Synergistic effect of leptin and lipidized PrRP on metabolic pathways in *ob/ob* mice

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Abstract

Lack of leptin production in *ob/ob* mice results in obesity and prediabetes that could be partly reversed by leptin supplementation. In the hypothalamus, leptin supports the production of prolactin-releasing peptide (PrRP), an anorexigenic neuropeptide synthesized and active in the brain. In our recent studies, the palmitoylated PrRP analog palm¹¹-PrRP31 showed a central anorexigenic effect after peripheral administration. This study investigates whether PrRP could compensate for the deficient leptin in *ob/ob* In two separate experiments, palm¹¹-PrRP31 (5 mg/kg) and leptin (5 or 10 g/kg) were administered subcutaneously twice daily for 2 or 8 weeks to 8- (younger) or 16-(older) week-old *ob/ob* mice, respectively, either separately or in combination. The body weight decreasing effect of palm¹¹-PrRP31 in both younger and older *ob/ob* mice was significantly powered by a subthreshold leptin dose, the combined effect could be then considered synergistic. Leptin and palm¹¹-PrRP31 also synergistically lowered liver weight and blood *ob/ob* mice. Reduced liver weight was linked to decreased mRNA expression of lipogenic enzymes. In the hypothalamus of older *ob/ob* mice, two main leptin anorexigenic signaling pathways, namely, Janus kinase, signal transducer and activator of transcription-3 activation and AMP-activated protein kinase de-activation, were induced by leptin, palm¹¹-PrRP31, and their combination. Thus, palm¹¹-PrRP31 could partially compensate for leptin deficiency in *ob/ob* mice. In conclusion, the results demonstrate a synergistic effect of leptin and our lipidized palm¹¹-PrRP31 analog.

Key Words

- prolactin-releasing
-
- *ob/ob*
- hypothalamic leptin signaling

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Introduction

Leptin has an appetite-regulating effect that occurs in the arcuate nucleus of the hypothalamus, where it activates anorexigenic proopiomelanocortin (POMC) and inhibits orexigenic neuropeptide Y (NPY) neurons and thus induces a decrease in food intake and an increase in energy consumption (Schwartz *et al.* 1996, Kwon *et al.* 2016). In addition, leptin action in the hypothalamus is connected with other anorexigenic neuropeptides, such as prolactin-releasing peptide (PrRP) (Ellacott *et al.* 2002).

PrRP was described to be produced in the nucleus tractus solitarius (NTS), ventrolateral medulla (VLM), and dorsomedial hypothalamus (DMH) (Maruyama *et al.* 1999, Dodd & Luckman 2013). It was suggested that PrRP neurons project from the NTS, where PrRP was detected

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in neuron bodies, to the hypothalamus, where PrRP was found in neuron fibers (Hinuma *et al.* 1998). In the paraventricular nucleus of the hypothalamus (PVN), PrRP fibers were shown to target anorexigenic corticotropin-releasing hormone (CRH) (Matsumoto *et al.* 1999). CRH production was also proven to be promoted by leptin (Schwartz *et al.* 1996).

Most PrRP neurons in VLM and DMH were shown to express leptin receptor (Ellacott *et al.* 2002); co-localization of PrRP and leptin receptor was questioned in the brainstem (Garfield *et al.* 2012). The combined central administration of leptin and PrRP had an additive anorexigenic effect on nocturnal food intake in freely fed rats. Moreover, in obese Zucker *fa/fa* rats lacking functional leptin receptor signaling, negligible PrRP mRNA expression was detected (Ellacott *et al.* 2002).

Leptin deficiency in *ob/ob* mice causes obesity based on hyperphagia and decreased energy expenditure; in addition, type 2 diabetes and hyperlipidemia are characteristic of *ob/ob* mice (Zhang *et al.* 1994).

Productive binding of leptin to its receptor affects two main anorexigenic pathways in the hypothalamus: JAK-STAT3 signaling (Janus kinase 2, signal transducer and activator of transcription-3) (Vaisse *et al.* 1996, Maniscalco & Rinaman 2014) and AMP-activated protein kinase (AMPK) (Minokoshi *et al.* 2004, Kahn 2019). AMPK is a serine/threonine kinase activated by an increase in the AMP:ATP ratio that reflects energy deficiency (Hardie 2008). In peripheral tissues, leptin-induced activation of AMPK results in lipid oxidation (Minokoshi *et al.* 2004) and attenuation of stored triglycerides (Unger *et al.* 1999). On the other hand, inhibition of hypothalamic AMPK is necessary for the anorexigenic and body weight-lowering effects of leptin; leptin inhibits AMPK specifically in the PVN and arcuate nucleus (Arc) of the hypothalamus (Minokoshi *et al.* 2004).

PrRP-deficient mice displayed late-onset obesity and adiposity, resulting from increased meal size, hyperphagia, and attenuated responses to cholecystokinin and leptin (Takayanagi *et al.* 2008). As PrRP is a brain-born and brain-acting neuropeptide, it cannot be administered peripherally to achieve central anorexigenic effects. Attachment of palmitic acid to PrRP enabled the implementation of the anorexigenic effect of PrRP after peripheral administration and also stabilized the palm^{11}-PrRP31 molecule (Maletinska *et al.* 2015, Prazienkova *et al.* 2017).

In this study, both leptin and palm^{11}-PrRP31 were repeatedly peripherally administered separately or in combination to *ob/ob* mice to explore the potential interaction between leptin and PrRP regarding their anorexigenic effect and impact on metabolic disturbances in *ob/ob* mice. If the palm^{11}-PrRP31 effect was powered by a subthreshold leptin dose, the combined effect could be considered synergistic. The impact of treatment with leptin, palm^{11}-PrRP31, and their combination on hypothalamic signaling was then investigated to determine whether PrRP followed leptin anorexigenic pathways.

Ob/ob mice of two ages were utilized: younger mice (treatment from 8 to 10 weeks old) in a metabolically active state, when the effect of lipidized PrRP on liver lipid metabolism could be expected, as in our previous study with diet-induced obese mice (Maletinska *et al.* 2015), and older *ob/ob* mice (treatment from 16 to 24 weeks old) with established morbid obesity.

Materials and methods

Animals

Ob/ob male mice and their wild-type (WT) littermates (5 weeks old) were obtained from ENVIGO (Correzzana, Italy). The mice were housed under controlled conditions at a constant temperature of 22±2°C, a relative humidity of 45 – 65% and a fixed daylight cycle (lights on: 6:00 h – 18:00 h), with two mice per cage. The animals were provided free access to water and the standard rodent chow diet Ssniff® R/M-H (Ssniff Spezialdiäten GmbH, Soest, Germany) containing 33, 9 and 58% of calories from proteins, fats and carbohydrates, respectively.

All of the animal experiments followed the ethical guidelines for animal experiments and the Act of the Czech Republic Nr. 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the Academy of Sciences of the Czech Republic.

Substances

Palm^{11}-PrRP31, an analog of human prolactin-releasing peptide palmitoylated at position 11, was synthesized at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague, Czech Republic, as previously described (Prazienková *et al.* 2017). The structure of palm^{11}-PrRP31 was SRTHRHSMEIK(N- -E(N-palmitoyl)) TPDINPAWYASRGIRPVGRF-NH2. Mouse leptin was obtained from Sigma-Aldrich.

Dosing of substances

Leptin doses (5 and 10 g/kg twice daily) were chosen as a subthreshold according to a daily dose of 1 g/mouse that was proven to be the minimum to achieve the anorexigenic

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effect in *ob/ob* mice (Harris *et al*. 1998). The palm^{11}-PrRP31 dose used (5 mg/kg twice daily) was chosen according to our previous studies, where it consistently decreased food intake in lean C57BL/6J mice after acute administration and food intake and body weight in C57BL/6J mice with diet-induced obesity after sub chronic administration (Prazienkova *et al*. 2017, Holubova *et al*. 2018).

Experimental design

The schema of the experimental design is shown in Fig. 1.

In Experiment 1, *ob/ob* and WT male mice (5 weeks old) were randomized into groups of 8–10 animals. After 8 weeks of age (younger mice), the mice were treated for 2 weeks as follows: 1. WT saline, 2. *ob/ob* saline, 3. *ob/ob* leptin, (5 µg/kg), 4. *ob/ob* palm^{11}-PrRP31 (5 mg/kg), and 5. *ob/ob* leptin+palm^{11}-PrRP (5 µg/kg+5 mg/kg). The compounds were dissolved in saline and administered subcutaneously twice a day. Food intake (FI) and body weight (BW) were monitored daily during the dosing period.

In Experiment 2, *ob/ob* and WT male mice (6–8 weeks old) were randomized into groups of ten animals. After 16 weeks of age (older mice), the mice were treated for 8 weeks as follows: 1. WT saline, 2. *ob/ob* saline, 3. *ob/ob* leptin (10 µg/kg), 4. *ob/ob* palm^{11}-PrRP31 (5 mg/kg), and 5. *ob/ob* leptin+palm^{11}-PrRP (10 µg/kg+5 mg/kg).

The compounds were dissolved in saline and administered subcutaneously twice a day. FI and BW were monitored daily during the dosing period.

The oral glucose tolerance test (OGTT) was measured in Experiment 2 (Fig. 1): 6-h-fasted mice were administered a glucose solution at a dose of 2 g/kg BW by gavage. Blood samples were obtained from the tail vessels. The blood glucose concentrations were measured using a glucometer (Arkray, Tokyo, Japan) at 0, 30, 60, 90, 120, and 180 min after glucose administration.

In the open field test in Experiment 2, fed mice were placed individually in an open field (TSE Systems, Bad Homburg, Germany), and their locomotor activity (velocity, total distance traveled, percentage of area visited and distance from the closest wall) was measured as described previously (Maletínská *et al*. 2008, 2015).

One week before the end of both experiments, rectal temperature was measured (Rodent thermometer BIO-TK9882, Bioseb, Pinellas Park, FL, USA).

At the end of both experiments, blood samples were collected from the tail veins of 12-h fasted mice, and blood plasma was separated and stored at −80°C. The mice were then deeply anesthetized with pentobarbital (170 mg/kg of body weight, Sigma-Aldrich) and transcardially perfused with ice-cold 0.01 mol/L pH 7.4 phosphate buffered saline (PBS) supplemented with heparin (10 U/mL, Zentiva, Prague, Czech Republic). The brains were removed, and the hypothalami were dissected and lysed in lysis buffer (Špolcová *et al*. 2015). During the dissections, the brains were maintained on ice to prevent tissue degradation. Subcutaneous adipose tissue (SCAT), intraperitoneal adipose tissue (IPAT) and livers of all of the mice were dissected and weighed. The liver was dissected, and the caudate lobes of each liver were fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.4. After 24 h of fixation, the liver was stored in 70% ethanol at 4°C until tissue processing in the Leica ASP200S tissue processor (Leica Biosystems Inc.). The paraffin embedding station Leica EG1150H (Leica Biosystems Inc.) was used to create paraffin blocks from the wax-penetrated liver samples. Another part of liver tissue, IPAT and SCAT were flash-frozen in liquid nitrogen and stored at −80°C for later extraction of mRNA.

Determination of hormonal and biochemical parameters

The blood glucose levels were measured using a glucometer (Arkray, Kyoto, Japan). Glycated hemoglobin (HbA1c) was measured using Afinion kits (Afinion AS100, Axis-Shield PoC-AS, Oslo, Norway). The plasma insulin concentrations

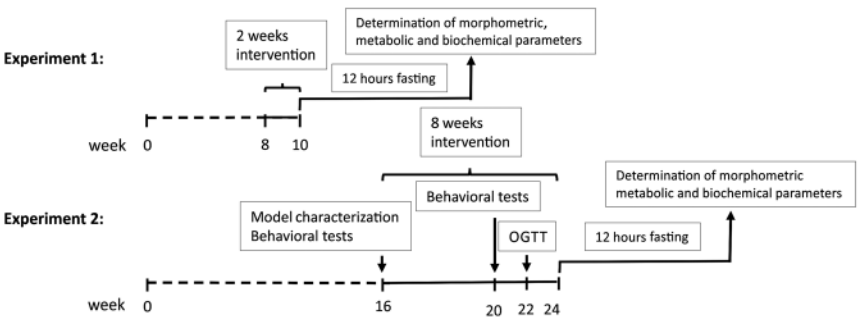

Figure 1
Schema of experimental design.

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were measured using an RIA assay (Millipore). Cholesterol was determined by colorimetric assay (Erba Lachema, Brno, Czech Republic). The plasma triglyceride (TAG) levels were measured using a quantitative enzymatic reaction (Sigma-Aldrich), and the free fatty acid (FFA) levels were determined using a colorimetric assay (Roche). All measurements were performed according to the manufacturer's instructions.

Liver histology

Liver samples in paraf□n blocks were cut on a Leica RM2255 microtome (Leica Biosystems Inc.) to slices of 5 m thickness. Deparaf□nization in xylene and rehydration in an ethanol range was performed. Slices were stained in hematoxylin using Weigert's iron hematoxylin solution set (HT1079-1Set, Sigma-Aldrich). Slices were washed with tap water and subsequently stained for 1 min in 0.5% eosin Y (C.I. 45380, Carl Roth GmbH+Co. KG, Karlsruhe, Germany). After washing with tap water, the samples were dehydrated and covered with DPX mounting medium (06522, Sigma-Aldrich). Histological images were performed at 200× magni□cation.

Western blotting

Hypothalami were processed, and Western blotting was performed as previously described (Špolcová *et al.* 2015). The following primary antibodies were used: total STAT3, phospho-STAT3 (Y705), phospho-STAT3 (S727), SOCS3, total AMPK, phospho-AMPK, phosphoinositide-3-kinase (PI3 kinase), total AKT, phospho-AKT (S473) (Cell Signaling Technology) and beta-actin (Sigma-Aldrich). The following secondary antibodies were used: anti-mouse IgG HRP-linked antibody and anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology).

Determination of mRNA expression

The mRNA expression of the genes of interest in liver (acetyl-CoA carboxylase 1 (*Acaca*), peroxisome proliferator-activated receptor (*Prarg* and *Ppara*), sterol regulatory element-binding protein 1 (*Srepf1*), fatty acid synthase (*Fasn*), phosphoenolpyruvate carboxykinase 1 (*Pck1*), carnitine palmitoyltransferase 1a (*Cpt1a*), and glucose-6-phosphatase (*G6pc*)) was determined using an ABI PRISM 7500 instrument (Applied Biosystems) in samples from the mouse liver as described previously (Prazienkova *et al.* 2017). The expression of beta-2-microglobulin (*B2m*) was used to compensate for variations in input mRNA amounts and the ef□ciency of RT. The formula 2-dCt was used to calculate relative gene expression.

Statistics

The data are presented as the means±S.E.M. Statistical analysis was performed using unpaired *t*-test or one-way or two-way ANOVA followed by Bonferroni's *post hoc* test as indicated in Figure legends and Tables with Graph-Pad Prism Software, and $P<0.05$ was considered statistically signi□cant.

Results

Experiment 1: Treatment with leptin, palm^{11}-PrRP31, and their combination in younger *ob/ob* mice

The leptin + palm^{11}-PrRP31 combination attenuated food intake and body weight in a synergistic manner in younger mice

A decreasing effect of the leptin+palm^{11}-PrRP31 combination on cumulative food intake was signi□cant after the □rst day of treatment compared to both the *ob/ob* saline and *ob/ob* leptin; between days 11 and 14, the effect of the combined treatment was signi□cant compared to that in the *ob/ob* saline. There were signi□cant differences between phenotypes (ob/ob saline versus WT saline) between days 5 and 14. (Fig. 2A and B). The body weight change caused by the treatment was signi□cant only for the leptin+palm^{11}-PrRP31 combination since day 12 compared to both *ob/ob* saline and *ob/ob* leptin (Fig. 2C); it was obvious that *ob/ob* mice did not gain weight during the treatment with the leptin+palm^{11}-PrRP31 combination. The □nal body weight of *ob/ob* saline was signi□cantly higher than that of WT saline (Fig. 2D); only the leptin+palm^{11}-PrRP31 combination signi□cantly lowered the □nal body weight compared to *ob/ob* saline (Fig. 2E).

Leptin and palm^{11}-PrRP31 synergistically decreased the liver weight of younger ob/ob mice

As expected, WT saline group had signi□cantly lower weights of SCAT and IPAT compared to *ob/ob* saline group. None of the treatments affected adipose tissue weights compared to *ob/ob* saline (Table 1A).

The liver weight of WT saline group was signi□cantly higher than that of *ob/ob* saline group (Fig. 3A and Table 1A). Leptin signi□cantly lowered liver weight in *ob/ob*

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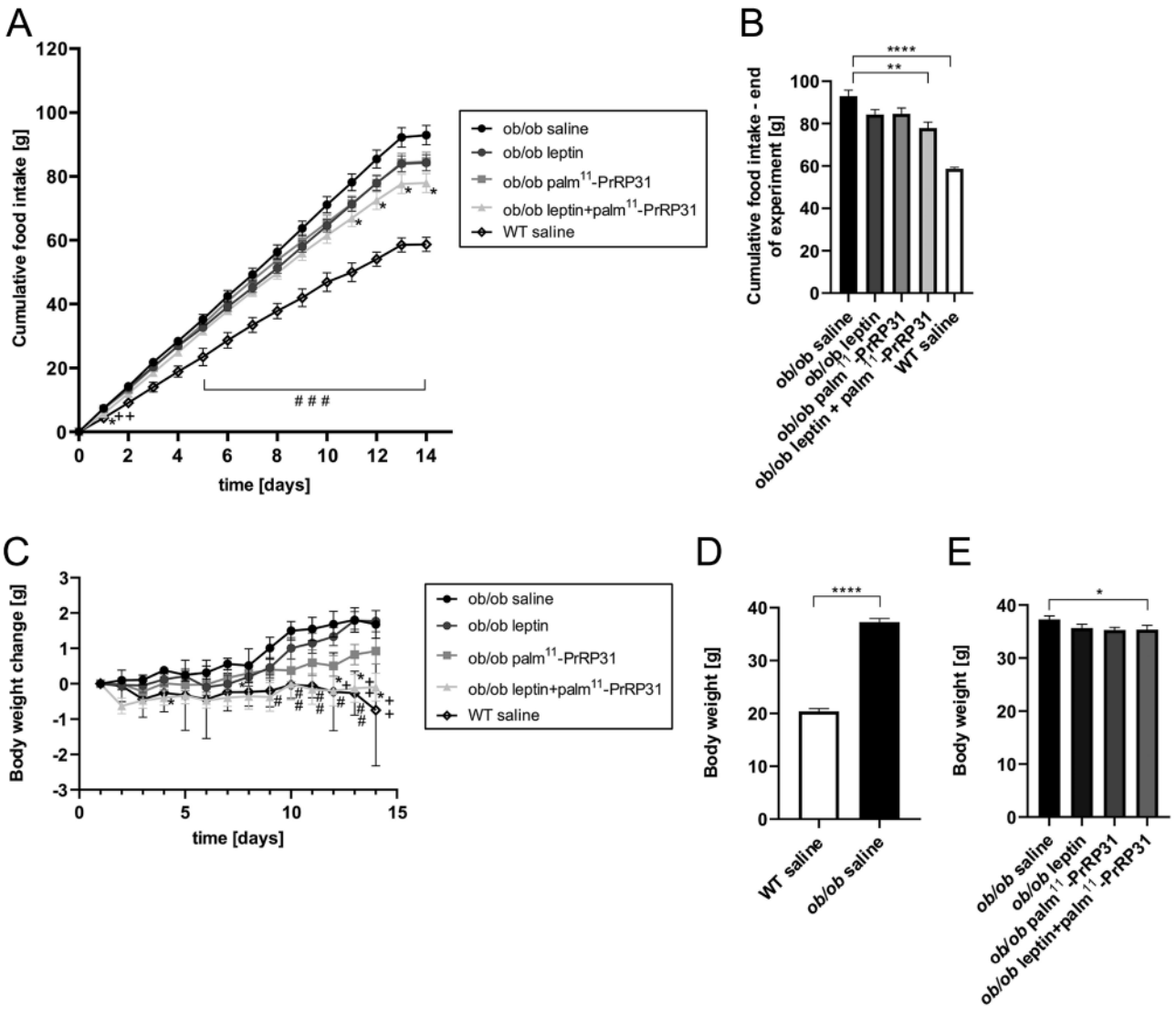

Figure 2
Food intake and body weight change of *ob/ob* mice in Experiment 1. (A) Food intake and (B) cumulative food intake at the end of experiment: *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (C) Body weight change of *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (D) Body weight at the end of experiment of WT saline and *ob/ob* saline mice. (E) Body weight at the end of experiment of *ob/ob* treated mice with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. Data are means ± S.E.M. (n = 8–10). Significance is $^{*/+}P < 0.05$, $^{**/++}P < 0.01$, $^{****/++++}P < 0.0001$ *vs ob/ob* saline or *ob/ob* leptin group, respectively (*t*-test or one-way ANOVA + Bonferroni *post hoc* test). Magnification was 200×.

mice; its effect was pronounced in the leptin + palm^{11}-PrRP31 combination (Fig. 3A and Table 1A). As single leptin did not cause any significant effect, the combined action of leptin and palm^{11}-PrRP31 seems synergistic (Fig. 3B). Histology of liver slices demonstrated regression of fat droplets in the liver tissue of all treated *ob/ob* groups compared to *ob/ob* saline toward the image of WT saline (Fig. 3C).

Body temperature was significantly lower in *ob/ob* saline compared to WT saline; none of the treatments affected body temperature in *ob/ob* mice (Table 1A).

Leptin and palm^{11}-PrRP31 synergistically attenuated blood glucose in younger ob/ob mice

Hyperglycemia was obvious in *ob/ob* saline compared to WT saline (Table 1B). Blood glucose was significantly lowered by both leptin and palm^{11}-PrRP31, and the leptin + palm^{11}-PrRP31 combination normalized glycemia to the level of WT saline (Table 1B); this result again pointed to a synergistic action of leptin and palm^{11}-PrRP31. *Ob/ob* saline was very significantly hyperinsulinemic compared to WT saline (Table 1B); neither treatment significantly affected the insulin level of *ob/ob* mice (Table 1B).

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Table 1 Morphometric and metabolic parameters in Experiment 1.

	WT saline	*ob/ob* saline	*ob/ob* leptin	*ob/ob* palm^{11}-PrRP31	*ob/ob* leptin+palm^{11}-PrRP31
(A) Morphometric parameters					
BW (g)	20.38 ± 0.54****	37.28 ± 0.67	35.64 ± 0.75	35.30 ± 0.49	35.34 ± 0.85
SCAT (g)	0.28 ± 0.05****	4.08 ± 0.28	3.74 ± 0.28	3.60 ± 0.29	3.68 ± 0.24
IPAT (g)	0.28 ± 0.04****	2.83 ± 0.11	2.57 ± 0.09	2.51 ± 0.10	2.60 ± 0.10
Liver weight (g)	0.97 ± 0.24****	2.73 ± 0.11	2.36 ± 0.09*	2.36 ± 0.12	2.08 ± 0.09***
Rectal temp (°C)	36.75 ± 0.17****	35.00 ± 0.21	35.64 ± 0.28	35.22 ± 0.32	35.33 ± 0.36
(B) Metabolic parameters					
Glucose (mmol/l)	5.65 ± 0.18***	14.19 ± 1.82	9.38 ± 1.16*	9.71 ± 1.21*	5.97 ± 0.35***
Insulin (ng/ml)	1.47 ± 0.51****	21.02 ± 3.53	15.35 ± 3.34	24.03 ± 4.41	19.27 ± 3.06
CHOL (mmol/l)	2.07 ± 0.10***	3.43 ± 0.22	3.13 ± 0.10	3.18 ± 0.21	3.11 ± 0.12
TAG (mmol/l)	1.02 ± 0.12	0.90 ± 0.09	1.02 ± 0.07	0.79 ± 0.04	1.11 ± 0.08
FFA (mmol/l)	0.12 ± 0.01***	0.32 ± 0.04	0.39 ± 0.05	0.34 ± 0.07	0.46 ± 0.04

(A) Morphometric parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (B) Metabolic parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. Data are means ± S.E.M. (n = 8–10). Significance is *P < 0.05, **P < 0.01, ***P < 0.001 *vs ob/ob* saline (t-test or one-way ANOVA + Bonferroni *post hoc* test).

Increased cholesterol but comparable TAG and FFA levels were detected in *ob/ob* saline compared to WT mice. None of the treatments affected cholesterol or TAG or FFA levels in *ob/ob* mice (Table 1B).

The leptin + palm^{11}-PrRP31 combination lowered liver mRNA expression of enzymes regulating lipid metabolism in younger mice

Liver mRNA expression levels of *Acaca* (Fig. 4A), which catalyzes the rate-limiting step in fatty acid synthesis, and *Fasn* (Fig. 4B), which catalyzes the next step of *de novo* lipogenesis, were higher in *ob/ob* saline than those in WT saline and were attenuated similarly by the treatment with the leptin + palm^{11}-PrRP31 combination (Fig. 4A and B). Single leptin treatment also lowered *Fasn* mRNA liver expression in *ob/ob* mice (Fig. 4B). However, no difference in the mRNA expression of *Srebf1*, which controls the expression of *Acaca* and *Fasn*, was found between the *ob/ob* saline and WT saline groups, and no treatment affected it signi cantly (Fig. 4C). The mRNA expression levels of *Pck1* and *Cpt1a*, both enzymes regulating fatty acid oxidation, were also signi cantly enhanced in *ob/ob* saline compared to WT saline, but no treatment affected their expression (Fig. 4D and E). A similar pattern was observed for peroxisome proliferator-activated receptors *Ppara* and *Pparg*, which are other regulators of fatty acid oxidation (Fig. 4F and G).

The mRNA expression of *G6pc*, a key enzyme catalyzing gluconeogenesis and glycogenolysis, was enhanced in the *ob/ob* saline compared to WT saline and was attenuated by leptin + palm^{11}-PrRP31 combination treatment (Fig. 4H).

Ucp-1 mRNA expression was determined in Experiment 1. There were no signi cant differences (Supplementary Fig. 4A, see section on supplementary materials given at the end of this article).

Experiment 2: Treatment with leptin, palm^{11}-PrRP31, and their combination in older *ob/ob* mice

Treatment did not affect the behavior of older ob/ob mice

The open eld test clearly showed lowered velocity, traveled distance and area covered by 16-week-old *ob/ob* mice compared to the age-matched WT mice. Analogously, after treatment at the age of 16–24 weeks, *ob/ob* saline showed a signi cant decrease in all of the above-mentioned parameters, and no effect of treatment on locomotor activity was found. Since the average distance from the maze wall (wall distance) did not differ between *ob/ob* mice and WT mice at either 16 or 24 weeks, we could assume that anxiety was not a probable cause of locomotor activity attenuation (Supplementary Fig. 1). The lower mobility could be attributed to the morbid obesity of *ob/ob* mice and leptin de ciency-related behavioral changes.

Morphometric and metabolic parameters before the treatment of older mice

Before starting the experiment, at the age of 16 weeks, ten *ob/ob* and ten WT mice were taken out from their particular cohort to characterize them before the treatment. Body, SCAT, and liver weights were signi cantly lower in WT mice compared to *ob/ob* mice (Supplementary Table 1A).

Analogously to Experiment 1, *ob/ob* mice had high hyperinsulinemia, but unlike in Experiment 1, they were normoglycemic similarly as WT mice (Supplementary Table 1B). Similar to Experiment 1, *ob/ob* mice had a signi cantly

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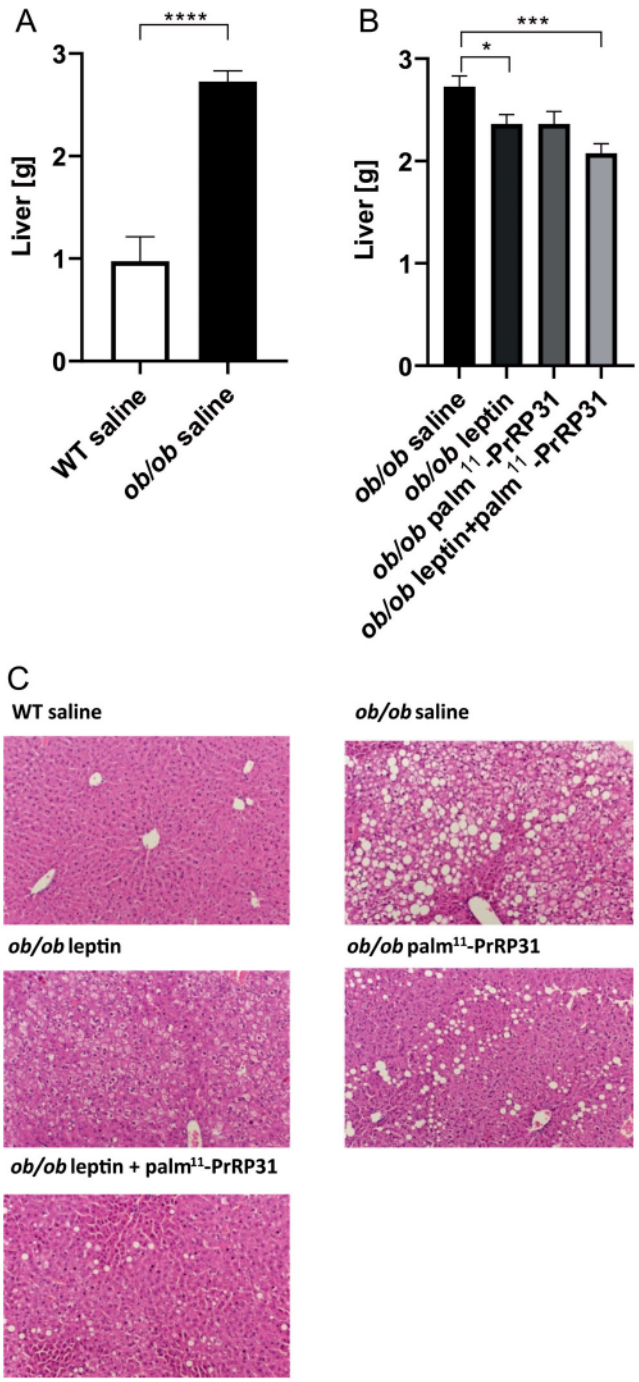

Figure 3
Liver weight and liver histology of *ob/ob* mice in Experiment 1. (A) Liver weight of WT saline and *ob/ob* saline mice. (B) Liver weight of *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (C) Liver histology of WT saline, *ob/ob* saline, *ob/ob* leptin, *ob/ob* palm^{11}-PrRP31 and *ob/ob* leptin + palm^{11}-PrRP31. Data are means ± S.E.M. (n = 8–10). Significance is $^{*/+}P < 0.05$, $^{***/+++}P < 0.001$, $^{****/++++}P < 0.0001$ *vs* *ob/ob* saline or *ob/ob* leptin group, respectively (t-test or one-way ANOVA + Bonferroni *post hoc* test).

increased cholesterol level, but TAG and FFA levels did not differ from those of WT mice (Supplementary Table 1B).

Leptin and palm^{11}-PrRP31 synergistically lowered body weight in older ob/ob mice

None of the treatments affected cumulative food intake compared to *ob/ob* saline (Fig. 5A and B). Until day 22 of the treatment, the leptin + palm^{11}-PrRP31 combination caused a negative change in body weight in *ob/ob* mice (Fig. 5C). The change in body weight caused by the leptin + palm^{11}-PrRP31 combination was significant compared to that of the *ob/ob* saline after day 20 and compared to that of *ob/ob* leptin after day 22 of the treatment (Fig. 5C). Figure 5C showed a decline in body weight at day 20, this could happen because anorexigenic substances in mice with DIO were effective until certain time of the treatment and then the effect of the substance ceased most probably because of the compensatory mechanisms that attenuated anorexigenic effect of the substances. This is also known from the treatment of obesity in humans as well. The final body weight of *ob/ob* saline was significantly higher than that of WT saline (Fig. 5D); treatment with the leptin + palm^{11}-PrRP31 combination lowered the final body weight significantly compared to *ob/ob* saline (Fig. 5E). All results point to a synergistic effect of leptin and palm^{11}-PrRP31 on body weight in *ob/ob* mice.

Leptin and palm^{11}-PrRP31 normalized body temperature synergistically in older ob/ob mice

As expected, SCAT weight was significantly higher in *ob/ob* saline compared to WT saline. Only leptin + palm^{11}-PrRP31 significantly lowered SCAT weight (Table 2A). Similar to Experiment 1, WT saline group had a significantly lower liver weight than *ob/ob* mice. Unlike Experiment 1, no treatment significantly affected the liver weight of *ob/ob* mice (Table 2A).

Similar to Experiment 1, *ob/ob* saline were hypothermic compared to WT saline. Although single leptin and single palm^{11}-PrRP31 did not affect body temperature, the leptin + palm^{11}-PrRP31 combination significantly upregulated the body temperature of *ob/ob* mice (Table 2A); these findings again could point to their synergistic action.

The treatment did not affect glucose resistance but lowered cholesterol in older ob/ob mice

The glucose level in *ob/ob* saline did not differ from the normoglycemic WT saline; no treatment affected blood

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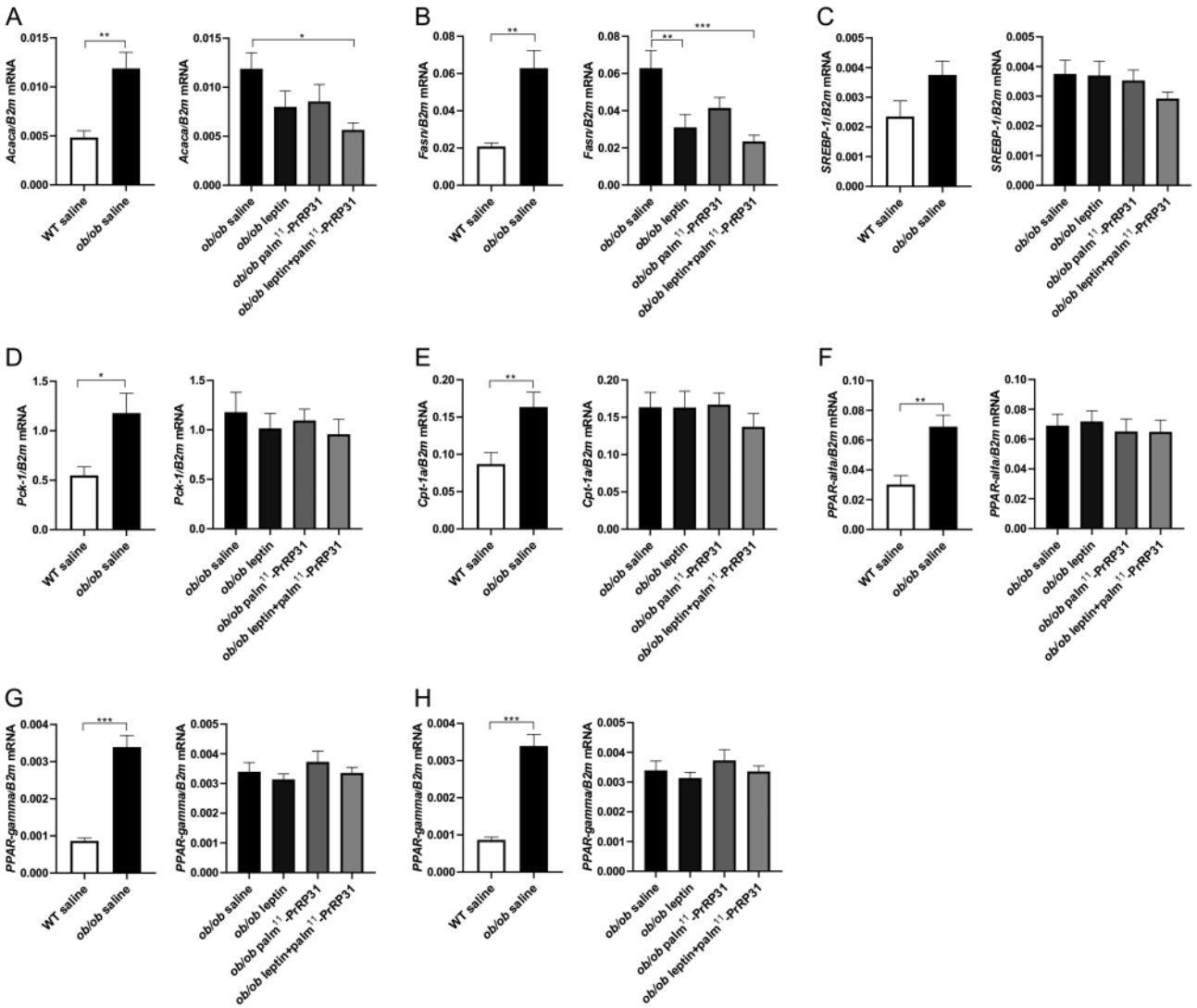

Figure 4
mRNA expression in liver in Experiment 1. *Ob/ob* mice were treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (A) *Acaca*. (B) *Fasn*. (C) *Srebf1*. (D) *Pck1*. (E) *Cpt1a*. (F) *Ppara*. (G) *Pparg*. (H) *G6pc*. Data are means ± S.E.M. (n = 8–10). Significance is *P < 0.05, **P < 0.01, ***P < 0.001 *vs ob/ob* saline (t-test or one-way ANOVA + Bonferroni *post hoc* test).

glucose measured at the end of Experiment 2 (Table 2B). On the other hand, the *ob/ob* saline had significantly higher HbA1c levels compared to WT saline; no treatment affected HbA1c levels (Table 2B). The course of OGTT was similar for all treated and *ob/ob* saline, and compared to that of WT mice, it pointed to glucose intolerance in *ob/ob* mice untreatable by leptin and PrRP analog at doses used (Fig. 6). Again, *ob/ob* mice were hyperinsulinemic compared to WT mice (Table 2B).

Similar to Experiment 1, cholesterol levels were significantly higher in *ob/ob* saline than in WT saline at the end of Experiment 2. Unlike Experiment 1, treatment with palm^{11}-PrRP31 and its combination with leptin attenuated cholesterol levels toward that of WT saline (Table 2B). The TAG and FFA levels did not differ between *ob/ob* saline and WT saline; neither were affected by any treatment (Table 2B).

Liver mRNA expression of proteins regulating lipid metabolism was not affected by the treatment in older ob/ob mice

Unlike Experiment 1, no difference was found in liver mRNA expression of enzymes and transcription factors regulating lipid metabolism between *ob/ob* saline and WT saline, and no treatment affected these mRNA liver expression levels (Supplementary Fig. 2).

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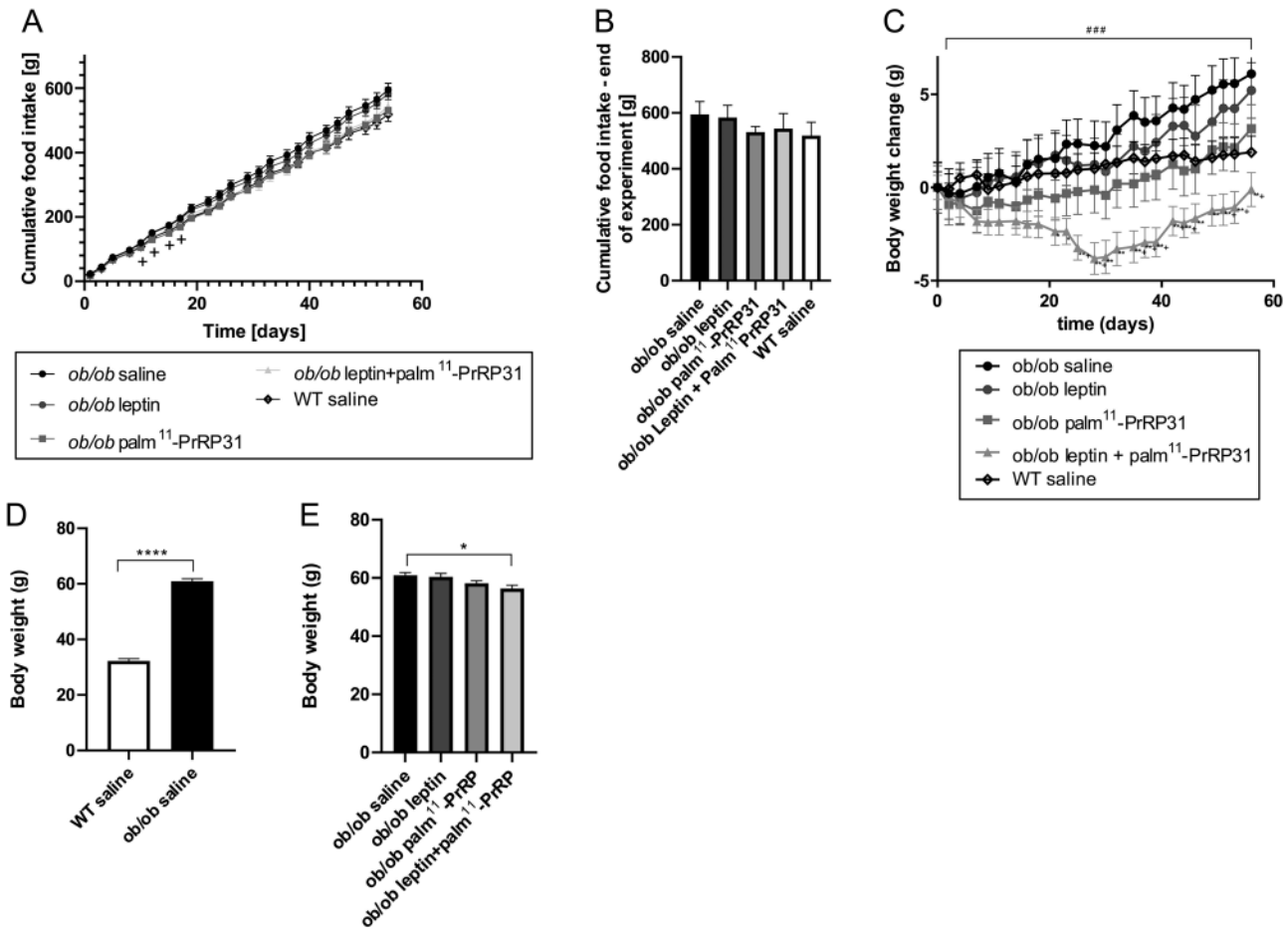

Figure 5
Food intake and body weight change of *ob/ob* mice in Experiment 2. (A) Food intake and cumulative food intake at the end of experiment of *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (B) Body weight change of *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (C) Body weight at the end of experiment of WT saline and *ob/ob* saline mice. (D) Body weight at the end of experiment of *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. Data are means ± S.E.M. (n = 8–10). Significance is $^{*/+}P < 0.05$, $^{**/++}P < 0.01$, $^{****/++++}P < 0.0001$ *vs ob/ob* saline or *ob/ob* leptin group, respectively (*t*-test or one-way ANOVA + Bonferroni *post hoc* test).

Ucp-1 mRNA expression was determined in Experiment 2, and there were only significant differences between phenotypes (*ob/ob* saline versus WT saline), but the treatment did not cause any significant change compared to *ob/ob* saline (Supplementary Fig. 3B).

Leptin and palm^{11}-PrRP31 synergistically attenuated SOCS3, and both leptin and palm^{11}-PrRP31 lowered AMPK phosphorylation in the hypothalami of ob/ob mice

Activation of the anorexigenic hypothalamic pathways JAK-STAT and AMPK was followed after treatment with leptin, palm^{11}-PrRP31, and their combination and compared to *ob/ob* saline group (Fig. 7A). STAT3 protein was enhanced after leptin treatment in *ob/ob* mice (Fig. 7D); Tyr705 p-STAT did not differ significantly between saline and compound-treated *ob/ob* groups (Fig. 6B), but Ser727 p-STAT was increased by all three treatments (Fig. 7C). SOCS3 protein, which negatively regulates leptin receptor signaling, was significantly lowered by palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31 combination treatment, but not by leptin; this finding could point to leptin + palm^{11}-PrRP31 synergistically attenuating the effect of SOCS3 production (Fig. 7E).

Regarding the common leptin and insulin pathway, PI3K protein was increased significantly by palm^{11}-PrRP31 but not by single leptin or its combination with palm^{11}-PrRP31 in *ob/ob* mice (Fig. 7F); phosphorylation of AKT at Ser473 was not affected significantly by any treatment and only tended to increase (Fig. 7G).

AMPK phosphorylation was significantly lowered after all three treatments (Fig. 7I).

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Table 2 Morphometric and metabolic parameters Experiment 2.

	WT saline	*ob/ob* saline	*ob/ob* leptin	*ob/ob* palm^{11}-PrRP	*ob/ob* leptin+palm^{11}-PrRP
(A) Morphometric parameters					
BW (g)	32.23 ± 0.80****	60.90 ± 0.93	60.36 ± 1.18	58.15 ± 0.87	56.36 ± 1.16*
SCAT (g)	0.39 ± 0.06****	6.45 ± 0.33	6.23 ± 0.24	5.57 ± 0.31	5.19 ± 0.36*
IPAT (g)	0.49 ± 0.05****	1.98 ± 0.06	1.99 ± 0.13	2.37 ± 0.16	2.31 ± 0.13
Liver weight (g)	1.35 ± 0.04****	4.42 ± 0.14	4.35 ± 0.20	3.93 ± 0.06	4.08 ± 0.18
Rectal temp (°C)	37.84 ± 0.17****	35.42 ± 0.18	35.09 ± 0.26	34.93 ± 0.32	36.52 ± 0.14*
(B) Metabolic parameters					
Glucose (mmol/l)	8.05 ± 0.27	7.96 ± 0.38	6.78 ± 0.49	8.29 ± 0.40	8.99 ± 0.97
Hb1Ac (mmol/mol)	23.8 ± 0.36****	33.2 ± 1.28	35.5 ± 1.09	32 ± 2.04	28.1 ± 2.18
Insulin (ng/ml)	0.11 ± 0.04***	9.43 ± 1.78	9.90 ± 2.82	16.77 ± 4.98	9.74 ± 3.74
CHOL (mmol/l)	1.75 ± 0.07****	5.00 ± 0.24	4.50 ± 0.17	3.66 ± 0.22***	3.82 ± 0.18**
TAG (mmol/l)	0.9 ± 0.09	0.82 ± 0.03	0.98 ± 0.05*	0.82 ± 0.04	0.83 ± 0.04
FFA (mmol/l)	1.33 ± 0.08	1.42 ± 0.06	1.59 ± 0.11	1.64 ± 0.10	1.59 ± 0.12

(A) Morphometric parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (B) Metabolic parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. Data are means ± S.E.M. (n = 8–10). Significance is *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 *vs ob/ob* saline (t-test or one-way ANOVA + Bonferroni *post hoc* test).

Discussion

Leptin and PrRP were shown to cooperate in decreasing food intake and increasing energy expenditure in rodents after their central administration (Ellacott *et al.* 2002). In our previous studies, lipidized PrRP analogs were described to attenuate obesity and beneficially affect related metabolic disturbances in mice with diet-induced obesity (Maletinska *et al.* 2015, Holubova *et al.* 2018) and improve tolerance to glucose in Zucker diabetic (ZDF) rats and Koletsky spontaneously hypertensive obese rats (SHROB) with impaired leptin signaling (Holubova *et al.* 2016, Mikulaskova *et al.* 2018). This study aimed to further elucidate the role of leptin and PrRP in energy metabolism and to identify possible molecular mechanisms underlying these effects.

Two experiments with leptin, palm^{11}-PrRP31, and leptin + palm^{11}-PrRP31 combined with two treatment periods (2 and 8 weeks) were performed in *ob/ob* mice at two ages: a younger one (8–10 weeks), which is metabolically active, and an older one (16–24 weeks), with established morbid obesity. The morphometric and metabolic parameters of single leptin at a subthreshold dose, single palm^{11}-PrRP31, and their combination at the end of both experiments were studied for their potential synergistic effect as well as an evaluation of the impact of both treatments on lipid metabolism and of the longer treatment on the main signaling pathways of leptin. At two ages, *ob/ob* mice differed in metabolic parameters and their susceptibility to treatment.

As hypothesized, in both experiments, food intake and body weight were negatively affected by both leptin and palm^{11}-PrRP31, but the body weight change was significantly lowered only after combined leptin + palm^{11}-PrRP31 treatment. This indicates an anorexigenic synergistic effect of leptin and palm^{11}-PrRP31 on body weight in *ob/ob* mice and a possible influence of this combination on the metabolic state and signaling pathways.

As *ob/ob* mice are known to be hypothermic (Ohtake *et al.* 1977), both in younger and older age (Gratuze *et al.* 2017), body temperature was also followed at the end of both experiments. *Ob/ob* mice were hypothermic compared to WT mice in both experiments, but only in older mice did the leptin + palm^{11}-PrRP31 combination significantly upregulate the body temperature of *ob/ob* mice, indicating their synergistic action.

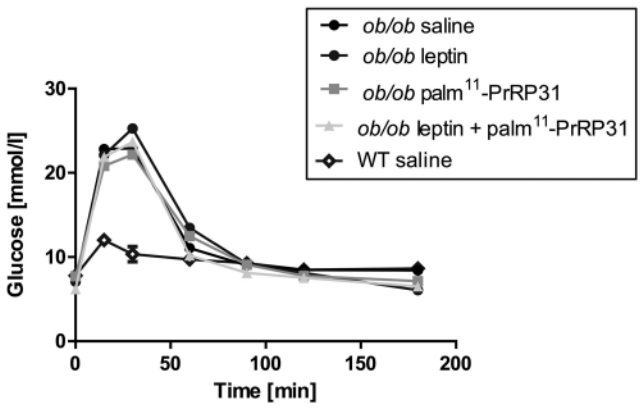

Figure 6
Oral glucose tolerance test in Experiment 2. WT mice were treated with saline and *ob/ob* mice were treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. Blood glucose was measured after oral glucose load 2 weeks before the end of treatment. Data are means ± S.E.M. (n = 8–10) (Two-way ANOVA + Bonferroni *post hoc* test).

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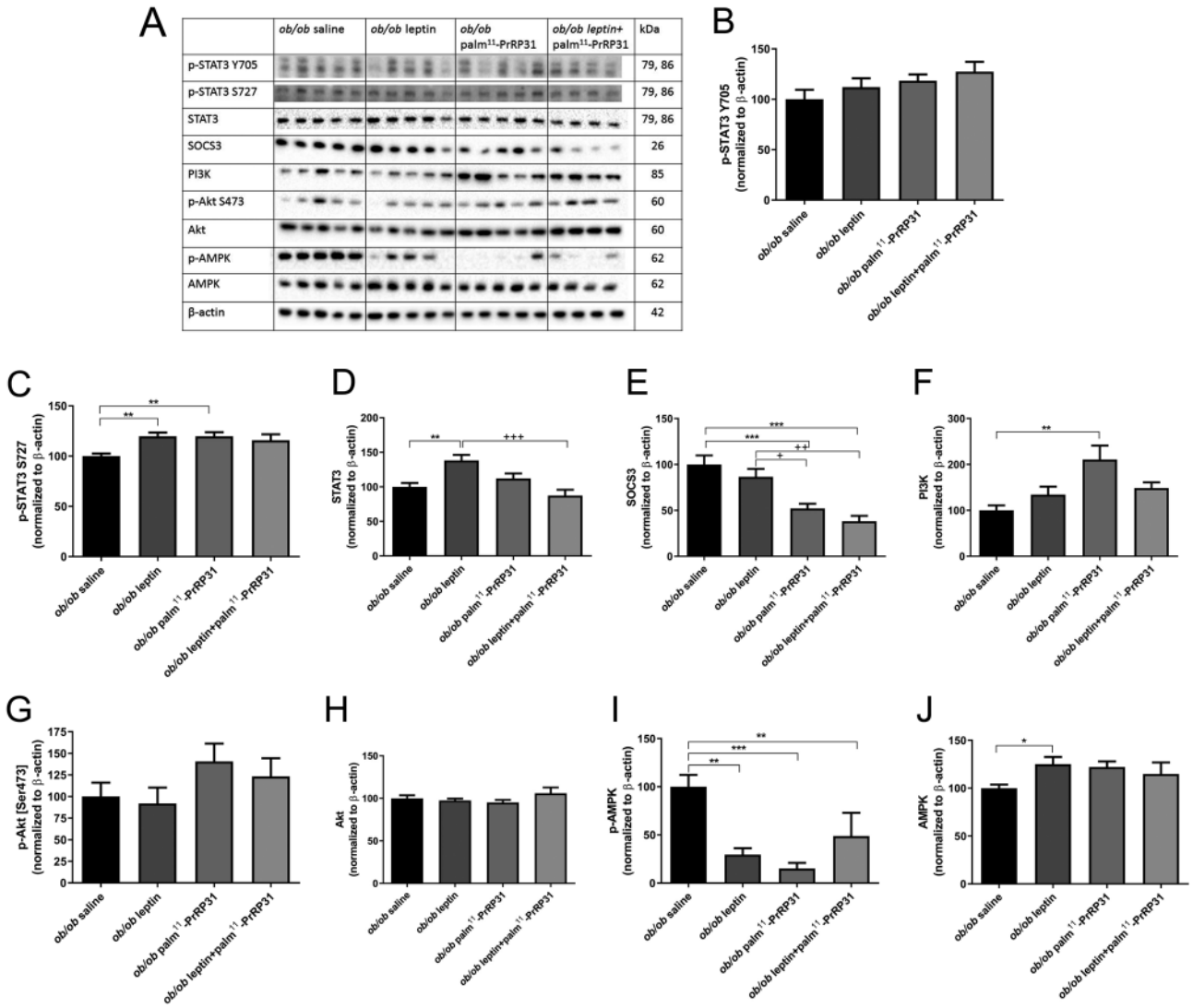

Figure 7
Hypothalamic signalling in Experiment 2. Western blot analyses in hypothalami of *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31. (A) Overview of Western blots for specific proteins. (B) p-STAT3 (Y705). (C) p-STAT3 (S727). (D) STAT3. (E) SOCS3. (F) PI3K. (G) p-AKT (S473). (H) AKT. (I) p-AMPK. (J) AMPK. Data are means ± S.E.M. (n = 8–10). Significance is $^{*/+}P < 0.05$, $^{**/++}P < 0.01$, $^{***/+++}P < 0.001$ *vs ob/ob* saline or *ob/ob* leptin group, respectively (one-way ANOVA + Bonferroni *post hoc* test).

Hyperglycemia was reported in *ob/ob* mice a long time ago (Enser 1972), and later on, it was specifically found only between 5 and 16 weeks of age (Menahan 1983). Moreover, *ob/ob* mice at 26 weeks old were found to be normoglycemic compared to both WT and *ob*+ controls (Gratuze *et al.* 2017). In this study, *ob/ob* saline mice aged 10 weeks were hyperglycemic; 2 weeks of treatment with leptin, palm^{11}-PrRP31 and their combination attenuated blood glucose significantly. On the other hand, at 16 weeks of age (start of Experiment 2), all *ob/ob* mice were normoglycemic, and no treatment affected blood glucose. However, the HbA1c level, which is considered a long-term measure of glucose control (Sacks 2013), was significantly higher in the older *ob/ob* saline than in WT saline and was not affected by any treatment. In addition, the OGTT demonstrated impaired glucose tolerance in older *ob/ob* mice when compared to WT saline and was not affected by any treatment. Similarly, a glucose tolerance test after an intraperitoneal glucose load showed impaired glucose tolerance in *ob/ob* mice at 26 weeks old (Gratuze *et al.* 2017). This suggests that even though *ob/ob* mice at the older age of 24 weeks were normoglycemic, their regulation of blood glucose was impaired.

A significantly enhanced insulin level in *ob/ob* mice at 8 and 16 weeks of age was reported a long time ago (Beloff-Chain *et al.* 1975), and *ob/ob* mice were found

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to be hyperinsulinemic until 63 weeks of age (Menahan 1983). Similarly, in this study, *ob/ob* mice aged 10, 16, and 24 weeks had hyperinsulinemia that was resistant to any treatment. Likewise, hyperinsulinemia and an enhanced HOMA index were reported at 4 weeks and then at 26 weeks of age in *ob/ob* mice (Gratuze *et al.* 2017).

Increased cholesterol but similar TAG levels in *ob/ob* mice compared to WT mice were also reported a while ago (Enser 1972). Similarly, in this study, cholesterol levels were found to be significantly increased in *ob/ob* mice at the ages of 10, 16, and 24 weeks compared to their respective WT controls. Two-week-long treatment did not affect cholesterol levels in younger *ob/ob* mice, but an 8-week-long intervention with palm^{11}-PrRP31 and its combination with leptin attenuated cholesterol levels in 24-week-old *ob/ob* mice very significantly. Analogously to Enser *et al.* (1972), the TAG level did not differ between the *ob/ob* saline and WT saline at 10, 16, and 24 weeks of age and was not affected by any treatment.

As liver weight was significantly higher in 10-week-old *ob/ob* saline compared to WT saline and was attenuated by all treatments and histological images of liver steatosis followed this pattern in Experiment 1, an effect of treatment on liver lipid metabolism came into question. Similarly, the increased liver weight of 14-week-old *ob/ob* mice was linked to *de novo* lipogenesis (Penfield *et al.* 2013). In this study, *ob/ob* controls aged 10 weeks showed increased hepatic *de novo* lipogenesis through Acaca and Fasn mRNA expression compared to WT controls that was attenuated by treatment with the leptin + palm^{11}-PrRP31 combination. Lipid oxidation as evaluated through mRNA expression of *Cpt1a* and *Pck1*, which catalyze fatty acid oxidation, was enhanced in *ob/ob* saline compared to WT saline but remained unaffected by any treatment. In *ob/ob* mice, the leptin + palm^{11}-PrRP31 combination also lowered hepatic synthesis of glucose, the main substrate for lipogenesis, through attenuation of *G6pc* mRNA, a key enzyme catalyzing gluconeogenesis and glycogenolysis. Although liver weight was also significantly higher at 16 and 24 weeks of age in *ob/ob* mice compared to WT mice in Experiment 2, no differences between *ob/ob* saline and WT saline in liver mRNA expression of genes regulating lipid metabolism were registered, and no effect of the treatment was found. This could be explained by a gradual decrease in hepatic fatty acid synthesis from 7 to 16 weeks of age in *ob/ob* mice (Kaplan & Leveille 1981). Rodriguez *et al.* (2015) found mRNA expression of aquaglyceroporin 9 (AQP9), the channel for glycerol influx into liver, to be positively correlated with hepatic steatosis in ob/ob mice. It points to glycerol abundance as another driving force of liver steatosis, besides *de novo* lipogenesis.

Lipidized PrRP was shown to target and activate the PVN, DMN, arcuate nucleus, and lateral hypothalamic area (Maletinska *et al.* 2015, Pirnik *et al.* 2015), and PI3K activation was detected after sub chronic palm^{11}-PrRP31 administration in the hypothalamus (Holubova *et al.* 2018). In this study, two main anorexigenic hypothalamic leptin pathways, JAK-STAT and AMPK, were followed in hypothalami from Experiment 2, where body weight change after leptin + palm^{11}-PrRP31 treatment was significant compared to *ob/ob* saline and single leptin. Regarding JAK/STAT, phosphorylation of STAT3 was found to be increased only at Ser727 but not at Tyr705 after treatment with leptin, palm^{11}-PrRP31, and the leptin-palm^{11}-PrRP31 combination. Although Tyr705 is considered the primary phosphorylation epitope in STAT3, direct Ser727 phosphorylation by insulin and other ligands was reported (Zhang *et al.* 2001). Positively regulated STAT3 and downregulated SOCS3 at the same time suggested that both leptin and palm^{11}-PrRP31 support JAK/STAT signaling in the hypothalami of *ob/ob* mice.

Hypothalamic AMPK phosphorylation was significantly lowered after all three treatments. This finding suggests that the AMPK anorexigenic pathway in the hypothalami of *ob/ob* mice was restored not only by leptin supplementation but also by palm^{11}-PrRP31 treatment. As inhibition of hypothalamic AMPK activity is necessary for the anorexigenic effects of leptin (Minokoshi *et al.* 2004), this study suggests that palm^{11}-PrRP31 could compensate for deficient leptin in *ob/ob* mice regarding leptin anorexigenic action in the hypothalamus. Moreover, a significant decrease in SOCS3 by palm^{11}-PrRP31 was not reached by a subthreshold dose of single leptin but by the leptin + palm^{11}-PrRP31 combination.

The synergistic effect of leptin and palm^{11}-PrRP31 was proven by several parameters: the decrease in liver weight and glucose levels after a shorter treatment in younger mice, and the body weight change, increase in body temperature, lowered cholesterol level and SOCS3 production with a longer treatment of older mice.

In conclusion, leptin and palm^{11}-PrRP31 synergistically lowered body weight and synergistically increased body temperature in older *ob/ob* mice with established morbid obesity. Regarding PrRP, this finding is novel and could point to other potent beneficial PrRP effects in the brain as obesity and hypothermia were recently linked to neurodegeneration.

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Supplementary materials

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Cholecystokinin System Is Involved in the Anorexigenic Effect of Peripherally Applied Palmitoylated Prolactin-Releasing Peptide in Fasted Mice

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Summary

Prolactin-releasing peptide (PrRP) has been proposed to mediate the central satiating effects of cholecystokinin (CCK) through the vagal CCK1 receptor. PrRP acts as an endogenous ligand of G protein-coupled receptor 10 (GPR10), which is expressed at the highest levels in brain areas related to food intake regulation, e.g., the paraventricular hypothalamic nucleus (PVN) and nucleus of the solitary tract (NTS). The NTS and PVN are also significantly activated after peripheral CCK administration. The aim of this study was to determine whether the endogenous PrRP neuronal system in the brain is involved in the central anorexigenic effect of the peripherally administered CCK agonist JMV236 or the CCK1 antagonist devazepide and whether the CCK system is involved in the central anorexigenic effect of the peripherally applied lipidized PrRP analog palm-PrRP31 in fasted lean mice. The effect of devazepide and JMV236 on the anorexigenic effects of palm-PrRP31 as well as devazepide combined with JMV236 and palm-PrRP31 on food intake and Fos cell activation in the PVN and caudal NTS was examined. Our results suggest that the anorexigenic effect of JMV236 is accompanied by activation of PrRP neurons of the NTS in a CCK1 receptor-dependent manner. Moreover, while the anorexigenic effect of palm-PrRP31 was not affected by JMV236, it was partially attenuated by devazepide in fasted mice. The present findings indicate that the exogenously influenced CCK system may be involved in the central anorexigenic effect of peripherally applied palm-PrRP31, which possibly indicates some interaction between the CCK and PrRP neuronal systems.

Key words

Lipidized prolactin-releasing peptide analog • Cholecystokinin system • Cholecystokinin 1 receptor • Hypothalamic paraventricular nucleus • Nucleus of the solitary tract • c-Fos

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Introduction

Anorexigenic neuropeptide prolactin-releasing peptide (PrRP), which has a structural RF-amide motif at the C-terminus that is important for its biological activity, is an endogenous ligand of G protein-coupled receptor 10 (GPR10), although it also has high binding affinity for neuropeptide FF receptor type 2 (NPFF-2R) (Kuneš *et al.* 2016, Pražienková *et al.* 2019). PrRP is mainly expressed in the nucleus of the solitary tract (NTS) of the brainstem, ventrolateral medulla and dorsomedial hypothalamic nucleus (DMN), and PrRP-positive fibers are found in the hypothalamus, amygdala and area postrema (AP), which are all areas connected with food intake regulation, as previously reviewed (Dodd and Luckman 2013, Pražienková *et al.* 2019, Quillet *et al.* 2016). The highest expression of GPR10 mRNA in rats was detected in the

reticular nucleus of the thalamus, paraventricular hypothalamic nucleus (PVN), periventricular hypothalamic nucleus, DMN, AP and NTS. A moderate level of GPR10 expression was also found in the anterior pituitary and ventromedial nuclei (Ibata *et al.* 2000, Roland *et al.* 1999).

Initial studies showed that intracerebroventricular (i.c.v.) administration of PrRP reduced food intake and increased energy expenditure in rodents (Lawrence *et al.* 2000, Lawrence *et al.* 2002). Moreover, Ellacott *et al.* (2002) suggested that the anorexigenic action of PrRP is regulated by the adiposity signal leptin. Moreover, i.c.v. co-administration of PrRP and leptin to rats lowered food intake by an additive effect and increased body temperature more than either peptide alone. PrRP was also proposed to mediate some of the central satiating actions of the peptide hormone cholecystokinin (CCK) released from the gastrointestinal tract (GIT) (Lawrence *et al.* 2002). CCK is released from the GIT following the consumption of a meal and exerts its short-term anorexigenic effect via CCK1 receptors located on vagal afferents. Vagal efferents synapse to second-order neurons in the NTS by releasing neurotransmitters and neuropeptides (Luckman 1992, Peters *et al.* 2006). In such a route, the intraperitoneal (i.p.) injection of CCK activated c-Fos expression as a marker of neuronal activation in NTS neurons producing PrRP (Lawrence *et al.* 2002). CCK-induced hypothalamic c-Fos activation is strongly associated with the activation of noradrenergic A2 neurons within the NTS that express c-Fos even after peripheral injection of low doses of CCK (Maniscalco and Rinaman 2013, Wall *et al.* 2020). In fasted or satiated GPR10 KO mice, i.c.v. administration of PrRP did not reduce food intake compared to their wild-type controls. The administration of CCK did not result in the inhibition of food intake in GPR10 KO mice, suggesting that PrRP is involved in the central satiating actions of CCK (Bechtold and Luckman 2006). In addition, Cre recombinase-mediated reactivation of PrRP in the brainstem rescued the anorectic action of CCK (Dodd *et al.* 2014). On the other hand, endogenous CCK is physiologically involved in feeding control during fasting via the hypothalamic PVN (Cano *et al.* 2003), where CCK1 receptors are abundant (Woodruff *et al.* 1991). In addition, the activation of CCK neurons of the NTS that innervate the PVN stimulates appetite (D'Agostino *et al.* 2016, Roman *et al.* 2017).

PrRP acts centrally; therefore, the potential of PrRP to decrease food intake after peripheral administration depends on its ability to reach receptors in the brain and thus facilitate its central effect. Our group designed analogs of PrRP lipidized at the N-terminus, myristoylated PrRP20 (myr-PrRP20) and palmitoylated PrRP31 (palm-PrRP31), and PrRP31 palmitoylated at position 11 were shown to significantly lower food intake in fasted lean mice after subcutaneous (s.c.) administration and to lower body weight and improve metabolic parameters in diet-induced obese mice (Maletínská *et al.* 2015, Pražienková *et al.* 2017). Moreover, only palm-PrRP31 and myr-PrRP20, but not natural PrRP20, PrRP31 or octanoylated PrRP31, showed longer stability in rat plasma and after s.c. administration significantly increased c-Fos immunoreactivity in hypothalamic and brainstem nuclei involved in food intake regulation, such as the PVN, arcuate hypothalamic nucleus (Arc) and NTS (Maletínská *et al.* 2015). Finally, palm-PrRP31 administration resulted in significantly increased c-Fos levels in lateral hypothalamic area (LHA) hypocretin neurons and PVN oxytocin neurons (Pirnik *et al.* 2015).

Although PrRP mediates some of the central satiating actions of CCK and endogenous CCK is physiologically involved in feeding control during fasting via the hypothalamic PVN, there is no information to date regarding whether the CCK system may also be involved in the central anorexigenic effect observed after peripherally administered lipidized PrRP analog. Therefore, the anorexigenic activity of palm-PrRP31 with neuronal activity of the NTS and PVN, brain structures involved in homeostatic food control, was studied in overnight-fasted mice in which the "silent" CCK system, i.e., minimal endogenous peripheral CCK activity caused by fasting, was pharmacologically influenced by devazepide (Dev), a CCK1 receptor antagonist, and JMV236, a stable anorexigenic CCK analog (Maletínská *et al.* 1992).

Methods

Applied drugs

The cholecystokinin analog JMV236 (Asp-Tyr (SO3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH2, PolyPeptide, Strasbourg, France), CCK-1 receptor antagonist Dev (L364,718) (gift from ML Laboratories, Liverpool, UK) and human palmitoylated PrRP analog palm-PrRP31 (N-palm-SRTHRHSMEIRTPDINPAWAYSRGIRPVGRF-NH2) were used in the experiments. Palm-PrRP31 was

synthesized and purified at the Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic, as described previously (Maletínská *et al.* 2015, Popelová *et al.* 2018). JMV236 and palm-PrRP31 were dissolved in saline (Sal), while Dev was dissolved in Sal containing 15 % DMSO.

Experimental design

Male C57BL/6J mice from Charles River Laboratories (Sulzfeld, Germany) were housed at a temperature of 23 °C and a relative humidity of 45-65 % with a daily 12 h light/dark cycle (lights on at 6:00 am). The mice were given *ad libitum* water and a standard rodent chow diet Ssniff R/M-H (Ssniff Spezialdiäten GmbH, Soest, Germany) and were housed at five mice per cage until three months of age. All animal experiments followed the ethical guidelines for animal experiments of the European Union Directive (2010/63/EU) and the Act of the Czech Republic Nr. 246/1992 and were approved by the Committee for experiments with Laboratory Animals of the Academy of Sciences of the Czech Republic.

In both experiments, individually housed overnight-fasted (16 h) mice (body weight 27.2 g ± 0.27 g) provided *ad libitum* to water were used. In both experiments, fasted mice were randomly divided into eight experimental groups: 1) Sal/Sal, 2) Sal/palm-PrRP31, 3) JMV236/Sal, 4) JMV236/palm-PrRP31, 5) Dev/Sal, 6) Dev/palm-PrRP31, 7) Dev/JMV236 and 8) Dev/JMV236 and palm-PrRP31. The first intraperitoneal (i.p., 0.15 ml) administration of Sal, Dev (1 mg/kg) and JMV236 (7 μg/kg) was thirty minutes later followed by a second subcutaneous (s.c., 0.15 ml) treatment with Sal and palm-PrRP31 (1 mg/kg) or i.p. treatment by JMV236 (7 μg/kg). The applied doses of JMV236, Dev and palm-PrRP31 were selected according to previously published data (Maletínská *et al.* 1992, Maletínská *et al.* 2008, Maletínská *et al.* 2015).

Food intake study

Rodent chow pellets were given to fasted mice (n=6 per group) 30 min after the second injection. The pellets were weighed every 30 min (noncumulative food intake) for the next 5 hours. The cumulative food intake was calculated by progressive summation of noncumulative food intakes during the individual time intervals. In all animals, the residual effect of the applied drugs was excluded based on nonsignificant differences in the amount of food intake during the first 24 h after drug administration compared to Sal/Sal treatment. For ethical reasons and for the validity, reproducibility, and respectability of the obtained data, the food intake study was repeated after 7 days in the same mice, and all data were pooled.

Immunohistochemical study

Fasted mice (n=5 per group) were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) ninety minutes after the second injection. The mice were transcardially perfused with ice-cold saline with heparin (10 U/ml, Zentiva, Prague, Czech Republic), and the brains were removed and postfixed in 0.1 M phosphate buffer (PB, pH 7.4) containing 4 % paraformaldehyde. To prevent diurnal variations in c-Fos expression, this part of the experiment was performed between 7:00 and 12:00 a.m.

Immunohistochemical staining

After 24 h of fixation, the brains were stored in a 20 % sucrose solution in PB with 0.1 % sodium azide at 4 °C and cut into 30 μm coronal sections using a cryostat (CM1950; Leica Biosystems, Germany). c-Fos immunohistochemistry was performed with rabbit c-Fos monoclonal antibody (1:2000; #2250S; Cell Signaling Technology, Inc., Danvers, MA, USA) according to the protocol described earlier (Pirnik *et al.* 2018). Consecutive PrRP immunostaining with rabbit polyclonal PrRP-31 antibody (1:500; #H-008-52; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) was performed according to the same procedure as described above. The final PrRP immunoreactions were visualized by a single 0.01 % 3,3′-diaminobenzidine tetrahydrochloride (DAB; Millipore Sigma, Burlington, MA, USA) solution in 0.05 M Tris buffer (pH 7.4) and 0.0006 % H_2O_2. The development time (6 min) of the DAB immunostaining was monitored under a light microscope to reach the appropriate yellow-brownish color. Finally, the sections were mounted into 0.5 % gelatin dissolved in 0.05 M sodium acetate buffer (pH 6.0), air-dried and cover-slipped with Permount (Millipore Sigma, Burlington, MA, USA). The immunostaining of the negative control, which did not show any antiserum immunolabeling, included the substitution of the primary antisera with normal rabbit serum and the sequential elimination of the primary and secondary antibodies from the staining series.

The c-Fos immunoreactive cells were counted separately on each side of the appropriate coronal brain

sections (n=3–4 sections/mouse) within the PVN (from bregma −0.7 mm to −0.94 mm) according to the mouse brain atlas (Paxinos 2004). In the same way, PrRP-, Fos-PrRP- and Fos-immunopositive cells were counted within the caudal NTS (from bregma -7.48 to -7.76 mm) according to the same mouse brain atlas based on (Ganchrow *et al.* 2014). Quantitative assessment of the immunostained cells was performed manually in CellCounter1.2. from digital images of the selected areas captured with an Olympus AX70 light microscope and digital camera (Olympus DP70; Olympus Europa SE & Co. KG, Hamburg, Germany). Images of representative sections were captured by the same microscope and digital camera.

Statistical analysis

All obtained data were first checked for normal distribution by the Shapiro-Wilks test in Statistica 7.0 (StatSoft) and SigmStat 4.0 (Systat). Square root normalized data from Fos immunoreactive cell counts in the PVN were analyzed by two-way ANOVA followed by Tukey's post hoc comparison (SigmStat 4.0, Systat). If the distribution of the data was also nonnormal after square root data normalization (data obtained from noncumulative and cumulative food intake, data obtained from the immunohistochemical studies related to cNTS), then nonparametric Kruskal-Wallis analysis followed by Student-Newman-Keuls multiple comparison was performed. All the results are reported as the mean ± SEM. Differences were considered significant at $p<0.05$.

Results

Food intake and Fos neuronal activation in fasted mice did not differ between mice treated with Sal and the CCK1 receptor antagonist Dev alone.

In overnight-fasted Sal mice, the maximal food intake was observed during the first half-hour interval (Fig. 1). The second maximal peak of noncumulative food intake during the second half-hour interval did not exceed 40 % of its noncumulative food intake in the first time interval (Fig. 1). Dev alone did not significantly influence the normal pattern of food consumption in overnight-fasted mice compared to the Sal group (Fig. 1, Fig. 2).

In the caudal NTS (cNTS), almost no c-Fos-immunopositive cells (Fig. 3A, C) or c-Fos-immunopositive PrRP neurons (Fig. 3B, C) were detected in the Sal or Dev alone group. In the PVN, only a minimal number of c-Fos-immunopositive cells was found after Sal or Dev administration alone (Fig. 4A,B). Moreover, the number of Fos-immunoreactive cells in the PVN did not significantly differ between Sal and Dev alone (Fig. 4A,B).

Onset of the anorexigenic effect of the CCK analog JMV236 is accompanied by Fos activation of PrRP neurons of the cNTS *via* CCK1 receptors.

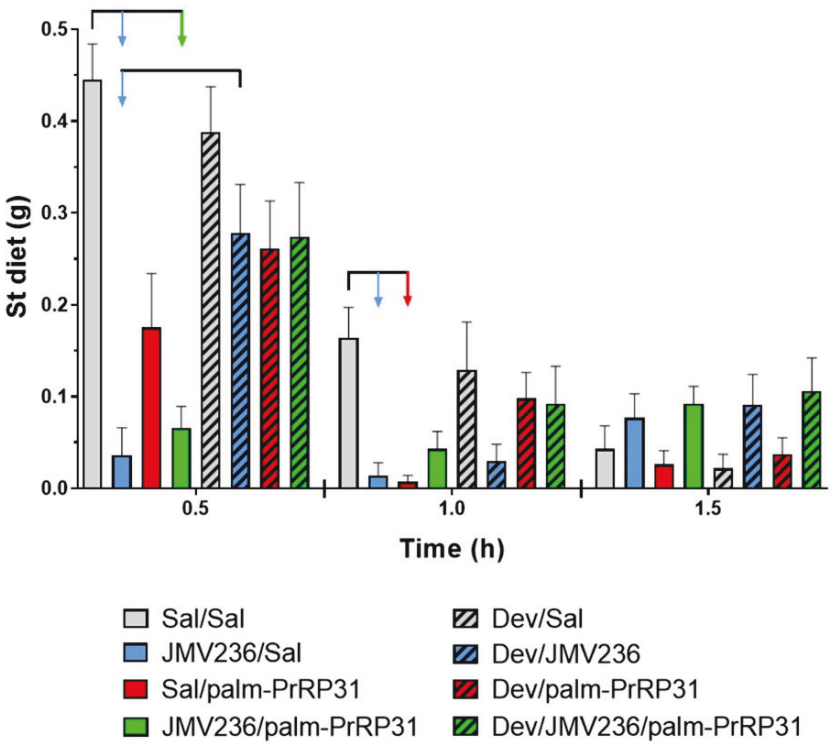

Fig. 1. Non-cumulative food intake of fasted mice (n=12 per group) treated by Sal or Dev (1 mg/kg) and followed by Sal, JMV236 (7 μg/kg), palm-PrRP31 (1 mg/kg) and JMV236 (7 μg/kg)/palm-PrRP31 (1 mg/kg). Dev pretreatment of mice suppressed anorexigenic effect of JMV236 and was able to partially block anorexigenic effect of palm-PrRP31 and JMV236/palm-PrRP31 in time interval which covered time interval of immunohistochemical study. $p<0.05$ for each group represented by arrow [nonparametric Kruskal-Wallis analysis, $H(7,N=96)=42.728$, $p<0.0001$], Sal – saline, Dev – devazepide, palm-PrRP31 - palmitoylated prolactin-releasing peptide 31

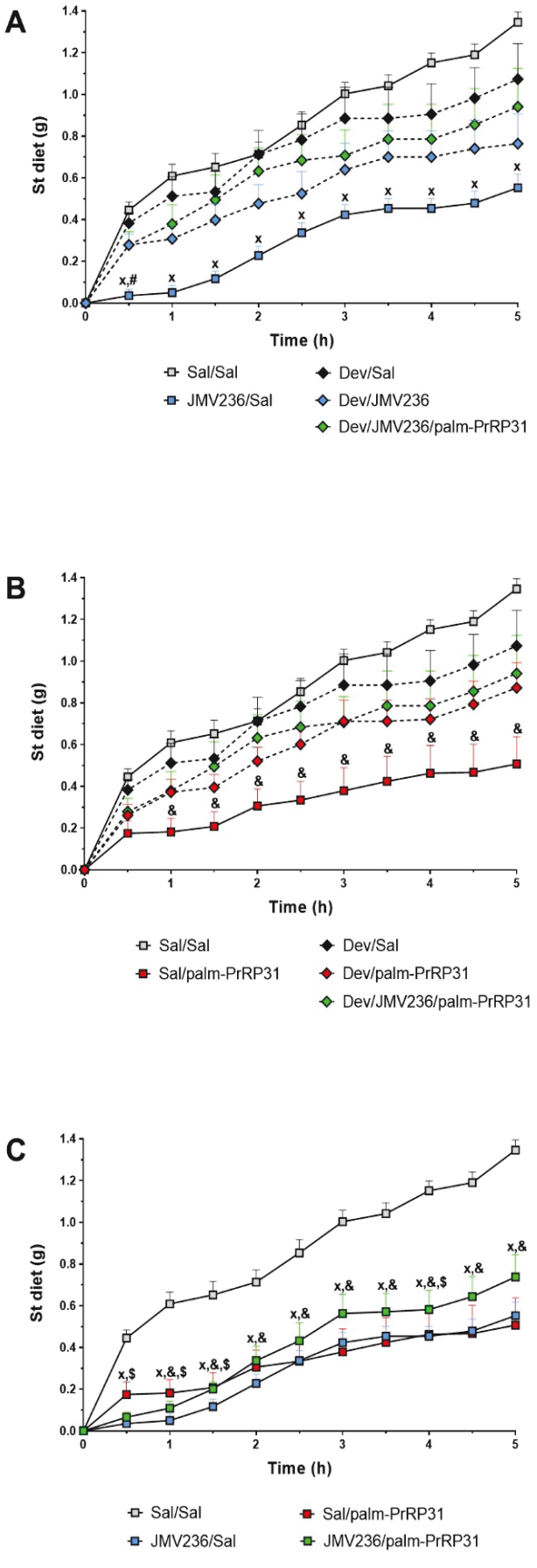

Fig. 2. Cumulative food intake of fasted mice (n=12 per group) treated by Sal or Dev (1 mg/kg) and followed by Sal, JMV236 (7 μg/kg), palm-PrRP31 (1 mg/kg) and JMV236 (7 μg/kg)/palm-PrRP31 (1 mg/kg). (**A**) Dev pretreatment of mice suppressed anorexigenic effect of JMV236, (**B**) In Dev pretreated group of mice no significant differences between Dev alone and Dev/palm-PrRP31 was achieved on food intake, (**C**) The anorexigenic effect of palm-PrRP31 after JMV236 treatment. Please note that in Dev pretreated group of mice no significant anorexigenic effect of JMV236/palm-PrRP31 was achieved as in Dev alone. $^{x}p<0.05$ for JMV236/Sal vs Sal/Sal, $^{\#}p<0.05$ for JMV236/Sal vs Dev/JMV236, $^{\&}p<0.05$ for Sal/palm-PrRP31 vs Sal/Sal and $^{\$}p<0.05$ for JMV236/palm-PrRP31 vs Sal/Sal [nonparametric Kruskal-Wallis analysis, $H(7,N=96)=42.728$, $p<0.0001$], Sal – saline, Dev – devazepide, palm-PrRP31 - palmitoylated prolactin-releasing peptide 31

Peripheral administration of JMV236 to overnight-fasted mice significantly lowered noncumulative food intake during the first and second half-hour intervals by almost 92 % compared to Sal alone (Fig. 1, $p<0.05$). This anorexigenic effect of JMV236 was significantly attenuated by Dev pretreatment (Fig. 1, $p<0.05$). In overnight-fasted mice, compared to Sal alone, a significant anorexigenic effect of JMV236 on cumulative food intake during all the measured time intervals was observed (Fig. 2A, $p<0.05$). No significant differences between Dev/JMV236 and Dev alone were found in noncumulative or cumulative food intake (Fig. 1, Fig. 2A).

The onset of the JMV236 effect on food intake was accompanied by a significantly increased number of Fos-immunopositive cells in the cNTS (Fig. 3A,C, $p<0.05$) and PVN compared to Sal alone (Fig. 4A,B, $p<0.05$). At the same time, approximately one-third of PrRP neurons in the cNTS were significantly activated after JMV236 administration compared to Sal alone (Fig. 3B,C, $p<0.05$). JMV236-induced c-Fos expression in PrRP cNTS neurons and PVN cells was almost completely suppressed by Dev pretreatment (Fig. 3B, C, $p<0.05$, Fig. 4A, B, $p<0.05$).

Pretreatment with Dev partially blocked the anorexigenic effect of palm-PrRP31

During the first and second half-hour intervals, palm-PrRP31 reduced noncumulative food intake by almost 61 % (Fig. 1, $p=0.055$) and 96 %, respectively (Fig. 1, $p<0.05$), compared to Sal alone. Except for the first half-hour (Fig. 2B, $p=0.055$), palm-PrRP31 also had a significant suppressive effect on cumulative food intake compared to Sal alone (Fig. 2B, $p<0.05$). On the other hand, no significant differences between Dev/palm-PrRP31 and Dev alone on food intake were achieved (Fig. 1, Fig. 2B).

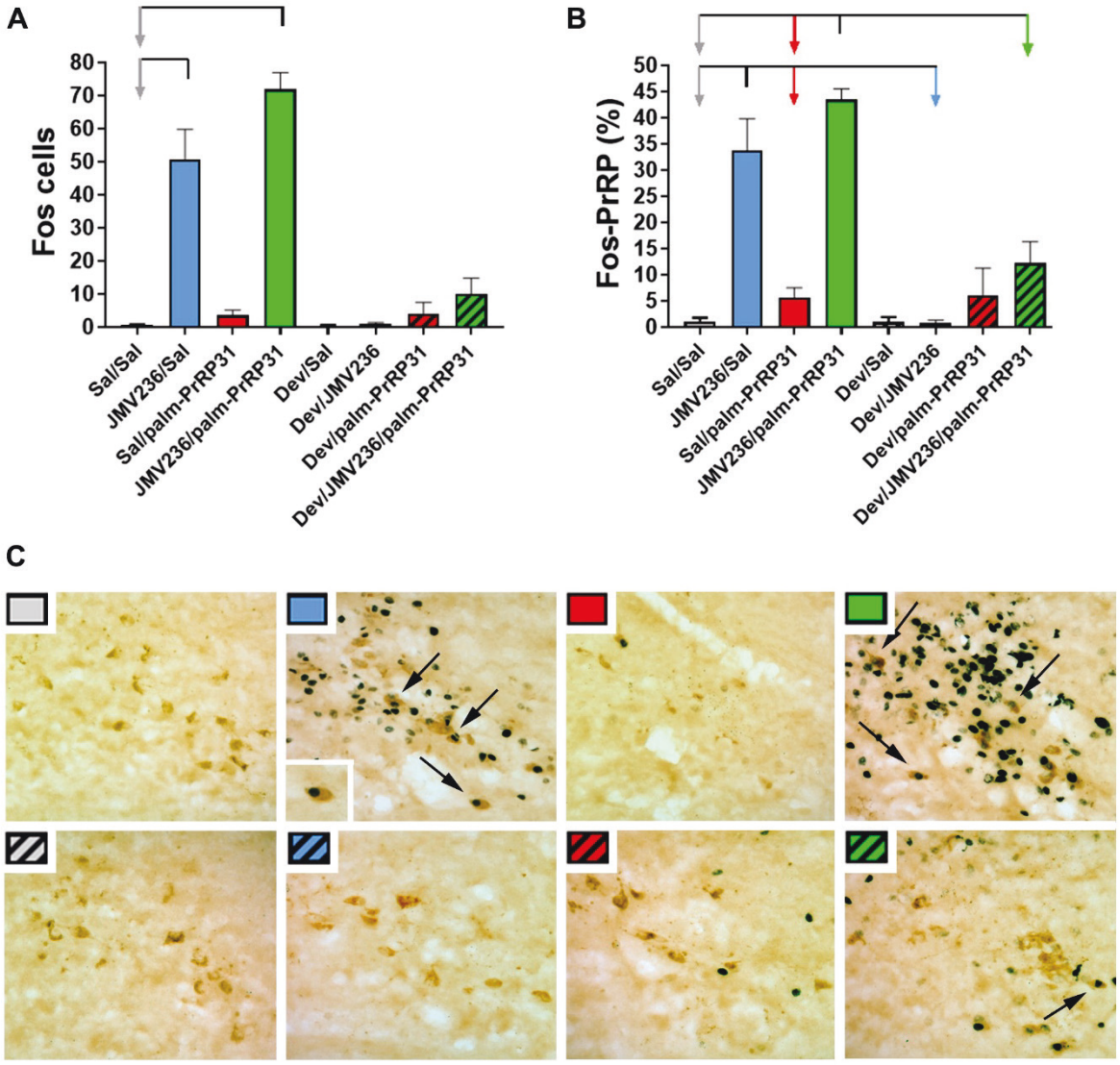

Fig. 3. cNTS: Fos cell immunoreactivity in brainstem cNTS 90 min after last injection in mice (n=5 per group) treated by Sal or Dev (1 mg/kg) and followed Sal, JMV236 (7 μg/kg), palm-PrRP31 (1 mg/kg) and JMV236 (7 μg/kg)/palm-PrRP31 (1 mg/kg). (**A**) In Sal pretreated group of mice JMV236 and JMV236/palm-PrRP31 significantly increased the number of Fos immunopositive cells, (**B**) Dev pretreatment significantly reduced percentage of Fos-PrRP immunostained neurons in mice treated JMV236 and JMV236/palm-PrRP31, (**C**) Representative photographs of Fos immunostained cells and Fos-PrRP immunostained neurons (black arrows) with detail photograph of one colocalization. $p<0.05$ for each group represented by arrow [Fos: nonparametric Kruskal-Wallis analysis, $H(7,N=5)=28.356$, $p<0.001$, percentage of Fos-PrRP: nonparametric Kruskal-Wallis analysis, $H(7,N=5)=31.388$, $p<0.001$], Sal – saline, Dev – devazepide, palm-PrRP31 - palmitoylated prolactin-releasing peptide 31, cNTS - caudal nucleus of the solitary tract

The onset of the anorexigenic effect of palm-PrRP31 was not accompanied by a significantly changed number of c-Fos-immunopositive cells in either the cNTS (Fig. 3A,C) or PVN (Fig. 4A,B) or Fos-PrRP neurons in the cNTS (Fig. 3B,C) compared to Sal alone. Dev in combination with palm-PrRP31 did not affect the number of Fos- or Fos-PrRP-immunopositive cells in the cNTS compared to Dev alone (Fig. 4A,B,C). In the PVN, Dev/palm-PrRP31-treated animals exhibited a significantly increased number of Fos immunopositive cells compared to Dev alone (Fig. 4A,B, $p<0.05$).

Anorexigenic effect of palm-PrRP31 in fasted mice was not affected by the CCK analog JMV236, but pretreatment with Dev partially blocked the anorexigenic effect of JMV236-palm-PrRP31

In the Sal pretreatment group, the anorexigenic effects achieved by JMV236, palm-PrRP31 and JMV236/palm-PrRP31 did not differ significantly from each other at any time (Fig. 2C). The onset of the anorexigenic activity of JMV236/palm-PrRP31 was achieved at the same time as JMV236 and earlier than the onset of the anorexigenic activity of palm-PrRP31 (Fig. 1).

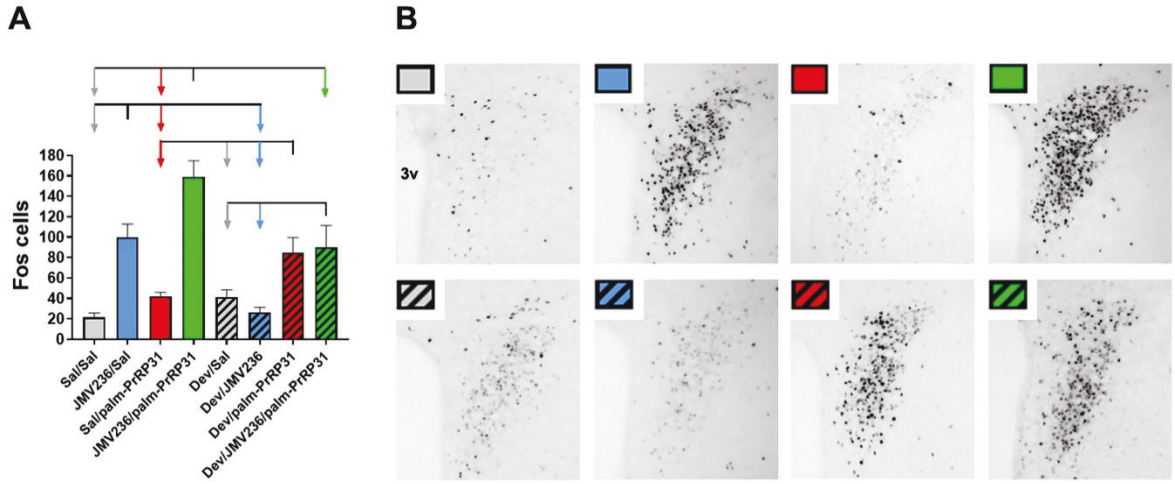

Fig. 4. PVN: Fos cell immunoreactivity in PVN 90 min after last injection in mice (n=5 per group) treated by Sal or Dev (1 mg/kg) and followed Sal, JMV236 (7 μg/kg), palm-PrRP31 (1 mg/kg) and JMV236 (7 μg/kg)/palm-PrRP31 (1 mg/kg). (**A**) Dev pretreatment significantly reduced number of Fos immunopositive cells in mice treated JMV236 and JMV236/palm-PrRP31 but significantly increased number of Fos immunopositive cells in mice treated palm-PrRP31, (**B**) Representative photographs of Fos immunostained cells. $p<0.05$ for each group represented by arrow [two-way ANOVA, treatment: $F(1,3)=20.438$, $p<0.001$, pretreatment x treatment: $F(1,3)=13.93$, $p<0.001$], Sal – saline, Dev – devazepide, palm-PrRP31 - palmitoylated prolactin-releasing peptide 31, PVN - paraventricular hypothalamic nucleus, 3v – third brain ventricle

No significant differences between Dev alone and Dev combined with JMV236/palm-PrRP31, JMV236 and palm-PrRP31 in effect on food intake were found (Fig. 1, Fig. 2).

In the Sal-pretreated groups, the onset of the anorexigenic effect of JMV236/palm-PrRP31 and JMV236 was associated with a significantly increased number of Fos-immunolabeled PrRP neurons in the cNTS compared to palm-PrRP31 (Fig. 3B,C, $p<0.05$). A similar effect was detected in the PVN: JMV236/palm-PrRP31 and JMV236 exhibited significantly more activated cells than palm-PrRP31 (Fig. 4A, B, $p<0.05$). No significant differences in the number of Fos-immunolabeled cells in either the cNTS or PVN or in the number of Fos-immunolabeled PrRP neurons in the cNTS were found between the JMV236/palm-PrRP31 and JMV236 groups (Fig. 3A,B,C, Fig. 4A,B).

Dev in combination with JMV236/palm-PrRP31 significantly reduced the number of Fos-immunolabeled PrRP neurons in the cNTS (Fig. 3B, C, $p<0.05$) and the number of Fos-immunolabeled cells in the PVN compared to those of the JMV236/palm-PrRP31 group (Fig. 4A,B, $p<0.05$). In addition, a significant parallel decrease in the number of Fos-PrRP neurons in the cNTS and Fos-immunolabeled cells in the PVN also occurred in the Dev/JMV236 group compared to the JMV236 alone group (Fig. 3B,C, $p<0.05$, Fig. 4A,B, $p<0.05$). On the other hand, Dev pretreatment with palm-PrRP31 significantly increased the number of Fos-immunolabeled cells in the PVN compared to palm-PrRP31 alone (Fig. 4A,B, $p<0.05$).

In the Dev-pretreated groups, JMV236, palm-PrRP31 or JMV236/palm-PrRP31 did not affect the number of Fos- or Fos-PrRP neurons in the cNTS compared to Dev alone (Fig. 3). On the other hand, the number of Fos immunolabeled cells in the PVN of Dev/JMV236/palm-PrRP31 was comparable to that of Dev/palm-PrRP31 group and was significantly higher than in the Dev/JMV236 and Dev alone (Fig. 4A,B, $p<0.05$).

Discussion

The present study aimed to elucidate the anorexigenic effect of peripherally applied palmitoylated prolactin-releasing peptide in the context of pharmacological manipulation of the "silent" cholecystokinin system in fasted mice.

Our data showed that the 1) onset of the anorexigenic effect of peripherally applied CCK agonist JMV236 was accompanied by significant Fos activation of PrRP neuronal population of the caudal NTS and by significant Fos cell activation in the PVN; 2) peripheral palm-PrRP31 administration in low anorexigenic dose was not associated with significant Fos activation of PrRP neuronal population in cNTS or Fos cell activation in PVN; 3) pretreatment with Dev was able to partially block anorexigenic effect of palm-PrRP31 and increase

Fos cell activation in PVN; and 4) although combination of peripherally administered JMV236 and palm-PrRP31 did not show additive anorexigenic effect in fasted mice, it caused enhancement of Fos neuronal activity both in NTS and PVN which was partially suppressed by Dev pretreatment.

In our study, Fos cell activation in the NTS and PVN was studied after peripheral administration of the CCK analogs JMV236 and palm-PrRP31 for several reasons. First, among the key brainstem and hypothalamic structures that are involved in homeostatic food intake regulation, only the NTS and PVN were found to be significantly activated after acute peripheral CCK administration (Lawrence *et al.* 2002, Maletínská *et al.* 2008, Pirnik *et al.* 2010). Second, the NTS and the hypothalamic DMN are the main locations of brain PrRP neurons (Maruyama *et al.* 1999, Morales *et al.* 2000, Roland *et al.* 1999); however, only PrRP neurons in the NTS were shown to be involved in mediating the central satiety effect of CCK (Bechtold and Luckman 2006, Lawrence *et al.* 2002) while PrRP neurons in the DMN seem to be regulated by energy status (Lawrence *et al.* 2000). Third, prominent PrRP fiber innervation from the NTS to the PVN was described (Morales *et al.* 2000), and the CCK-PrRP-oxytocin PVN pathway was identified as critical for controlling meal size (Yamashita *et al.* 2013). In addition, although the PVN contains a lower number of GPR10 receptors than the DMN and NTS (Roland *et al.* 1999), our data with another palmitoylated PrRP analog indicated that while DMN cells might be associated with the process of long-term adaptation to modified energy homeostasis, activated PVN and NTS cells are instead also associated with the anorexigenic effect of lipidized analogs of PrRP (Pirník *et al.* 2018).

Our data showed that the anorexigenic effect of JMV236, which was accompanied by cell activation in the NTS and PVN, was inhibited by the selective CCK1 receptor antagonist Dev. Dev is able to cross the blood-brain barrier (BBB) (Woltman *et al.* 1999) and thus could be able to inhibit the anorexigenic effect of JMV236 *via* central CCK1 receptors. On the other hand, peripheral CCK1 receptors are involved in the central anorexigenic effect of CCK (Reidelberger *et al.* 2003), and JMV236 is analogously a CCK peptide agonist, as endogenous CCK is unable to cross the BBB (Passaro *et al.* 1982), and its central anorexigenic effect is probably indirect. The anorexigenic effect of JMV236 lasted several hours after its i.p. administration (Gourch *et al.* 1990, Maletínská *et al.* 1992). Moreover, JMV236 can inhibit food intake in rats only after its peripheral i.p. but not central i.c.v. administration (Gourch *et al.* 1990). Our study indicates that significant activation of PVN cells after peripheral JMV236 administration may be a consequence of the activation of peripheral CCK1 receptors and is probably associated with PrRP neurons of the NTS. On the other hand, the activation of cocaine- and amphetamine-regulated transcript (CART)-positive neurons in the PVN was also associated with the activation of NTS cells after peripheral CCK administration (Peter *et al.* 2010). In addition, a significant synergistic anorexigenic effect of peripheral CCK and central CART peptide in mice was also described (Maletínská *et al.* 2008). From this point of view, another neuronal system may also participate in the central anorexigenic effect observed after peripheral JMV236 administration.

In addition, the onset of the anorexigenic effect of JMV236/palm-PrRP31 and JMV236 was associated with a significantly increased number of Fos-immunolabeled PrRP neurons in the cNTS compared to that of palm-PrRP31. In addition, peripheral palm-PrRP31 administration at a dose of 1 mg/kg was associated only with nonsignificant PVN cell activation in our study. Recently, we showed that the central anorexigenic effect of peripherally administered palm-PrRP31 (5 mg/kg, s.c.) in mice was accompanied by significant Fos cell activation of the brainstem cNTS as well as hypothalamic PVN-Arc-DMN activation (Pirnik *et al.* 2015). In addition, a significant number of oxytocin PVN neurons and hypocretin LHA neurons were also activated after s.c. administration of palm-PrRP31 at dose of 5 mg/kg (Pirnik *et al.* 2015). In our study, only a nonsignificant increase in Fos cell activity in the cNTS and PVN after a lower palm-PrRP31 dose (1 mg/kg, s.c.), despite its significant anorexigenic effect, could be explained by a delayed onset of its anorexigenic activity that was already published for both doses (Maletínská *et al.* 2015).

This study showed that Dev alone did not significantly influence food intake and cell activation in the cNTS and PVN in overnight-fasted mice compared to Sal, but Dev pretreatment was able to significantly diminish food intake and Fos activation in both the NTS and PVN after JMV236 administration. Dev pretreatment was also able to partially reduce palm-PrRP-mediated food intake inhibition and to decrease Fos activation in response to combined JMV236 and palm-PrRP treatment in both the NTS and PVN. Moreover, our data also showed that peripheral Dev administration in fasted

animals increased the number of Fos immunopositive cells in the PVN after palm-PrRP administration at a dose of 1 mg/kg. It must be mentioned that the plasma level of endogenous CCK in fasted animals is low (Playford *et al.* 1993), and in our study, Dev did not affect food intake when administered to overnight-fasted animals. In addition, even though Dev was able to increase CCK synthesis in the gastrointestinal tract of fasted animals, it was not able to increase plasma CCK levels (Playford *et al.* 1993). Although our data indicated that Dev could act peripherally to antagonize the action of JMV236, due to the low endogenous peripheral plasma CCK levels in fasted animals, the central effect of Dev should probably be considered. As shown previously, Dev can bind to CCK1 receptors located in the vagus nerve as well as in NTS neurons (Corp *et al.* 1993) and is able to cross the BBB (Woltman *et al.* 1999). On the other hand, food deprivation selectively increases the number of CCK1 receptors in the hypothalamus (Saito *et al.* 1981), and endogenous CCK is physiologically involved in feeding control during fasting via the hypothalamic PVN (Cano *et al.* 2003). As previously reported, CCK1 receptors are abundant in the PVN (Woodruff *et al.* 1991), and activation of CCK neurons of the NTS that innervate the PVN stimulates appetite (D'Agostino *et al.* 2016, Roman *et al.* 2017). In addition, i.p. administration of Dev did not alter food intake, although central i.c.v. administration of Dev increased food intake in fasted animals (Ebenezer 2002). Finally, i.p. Dev administration also resulted in almost complete loss of the anorexigenic effect of i.c.v. administered the CART peptide, whose anorexigenic effect was associated with PVN cell activation (Maletínská *et al.* 2008).

Thus, our data may indicate that the pharmacologically influenced CCK system may be involved in the central anorexigenic effect of peripherally applied palm-PrRP31. According to the Dev pretreatment, it cannot be excluded that both peripheral (mediated via brainstem NTS) and central mechanisms (mediated via hypothalamic PVN) may be involved in this effect. The central mechanism of the interaction between CCK and the PrRP neuronal system should be elucidated in future studies.

Abbreviations

AP, Area postrema; Arc, Arcuate hypothalamic nucleus; BBB, Blood-brain barrier; CART, Cocaine- and amphetamine-regulated transcript; CCK, Cholecystokinin; cNTS, Caudal nucleus of the solitary tract; DAB, 3,3′-diaminobenzidine tetrahydrochloride; Dev, Devazepide; DMN, Dorsomedial hypothalamic nucleus; GIT, Gastrointestinal tract; i.c.v., Intracerebroventricular; i.p., Intraperitoneal; LHA, Lateral hypothalamic area; myr, myristoylated; palm, palmytoylated; PB, Phosphate buffer; PrRP, Prolactin-releasing peptide; PVN, Paraventricular hypothalamic nucleus; Sal, Saline; s.c, Subcutaneous

Conflict of Interest

There is no conflict of interest.

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REVIEW
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Lipidized Prolactin-Releasing Peptide as a New Potential Tool to Treat Obesity and Type 2 Diabetes Mellitus: Preclinical Studies in Rodent Models

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Obesity and type 2 diabetes mellitus (T2DM) are preconditions for the development of metabolic syndrome, which is reaching pandemic levels worldwide, but there are still only a few anti-obesity drugs available. One of the promising tools for the treatment of obesity and related metabolic complications is anorexigenic peptides, such as prolactin-releasing peptide (PrRP). PrRP is a centrally acting neuropeptide involved in food intake and body weight (BW) regulation. In its natural form, it has limitations for peripheral administration; thus, we designed analogs of PrRP lipidized at the N-terminal region that showed high binding affinities, increased stability and central anorexigenic effects after peripheral administration. In this review, we summarize the preclinical results of our chronic studies on the pharmacological role of the two most potent palmitoylated PrRP31 analogs in various mouse and rat models of obesity, glucose intolerance, and insulin resistance. We used mice and rats with diet-induced obesity fed a high-fat diet, which is considered to simulate the most common form of human obesity, or rodent models with leptin deficiency or disrupted leptin signaling in which long-term food intake regulation by leptin is distorted. The rodent models described in this review are models of metabolic syndrome with different severities, such as obesity or morbid obesity, prediabetes or diabetes and hypertension. We found that the effects of palmitoylated PrRP31 on food intake and BW but not on glucose intolerance require intact leptin signaling. Thus, palmitoylated PrRP31 analogs have potential as therapeutics for obesity and related metabolic complications.

Keywords: prolactin-releasing peptide, rodent models, obesity, type 2 diabetes, leptin resistance

INTRODUCTION

Obesity, along with type 2 diabetes mellitus (T2DM), is reaching pandemic levels worldwide, and both are a prerequisite for the development of metabolic syndrome (MetS), which culminates in an increased risk of metabolic and cardiovascular diseases (Said, Mukherjee, and Whayne 2016; Engin 2017; Tune et al., 2017). Although it is clear that obesity is linked to an unhealthy lifestyle in today's society and that adjusting eating habits and lifestyle can partially address obesity issues, new pharmacological treatments are urgently required. Unfortunately, despite the huge efforts to find

weight-lowering pharmacotherapies, only a few anti-obesity drugs have recently become available (Rodgers, Tschöp, and Wilding 2012; Kumar 2019; Rose, Bloom, and Tan 2019; Williams, Nawaz, and Evans 2020). One of the promising tools for the treatment of obesity and other related metabolic complications is anorexigenic peptides that are synthetized endogenously in the brain or in the gastrointestinal tract and act centrally to decrease energy intake. As demonstrated in experimental models, these peptides have minimal side effects during long-term anti-obesity treatment (Arch 2015; Patel 2015; Bray et al., 2016).

In their natural form, anorexigenic peptides have several disadvantages for direct use in pharmacotherapy for obesity, mainly due to their chemical instability, short half-life and low brain penetrance through the blood–brain barrier (BBB) after peripheral application. A peptidomimetic approach to modify natural peptides is currently being used for the development of promising drugs (Kumar 2019). The problem of penetration through the BBB can be solved, for example, by coupling of peptides to fatty acids, e.g., palmitic acid, resulting in increased stability and half-life in organisms (Malavolta and Cabral 2011; Salameh and Banks 2014).

Some lipidized peptide-based drugs for treatment of diabetes or obesity have been introduced into the market, such as the insulin analog detemir, which employs myristic acid attached through an amide bound to insulin molecules (Havelund et al., 2004), and liraglutide, a palmitoylated agonist of glucagon-like peptide 1 (GLP-1) (Gault et al., 2011). In addition, liraglutide has been approved for anti-obesity treatment in the United States and Europe (Saxenda). Very recently, a once-weekly injection of the lipidized GLP-1 agonist semaglutide was approved by the FDA for treatment of obesity (Wegovy). Several other peptidomimetics, including multitargeted molecules—dual and triple agonists targeting GLP-1, glucagon and gastric inhibitory polypeptide receptors—are in clinical trials as possible future anti-obesity drugs (Williams, Nawaz, and Evans 2020).

Several neuropeptides of brain origin have been demonstrated to have an anorexigenic effect in animal models, such as prolactin-releasing peptide (PrRP), cocaine- and amphetamine-regulated transcript (CART) peptide, α-melanocyte-stimulating hormone (α-MSH) and melanin-concentrating hormone (MCH) (Kunes et al., 2016; Mikulaskova et al., 2016). A lipidized α-MSH analog has been shown to be stable and exert a strong anorexigenic effect (Fosgerau et al., 2014); however, further research was terminated because of its adverse effects on the skin (Royalty et al., 2014).

Prolactin-releasing peptide was initially isolated from the hypothalamus as a ligand for the human orphan G-protein coupled receptor (GPR10) (Hinuma et al., 1998) as a possible regulator of prolactin secretion from anterior pituitary cells. However, later findings suggested that prolactin release is likely not a primary function of PrRP (Jarry et al., 2000; Taylor and Samson 2001). Shortly after its discovery, it was established that PrRP has other physiological functions, particularly it has been found to be involved in food intake, body weight (BW) and energy expenditure regulation (Lawrence et al., 2000; Takayanagi et al., 2008; Atanes, Ashik, and Persaud 2021). There are two biologically active isoforms of PrRP, with either 20 (PrRP20) or 31 (PrRP31) amino acids. Both isoforms have a common C-terminal Arg-Phe-amide sequence, which is critical for their biological activity (Roland et al., 1999; Maletínská et al., 2011). The fact that PrRP is involved in food intake and BW regulation is supported by the detection of PrRP and its receptor GPR10 in brain areas involved in food intake regulation, such as several hypothalamic nuclei (e.g., nucleus arcuatus (Arc), paraventricular nucleus (PVN), dorsomedial nucleus (DMN)) and the brainstem (e.g., nucleus tractus solitarius (NTS)). PrRP was also found to have high affinity for the neuropeptide FF2 (NPFF2) receptor, resulting in anorexigenic effects (Engström et al., 2003). It has also been shown in rodents that intracerebroventricular injection of natural PrRP20 and PrRP31 decreased food intake and BW (Lawrence et al., 2000; Ellacott et al., 2003; Maixnerová et al., 2011). Coadministration of PrRP and adipose tissue-born long-term acting regulator of energy balance leptin in rats resulted in additive reductions in nocturnal food intake and BW gain and an increase in energy expenditure (Ellacott et al., 2002).

Furthermore, both GPR10 knockout mice and PrRP-deficient mice developed late-onset obesity and exhibited a significant decrease in energy expenditure compared to wild-type mice (Bjursell et al., 2007) as well as altered insulin sensitivity and lipid homeostasis (Prazienkova et al., 2021). Moreover, PrRP-deficient mice also displayed increased food intake and attenuated responses to food intake, lowering the cholecystokinin (CCK) and leptin signals (Takayanagi et al., 2008). Therefore, PrRP or its receptor(s) might be new targets in obesity treatment.

However, as a centrally released and centrally acting neuropeptide, natural PrRP has several limitations after peripheral administration: low stability in the organism to exert its central effect and inability to reach the target brain receptors. To overcome these disadvantages, we designed analogs of PrRP lipidized at the N-terminal region, which is not essential for biological activity (Maletínská et al., 2015; Kunes et al., 2016). Our earlier studies demonstrated that analogs lipidized by 8–18 carbon chain fatty acids at the N-terminus of PrRP20 or PrRP31 showed high binding affinities with a K_i in the nanomolar range for both GPR10 and the NPFF2 receptor, similar to analogs that were palmitoylated through linkers to Lys^{11} (e.g., $palm^{11}$-PrRP31) (Maletínská et al., 2015; Pražienková et al., 2017; Karnošová et al., 2021).

It was confirmed that lipidization increased the stability of these peptides, as palmitoylated PrRP31 (palm-PrRP31) and myristoylated PrRP20 (myr-PrRP31) were stable for more than 24 h in rat plasma (Zemenová et al., 2017). *In vivo* pharmacokinetics studies in mice also showed longer stability for lipidized analogs than for natural, nonlipidized PrRP31 (Maletínská et al., 2015). The long-lasting anorexigenic effect of lipidized analogs of PrRP could be explained by their prolonged stability owing to binding to serum albumin, similar to liraglutide, semaglutide or palmitoylated gastric inhibitory polypeptide (Gault et al., 2011; Lau et al., 2015; Bech et al., 2017).

Acute *in vivo* experiments demonstrated that lipidized PrRP analogs have central anorexigenic effects after peripheral administration. Our work further supports several indirect studies confirming that the food intake-lowering effect of these analogs is mainly central. There was a significant and dose-dependent decrease in food intake in lean overnight-fasted or freely fed mice after subcutaneous (SC) injection of palm-PrRP31, myr-PrRP20 (Maletínská et al., 2015) or palm^{11}-PrRP31 (Pražienková et al., 2017; Pirnik et al., 2021), while analogs lipidized with shorter carbon chains or natural PrRP20 or PrRP31 had no effect on food intake (Maletínská et al., 2015). Moreover, neuronal activity (manifested by increased expression of the immediate early gene c-Fos in brain areas related to food intake regulation) was significantly increased in specific brain nuclei or in areas such as the Arc, PVN, DMN and NTS 90 min after SC application of myr-PrRP20, palm-PrRP31 and palm^{11}-PrRP31 but not after natural PrRP31 or octanoyl-PrRP31 administration (Maletínská et al., 2015; Pražienková et al., 2017; Pirník et al., 2018). The central neuronal activation of c-Fos after peripheral application of palmitoylated PrRP is also supported by the selective activation of specific hypothalamic oxytocin and hypocretin neuronal subpopulations both involved in food intake regulation (Pirnik et al., 2015). Furthermore, double c-Fos-GPR10 immunostaining in the brainstem C1/A1 cell group indicated that neurons containing GPR10 receptors are activated after administration of palmitoylated PrRP (Mikulášková et al., 2016).

In the hypothalamus, leptin receptor and PrRP are colocalized and have additive anorexigenic effects. Intracerebroventricular coadministration of PrRP and leptin in rats resulted in additive decrease in food intake and BW loss and an increase in energy expenditure (Ellacott et al., 2002). Furthermore, PrRP-expressing neurons in brain regions involved in food intake regulation (ventromedial nucleus of hypothalamus and ventrolateral medulla and NTS of brainstem) also contain leptin receptors (Ellacott et al., 2002). An anorexigenic effect of PrRP independent of leptin but dependent on the peripheral short-term anorexigenic hormone CCK was suggested in the brainstem. CCK was shown to have no effect on food intake in GPR10-knockout mice. This finding suggests that PrRP acting through its receptor may be a key mediator in the central satiating action of CCK (Bechtold and Luckman 2006).

An exogenously influenced CCK system was also shown to be involved in the central anorexigenic effect of peripherally applied palm-PrRP (Pirnik et al., 2021). We can thus hypothesize that peripheral signals (leptin, CCK) and the central neuropeptide PrRP cooperate in the stimulation of food intake-regulating pathways, leading to a decrease in food intake.

In this review, we summarize the preclinical results of our chronic studies on the pharmacological role of the two most potent palmitoylated PrRP31 analogs with the following sequences: palm-PrRP31 (N-palm) SRAHQHSNleETRTPDINPAWYTGRGIRPVGRF-NH_2) and palm^{11}-PrRP31 (SRTHRHSMEIK(N-γ-E (N-palm)) TPDINPAWYASRGIRPVGRF-NH_2).

These analogs were tested in various mouse and rat models of obesity, glucose intolerance/insulin resistance and T2DM resulting from high-fat (HF) diet feeding (diet-induced obesity (DIO) models) or in rodents with nonfunctional leptin signaling due to a spontaneous mutation in the leptin receptor.

Each of these rodent models represents different types and severities of pathological features of MetS, i.e., 1/obesity as shown by increased BW, triacylglycerides, free fatty acids, cholesterol and/or liver steatosis, 2/prediabetes or T2DM as shown by increased glucose and insulin levels and glucose intolerance, 3/ leptin and/or insulin resistance as shown by disrupted peripheral and central leptin or insulin signaling and 4/hypertension as shown by increased blood pressure. All pathologies were compared with that of age-matched control rodents. Chronic peripheral interventions with both palmitoylated PrRP31 analogs in different models allowed us to describe different metabolic changes in these models and to clarify the interactions with other systems involved in food intake regulation, such as the leptin system.

CHRONIC TREATMENT WITH PALMITOYLATED PrRP31 ANALOGS IN MOUSE AND RAT MODELS OF METABOLIC DISEASES

One of the major risks for the development of cardiovascular and metabolic dysfunction, including obesity, prediabetes and hypertension, is high dietary fat intake. Hypercaloric diets rich in lipids are widely used in experimental studies to induce metabolic disorders commonly found in humans (Dourmashkin et al., 2005; Buettner, Schölmerich, and Bollheimer 2007; Agahi and Murphy 2014). Most rodents tend to become obese and develop pathologies of MetS when fed specific calorie-rich diets (Shafrir, Ziv, and Mosthaf 1999; Bergman et al., 2006; Varga et al., 2010). Frequently used models are mice or rats fed a HF diet.

On the other hand, genetic factors undoubtedly play an important role in obesity development, and it is important to better understand the role of specific factors in food intake regulation using models with genetically disrupted production or signaling of these factors. One of the most important hormones regulating long-term energy balance in organisms is leptin, and the most widely used rodent models of spontaneous genetic obesity and related complications are congenital leptin- or leptin receptor-deficient mice and rats (Varga et al., 2010; Wang, Chandrasekera, and Pippin 2014; Fuchs et al., 2018).

In our studies summarized in this review, various mouse and rat models with different features of MetS were used to investigate the effects of palmitoylated PrRP analogs as potential anti-obesity and antidiabetic compounds and to explore their mechanism of action. Each of these models show a variety of pathologies, and the basic characterization of each model is shown in **Table 1**.

DIO Models

DIO rodents are considered models of the most common type of human obesity, which is associated with overconsumption of HF food (Bagnol et al., 2012). To test the effect of chronic treatment

TABLE 1 | Characterization of rodent models used in studies of interventions with palmitoylated PrRP31 analogs.

Model	Characterization	References
DIO mice C57BL/6J	Obesity, prediabetes, disturbed central leptin and insulin signaling, liver steatosis	(Maletínská et al., 2015; Pražienková et al., 2017; Holubová et al., 2018)
DIO rats Sprague-Dawley and Wistar Kyoto	Obesity, diabetes, glucose intolerance	Holubova et al. (2016); Čermáková et al. (2019)
Ob/ob mice	Severe early onset obesity, disrupted production of leptin, severe liver steatosis, glucose intolerance, disturbed central leptin and insulin signaling	Korinkova et al. (2020)
MSG mice	Obesity, glucose intolerance, hormone disbalance, disrupted hypothalamic leptin and insulin signaling	Špolcová et al. (2015)
ZDF rats	Lean, severe T2DM	Holubova et al. (2016)
Koletsky rats	Obesity, prediabetes, hypertension, liver steatosis, disrupted central leptin and insulin signaling	Mikulaskova et al. (2018)

TABLE 2 | Summary of metabolic and morphometric parameters in DIO models and impact of treatment with palm-PrRP31 or palm^{11}-PrRP31.

Model	Characterization/treatment	BW change	Cumulative food intake	Liver weight	Glucose	Insulin	Leptin	TAG	CHOL	FFA
DIO C57	HF vs LF	↑ 63%	NT	NT	↑	↑	↑	↑	NT	NS
DIO Sprague Dawley	HF vs LF	↑ 22%	↓	NS	NS	↑	↑	NS	NT	↑
DIO Wistar Kyoto	HF vs LF	↑ 10%	NT	NS	↑	NS	NS	NS	NS	NS
DIO C57	palm-PrRP31	↓13%	↓	NS	NS	↓	↓	NT	NT	NT
	palm^{11}-PrRP31	↓12%	NS	↓	NS	↓	↓	↓	↓	↓
	palm^{11}-PrRP31	↓13, 6%	↓	NS	NS	NS	↓	NS	↓	NS
DIO Sprague Dawley	palm-PrRP31	↓8%	↓	NS	↑	NS	NS	NS	NS	NS
DIO Wistar Kyoto	palm^{11}-PrRP31	↓7, 7%	NT	NS	NS	NS	NS	NS	NS	NS

Statistical analysis was performed by unpaired t-test, significance is shown as increased (↑) or decreased (↓) vs LF or treatment vs HF saline treated group. Cummulative food intake and body weight (BW) change measured at the end of experiment. Cholesterol (CHOL), free fatty acid (FFA) and triacylglycerides (TAG) measured from the plasma. Non-significant (NS), not-tested (NT) (Maletínská et al., 2015; Holubova et al., 2016; Pražienková et al., 2017; Holubová et al., 2018; Mikulaskova et al., 2018; Čermáková et al., 2019; Korinkova et al., 2020).

with palmitoylated PrRP31 analogs on obesity and prediabetes parameters and on temporarily disturbed central leptin and insulin signaling, we used several mouse and rat models in our studies.

C57BL/6 mice fed a HF diet containing 60% fat based on lard from 8 to 19 weeks of age developed severe obesity and prediabetes (Pelantová et al., 2016). Consumption of the HF diet resulted in significant BW gain in the mice, mediated by an increase in body fat and liver weight and an increased level of leptin, as shown in **Table 2**. HF diet feeding induced an increase in the mRNA expression of genes involved in lipogenesis in adipose tissue but did not affect the mRNA expression of genes involved in lipolysis. The HF diet also increased the blood glucose level and the insulin and triacylglycerides (TAG) levels in plasma compared to mice on a standard chow diet (LF—low fat diet) (Pelantová et al., 2016).

In the studies of Maletínská (Maletínská et al., 2015) and Pražienkova (Pražienková et al., 2017), C57BL/6 male mice were provided with a HF diet from 8 to 19 weeks of age to induce obesity. Subsequently, mice were treated SC with saline or palmitoylated analogs of PrRP, palm-PrRP31 or palm^{11}-PrRP31 twice a day for 2 weeks. Palm-PrRP31 treatment significantly decreased cumulative food intake. Both palm-PrRP31 and palm^{11}-PrRP31 significantly decreased BW, which was primarily mediated by a reduction in body fat and liver, accompanied by a decrease in leptin levels (**Table 2**).

Due to the decrease in mRNA expression of fatty acid synthase (*Fasn*) in both adipose tissue and the liver along with decreased expression of acetyl-CoA carboxylase (*Acaca*) and sterol regulatory element-binding protein (*Srebp*) in the liver, BW reduction most likely resulted from decreased *de novo* lipogenesis, owing primarily to negative energy balance due to reduced food intake (Maletínská et al., 2015; Pražienková et al., 2017). Moreover, increased uncoupling protein 1 (*UCP-1*) mRNA in brown adipose tissue (BAT) after palm^{11}-PrRP31 treatment points to a possible increase in energy expenditure. Furthermore, treatment with both palm-PrRP31 and palm^{11}-PrRP31 significantly lowered insulin levels in the blood of DIO mice, and the levels of free fatty acids (FFAs), cholesterol (CHOL) and TAG were significantly reduced after palm^{11}-PrRP31 treatment (**Table 2**).

The next study of Holubová (Holubová et al., 2018) aimed at palm^{11}-PrRP31 posttreatment regarding a possible yo-yo effect after drug termination. C57BL/6 mice were fed for 12 weeks with a HF diet. At the age of 19 weeks, mice were SC injected twice a day with saline for 4 weeks, with palm^{11}-PrRP31 for 4 weeks or with palm^{11}-PrRP31 for 2 weeks and with saline for the following 2 weeks. DIO mice treated for 4 weeks with palm^{11}-PrRP31 and those treated with palm^{11}-PrRP31 for 2 weeks and then with saline for 2 weeks reached a similar decrease in BW and body fat and attenuated plasma leptin, which continued for 2 weeks after termination of the 2 weeks-long administration of palm^{11}-PrRP31 (**Figure 1**).

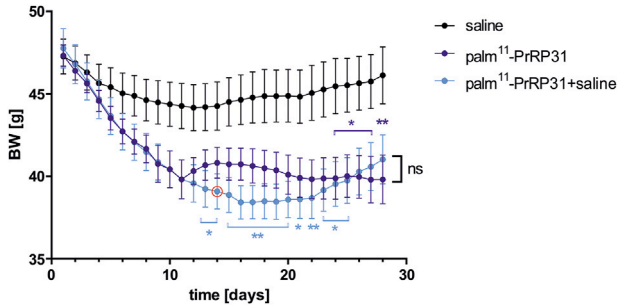

FIGURE 1 | Effect of chronic treatment with palm^{11}-PrRP31 on BW in DIO mice. Mice were treated with palm^{11}-PrRP31 for 4 weeks (palm^{11}-PrRP31 group) or with palm^{11}-PrRP31 for 2 weeks and for the following 2 weeks with saline (palm^{11}-PrRP31 + saline group), change of the treatment marked by red circle , modified from (Holubová et al., 2018). Data are presented as means ± S.E.M. Statistical analysis was performed by Two-way ANOVA with Tukey *post hoc* test, significance is $p<0.05$, **<0.01 vs saline treated group. Body weight (BW) measured during the treatment, diet-induced obesity (DIO).

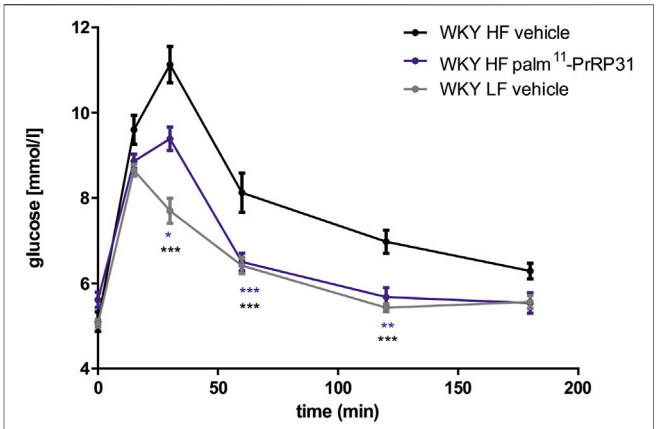

FIGURE 2 | Effect of chronic treatment with palm^{11}-PrRP31 on glucose tolerance response in DIO Wistar Kyoto (WKY) rats. Rats were treated with palm^{11}-PrRP31 for 3 weeks (WKY HF palm^{11}-PrRP31 group). Oral glucose tolerance test (OGTT) was performed after overnight fasting at the end of experiment. Results are shown as a glucose profile modified from (Čermáková et al., 2019). Data are presented as means ± S.E.M. Statistical analysis was performed by Two-way ANOVA with Tukey *post hoc* test, significance is $p<0.05$, **<0.01, ***<0.001 vs WKY HF vehicle treated group. DIO Diet induced obesity, HF high fat, LF low fat.

mRNA expression of lipolytic enzymes was significantly lowered by the action of palm^{11}-PrRP31 in the liver, suggesting complexly attenuated liver lipid metabolism. Furthermore, similar to our previous study, UCP-1 in BAT points to increased energy expenditure. Under both treatment modes, neuronal activity was increased in food intake-regulating neurons, as determined by FosB expression, a marker of long-term neuronal potentiation (Nestler 2001). Blood glucose, insulin, TAG, FFA and CHOL in plasma were not significantly affected by any of the treatments.

Furthermore, in this study, palm^{11}-PrRP31 impacted hypothalamic signaling by restoring the leptin receptor-induced phosphatidylinositol-3-kinase (PI3K) pathway and increasing extracellular signal regulated kinase (ERK) 1/2 phosphorylation as a result of increased leptin or PrRP receptor signaling (Balland and Cowley 2015). Moreover, in this study, palm^{11}-PrRP31 lowered the phosphorylation of both c-Jun and c-Jun N-terminal kinases (JNKs), generally activated by HF feeding in DIO mice, both in the periphery and the brain (De Souza et al., 2005; Holubová et al., 2018).

Collectively, studies in DIO mouse models revealed a long-lasting effect of palmitoylated analogs of PrRP31 on BW lowering, accompanied by increased neuronal signaling in the hypothalamus, even after discontinuation of treatment.

In the following studies, we aimed to examine the effects of intraperitoneal (IP) administration of palmitoylated analogs of PrRP31 in rats fed a HF diet that developed not only severe obesity and prediabetes but also glucose intolerance. Sprague–Dawley rats were provided a HF diet from 8 to 32 weeks and subsequently treated with either saline or palm-PrRP31 for 2 weeks (Holubova et al., 2016). Wistar Kyoto rats were fed a HF diet from 8 to 23 weeks of age. At the age of 23 weeks, the mice were IP injected for 3 weeks with either saline or palm^{11}-PrRP31 (Čermáková et al., 2019).

The HF diet resulted in significant BW gain, mediated by an increase in body fat and liver weight and an increased level of leptin, as shown in **Table 2** (Holubova et al., 2016; Čermáková et al., 2019). Furthermore, the consumption of the HF diet significantly increased intolerance to glucose, determined by an oral glucose tolerance test (OGTT) in both rat strains, with significantly increased fasting blood glucose in Sprague–Dawley rats (Holubova et al., 2016) and increased insulin levels in Wistar Kyoto rats compared to those in the low-fat (LF) diet-fed group.

Similar to mice with DIO, treatment with palm-PrRP31 significantly decreased cumulative food intake, corresponding to a significant decrease in BW in DIO rats after treatment with both palmitoylated analogs (**Table 2**), primarily mediated by a reduction in body fat and liver weight. In these studies, a significant glucose-lowering effect of both PrRP31 analogs was found in DIO rats after the OGTT but not in the saline-treated control group (**Figure 2**). Treatment with PrRP31 analogs significantly decreased expression of the enzymes that catalyze *de novo* lipogenesis in both the liver (Holubova et al., 2016; Čermáková et al., 2019) and adipose tissue (Čermáková et al., 2019), while the mRNA expression of lipolytic enzymes was increased after palm^{11}-PrRP31 treatment, supporting previous results of complexly affected lipid metabolism. Furthermore, the expression of insulin receptor substrate (Irs) 1 and Irs-2 was increased after palm^{11}-PrRP31 treatment. Insulin, TAG, FFA and CHOL in plasma were not significantly affected by any treatment.

In conclusion, in DIO rat models, both palmitoylated analogs of PrRP31 exhibited not only a strong effect on BW lowering but also a great glucose-lowering effect.

Rodent Models With Leptin Deficiency or Disrupted Leptin Signaling

To test the chronic effect of palmitoylated PrRP31 analogs on obesity and prediabetes or diabetes parameters in relation to the

TABLE 3 | Summary of metabolic and morphometric parameters in rodent models of leptin deficient or leptin signaling disturbances and impact of treatment with palm-PrRP31 or palm^{11}-PrRP31.

Model	Characterization	BW change	Cumulative food intake	Liver weight	Glucose	Insulin	Leptin	TAG	CHOL	FFA
ob/ob	*ob/ob* vs WT	↑ 82%	NS	↑	NS	↑	NT	NS	↑	NS
MSG	MSG vs controls	$\uparrow^{ns}$ 7.5%	NT	NT	NS	↑	↑	NT	NT	NT
ZDF	Diabetic ZDF vs non-diabetic ZDF	↑ 10%	↑	↑	↑	NS	↑	↑	↑	NS
SHROB	SHROB vs SHR	↑ 38%	NS	↑	NS	↑	↑	↑	NS	↓
ob/ob	palm^{11}-PrRP31	$\downarrow^{ns}$ 4%	NS	NS	NS	NS	NT	NS	↓	NS
MSG	palm-PrRP31	$\downarrow^{ns}$ 5.6%	↓	NT	NS	NS	NS	NT	NT	NT
ZDF	palm-PrRP31	$\downarrow^{ns}$ 2%	↓	NS	NS	NS	NS	NS	↓	NS
SHROB	palm^{11}-PrRP31	$\downarrow^{ns}$ 1.5%	↓	NS	NS	↓	NS	NS	NS	↑

Statistical analysis was performed by unpaired t-test, significance is shown as increase (↑) or decrease (↓) vs their age-matched controls or treatment vs saline treated group. Cummulative food intake and body weight (BW) change measured at the end of experiment. Cholesterol (CHOL), free fatty acid (FFA) and triacylglycerides (TAG) measured from the plasma. Koletsky rats or spontaneously hypertensive obese rats (SHROB), monosodiumm glutamate (MSG), Non-significant (NS), not-tested (NT), Zucker diabetic fa/fa rats (ZDF), (Maletínská et al., 2015; Holubova et al., 2016; Pražienková et al., 2017; Holubová et al., 2018; Mikulaskova et al., 2018; Čermáková et al., 2019; Korinkova et al., 2020).

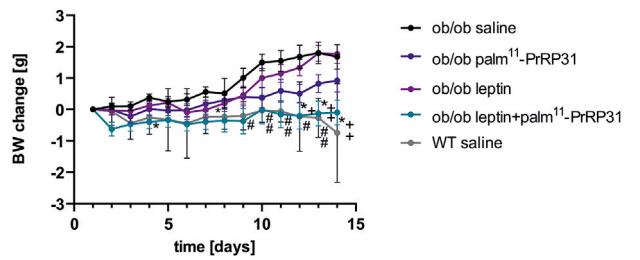

FIGURE 3 | Chronic effect of palm^{11}-PrRP31, leptin and their combination on BW change in *ob/ob* mice modified from (Korinkova et al., 2020) Mice were treated fo 2 weeks. Data are presented as means ± S.E.M. Statistical analysis was performed by Two-way ANOVA with Tukey *post hoc* test, significance is $^{\#}$<0.05, $^{\#\#}$<0.01 *ob/ob* saline vs wild type (WT) saline, *p*<0.05, **<0.01 *ob/ob* leptin + palm^{11}-PrRP31 vs *ob/ob* saline, $^{+}$<0.05, $^{++}$<0.01 *ob/ob* leptin + palm^{11}-PrRP31 vs *ob/ob* leptin. Body weight (BW) measured during the treatment, wild-type.

important long-term food intake regulator leptin, we used several mouse and rat models with spontaneous leptin deficiency or disrupted leptin signaling.

Leptin-deficient o*b/ob* mice were used to explore the potential interaction between leptin and PrRP with regard to their anorexigenic effect and impact on metabolic disturbances (Korinkova et al., 2020). In this study, younger mice (treated from 8 to 10 weeks of age) and older mice (treated from 16 to 24 weeks of age) were used. Younger mice were used because they are in a metabolically active state, and older mice have established morbid obesity.

Ob/ob mice of both ages had significantly higher BW, body fat and liver weight than wild-type (WT) mice. As *ob/ob* mice are known to be hypothermic (Ohtake, Bray, and Azukizawa 1977), their rectal temperature was significantly lower at both 10 and 24 weeks of age. Older *ob/ob* mice had high hyperinsulinemia and significantly increased cholesterol levels, but TAG and FFA levels did not differ from those of WT mice (**Table 3**). Our results supported the study of Enser (Enser 1972), who found that *ob/ob* mice are hyperglycemic only between 5 and 16 weeks of age; however, 24 week-old *ob/ob* mice were normoglycemic in our study (Korinkova et al., 2020). It was demonstrated that nonfunctional leptin receptor signaling leads to negligible PrRP mRNA expression (Ellacott et al., 2002), suggesting interaction of both systems. In this study, neither palm^{11}-PrRP31 nor leptin alone significantly decreased the BW, body fat or liver weight of *ob/ob* mice, but their combination significantly lowered all these parameters (**Figure 3**). Moreover, an increase in the rectal temperature in older *ob/ob* mice was detected after treatment with a combination of leptin and palm^{11}-PrRP. Reduced liver weight in younger *ob/ob* mice treated with a leptin and palm^{11}-PrRP31 combination was linked to decreased mRNA expression of lipogenic enzymes in the liver and with regression of fat droplets in liver tissue in all groups of younger peptide-treated *ob/ob* mice compared to *ob/ob* saline-treated mice (Korinkova et al., 2020). Treatment with leptin and the combination of palm^{11}-PrRP31 + leptin also had a significant decreasing effect on cumulative food intake and total plasma cholesterol levels. In the hypothalamus of older *ob/ob* mice, two main leptin anorexigenic signaling pathways, namely, Janus kinase (JNK)/signal transducer and activator of transcription-3 (STAT3) activation and AMP-activated protein kinase (AMPK) deactivation, were induced by leptin, palm^{11}-PrRP31, and their combination.

Our study (Korinkova et al., 2020) clearly showed that palm^{11}-PrRP31 and leptin synergistically lowered BW (**Figure 3**) and increased body temperature in older *ob/ob* mice with established morbid obesity. However, the effect of the combination of both drugs on liver weight was only seen in younger *ob/ob* mice. We can conclude that palm^{11}-PrRP31 might be a potential anti-obesity drug in the case of a functional leptin system.

MSG mice are a widely used rodent model of obesity and prediabetes. This model is induced by subcutaneous injections of monosodium glutamate (MSG) administered to newborns, resulting in specific lesions in the Arc of the hypothalamus (Takasaki 1978), leading to prediabetes with mild hyperglycemia, hyperinsulinemia and hyperleptinemia (Cameron, Poon, and Smith 1976; Matysková et al., 2008). The obesity of these animals is characterized by increased

adiposity (Djazayery, Miller, and Stock 1979) because of a lower metabolic rate rather than elevated food intake (Maletínská et al., 2006). We tested whether treatment with palm-PrRP31 influenced the metabolic parameters of the MSG model at 6 months of age when the total adipose tissue weight and plasma level of leptin were significantly higher.

Two weeks of SC treatment with palm-PrRP31 did not significantly change BW or plasma leptin levels, while the white adipose tissue weight tended to decrease after treatment (Špolcová et al., 2015). While MSG mice were normoglycemic, plasma insulin levels were significantly higher in the MSG mice than in age-matched controls (**Table 3**). The cumulative food intake was significantly decreased after treatment with palm-PrRP31, but the fasting glucose and insulin levels did not differ from those in the saline-treated controls (**Table 3**). An intraperitoneal glucose tolerance test (IPGTT) showed that only the final glucose level was significantly lower in MSG mice treated with palm-PrRP31 than in MSG mice treated with saline (Špolcová et al., 2015). Moreover, palm-PrRP31 appeared to exert a central anorexigenic effect, resulting in increased phosphorylation of the insulin cascade kinases phosphoinositide-dependent protein kinase 1 (PDK1), protein kinase B (Akt) and glycogen synthase kinase-3β (GSK-3β).

We can conclude that palm-PrRP31 affects metabolic parameters connected with prediabetes in the periphery of MSG mice and insulin signaling in the hippocampus without an effect on BW.

Zucker diabetic rats, which are a model of impaired leptin receptor signaling (Fellmann et al., 2013), are frequently used for studying the potential of antiobesity and antidiabetic peptidic drugs (Andreassen et al., 2014; Skarbaliene et al., 2015). Thus, we used this model to evaluate the chronic antidiabetic potency of palm-PrRP31 and the involvement of the leptin signaling pathway in these effects.

As evident from the definition of this model, ZDF rats were slightly overweight and highly hyperglycemic compared to controls (Holubova et al., 2016). Diabetic ZDF rats had significantly increased cumulative food intake and hyperglycemia and exhibited markedly lowered glucose tolerance during the OGTT in comparison with controls. Hyperlipidemia was also found in diabetic ZDF rats *via* significantly increased plasma cholesterol and TAG in comparison with controls (**Table 3**) (Holubova et al., 2016). In this model, 2 weeks of treatment with palm-PrRP31 did not affect BW but had a tendency to improve tolerance to glucose but did not affect fasting glucose. However, the treatment lowered food intake and significantly decreased plasma cholesterol and nonsignificantly decreased plasma free fatty acids, triglycerides, leptin and insulin levels (**Table 3**).

This study clearly demonstrated that despite the food intake-lowering effect, palm-PrRP31 failed to decrease BW or improve glucose tolerance in this model, probably again due to a lack of functional leptin receptors and therefore the impossibility of an interaction of leptin and PrRP systems in the brains of ZDF rats.

The Koletsky rat strain of genetically obese hypertensive rats develops obesity, hyperinsulinemia, hyperlipidemia and spontaneous hypertension, which are the main symptoms of MetS (Koletsky 1973; Xu et al., 2008). These rats showed elevated fasting insulin levels compared to lean spontaneously hypertensive rats (SHRs), which were used as a control. OGTTs also demonstrated glucose intolerance; however, the rats were not diabetic, as previously reported (Friedman et al., 1997). Therefore, we tested parameters and insulin signaling in SHROB rats and their SHR controls.

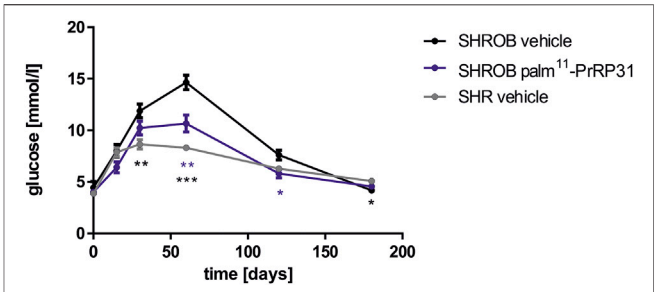

FIGURE 4 | Effect of chronic treatment with palm^{11}-PrRP31 on glucose tolerance response in SHROB rats. Rats were treated with palm^{11}-PrRP31 for 3 weeks (SHROB palm^{11}-PrRP31 group). Oral glucose tolerance test (OGTT) was performed after overnight fasting at the end of experiment. Results are shown as a glucose profile modified from (Mikulaskova et al., 2018). Data are presented as means ± S.E.M. Statistical analysis was performed by Two-way ANOVA with Tukey *post hoc* test, significance is $p<0.05$, **<0.01, ***<0.001 vs. the vehicle treated control SHR group.

As expected, SHROB rats were obese and had higher leptin, cholesterol and triglyceride levels than SHR controls (**Table 3**). The level of insulin was significantly higher than that in controls, while both genotypes were normoglycemic (**Table 3**). SHROB rats showed significantly higher liver weights than SHRs, but kidney and heart weights did not show differences between genotypes in which both were hypertensive (Mikulaskova et al., 2018). The mRNA expression levels of several genes related to lipogenesis in the liver or in adipose tissue were significantly higher in SHROB rats than in SHR controls. Stearoyl-CoA desaturase 1 (Scd-1) contributes to the development of obesity and is suppressed by functional leptin (Biddinger et al., 2006); thus, in this strain with a mutation in the leptin receptor, subcutaneous adipose tissue (SCAT) mRNA expression of Scd-1 was significantly higher in SHROB rats than in SHR controls (Mikulaskova et al., 2018). Treatment with palm^{11}-PrRP31 for 3 weeks lowered food intake in both genotypes; however, an effect on BW was seen only in the SHR group with intact leptin signaling. While fasting plasma glucose levels were not affected by treatment in either genotype, based on OGTT results, palm^{11}-PrRP31 administration significantly improved tolerance to glucose (**Figure 4**) in both groups and improved insulin signaling in the hypothalamus (Mikulaskova et al., 2018). The treatment did not have any effect on hypertension in either strain.

The most important result of this study was the marked improvement in glucose tolerance after palm^{11}-PrRP treatment in both genotypes, while fasting normoglycemia was not altered. This improvement in glucose tolerance was accompanied by a significant decrease in plasma insulin levels and improved central insulin signaling in SHROB rats. The results also suggested that intact leptin signaling is needed for the BW-lowering effect.

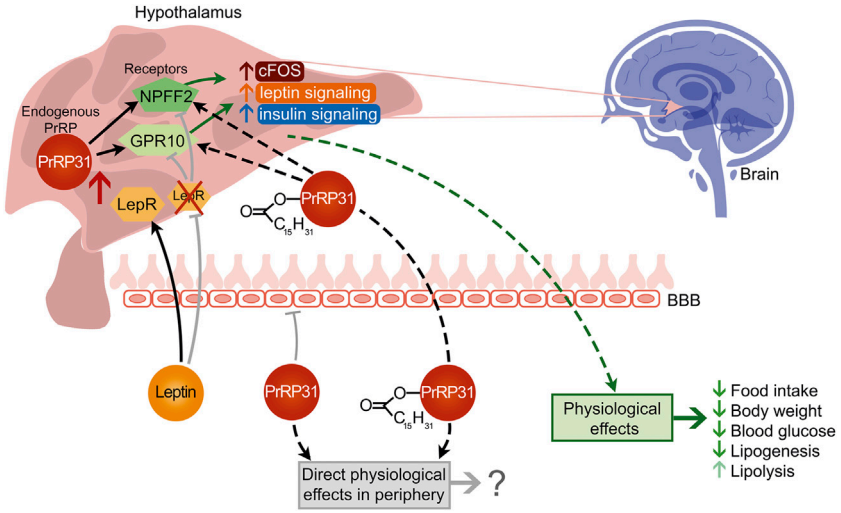

FIGURE 5 | Proposed peripheral and central action of natural PrRP31 and its palmitoylated analog.

CONCLUSION

This review summarizes our results with a novel potential anorexigenic drug, palmitoylated PrRP, showing its effects on several parameters characterizing obesity or T2DM in different rodent models. Each of these models has specific features and might help us to analyze the particular effects of anorexigenic palm-PrRP analogs and to depict their mechanism of action as potential antiobesity and antidiabetic compounds.

DIO rodent models developed severe obesity, prediabetes or diabetes, resulting in BW gain that was mediated by an increase in body fat and liver weight; in addition, these models showed an increased level of leptin, with disturbed metabolic parameters and increased lipogenesis in adipose tissue. Palm-PrRP31 and palm^{11}-PrRP31 seems to reverse the effects of a HF diet. A decrease in food intake resulted in attenuated fat storage and body and liver weight, accompanied by a decrease in leptin levels. Furthermore, palmitoylated analogs of PrRP affected lipid metabolism in adipose tissue and the liver by suppressing lipid synthesis and increasing lipid degradation. Moreover, increased mRNA expression of UCP-1 in BAT points to increased energy expenditure. A very interesting result was also demonstrated in the study after the treatment was discontinued: no yo-yo effect was observed after palm^{11}-PrRP31 treatment termination.

The rodent models of leptin deficiency or disturbances in leptin signaling mentioned in this review developed obesity or morbid obesity, but treatment with palm-PrRP31 or palm^{11}-PrRP31 did not significantly decrease BW or related metabolic parameters. On the other hand, treatment of *ob/ob* mice with a combination of leptin and palm^{11}-PrRP31 synergistically decreased BW. This synergistic effect was also confirmed by a lower liver weight and body fat and increased body temperature. In two rat strains with nonfunctional leptin signaling, ZDF diabetic rats and Koletsky rats, monotherapy with palm^{11}-PrRP31 or palm-PrRP did not have an antiobesity effect, but there were significant glucose-lowering effects. These results suggest that to achieve the full anti-obesity effects of PrRP, intact leptin signaling is needed, but the effect on glucose tolerance could be independent of leptin signaling. The central effect of both palmitoylated PrRP analogs was demonstrated by increased leptin and insulin signaling in the brain.

Overall, based on the results described in this review and in our other studies, the effects of palmitoylated PrRP analogs are summarized in **Figure 5**. It is evident that natural PrRP is not able to act centrally after peripheral administration and thus affects BW and related metabolic parameters. On the other hand, palmitoylated PrRP stimulates anorexigenic pathways in the hypothalamus. However, our results clearly suggest that the central effects of peripherally applied palm-PrRP on food intake and BW are possible only in the presence of intact leptin signaling. Despite this, palmitoylated PrRP has the potential to be an attractive candidate for obesity therapy.

AUTHOR CONTRIBUTIONS

LMr, BN, AM did the review search and wrote the manuscript, AP, VS substantially contributed to the writting and editing of manuscript, LH created the summary figure, BŽ, JK, LMa designed the review, contributed to the writting and edited the manuscript.

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GLOSSARY

Acaca acetyl-CoA carboxylase
Akt protein kinase B
AMPK AMP-activated protein kinase
Arc nucleus arcuatus
BAT brown adipose tissue
BBB blood brain barrier
BW body weight
CART cocaine- and amphetamine-regulated transcript
CCK cholecystokinin
CHOL cholesterol
DIO diet induced obesity
DMN dorsomedial nucleus
ERK extracellular signal regulated kinase
Fasn fatty-acid synthase
FFA free fatty acid
GLP-1 glucagon-like peptide 1
GPR10 G-protein coupled receptor
GSK-3β glycogen synthase kinase-3β
HF high fat
IP intraperitoneal
IPGTT intraperitoneal glucose tolerance test
Irs insulin receptor substrate
JNK c-Jun N-terminal kinase
LF low fat
MCH melanin-concentrating hormone
MetS metabolic syndrome
Myr-PrRP31 myristoylated PrRP
NPFF neuropeptide FF
NPY neuropeptide Y
NTS nucleus tractus solitarii
OGTT oral glucose tolerance test
Palm^{11}-PrRP31 palmitoylated PrRP at position 11
Palm-PrRP31 palmitoylated PrRP
PDK1 phosphoinositide-dependent protein kinase 1
PI3K phosphatidylinositol-3-kinase
PrRP prolactin releasing peptide
PVN paraventricular nuclei
SC subcutaneous
SCAT subcutaneous adipose tissue
Scd-1 stearoyl-CoA desaturase-1
Srebp sterol regulatory element-binding protein
STAT3 signal transducer and activator of transcription-3
T2DM type 2 diabetes mellitus
TAG triacylglycerides
UCP-1 uncoupling protein 1
WT wild type
α-MSH α-melanocyte-stimulating hormone

Palmitoylated prolactin-releasing peptide treatment had neuroprotective but not anti-obesity effect in fa/fa rats with leptin signaling disturbances

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Running title: Effect of palmitoylated PrRP in fa/fa rats

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Abstract

Background/Objective

Anorexigenic palmitoylated prolactin-releasing peptide (palm^{11}-PrRP) is able to act centrally after peripheral administration in rat and mouse models of obesity, type 2 diabetes and/ or neurodegeneration. Functional leptin and intact leptin signaling pathways are necessary for the body weight reducing and glucose tolerance improving effect of palm^{11}-PrRP. We have previously shown that palm^{11}-PrRP31 had glucose-lowering properties but not anti-obesity effect in Koletsky rats with leptin signaling disturbances, so improvements in glucose metabolism appear to be completely independent of leptin signaling. The purpose of this study was to describe relationship between metabolic and neurodegenerative pathologies and explore if palm^{11}-PrRP31 could ameliorate them in obese fa/fa rat model with leptin signaling disruption.

Subject/Methods

The fa/fa rats and their age-matched lean controls at the age 32 weeks were used for this study. The rats were infused for 2 months with saline or palm^{11}-PrRP31 (n=7-8 per group) at a dose of 5 mg/kg per day using Alzet osmotic pumps. During the dosing period food intake and body weight were monitored. At the end of experiment the oral glucose tolerance test was performed; plasma and tissue samples were collected and arterial blood pressure was measured. Then, markers of leptin and insulin signaling, Tau phosphorylation, neuroinflammation and synaptogenesis were measured by western blotting and immunohistochemistry.

Results

Fa/fa rats developed obesity, mild glucose intolerance and peripheral insulin resistance but not hypertension while palm^{11}-PrRP31 treatment neither lowered body weight nor attenuated

glucose tolerance but ameliorated leptin and insulin signaling and synaptogenesis in hippocampus.

Conclusion

We demonstrated that palm^{11}-PrRP31 had neuroprotective features without anti-obesity and glucose lowering effects in fa/fa rats. This data suggest that this analog has the potential to exert neuroprotective effect despite of leptin signaling disturbances in this rat model.

Introduction

The incidence of so-called diseases of civilization, such as obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM), and hypertension, is constantly rising, creating major health problems for developed and also developing countries [1-3]. Unfortunately, an unsatisfactory number of anti-obesity drugs are currently available. This dearth is related to the fact that a broad range of factors have been implicated in the progression of obesity and related diseases; therefore, it is difficult to find a suitable animal model with similar pathology to that of humans. It was suggested that obesity, together with T2DM and IR, can play a significant role in the pathology of neurodegenerative disorders, including Alzheimer's disease (AD) [4]. The accuracy of this description is supported by the fact that cerebral glucose utilization and energy metabolism worsen with the progression of cognitive impairment, and the accumulation of amyloid β (Aβ) and Tau protein hyperphosphorylation, two main hallmarks of AD, are increased. Since increased Tau hyperphosphorylation was found in db/db mice with a nonfunctional leptin receptor [5], leptin was determined to play an important role in Tau pathology [1, 6].

Zucker fatty (fa/fa) rats are a model of genetic obesity. The single nucleotide mutation of the *fa* gene (Gln269Pro) leads to a mutation in the extracellular domain of all isoforms of the leptin Ob-R receptor that is critical for the obese phenotype [7-9]. As a consequence, fa/fa animals have elevated plasma leptin levels and are resistant to exogenous leptin administration [10]. The obesity of fa/fa rats was correlated with hyperphagia and decreased energy expenditure [11]. Moreover, fa/fa animals with normoglycemia and high lipid levels were found to exhibit IR, as shown by increased plasma cholesterol (CHOL), triglycerides (TG) and free fatty acid (FFA) levels compared to lean controls [12]. Multiple abnormalities in the cellular physiology of insulin-mediated glucose clearance have been associated with IR,

77 including alterations in insulin binding and cellular signaling at its receptor, glucose
78 metabolism, and glucose transport [13, 14].

79 Although the mechanism by which diabetes reduces cognitive function is not
80 completely clear, research in several rodent models has led to the identification of numerous
81 correlations between hippocampal functional impairment, obesity and diabetes [1, 15]. In our
82 previous study with 12- and 33-week-old Zucker fa/fa rats, we showed that aging and obesity
83 significantly contributed to increased peripheral IR, which further worsened the activation of
84 the hippocampal insulin signaling cascade. This effect resulted in decreased phosphorylation
85 at the inhibitory epitope Ser 9 of glycogen-synthase kinase 3β (GSK-3β), one of the main
86 kinases of Tau protein phosphorylation. Subsequently, an increase in the pathological
87 hyperphosphorylation of Tau protein was observed in the hippocampi of fa/fa rats; thus,
88 peripheral IR resulted in central insulin resistance and Tau hyperphosphorylation [16].
89 Moreover, a balance between kinases and phosphatases regulates the Tau phosphorylation
90 state. The main phosphatase is protein phosphatase 2A (PP2A), while cyclin-dependent kinase
91 5 (Cdk5) is another important Tau kinase previously shown to be involved in abnormal Tau
92 phosphorylation and activated in the brains of AD patients [17-19]. Beside Aβ plaques and
93 increased Tau phosphorylation, increased neuroinflammation manifested by increased level of
94 astrocytes or microglia, and decreased synaptogenesis and neurogenesis were described to
95 contribute to development of AD and other neurodegenerative disordes [20 21 22]. Subsequently,
96 neuroinflammation was recently demonstrated in the Zucker fa/fa rats brain [23].

97 It has been recently suggested that some food intake-regulating peptides may be
98 promising candidates for obesity and T2DM treatment and for alleviating cognitive deficits in
99 neurodegenerative disorders [1]. One of these peptides is the anorexigenic prolactin-releasing
100 peptide (PrRP), centrally released and acting to regulate energy metabolism in cooperation
101 with leptin [24]. The lipidization of its N-terminus enables its central effect after peripheral

administration in rodents [25, 26]. Recently we have developed a novel PrRP analog palmitoylated at position 11 (palm^{11}-PrRP31) with improved bioavailability, demonstrated that it is able to bind to PrRP receptor with high affinity in vitro and shown that PrRP analog decreased BW and food intake in DIO mice [27-29]. In our previous studies, we demonstrated that functional leptin and intact leptin signaling pathways are necessary for the body weight (BW)-reducing properties of palmitoylated PrRP in ob/ob mice, which are unable to produce leptin [30]; in Koletsky obese rats, which lack functional leptin receptors [31]; and in Zucker diabetic fatty (ZDF) rats [32]. Moreover, palmitoylated PrRP analog has been shown to have both anti-obesity and glucose lowering properties, and it has also been shown to act as a neuroprotective compound in mouse models of Aβ or Tau pathology [33, 34].

Therefore, we hypothesize that aged fa/fa rats might be a suitable model to study the relationship between metabolic and neurodegenerative disorders. The aim of this study was to examine whether obesity and diabetic metabolic parameters of fa/fa rats relate not only to IR and Tau hyperphosphorylation in the brain but also to impaired synaptogenesis and neuroinflammation which are important players in AD progression. Then, the impact of treatment with a palmitoylated PrRP analog (palm^{11}-PrRP31) on all these parameters was studied to examine its potential neuroprotective effects in this model of leptin and insulin resistance.

Material and methods

Synthesis of PrRP analog

A human palmitoylated analog of PrRP (palm^{11}-PrRP31) with the sequence SRTHRHSMEIK (N-γ-E(N-palmitoyl)) TPDINPAWYASRGIRPVGRF-NH_2 was synthesized and purified at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague (CAS), Czech Republic, as previously described [35].

Animals and diet

All animal experiments were performed following the ethical guidelines for work with animals by the Act of the Czech Republic Nr. 246/1992 and were approved by the Committee for Experiments with Laboratory Animals of the CAS.

Experiments were conducted on homozygous Zucker *fa/fa* male rats (fa/fa) and their lean counterparts *fa*/+- (control) rats. Six-week-old male rats of both genotypes were purchased from Charles River (Lecco, Italy). The rats were provided with a standard Ssniff diet (Spezialdiäten GmbH, Soest, Germany) (58% carbohydrates, 9% fat, and 33% protein) and water *ad libitum*. Animals were on a 12:12-h light:dark cycle (lights on from 5:00) and maintained at a constant temperature of 22 ± 2°C.

Study design, drug administration and dosing

The schema of the experimental design is shown in Figure 1. Before the start of treatment, the body weight (BW) and food intake (FI) of the fa/fa and control rats were monitored once per week. Fasted blood samples were collected from tail vessels of rats 32 weeks of age to determine the basic biochemical profile of the rat plasma. Beginning at 32 weeks of age, the fa/fa rats were infused for 2 months with palm^{11}-PrRP31 dissolved in saline (fa/fa palm^{11}-PrRP31 group, n= 8) at a dose of 5 mg/kg BW per day using Alzet osmotic pumps (Alzet, Cupertino, CA, USA), which were certified to infuse 6 µl of solution daily. The dose used in this study was chosen according to our previous studies [31, 32]. Two other groups, lean controls

fa/+- (control saline group, n=8) and fa/fa rats (fa/fa saline group, n=7), were infused with saline. All Alzet osmotic pumps were replaced after 4 weeks with new pumps. Alzet osmotic pumps were implanted intraperitoneally (IP) under short-term ether anesthesia. During the treatment, the BW and FI were measured twice per week. At the end of the experiment, the rats were fasted overnight, blood plasma was collected from tail vessels for determination of the biochemical parameters, and an oral glucose tolerance test (OGTT) was performed. Thereafter, arterial blood pressure was measured by direct puncture of the carotid artery under light ether anesthesia. The animals were sacrificed by decapitation, and tissue samples were collected. The brain, liver, kidney and heart were dissected and kept frozen at -80°C until use.

Oral glucose tolerance test

Rats fasted overnight were subjected to OGTT. At time point 0, blood glucose was measured in the tail vessels blood, and then glucose, at a dose of 2 g/kg of BW, was administered perorally by gavage. Blood glucose concentrations in whole blood were determined using a glucometer (Arkray, Tokyo, Japan) at 15, 30, 60, 90, 120 and 180 min after glucose gavage. EDTA plasma samples were collected at 0, 30, 60, and 120 min intervals to determine insulin levels.

Determination of biochemical parameters

Plasma insulin concentration was measured using a radioimmunoassay (RIA) kit (Millipore, St. Charles, MI, USA). Colorimetric assays were used to determine plasma levels of CHOL, TG (Erba Lachema, Brno, Czech Republic) and FFA (Roche, Mannheim, Germany). All measurements were performed according to the manufacturer's instructions. Quantitative insulin sensitivity check index (QUICKI) was measured from fasting glucose and fasting insulin ($QUICKI = 1/[(\log I_0) + (\log G_0)]$, where I_0 is fasting insulin in μU/ml and G_0 is fasting glucose in mg/dl).

Drug exposure

The blood plasma concentration of palm^{11}-PrRP31 at the end of the experiment was determined with a rat PrRP(1-31) EIA high-sensitivity kit (Peninsula Laboratories, San Carlos, CA, USA) according to the manufacturer's instructions.

Western blot analysis

Hippocampal samples were processed, and western blotting (WB) was performed as previously described [30]. The primary antibodies used are shown in supplementary Table 1. The following secondary antibodies were used: anti-mouse or anti-rabbit IgG HRP-linked antibody (both Cell Signaling Technology, Beverly, MA, USA).

Chromogenic immunohistochemistry (IHC)

One-half of the rat brains were processed as previously described [33]. The sections were incubated in anti-ionized calcium-binding adaptor molecule 1 (Iba1) (Wako, Osaka, Japan), anti- glial fibrillary acidic protein (GFAP) (Thermo Fisher Scientific, Waltham, MA, USA) or anti-doublecortin (DCX) (Cell Signaling Technology) primary rabbit antibodies. A biotinylated goat anti-rabbit secondary antibody was used for chromogenic IHC.

Statistical analyses

The data are presented as the means ± S.E.M as analyzed with GraphPad Software (San Diego, CA, USA). The data were evaluated byone-way or two-way ANOVA followed by Bonferroni *post hoc* test. $P<0.05$ was considered statistically significant.

Results

Palm^{11}-PrRP31 had no effect on morphometric and metabolic parameters

The BW was significantly higher in the fa/fa rats compared to controls before treatment, at 32 weeks of age. At the end of the experiment (40 weeks of age), the BW and liver weight were also increased in fa/fa rats (Table 1). Treatment with palm^{11}-PrRP31 did not significantly

decrease the BW of the fa/fa rats. At age 32 and 40 weeks, the fa/fa rats had a significantly higher fasted glucose than the age-matched controls. After treatment with palm^{11}-PrRP31, fa/fa rats had slightly increased glucose level, but still normoglycemic [31]. Plasma levels of insulin, leptin, CHOL and TG were significantly higher in the fa/fa group than in the control group before the treatment and at the end of the experiment. Moreover, QUICKI was significantly decreased in fa/fa saline rats compared to lean controls. Treatment with palm^{11}-PrRP31 had no effect on the measured metabolic parameters (Table 1). There were no significant changes in blood pressure among the groups (Table 1). The plasma concentration of palm^{11}-PrRP31 in the fa/fa palm^{11}-PrRP31 rats was 30.9 ± 8.7 ng/ml (n=8) at the end of the experiment.

No significant change in FI was registered among control saline, fa/fa saline, or fa/fa palm^{11}-PrRP31 rats during the treatment (Figure 2A). Similarly, the change in BW over the course of the treatment did not differ among the groups (Figure 2B). As indicated by the OGTT, the glucose level was significantly higher at 30, 120 and 180 min in the fa/fa saline group than in the control saline group (Figure 2C), resulting in a significantly increased area under the curve of the fa/fa saline group (Figure 2D). Palm^{11}-PrRP31 treatment did not affect glucose tolerance in fa/fa rats (Figure 2C, 2D). The results of the OGTT showed that the fa/fa saline group exhibited a significantly higher plasma insulin level than the control saline group; the insulin levels of the fa/fa palm^{11}-PrRP31 group and the fa/fa saline group did not differ (Figure 2E).

Palm^{11}PrRP31 attenuated IR and sporadic neuroinflammation and increased mildly synaptogenesis in fa/fa rats.

WB was performed with the hippocampi of control saline, fa/fa saline and fa/fa palm^{11}-PrRP31 rats (n=7-8 rats per group). The fa/fa saline group showed a trend toward a decrease

220 in phosphoinositide 3-kinases (PI3K), pAkt (Ser473), and pGSK-3β (Ser9) levels and a
221 significantly lower pAkt (Thr308) level compared to the control saline group suggesting IR in
222 the hippocampus. Palm^{11}-PrRP31 treatment significantly increased PI3K and pGSK3β (Ser9)
223 levels, and there was a trend toward an increase in pAkt (Thr308) and pAkt (Ser473) levels
224 (Figure 3A).
225 There was non-significant change in methyl-PP2A subC level, but the Cdk5 level was
226 significantly increased in the fa/fa saline group compared to the control saline group. The
227 fa/fa saline group showed non-significant increase in pTau (Thr231) and pTau (Thr212)
228 levels, and a significant increase in pTau (Ser199) and pTau (Ser396) levels, compared to the
229 control saline group. Palm^{11}-PrRP31 treatment significantly decreased Cdk5 and significantly
230 increased methyl-PP2A subC, but it did not affect Tau phosphorylation at any epitope
231 measured (Figure 3B).
232 In the extracellular signal-regulated kinases (ERK) and signal transducer and activator of
233 transcription 3 (STAT3) signaling pathways, no significant differences in phosphorylation
234 were found between the fa/fa saline group and the control saline group but a trend toward a
235 decrease in STAT3 phosphorylation at Tyr705 was evident. STAT3 phosphorylation at
236 Tyr705 was increased in the palm^{11}-PrRP31-treated fa/fa rat group compared to the fa/fa
237 saline group (Figure 4).

238 Synaptogenesis in the hippocampus was detected *via* the presynaptic markers synaptophysin
239 and Syntaxin 1A. Compared to the level in the control saline group, synaptophysin showed a
240 trend toward a decrease in the fa/fa saline group, and Syntaxin 1A was significantly lower in
241 the fa/fa saline group. Both the presynaptic markers synaptophysin and Syntaxin 1A in the
242 hippocampus were significantly increased in the fa/fa palm^{11}-PrRP31 group compared to the
243 fa/fa saline group (Figure 5A).

NeuN is a neuronal marker; there was no significant change between the fa/fa saline and control saline group (Figure 5B). Moreover, treatment with palm^{11}-PrRP31 had no effect on the neuronal marker NeuN. There were no significant changes in the number of DCX^{+} positive cells in the granular layer of the dentate gyrus (DG) (Figure 5C) as well as in GFAP, a marker of astrocytosis, in the hippocampus of the control saline and fa/fa saline rats as detected by both IHC and by WB (Figure 5D, E). There was no difference in hippocampal GFAP level in the fa/fa palm^{11}-PrRP31 and fa/fa saline rats. IHC staining with the anti-Iba1 antibody showed a slight indication of activated microglia in the fa/fa saline group compared to the control saline group, mainly in the CA1 and DG regions of the hippocampus (Figure 5E). However, these changes were not significant.

Discussion

Obese Zucker rats develop the following symptoms of metabolic syndrome: obesity, hyperinsulinemia, IR, glucose intolerance, hyperlipidemia and arterial hypertension [36]. The recessive mutant gene "fatty" (*fa*) of the leptin receptor is the reason for the impaired leptin signaling [8] and extreme obesity with juvenile onset [12].

The design of this study was based on our previous study with 12- and 33-week-old male fa/fa rats [16], where age- and obesity-induced peripheral IR under normoglycemia was described and linked to central IR and pathological Tau hyperphosphorylation.

Therefore, in this study, we presumed that at age of 32-weeks, all these pathologies would be present. We also aimed to determine whether chronic treatment with a palmitoylated PrRP analog administered peripherally can influenced obesity and IR as well as Tau phosphorylation, neurogenesis, synaptogenesis and neuroinflammation in the hippocampus, as it is the first brain structure affected by neurodegenerative changes.

The main difference between the two studies was the source of rats used in our previous study [16], we used fa/fa rats obtained from Harlan, now Envigo (Italy), and in the present study, we used fa/fa rats obtained from Charles River (Italy).

In obese Zucker fa/fa rats obtained from Envigo, obesity, IR and hyperlipidemia were reported at 12- [37] or 16 weeks old [38]. In our previous study [16], the Envigo fa/fa rats developed obesity accompanied by significantly increased plasma insulin, glucose intolerance, and IR at 33 weeks of age. In the present study with Charles River rats, these parameters were much less pronounced. Obesity in the Charles River fa/fa rats at 32 and 40 weeks of age was accompanied by mild glucose intolerance and increased insulin compared to the controls; however, insulin level did not reach the same values as in the fa/fa rats from Envigo, and also QUICKI showing peripheral IR was not as decreased, even it was significantly decreased

279 compared to control saline group [16]. Moreover, the Charles River fa/fa rats in this study were
280 not hypertensive. However, elevated blood pressure was reported in Envigo fa/fa rats older
281 than 16 weeks [38], and in 22-week-old fa/fa rats [36] but not in Charles River fa/fa rats [39]. The
282 strain variability in the Charles River and Envigo obese fa/fa rats was previously identified in
283 8-week-old fa/fa rats and their lean controls. Total lipid, TG and glucose levels were higher in
284 Charles River rats, while BW, CHOL and insulin levels were higher in the Harlan rats [40]. In
285 our present study, the Charles River fa/fa rats at 32 and 40 weeks of age had a higher plasma
286 TG level but lower BW, CHOL, glucose, and insulin levels than the Envigo rats of a similar
287 age examined in our previous study [16]. We can speculate that fa/fa rats from Charles River
288 used in this study with milder phenotype were too young to develop neurodegenerative
289 features and that older animals could have more pronounced phenotype concerning both
290 metabolic disturbances and neurodegeneration. There is also a possibility that because
291 metabolic disturbances were only weakly developed in fa/fa rats from Charles River, the
292 effect of palm^{11}-PrRP31 did not appear clearly in this model.

293 In our previous study with ZDF rats, we found that intact leptin signaling was necessary for
294 the full effect of palm-PrRP31 on metabolic parameters such as BW-lowering or glucose
295 tolerance; therefore, its antidiabetic and anti-obesity effects were not realized [32]. In Koletsky
296 (spontaneously hypertensive obese, SHROB) rats with a nonsense mutation in the leptin
297 receptor, palm^{11}-PrRP31 improved glucose tolerance, but its effect on BW change was
298 minimal [31]. On the other hand, in rat models of high-fat diet-induced obesity that have
299 functioning leptin receptors, such as Sprague-Dawley rats [32] and Wistar Kyoto rats [27],
300 treatment with palmitoylated PrRP significantly decreased BW and blood glucose, as
301 indicated by OGTTs. In this study, treatment with palm^{11}-PrRP31 neither affected BW nor
302 attenuated tolerance to glucose. This outcome could have been a result of disrupted leptin

303 receptor signaling in the fa/fa rats, similar to our aforementioned studies with ZDF and
304 Koletsky rats.

305 Obesity, IR, hyperinsulinemia and hypertension are features associated with an increased risk
306 of AD, vascular dementia and impaired cognitive function [41]. In our previous study, we
307 demonstrated that phosphorylation of hippocampal Tau protein at Ser396 and Thr231 was
308 significantly increased in 33-week-old fa/fa rats compared to age-matched controls, and this is
309 a possible consequence of peripheral IR and decreased insulin signaling in the hippocampus
310 [16]. As a consequence of lower obesity and mild peripheral IR observed in the Charles River
311 fa/fa rats compared to the Envigo fa/fa rats manifested by decreased QUICKI, there was only
312 a mild impairment of hippocampal insulin signaling. There was a significant decrease in pAkt
313 (Thr308) and a trend toward a decrease in PI3K and pAkt (Ser473) levels in the fa/fa saline
314 rats compared to the control saline rats. Additionally, we observed a slight decrease in
315 phosphorylation of GSK-3β at inhibitory epitope Ser9, which led to increased kinase activity
316 toward Tau protein. Moreover, Tau kinase Cdk5 was significantly increased; thus, we
317 observed a significant increase in the phosphorylation of Tau at Ser199 and Ser396, both of
318 which had been previously connected to the early stages of neurodegeneration [42, 43]. After the
319 2-month-long treatment with palm^{11}-PrRP31, we observed ameliorated activation of the
320 hippocampal insulin signaling cascade, resulting in significantly increased phosphorylation of
321 GSK-3β at Ser9, which showed inhibited kinase activity toward the Tau protein. We also
322 observed a decrease in the levels of Cdk5, another important Tau kinase. Moreover, activation
323 of methyl-PP2A subC, which is a major Tau phosphatase, was increased. However, Tau
324 hyperphosphorylation at three measured epitopes was not affected; this could have been a
325 result of defective leptin signaling or an insufficient duration of the treatment with palm^{11}-
326 PrRP.

It has also been previously shown that intact leptin signaling in the hypothalamus was important for the anorectic effect of leptin in fa/fa rats [44] and proper hippocampal leptin signaling manifested by activation of STAT3, which further can activates Akt, acts as neuroprotective agent and increase survival of hippocampal neurons [45], as well as supports memory formation [46]. In concordance with these observation, increased phosphorylation of STAT3 at Tyr705 after the treatment of fa/fa rats with palm[11]-PrRP31 was observed pointing to potential neuroprotective properties of the compound. This finding confirms our previous studies [28, 31], where palmitoylated PrRP analogs activated STAT3 and ERK signaling.

Hippocampal synaptogenesis was reduced in db/db mice lacking functional leptin receptors by an ERK-dependent pathway [47]. In our study, a reduction in synaptogenesis was shown via significantly decreased presynaptic marker Syntaxin 1A in the fa/fa saline rats compared to the saline control rats, however, no changes were observed in hippocampal activation of ERK. Similar to db/db mice previously studied, the levels of synaptophysin and the neuronal marker NeuN did not differ [48]. Moreover, age-related changes linked to metabolic syndrome were described in the cortex and hippocampus of 12-, 16- and 20-week-old fa/fa rats from Envigo [23]; a decrease in a number of neurons, enhancement of gliosis, disruption of the blood brain barrier, and cognitive alterations. In addition, the number of GFAP-immunoreactive astrocytes have been found to be increased in the hippocampi and cortex of fa/fa rats [37]. We observed no changes in astrocytosis and only sporadic signs of gliosis in the fa/fa rats. These outcomes could be results of the moderate metabolic syndrome found in the fa/fa rats from Charles River with only mild obesity, and peripheral and central IR [41]. Stranahan et al.[15] described decreased neurogenesis in db/db mice using BrdU staining; in our study, we did not observe any changes in neurogenesis in the hippocampus of the fa/fa saline rats compared to the control rats using DCX.

From all the results obtained from this and previous studies , it is possible to summarize that palm^{11}-PrRP31 can ameliorate neurodegeneration by an increase of insulin and leptin signaling, as well as by increase of synaptogenesis and neurogenesis in brain of treated animals.

In conclusion, this study demonstrates that fa/fa rats from Charles River developed obesity, mild glucose intolerance, and mild central and peripheral IR but not hypertension. Mildly worsened synaptogenesis with no difference in neurogenesis and sporadic neuroinflammation was observed in the fa/fa saline rats compared to the saline controls. Eight-week treatment with palm^{11}-PrRP31 neither lowered BW nor attenuated glucose tolerance, suggesting that intact leptin signaling was necessary for the anti-obesity effect of palm^{11}-PrRP31, similar to our previous studies. Treatment with palm^{11}-PrRP31 had no effect on Tau phosphorylation or neuroinflammation; however, it ameliorated insulin signaling, increased activation of PP2A, decreased activation of Tau kinases, and increased synaptogenesis in the hippocampus. This mild beneficial effect of palm^{11}-PrRP31 treatment was probably due to the lack of leptin receptor activity, but despite of no improvement in metabolic profile, some neuroprotective effects were observed. These results indicate the preserved neuroprotective effects of palm^{11}-PrRP despite of disrupted leptin signaling in this rat model.

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Author contribution

L Ma and J K designed the experiments; L Ma, J K, L Mr, S H, B N, V S and L H performed the experiments; L Mr, S H and B N analyzed the data; L Ma, L Mr and J K wrote the manuscript; L Ma, J K, A P and B Ž edited the manuscript; L Ma, J K, A P and B Ž read and approved the final manuscript

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Abbreviations

AD	Alzheimer disease
Akt	Protein kinase B (Serine/Threonine Kinase 1)
AUC	area under a curve
Aβ	amyloid β
BW	body weight
CAS	Czech Academy of Sciences
Cdk5	Cyclin-dependent kinase 5
DCX	doublecortin
EDTA	Ethylenediaminetetraacetic acid
Fa	fatty

FFA	free fatty acid
FI	food intake
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
Gsk3β	glycogen synthase kinase 3β
Iba1	ionized calcium-biding adaptor molecule 1
IHC	immunohistochemistry
IPAT	intraperitoneal adipose tissue
IR	insulin resistance
Irs1	insulin receptor substrate
Lep	leptin
NAFLD	nonalcoholic fatty liver disease
NT	not tested
$Palm^{11}PrRP31$	palmitoylated analog of PrRP
PI3K	phosphoinositide 3-kinase
PP2A	protein phosphatase 2A
RIA	radioimmunoassay
SCAT	subcutaneous adipose tissue
STAT3	signal transducer and activator of transcription 3
T2DM	type 2 diabetes mellitus
ZDF	Zucker Diabetic Fatty

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Figure and Table legends

Table 1 Metabolic parameters in fasted blood plasma and in liver, organs weight and blood pressure of control and fa/fa group before treatment (32 weeks of age) and at the end of experiment (40 weeks of age) in control saline and fa/fa treated with saline or palm^{11}-PrRP31.

Data are presented as means ± S.E.M. Statistical analysis was performed by t-test or One-way ANOVA.

Significance is *P<0.05 **P<0.01 ***P<0.001 and ****P <0.0001 by t-test fa/fa vs control (n=7-8 before the treatment) and One-way ANOVA fa/fa saline vs control saline (n=7-8 at the end of experiment), #P<0.5 fa/fa palm^{11}-PrRP31 vs fa/fa saline (n=8 at the end of experiment)

Figure 1 Experimental design

Figure 2 FI **(A)** and BW **(B)** measured during the treatment of control saline, fa/fa saline and fa/fa palm^{11}-PrRP31 rats. Glucose **(C, D)** and insulin **(E,F)** measured during the OGTT performed at the end of experiment. Data are presented as means ± S.E.M. Statistical analysis of AUC was performed by One-way ANOVA. Two-way ANOVA performed statistical

analysis of BW change, FI, glucose and insulin. Significance is *P<0.05 **P<0.01 ***P<0.001 and ****P <0.0001 fa/fa saline vs control saline (n= 7-8).

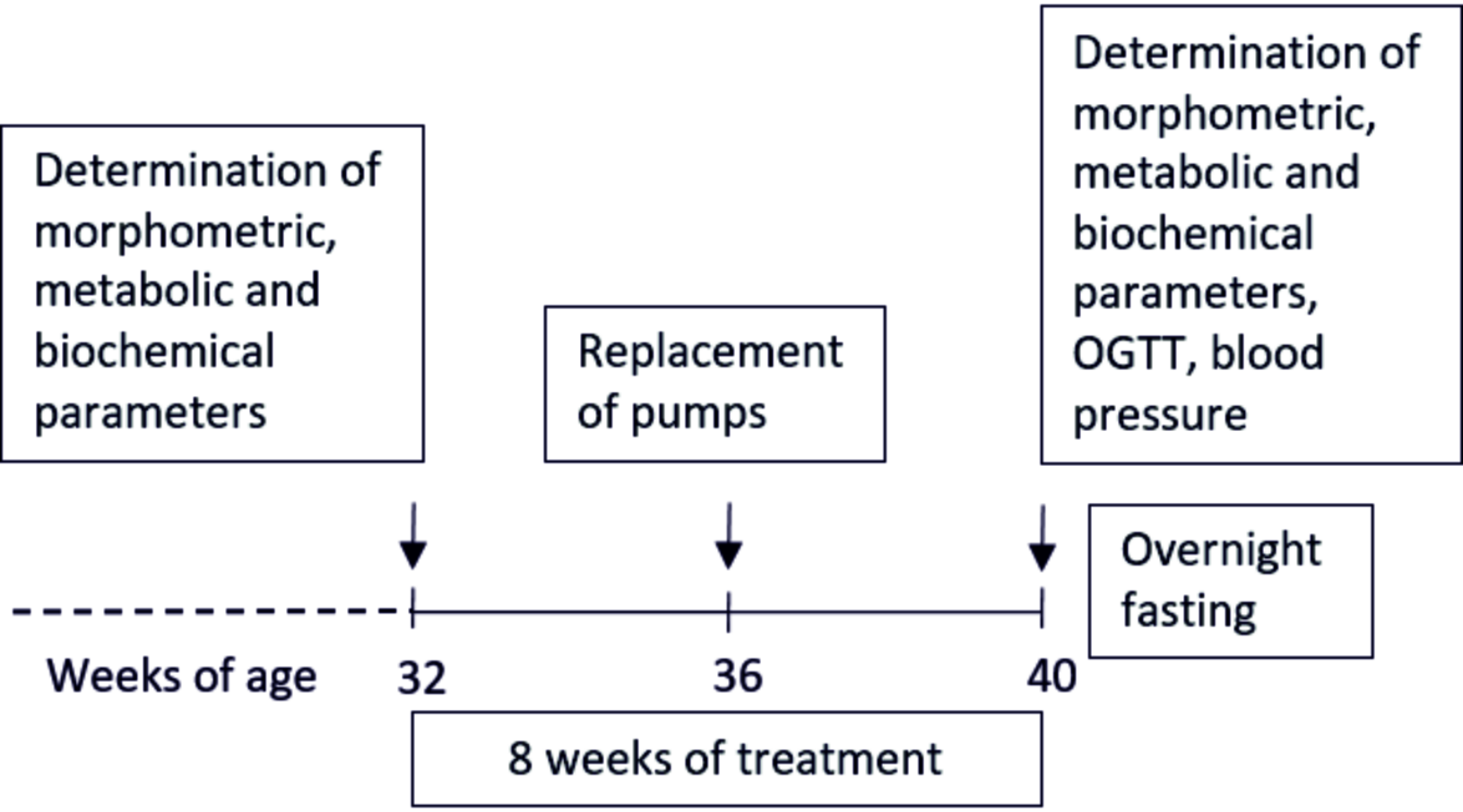
Figure 3 Insulin signaling pathway, **(A)** Tau phosphorylation **(B)** measured in hippocampi by western blot at the end of experiment. Data are presented as means ± S.E.M. Statistical analysis was performed by One-way ANOVA . Significance is *P <0.05 and **P <0.01 fa/fa saline vs control saline (n=7-8).

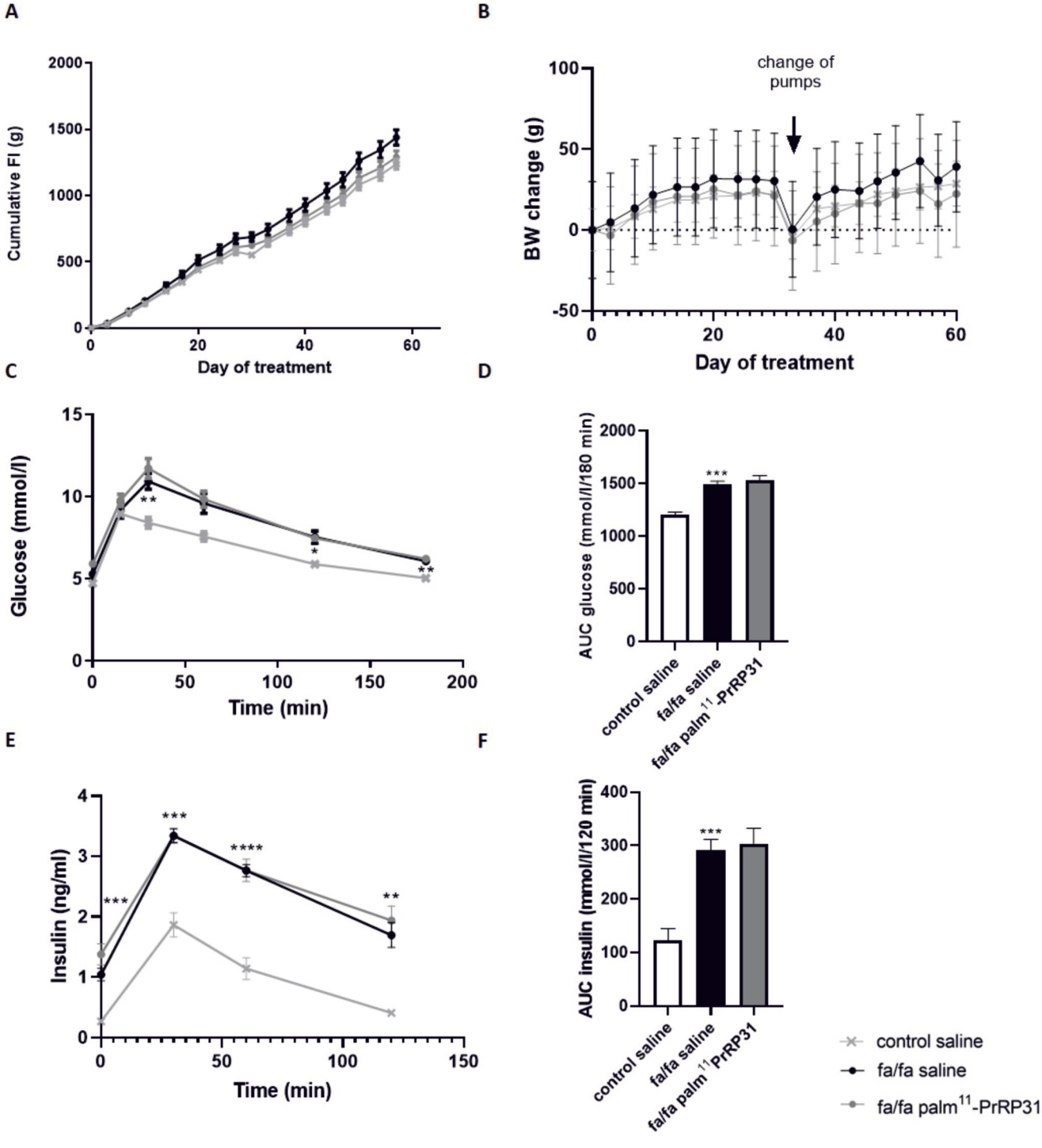
Figure 4 ERK and STAT3 signaling pathway measured in hippocampi by western blot at the end of experiment. Data are presented as means ± S.E.M. Statistical analysis was performed by One-way ANOVA. Significance is *P <0.05 (n=7-8).

Figure 5 Synaptophysin and syntaxin 1A as a marker of synaptogenesis **(A)** and neuronal marker NeuN **(B)** in hippocampi detected by western blot. Marker of neurogenesis doublecortin in hippocampi **(C)** detected immunohistochemically. Marker of neuroinflammation GFAP detected by western blot **(D)** and representative photomicrograph of neuroinflammatory markers GFAP and Iba1 **(E)** at the end of experiment in control saline and fa/fa saline. Data are presented as means ± S.E.M. Statistical analysis was performed by One-way ANOVA. Significance is *P<0.05 **P<0.01 ***P<0.001 and ****P <0.0001 (n=7-8).

Supplementary Table 1 List of primary antibodies and their appropriate dilution used for western blot.

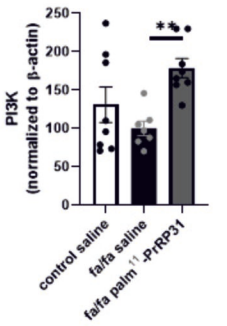
PI3K total phosphoinositide 3-kinase, ERK extracellular signal-regulated kinase 1/2, STAT3 total signal transducer and activator of transcription 3, GFAP glial fibrillary acidic protein, Cdk5 cyclin-dependent kinase 5, methyl-PP2A subC methyl protein phosphatase 2A subunit C

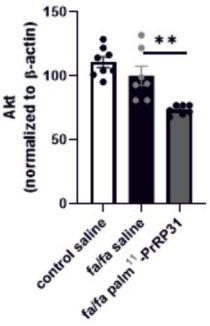

Determination of morphometric, metabolic and biochemical parameters
Replacement of pumps
Determination of morphometric, metabolic and biochemical parameters, OGTT, blood pressure
Overnight fasting
Weeks of age
32
36
40
8 weeks of treatment

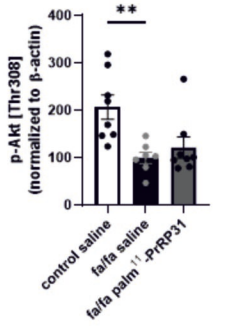

A
Cumulative FI (g)
2000
1500
1000
500
0
0
20
40
60
Day of treatment
B
change of
pumps
BW change (g)
100
50
0
-50
0
20
40
60
Day of treatment
C
Glucose (mmol/l)
15
10
5
0
0
50
100
150
200
Time (min)
**
*
**
D
AUC glucose (mmol/l/180 min)
2000
1500
1000
500
0
***
control saline
fa/fa saline
fa/fa palm 11-PrRP31
E
Insulin (ng/ml)
4
3
2
1
0
***
***
****
**
0
50
100
150
Time (min)
F
AUC insulin (mmol/l/120 min)
400
300
200
100
0
***
control saline
fa/fa saline
fa/fa palm 11-PrRP31
control saline
fa/fa saline
fa/fa palm11-PrRP31

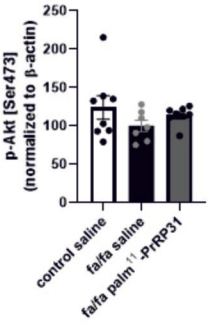
A

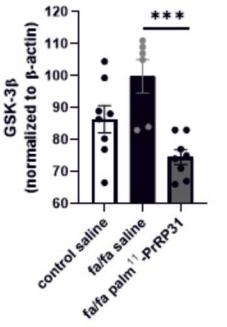
	control saline	fa/fa saline	fa/fa palm^{11}-PrRP31	kDa
PI3K				85
Akt				60
p-Akt [Thr 308]				60
p-Akt [Ser 473]				60
GSK-3β				46
p-GSK-3β S9				46
β-actin				42

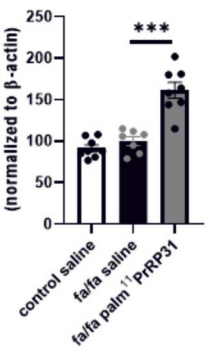






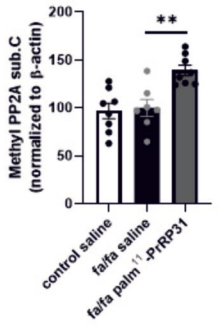


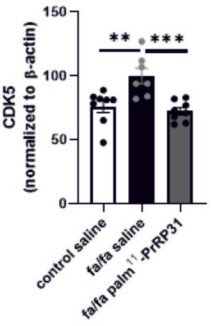


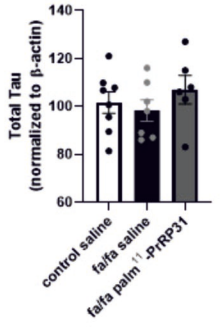


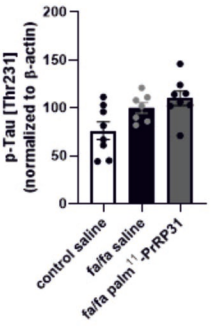
B

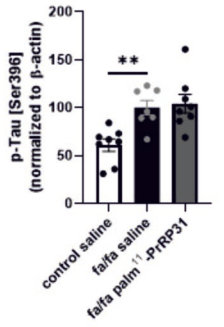
	control saline	fa/fa saline	fa/fa palm^{11}-PrRP31	kDa
Methyl-PP2A subC				36
Cdk5				33
Total Tau				50
p-Tau [Thr231]				50 (60)
p-Tau [Ser396]				50 (60)
p-Tau [Ser199]				50 (60)
p-Tau [Thr212]				50 (60)
β-actin				42

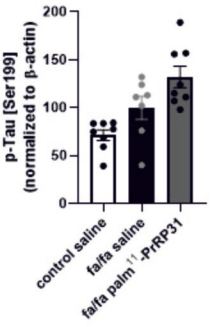


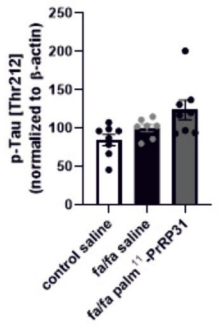




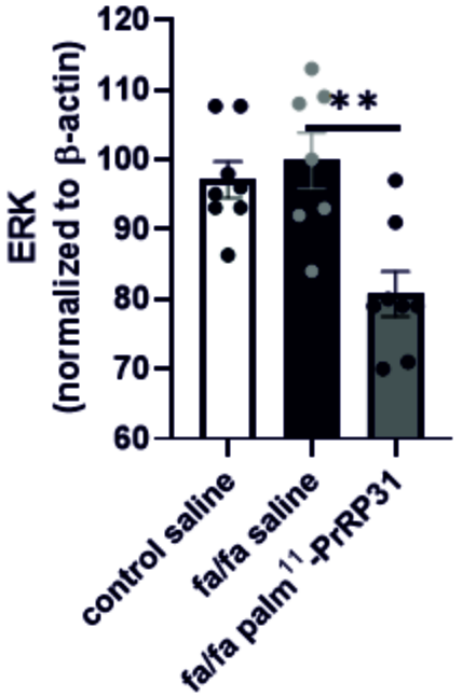


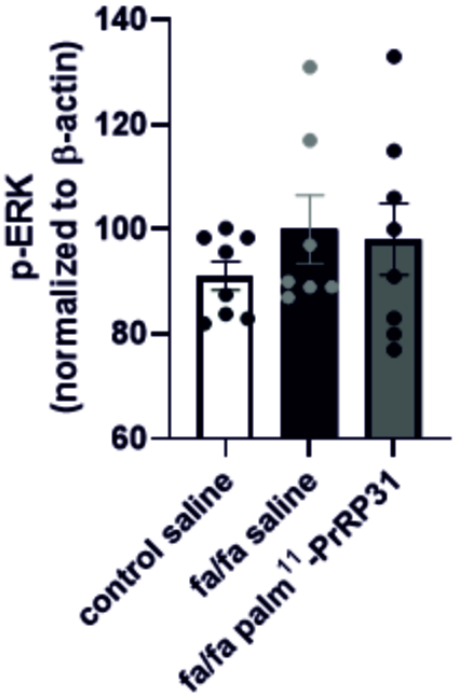


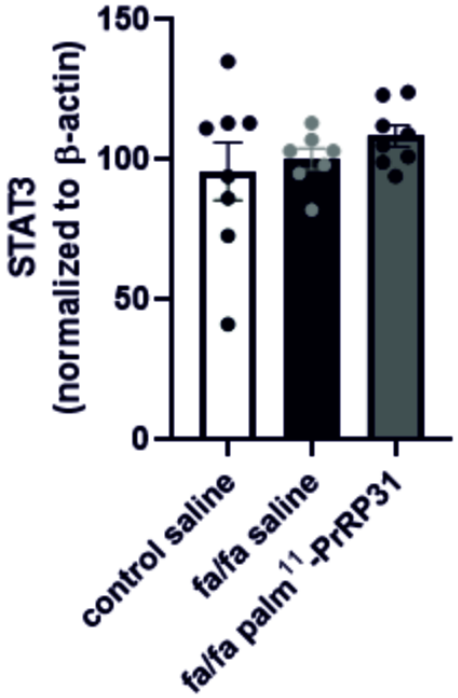


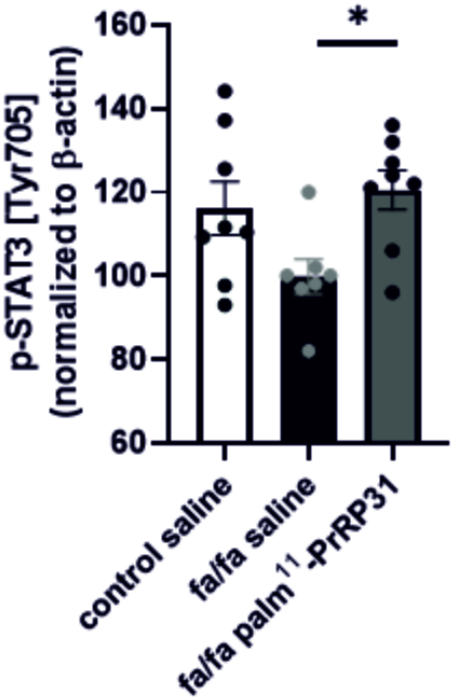


	control saline	fa/fa saline	Fa/fa palm^{11}-PrRP31	kDa
ERK				42, 44
p-ERK				42, 44
STAT3				79
p-STAT3 [Tyr 705]				79
β-actin				42

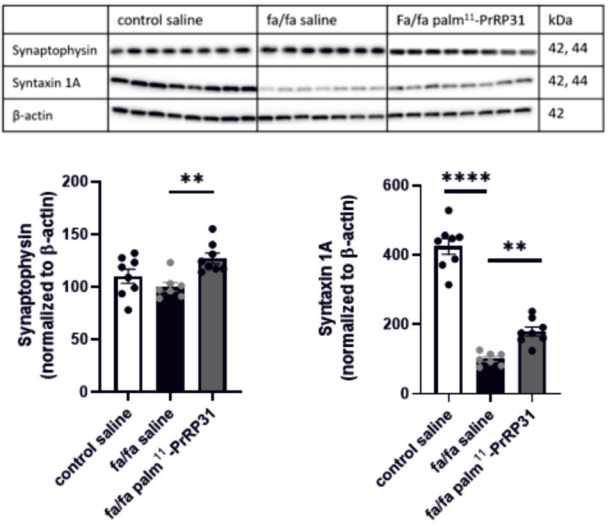




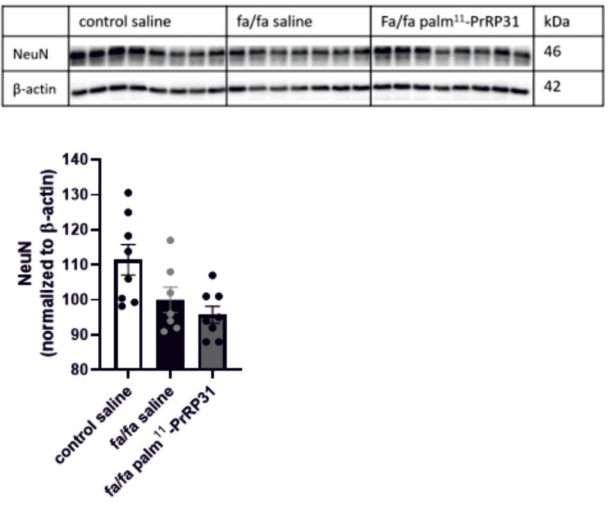




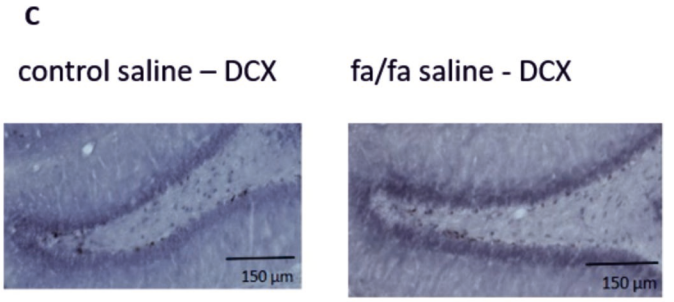
A


control saline
fa/fa saline
Fa/fa palm11-PrRP31
kDa
Synaptophysin
42, 44
Syntaxin 1A
42, 44
β-actin
42
Synaptophysin (normalized to β-actin)
Syntaxin 1A (normalized to β-actin)
**
****
**
control saline
fa/fa saline
fa/fa palm 11-PrRP31

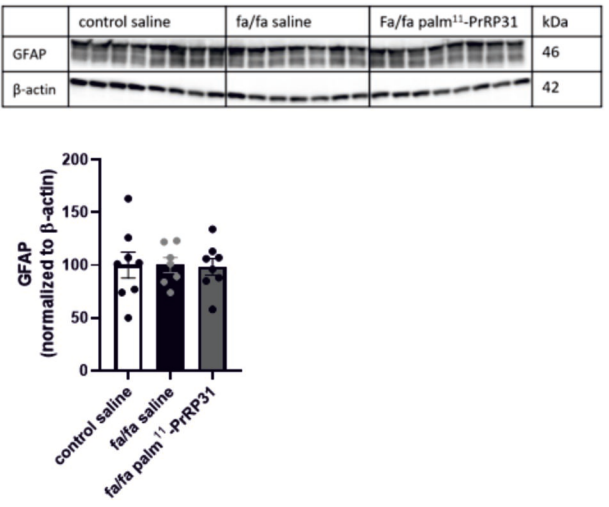
B


control saline
fa/fa saline
Fa/fa palm11-PrRP31
kDa
NeuN
46
β-actin
42
NeuN (normalized to β-actin)
control saline
fa/fa saline
fa/fa palm 11-PrRP31

C


control saline – DCX
fa/fa saline - DCX
150 µm
150 µm

D


control saline
fa/fa saline
Fa/fa palm11-PrRP31
kDa
GFAP
46
β-actin
42
GFAP (normalized to β-actin)
control saline
fa/fa saline
fa/fa palm 11-PrRP31

E

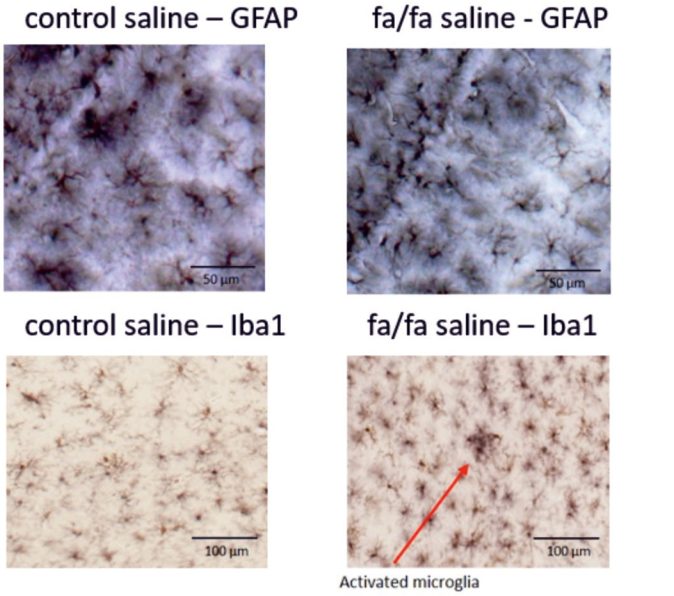

control saline – GFAP
fa/fa saline - GFAP
50 µm
50 µm
control saline – Iba1
fa/fa saline – Iba1
100 µm
100 µm
Activated microglia

Table 1

Age	32 weeks		40 weeks		
Group	control	fa/fa	control saline	fa/fa saline	fa/fa palm^{11}-PrRP31
BW (g)	441 ± 9	572 ± 11****	456 ± 9	592 ± 18***	572 ± 26
Glucose (mmol/l)	4.95 ± 0.13	5.38 ± 0.06**	4.73 ± 0.05	5.29 ± 0.14**	5.91 ± 0.16$^{\#}$
TG (mmol/l)	1.11 ± 0.08	4.30 ± 0.28****	1.24 ± 0.15	6.78 ± 1.15***	5.09 ± 0.74
CHOL (mmol/l)	3.37 ± 0.27	7.83 ± 0.51****	2.08 ± 0.15	3.77 ± 0.22****	3.75 ± 0.27
FFA (mmol/l)	0.66 ± 0.04	0.58 ± 0.04	1.79 ± 0.10	1.42 ± 0.18	1.53 ± 0.18
Leptin (ng/ml)	4.02 ± 0.61	46.55 ± 1.61****	3.74 ± 0.51	46.80 ± 1.90****	49.57 ± 2.20
Insulin (ng/ml)	0.49 ± 0.06	1.24 ± 0.02****	0.27 ± 0.06	1.04 ± 0.11***	1.38 ± 0.18
QUICKI			0.374 ± 0.013	0.296 ± 0.005****	0.283 ± 0.004
Liver (g)			15.00 ± 0.65	23.14 ± 0.70****	21.50 ± 0.71
Heart (g)			1.10 ± 0.03	1.19 ± 0.04	1.16 ± 0.03
Kidney (g)			3.18 ± 0.16	3.46 ± 0.16	3.34 ± 0.15
MAP (mmHg)			105.75 ± 6.39	117.67 ± 7.01	125.13 ± 1.57
HR (beat/min)			389.05 ± 13.00	371.07 ± 38.80	378.80 ± 16.37