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Účinky stabilních analogů anorexigenních neuropeptidů v modelech metabolického syndromu

Effects of stable analogs of anorexigenic neuropeptides in models of metabolic syndrome

Disertační práce

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ABSTRAKT

Obezita je celosvětový zdravotní problém, ale účinná léčba je nedostatečná. Anorexigenní neuropeptidy, jako je peptid uvolňující prolaktin (PrRP), mají potenciál pro léčbu obezity a s ní spojených komplikací. Ve své přirozené formě mají tyto látky určité nevýhody, například: špatnou biologickou dostupnost, nízkou stabilitu a neschopnost procházet hematoencefalickou bariérou po periferním podání. V naši laboratoři byly navrženy lipidizované analogy PrRP. Lipidizace činí tento peptid stabilnějším a schopným působit centrálně po periferním podání.

Cílem této disertační práce bylo prozkoumat dlouhodobý antiobezitní účinek PrRP palmitoylovaného na pozici 11 (palm^{11}-PrRP31) a objasnit tak mechanismy účinku tohoto peptidu. Ke studiu obezity a metabolických parametrů souvisejících s obezitou byly použity tři modely: Wistar Kyoto (WKY) potkani s obezitou navozenou vysokotukovou dietou (DIO) s intaktním leptinem a leptinovým receptorem, a experimentální modely s narušenou funkcí leptinu: *ob/ob* myši s deficitem leptinu a fa/fa potkani s narušenou signalizací leptinu.

Příjem vysokotukové diety zvýšil u WKY potkanů tělesnou hmotnost. Tito potkani vykazovali silnou glukózovou intoleranci a zvýšenou mRNA expresi enzymů regulujících *de novo* lipogenezi. Léčba palm^{11}-PrRP31 vedla k signifikantnímu snížení kumulativního příjmu potravy, tělesné hmotnosti a plazmatické hladiny leptinu, snížení glukózové intolerance a exprese lipogenetických enzymů. U *ob/ob* myší s deficitem leptinu kombinace palm^{11}-PrRP31 a leptinu v chronickém podání vykazovala synergistický účinek. V mladším věku kombinovaná léčba snížila glykémii a hmotnost jater, ve starším věku vedla ke snížení tělesné hmotnosti a hladiny cholesterolu a zvýšení tělesné teploty. Naopak léčba palm^{11}-PrRP31 nesnížila tělesnou hmotnost ani z ní vyplývající poruchy u potkanů *fa/fa* s poruchou signalizace leptinu.

Palm^{11}-PrRP31 prokázal antiobezitní a antidiabetické účinky u potkanů s obezitou indukovanou vysokotukovou dietou, kteří měli intaktní leptin a leptinový receptor. V modelech s porušenou leptinovou signalizací jsme zjistili, že pro anorexigenní účinky palm^{11}-PrRP31 je nezbytná funkční leptinová signalizace. Palmitoylované analogy PrRP jsou tedy atraktivními kandidáty pro léčbu obezity indukované vysokoenergetickou dietou a z ní vyplývajících poruch.

KLÍČOVÁ SLOVA

metabolický syndrom, peptid uvolňující prolaktin, lipidizace, myší a potkaní modely

ABSTRACT

Obesity is a worldwide health problem and an effective treatment is still scarce. Anorexigenic neuropeptides, such as prolactin-releasing peptide (PrRP), have a potential for the treatment of obesity and its complications, but in their natural form they have several limitations such as poor bioavailability, low stability and inability to cross the blood-brain barrier after peripheral administration. Recently we have designed lipidized analogs of PrRP. Lipidization makes this peptide more stable and able to act centrally after peripheral administration.

The aim of this study was to investigate the chronic effect of PrRP palmitoylated at position 11 (palm^{11}-PrRP31) on obesity and obesity-related metabolic parameters and to clarify mechanisms of its action. We used three rodent models of obesity: Wistar Kyoto (WKY) rats with high-fat diet-induced obesity (DIO) having intact leptin and leptin receptor as well as rodents with disrupted leptin function: leptin deficient *ob/ob* mice and *fa/fa* rats with a disturbed leptin signaling.

Consumption of a high-fat diet in DIO WKY rats increased their body weight, caused strong glucose intolerance and increased liver mRNA expression of enzymes of *de nov*o lipogenesis. Palm^{11}-PrRP31 treatment significantly decreased cumulative food intake, body weight, plasma leptin level, attenuated glucose intolerance as well as expression of liver lipogenesis enzymes. In leptin deficient *ob/ob* mice, palm^{11}-PrRP31 and leptin showed a synergistic effect in chronic treatment at a younger age on attenuating hyperglycemia and liver weight. At an older age it showed a decrease in body weight, cholesterol level and an increase in body temperature. On the other hand, there was a beneficial effect on obesity and related disturbances occurred in *fa/fa* rats with leptin signaling disruption after palm^{11}-PrRP31 treatment.

Our data suggest a good efficacy of palm^{11}-PrRP31 with diet-induced obesity with intact leptin and leptin receptor. Through the rodents with disturbed leptin signaling, we showed that leptin signaling is necessary for palm^{11}-PrRP31 anorexigenic and related effects. Thus, palmitoylated PrRP analogs are attractive candidates for treatment of humans with high energy diet-induced obesity and derived disturbances.

KEY WORDS

metabolic syndrome, prolactin-releasing peptide, lipidization, mouse and rat models

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CONTENTS

ABBREVIATIONS

Acaca	acetyl-CoA carboxylase alpha
AgRP	agouti-releated protein
Akt	protein kinase B
AMP	adenosine monophosphate
AMPK	5´adenosine monophosphate activated protein kinase
ARC	arcuate nukleus
ATP	adenosine triphosphate
AUC	area under the curve
B2M	beta-2-microglobulin
BAT	brown adipose tissue
BBB	blood brain barrier
BW	body weight
CART	cocaine and amphetamine – regulated transcript
CCK	cholecystokinin
Cpt-1a	carnitine palmitoyltransferase 1a
CRH	corticotrophin-releasing hormone
DIO	diet induced obesity
DMN	dorsomedial nucleus
fa/fa	obese Zucker *fa/fa* rats
FABP-4	fatty acid-binding protein 4
Fasn	fatty acid synthase
FFA	free fatty acid
FFC	high-fat, fructose and cholesterol diet
FGF21	fibroblast growth factor 21
FI	food intake
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G6pc	glucose-6-phosphatase
GHSR	growth hormone-secretagogue receptor

GLP-1	glucagon-like peptide 1
GLUT 4	glucose transporter 4
Gsk-3β	glycogen synthase kinase 3 beta
GUSB	glucuronidase beta
HbA1c	glycated hemoglobin
HF diet	high-fat diet
HOMA	homeostasis model assessment
HPA	hypothalamus-pituitary-adrenal axis
HSD	high-sugar diet
CHOL	cholesterol
ChREBP	carbohydrate response element binding protein
IP	intraperitoneal
IPAT	intraperitoneal adipose tissue
IR	insulin receptor
IRS	insulin receptor substrate
JAK 2	Januse kinase 2
Lep	leptin
LHA	lateral hypothalamic nucleus
Lipe	lipase E
Lpl	lipoprotein lipase
MAPK	mitogen activated protein kinase
MCH	melanin-concentrating hormone
MCH-R	melanin-concentrating hormone receptor
NPFF	neuropeptide FF
NPFF-2R	neuropeptide FF 2 receptor
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
OGTT	oral glucose tolerance test
palm^{11}-PrRP31	PrRP analog palmitoylated in position 11
Pck-1	phosphoenol pyruvate carboxykinase 1
PDK	phosphoinositide dependent protein kinase

PFA	paraformaldehyde
PI3K	phosphoinositol 3 kinase
POMC	pro-opiomelanocortin
Ppar-α/γ	peroxisome proliferatore-activated receptor alpha/gamma
PrRP	prolactin-releasing peptide
PTP1B	phosphotyrosine phosphatase 1B
PVN	paraventricular nucleus
PYY	peptide YY
RISK	reperfusion injury salvage kinase
SC	subcutaneous
SCAT	subcutaneous adipose tissue
Scd-1	stearoyl-CoA desaturase 1
SD rats	Sprague Dawley rats
SOCS3	suppressor of cytokine signaling 3
SREBP-1	sterol regulatory binding protein 1
STAT3	signal transducer and activator of transcription 3
STAT5	signal transducer and activator of transcription 5
T2DM	type 2 diabetes mellitus
TG	triglycerides
TRH	thyrotropin-releasing hormone
Ucp-1	uncoupling protein 1
VMH	ventromedial nucleus
WKY rats	Wistar Kyoto rats
WT	wild type
ZDF	Zucker diabetic rats
α-MSH	α-melanocyte-stimulating hormon

1. INTRODUCTION

1.1 Obesity and its possible treatment

Obesity is becoming a major public problem worldwide. Abdominal obesity in combination with dyslipidemia and/or hyperglycemia (impaired glucose tolerance), as well as insulin resistance and hypertension are the most observed components of metabolic syndrome, which are risk factors in various chronic diseases including type 2 diabetes mellitus (T2DM), cardiovascular diseases, psychological deficits or fatty liver disease (Guo, 2014;Pan et al., 2014;Engin, 2017;Tune et al., 2017;Blüher, 2019). The prevalence of the metabolic syndrome is estimated to be 25% in adults. In children and young adults, it is estimated to be 6,5% (Kwitek, 2019).

Obesity stems from a long-term energy disbalance of overeating, low energy expenditure, and physical inactivity, resulting in excessive body fat storage. Obesity is a disease caused by a combination of behavioural, environmental, and genetic factors and could be defined by a body mass index (BMI) ≥ 30 kg/m2. BMI is calculated by a person's weight in kilograms divided by the square of their height in meters. However, BMI does not give us comprehensive information about body weight (BW) composition or distribution of fat or muscle. On the other hand, there are rare monogenetic causes of obesity like a mutation in genes coding leptin or the leptin receptor resulting in early onset obesity (Engin, 2017;Blüher, 2019). Mutations in gene coding leptin are more common when compared to its receptor's gene mutations. In 2016, there were 11 mutations reported in gene coding leptin. Both congenitally leptin deficient individuals and patients with leptin receptor disturbances exhibited rapid weight gain in the first few months of life, with severe hyperphagia and endocrine abnormalities (Dubern and Clement, 2012;Wasim et al., 2016).

Potential treatments of obesity include lifestyle modification, bariatric surgery, pharmacotherapy or a combination of these. Lifestyle modification, dieting and exercise are the first-line treatment of obesity but usually do not result in sustainable weight loss. Bariatric surgery may be used as a treatment for extremely or morbidly obese patients and is much more effective in terms of weight loss; however, there is the possibility of surgical complications and the frequent need for reoperation (Patel, 2015).

In 2015, there were only four drugs for short-term pharmacotherapy in the world that act as an appetite suppressant: Phentermine, Diethylpropion, Phendimetrazine and Benzphetamine. These four drugs were approved by the FDA and they are usually only considered for less than 12 weeks of use. Recently, several long-term pharmacotherapies like Orlistat, Lorcaserin and Liraglutide and combination therapies like phentermine/topiramate and naltrexone/bupropion were established as chronic anti-obesity treatments.

Anorexigenic peptides synthetized endogenously in the brain or in the gastrointestinal tract act centrally to decrease energy intake which prove to be promising tools for the treatment of obesity. These peptides are usually synthetically lipidized e.g., by palmitic acid which leads to increasing stability and half-life in organisms. Liraglutide, a lipidized peptide-based drug is a glucagon-like peptide-1 (GLP-1) receptor agonist that was introduced into the market originally as an antidiabetic drug but is now also used as a treatment of obesity approved by the FDA. Semaglutide, another lipidized GLP-1 agonist, was also approved by the FDA for the treatment of obesity with application only once-weekly. There are also several neuropeptides of brain origin with an anorexigenic effect in animal models as a potential future obesity treatment such as prolactin-releasing peptide (PrRP), cocaine- and amphetamine-regulated transcript (CART) peptide, and α-melanocyte-stimulating hormone (α-MSH) (Patel, 2015;Meneguetti et al., 2019;Gao et al., 2020;Williams et al., 2020).

1.2 Leptin and leptin resistance

Five single-gene mutations in mice leading to the obese phenotype were described in 1950 (Ingalls et al., 1950). After many years, in 1994, a messenger RNA from adipose tissue with a 167-amino acid reading frame was discovered in the laboratory of J. F. Friedman and this product was named leptin (Zhang et al., 1994). Leptin is an adipocyte-derived hormone produced and secreted from adipose tissue and is derived from the *lep* gene. Leptin is involved in the regulation of BW and energy homeostasis, but also in lipid metabolism and insulin sensitivity (Zhang et al., 1994;Wasim et al., 2016).

Leptin is transported across the blood-brain barrier (BBB) by a saturable transport system. Circulating concentrations of leptin correlate with total adipose tissue mass when fasting

decreases circulating leptin levels, while feeding or obesity increases them (Münzberg and Morrison, 2015).

Leptin receptors are expressed in brain and peripheral tissues and six leptin receptor isoforms are known: LepRa, b, c, d, e, and f. LepRe is a soluble LepR isoform that allows the binding of circulating leptin and inhibits central leptin transport. On the other hand, LepRb is a long form of the LepR responsible for the main effects of leptin on energy homeostasis or other neuroendocrine functions. The hypothalamus is the major site of leptin action in food intake (FI) regulation. It is the place where leptin binds to the leptin receptors which leads to the activation of receptors associated with Janus kinase 2 (JAK 2). Phosphorylation of JAK2 leads to the activation of mitogen-activated-protein-kinase (MAPK), signal transducer and activator of transcription 5 (STAT5), or STAT3 activation. On the other hand, suppressors of cytokine signaling-3 (SOCS3) and phosphotyrosine phosphatase 1B (PTP1B) are negative regulators of leptin signaling. Leptin induces phosphoinositol 3 kinase (PI3K) and regulates 5′-adenosine monophosphate activated protein kinase (AMPK). In peripheral tissues, leptin activates AMPK activity while AMPK activity is inhibited in the brain (Bjorbak et al., 2000;Minokoshi et al., 2002;Morton et al., 2005;Münzberg and Morrison, 2015). Leptin signaling pathways are shown in Figure 1.

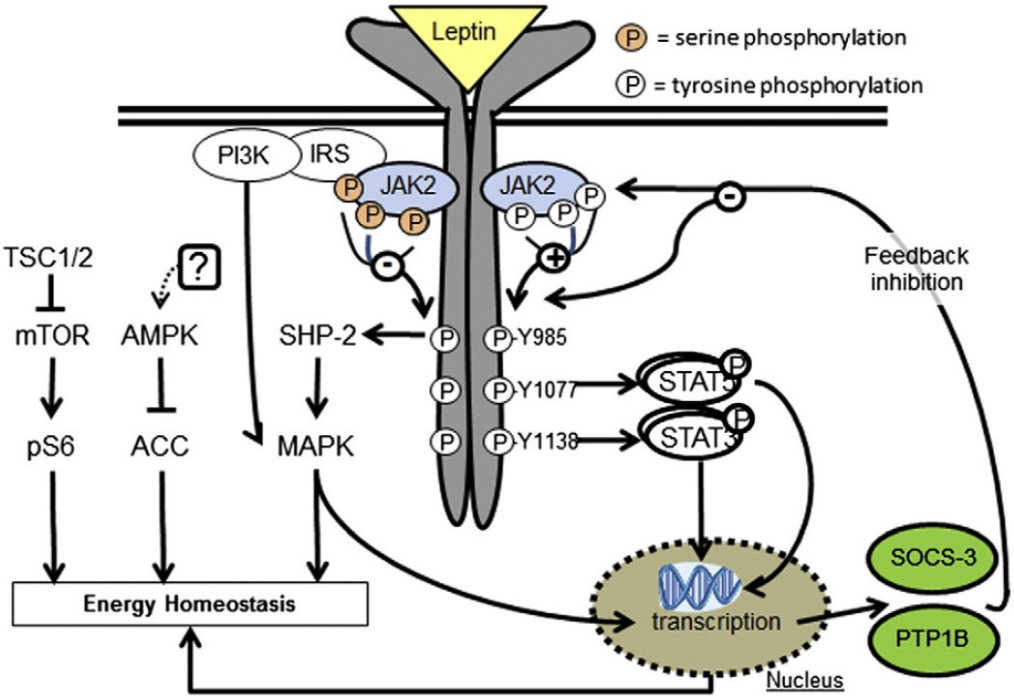


Figure 1: Leptin signaling pathways (modified from (Münzberg and Morrison, 2015))

ACC acetyl CoA carboxylase, AMPK 5´AMP-activated protein kinase, IRS insulin receptor substrate, JAK2 Janus kinase-2, MAPK mitogen activated protein kinase, mTOR mammalian target of rapamycin, PI3K total phosphoinositide 3-kinase, pS6 phosphorylated ribosomal protein S6, PTP1B phosphotyrosine phosphatase 1B, SHP-2 src-homology-2 containing phosphotyrosine phosphatase 2, SOCS-3 suppressor of cytokine signaling 3, STAT total signal transducer and activator of transcription, TSC1/2 tuberous-sclerosis1/2.

Leptin deficiency or non-functional signaling of leptin receptors lead to obesity. For example, leptin receptor deficient db/db mice are morbidly obese, as well as leptin deficient *ob/ob* mice which are also morbidly obese due to hyperphagia. Moreover, these mice are also insulin resistant and glucose-intolerant, so they could serve as a good experimental model for the study of obesity and energy metabolism (Chen et al., 1996;Chua et al., 1997;Mistry et al., 1997;Wasim et al., 2016;Zhang and Chua, 2017). Activation of leptin receptors in the arcuate nucleus (ARC) leads to up-regulation of pro-opiomelanocortin (POMC), and CART, in

POMC/CART neurons but down-regulation of agouti-related-protein (AgRP) and neuropeptide Y (NPY) in other neurons. When leptin levels are high, leptin activates POMC neurons which suppress FI (anorexigenic effect of leptin). NPY activation is due to low levels of leptin when FI is increased (hyperphagia) (Schwartz et al., 1996;Schwartz et al., 1997;Münzberg and Morrison, 2015).

At the beginning, it was thought that leptin could be an effective treatment of obesity. But nowadays, it is known that leptin can reverse leptin deficiency-induced obesity, a very rare case of human obesity, and effectively treats lipodystrophy, a partial or complete lack of fat. On the other hand, the typical or most common type of obesity is characterized by hyperleptinemia and so-called leptin resistance. Leptin treatment is ineffective in this case (Moon et al., 2011;Farooqi and O'Rahilly, 2014;Farr et al., 2015). Leptin resistance is defined as elevated circulating leptin levels and decreased leptin sensitivity. Decreased leptin transport to the brain, impaired leptin signal transduction in target neurons or hypothalamic inflammation, and endoplasmic reticulum stress have been suggested to be involved in obesity-associated leptin resistance (Figure 2). Mechanisms involved in leptin resistance are not clear yet and need to be studied further (Jung and Kim, 2013;Pan et al., 2014;Izquierdo et al., 2019).

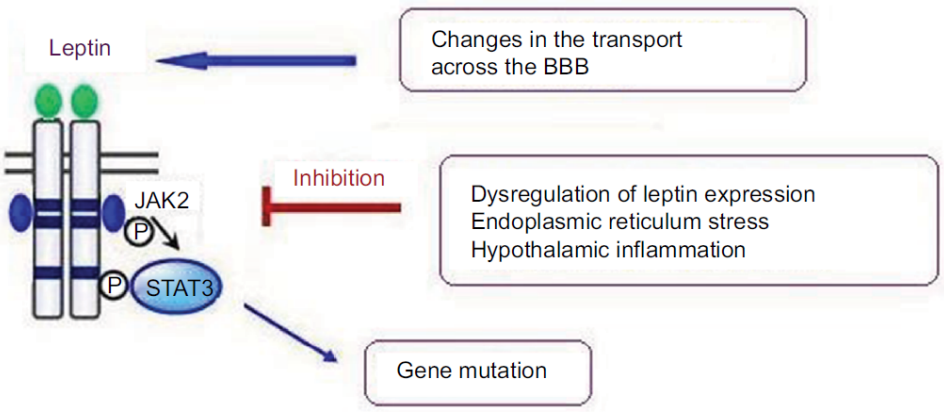


Figure 2: Mechanisms of leptin resistance (modified from (Gruzdeva et al., 2019))

BBB blood brain barrier, JAK2 Janus kinase-2, STAT3 signal transducer and activator of transcription 3.

1.3 Type 2 diabetes mellitus

Diabetes Mellitus (DM) is characterized by dysregulated glucose homeostasis. There are two types of DM: T1DM, or insulin-dependent DM, is due to autoimmune response-mediated destruction of the pancreatic β-islets resulting in insulin deficiency for which patients must take exogenous insulin for survival. T2DM, or noninsulin-dependent DM, is characterized by insulin resistance and is associated with abnormal insulin secretion. Moreover, T2DM is usually the result of polygenetic but also environmental influences (Quinn, 2002;Bhattacharya et al., 2007;Pandey et al., 2015). DM is a risk factor for cardiovascular disease development, but also for other diseases such as retinopathy, neuropathy or nephropathy. The global prevalence of DM is increasing at an alarming rate, and 90-95% of the global DM population are patients with T2DM (Quinn, 2002;Henning, 2018).

Insulin and glucagon are two major hormones responsible for normal plasma glucose concentration. While insulin stimulates glucose uptake, utilization, and storage, glucagon is responsible for the release of newly synthesized and stored glucose. There are three distinct stages of T2DM. At the end of the first stage, temporary restoration of normal glucose homeostasis appears. In the second stage, fasting plasma glucose levels remain normal, but postprandial plasma glucose levels rise. In the third stage, fasting and postprandial hyperglycemia result from increased insulin resistance, unrestrained hepatic glucose production, and glucose toxicity (Saltiel, 2001;Quinn, 2002).

1.4 Insulin and insulin resistance

Insulin, a hormone with a 51-amino acid reading frame containing two chains (A and B) connected with two disulfide bonds was discovered in 1921 (Mayer et al., 2007). Insulin plays a central role in the regulation of human metabolism and the best-known action to take is to increase glucose uptake in peripheral tissues while the glucose level is lowered in the blood. Insulin is also involved in promoting cellular uptake, stimulating nutrient transport into cells, regulating metabolic enzyme activity, controlling transcription of metabolic genes, regulating cellular growth and differentiation. All of these actions are mediated through activation of its insulin receptor (IR) (Woods et al., 2006;Saltiel, 2021).

IR is a glycoprotein with an extracellular α-subunit and the extracellular β-subunit and belongs to a subfamily of receptor tyrosine kinases. Insulin binds to the α-subunit of its receptor and activates the tyrosine kinase of the β-subunits which leads to receptor dimerization. Receptor activation is mediated by a cascade of phosphorylations or dephosphorylations. The IR activation leads to the phosphorylation of several substrates including IR substrate 1-4 (IRS1-4), leading to the activation of MAPKs, which mediates the effect on cellular growth, and PI3K which leads to activation of 3-phosphoinositide dependent protein kinase 1 and 2 (PDK1 and PDK2). PDK1 phosphorylates protein kinase B (Akt) at T308 which has an effect on cellular survival and metabolism while PDK2 phosphorylates Akt at S473 (Alessi et al., 1997;White, 2003;Kikani et al., 2005). Akt stimulates glucose uptake through glucose transporter 4 (GLUT4) (Cong et al., 1997). The insulin signaling pathway is shown in Figure 3.

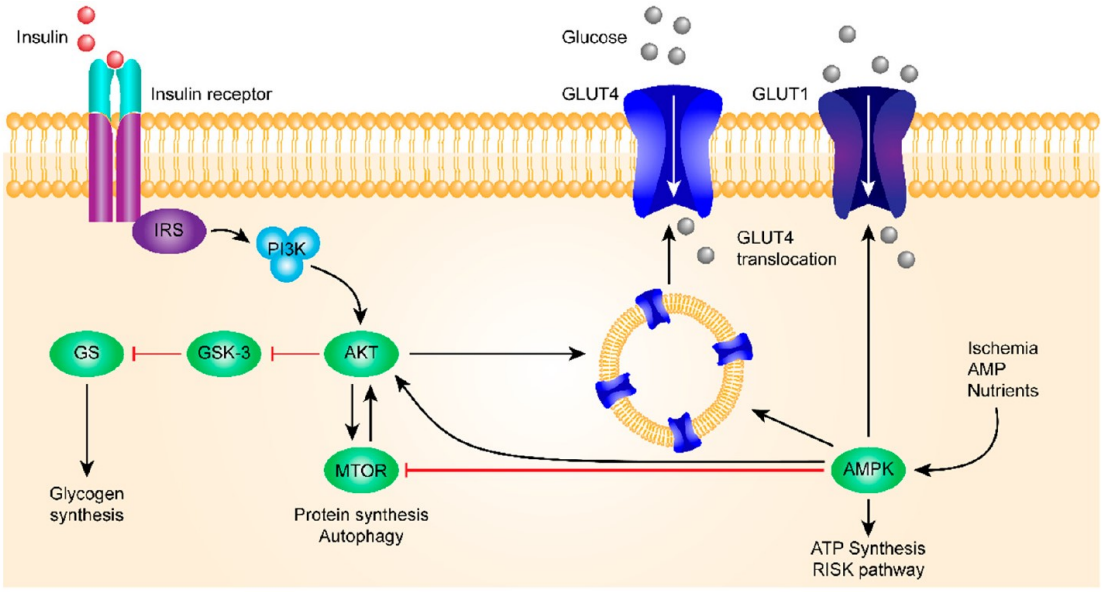


Figure 3: Insulin signaling pathway (modified from (Arneth et al., 2019))

Akt protein kinase B, AMP adenosine monophosphate, AMPK 5′AMP-activated protein kinase, ATP adenosine triphosphate, GLUT glucose transporter, GS glycogen synthase, GSK-3 glycogen synthase kinase-3, IRS insulin receptor substrate, mTOR mammalian target of rapamycin, PI3K total phosphoinositide 3-kinase, RISK reperfusion injury salvage kinase.

Abnormalities in the IR function could lead to insulin resistance. Defects of IR structure, loss of IRS1 and IRS2, Akt inactivation, binding affinity, or signaling capacity in insulin-responsive tissues such as brain, liver, adipose tissue and muscle could also lead to insulin resistance. Insulin resistance is an early stage of T2DM development but it is also linked with other diseases such as obesity or hypertension (Mayer et al., 2007;Guo, 2014;Mancusi et al., 2020).

1.5 Food intake regulation

FI regulation is a complex system where peripheral and central signals are involved. Peripheral signals or peripheral hormones involved in FI regulation are e.g., leptin, insulin and ghrelin, while central signals involved in FI regulation are so-called neuropeptides.

The term "neuropeptides" was first introduced in the 1970s and defined as small protein-like substances produced and released by neurons. To modulate neuronal activity, neuropeptides act via G-protein coupled receptors (GPCRs) (Hillebrand et al., 2002). Hypothalamus is the main center of FI regulation and consists of several nuclei such as ARC, the paraventricular nucleus (PVN), the lateral hypothalamic nucleus (LHA), the ventromedial nucleus (VMH), and the dorsomedial nucleus (DMN). The ARC is a key hypothalamic nucleus in the regulation of appetite where anorexigenic neuropeptides that reduce appetite are released: POMC and CART, as well as orexigenic neuropeptides that increase appetite, AgRP and NPY.

ARC neurons, also called first order neurons, are located at the bottom of the hypothalamus, so they are in close contact with peripheral satiety factors such as leptin and insulin, and project to second order neurons in PVN, VMH, DMH and LHA and also to neurons in other brain areas such as the nucleus tractus solitary (NTS) in the brainstem. The communication between hypothalamic neurons and the caudal brainstem is essential for the long-term regulation of energy homeostasis. (Rossi et al., 1998;Schwartz et al., 2000;Hillebrand et al., 2002). The factors involved in FI regulation are shown in Figure 4.

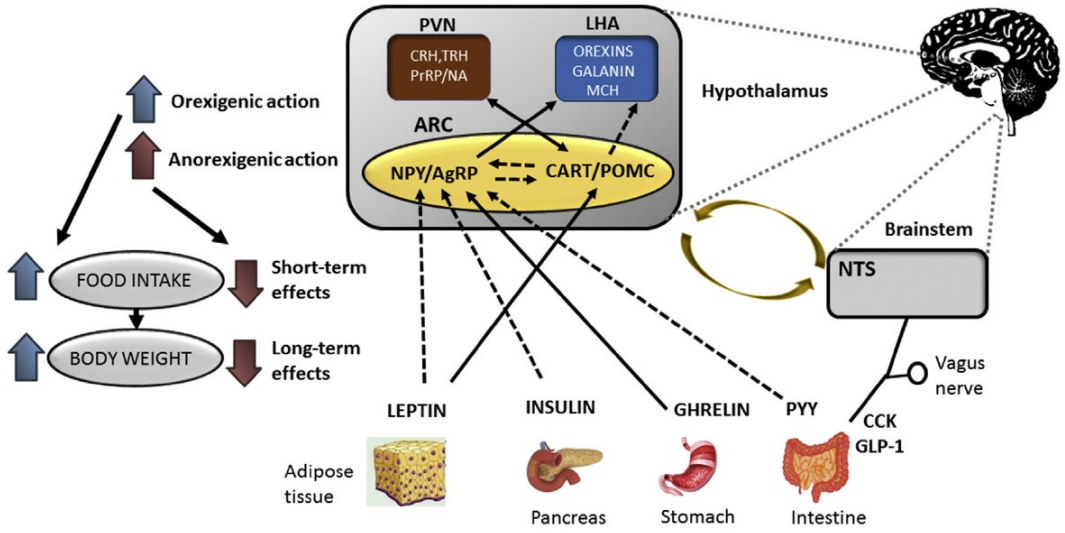


Figure 4: The scheme of central and peripheral factors regulating food intake (modified from (Mikulášková et al., 2016))

AgRP agouti-related peptide, ARC arcuate nucleus, CART cocaine- and amphetamine-regulated transcript peptide, CCK cholecystokinin, CRH corticotrophin-releasing hormone, GLP-1 glucagon-like peptide-1, LHA lateral hypothalamic area, MCH melanin-concentrating hormone, NPY neuropeptide Y, NTS nucleus tractus solitarius, POMC pro-opiomelanocortin, PrRP prolactin-releasing peptide, PVN paraventricular nucleus, TRH thyrotropin-releasing hormone.

1.5.1 Peripheral hormones involved in food intake regulation

Several peripheral hormones are involved in FI regulation. Leptin and insulin described in chapters 1.1.2 and 1.1.4 are involved in long-term FI regulation, while cholecystokinin (CCK), GLP-1, and peptide YY (PYY) are involved in short-term FI regulation. On the other hand, ghrelin is both a short-term and long-term peripheral hormone of FI regulation.

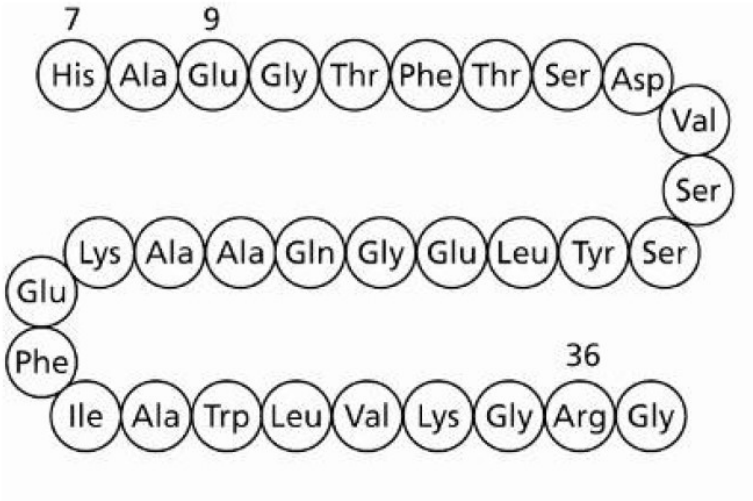
CCK is a member of the gut-brain family of peptide hormones and there are several forms of CCK. CCK-58 is the largest circulating form, while CCK-8 is the smallest fragment with full biological activity. There are two types of CCK receptors: CCK-1 primarily expressed in the gastrointestinal tract and CCK-2 primarily expressed in the brain (Chandra and Liddle,

2007). CCK is a key factor involved in the activation of intestinal feedback control mediated by the vagal afferent pathway. CCK is also involved in the stimulation of the exocrine pancreas and gallbladder and in the inhibition of FI (Raybould, 2007). Leptin could also act peripherally to increase vagal afferent activity and there is a synergistic interaction between leptin and CCK (Barrachina et al., 1997;Wang et al., 2000).

GLP-1, a gut-brain hormone, is secreted by intestinal L-cells, but also expressed by neurons within the NTS of the brainstem. GLP-1 plays an important role in glucose homeostasis, gastrointestinal motility and in appetite. The biologically active forms of GLP-1 are GLP-1 (7-36) amide and GLP-1 (7-37) and the truncated form of GLP-1 (7-36) amide is the major circulating bioactive species in humans (Figure 5A). The action of GLP-1 is mediated by the activation of the GLP-1 receptor (GLP-1R), expressed throughout the periphery but also within the brain. GLP-1 inhibits gastric emptying and glucagon secretion and stimulates glucose-dependent insulin secretion and biosynthesis though this molecule is limited by its short half-life. Therefore, long-acting GLP-1 agonists were developed (Dailey and Moran, 2013;Krieger, 2020).

Liraglutide (Figure 5B), a long-acting GLP-1 receptor agonist, is modified with an amino acid substitution and the addition of a fatty acid using a linear monocarboxylic acid chain that causes longer half-life. Liraglutide is successful for T2DM treatment as well as obesity treatment shown by decreased FI, BW reduction and improvements in metabolic parameters in animal models and in human studies. Another long-acting GLP-1 receptor agonist is semaglutide with once-weekly administration in humans, which is more convenient for patients (Dailey and Moran, 2013;Ladenheim, 2015;Knudsen and Lau, 2019;Krieger, 2020).

A



B

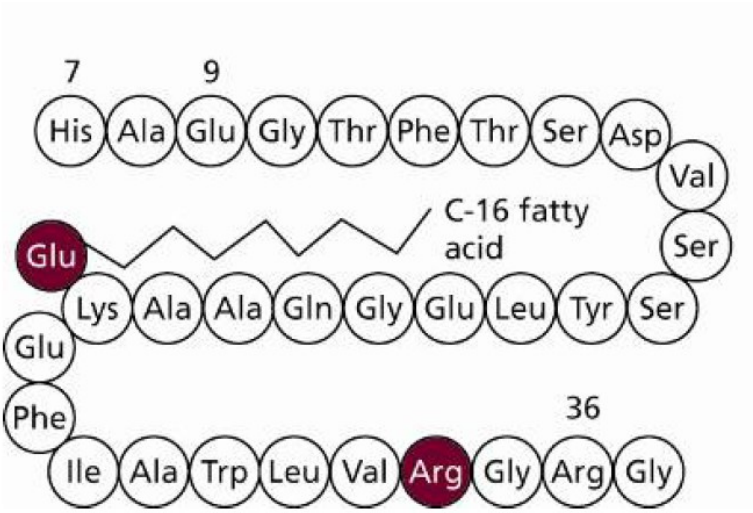


Figure 5: The amino acid sequence of GLP-1 (A) and liraglutide (B) (modified from (Sjöholm, 2010)).

PYY is a 36 amino acid peptide, secreted primarily from L-cells. It belongs to a family of peptides which includes neuropeptide Y (NPY) and pancreatic polypeptide. Into the circulation, PYY is released as PYY_{1-36} and PYY_{3-36}. Five receptors are known for PYY: Y1,

Y2, Y4, Y5, Y6. PYY_{1-36} binds to all Y receptors while Y_{3-36} has a high affinity for the Y2 receptor and a moderate affinity for Y1 and Y5 receptors. PYY secretion is stimulated by neuronal and humoral factors, and plasma PYY levels are lower in obese subjects compared to normal-weight subjects. PYY_{1-36} as well as PYY_{3-36} should have a local effect on gut motility and inhibit gallbladder emptying and secretion of gastric acid and pancreatic enzymes. Some published data indicate that peripheral administration of PYY_{3-36} reduces FI in rodents and in humans, and that PYY knockout mice developed obesity (le Roux and Bloom, 2005;Ueno et al., 2008;Valassi et al., 2008;Simpson et al., 2009).

Ghrelin is a 28 amino acid peptide, one of the key hormones regulating food intake. Ghrelin is an orexigenic peptide mainly synthetized by the stomach which represents endogenous ligand for growth hormone-secretagogue receptors (GHSRs). An acyl functional group attached to the serine-3 of ghrelin is essential for binding to receptor GHSR-1a. GHSR-1a mediates activation of NPY/AgRP and inhibits POMC neurons in a way opposite to that of leptin. Peripheral and central administration of ghrelin increases c-fos expression in ARC. In the periphery, ghrelin influences pancreatic function, glucose metabolism, gastric motility and gastric acid secretion. Ghrelin is also involved in reward processes, memory and learning, mood and stress response, sleep and reproduction. Peripheral chronic administration of ghrelin in rodents results in an increase in cumulative FI and BW, so antagonists of ghrelin should have a potential to decrease food intake and reduce fat mass in obesity. Circulating ghrelin increases before a meal and rapidly falls down after meals. Because of that, ghrelin is also called the hungry hormone. Ghrelin-deficient mice fed a standard diet showed normal appetite and BW, however they are resistant to diet-induced obesity (DIO) (Hillebrand et al., 2002;Valassi et al., 2008;Simpson et al., 2009;Howick et al., 2017).

1.5.2 Neuropeptides involved in food intake regulation

There are many anorexigenic or orexigenic neuropeptides involved in FI regulation e.g., MSH, CART peptide, PrRP, corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), oxytocin, NPY, melanin-concentrating hormone (MCH), orexins or galanin and some of them are described in this chapter.

Several forms of CART peptide were identified: $CART_{(55-86)}$, $CART_{(54-102)}$, $CART_{(55-102)}$, $CART_{(61-102)}$ and $CART_{(62-102)}$. CART mRNA is mainly expressed in hypothalamic ARC, PVN, DMN and LHA and CART is co-localized with anorexigenic neuropeptides as well as with orexigenic neuropeptides e.g., with anorexigenic POMC in the ARC or with orexigenic MCH in LHA. CART peptide is involved in FI regulation. The central administration of CART peptide decreased FI and BW, while central administration of CART peptide antibody increased FI in rodents. CART peptide also induced activation of the hypothalamic-pituitary-adrenal (HPA) axis and plays an important role in energy metabolisms (Hillebrand et al., 2002;Lau and Herzog, 2014;Singh et al., 2021). CART peptide receptor has been sought for many years, and although GPR160 has recently been suggested, further studies are needed (Samson et al., 2021).

Neuropeptide FF (NPFF), an 8 amino acid containing peptide, belongs to the family of so-called RF amide peptides with C-terminal arginine and phenylalanine amide sequence, which is very important for receptor binding. NPFF binds to an NPFF receptor 1 (NPFF-R1) and NPFF-R2. NPFF plays an important role in FI regulation, pain perception or in blood pressure (Elshourbagy et al., 2000;Quillet et al., 2016).

MCH, a 19 amino acid orexigenic neuropeptide is expressed in LHA. MCH receptor 1 (MCH-R1) is expressed in hypothalamic VMN, DMN and ARC as well as in hippocampi and olfactory regions. On the other hand, MCH-R2 is expressed in VMN, LHA and ARC, in hippocampus and in olfactory regions. MCH plays an important role in FI regulation by acting downstream of the leptin and melanocortin pathways and MCH expression in LHA is increased after fasting. Central administration of MCH induces hyperphagia with an increase in BW and decreased energy expenditure. Several antagonists of MCH-R1 are studied as anti-obesity agents (Hillebrand et al., 2002;Valassi et al., 2008).

Orexins, orexin A (33 amino acid peptide) and orexin B (28 amino acid peptide), are orexigenic neuropeptides involved in FI regulation and body temperature regulation. Orexins are produced in neurons of LHA, but also in peripheral organs like the liver and heart. Orexins

act on two closely related GPCRs: orexin 1 receptor (Ox1-R) mainly expressed in the VMH and Ox2-R mainly expressed in PVN. The activity of orexins is increased by low glucose and decreased by signals related to nutrient ingestion. Orexin A is involved in FI regulation and temperature regulation but also in short-term regulation of energy homeostasis, while the role of orexin B is more often questioned. Antagonists of orexin A are a potential use for reducing appetite in obese patients (Yamanaka et al., 2000;Hillebrand et al., 2002;Valassi et al., 2008) (A. Yamanaka 2000, E Valassi 2008, J.J.G. Hillebrand 2002).

NPY, a 36 amino acid orexigenic neuropeptide, is a member of the pancreatic polypeptide family. NPY is expressed in hypothalamus mainly in ARC from which NPY neurons project to second-order neurons located in PVN, VMN, DMN and other brain regions. NPY is involved in FI stimulation, but also in learning and memory, anxiety and cardiovascular function. Six different NPY receptors, which mediate the effects of NPY have been known, but NPY prefers to bind at the Y1, Y2 and Y5 receptors. Central administration of NPY increases FI, inhibits thermogenesis and increases lipogenesis, thus central administration of NPY leads to an increase of fat storage and positive energy balance. Antagonists of Y1 and Y5 receptors are studied as potential anti-obesity agents (Hillebrand et al., 2002;Valassi et al., 2008;Mercer et al., 2011).

1.5.3 Prolactin-releasing peptide

PrRP was first isolated in 1998 by Hinuma (Hinuma et al., 1998), and the name was suggested based on its prolactin-releasing activity in a rat pituitary adenoma-derived cell line and in pituitary cells of lactating rats. Nevertheless, this activity was later questioned because of non-typical features for hypophysiotropic hormone (Samson et al., 1998;Lawrence et al., 2002). PrRP is an anorexigenic neuropeptide involved mainly in FI regulation and energy expenditure, but also in stress regulation, the cardiovascular system and its potential neuroprotective properties were shown. (Samson et al., 1998;Maruyama et al., 2001;Zhang et al., 2001;Lawrence et al., 2002;Onaka et al., 2010;Holubová et al., 2019).

PrRP neurons are localized mostly in NTS and slightly in hypothalamic DMN, while immunoreactive cell bodies were detected in DMN, VMN, NTS, and the ventrolateral medulla oblongata (ME) and immunoreactive fibers were found in high concentration in the posterior pituitary (Takayanagi and Onaka, 2010;Pražienková et al., 2019). PrRP belongs to so-called RF amide peptides and it binds with a high affinity to a GPR10 receptor and with a lesser affinity to a NPFF2 receptor. GPR10 mRNA expression was detected in several parts of the brain, mostly in the reticular nucleus of the thalamus, PVN, DMN, NTS and the area postrema (Takayanagi and Onaka, 2010;Pražienková et al., 2019). Two isoforms, a 20 amino acid peptide named PrRP20 and 31 amino acid peptide named PrRP31, are derived from the same preproprotein and they share an identical C-terminus (Figure 6) (Lin, 2008;Pražienková et al., 2019).

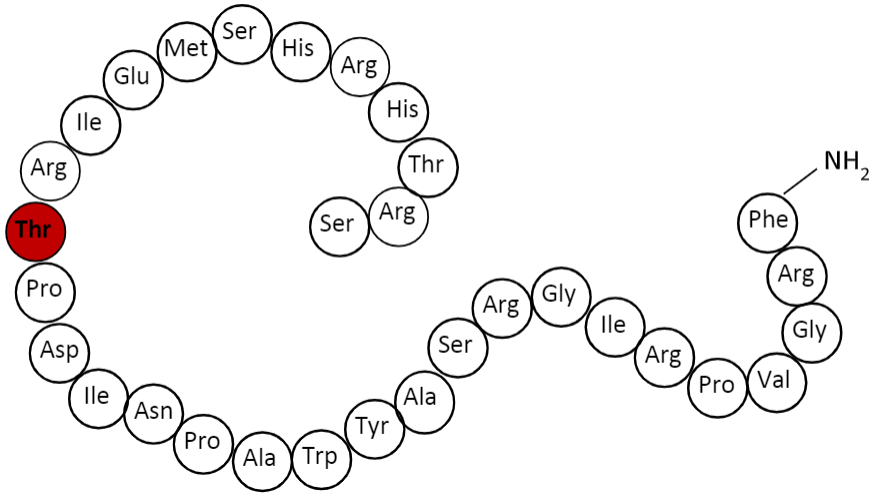


Figure 6: Structure of human prolactin-releasing peptide

Human PrRP31 peptide; threonine (in red) is the beginning of PrRP20.

PrRP is involved in various brain functions such as FI regulation, energy homeostasis, stress modulation, sleep and cardiovascular regulation. In addition, cooperation with other neuropeptides, mainly with leptin, CCK or NPY is very important for the effects of PrRP. Central administration of PrRP caused a decrease in FI and BW while body temperature increased in fasted and free-fed rats (Lawrence et al., 2000;Ellacott et al., 2002;Fukusumi et

al., 2006). The interaction between CCK and PrRP was studied in short-term regulation of energy balance. Peripheral administration of CCK activated neurons containing PrRP in NTS. Anorexigenic effect of CCK is reduced in mice with GPR10 or PrRP deficiency, suggesting that PrRP signaling may be important for the transmission of peripheral signals, such as CCK, to the brain (Lawrence et al., 2002;Bechtold and Luckman, 2006;Pražienková et al., 2019). On the other hand, central administration of PrRP and leptin have an additive effect on reducing FI and BW, suggesting an interaction between PrRP and leptin in the metabolism and in FI regulation (Ellacott et al., 2002).

PrRP, an anorexigenic neuropeptide, has potential as an anti-obesity treatment but in its natural form has low stability and is not able to cross BBB after peripheral administration. Modification such as lipidization makes this peptide more stable and able to act centrally after peripheral administration. In our previous studies, we demonstrated that analogs lipidized by 8-18 carbon chain fatty acids at N-terminus showed high binding affinities for both GPR10 and NPFF-2R similar to the analogs palmitoylated through linkers to Lys^{11} e.g., $palm^{11}$-PrRP31 (Maletínská et al., 2015;Pražienková et al., 2017;Karnošová et al., 2021). Nevertheless, natural PrRP20 or PrRP31 had no effect on FI after peripheral administration, as well as analogs lipidized with shorter carbon chains. But palm-PrRP31, myr-PrRP20 or $palm^{11}$-PrRP31 significantly and dose-dependently decreased FI in lean overnight fasted or freely fed mice after peripheral administration (Maletínská et al., 2015;Pražienková et al., 2017;Pirník et al., 2021). $Palm^{11}$-PrRP31 is a promising tool in obesity treatment and the structure of $palm^{11}$-PrRP31 is shown in Figure 7.

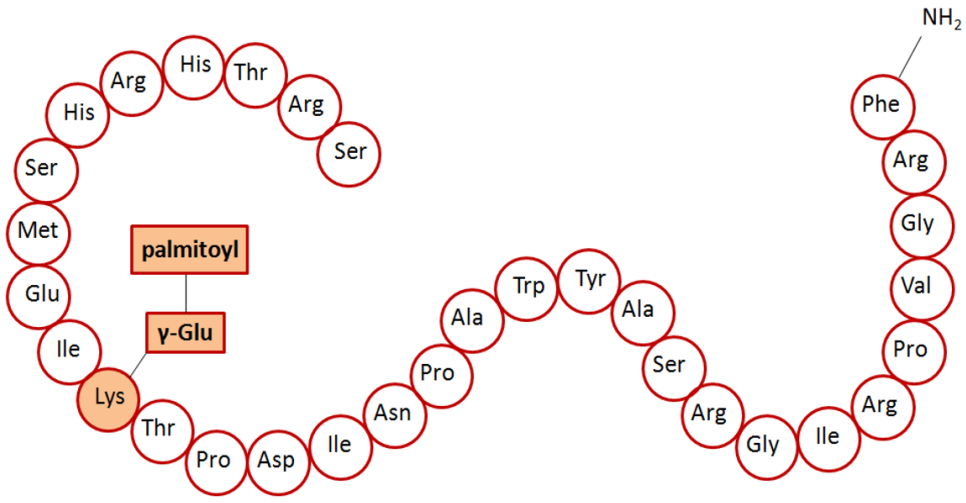


Figure 7: Structure of palm^{11}-PrRP31

PrRP palmitoylated in position 11, γ-Glu – γ- glutamic acid, linker in (palm^{11}-PrRP31).

1.6 Rodent models of metabolic syndrome

Knowledge about animal models for metabolic studies is very important and rodent models are the most commonly used. However, there is no fully effective model to understand all disease mechanisms connected with metabolic syndrome. Nevertheless, DIO models when a high-fat (HF) diet is employed allow us a better understanding of the promoting metabolic syndrome, which is a multifactorial disease, and are closer to the human obesity and metabolic syndrome. On the other hand, genetic factors also play an important role in obesity development. Rodent models with genetically disrupted production or signaling of some specific factors of FI regulation develop more severe pathology, where long feeding (diet) programs are not needed to induce obesity, but they are not so close to the human metabolic syndrome and obesity (Srinivasan and Ramarao, 2007;Nilsson et al., 2012;Fuchs et al., 2018;Mráziková et al., 2021).

Rodent DIO studies are based on specifically defined diets and the most frequently used are an HF diet, high-sugar diet (HSD), or their combination, and a high-fat, fructose and cholesterol diet (FFC) is also used for metabolic syndrome development. The most used

rodents for DIO are C57BL6J mice as well as Sprague Dawley (SD) rats. In these rodent models, most of the metabolic syndrome features were found e.g., obesity, prediabetes or diabetes, glucose intolerance and disrupted central leptin and insulin signaling (Fuchs et al., 2018;Kwitek, 2019;Preguiça et al., 2020).

Rodent models of leptin-deficiency or leptin signaling disturbances are widely used as spontaneous genetic models of obesity and related complications. Leptin deficient ob/ob mice do not produce leptin. These mice develop severe early-onset obesity from 4 weeks of age and have a defect in the thermogenesis of brown adipose tissue (BAT) which could lead to an increased hepatic lipogenesis. Leptin deficiency in ob/ob mice also leads to a hyperinsulinemia and mild hyperglycemia, apparent from 8 to 12 weeks of age. The mice have severe liver steatosis but do not develop steatohepatitis (Kennedy et al., 2010;Fuchs et al., 2018;Kořínková et al., 2020). Leptin receptor-deficient db/db mice are obese, hyperinsulinemic, and hyperglycemic (depending on the strain and age) and are widely used for the study of T2DM and its complications. The Obese Zucker *fa/fa* rats similarly as db/db mice develop obesity because of a defect in the leptin receptor and also could be hyperinsulinemic and hyperglycemic depending on the strain and age. These models are mainly used for pharmacological studies of anti-obesity drugs (Ramarao and Kaul, 1999;Kwitek, 2019). Zucker diabetic (ZDF) rats are lean and diabetic. These rats are used to study potential antidiabetic compounds mainly (Wang et al., 2014).

1.7 Effect of palmitoylated analogs of PrRP in diet-induced obesity rodent models

Chronic administration of palm-PrRP31 was studied in (Maletínská et al., 2015) while chronic administration of palm^{11}-PrRP31 was studied in (Pražienková et al., 2017). In both cases, obese C57BL/6 male mice with an HF diet were used. A two-week treatment with palm-PrRP31 significantly decreased FI and both used analogs significantly decreased BW. Palm-PrRP31 significantly decreased the weight of subcutaneous adipose tissue (SCAT), while palm^{11}-PrRP31 significantly decreased liver weight, as well as the weight of SCAT. Palm-PrRP31, as well as palm^{11}-PrRP31, significantly decreased insulin and leptin plasma levels while palm^{11}-PrRP31 also significantly decreased triglycerides (TG), cholesterol (CHOL) and free fatty acid (FFA). Palm-PrRP31 significantly attenuated mRNA expression of fatty acid

synthase *Fasn* in SCAT, intraperitoneal adipose tissue (IPAT) and moreover in the liver, significantly reduced acetyl-CoA carboxylase alpha (*Acaca*) and sterol regulatory binding protein 1 (*SREBP*-1) mRNA expression. Nevertheless, palm^{11}-PrRP31 significantly decreased mRNA expression of *Fasn* in SCAT and *SREBP-1* in the liver. Expression of *Ucp-1* was not changed after palm-PrRP31 treatment in BAT, but palm^{11}-PrRP31 significantly increased mRNA expression of *Ucp-1* in BAT. These studies demonstrated that lipidization enabled a central anorexigenic effect of PrRP after its peripheral administration. Moreover, chronic two-week administration of PrRP palmitoylated analogs significantly decreased BW, improved multiple metabolic parameters related to obesity, attenuated lipid metabolisms and showed a potential increase in energy expenditure after palm^{11}-PrRP31 administration (Maletínská et al., 2015;Pražienková et al., 2017).

Chronic two-week intraperitoneal (IP) administration of palm-PrRP31 was studied in DIO Sprague-Dawley rats fed an HF diet (Holubová et al., 2016). Palm-PrRP31 lowered FI and BW. This palmitoylated analog also improved tolerance to glucose and tended to decrease adipose tissue masses and leptin levels (Holubová et al., 2016). On the other hand, the chronic effect of palm^{11}-PrRP31 was studied in DIO WKY rats fed 15 weeks with an HF diet. These rats were obese with prediabetes. Three weeks with palm^{11}-PrRP31 significantly decreased BW, and measured metabolic parameters. Moreover, improvement in glucose tolerance was also found (Čermáková et al., 2019).

1.8 Effect of palmitoylated analogs of PrRP in rodents with leptin signaling disturbances

These studies were aimed at the chronic effects of palm-PrRP31 or palm^{11}-PrRP31 in rat models. In the first study, described in (Holubová et al., 2016), the potential antidiabetic and anti-obesity effects were studied in ZDF rats with leptin signaling disturbances. ZDF rats were slightly overweight and highly hyperglycemic compared to controls, moreover these rats had a significantly increased cumulative FI and plasma levels of CHOL and TG and exhibited markedly lowered glucose intolerance during oral glucose tolerance tests (OGTT) in comparison with controls. A two-week treatment with palm-PrRP31 significantly decreased cumulative FI and plasma levels of CHOL. Thus, even if palm-PrRP31 significantly decreased

FI, it failed to decrease BW or improve glucose tolerance, probably due to a lack of functional leptin receptor (Holubová et al., 2016).

In the second study, (Mikulášková et al., 2018), the Koletsky rat strain of genetically obese and hypertensive rats (SHROB) was studied. SHROB rats were obese and had significantly increased metabolic parameters in plasma such as TG, FFA, leptin and insulin compared to their SHR controls. Both genotypes were normoglycemic, but SHROB exhibited glucose intolerance during OGTT compared to SHR rats. The mRNA expression levels of several genes related to lipogenesis were significantly increased in the liver and in adipose tissue of SHROB compared to control SHR rats. Three weeks of treatment with palm^{11}-PrRP31 significantly decreased FI, but did not decrease BW in SHROB rats compared to SHR rats. Insulin plasma level was significantly decreased by the palm^{11}-PrRP31 treatment while fasted plasma was not affected by the treatment. Palm^{11}-PrRP31 significantly improved tolerance to glucose in SHROB palm^{11}-PrRP31, which is a very important result of this study. Palm^{11}-PrRP31 treatment also improved insulin signaling in the hypothalamus. These data indicated that intact leptin and leptin receptor are needed for the BW-lowering effect of palm^{11}-PrRP31 treatment, while improving glucose tolerance is mostly independent of anti-obesity effects (Mikulášková et al., 2018).

2. AIMS OF THE THESIS

Anorexigenic neuropeptide, PrRP, has a potential as a treatment for obesity but in its natural form has low stability and is not able to cross BBB after peripheral administration. Modification, such as lipidization makes this peptide more stable and able to act centrally after peripheral administration. Lipidized analogs of PrRP were previously tested in our laboratory at IOCB. The most potent analogs, palm-PrRP31 and palm^{11}-PrRP31, were then tested in rodent models for their potential anti-obesity and glucose-lowering properties. In my thesis, studies of rodent models with features of metabolic syndrome are described.

The aims of my thesis were:

Investigation of chronic effect of palm^{11}-PrRP31 and liraglutide in WKY rats fed an HF diet.

The first aim of my thesis was to evaluate an effect of palm^{11}-PrRP31 and liraglutide in WKY rats fed an HF diet for one year (as the most common type of human-like obesity) by monitoring the metabolic parameters related to obesity and glucose intolerance.

The study of chronic effect of palm^{11}-PrRP31, leptin and their combination in *ob/ob* leptin deficient mice.

The second aim was to determine the long-time effect of palm^{11}-PrRP31 alone or in combination with leptin in leptin deficient mice that develop early onset obesity. We have hypothesized that supplementation of leptin will influence the effect of palm^{11}-PrRP31, namly: metabolic parameters as well as signaling in the brain.

Research of chronic effect of palm^{11}-PrRP31 in *fa/fa* rats with leptin signaling disturbances.

The third aim was to study anorexigenic impact of palm^{11}-PrRP31 in *fa/fa* rats with non-functional leptin receptor in order to investigate a potential involvement of leptin signaling in the effect of palm^{11}-PrRP31.

3. MATERIALS AND METHODS

3.1 Substances

Analog of human PrRP palmitoylated at position 11 (palm^{11}-PrRP31) with the sequence SRTHRHSMEIK(N-γ-E(N-palmitoyl)) TPDINPAWYASRGIRPVGRF-NH2 was synthesized at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences (IOCB CAS), Prague, Czech Republic, in the peptide synthesis laboratory by Miroslava Blechová, as previously described (Pražienková *et al*. 2017). Mouse leptin was obtained from Sigma-Aldrich. Liraglutide was obtained from Pharmacy (Victoza®, Novo Nordisk A/S, Bagsværd, Denmark). IP administration of palm^{11}-PrRP31 was used in rats while subcutaneous (SC) administration of palm^{11}-PrRP31 was used in mice. Palm^{11}-PrRP31 was dissolved in saline. SC administration of leptin at a dose of 5 and 10 μg/kg was chosen as a subthreshold dose (Kořínková et al., 2020) to achieve the anorexigenic effect in mice. IP administration of liraglutide diluted in saline was used in rats.

3.2 Experimental animals

Three rodent models of obesity: Wistar Kyoto (WKY) rats fed an HF diet, leptin deficient *ob/ob* mice and rats with leptin signaling disturbance (*fa/fa* rats) were used to study the effects of palm^{11}-PrRP31.

The mice and rats were housed under controlled conditions at a constant temperature of 22 ± 2°C and a fixed 12:12 light:dark cycle. The animals were provided free access to water and a standard rodent chow diet Ssniff® R/M-H (Ssniff Spezialdiäten GmbH, Soest, Germany) containing 33% protein, 9% fat and 58% of carbohydrates or, in the case of WKY rats, an HF diet containing 60% fat, 20% carbohydrates, and 20% protein (D12492, Research Diets Inc., New Brunswick, NJ, USA). 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.

3.2.1 Diet-induced obese Wistar Kyoto rats

Eight-week-old WKY rats were obtained from Charles River (Wilmington Massachusetts, USA). The design of experiment is shown in Figure 8. Ten rats were fed the Ssniff® diet (WKY LF) while 40 rats were fed an HF diet (WKY HF) for 52 weeks from eight weeks of age. Before the start of treatment, BW was monitored once a week. OGTT was performed and plasma samples were collected from tail vessels at 60 weeks of age for determination of the biochemical parameters. At the age of 60 weeks, 24 WKY rats on an HF diet with the highest BW were selected and divided into three experimental groups. The rats were treated from Monday to Friday with saline (WKY HF saline, n=8) and palm^{11}-PrRP31 at a dose of 5 mg/kg (WKY HF palm^{11}-PrRP31, n=8) or with liraglutide at a dose of 0,2 mg/kg (WKY HF liraglutide, n=8). Rats fed with the Ssniff® diet formed the control group (WKY LF saline, n=8). During the six-week dosing period, the BW and FI were measured twice per week. At the end of the experiment, the rats were fasted overnight, plasma samples were collected from the tail veins for determination of the biochemical parameters, and an OGTT was performed. The animals were deeply anesthetized with pentobarbital (170 mg/kg of BW, Sigma-Aldrich) and transcardially perfused with ice-cold 0.01 mol/l pH 7.4 phosphate buffered saline (PBS) supplemented with heparin (10U/mL, Zentiva, Prague, Czech Republic). Tissue samples, SCAT, IPAT, livers and brains of all rats were dissected and stored in -80°C until use. The caudate lobes of each liver were used for liver histology described in chapter 3.5. The hypothalamics were separated from the dissected brains and homogenized in a Bullet Blender (Next Advance Inc., Averill Park, NY, USA) using a lysis buffer (Špolcová et al., 2015) and stored at -20°C until use.

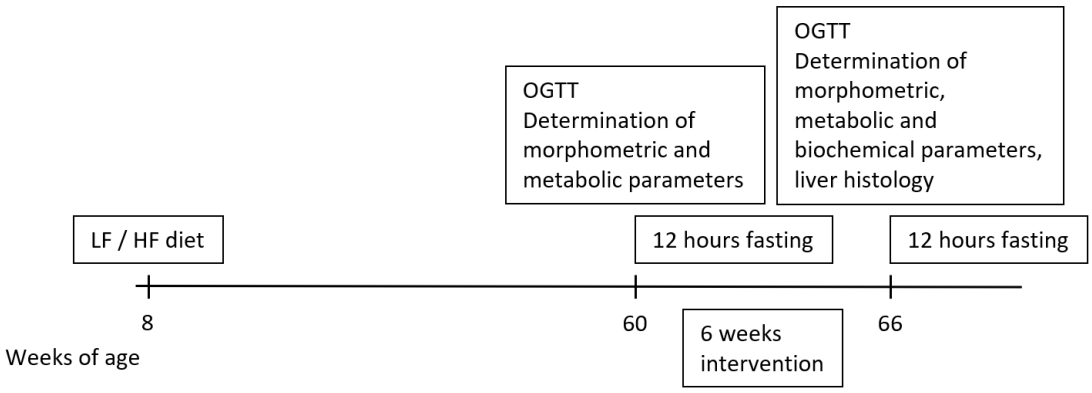


Figure 8: Design of experiment of WKY rats fed HF diet.

3.2.2 *ob/ob* mice

Five-week-old male *ob/ob* mice and their wild-type (WT) controls were obtained from ENVIGO (Correzzana, Italy). Mice on the Ssniff® diet were randomly divided into two experiments (experiment 1 and experiment 2) in groups of 8-10 animals. The design of experiments 1 and 2 are shown in Figure 9. In this study we wanted to explore the potential interaction between leptin and PrRP in younger mice treated for two weeks (experiment 1) and in older mice treated for eight weeks (experiment 2). Saline, leptin (5 or 10 μg/kg twice a day) and palm^{11}-PrRP31 (5 mg/kg twice a day) were used as a treatment. In experiment 1, mice were treated for two weeks from 8 weeks of age. The following groups were used (n=8-10): WT saline, *ob/ob* saline, *ob/ob* leptin (5 μg/kg), *ob/ob* palm^{11}-PrRP31 (5 mg/kg) and *ob/ob* leptin + palm^{11}-PrRP31 (5 μg/kg + 5 mg/kg). In experiment 2, mice were treated for eight weeks from 16 weeks of age and the following groups of 10 animals were used: WT saline, *ob/ob* saline, *ob/ob* leptin (10 μg/kg), *ob/ob* palm^{11}-PrRP31 (5 mg/kg) and *ob/ob* leptin + palm^{11}-PrRP31 (5 μg/kg + 5 mg/kg). During the dosing period, FI and BW were monitored daily. Two weeks before the end of experiment 2, an OGTT was performed and one week before the end of both experiments, rectal temperature was measured. At the end of both experiments, plasma samples were collected from the tail veins for determination of the biochemical parameters, the mice were deeply anesthetized with pentobarbital (170 mg/kg of BW, Sigma-Aldrich) and transcardially perfused with ice-cold 0.01 mol/l pH 7.4 phosphate buffered saline (PBS) supplemented with heparin (10U/mL, Zentiva, Prague, Czech Republic).

Tissue samples, SCAT, IPAT, livers and brains of all mice were dissected and stored in -80°C until use. The caudate lobes of each liver were used for liver histology, described in chapter 3.5. The hypothalamics were separated from the dissected brains and homogenized in a Bullet Blender using a lysis buffer and stored at -20 °C until use (Kořínková et al., 2020).

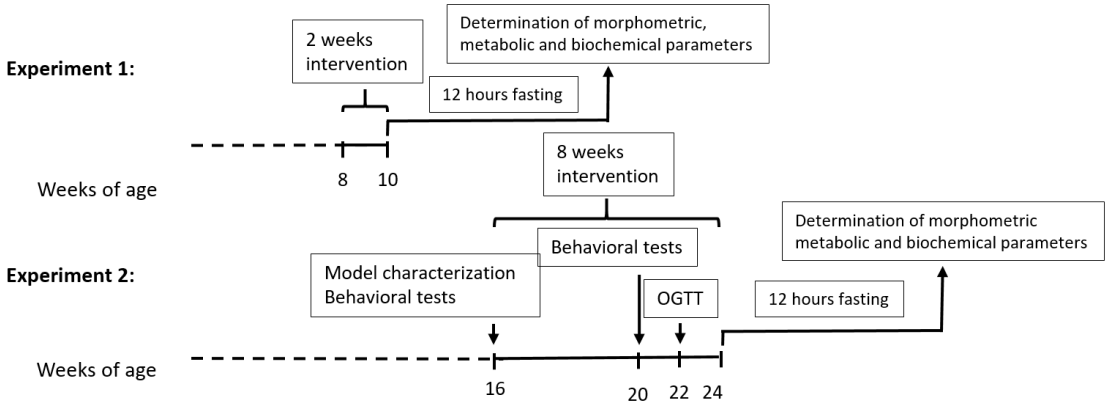


Figure 9: Design of experiments 1 and 2 of *ob/ob* mice.

3.2.3 *fa/fa* rats

Homozygous Zucker *fa/fa* male rats (fa/fa) and their lean littermates, *fa*/+- (control) rats, were purchased from Charles River (Lecco, Italy). The rats were provided with a standard Ssniff® diet. The design of the experiment is shown in Figure 10. Before the start of treatment, BW of fa/fa and control rats was monitored once per week. At the age of 32 weeks, fasted blood samples were collected from the tail veins to determine the basic biochemical parameters of the rat plasma. From 32 weeks of age, control rats were infused with saline (control saline group, n=8) while fa/fa rats were infused with saline (fa/fa saline group, n=7) or with palm^{11}-PrRP31 (5 mg/kg BW per day) (fa/fa palm^{11}-PrRP31 group, n=8) for two months. All three of these groups were infused using Alzet osmotic minipumps (Alzet, Cupertino, CA, USA), which were certified to infuse 6 µl of solution daily. Alzet osmotic minipumps were implanted IP under a short-term ether anesthesia and were replaced after 4 weeks with the new ones. During the dosing period, BW and FI were measured twice per week. At the end of the experiment, the rats were fasted overnight, blood plasma was collected from the tail veins for

determination of the biochemical parameters and OGTT was performed. The animals were sacrificed by decapitation and plasma and tissue samples: SCAT, IPAT, livers and brains were collected and stored in -80°C until use. The caudate lobes of each liver were used for liver histology described in chapter 3.5. The hypothalamics were separated from the dissected brains and homogenized in a Bullet Blender using a lysis buffer and stored at -20 °C until use.

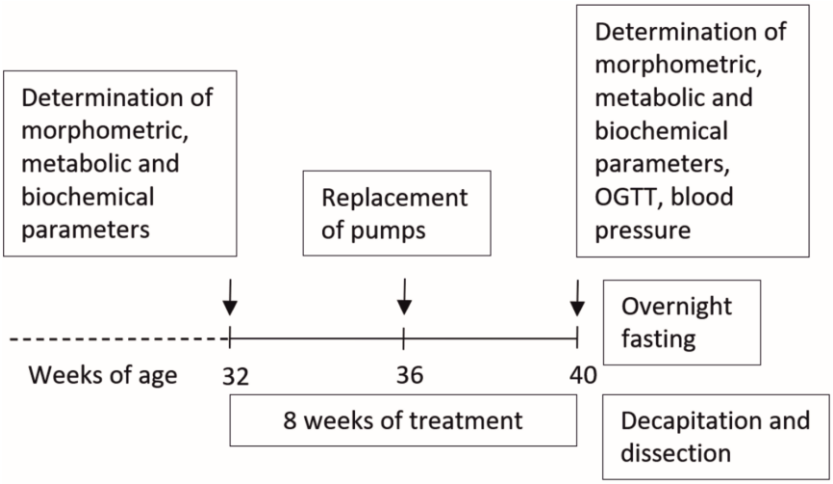


Figure 10: Design of experiment with *fa/fa* rats.

3.3 Oral glucose tolerance test

OGTT was performed after overnight fasting. At the time point 0, blood was drawn from the tail vein and a glucose solution at a dose of 2 g/kg BW was loaded perorally by gavage. The blood glucose concentration was determined in whole blood at 0, 30, 60, 90, 120 and 180 minutes using a glucometer (Arkray, Tokyo, Japan) and the delta of the area under the curve (AUC) was calculated.

3.4 Determination of biochemical parameters

Colorimetric assays were used to determine plasma levels of CHOL, TG (Erba Lachema, Brno, Czech Republic) FFA (Roche, Mannheim, Germany). Plasma insulin concentration was determined using a radioimmunoassay (RIA) kit (Millipore, St. Charles, MI, USA). Leptin was determined using mouse and rat leptin ELISA kits (Millipore, St. Charles, MI, USA).

Glycated hemoglobin (HbA1c) was determined using the Tina-quant HbA1c Gen. 3 kit (Roche, Mannheim, Germany). All measurements were performed according to the manufacturer´s instructions.

3.5 Liver histology

The caudate lobes of each liver were fixed in 4% paraformaldehyde (PFA) in 0,1% mol/l phosphate buffer at pH 7.4. After 24 hours in PFA, the liver samples were stored in 70% ethanol at 4°C until the tissue was processed on a Leica ASP200S tissue processor (Leica Biosystems Inc.). Liver samples were wax-penetrated to create paraffin blocks using the paraffin embedding station Leica EG1150H (Leica Biosystems Inc.) and then were cut on a Leica RM2255 microtome (Leica Biosystems Inc.). Five µm thick slices of liver were deparaffinized in xylene and rehydrated in ethanol range. Slices were stained in hematoxylin and eosin as described in previous papers (Kořínková et al., 2020;Pražienková et al., 2021).

3.6 Western blotting

Hypothalamic samples were processed and western blotting (WB) was performed as previously described (Špolcová et al., 2015). The following primary antibodies were obtained from Cell Signaling Technology, Beverly, MA, USA, and 1:1000 dilution in 5% milk TBS/tween-20was used: protein kinase B (Akt), pAkt (Thr308), pAkt (Ser473), suppressor of cytokine signaling (SOCS3), total signal transducer and activator of transcription 3 (STAT3), pSTAT (Tyr705), pSTAT (Ser727), extracellular signal-regulated kinase ½ (ERK), pERK, 5´AMP-activated protein kinase (AMPK), pAMPK (Tyr172). Total phosphoinositide 3-kinase (PI3K) was also obtained from Cell Signaling Technology, Beverly, MA, USA, and diluted 1:1000 in 5% BSA TBS/tween-20. β-actin was obtained from Sigma, St. Louis, MO, USA, and diluted 1:10000 in 5% milk TBS/tween-20. The following secondary antibodies were used: anti-mouse or anti-rabbit IgG HRP-linked antibody (both from Cell Signaling Technology, Beverly, MA, USA).

3.7 Determination of mRNA expression

The liver samples for mRNA determination were processed at the Institute for Clinical and Experimental Medicine (IKEM), Prague, Czech Republic, by Zdena Lacinová and Miloslava Čechová as previously described (Maletínská et al., 2015). The mRNA gene expressions of interest are shown in Table 1. The expression of beta-2-microglobulin (*B2m*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) or glucuronidase Beta (*GUSB*) were used to compensate for variations in input RNA amounts and the efficiency of reverse transcription.

Table 1: List of genes analyzed in adipose tissues and in the liver

Adipose tissue	***Acaca***	**Liver**	***Acaca***
	Cpt-1		*Cpt-1*
	FABP-4		*FABP-4*
	Fasn		*Fasn*
	GLUT-4		*G6pc*
	Gsk-3β		*Gsk-3β*
	Chrebp		*Lpl*
	Irs-1		*Pck-1*
	Lep		*Ppar-α*
	Lipe		*Ppar-γ*
	Lpl		*Scd-1*
	Pck-1		*SREBP-1*
	Ppar-γ		
	Scd-1		
	SREBP-1		

Acaca acetyl-CoA carboxylase, *FABP-4* fatty acid-binding protein 4, *Fasn* fatty acid synthase, *Cpt-1a* carnitine palmitoyltransferase 1a, *G6pc* glucose-6-phosphatase, *GLUT-4* glucose transporter type 4, *Gsk-3β* glycogen synthase kinase 3 beta, *ChREBP* carbohydrate response

element binding protein, *Irs-1* insulin receptor substrate 1, *Lep* leptin, *Lipe* lipase E, *Lpl* lipoprotein lipase, *Pck-1* phosphoenol pyruvate carboxykinase 1, *Ppar-α/γ* peroxisome proliferatore-activated receptor alpha/gamma, *Scd-1* stearoyl-CoA desaturase 1, *SREBP-1* sterol regulatory element binding protein 1.

3.8 Statistical analysis

The data are presented as means ± S.E.M. Statistical analysis was performed using an unpaired *t*-test or one-way followed by Dunnett´s multiple comparisons test or two-way ANOVA followed by Bonferroni´s multiple comparisons test as indicated in Figures legends and Tables with Graph-Pad Prism software (Graph-Pad Software, San Diego, CA, USA). The differences were considered significant at $P<0.05$.

The rate of insulin resistance was expressed with a homeostatic model assessment (HOMA) index calculated as (fasting glucose level, mmol/l) x (fasting insulin level, pmol/l) divided by 22.5 (Lansang et al., 2001).

4. RESULTS

4.1 Palm^{11}-PrRP31 significantly decreased body weight and improved glucose intolerance in obese WKY rats

The results written in this chapter have not been published yet but a manuscript is in preparation.

The consumption of an HF diet for 52 weeks resulted in significantly higher BW as well as plasma levels of leptin and insulin in WKY HF compared to WKY LF, while there were no significant changes in TG and CHOL measured in plasma between WKY HF and WKY LF (Table 2).

Table 2: Morphometric and metabolic parameters analyzed in fasted plasma of WKY LF and WKY HF before the treatment (60 weeks of age).

Age	60 weeks	
Group	WKY LF	WKY HF
BW (g)	439.25 ± 6.85	589.42 ± 6.81****
Glucose (mmol/l)	5.00 ± 0.25	6.11 ± 0.09****
Insulin (ng/ml)	0.53 ± 0.12	2.95 ± 0.19***
HOMA index	20.85 ± 5.07	133.46 ± 11.60****
Leptin (ng/ml)	2.29 ± 0.28	33.02 ± 1.75****
TG (mmol/l)	0.46 ± 0.06	0.47 ± 0.02
CHOL (mmol/l)	3.51 ± 0.25	3.20 ± 0.09

Data are presented as means ± S.E.M. Statistical analysis was performed by t-test. BW body weight, TG triglycerides, CHOL cholesterol. Significance is ***p<0.001, ****p<0.0001 *vs* WKY LF (n= 8, 24).

WKY HF had significantly increased fasted plasma glucose level and also showed insulin resistance analyzed by HOMA index (Table 2). WKY HF had significantly worsened glucose intolerance compared to WKY LF in OGTT (Figure 11A) and significantly increased corresponding AUC (Figure 11B).

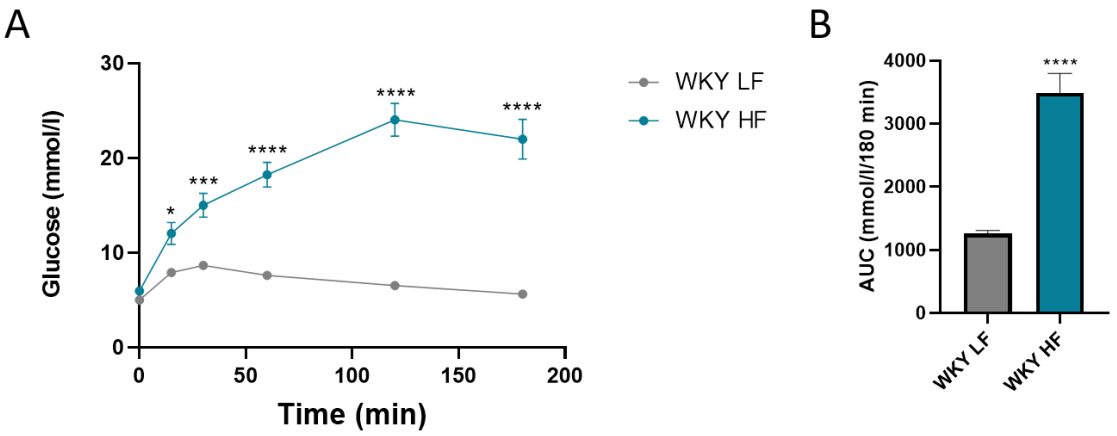


Figure 11: OGTT (A) and corresponding AUC (B) measured in WKY on LF diet and on HF diet before the treatment (60 weeks of age).

Data are presented as means ± S.E.M. Statistical analysis was performed by t-test or two-way ANOVA with Bonferroni´s *post hoc* test. AUC area under the curve, Significance is *p<0.05, ***p<0.001, ****p<0.0001 *vs* WKY LF (n= 8).

During the six weeks of IP treatment with saline, palm^{11}-PrRP31 or liraglutide, FI and BW change of WKY HF saline rats were similar to WKY LF saline rats, while both peptide treatments significantly lowered BW and FI compared to the saline-treated WKY HF group (Figure 12).

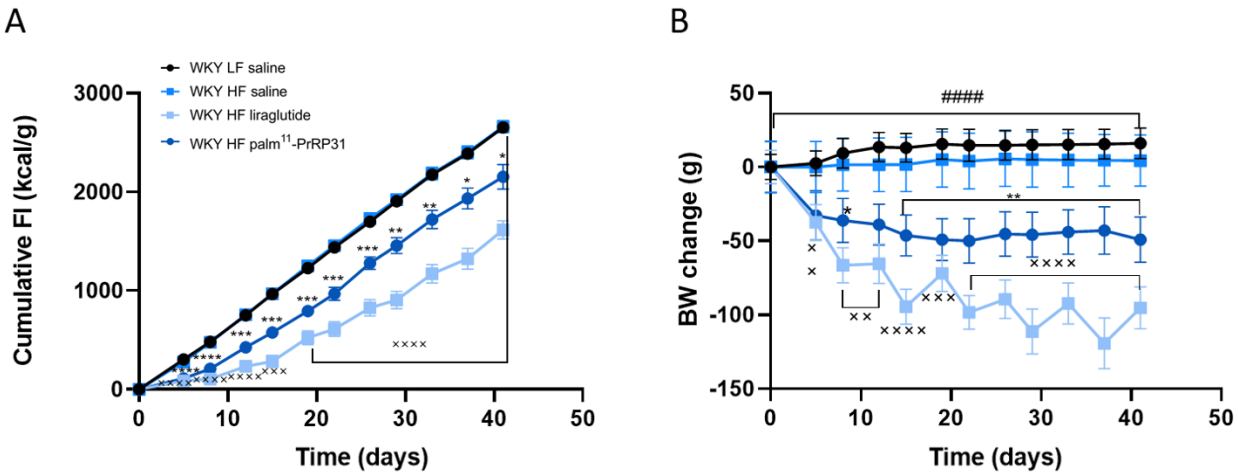


Figure 12: Chronic effect of palm^{11}-PrRP31 and liraglutide on cumulative FI (A) and BW change (B) in DIO WKY rats during the treatment (60-66 weeks).

Data are presented as means ± S.E.M. Statistical analysis was performed by two-way ANOVA with Bonferroni´s *post hoc* test. BW body weight, FI food intake. Significance is $^{\#}p<0.05$, $^{\#\#\#\#}p<0.0001$ WKY HF saline *vs* WKY LF saline; $^{x}p<0.05$, $^{xx}p<0.01$, $^{xxx}p<0.001$, $^{xxxx}p<0.0001$ WKY liraglutide *vs* WKY HF saline; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 WKY palm^{11}-PrRP31 *vs* WKY HF saline (n= 6-8).

At the end of the experiment, BW, plasma levels of insulin, leptin and fibroblast growth factor 21 (FGF21) were significantly increased in WKY HF saline compared to WKY LF saline. There were no significant differences in liver weight, plasma TG and CHOL between WKY HF saline and the WKY LF saline group (Table 3). BW at the end of the experiment was significantly decreased after both treatments with palm^{11}-PrRP31 and liraglutide compared to WKY HF saline rats (Table 3). WKY HF palm^{11}-PrRP31 and WKY HF liraglutide groups had significantly decreased plasma levels of leptin in comparison with WKY HF saline while neither palm^{11}-PrRP31 nor liraglutide changed the plasma level of insulin, CHOL or liver weight. WKY HF liraglutide group had a significantly decreased plasma level of TG and FGF21 compared to WKY HF saline (Table 3).

Table 3: Morphometric and metabolic parameters analyzed in fasted plasma of WKY LF saline, WKY HF saline, WKY HF liraglutide and WKY HF palm^{11}-PrRP31 at the end of intervention (66 weeks of age).

Age	66 weeks			
Group	**WKY LF saline**	**WKY HF saline**	**WKY HF Liraglutide**	**WKY HF palm^{11}-PrRP31**
BW (g)	444.00 ± 8.55****	591.00 ± 12.37	456.83 ± 11.77****	519.14 ± 9.05***
Liver weight (g)	10.81 ± 0.55	11.20 ± 0.435	9.76 ± 0.40	10.42 ± 0.34
Glucose (mmol/l)	5.49 ± 0.17	5.98 ± 0.17	6.62 ± 0.65	6.56 ± 0.28
HbA1c (mmol/mol)	2.00 ± 0.09**	2.62 ± 0.13	1.86 ± 0.13***	2.27 ± 0.08
Insulin (ng/ml)	0.48 ± 0.16****	2.77 ± 0.27	2.07 ± 0.25	2.09 ± 0.35
HOMA index	20.65 ± 6.69****	127.25 ± 13.29	104.73 ± 15.00	116.58 ± 19.38
Leptin (ng/ml)	3.13 ± 0.60****	24.52 ±2.22	12.60 ± 2.13***	15.14 ± 2.07**
TG (mmol/l)	0.54 ± 0.06	0.56 ± 0.03	0.38 ± 0.03	0.59 ± 0.05
CHOL (mmol/l)	2.79 ± 0.12	2.64 ±0.15	2.47 ± 0.13	2.50 ± 0.15
FGF21 (pg/ml)	262.96 ± 36.90*	607.63 ± 147.23	173.85 ± 31.75*	512.64 ± 149.38

Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test. BW body weight, FGF21 fibroblast growth factor 21, HbA1c glycated hemoglobin, TG triglycerides, CHOL cholesterol. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 *vs* WKY HF saline (n= 6-8).

At the end of the experiment, there were no significant changes in fasted glucose levels between the WKY LF saline and WKY HF saline groups, while the HbA1c and HOMA indexes were significantly increased in WKY HF saline compared to the WKY LF saline group (Table 3). WKY HF saline revealed strong glucose intolerance based on OGTT results compared to WKY LF saline (Figure 13). The treatment with both palm^{11}-PrRP31 and liraglutide had no effect on fasted glucose in comparison with WKY HF saline, but HbA1c was significantly decreased in WKY HF liraglutide compared to WKY HF saline (Table 3). HOMA index was non-significantly decreased after both treatments but palm^{11}-PrRP31 as well as liraglutide significantly improved glucose intolerance and lowered corresponding AUC in WKY on an HF diet (Figure 13).

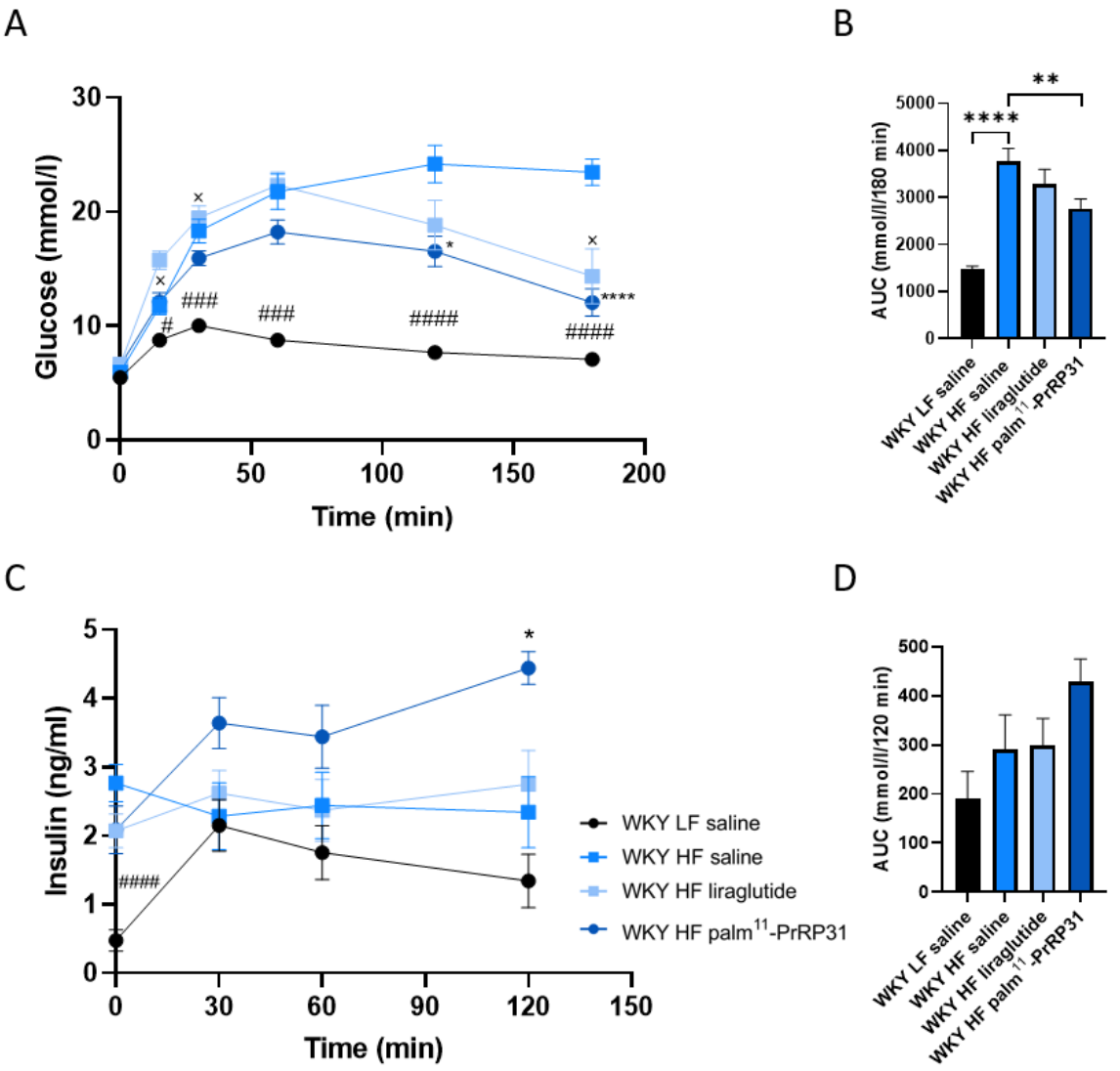


Figure 13: Chronic effect of palm^{11}-PrRP31 and liraglutide on OGTT (A), corresponding AUC (B), plasma levels of insulin during OGTT (C) and corresponding AUC (D) in DIO WKY rats at the end of the experiment (66 weeks).

Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test or two-way ANOVA with Bonferroni´s *post hoc* test. AUC area under the curve. Significance is $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, $^{\#\#\#\#}p<0.0001$ WKY LF saline *vs* WKY HF saline; $^{x}p<0.05$ WKY liraglutide *vs* WKY HF saline; *p<0.05, ****p<0.0001 WKY palm^{11}-PrRP31 *vs* WKY HF saline (n= 6-8).

The liver histology of WKY LF saline and WKY HF saline is shown in figure 14. No accumulation of fat droplets and no liver steatosis was detected in these two groups: WKY LF saline and WKY HF saline rats (Figure 14).

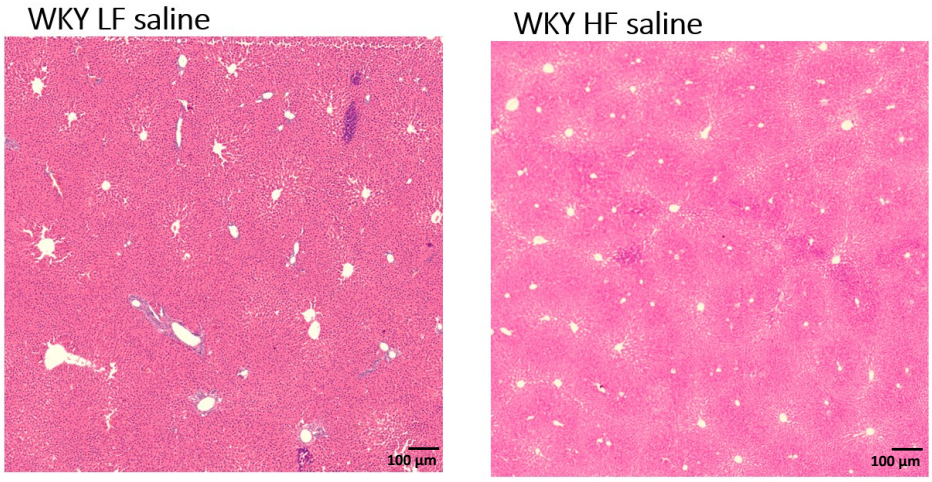


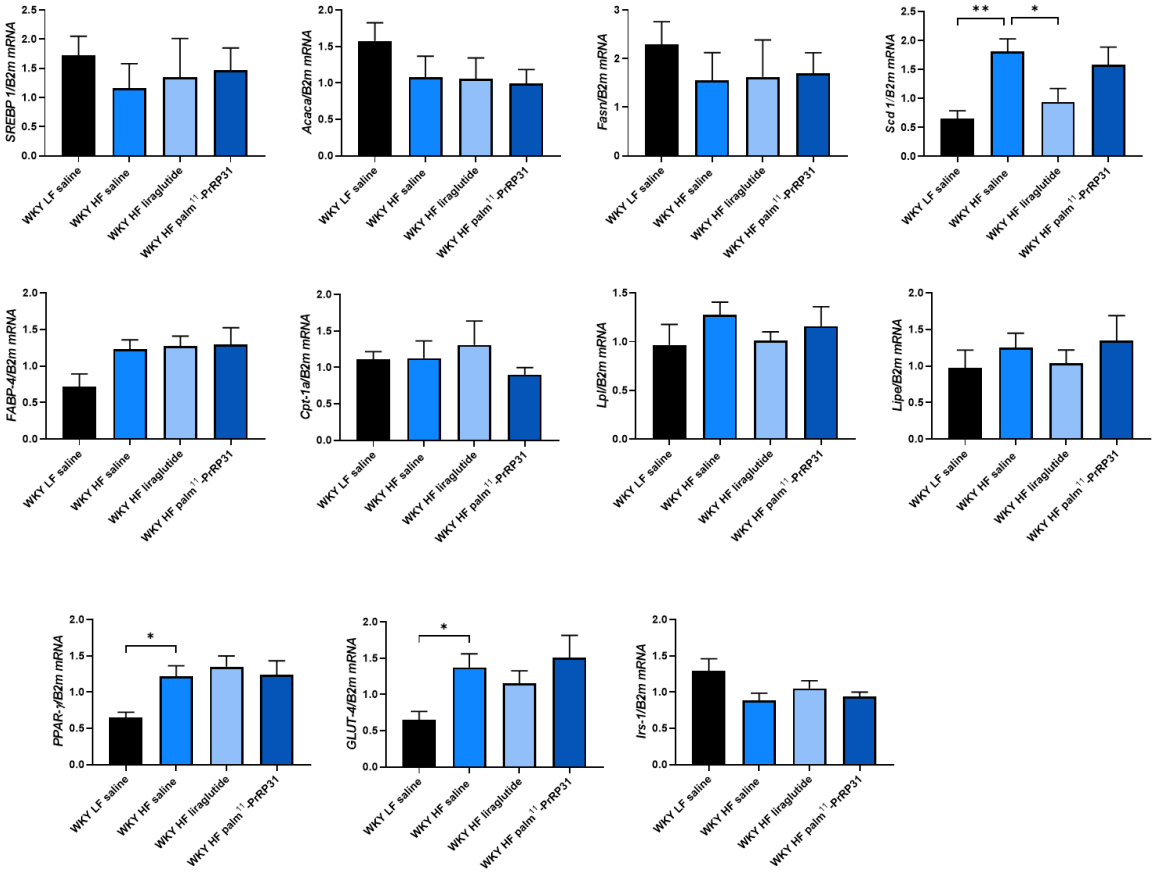
Figure 14: Liver histology, representative photomicrographs of WKY LF saline and WKY HF saline (n= 6).

Eosin-hematoxylin staining. The magnification of photomicrographs is 200x.

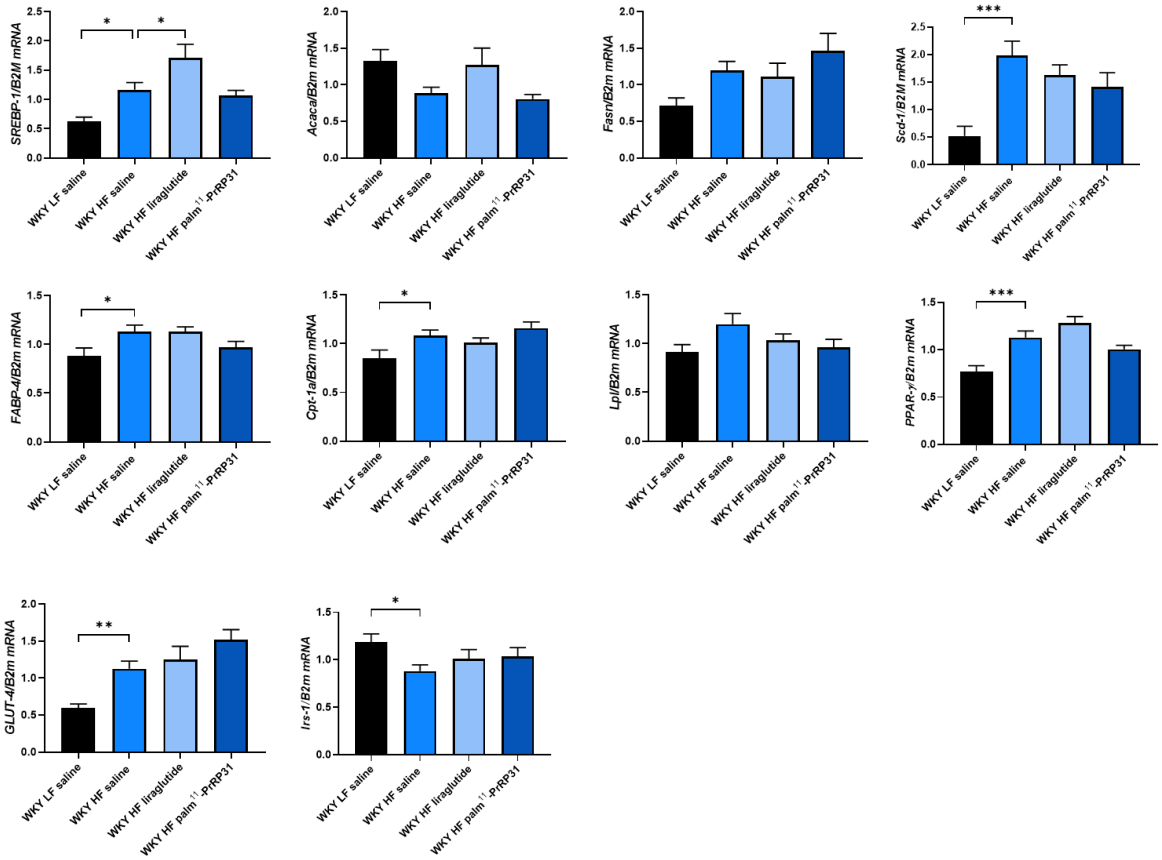
At the end of the experiment, lipid metabolism in adipose tissue was slightly affected in WKY HF saline compared to WKY LF saline. Regarding lipogenesis, mRNA expression of stearoyl-CoA desaturase 1 (*Scd-1*) was significantly increased in both SCAT and IPAT while that of *SREBP-1* was significantly increased only in IPAT of WKY HF saline compared to WKY LF saline. mRNA expression of fatty acid-binding protein 4 (*FABP-4*), *Cpt-1a*, peroxisome proliferator-activated receptor γ (*PPAR-γ*), and genes involved in lipolysis were significantly higher in WKY HF compared to WKY LF in IPAT, while in SCAT only mRNA expression of *PPAR-γ* was significantly increased in WKY HF saline compared to WKY LF saline. mRNA expression of *GLUT-4* was significantly higher in WKY HF saline in comparison with WKY LF saline both in SCAT and IPAT (Figure 15 A, B). The treatment with liraglutide significantly decreased mRNA expression of *Scd-1* in SCAT of WKY HF compared to WKY HF saline (Figure 15 A, B).

At the end of the experiment, liver lipogenesis was increased by an HF diet. mRNA expression of lipogenetic enzymes *Acaca*, *Fasn*, *SREBP-1* and *Scd-1* were significantly increased in WKY HF saline in comparison to WKY LF saline (Figure 15C). Treatment with palm^{11}-PrRP31 significantly attenuated mRNA expression of *Acaca*, *Fasn* and *Scd-1*, while only *Scd-1* mRNA expression was significantly decreased by liraglutide treatment (Figure 15C).

A



B


SREBP-1/B2M mRNA
Acaca/B2m mRNA
Fasn/B2m mRNA
Scd-1/B2M mRNA
FABP-4/B2m mRNA
Cpt-1a/B2m mRNA
Lpl/B2m mRNA
PPAR-γ/B2m mRNA
GLUT-4/B2m mRNA
Irs-1/B2m mRNA
WKY LF saline
WKY HF saline
WKY HF liraglutide
WKY HF palm11-PrRP31

C

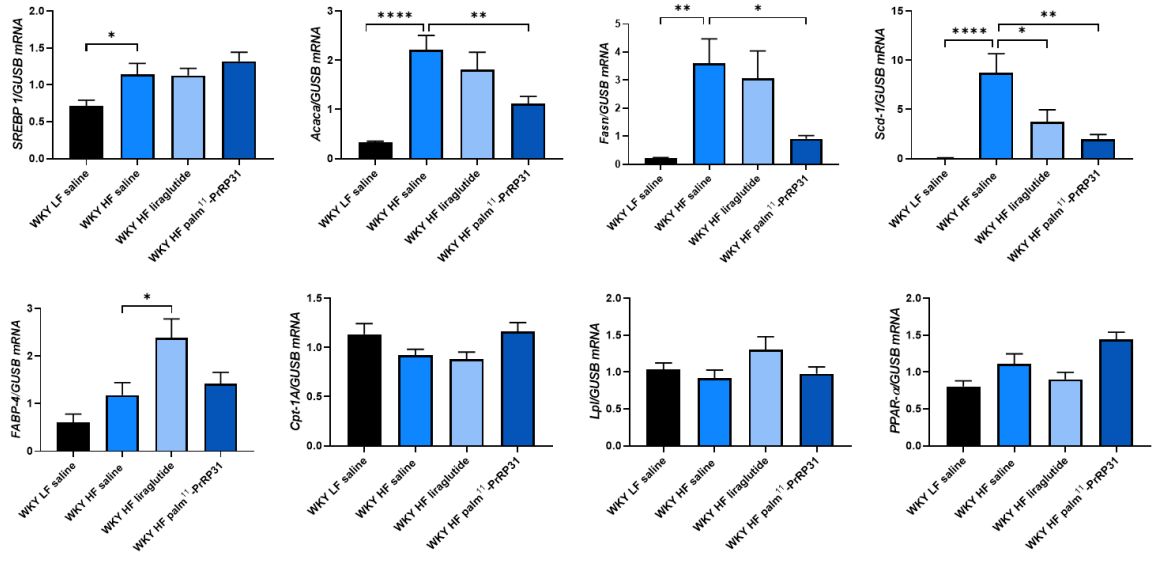

SREBP 1/GUSB mRNA
Acaca/GUSB mRNA
Fasn/GUSB mRNA
Scd-1/GUSB mRNA
FABP-4/GUSB mRNA
Cpt-1A/GUSB mRNA
Lpl/GUSB mRNA
PPAR-α/GUSB mRNA
WKY LF saline
WKY HF saline
WKY HF liraglutide
WKY HF palm11-PrRP31

Figure 15: Chronic effect of palm^{11}-PrRP31 and liraglutide on mRNA expression in SCAT (A), IPAT (B) and liver (C) in DIO WKY rats at the end of the experiment (66 weeks)

Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test. *Acaca* acetyl-CoA carboxylase*, FABP-4* fatty acid-binding protein 4, *Fasn* fatty acid synthase, *Cpt-1a* carnitine palmitoyltransferase 1a, *GLUT-4* glucose transporter type 4, *Gsk-3β* glycogen synthase kinase 3 beta, IPAT intraperitoneal adipose tissue, *Irs-1* insulin receptor substrate 1, *Lipe* lipase E, *Lpl* lipoprotein lipase, *Ppar-α/γ* peroxisome proliferatore-activated receptor alpha/gamma, SCAT subcutaneous adipose tissue, *Scd-1* stearoyl-CoA desaturase 1, *SREBP-1* sterol regulatory element binding protein 1. Significance is *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 WKY *vs* WKY HF saline (n=6).

The hypothalamic insulin signaling pathway was not affected by an HF diet in WKY HF saline compared to WKY LF saline, while ERK phosphorylated at Thr202/Tyr204 was significantly decreased in WKY HF saline in comparison with WKY LF saline. Both treatments did not significantly affect insulin signaling but significantly decreased total STAT3 in comparison with WKY HF saline rats. Palm^{11}-PrRP31 significantly increased pERK (Thr202/Tyr204) in WKY HF palm^{11}-PrRP31 compared to WKY HF saline (Figure 16).

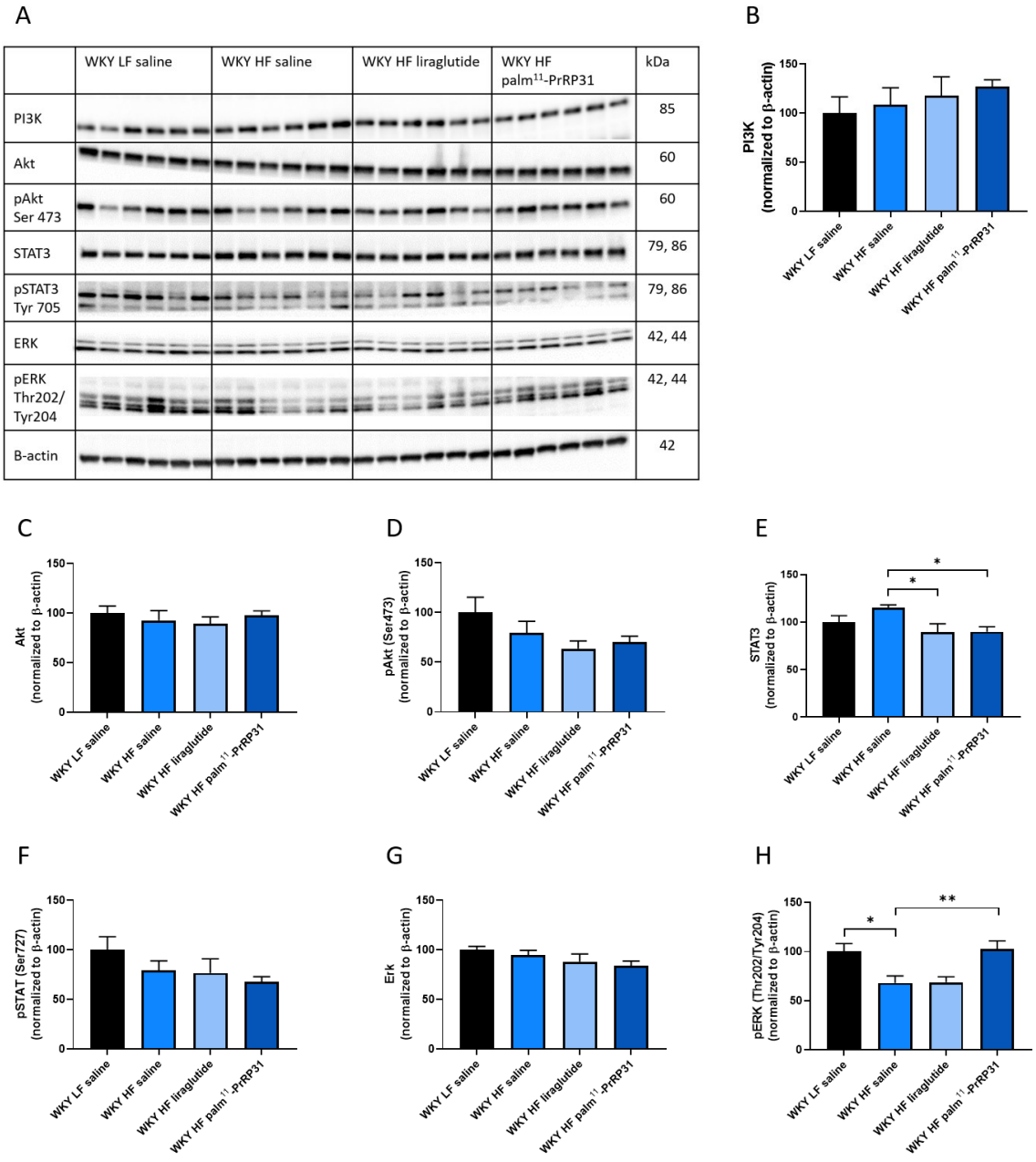


Figure 16: Insulin and leptin signaling pathways in hypothalamus of WKY rats

Overview of western blots for specific proteins (A), quantification of western blots normalized to β-actin: PI3K (B), Akt (C) pAkt (Ser473) (D), STAT3 (E), pSTAT (Tyr705) (F), ERK (G), pERK (Thr202/Tyr204). Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test. PI3K total phosphoinositide 3-kinase, Akt protein kinase B, STAT3 total signal transducer and activator of transcription 3,

ERK extracellular signal-regulated kinase. Significance is *p<0.05, **p<0.01 *vs* WKY HF saline (n= 6).

4.2 Synergistic effect of palm^{11}-PrRP31 and leptin in leptin deficient *ob/ob* mice

The results obtained in the experiments performed in *ob/ob* mice presented in this chapter were published in the Journal of Molecular Endocrinology (Kořínková et al., 2020).

Five-week old *ob/ob* mice and their wild-type littermates were randomly divided into two experiments (experiment 1 and experiment 2). In experiment 1, mice were treated with saline, palm^{11}-PrRP31, leptin and palm^{11}-PrRP31 + leptin for two weeks from 8 weeks of age, while in experiment 2, mice were treated for eight weeks from 16 weeks of age exactly as in experiment 1. In both experiment 1 and experiment 2, *ob/ob* saline mice had significantly increased BW, liver, SCAT and IPAT weight compared to WT saline mice. Levels of plasma insulin and CHOL were also significantly increased in *ob/ob* saline mice in both experiments compared to WT saline mice (Table 4).

Table 4: Morphometric and metabolic parameters of WT saline and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31 analyzed in experiment 1 (10-week-old mice) (A) and in experiment 2 (24-week-old mice) (B)

A

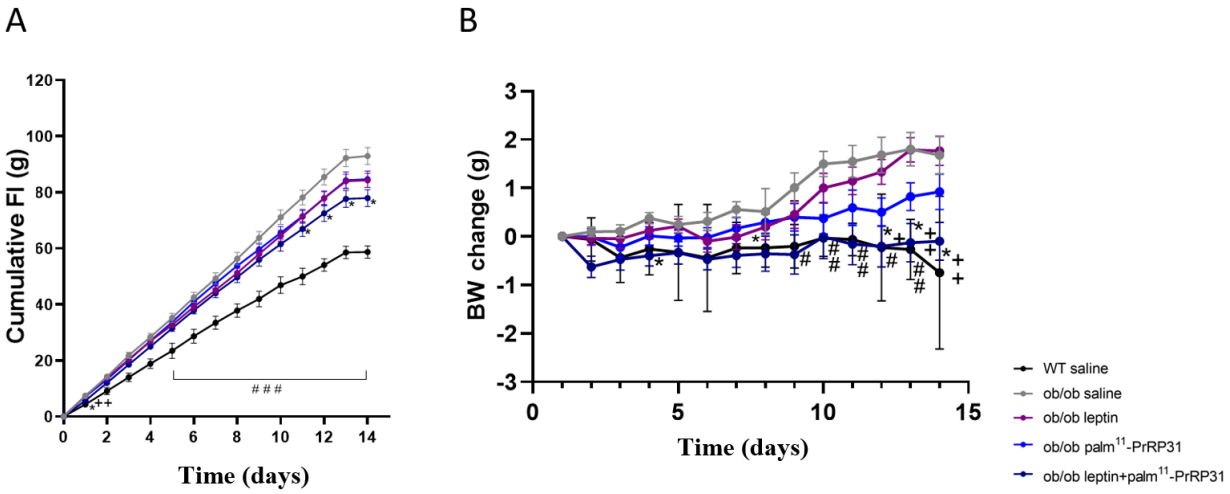
	WT saline	*ob/ob* saline	*ob/ob* leptin	*ob/ob* palm^{11}-PrRP31	ob/ob leptin+palm^{11}-PrRP31
BW (g)	20,38 ± 0,54****	37,28 ± 0,67	35,64 ± 0,75	35,30 ± 0,49	35,34 ± 0,85
SCAT (g)	0,28 ± 0,05****	4,08 ± 0,28	3,74 ± 0,28	3,60 ± 0,29	3,68 ± 0,24
IPAT (g)	0,28 ± 0,04****	2,83 ± 0,11	2,57 ± 0,09	2,51 ± 0,10	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***
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
Rectal temp (˚C)	36,75 ± 0,17****	35,00 ± 0,21	35,64 ± 0,28	35,22 ± 0,32	35,33 ± 0,36

B

	WT saline	*ob/ob* saline	*ob/ob* leptin	*ob/ob* palm^{11}-PrRP31	ob/ob leptin+palm^{11}-PrRP31
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
Glucose (mmol/l)	8,05 ± 0,27	7,96 ± 0,38	6,78 ± 0,49	8,29 ± 0,40	8,99 ± 0,97
HbA1c (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**
Rectal temp (˚C)	37,84 ± 0,17****	35,42 ± 0,18	35,09 ± 0,26	34,93 ± 0,32	36,52 ± 0,14*

Data are presented as means ± S.E.M. Statistical analysis was performed by t-test or one-way ANOVA with Dunnett´s *post hoc* test. BW body weight, SCAT subcutaneous adipose tissue, IPAT intraperitoneal adipose tissue, HbA1c glycated hemoglobin, CHOL cholesterol. Significance is * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ *vs ob/ob* saline (n= 8-10).

FI was significantly decreased in experiment 1, but not in experiment 2, after leptin + palm^{11}-PrRP31 treatment compared to *ob/ob* saline group, but leptin and palm^{11}-PrRP31 itself did not significantly change FI (Figure 17). Analogously, treatment with the combination of palm^{11}-PrRP31 and leptin significantly decreased BW in *ob/ob* mice in both experiment 1 and experiment 2 while leptin or palm^{11}-PrRP31 alone did not significantly attenuate BW. Palm^{11}-PrRP31 itself and also in combination with leptin significantly decreased level of plasma CHOL compared to *ob/ob* saline group in experiment 2. It is generally known that *ob/ob* mice are hypothermic, and our measurement of their rectal temperature confirmed it. There was a significant difference in the rectal temperature between *ob/ob* and WT saline mice. On the other hand, the combined treatment of leptin and palm^{11}-PrRP31 significantly increased body temperature in experiment 2 (Table 4).



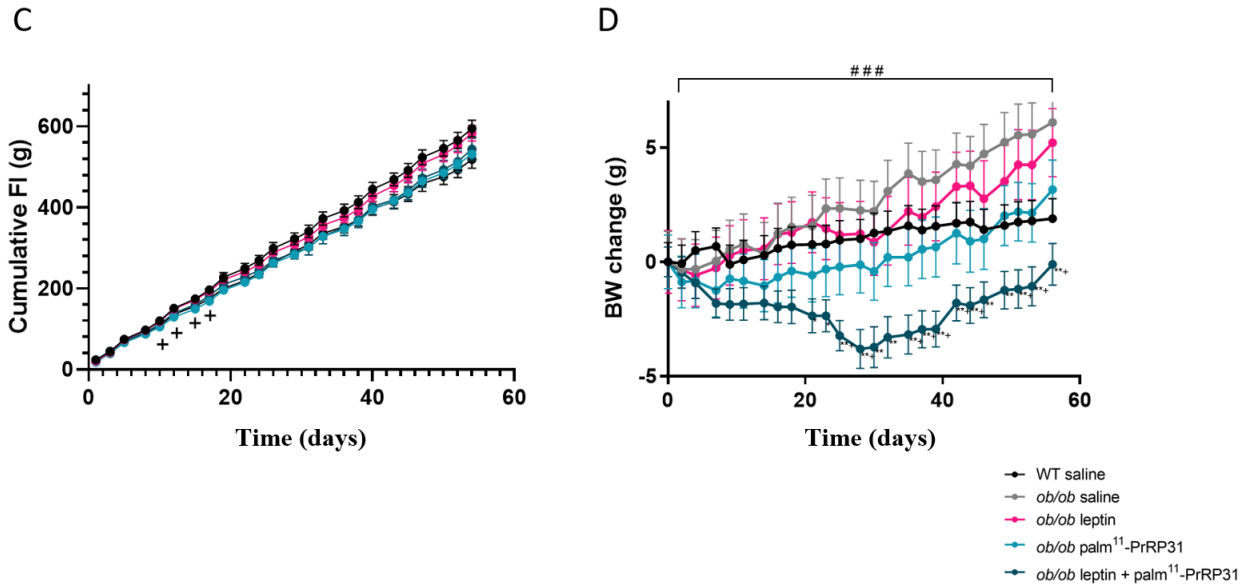


Figure 17: Chronic effect of palm^{11}-PrRP31, leptin and leptin + palm^{11}-PrRP31 on the FI and BW in experiment 1 (A, B) and in experiment 2 (C, D)

Data are presented as means ± S.E.M. Statistical analysis was performed by two-way ANOVA with Bonferroni´s *post hoc* test. FI food intake, BW body weight. Significance is *p<0.05, **p<0.01 *ob/ob* saline *vs* WT saline; $^{\#}$p<0.05, $^{\#\#}$p<0.01 $^{\#\#\#}$p<0.001 *ob/ob* leptin + palm^{11}-PrRP31 *vs ob/ob* saline; $^{+}$p<0.05, $^{++}$p<0.01. *ob/ob* leptin + palm^{11}-PrRP31 *vs ob/ob* leptin (n= 8-10).

The fasting glucose level before OGTT was significantly higher in *ob/ob* saline than WT saline (experiments 1 and 2) and HbA1c was significantly increased in *ob/ob* saline compared to WT saline in experiment 2 (Table 4). Saline treated *ob/ob* mice developed intolerance to glucose during OGTT in comparison with WT saline in experiment 2 (Figure 18). Leptin and palm^{11}-PrRP31 significantly decreased blood glucose, however only their combination significantly decreased blood glucose to the level of wild-type mice in experiment 1. In experiment 2, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31 caused neither change in blood glucose (Table 4) nor improvement of glucose intolerance (Figure 18).

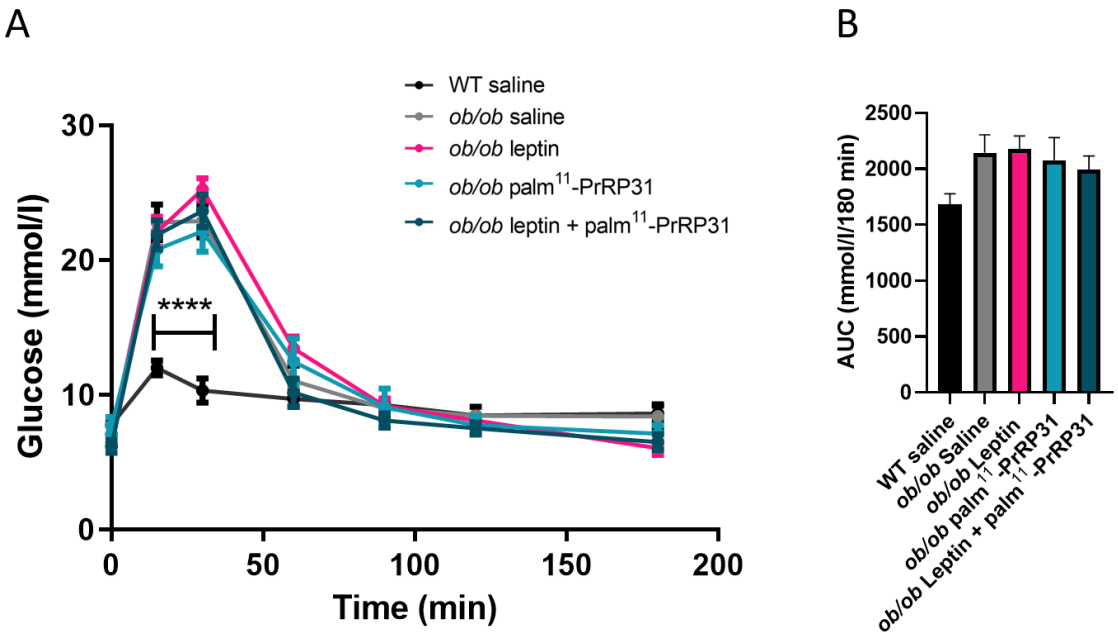


Figure 18: Chronic effect of palm^{11}-PrRP31, leptin and palm^{11}-PrRP31 + leptin on OGTT (A) AUC (B) in experiment 2

Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test or two-way ANOVA with Bonferroni´s *post hoc* test. AUC area under the curve. Significance is ****p<0.0001 WT saline *vs ob/ob* saline (n= 8-10).

The treatment with leptin and leptin + palm^{11}-PrRP31 combination significantly decreased liver weight compared to *ob/ob* saline group in both experiments (Table 4). *Ob/ob* saline mice showed mild liver steatosis compared to WT saline, and all treated *ob/ob* groups showed regression of fat droplets in liver tissue after hematoxylin-eosin staining (Figure 19).

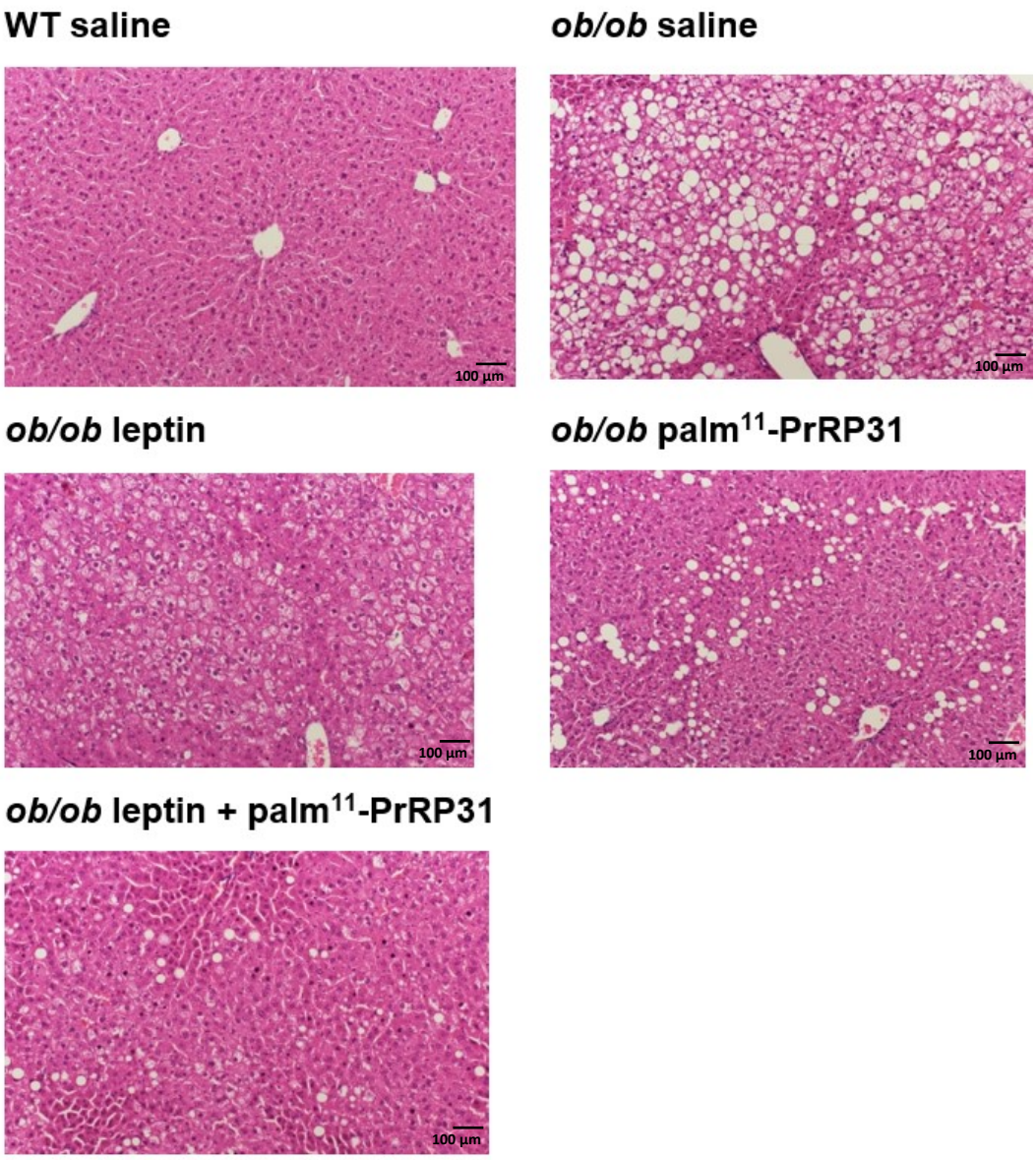
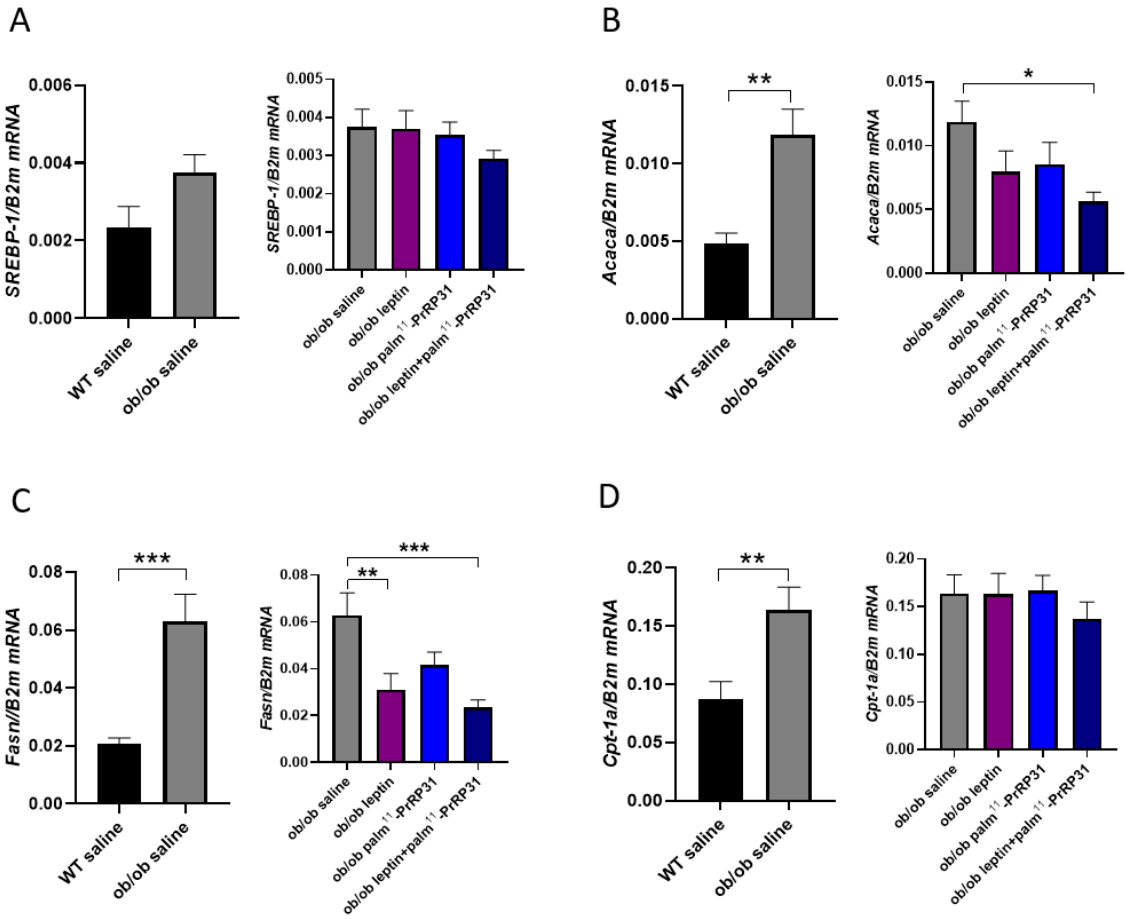


Figure 19: **Liver histology, representative photomicrographs of WT saline mice and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31 analyzed in experiment 2 (n= 8-10).**

Eosin-hematoxylin staining. The magnification of photomicrographs is 200x.

In experiment 1, liver mRNA expression of *SREBP-1*, which controls the expression of *Acaca* and *Fasn*, did not differ between *ob/ob* saline and WT saline groups and no treatment affected it significantly. mRNA expression of *Acaca* and *Fasn* were significantly increased in *ob/ob* saline compared to WT saline and leptin treatment significantly attenuated *Fasn* mRNA liver expression, moreover treatment with a leptin + palm^{11}-PrRP31 combination significantly lowered both *Acaca* and *Fasn* mRNA expression. The mRNA expression levels of enzymes regulating fatty acid oxidation, such as *Cpt-1a*, *Pparα* and *Pparγ*, were significantly increased in *ob/ob* saline compared to WT saline, but no treatment affected the expression (Figure 20).



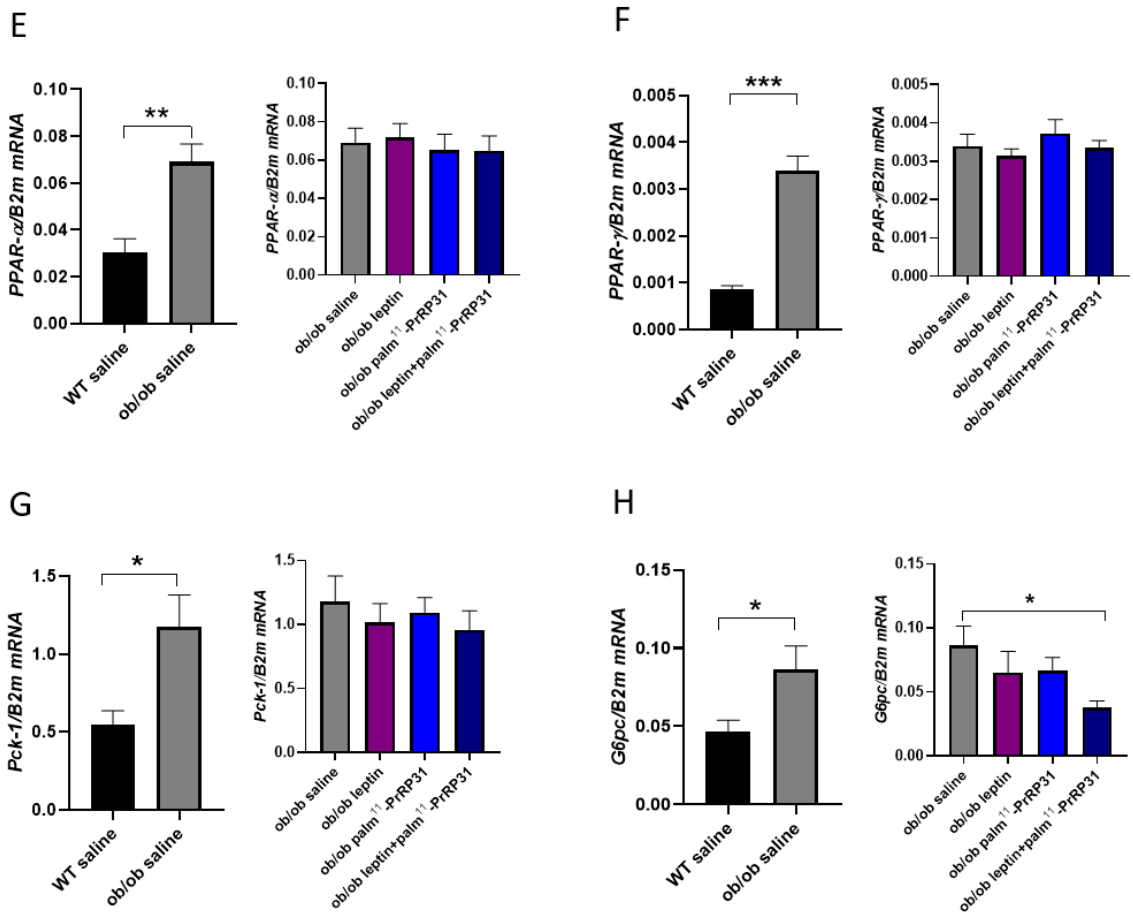
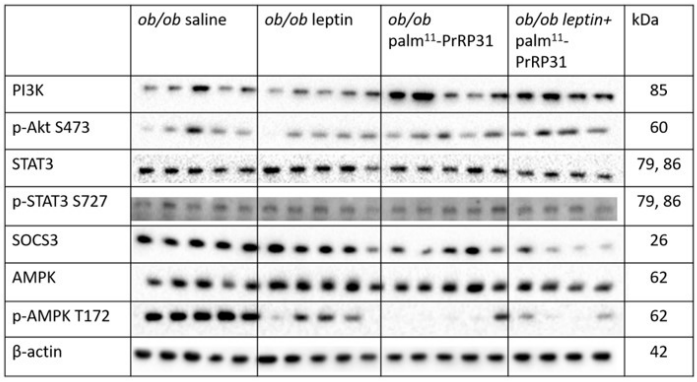


Figure 20: mRNA expression in the liver of WT saline and *ob/ob* mice treated with saline, leptin, palm^{11}-PrRP31, leptin + palm^{11}-PrRP31 measured in experiment 1. (A) *SREBP-1* (B) *Acaca* (C) *Fasn* (D) *Pck-1* (E) *Cpt-1a* (F) *PPAR-α* (G) *PPAR-γ* (H) *G6pc*.

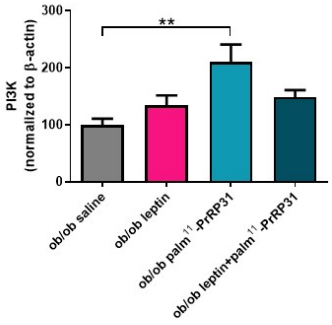
Data are presented as means ± SEM. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test. *SREBP-1* sterol regulatory element binding protein, *Acaca* acetyl-CoA carboxylase, *Fasn* fatty acid synthase, *Pck-1* phosphoenol pyruvate carboxykinase 1, *Cpt-1a* carnitine palmitoyltransferase 1a, *Ppar-α/γ* peroxisome proliferatore-activated receptor alpha/gamma, G6pc glucose-6-phosphatase. Significance is *p<0.05, **p<0.01, ***p<0.001 *vs ob/ob* saline (n= 8-10).

Leptin, palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31 activated JAK-STAT and AMPK pathways in the hypothalamus of *ob/ob* mice were measured in experiment 2 (Figure 21). PI3K protein was significantly increased in *ob/ob* palm^{11}-PrRP31. The treatment with palm^{11}-PrRP31, leptin and leptin + palm^{11}-PrRP31 tended to increase pAkt (Ser473). STAT3 protein was significantly increased in *ob/ob* leptin compared to *ob/ob* saline. pSTAT (Ser727) was increased by all three treatments. SOCS3 protein was significantly lowered by a palm^{11}-PrRP31 and leptin + palm^{11}-PrRP31 combination treatment. Both, leptin and palm^{11}-PrRP31 treatment significantly increased total AMPK, but AMPK phosphorylation at Tyr172 was significantly lowered after all three treatments (Figure 21).

A



B



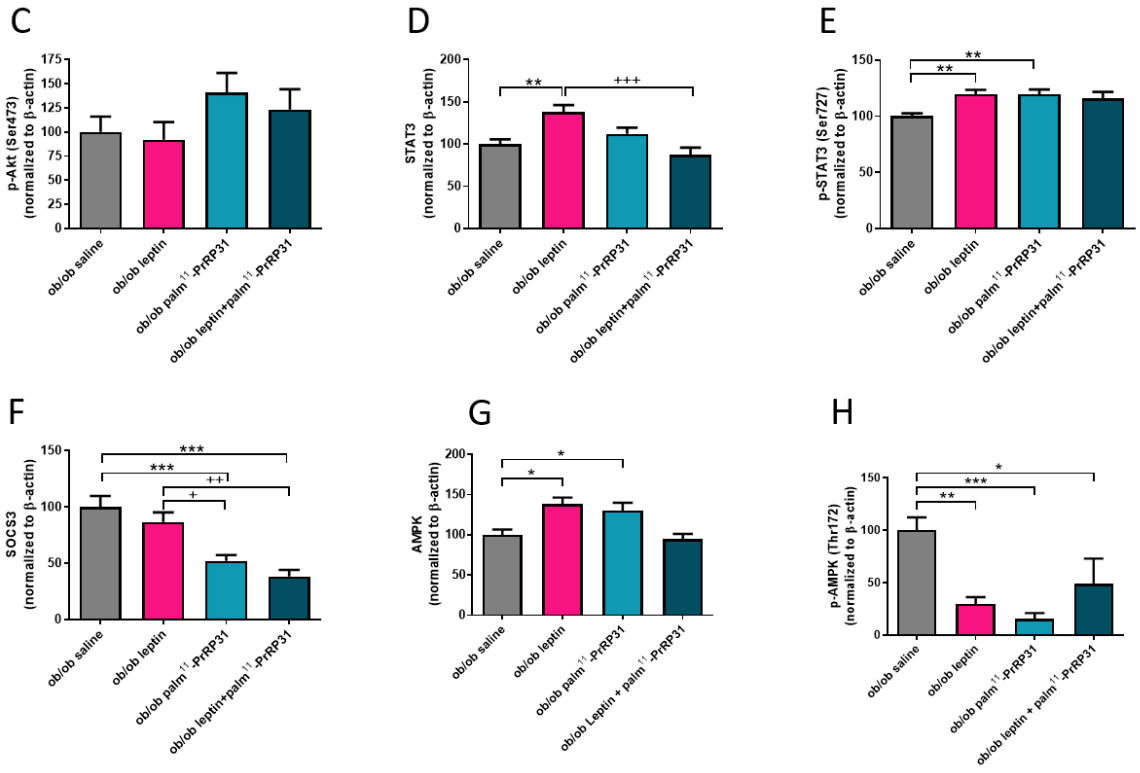


Figure 21: **Insulin and leptin signaling pathways in hypothalamus of *ob/ob* mice**

Overview of western blots for specific proteins (A), quantification of western blots normalized to β-actin PI3K (B), pAkt(Ser473) (C), STAT3 (D), pSTAT3(Ser727) (E), SOCS3 (F), AMPK (G) and pAMPK(Thr172) (H). Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test. PI3K total phosphoinositide 3-kinase, Akt protein kinase B, STAT3 total signal transducer and activator of transcription 3, SOCS3 suppressor of cytokine signaling, AMPK 5´AMP-activated protein kinase. Significance is *p<0.05, **p<0.01, ***p<0.001 *vs ob/ob* saline; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 *vs ob/ob* leptin (n= 8-10).

4.3 Palm^{11}-PrRP31 treatment neither lowered body weight nor ameliorated glucose tolerance in *fa/fa* rats

The results obtained in this chapter are after major revision in Nutrition and Diabetes.

Before the treatment, 32-weeks-old fa/fa rats had significantly increased BW, glucose and plasma TG, CHOL, leptin and insulin compared to control rats. After the treatment, 40-week-old fa/fa saline rats had significantly increased BW, glucose and all measured plasma metabolic parameters compared to control saline rats. On the other hand, the treatment with palm^{11}-PrRP31 neither lowered BW nor improved all measured metabolic parameters in fa/fa rats (Table 5).

Table 5: Morphometric and metabolic parameters analyzed in fasted blood plasma and in liver of control and fa/fa group before treatment (32 weeks of age) and at the end of experiment (40 weeks of age) treated with saline or palm^{11}-PrRP31.

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
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
Liver (g)			15.00 ± 0.65****	23.14 ± 0.70	21.50 ± 0.71
Liver TG (µmol/g)			20.14 ± 1.70****	46.92 ± 3.06	NT
Liver CHOL (µmol/g)			7.21 ± 0.16	8.08 ± 0.45	NT

Data are presented as means ± S.E.M. Statistical analysis was performed by t-test or one-way ANOVA with Dunnett´s *post hoc* test. BW body weight, TG triglycerides, CHOL cholesterol, NT non-tested. Significance is **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 with Dunnett´s *post hoc* test (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).

During the two-month treatment with palm[11]-PrRP31, there were no significant differences in cumulative FI and BW between fa/fa saline rats and control saline rats. Moreover, there were no significant changes in BW and FI between fa/fa palm[11]-PrRP31 and fa/fa saline rats (Figure 22).

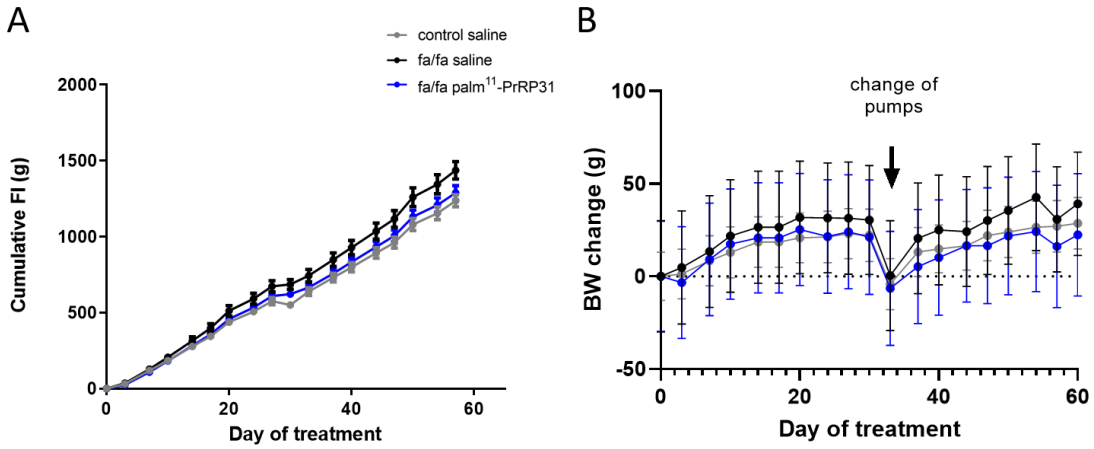


Figure 22: Chronic effect of palm[11]-PrRP31 on Cumulative FI (A) and BW change (B) in fa/fa rats during the treatment (32 - 40 weeks)

Data are presented as means ± S.E.M. Statistical analysis was performed by two-way ANOVA with Bonferroni´s *post hoc* test. FI food intake, BW body weight (n= 7, 8).

All saline and palm[11]-PrRP31 treated fa/fa rats were normoglycemic, however, fa/fa saline rats were glucose intolerant based on the OGTT result and the treatment with palm[11]-PrRP31 did not improve their tolerance to glucose (Figure 23). The insulin plasma level measured during OGTT was significantly increased in fa/fa saline rats compared to control saline rats, and treatment with palm[11]-PrRP31 did not affect insulin level (Figure 23).

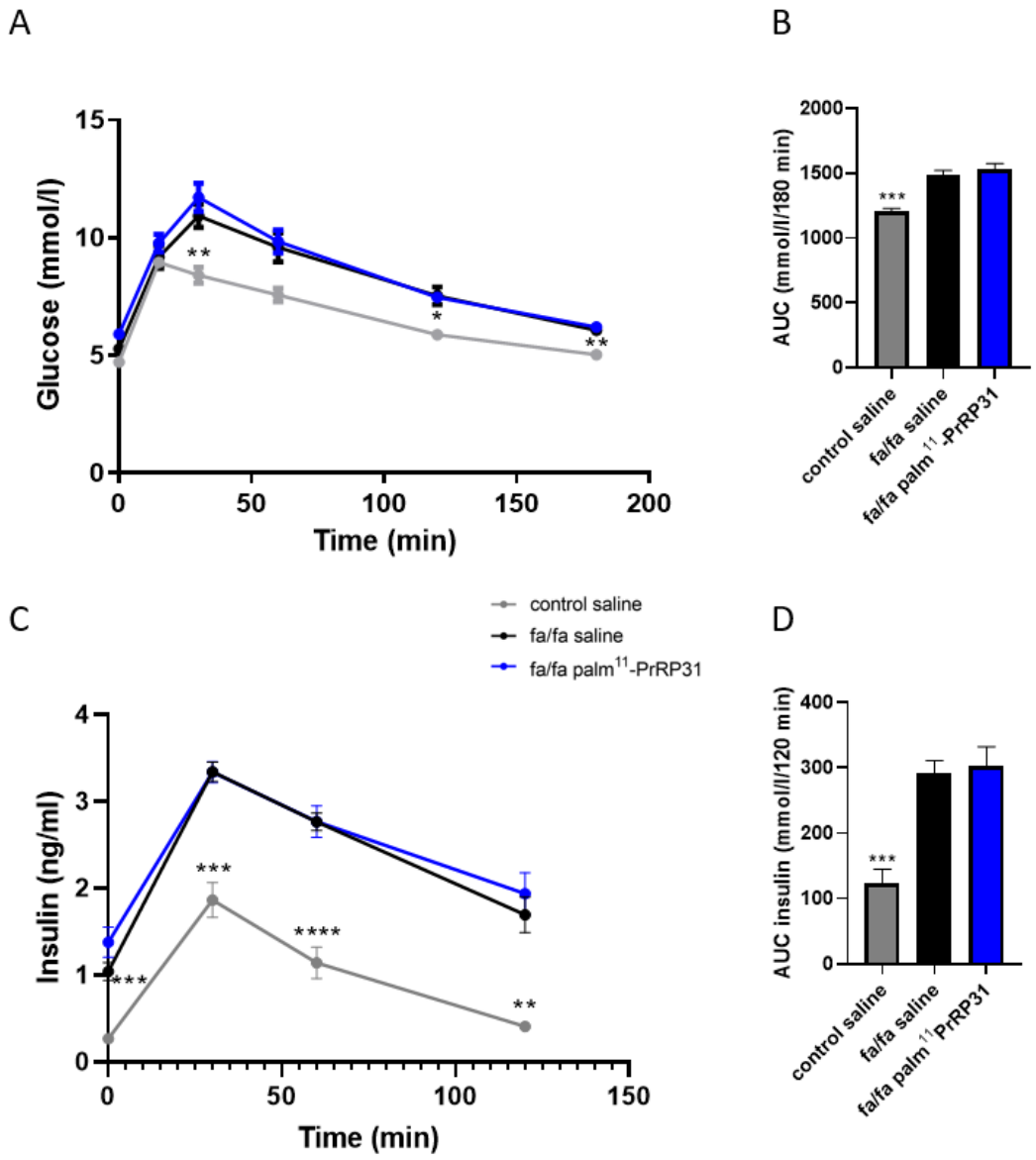


Figure 23: Chronic effect of palm^{11}-PrRP3 on OGTT (A), AUC (B) and plasma levels of insulin during OGTT (C) and corresponding AUC (D) in fa/fa rats at the end of the experiment (40 weeks)

Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test or two-way ANOVA with Bonferroni´s *post hoc* test. AUC area under the curve. Significance is **p<0.01, ***p<0.001, ****p<0.0001 fa/fa saline *vs* control saline (n= 7, 8).

The liver histology of the fa/fa saline and control saline rats is shown in Figure 24. Livers from the fa/fa saline group had mild liver steatosis manifested by significantly enhanced accumulation of fat droplets compared to their respective controls (Figure 24).

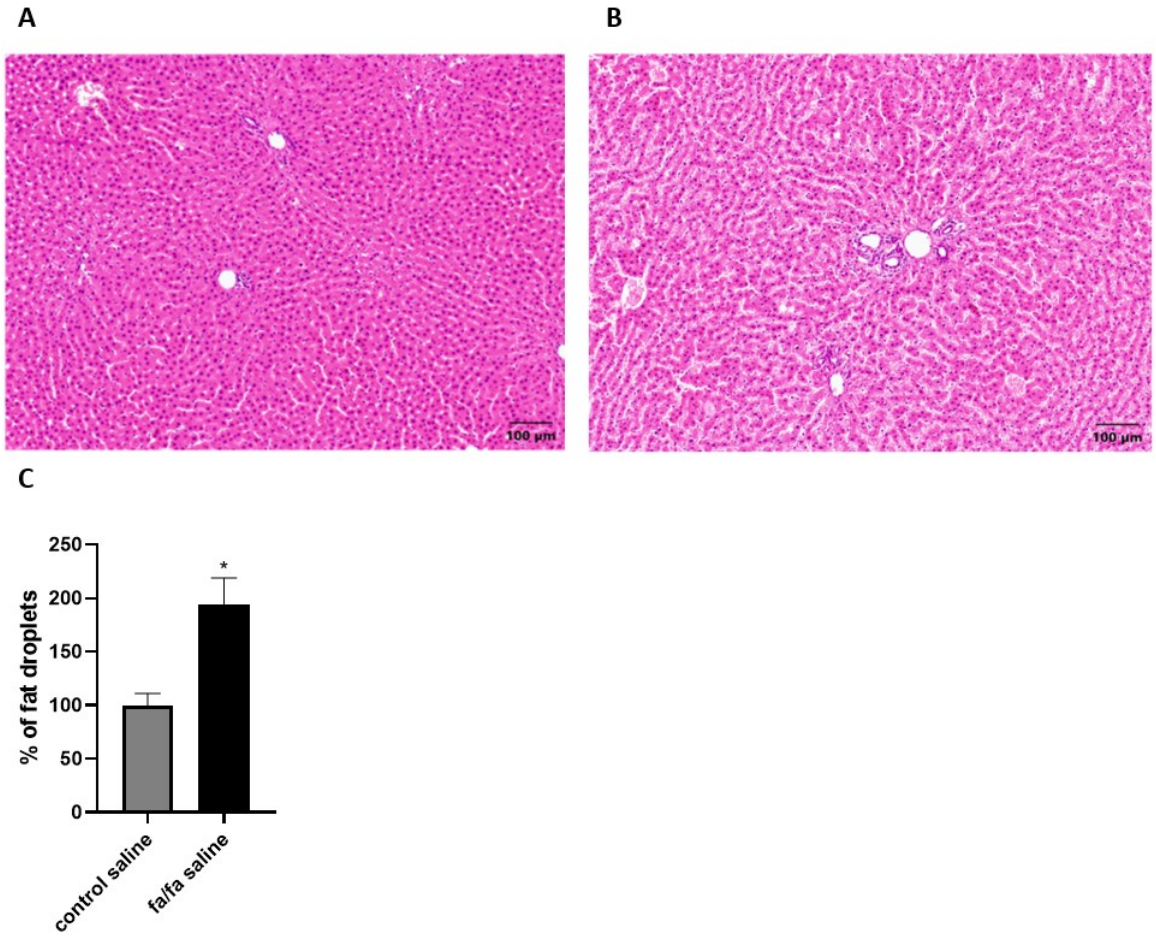
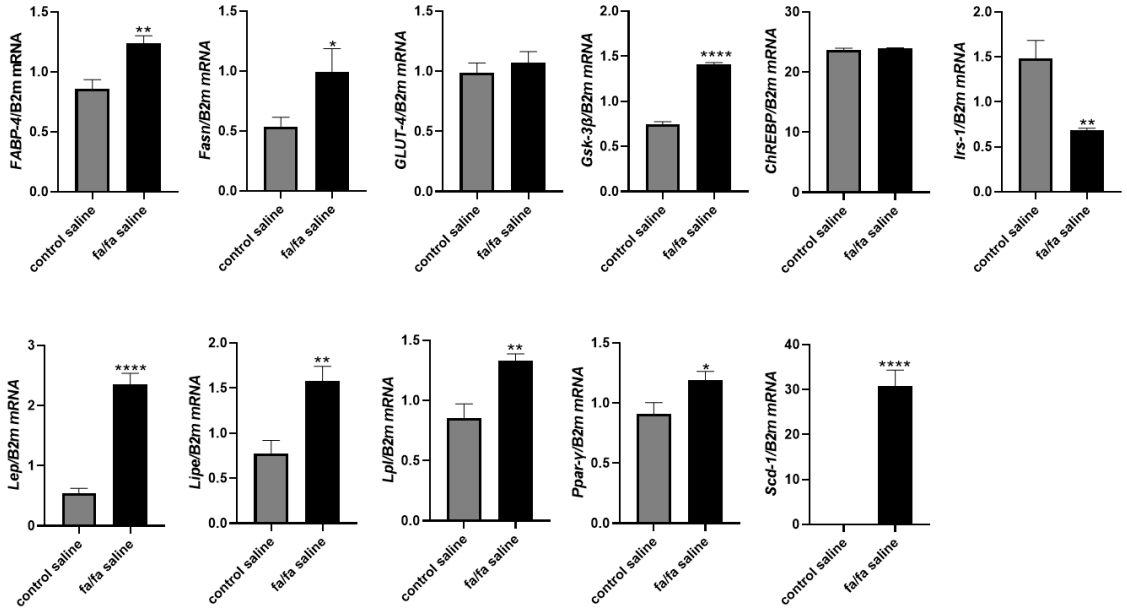


Figure 24: Liver histology, representative photomicrographs of control saline (A) and fa/fa saline (B). Quantification of liver steatosis (C) represented by percentage of fat droplets.

Eosin-hematoxylin staining. The magnification of photomicrographs is 200x. Data are means ± SEM determined by t-test. Significance is *$p < 0.05$ fa/fa saline *vs* control saline (n= 4).

mRNA expression of genes related to obesity and IR was measured in SCAT, IPAT and the liver of fa/fa saline and control saline rats and is shown in Figure 25 A-C. In SCAT, mRNA expression levels of lipases lipoprotein lipase (*LPL*) and lipase E (*LIPE*) were significantly higher in the fa/fa saline group than in the control saline group, and so was SCAT mRNA expression of *FABP4* and *PPARγ*. *Scd-1* mRNA expression was significantly higher in both SCAT and IPAT of the fa/fa saline group than in the control saline group, as was *Lep* mRNA expression. SCAT *Irs1* mRNA expression was significantly lower in fa/fa saline group than in the control saline group. In the liver, *Scd-1, SREBP-1* and *FABP4* mRNA expression was significantly increased and *PPARγ* expression was lower in the fa/fa saline group compared to the control saline group (Figure 25 A-C).

A



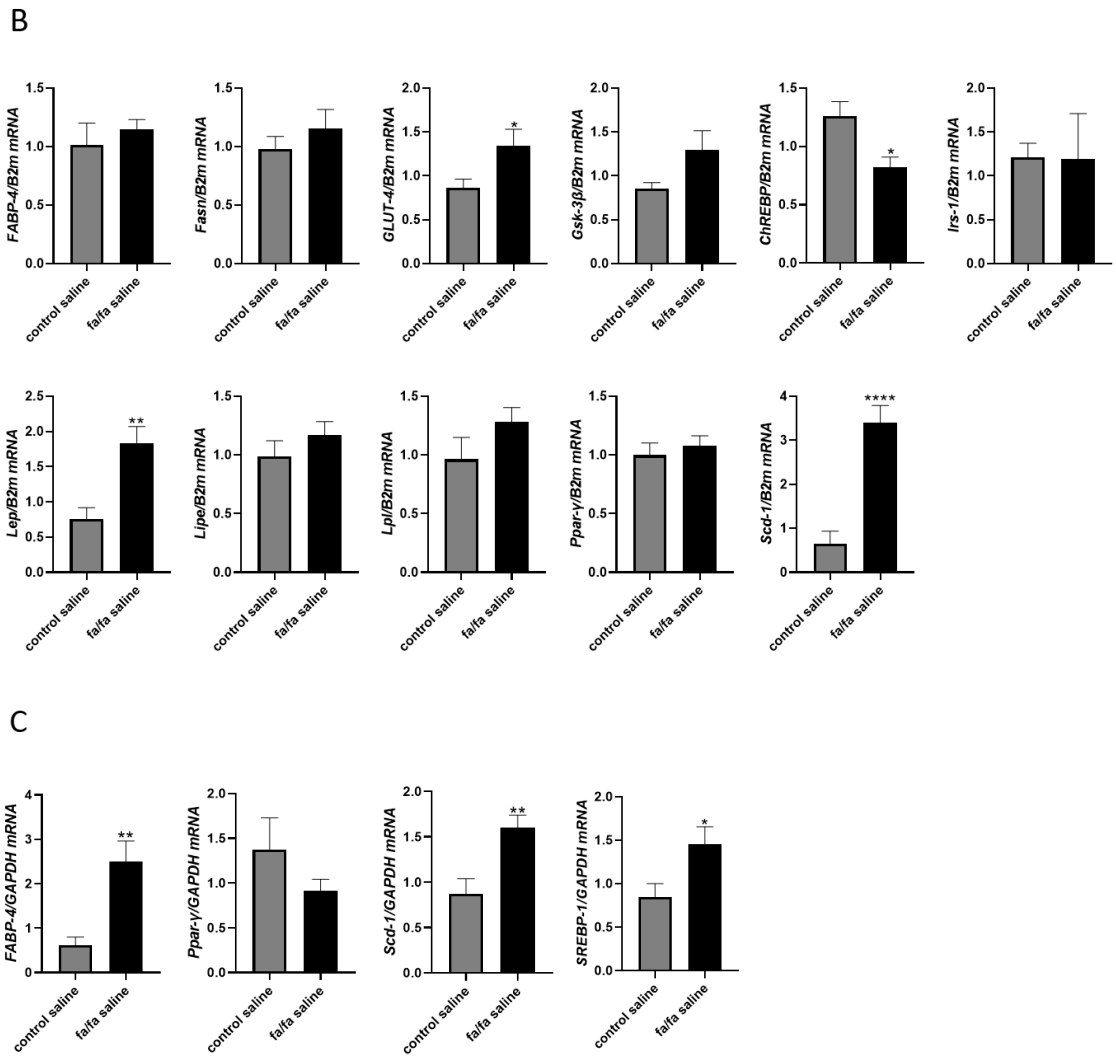


Figure 25: mRNA expression in SCAT (A), IPAT (B) and liver (C) of control saline and fa/fa saline rats.

Data are presented as means ± S.E.M. The data were normalized to *B2m* or to *GAPDH* and statistical analysis was performed by t-test. *FABP-4* fatty acid-binding protein 4, *Fasn* fatty acid synthase, *GLUT-4* glucose transporter type 4, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *Gsk-3β* glycogen synthase kinase 3 beta, *ChREBP* carbohydrate response element binding protein, *Irs-1* insulin receptor substrate 1, *Lep* leptin, *Lipe* lipase E, *Lpl* lipoprotein lipase, *Ppar-γ* peroxisome proliferator-activated receptor gamma, *Scd-1* stearoyl-CoA desaturase 1, *SREBP-1* sterol regulatory element binding protein. Significance is *p<0.05, **p<0.01, ****p<0.0001 fa/fa saline *vs* control saline (n= 7, 8).

In the hypothalamus, fa/fa saline rats had mildly worsened insulin resistance compared to the saline controls via significantly decreased pAkt (Ser473). Treatment with palm^{11}-PrRP31 reversed it to the saline control level. STAT3 phosphorylated at Tyr705 tended to be increased in the fa/fa palm^{11}-PrRP31 group compared to the fa/fa saline group (Figure 26).

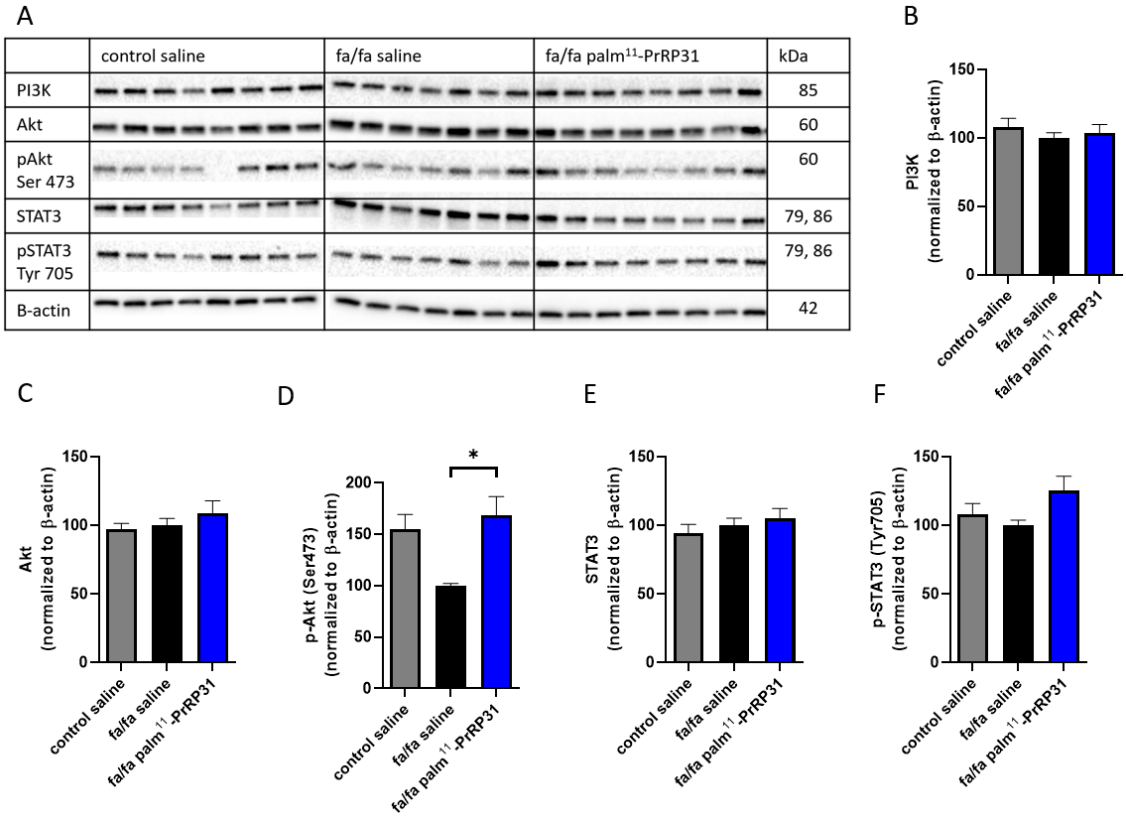


Figure 26: Insulin and STAT3 signaling pathways in hypothalamus of fa/fa rats.

Overview of western blots for specific proteins (A), quantification of western blots normalized to β-actin PI3K (B), Akt (C) pAkt (Ser473) (D), STAT3 (E), pSTAT (Tyr705) (F). Data are presented as means ± S.E.M. Statistical analysis was performed by one-way ANOVA with Dunnett´s *post hoc* test. PI3K total phosphoinositide 3-kinase, Akt protein kinase B, STAT3 total signal transducer and activator of transcription 3. Significance is *p<0.05 fa/fa saline *vs* control saline (n= 7, 8).

5. DISCUSSION

A fully effective preclinical model to understand pathophysiological mechanisms connected to obesity and related metabolic complications is still missing. DIO rodent models are similar to human obesity and metabolic syndromes, but genetic factors also play an important role in obesity development. Rodent models of obesity with disrupted leptin function develop more severe pathology, where long-term feeding with specific obesogenic diets is not needed. However, they are not as close to human obesity (Srinivasan and Ramarao, 2007;Fuchs et al., 2018) as DIO models.

Anorexigenic peptides, such as PrRP are promising tools for the treatment of obesity. Lipidization, such as palmitoylation of PrRP, leads to increased stability and half-life in organisms and allows its central anorexigenic effect after peripheral administration. Thus, palmitoylated PrRP analogs are good candidates for obesity treatment (Maletínská et al., 2015;Špolcová et al., 2015;Holubová et al., 2016;Pražienková et al., 2017;Čermáková et al., 2019).

In my Ph.D. thesis, the analog of prolactin-releasing peptide palmitoylated at position 11 (palm^{11}-PrRP31) was used for metabolic studies in three rodent models: DIO WKY rats with intact leptin and leptin receptor and in rodents with disrupted leptin signaling: in leptin deficient *ob/ob* mice and in *fa/fa* rats with leptin signaling disturbances.

5.1 Chronic effect of palm^{11}-PrRP31 on obesity and obesity-related metabolic parameters in DIO rodents or in rodents with disrupted leptin function

The design of the study with WKY rats fed an HF diet for one year was based on our previous two studies. In the study of (Holubová et al., 2016), SD rats were fed an HF diet for 24 weeks and treated with palm-PrRP31 for two weeks. During the intervention, palm-PrRP31 significantly decreased BW and FI, but at the end of the experiment, DIO SD rats treated with palm-PrRP31 showed only a non-significant decrease in obesity-related metabolic parameters. Similarly, in the following study WKY rats fed an HF diet for 15 weeks developed obesity and had worsened all measured metabolic parameters related to obesity compared to the WKY fed an LF diet before the intervention (Čermáková et al., 2019). Three weeks of treatment with

palm^{11}-PrRP31 significantly decreased BW, but only non-significantly improved insulin, leptin, and TG plasma levels (Čermáková et al., 2019). Therefore, we assumed that WKY fed for 52 weeks with an HF diet would develop more robust obesity, as well as metabolic syndrome features and hoped that the effect of palm^{11}-PrRP31 treatment would be more distinct. As we expected, WKY HF rats fed an HF diet for one year developed very significant obesity with increased BW and hyperleptinemia in comparison with those fed an HF diet for only 15 weeks (Čermáková et al., 2019). Moreover, six weeks of intervention with palm^{11}-PrRP31 in this study significantly decreased BW and FI and also significantly decreased plasma leptin levels (Table 6). Thus, in these two experiments with DIO rats with intact leptin and leptin receptor, the anorexigenic effect of palmitoylated PrRP31 analogs was demonstrated.

Table 6: Summary of morphometric and metabolic parameters in rats with HF diet induced obesity with intact leptin and leptin receptor or in rodents with disrupted leptin function (*ob/ob* mice and *fa/fa* rats) and impact of treatment with palm^{11}-PrRP31.

Parameters	WKY HF *vs* WKY LF	*ob/ob vs* WT	fa/fa *vs* controls	WKY palm^{11}-PrRP31 *vs* WKY HF	*ob/ob* palm^{11}-PrRP31 *vs* *ob/ob* saline	fa/fa palm^{11}-PrRP31 *vs* fa/fa saline
BW change	↑	↑	↑	↓	NS	NS
Cumulative FI	NS	NS	NS	↓	NS	NS
Liver weight	NS	↑	↑	NS	NS	NS
Leptin	↑	NT	↑	↓	NT	NS
TG	NS	NS	↑	NS	NS	NS
CHOL	NS	↑	↑	NS	↓	NS
Rectal temp.	NT	↓	NT	NT	NS	NT

Statistical analysis was performed by unpaired t-test, significance is shown as increase (↑), decrease (↓). BW body weight, FI food intake, TG triglycerides, CHOL cholesterol, NS not significant, NT non-tested.

It was previously demonstrated that central administration of PrRP and leptin has an additive effect on reduction of FI and BW, suggesting an interaction between PrRP and leptin in the metabolism and in FI regulation (Ellacott et al., 2002). To better explore the interaction between leptin and PrRP with regard to their anorexigenic effect, leptin deficient *ob/ob* mice were used in our study (Kořínková et al., 2020). Two experiments with mice of two different ages were performed. In experiment 1, younger mice in a metabolically active state were treated from 8 to 10 weeks of age. In experiment 2, older mice with established morbid obesity were treated from 16 to 24 weeks of age. In younger *ob/ob* mice, the combined treatment lowered liver weight but not body weight. In older ones, there was a change in continuous body weight, final body weight and subcutaneous fat weight. Treatment with one substance did not cause such effects. (Kořínková et al., 2020).

It is known that *ob/ob* mice are hypothermic (Ohtake et al., 1977;Gratuze et al., 2017), and in both experiments, *ob/ob* mice were more hypothermic compared to WT mice (Table 6) (Kořínková et al., 2020). Both leptin and palm^{11}-PrRP31 treatment alone did not affect the temperature but the combination leptin + palm^{11}-PrRP31 significantly upregulated the body temperature in older *ob/ob* mice, also indicating their synergistic action (Table 6).

Obese Zucker rats were first described in 1965 (Zucker, 1965) as the progeny of cross-bred Sherman and Merck Stock M 13 rat strains (da Silva et al., 1998;Cai et al., 2000). These rats develop the following symptoms: obesity as a result of hyperphagia and low physical activity, hyperinsulinemia, IR, glucose intolerance, hyperlipidemia and arterial hypertension (Van Zwieten et al., 1996). The recessive mutant gene "fatty" (*fa*) of the leptin receptor is the reason for the impaired leptin signaling (Takaya et al., 1996) and extreme obesity with juvenile-onset (Zucker and Antoniades, 1972).

The design of our study was based on our previous study with 12- and 33-week-old male *fa/fa* rats (Spolcova et al., 2014), where age- and obesity-induced peripheral insulin resistance under normoglycemia was described.

The main difference between the two studies was the source of rats used in the previous study (Špolcová, 2014). *fa/fa* rats from Harlan, now Envigo, Italy

(https://www.envigo.com/model/hsdhlr-zucker-leprfa) were used, and in the present study, we used *fa/fa* rats obtained from Charles River, France (https://www.criver.com/products-services/find-model/zucker-rat). Even though both providers declared the genetic background of both strains should be identical, appearance of the animals was different. The *fa/fa* rats obtained from Harlan were black-hooded, while *fa/fa* rats obtained from Charles River were agouti colored. Strain variability in Zucker rats has already been reported (Díaz-Silva et al., 2004), and considerable differences in phenotype and metabolic parameters of the strains from Harlan (Envigo) and Charles River with the same *fa/fa* mutation (*lepr*^{fa}) were described.

In obese Zucker *fa/fa* rats obtained from Harlan, obesity and hyperlipidemia were reported at 12- (Tomassoni et al., 2013) or 16- weeks old (Martinelli et al., 2020a). In the previous study (Spolcova et al., 2014), Harlan *fa/fa* rats developed obesity, but in the present study with Charles River rats, obesity was much less pronounced. The difference between strain variability in the Charles River (Martinelli et al.) and Harlan (Italy) obese *fa/fa* rats was previously identified in 8-week-old *fa/fa* rats and their lean controls. Total lipid and TG were higher in Charles River rats, while BW and CHOL levels were higher in the Harlan rats than in their respective controls (Díaz-Silva et al., 2004). In our present study, the Charles River *fa/fa* rats at 32 and 40 weeks of age had a higher plasma TG level but lower BW and CHOL levels (Table 6) than the Harlan rats of a similar age examined in our previous study (Spolcova et al., 2014).

In the study with ZDF rats (Holubová et al., 2016) with a non-functional leptin receptor, anti-obesity effect was not accomplished and only cholesterol was significantly decreased after palm-PrRP31 treatment (Holubová et al., 2016). In Koletsky SHROB rats (Mikulášková et al., 2018) with a nonsense mutation in leptin receptor, palm^{11}-PrRP31 affected neither BW nor related metabolic parameters (Mikulášková et al., 2018).

These data altogether suggest that intact leptin and leptin receptor are necessary for anorexigenic effect of lipidized PrRP analogs (Holubová et al., 2016;Mikulášková et al., 2018) and our study with *fa/fa* rats.

5.2 Chronic effect of palm^{11}-PrRP31 on type 2 diabetes-related biochemical parameters in DIO rodents or in rodents with disrupted leptin function

In our study, saline-treated WKY HF rats were normoglycemic, but had very significant glucose intolerance shown by OGTT which persisted even after 180 min after glucose load. Insulin resistance represented by HOMA index was significantly increased by an HF diet. A six-week treatment with palm^{11}-PrRP31 and liraglutide was able to ameliorate glucose intolerance, thus revealing strong antidiabetic effects of both peptides. Similarly as in our previous study with WKY rats fed only 15 weeks with an HF diet, that were also normoglycemic but glucose intolerant, and a 3-week treatment with palm^{11}-PrRP31 improved their tolerance to glucose (Čermáková et al., 2019). On the other hand, in the previous study, SD DIO rats were also normoglycemic but HF feeding significantly increased their glucose level without causing glucose intolerance. Insulin plasma levels were significantly increased in our present study as well as in the previous study (Čermáková et al., 2019) with WKY rats fed an HF diet. In both studies, palm^{11}-PrRP31 treatment did not significantly decrease the insulin level (Table 7). In the present study, HbA1c as a marker of long-lasting hyperglycemia was significantly higher in WKY HF saline compared to WKY LF saline and the treatment with palm^{11}-PrRP31 only tended to decrease it (Table 7).

In WKY HF rats, a glucose-lowering effect of palm^{11}-PrRP31 was found after an OGTT test in our present study, similarly as in the previous study (Čermáková et al., 2019).

Table 7: Summary of type 2 diabetes-related biochemical parameters in rodent model with intact leptin and leptin receptor, or in rodent models with disrupted leptin system function and impact of treatment with palm^{11}-PrRP31.

Parameters	WKY HF *vs* WKY LF	*ob/ob vs* WT	fa/fa *vs* controls	WKY palm^{11}-PrRP31 *vs* WKY HF	*ob/ob* palm^{11}-PrRP31 *vs* *ob/ob* saline	fa/fa palm^{11}-PrRP31 *vs* fa/fa saline
Glucose	NS	NS	↑	NS	NS	↑
HbA1c	↑	↑	NT	NS	NS	NT
Insulin	↑	↑	↑	NS	NS	NS
HOMA index	↑	NT	NT	NS	NT	NT

Statistical analysis was performed by unpaired t-test, significance is shown as increase (↑), decrease (↓). HbA1c glycated hemoglobin, NS not significant, NT non-tested.

In *ob/ob* mice, hyperglycemia was previously detected specifically between 5 and 16 weeks of age (Enser, 1972;Menahan, 1983), but 26-week-old *ob/ob* mice were normoglycemic (Gratuze et al., 2017). In the present study, (Kořínková et al., 2020), 10 weeks old *ob/ob* mice were hyperglycemic and a 2-week treatment with leptin, palm^{11}-PrRP31 and the combination of both lowered blood glucose significantly. On the other hand, 24-week-old *ob/ob* mice were normoglycemic and any treatment did not affect their blood glucose level (Table 7). HbA1c was significantly increased in 24-week-old *ob/ob* mice compared to the WT mice and it was not affected by any treatment (Table 7) (Kořínková et al., 2020). In older *ob/ob* mice, glucose intolerance was registered and no treatment improved it, similarly as in the study with 26-week-old *ob/ob* mice (Gratuze et al., 2017). It was previously reported that *ob/ob* mice were hyperinsulinemic (Menahan, 1983) and in the present study (Kořínková et al., 2020) *ob/ob* mice ages 10-24 weeks were hyperinsulinemic, while insulin plasma level was not affected by any treatments (Table 7).

Obese Zucker *fa/fa* rats obtained from Harlan, in a study by (Martinelli et al., 2020b) at 12-, 16-, and 20 weeks of age were normoglycemic, with significantly increased insulin plasma levels. On the other hand, 12-week-old *fa/fa* rats obtained from Harlan (Tomassoni et al., 2013) were hyperglycemic and had also significantly increased insulin levels. In our previous study (Spolcova et al., 2014), Harlan *fa/fa* rats developed obesity accompanied by significantly increased plasma insulin at normoglycemia, but glucose intolerance at 33 weeks of age (Spolcova et al., 2014). In the present study with Charles River rats, these parameters (glucose and insulin) were much less pronounced. Mild glucose intolerance and increased insulin levels were found in *fa/fa* rats compared to the controls (Table 7). The treatment with palm^{11}-PrRP31 did not improve tolerance to glucose and did not significantly decrease plasma insulin levels in *fa/fa* rats from Charles River. On the other hand, in two other rat strains with nonfunctional leptin signaling, ZDF rats (Holubová et al., 2016) and SHROB rats (Mikulášková et al., 2018),

glucose intolerance was obvious in comparison with their controls. Palm-PrRP31 treatment did not improve tolerance to glucose in ZDF rats, but palm^{11}-PrRP31 markedly improved glucose tolerance in SHROB rats (Mikulášková et al., 2018).

Thus, we can conclude that functional leptin signaling is necessary for the anorexigenic but not for anti-diabetic effects of palmitoylated PrRP31 analogs.

5.3 Effect of palm^{11}-PrRP31 on mRNA expression of enzymes regulating lipid and glucose metabolism in the adipose tissue and in the liver in DIO rodents or in rodents with disrupted leptin function

Lipid metabolism in IPAT and SCAT was slightly affected in the WKY HF saline group compared to WKY LF saline (Table 8A, B) in the present study. mRNA expression of transcription factor of lipogenic enzymes, *SREBP-1* was significantly increased in WKY HF saline compared to WKY LF saline in IPAT. *Scd-1* plays a major role in lipid biosynthesis and its mRNA expression was significantly increased in WKY HF saline in comparison with WKY LF saline in IPAT and in SCAT, but it was not affected by palm^{11}-PrRP31 treatment. Similarly, mRNA expression of genes involved in lipogenesis were increased in SCAT of WKY on an HF diet in our previous study but palm^{11}-PrRP31 treatment decreased it (Čermáková et al., 2019). Even though no liver steatosis was found in the WKY HF saline group, liver lipid metabolism was shown to be affected in WKY HF saline in the present study (Table 8C). mRNA expression of genes involved in lipogenesis, *SREBP-1*, *Acaca*, *Fasn*, *Scd-1* was significantly increased in the liver in WKY HF saline similarly as in our previous study (Čermáková et al., 2019). The treatment with PrRP palmitoylated analogs significantly attenuated mRNA expression of these genes similarly as in our previous studies (Holubová et al., 2016;Čermáková et al., 2019).

Thus, palmitoylated PrRP31 analogs decreased *de novo* lipogenesis in the liver and in adipose tissues in rodent models with DIO.

Table 8 Summary of mRNA expression analyzed in IPAT (A), SCAT (B) and in the liver (C) in DIO rodents or in rodents with disruption in leptin system function

A) IPAT

Gene	WKY HF vs WKY LF	*ob/ob* vs WT	fa/fa vs controls	WKY palm^{11}-PrRP31 vs WKY HF	*ob/ob* palm^{11}-PrRP31 vs *ob/ob* saline	fa/fa palm^{11}-PrRP31 vs fa/fa saline
Acaca	NS	NT	NT	NS	NT	NT
Cpt-1	↑	NT	NT	NS	NT	NT
FABP-4	↑	NT	NS	NS	NT	NT
Fasn	NS	NT	NS	NS	NT	NT
GLUT-4	↑	NT	↑	NS	NT	NT
Gsk-3β	NT	NT	NS	NT	NT	NT
Chrebp	NT	NT	↓	NT	NT	NT
Irs-1	↓	NT	NS	NS	NT	NT
Lep	NT	NT	↑	NT	NT	NT
Lipe	NT	NT	NS	NT	NT	NT
Lpl	NS	NT	NS	NS	NT	NT
Pck-1	NT	NT	NS	NT	NT	NT
Ppar-γ	↑	NT	NS	NS	NT	NT
Scd-1	↑	NT	↑	NS	NT	NT
SREBP-1	↑	NT	NT	NS	NT	NT

B) SCAT

Gene	WKY HF vs WKY LF	*ob/ob* vs WT	fa/fa vs controls	WKY palm^{11}-PrRP31 vs WKY HF	*ob/ob* palm^{11}-PrRP31 vs *ob/ob* saline	fa/fa palm^{11}-PrRP31 vs fa/fa saline
Acaca	NS	NT	NT	NS	NT	NT
Cpt-1	NS	NT	NT	NS	NT	NT
FABP-4	NS	NT	↑	NS	NT	NT
Fasn	NS	NT	↑	NS	NT	NT
GLUT-4	↑	NT	NS	NS	NT	NT
Gsk-3β	NT	NT	↑	NT	NT	NT
Chrebp	NT	NT	NS	NT	NT	NT
Irs-1	NS	NT	↓	NS	NT	NT
Lep	NT	NT	↑	NT	NT	NT
Lipe	NS	NT	↑	NS	NT	NT
Lpl	NS	NT	↑	NS	NT	NT
Pck-1	NT	NT	NT	NT	NT	NT
Ppar-γ	↑	NT	↑	NS	NT	NT
Scd-1	↑	NT	↑	NS	NT	NT
SREBP-1	NS	NT	NT	NS	NT	NT

C) Liver

Gene	WKY HF vs WKY LF	*ob/ob* vs WT	fa/fa vs controls	WKY palm^{11}-PrRP31 vs WKY HF	*ob/ob* palm^{11}-PrRP31 vs *ob/ob* saline	fa/fa palm^{11}-PrRP31 vs fa/fa saline
Acaca	↑	↑	NT	↓	NS	NT
Cpt-1	NS	↑	NT	NS	NS	NT
FABP-4	NS	NT	↑	NS	NT	NT
Fasn	↑	↑	NT	↓	NS	NT
G6pc	NT	↑	NT	NT	NS	NT
Lpl	NS	NT	NT	NS	NT	NT
Pck-1	NT	↑	NT	NT	NS	NT
Ppar-α	NS	↑	NT	NS	NS	NT
Ppar-γ	NT	↑	NS	NT	NS	NT
Scd-1	↑	NT	↑	↓	NT	NT
SREBP-1	↑	NS	↑	NS	NS	NT

Statistical analysis was performed by unpaired t-test, significance is shown as increase (↑), decrease (↓). *Acaca* acetyl-CoA carboxylase, *FABP-4* fatty acid-binding protein 4, *Fasn* fatty acid synthase, *Cpt-1a* carnitine palmitoyltransferase 1a, *G6pc* glucose-6-phosphatase, *GLUT-4* glucose transporter type 4, *Gsk-3β* glycogen synthase kinase 3 beta, *ChREBP* carbohydrate response element binding protein, *Irs-1* insulin receptor substrate 1, *Lep* leptin, *Lipe* lipase E, *Lpl* lipoprotein lipase, *Pck-1* phosphoenol pyruvate carboxykinase 1, *Ppar-α/γ* peroxisome proliferatore-activated receptor alpha/gamma, *Scd-1* stearoyl-CoA desaturase 1, *SREBP-1* sterol regulatory element binding protein 1, NS not significant, NT non-tested.

Regarding *ob/ob* mice, liver steatosis at an increased liver weight was obvious at 14 weeks of age, and could be linked to *de novo* lipogenesis (Perfield et al., 2013). Similarly, younger mice in this study had significantly higher liver weight compared to control saline mice (Table 6) and showed fat droplets after hematoxylin-eosin staining. All leptin and palm^{11}-PrRP31 treated groups had regression of fat droplets but only leptin and combined leptin + palm^{11}-PrRP31 treatment significantly decreased liver weight (Kořínková et al., 2020). Increased hepatic *de novo* lipogenesis through increased mRNA expression of *Acaca* and *Fasn* was shown in *ob/ob* mice compared to WT controls (Table 8C). Leptin + palm^{11}-PrRP31 combination attenuated this *de novo* lipogenesis. Fatty acid oxidation demonstrated by mRNA expression of *Cpt1a* and *PCK1* was enhanced in *ob/ob* saline compared to WT saline and any treatment did not affect it. mRNA expression of *G6pc*, a key enzyme catalyzing glycogenolysis and gluconeogenesis was enhanced after combined leptin + palm^{11}-PrRP31 treatment (Table 8C).

The expression of genes related to lipid metabolism in SCAT and IPAT indicated increased lipid accumulation in the adipose tissue of the fa/fa saline rats compared with the control saline rats (Table 8A, B). In addition, the fa/fa saline rats showed significantly higher expression of *LPL,* which hydrolyses TG from extracellular lipoproteins translocated to adipocytes for storage, and *LIPE* mRNA expression in SCAT compared to control saline rats (Voruganti et al., 2010). Higher *FABP4* mRNA expression in fa/fa saline rats indicates an increase in the transfer of long-chain fatty acids into adipocytes which contribute to the import of fatty acids released by *LPL* from extracellular TG. Moreover, *Fasn* catalyzed the rate-limiting step of fatty acid synthesis *de novo,* and SCAT mRNA expression of *Fasn* was significantly higher in fa/fa saline rats than in controls, which could further enhance intracellular fatty acid levels (Voruganti et al., 2010). *Scd-1* plays a major role in lipid biosynthesis, catalyzing the rate-limiting step of monounsaturated fatty acid (MUFA) production, supporting TG deposition and thus contributing to the development of obesity. It is suppressed by functional leptin (Biddinger et al., 2006). Dramatically increased *Scd-1* mRNA expression was found in both the SCAT and IPAT of the fa/fa saline group (Table 8A, 8B). Similarly, in this study, obese fa/fa saline rats with a malfunctioning leptin receptor had significantly increased *lep* mRNA

expression in both SCAT and IPAT compared to the control saline group resulting from the inability of leptin to activate the leptin receptor properly. *Irs1* mRNA expression was significantly decreased in SCAT which indicates insulin resistance in SCAT, which precedes insulin resistance in the liver (Hotamisligil et al., 1993), (van der Heijden et al., 2015). Subsequently, mild liver steatosis was obvious through an increase in ectopic lipid storage resulting from higher *FABP4* mRNA expression in the fa/fa saline rats (Table 8C). This finding indicates an increase in the transfer of long-chain fatty acids into hepatocytes and an increase in mRNA expression of *Scd-1* in the fa/fa rats. These outcomes positively affected liver mRNA expression of *SREBP-1,* which is a transcription factor of lipogenic enzymes that contributes to increased production of fatty acids in the liver (Table 8C). Furthermore, low mRNA expression of *PPARγ*, a receptor activated by peroxisome proliferators, indicated attenuated peroxisomal beta-oxidation of fatty acids in the liver of fa/fa saline, which led to the accumulation of ectopic lipids in the liver (Biddinger et al., 2006).

5.4 Effect of palm^{11}-PrRP31 on hypothalamic signaling in DIO rodents or in rodents with disrupted leptin function

Although many brain systems participate in energy homeostasis, changes of hypothalamic function have great potential to participate in the genesis of obesity in DIO rodent models. It was previously shown that palmitoylated PrRP analogs impacted hypothalamic signaling by restoring the leptin receptor-induced PI3K pathway and increasing ERK phosphorylation as a result of increased leptin or PrRP receptor signaling (Holubová et al., 2016;Mikulášková et al., 2018). Similarly, as in our present study, palm^{11}-PrRP31 treatment increased ERK phosphorylation.

JAK-STAT and AMPK are two main anorexigenic hypothalamic pathways that were studied in hypothalamic signaling of *ob/ob* mice in experiment 2. Increased phosphorylation of STAT3 at Ser727 was observed after any peptide treatment compared to *ob/ob* saline (Kořínková et al., 2020). Simultaneously downregulated SOCS3 and positively regulated STAT3, suggested that leptin as well as palm^{11}-PrRP31 support JAK/STAT signaling in the hypothalami of *ob/ob* mice. Inhibition of AMPK activity is necessary for the anorexigenic

effects of leptin in the hypothalamus (Minokoshi et al., 2004). All three treatments significantly decreased hypothalamic AMPK phosphorylation in this study (Kořínková et al., 2020), which could mean that palm^{11}-PrRP31 could compensate for deficient leptin in *ob/ob* mice.

It has also been previously shown that intact leptin signaling in the hypothalamus was important for anorectic effect of leptin in *fa/fa* rats (Rahmouni et al., 2009). Activation of STAT3 which could further activate Akt was shown in the present study. *Fa/fa* rats had tendency to decrease phosphorylation of STAT at Tyr705 and Akt at Ser473 which could be an effect of insulin related signaling. It is accepted that leptin and insulin act together in the hypothalamus in order to target energy homeostasis (Thon et al., 2016). Tendency to increase phosphorylation of STAT3 at Tyr705 after treatment of *fa/fa* rats with palm^{11}-PrRP31 was observed with the following significantly increased phosphorylated Akt at Ser473 in *fa/fa* palm^{11}-PrRP31 compared to *fa/fa* saline rats.

6. SUMMARY

Anorexigenic neuropeptides, such as PrRP have potential as a treatment of obesity but in their natural form, they have several limitations after peripheral application, such as poor bioavailability, low stability and inability to cross BBB after peripheral administration. Modifications such as lipidization of PrRP make this peptide more stable and able to act centrally after peripheral administration.

Palmitoylated analogs of PrRP were previously designed and tested in our laboratory at IOCB. Their chronic effect was investigated in several mouse and rat models of obesity and glucose intolerance. Palm^{11}-PrRP31 exhibited strong anorexigenic and antidiabetic effects after peripheral administration as well as attenuated mRNA expression of lipogenetic enzymes in the liver and in adipose tissue.

Even though, it is evident that natural PrRP is not able to act centrally after peripheral administration, palmitoylated PrRP analogs such as palm^{11}-PrRP31 are able to accomplish their anorexigenic effect in the hypothalamus (Figure 27).

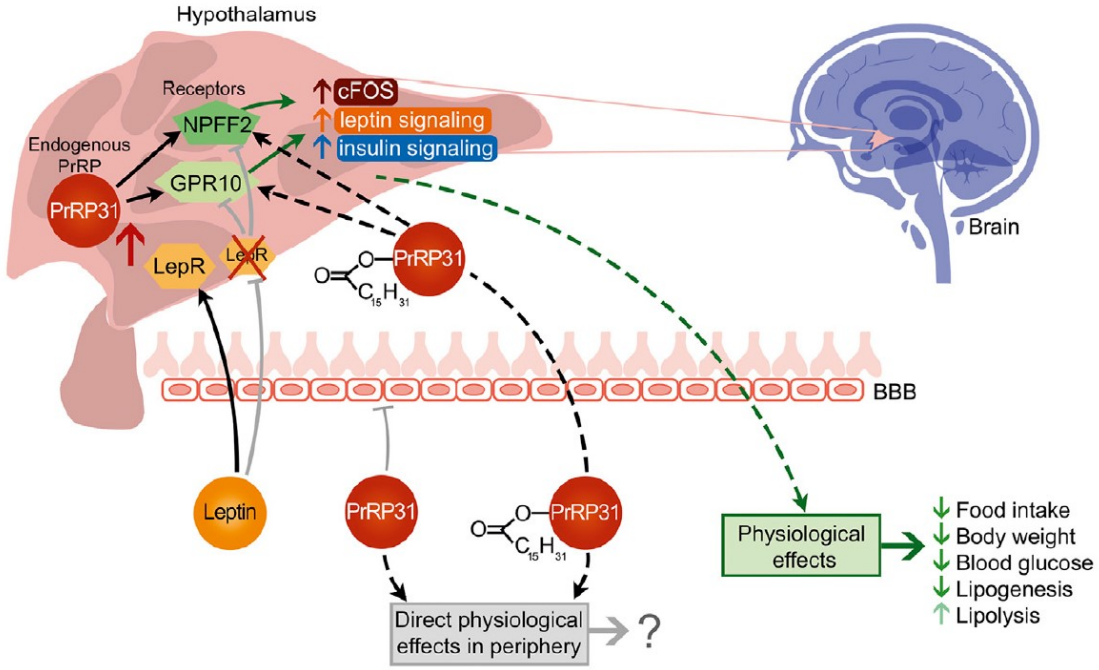


Figure 27: Proposed peripheral and central action of natural PrRP31 and its palmitoylated analog (modified from (Mráziková et al., 2021)).

WKY rats fed an HF diet develop DIO and are a model of the most common human obesity. After feeding with an HF diet for 52 weeks, WKY HF rats developed obesity with worsened metabolic parameters, strong glucose intolerance and increased mRNA expression of enzymes of *de nov*o liver lipogenesis. Chronic treatment with palm^{11}-PrRP31 had strong anorexigenic effect resulting in significantly decreased cumulative FI, BW and plasma leptin levels. A glucose-lowering effect of palm^{11}-PrRP31 was also found after the OGTT test, as well as decreased *de novo* liver lipogenesis.

Ob/ob leptin deficient mice develop early onset obesity with worsened related metabolic parameters and are hypothermic. Effects of chronic treatment of palm^{11}-PrRP31, leptin and the combination of these in *ob/ob* mice were studied at younger metabolically active age and *ob/ob* mice of older age. Leptin and palm^{11}-PrRP31 synergistically lowered liver weight and glucose levels in younger mice, decreased body and subcutaneous fat weight, decreased cholesterol level and increased body temperature in older mice. Production of SOCS3, an inhibitor of leptin signaling, was attenuated by action of the combined substances.

Fa/fa rats have obesity resulting from a disturbed leptin signaling. After palm^{11}-PrRP31 chronic treatment they neither lowered BW nor attenuated glucose tolerance and did not improve metabolic parameters related to obesity.

7. CONCLUSIONS

Action of palm^{11}-PrRP31, a lipidized analog of anorexigenic neuropeptide PrRP, on obesity and related metabolic disorders was followed in three rodent models of obesity: WKY rats with DIO resulting from HF diet having intact leptin and leptin receptor and two rodent models with obesity resulting from disrupted leptin function - leptin deficient *ob/ob* mice, and *fa/fa* rats with a disturbed leptin signaling.

Based on the results from this PhD thesis, we would like to conclude that the full effect of palm^{11}-PrRP31 on food intake and BW decrease depends on undisturbed leptin signaling. Palm^{11}-PrRP31 showed strong anorexigenic, body weight-reducing, glucose tolerance-improving and lipogenesis-attenuating effects in WKY rats fed with an HF diet for 52 weeks, where leptin resistance resulted from excessive adipose tissue but leptin signaling apparatus was functional. The fact that a BW-lowering effect of palm^{11}-PrRP31 was not accomplished in *fa/fa* rats with leptin signaling disruption supports this idea. Palm^{11}-PrRP31 and leptin showed synergistic effect in leptin-deficient *ob/ob* mice, both at a younger and older age where palm^{11}-PrRP31 could manifest its full anti-obesity effect only when leptin was supplemented simultaneously. Leptin and palm^{11}-PrRP31 synergistically lowered liver weight and glucose levels in younger mice, decreased body and subcutaneous fat weight, decreased cholesterol levels and increased body temperature in older mice. Production of SOCS3, an inhibitor of leptin signaling, was attenuated by action of the combined substances.

In conclusion, our data suggest a good efficacy of palmitoylated PrRP analogs in rodent models of diet-induced obesity with intact leptin and leptin receptor revealing an anti-obesity and antidiabetic effect together with decreased lipogenesis, increased lipolysis and hypothalamic insulin signaling. Thus, palmitoylated PrRP analogs are attractive candidates for anti-obesity and glucose-lowering treatment of the most common human – high energy diet-induced obesity.

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WEB SITES

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LIST OF MY PUBLICATIONS

Publications related to Ph.D. thesis:

1. **Kořínková L**, Holubová M, Neprašová B, Hrubá L, Pražienková V, Bencze M, Haluzík M, Kuneš J, Maletínská L, Železná B. Synergistic effect of leptin and lipidized PrRP on metabolic pathways in ob/ob mice. J Mol Endocrinol. 2020 Feb;64(2):77-90. doi: 10.1530/JME-19-0188. PMID: 31855558. **IF = 5.098**

2. Pirník Z, **Kořínková L**, Osacká J, Železná B, Kuneš J, Maletínská L. Cholecystokinin system is involved in the anorexigenic effect of peripherally applied palmitoylated prolactin-releasing peptide in fasted mice. Physiol Res. 2021 Aug 31;70(4):579-590. doi: 10.33549/physiolres.934694. Epub 2021 Jun 1. PMID: 34062082. **IF = 1.88**

3. **Mráziková L**, Neprašová B, Mengr A, Popelová A, Strnadová V, Holá L, Železná B, Kuneš J, Maletínská L. Lipidized Prolactin-Releasing Peptide as a New Potential Tool to Treat Obesity and Type 2 Diabetes Mellitus: Preclinical Studies in Rodent Models. Front Pharmacol. 2021 Nov 18;12:779962. doi: 10.3389/fphar.2021.779962. PMID: 34867411; PMCID: PMC8637538. **IF = 5.81**

4. **Mráziková L,** Hojná S, Popelová A, Hrubá L, Strnadová V, Neprašová B, Železná B, Kuneš J, Maletínská L. Palmitoylated prolactin-releasing peptide treatment had neuroprotective but not anti-obesity effect in fa/fa rats with leptin signaling disturbances. Nutr. Diabetes. After major revision. **IF = 5.097**

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2. Kořínková L, Pražienková V, Černá L, Karnošová A, Železná B, Kuneš J, Maletínská L. Pathophysiology of NAFLD and NASH in Experimental Models: The Role of Food Intake Regulating Peptides. Front Endocrinol (Lausanne). 2020 Nov 26;11:597583. doi: 10.3389/fendo.2020.597583. PMID: 33324348; PMCID: PMC7726422. **IF = 5.555**

3. Pražienková V, Funda J, Pirník Z, Karnošová A, Hrubá L, **Kořínková L**, Neprašová B, Janovská P, Benzce M, Kadlecová M, Blahoš J, Kopecký J, Železná B, Kuneš J, Bardová K, Maletínská L. GPR10 gene deletion in mice increases basal neuronal activity, disturbs insulin sensitivity and alters lipid homeostasis. Gene. 2021 Mar 30;774:145427. doi: 10.1016/j.gene.2021.145427. Epub 2021 Jan 12. PMID: 33450349. **IF = 3.688**

Commercial activities:

The research collaboration and license agreement between IOCB AS CR and IP AS CR and Novo Nordisk A/S was signed in August 2017, and I participated in this project.

SUPPLEMENT

1. Kořínková L, Holubová M, Neprašová B, Hrubá L, Pražienková V, Bencze M, Haluzík M, Kuneš J, Maletínská L, Železná B. Synergistic effect of leptin and lipidized PrRP on metabolic pathways in ob/ob mice. J Mol Endocrinol. 2020 Feb;64(2):77-90. doi: 10.1530/JME-19-0188. PMID: 31855558.

2. Pirník Z, Kořínková L, Osacká J, Železná B, Kuneš J, Maletínská L. Cholecystokinin system is involved in the anorexigenic effect of peripherally applied palmitoylated prolactin-releasing peptide in fasted mice. Physiol Res. 2021 Aug 31;70(4):579-590. doi: 10.33549/physiolres.934694. Epub 2021 Jun 1. PMID: 34062082.

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