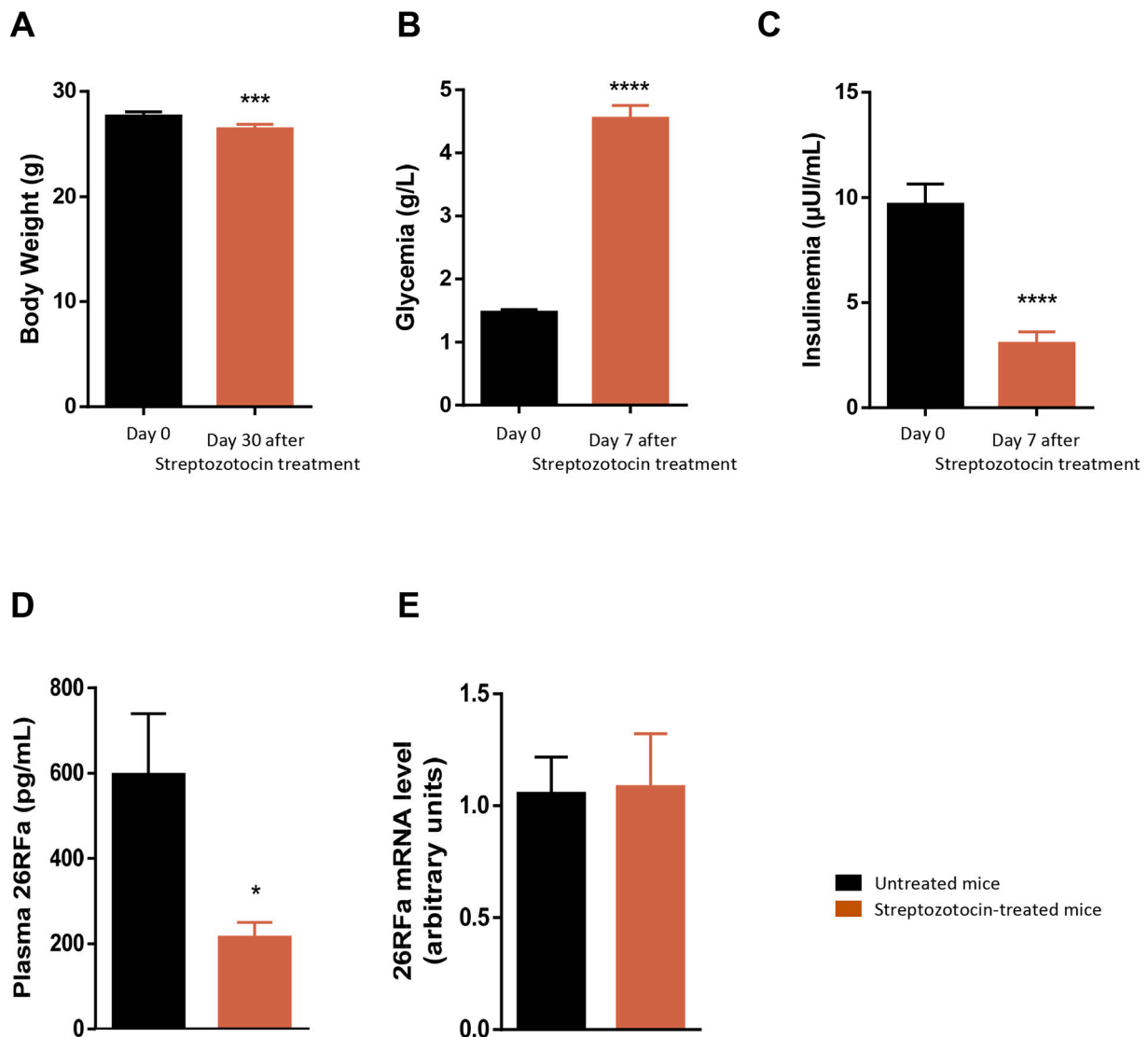


**Fig. 3.** Metabolic phenotype of high fat 26RFa-deficient mice. (A–C) Evaluation of liver (A), perigonadal adipose tissue (B) and interscapular brown adipose tissue (C) weight of 26RFa<sup>-/-</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>+/+</sup> mice after a 3-month HFD treatment ( $n = 8–10$  per group). (D–F) Quantitative analysis of the number of pancreatic islets (D), their areas (E) and their perimeters (F) in 26RFa<sup>-/-</sup>, 26RFa<sup>+/-</sup> and 26RFa<sup>+/+</sup> mice after a 3-month HFD treatment ( $n = 7$  per group). (G–I) Representative photomicrographs of pancreatic islets of 26RFa<sup>+/+</sup> (G), 26RFa<sup>+/-</sup> (H) and 26RFa<sup>-/-</sup> (I) mice labelled with an insulin antibody (green) and a glucagon antibody (red) showing no obvious difference in the distribution and intensity of insulin- and glucagon-like immunoreactivity between the 3 groups of mice. Data represent means  $\pm$  SEM. \*,  $p < 0.05$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4.** Effect of 26RFa on the glycemic phenotype of streptozotocin-induced diabetic mice. (A) Schematic representation of the experimental protocol used to evaluate the impact of 26RFa, injected intraperitoneally (500 µg/kg), on various metabolic and glycemic parameters in streptozotocin-induced diabetic mice. (B–F) Effect of i.p. administration of 26RFa in streptozotocin-treated mice on basal glycemia (B), during an i.p. glucose tolerance test (IPGTT) (C), an oral glucose tolerance test (OGTT) (D), an insulin tolerance test (ITT) (E) and an acute glucose-stimulated insulin secretion test (F) (n = 8–10 per group). Values represent the mean ± SEM and AUC the areas under the curves.





**Fig. 5.** Metabolic phenotype of streptozotocin-induced diabetic mice. (A-E) Evaluation of body weight (A), basal glycemia (B) and plasma insulin (C), plasma 26RFa (D) and duodenal 26RFa mRNA expression in streptozotocin (STZ)-treated mice compared to untreated animals ( $n = 6-10$  per group). Data represent means  $\pm$  SEM. \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

in mice, BAT is reported to inhibit obesity-induced inflammation and T-cell activation that may improve insulin sensitivity (Fitzgibbons et al., 2011; Ingram et al., 2017). Supporting this view, a previous study reported that chronic central infusion of 43RFa (the N-elongated form of 26RFa) was associated with a decrease of thermogenesis (Moriya et al., 2006). Unfortunately, no data about the BAT mass was available in this study (Moriya et al., 2006), but considering our present data, it is tempting to speculate that 26RFa treatment may decrease BAT mass and function.

The lack of impact of 26RFa deficiency on glucose homeostasis in obese/hyperglycemic mice may also be due to the occurrence of compensatory mechanisms previously described for the other incretins GLP-1 and GIP (Flamez et al., 1998; Miyawaki et al., 1999; Ayala et al., 2008). For instance, it was reported that, in GLP-1R<sup>-/-</sup> mice, the  $\beta$  cell sensitivity to the action of GIP is increased (Pederson et al., 1998), whereas the GIP-R<sup>-/-</sup> mice exhibit an enhanced insulin secretory response to GLP-1 (Pamir et al., 2003). Further studies with GLP1 and/or GIP antagonists in 26RFa-deficient mice are warranted to decipher potential compensatory mechanisms due to the other incretins. Finally, as we have previously showed that the effect of the 26RFa/GPR103 system on glucose metabolism is profoundly blunted in HFD mice

(Prévost et al., 2019a), it may be hypothesized that, in the HFD 26RFa-deficient mice, the glucose tolerance is not worsened because the peptide is inactive in lipo-glucotoxic conditions.

To complete the investigation of the interactions between the 26RFa/GPR103 system and insulin in a diabetic context, we decided to study a model of insulin-deficient mice, induced by the administration of streptozotocin. As expected, streptozotocin-treated mice exhibit severe hyperglycemia associated with severe insulinopenia. In these insulin-deficient mice, injection of 26RFa does not alter the glucose tolerance. This finding is consistent with our previous studies showing that 26RFa regulates glucose homeostasis at the periphery and centrally, mainly by stimulating insulin secretion in the pancreatic islets (Prévost et al., 2015; Prévost et al., 2019a; El-Mehdi et al., 2020). Similarly, the insulinotropic effect of GIP is also severely impaired in insulin deficient condition such as type 1 diabetic patient (Vilsbøll et al., 2003). By contrast, GLP-1 is still able to reduce postprandial plasma glucose levels in an insulinopenic context, but this effect of GLP-1 is rather attributed to an inhibitory action of the incretin on glucagon secretion (Kielgast et al., 2011). Our data also reveal that in streptozotocin-treated mice, plasma 26RFa levels are significantly lowered while the expression of the peptide in the duodenum is not modified. As 26RFa is also produced by the  $\beta$  cells of the

pancreatic islets in addition to the enterocytes of the duodenum (Prevost et al., 2015; Prévost et al., 2019a; Prévost et al., 2019b), it is reasonable to assume that the observed decrease of circulating 26RFa in streptozotocin-treated mice is mainly due to a reduced production of 26RFa by the non-functional  $\beta$  cells characterizing this model. However, although a direct action of insulin on the 26RFa production by the duodenum is not yet known, it cannot be ruled out that the impaired insulin secretion of the streptozotocin-treated mice alters 26RFa secretion by the gut. Interestingly, it is noteworthy that no significant alteration of plasma incretin concentrations (GLP-1 and GIP), which are only produced by the endocrine cells of the gut, is reported in type 1 diabetic patients (Kielgast et al., 2011).

To conclude, in the present study we show that dysregulation of glucose homeostasis in obese/hyperglycemic mice is not impaired by the absence of the incretin-like peptide 26RFa that is probably compensated by the increase of BAT mass and/or the other incretins i.e. GLP-1 and GIP. In diabetic insulin-deficient mice, we show that the anti-hyperglycemic effect of 26RFa is totally blunted probably as a result of the impaired insulin production characterizing this model of diabetes, avoiding therefore the action of the peptide. Finally, it is possible that the low plasma 26RFa levels, induced by the altered  $\beta$  cell physiology observed in the streptozotocin-treated mice, contribute to the development of the diabetic status in this model.

#### Author statement

MA.L.S., N.C. and G.P. contributed to the study design and interpretation, and wrote the manuscript. MA.L.S., S.T., A.A., J.M., M.D. and H.B. performed the in vivo experiments on mice. MA.L.S., S.T. and M.D. contributed to the immunohistochemical and PCR experiments. A. B. and E. N. performed the insulin assays. B.L. and J. L. produced synthetic 26RFa. M.P. and J.L. revised and approved the final version of the manuscript. N.C. 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.

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#### Declaration of Competing Interest

The authors have no competing interests to declare.

#### Data availability

Data will be made available on request.

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