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Autoreferát disertační práce

Lipidované analogy peptidu uvolňujícího prolaktin jako potenciální antiobezitika: zkoumání mechanizmu účinku

Lipidized analogs of prolactin-releasing peptide as potential agents for obesity therapy: search for mechanism of action

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

CONTENT

ABSTRAKT

Obezita je jedním z nejvíce rozšířených onemocnění na světě, ale její současná léčba má mnoho omezení. Peptid uvolňující prolaktin (PrRP) je neuropeptid snižující příjem potravy po podání do třetí mozkové komory, tuto schopnost však ztrácí po periferním podání. Lipidizace peptidů zvyšuje jejich stabilitu v krevním řečišti a usnadňuje jejich centrální účinek po periferním podání. V naší laboratoři byly vyvinuty lipidizované analogy PrRP, které mohou být perspektivním řešením pro léčbu obezity. Dříve jsme prokázali, že lipidizované analogy PrRP při periferním podání významně snižují příjem potravy a tělesnou hmotnost u myší. Palm-PrRP31 a palm^{11}-PrRP31 byly identifikovány jako klíčové analogy s těmito účinky a v této studii jsme se zaměřili na zkoumání mechanismu účinků těchto analogů in vitro.

Přirozený PrRP31 se váže ke svému receptoru GPR10 a s vysokou afinitou i na receptor pro neuropeptid FF 2. typu (NPFFR2), které jsou oba exprimovány v oblastech zapojených do regulace příjmu potravy. Palmitoylace PrRP31 zvýšila jeho vazebné a agonistické vlastnosti pro receptory GPR10 i NPFFR2. Lipidizované analogy také vykazovaly vyšší afinitu než přirozený PrRP31 k dalšímu receptoru neuropeptidu FF 1. typu (NPFFR1), což naznačuje, že NPFFR1 by mohl být novým potenciálním cílem lipidizovaných PrRP31 analogů. V buňkách exprimujících receptory GPR10, NPFFR2 a NPFFR1, byly studovány buněčné signální dráhy, které by mohly být základem účinků palmitoylovaných analogů PrRP31. Palmitoylované analogy PrRP31 stimulovaly aktivaci několika signalizačních kaskád, jako jsou MAPK, Akt a CREB, a transkripčních faktorů c-Foc a c-Jun, které se účastní regulace různých buněčných procesů, jako je progrese buněčného cyklu, migrace a diferenciace.

Druhá část práce byla zaměřena na *in vivo* experimenty zahrnující metabolickou fenotypizaci myší s deficitem NPFFR2 a myší s deficitem GPR10/NPFFR2, krmených buď standardní nebo vysokotukovou dietou, a jejich srovnáním s kontrolními myšmi. Delece receptoru NPFFR2 vedla ke glukózové intoleranci, která byla zhoršena vysokotukovou dietou. Navíc myši s vyřazeným NPFFR2 vykazovaly narušenou centrální signální dráhu PI3K/Akt. Delece receptorů GPR10 a NPPFR2 vedla ke změnám, které byly závislé na pohlaví a dietě vedoucím k prediabetickým symptomům. Při podávání stravy s vysokým obsahem tuků vykazovala obě pohlaví hyperinzulinémii. Navíc samice myší s deficitem GPR10/NPFFR2 také vykazovaly zhoršenou glukózovou toleranci a hyperglykémii, když byly krmeny vysokotukovou dietou.

ABSTRACT

Obesity is a common metabolic condition that is becoming more prevalent globally, but current treatments have limitations. Prolactin-releasing peptide (PrRP), a neuropeptide that reduces food intake after administration to the third ventricle, loses this ability when administered peripherally. However, lipidization of peptides enhances their stability in the bloodstream and facilitates their central effect after peripheral administration. We developed lipidized analogs of PrRP, which have high potential as a treatment option for obesity. We previously demonstrated that peripheral administration of lipidized PrRP analogs led to a substantial reduction in food intake and body weight in mice, with palm-PrRP31 and palm^{11}-PrRP31 emerging as key analogs. In this study, we aimed to further investigate the mechanisms underlying the effects of these two PrRP31 analogs *in vitro*.

Natural PrRP31 binds to its receptor GPR10 and with high affinity to neuropeptide FF receptor type 2 (NPFFR2), which are both expressed in regions involved in food intake regulation. The palmitoylation of PrRP31 increased their binding and agonist properties for both GPR10 and NPFFR2 receptors. Lipidized analogs exhibited a stronger affinity also for another neuropeptide FF receptor, NPFFR1, suggesting that NPFFR1 could be a new potential target for PrRP31 analogs. The molecular mechanisms underlying the effects of palmitoylated PrRP31 analogs on cellular signaling pathways were studied in cells expressing GPR10, NPFFR2, and NPFFR1. Palmitoylated PrRP31 analogs stimulated the activation of multiple signaling kinases such as MAPK, Akt, and CREB, and transcription factors c-Foc and c-Jun, which are involved in regulation of various cellular processes such as cell cycle progression, migration, and differentiation.

The second part of the thesis was focused on *in vivo* experiments involving metabolic phenotyping of NPFFR2-deficient and GPR10/NPFFR2-deficient mice, fed either standard or high-fat diets, and comparing them to age-matched wild type mice. We observed that deficiency of NPFFR2 resulted in glucose intolerance impaired on a high-fat diet. Moreover, NPFFR2 knock-out mice showed a disrupted central PI3K/Akt signaling pathway. Deletion of both GPR10 and NPFFR2 receptors resulted in sex-specific and diet-dependent changes leading to prediabetic symptoms. When fed a high-fat diet, both sexes exhibited hyperinsulinemia. Moreover, female GPR10/NPFFR2-deficient mice also showed impaired glucose tolerance and hyperglycemia when fed high-fat diet.

INTRODUCTION

Obesity is a prevalent and widespread disease caused by an abnormal or excessive accumulation of fat, with dysregulation of energy metabolism being a contributing factor. It is associated with several diseases, including inflammation, insulin resistance, and dyslipidemia (WHO European Regional Obesity Report, 2022). The central nervous system (CNS) is responsible for regulating the body's energy balance to maintain energy homeostasis (Morton et al., 2006).

The RF-amide neuropeptide family is characterized by the presence of a carboxy-terminal amino acid sequence (RF-NH_2), which is crucial for binding to receptors. The family shows N-terminal variation, resulting in a wide range of biological actions. RF-amide peptides have been shown to play a role in the regulation of food intake and energy homeostasis, making them potential targets for the treatment of obesity. The first RF-amide peptide discovered was FMRF-NH_2 in 1977, and the C-terminal amide sequence was later found in the neural systems of numerous species, including insects, fish, and mammals (Price and Greenberg, 1977). The mammalian RF-amide peptides family is subdivided into five groups encoded by five genes (Fukusumi et al., 2006).

NPFF (Neuropeptide FF) belongs into RF-amide neuropeptide family and it has 8 amino acids. NPFF specifically binds to the G-protein coupled receptors (GPCRs), NPFFR1 and NPFFR2, with a higher affinity towards NPFFR2 (Liu et al., 2001). NPFF was initially found to be involved in the regulation of pain and other physiological functions, including the development of tolerance to opioid drugs. Studies have shown that NPFF has both pro-opioid and anti-opioid effects, with some studies indicating that it can enhance the pain-relieving effect of morphine and others suggesting that it can reverse opioid analgesia induced by μ-receptor (MOR) (Desprat and Zajac, 1997; Liu et al., 2001; Yang et al., 1985). NPFF is produced in the hypothalamus, which suggests that it directly affects the hypothalamic neurons that regulate appetite and energy metabolism. NPFFR2 was found to be expressed mainly in the ascending tracts of the CNS and was detected in the hypothalamus, olfactory bulb, NTS, thalamus, amygdala, and spinal cord. The localization of NPFF and NPFFR2 expression suggests that the system is important for maintaining the energy homeostasis, stress response, and controlling the release of hormones (Bonini et al., 2000; Higo et al., 2021).

Studies have shown that central administration of NPFF decreases food intake and stimulates water intake in rats, while infusion of NPFF into the brainstem results in an increase in food intake (Murase et al., 1996; Sunter et al., 2001). NPFF also plays a role in insulin secretion and glucose tolerance, and NPFF-deficient mice show increased energy expenditure

and higher basal thermogenic activity. NPFF signaling is upregulated in energy deficiency situations, indicating its role in the regulation of energy metabolism (Zhang et al., 2021; Zhang et al., 2022). Study by Zhang *et* al. found that NPFFR2 has a role in controlling diet-induced adaptive thermogenesis through a hypothalamic neuropeptide Y (NPY) pathway which may have an impact on energy balance regulation (Zhang et al., 2018).

Prolactin releasing peptide (PrRP) is another member of RF-amide neuropeptide family. It was initially thought to stimulate prolactin release in mammals, but later studies showed that PrRP reduces food intake and increases energy expenditure in rodents when administered intracerebroventricularly (i.c.v) (Hinuma et al., 1998; Lawrence et al., 2000). PrRP binds to its receptor GPR10 and has a high binding affinity also to the NPFFR2 (Engstrom et al., 2003; Marchese et al., 1995). The prepropeptide of PrRP produces two peptides, PrRP31 and PrRP20, with identical C-terminal sequences that are highly conserved across mammalian species (Hinuma et al., 1998). PrRP-synthesizing neurons have been localized in various regions of the brain, including the NTS, thalamus, medulla oblongata, and hypothalamus (Hinuma et al., 1998; Iijima et al., 1999). GPR10 mRNA is widely distributed throughout the brain, including the thalamus, PVN, NTS, hypothalamus, and as well in the medulla oblongata (Fujii et al., 1999; Roland et al., 1999). Interestingly, PrRP expression was found to be increased in the NTS of mice fed a high-fat diet (HFD) and decreased in response to fasting (Zhang et al., 2018). I.c.v. administration of PrRP in male rats led to decreased food intake and body weight, and activated areas associated with feeding behavior (Bechtold and Luckman, 2006). PrRP and GPR10 were found to be important in cooperation with anorexigenic peptides cholecystokinin (CCK) and leptin in regulating food intake (Ellacott et al., 2003).

The role of PrRP and its receptor GPR10 in regulating body weight and food intake was investigated in various studies. Deficiency of PrRP led to obesity, while GPR10 KO mice showed increased food intake, elevated insulin levels, impaired glucose tolerance, and changes in gene expression connected to lipid metabolism (Bjursell et al., 2007; Gu et al., 2004; Prazienkova et al., 2021; Takayanagi et al., 2008). Centrally administered PrRP showed acute anorexigenic effect, but when administrated to GPR10 KO mice, no effect on food intake was observed (Bechtold and Luckman, 2006). These findings highlight the importance of PrRP and GPR10 in regulating energy balance and maintaining glucose and lipid homeostasis.

The use of peptides as potential therapeutics is limited by insufficient availability and low stability, and their modification is an essential strategy to improve their availability and stability. In the case of PrRP, multiple studies have been conducted to find the bioactive part of the peptide. The last seven amino acids at the C-terminal end are essential for PrRP peptide

function, while the Arg amino acids at positions 23 and 26 may be replaced without significantly reducing its affinity towards GPR10 (Boyle et al., 2005). To improve availability, stability, and prolong half-life of PrRP in an organism we decided to attach fatty acid. PrRP analogs lipidized with various fatty acids at N-terminus showed increased binding affinity towards receptors GPR10 and NPFFR2 (Maletinska et al., 2015). Moreover, previously we have shown that the peripheral administration of lipidized PrRP analogs led to a significant decrease in food intake in mice and increased c-Fos expression in various brain regions confirming their central effects (Pirnik et al., 2015; Pirnik et al., 2018; Prazienkova et al., 2017). Moreover, chronic studies in diet-induced obesity (DIO) mice peripherally treated with palm-PrRP31 (Fig. 1A) and palm^{11}-PrRP31 (Fig. 1B) for two weeks resulted in a significant decrease in cumulative food intake, body weight, fat mass, and plasma leptin levels (Maletinska et al., 2015; Prazienkova et al., 2017).

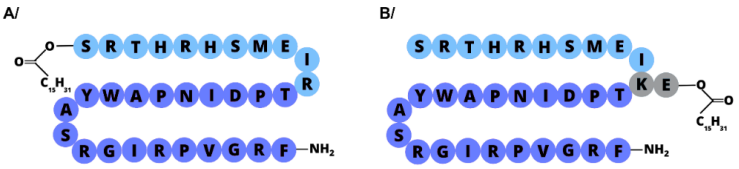

Fig. 1: Structure of lipidized PrRP31 analogs and human PrRP31 (purple and blue) and PrRP20 (purple). Palm-PrRP31 palmitoylated at position 1 (**A**), and palm^{11}-PrRP31 palmitoylated at position 11 on lysine though γ-glutamic acid (E) linker (**B**).

PrRP was found to have strong dual-agonist activity toward GPR10 and NPFFR2 receptors (Engstrom et al., 2003; Maletinska et al., 2015; Prazienkova et al., 2017). Even though PrRP was previously found to stimulate extracellular signal-regulated kinases (ERK) phosphorylation and the activation of cAMP response element-binding protein (CREB) in different cell lines, the mechanism of action of the PrRP signal transduction through GPR10 and NPFFR2 receptors is not fully understood yet (Kimura et al., 2000; Maixnerova et al., 2011; Maletinska et al., 2015).

1. AIMS OF THE THESIS

The first part of this thesis was dedicated to *in vitro* experiments where the goal was to specify the biological activity of lipidized PrRP31 analogs.

1. To compare the affinity of lipidized PrRP31 analogs and the natural PrRP31 peptide in cells transfected with GPR10 and NPFFR2.
2. To test and compare agonist properties of selected lipidized PrR31 analogs at GPR10 or NPFFR2 receptors.
3. To test and compare signaling transduction pathways of natural PrRP31 and its selected lipidized PrRP31 analogs in cells expressing GPR10 and NPFFR2.

4. To test the affinity of natural PrRP31 and its selected lipidized PrRP31 analogs for potential off-target receptors in cells transfected with these receptors.

The second part of this thesis was dedicated to *in vivo* experiments.

1. Metabolic phenotyping of NPFFR2-deficient mice of both sexes fed either standard diet (STD) or HFD and comparing them to age-matched wild-type (WT) control mice.
2. Metabolic phenotyping of GPR10/NPFFR2-deficient mice of both sexes fed either STD or HFD and comparing them to age-matched WT control mice.

2. METHODS

Human PrRP31, its lipidized analogs, and NPFF, and its stable analog 1DMe, were synthesized at the Institute of Organic Chemistry and Biochemistry as previously described (Prazienkova et al., 2017).

3.2 *In Vitro* Experiments

All cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2, and media were prepared according to the respective manufacturer's protocols.

The isolation of cell membranes from cells expressing either the NPFFR2, NPFFR1, and κ-opioid (KOR) receptor and was performed as described in (Karnosova et al., 2021). Shortly, pellets were homogenized using a DIAX 100 Homogenizer (Heidolph Instruments, Schwabach, DE) in ice-cold homogenizing buffer and centrifuged in an ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) at 26 000 x g for 15 minutes at 4°C. This process was repeated 2 times and final pellets were resuspended in ice-cold storage buffer and stored at -80°C. Isolated membranes were used at a concentration of 5 µg of protein/tube in saturation and competitive binding experiments. For saturation and competitive binding experiments with cells expressing GPR10 were cells seeded and the experiments were carried out in 24-well plates. Saturation and competitive binding experiments were carried out using iodinated [^{125}I]-labeled peptides and were performed according to (Maixnerova et al., 2011; Motulsky and Neubig, 2002). Isolated cell membranes or cells seeded in plate were incubated for 60 minutes at RT with 25 µl 0.1 nM [^{125}I]-labeled peptide and 25 µl peptide or lipidized analog at final concentrations from 10^{-12} to 10^{-5} M and for saturation experiments just concentration 10^{-6} M. Radioactivity was determined by a γ-counter Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Waltham, MA, USA).

The agonist properties of PrRP31 and lipidized PrRP31 analogs at final concentrations from 10^{-12} to 10^{-5} M were studied using cell lines stably expressing receptors GPR10, Y5, GHSR, and opioid receptors and containing beta-lactamase reporter genes to monitor β-arrestin recruitment. Experiments were performed according to (Prazienkova et al., 2017). The agonist

properties of PrRP31 and lipidized PrRP31 analogs at NPFFR2 were measured using the AequoScreen CHO-K1 cell line expressing aequorin and NPFFR2. The intracellular Ca^{2+} level was detected after the stimulation and luminescent light emission was recorded using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA, USA). The experiment was conducted according to Perkin Elmer's manufacturer protocol (Molecular Devices).

Activation of signaling pathways was studied in the Chinese Hamster Ovary (CHO)-K1 cell lines expressing GPR10 and NPFFR2. Cells were seeded and incubated with PrRP31, lipidized PrRP31 analogs, NPFF or 1DMe at final concentration 10^{-6} M for 5 minutes or 60 minutes at 37°C and washed with ice-cold phosphate-buffered saline pH 7.4 and subsequently lysed with Laemmli sample buffer. Electrophoresis and immunoblotting were performed as described previously in (Spolcova et al., 2015). The protein levels were normalized to the loading control protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.3 *In Vivo* Experiments

The animals were housed in the animal facility under standard conditions (12/12 light-dark cycle, temperature 22 ± 2°C) and had free access to water and either STD (Ssniff R/M-H diet; 8% kcal from fat, 21% kcal from protein, 71% kcal from carbohydrate; Ssniff Spezialdieten GmbH, Soest, Germany) or HFD (60% kcal from fats, 13% kcal from proteins and 27% kcal).

Mice with a genetic change that eliminates the functional *Npffr2* gene were created using the CRISPR genome-editing system. C57BL/6J *Gpr10*$^{-/-}$ (Prazienkova et al., 2021) and C57BL/6n *Npffr2*$^{-/-}$ mice, were crossbred to generate double knock-out mice (dKO) of both sexes. NPFFR2 KO and dKO mice were exposed to an STD or an HFD starting at the age of 9 and 8 weeks, respectively.

At 24 weeks of age, the NPFFR2 KO and GPR10/NPFFR2 KO mice were subjected to an oral glucose tolerance test (OGTT). At the end of the experiments at 26 weeks of age for NPFFR2 KO mice and 25 weeks of age for dKO mice, blood of mice in a randomly fed state was collected for plasma leptin detection, and mice were subsequently perfused with heparinized saline (20 U/ml) under pentobarbital anesthesia. Mice were dissected, and morphometric and biochemical analyses, as well as immunoblotting, were performed as previously described in (Prazienkova et al., 2021).

3. RESULTS

3.3 Search for Mechanism of Action of Lipidized PrRP31 Analogs *In Vitro*

We characterized lipidized PrRP31 analogs to find the best analog with strong dual GPR10-NPFFR2 agonist properties. Palm-PrRP31 and palm^{11}-PrRP31 were previously published in

(Maletinska et al., 2015; Prazienkova et al., 2017), and here we aimed to further characterized them.

Analogs palm^{11}-PrRP31 and palm-PrRP31 demonstrated a higher binding affinity for GPR10 and NPFFR2 compared to natural PrRP31 (Table 1). Additionally, it was found that palm^{11}-PrRP31 exhibited higher affinity towards GPR10 than for the NPFFR2 receptor. Furthermore, NPFF receptor, NPFFR1, was examined as a possible target or off-target of PrRP31 analogs. Palmitoylation increased the binding affinities of palm^{11}-PrRP31 and palm-PrRP31 for NPFFR1. Palm-PrRP31 displayed higher binding affinity then palm^{11}-PrRP31 not only towards NPFFR2 but also towards NPFFR1 receptor (Table 1).

Table 1: Binding affinities to CHO-K1 cell line expressing GPR10, NPFFR2, and NPFFR1.

Analog	GPR10 ^{125}I-PrRP31 K_i [nM]	% of PrRP31	NPFFR2 ^{125}I-1DMe K_i [nM]	% of PrRP31	NPFFR1 ^{125}I-1DMe K_i [nM]	% of PrRP31
PrRP31	4.58 ± 0.66	**100**	18.82 ± 2.28	**100**	40.39 ± 4.20	**100**
Palm-PrRP31	4.04 ± 0.01	**113**	0.45 ± 0.04	**4182**	0.78 ± 0.11	**5178**
Palm^{11}-PrRP31	3.52 ± 0.71	**130**	9.08 ± 1.14	**207**	21.04 ± 2.87	**192**
NPFF	>10 000	**-**	0.28 ± 0.06	**6721**	1.08 ± 0.09	**3740**
1DMe	>10 000	**-**	1.03 ± 0.23	**1827**	0.79 ± 0.06	**5113**

Data are presented as the mean ± SEM, performed in 2–5 independent experiments in duplicates, and analyzed in Graph-Pad Software. K_i values determined using Cheng and Prusoff equation (Cheng and Prusoff, 1973).

To determine the agonist characteristics of natural PrRP31 and its palmitoylated analogs, GPR10 activation was investigated using the beta-lactamase reporter gene assay. The 50% maximal effect (EC_{50}) of both PrRP31 analogs were in the range of 10^{-10} to 10^{-11} M. Lipidization increased the agonist properties of both palm-PrRP31 and palm^{11}-PrRP31. Palm^{11}-PrRP31 was found to have a stronger agonist activity at GPR10 than palm-PrRP31 (Table 2).

Both palmitoylated analogs showed increased agonist activity compared to natural PrRP31 at the NPFFR2 receptor, but agonists of NPFFR2 receptor NPFF and 1DMe promoted intracellular Ca^{2+} release at substantially lower concentrations (Table 2).

Table 2: Agonist properties of PrRP31 and its palmitoylated analogs at the GPR10 and NPFFR2.

Analogs	GPR10 EC_{50} [pM]	GPR10 % of PrRP31	NPFFR2 EC_{50} [nM]	NPFFR2 % of PrRP31
PrRP31	530 ± 70	**100**	89.33 ± 0.84	**100**
palm-PrRP31	72 ± 6	**736**	14.16 ± 1.52	**631**
palm^{11}-PrRP31	39 ± 5	**1359**	18.71 ± 1.31	**477**
NPFF	>10 000	**-**	0.24 ± 0.02	**37221**
1DMe	>10 000	**-**	0.82 ± 0.15	**10894**

Data presented as the means EC_{50} values ± SEM and analyzed by non-linear regression as log agonist versus response in Graph-Pad Prism and performed in 2–3 independent experiments.

The activation of potential off-target receptors was studied to establish agonist properties of natural PrRP31 and its two palmitoylated analogs using the beta-lactamase reporter gene assay. Natural PrRP31 displayed no agonist effect at KOR and other opioid receptors (Table 3). Moreover, for neither palm^{11}-PrRP31 nor palm-PrRP31 was observed agonist activity at the δ-opioid receptor (DOR), μ-opioid receptor (MOR), and opioid receptor like-1 (ORL-1). Palm^{11}-PrRP31 and palm-PrRP31 exert negligible agonist activity at the KOR (Table 3).

Table 3: Agonist properties of natural PrRP31 and its palmitoylated analogs at potential off-target receptors.

Receptor	KOR	DOR	MOR	ORL-1
		EC_{50} [nM]		
PYY				
U-50488	1.4 ± 1.0			
Deltorphin II		5.6 ± 9.9		
DAMGO			14.7 ± 1.9	
Nociceptin				3.8 ± 0.6
PrRP31	N	N	N	N
Palm^{11}-PrRP31	>10 000	N	N	N
Palm-PrRP31	>10 000	N	N	N

Data are presented as the mean ± SEM, performed in 2–3 independent experiments in duplicates, and analyzed analyzed by non-linear regression as log agonist versus response in Graph-Pad Software. N = no agonist properties.

In order to comprehend the mechanism of action of PrRP31 and its selected palmitoylated analogs to intracellular signaling pathways, CHO-K1 cells expressing GPR10 or NPFFR2 were employed.

The phosphorylation of protein kinase B (Akt) at Ser473 and Thr308 was measured in order to investigate the activation of Akt pathway. Interestingly, natural PrRP31 increased phosphorylation of Akt at both Ser473 (Fig. 2A) and Thr308 (Fig. 2B) in cells expressing NPFFR2. The phosphorylation of Akt at Ser473 and Thr308 was shown to be significantly increased by palm^{11}-PrRP31 and palm-PrRP31 in cells expressing GPR10 or NPFFR2 (Fig. 2).

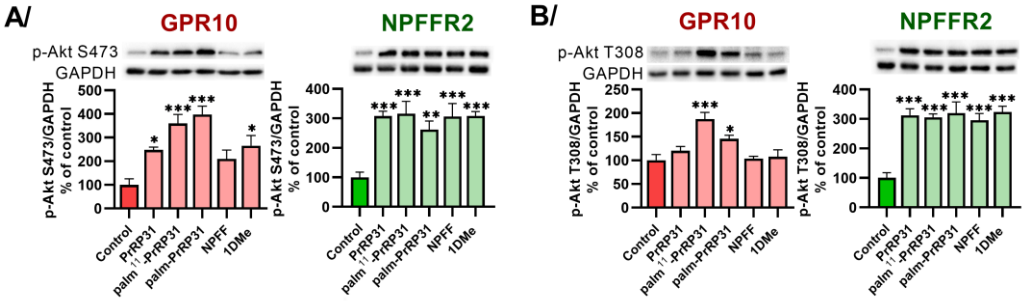

Figure 2: Akt activation by PrRP31 and its palmitoylated analogs in CHO-K1 cells expressing GPR10 and NPFFR2 receptors.
Phosphorylation of Akt (Ser473) (A) and Akt (Thr308) (B) after 5 minutes incubation at 37°C with peptides at final concentrations of 10-6 M. Densitometric quantification of the Western blots normalized to GAPDH and the phosphorylation level in the untreated control was standardized as 100%. Experiments were performed independently at least three times. Data are expressed as the mean ± SEM

and were determined by one-way ANOVA with Dunnett's post hoc test *p < 0.05, **p < 0.01, ***p < 0.001 for control vs stimulated cells.

The effects of PrRP31 and palmitoylated PrRP31 analogs on the activation of the mitogen-activated protein kinase (MAPK) pathway were investigated. Natural PrRP31 was able to activate MAPK intracellular signaling pathways in cells expressing GPR10 and NPFFR2. Stimulation with palmitoylated analogs palm-PrRP31 and palm^{11}-PrRP31 in cells expressing GPR10 and NPFFR2 receptors significantly increased the phosphorylation of extracellular signal-regulated kinase (ERK) (Fig. 3A) and the c-Jun N-terminal kinase (JNK) (Fig. 3B).

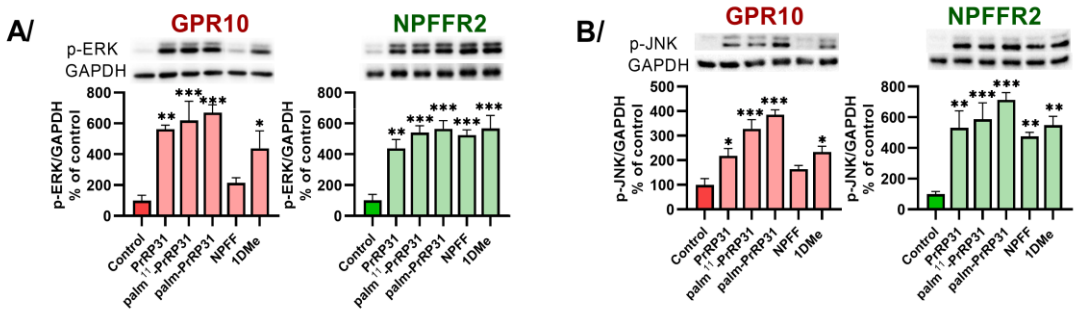

Figure 3: Activation of MAPK pathway by PrRP31 and its palmitoylated analogs in CHO-K1 cells expressing GPR10 and NPFFR2 receptors.
Phosphorylation of ERK (**A**) and JNK (**B**) after 5 minutes incubation at final concentrations of 10^{-6} M. Densitometric quantification of the Western blots normalized to GAPDH and the phosphorylation level in the untreated control was standardized as 100%. Experiments were performed independently at least three times. Data are expressed as the mean ± SEM and were determined by one-way ANOVA with Dunnett's post hoc test *p < 0.05, **p < 0.01, ***p < 0.001 for control vs stimulated cells.

The DNA-binding proteins c-Jun, and c-Fos protein, were examined for their potential to regulate cell proliferation, differentiation, and apoptosis. The results revealed that natural PrRP31 and both palmitoylated analogs, palm-PrRP31 and palm^{11}-PrRP31, showed significant activation of c-Jun (Fig. 4A) and c-Fos (Fig. 4B) in cells with GPR10 and NPFFR2.

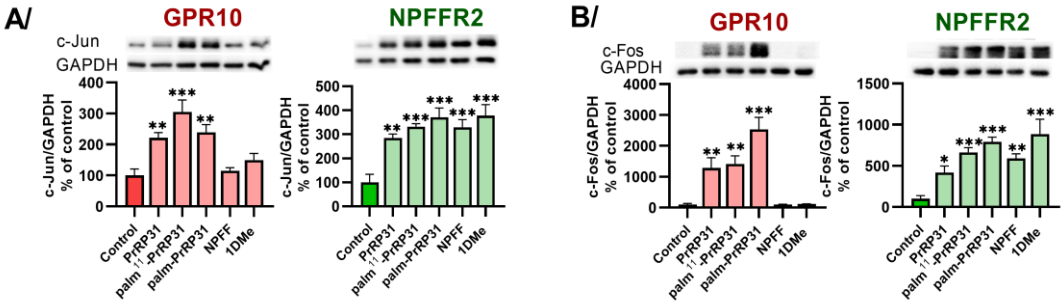

Figure 4: Activation of c-Fos and c-Jun by PrRP31 and its palmitoylated analogs in CHO-K1 cells expressing GPR10 and NPFFR2 receptors.
Activation of c-Jun (**A**) and c-Fos (**B**) after 60 minutes incubation at final concentrations of 10^{-6} M. Densitometric quantification of the Western blots normalized to GAPDH and the phosphorylation level in the untreated control was standardized as 100%. Experiments were performed independently at least three times. Data are expressed as the mean ± SEM and were determined by one-way ANOVA with Dunnett's post hoc test *p < 0.05, **p < 0.01, ***p < 0.001 for control vs stimulated cells.

3.4 Metabolic Phenotyping of NPFFR2-Deficient and GPR10/NPFFR2-Deficient Mice

The body weight was monitored throughout the entire experiment, and at the end of the study when the mice were 6 months old and in a state of unrestricted feeding with either STD or HFD, the weight of dissected organs was measured.

Male NPFFR2 KO mice showed significantly lower final body weight than WT, whereas female body weight did not differ between HFD-fed genotypes (Fig. 5A). NPFFR2 KO male mice fed HFD had significantly lower weights of both adipose tissues compared to their WT controls (Fig. 5B). These differences were not observed in the female mice (Fig. 5E). Moreover, the HFD led to a significant increase in plasma leptin levels (Fig. 5C, F). NPFFR2 KO male mice that were fed HFD had a lower plasma leptin level than the WT control mice (Fig. 5C). This is consistent with the observed lower body weights and adipose tissue weights.

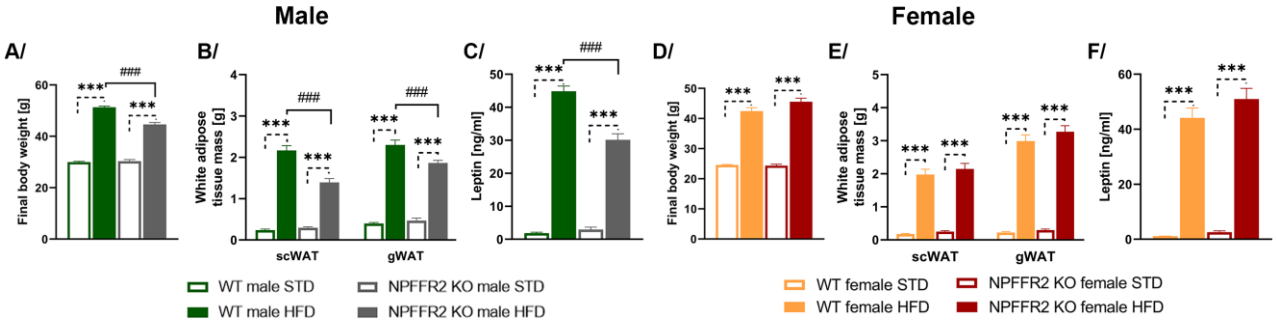

Figure 5: NPFFR2 KO male mice on HFD exhibit a reduced body weight compared to WT.
Final body weight (**A** and **B**) of NPFFR2 KO mice on a STD or HFD. Weights of dissected subcutaneous white adipose tissue (scWAT) and gonadal white adipose tissue (gWAT) in (**B**) males and (**E**) females. Serum leptin levels in (**C**) males and (**F**) females. All data are expressed as the mean ± SEM (n = 10). All Data were determined by one-way ANOVA with Bonferroni post hoc test. *** $p < 0.001$ for STD vs. HFD of the same genotype; ### $p < 0.001$ for NPFFR2 KO vs. WT mice on the same diet.

Glucose tolerance was measured by OGTT in NPFFR2-deficient mice. The absence of the *Npffr2* gene led to significantly higher glucose levels following glucose administration and higher AUC in both HFD-fed and STD-fed mice of both sexes (Fig. 6A, B, E, F). HFD-fed NPFFR2 KO mice showed significantly greater glucose excursions and AUC than their WT controls and NPFFR2 KO mice on STD (Fig. 6A, B, E, F). The fasted glucose levels were increased in WT and NPFFR2 KO mice on HFD, but no differences were observed between genotypes (Fig. 6C, G). Additionally, insulin levels were found to be increased in HFD-fed NPFFR2 KO and WT mice of both sexes than in their STD-fed controls (Fig. 6D, H).

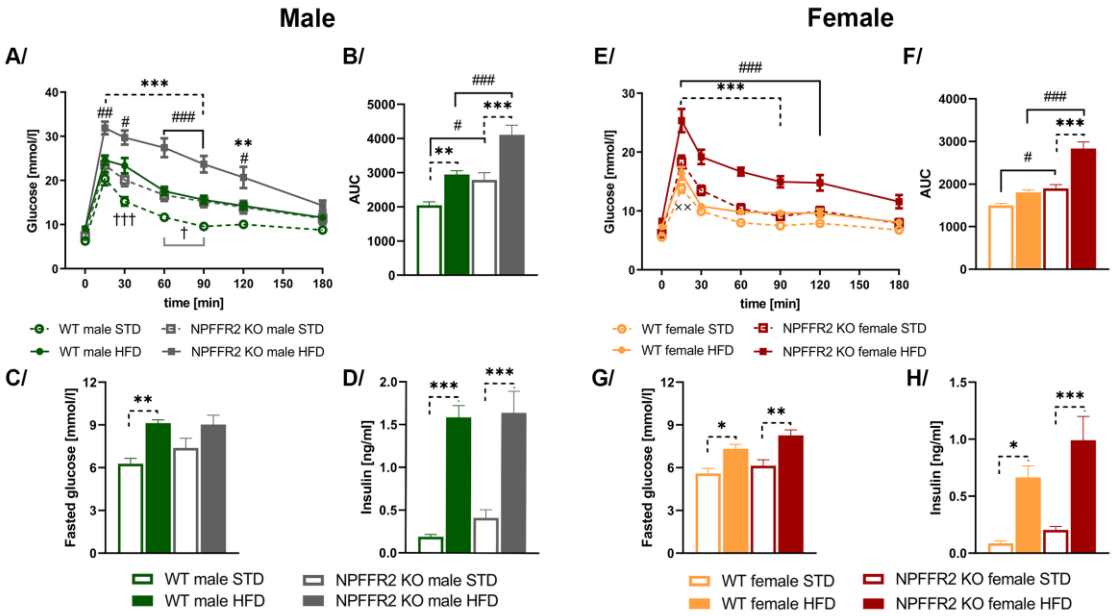

Figure 6: HFD-fed NPFFR2 KO mice exhibit severe glucose intolerance.
After oral glucose gavage (2 g/kg dosage), blood glucose excursions were seen in (**A**) males and (**E**) females. Data are expressed as the mean ± SEM (n = 10) and were determined by two-way ANOVA with Bonferroni post hoc test. † $p < 0.05$ and ††† $p < 0.001$ for WT mice fed a STD vs. HFD; ×× $p < 0.01$ for NPFFR2 KO vs. WT mice fed a STD; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ for WT mice vs. NPFFR2 KO mice fed a HFD; ** $p < 0.01$ and *** $p < 0.001$ for NPFFR2 KO mice fed a HFD vs. NPFFR2 KO mice fed a STD. Area under the curve (AUC) from OGTT for (**B**) males and (**F**) females. Fasted glucose levels in (**C**) males and (**G**) females and insulin levels in (**D**) males and (**H**) females. Data are expressed as the mean ± SEM (n = 7–10) and were determined by one-way ANOVA with Bonferroni post hoc test. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ for NPFFR2 KO vs. WT mice on the same diet; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for STD vs. HFD mice of the same genotype.

Male dKO mice fed STD had a considerably higher body weight than their WT controls (Fig. 7A). On the other hand, no significant changes were observed in body weight of dKO male mice fed HFD compared to their WT controls. Interestingly, dKO HFD-fed male mice exhibited significantly reduced white adipose tissue mass (Fig. 7B) and significantly decreased plasma leptin levels (Fig. 7C). Female dKO mice fed STD showed final body weight comparable to their WT control mice (Fig. 7D). However, dKO female mice fed HFD showed significantly increased body weight compared to their WT controls on HFD (Fig. 7D). Moreover, significantly increased gonadal white adipose tissue (gWAT) and subcutaneous white adipose tissue (scWAT) mass was observed in dKO female mice fed HFD (Fig. 7E) and as anticipated, they exhibited a significant increased plasma leptin levels (Fig. 7F).

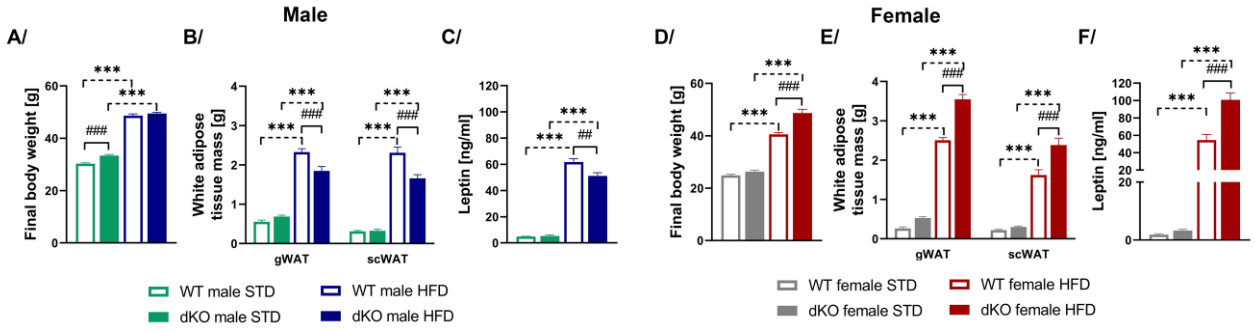

Figure 6: dKO female mice on HFD exhibit strong late-onset obesity.
Final body weight in (**A**) males and (**D**) females of GPR10/NPFFR2 KO (dKO) mice on a STD or HFD. Weights of dissected subcutaneous white adipose tissue (scWAT) and gonadal white adipose tissue (gWAT) in (**B**) males and (**E**) females. Serum leptin levels in (**C**) males and (**F**) females. Data are expressed as the mean ± SEM (n = 9-10) and determined by one-way ANOVA with Bonferroni post hoc test.

Interestingly, dKO male mice did not show significantly higher glucose excursion (Fig. 8A) and AUC (Fig. 8B) comparing to WT controls either on STD or HFD. Female dKO HFD-fed mice revealed significantly elevated blood glucose during the OGTT (Fig. 8C) and significantly higher AUC (Fig. 8E) compared to WT mice. Moreover, dKO female mice fed HFD showed significantly increased glucose levels after 6 hours of fasting followed by glucose gavage (Fig. 8F). This was not observed in dKO male mice (Fig. 8D). Furthermore, dKO mice of both sexes of dKO mice fed the HFD showed significantly elevated insulin levels in fasted plasma (Fig. 8G, H).

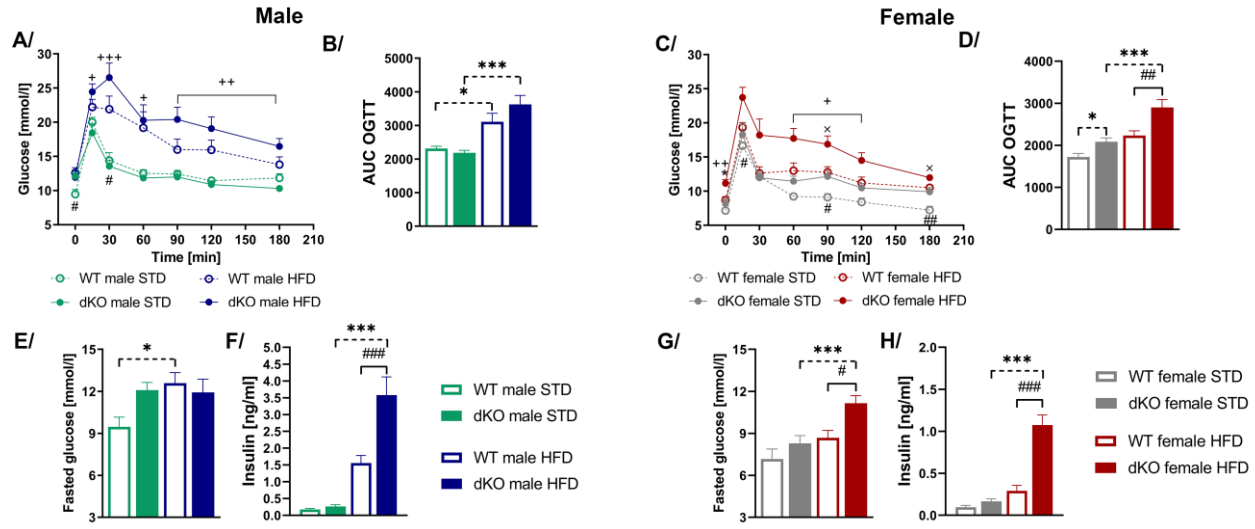

Figure 8: Female HFD-fed dKO mice exhibit severe glucose intolerance.
After oral glucose gavage (2 g/kg dosage) of WT and GPR10/NPFFR2 KO (dKO) mice, blood glucose excursions were seen in (**A**) males and (**C**) females. Data are expressed as the mean ± SEM (n = 10) and were determined by two-way ANOVA with Bonferroni post hoc test. × $p < 0.05$ for dKO vs. WT mice fed a STD; # $p < 0.05$, and ## $p < 0.01$ for WT mice fed a STD vs. WT fed a HFD; + $p < 0.05$, ++ $p < 0.01$ and +++ $p < 0.001$ for dKO mice fed a HFD vs. dKO mice fed a STD; * $p < 0.05$, for dKO mice fed a HFD vs. WT mice fed a HFD. Area under the curve (AUC) from OGTT for (**B**) males and (**E**) females. Fasted glucose levels in (**D**) males and (**F**) females. Data are expressed as the mean ± SEM (n =). Insulin levels in (**G**) males and (**H**) females and data are expressed as the mean ± SEM (n = 6–10).

Data were determined by one-way ANOVA with Bonferroni post hoc test. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ for dKO vs. WT mice on the same diet; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ for STD vs. HFD mice of the same genotype.

4. DISCUSSION

4.3 Search for Mechanism of Action of Lipidized PrRP31 Analogs *In Vitro*

Anorexigenic neuropeptides have a high potential to become a treatment option for obesity. Our previous acute studies have demonstrated a substantial reduction in food intake in mice after peripheral administration of lipidized PrRP (Maletinska et al., 2015; Prazienkova et al., 2017). Moreover, diet-induced obese (DIO) mice revealed reduced body weight after chronic administration of lipidized PrRP (Pirnik et al., 2018; Prazienkova et al., 2017). However, the exact mechanisms underlying the anorexigenic effect of lipidized PrRP31 analogs is not well understood. Therefore, we aimed to further investigate the mechanism of action of lipidized PrRP31 analogs.

PrRP31 has a high affinity for its receptor GPR10, but it also binds to another receptor from the RF-amide peptide family receptor, NPFFR2 (Engstrom et al., 2003; Maletinska et al., 2015). NPFFR1 and NPFFR2 share high amino acid sequence similarity (Bonini et al., 2000). Therefore, NPFFR1 was tested in this study as a potential target for the lipidized PrRP31 analogs. The palmitoylation enhanced the binding and agonist properties of both PrRP31 analogs to GPR10 and NPFFR2 receptors. The palm^{11}-PrRP31 analog displayed a stronger binding affinity for and agonist activity at the GPR10 receptor compared to the palm-PrRP31. They both revealed a stronger affinity towards NPFFR1 than natural PrRP31, hence NPFFR1 is now considered as a potential target of lipidized PrRP31 analogs. Targeting NPFFR1 with these analogs opened up new questions for the research of PrRP31 signaling and various physiological processes.

NPFF and its receptors are connected with regulation of pain nociception (Gicquel et al., 1992; Gouarderes et al., 1993). Although PrRP has a strong binding affinity for NPFF receptors, its ability to affect pain perception through NPFFR1 and NPFFR2 has not yet been determined. Previously, one study showed that PrRP reduced sensitivity to normally non-painful touch in rats (Kalliomaki et al., 2004). In our study, no agonist effect of PrRP31 palmitoylated analogs were seen at the MOR, DOR, and ORL-1 opioid receptors. Even though both analogs displayed very low affinity towards the KOR receptor, and they had only negligible ability to activate the KOR receptor, palm-PrRP31 showed stronger affinity and agonist activity towards KOR then palm^{11}-PrRP31. Overall, our results suggest that the potential pain-modulating effects of lipidized PrRP analogs would not be associated with opioid

receptors. Based on these result, palm^{11}-PrRP31 is a more suitable option than palm-PrRP31 for the potential treatment of obesity and neurodegenerative diseases due to its reduced potential for unwanted effects caused by increased off-target activity.

Using CHO-K1 cells transfected with GPR10 or NPFFR2 receptors the intracellular mechanism of action was studied. Hayakawa *et al.* previously demonstrated the activation of Akt in rat pituitary GH3 cells after 5 min of stimulation with PrRP (Hayakawa et al., 2002). In our study, we observed significant induction of Akt phosphorylation at T308 and S473 in CHO-K1 cells expressing GPR10 and NPFFR2 after 5 min of stimulation with palm^{11}-PrRP31 and palm-PrRP31. Previously it was reported that PrRP activates members of the MAPK family, JNK and ERK, in GH3 cells (Kimura et al., 2000). Our findings demonstrate that palmitoylated analogs stimulated the activation of ERK and JNK, members of the MAPK family, in cells expressing GPR10 and NPFFR2. MAPK are involved in a variety of cellular processes such as migration, cell growth, differentiation, and apoptosis (Sun et al., 2015). The phosphorylation of JNK and ERK can activate the transcription factors c-Fos and c-Jun, leading to the formation of the activator protein 1 (AP-1) complex. The formation of AP-1 complexes is required to permit G1/S transition and cell cycle progression and hereby can promote the cellular processes mentioned above (Cargnello and Roux, 2011). Significant activation of c-Fos and c-Jun was observed after the cells with GPR10 and NPFFR2 were stimulated with PrRP31 and its palmitoylated analogs (results published in (Karnosova et al., 2021)). The possible intracellular mechanism of action of lipidized PrRP31 analogs was suggested in the transduction pathway scheme (Fig. 9).

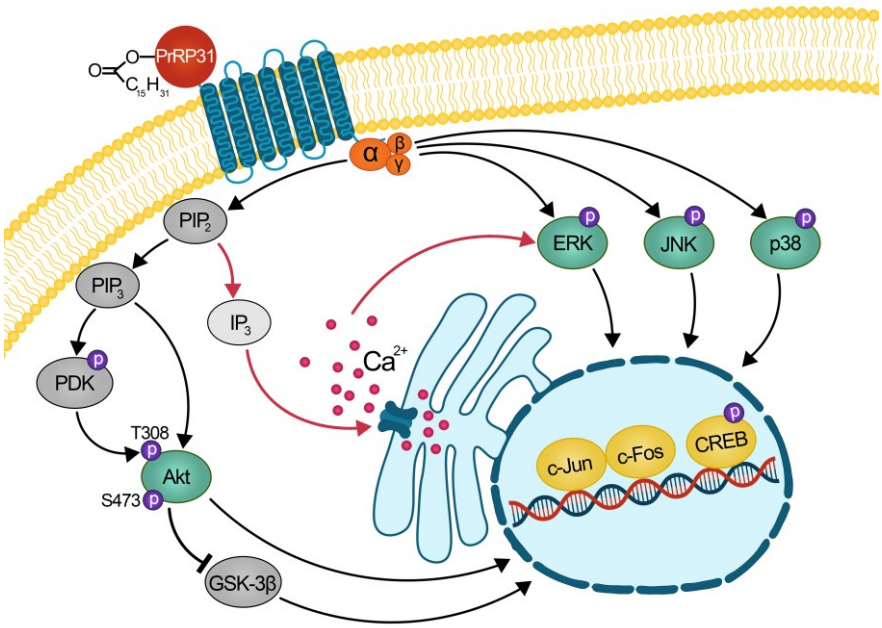

Figure 9: Scheme of mechanism of action of palmitoylated PrRP31 analogs (modified from (Karnosova et al., 2021)**.**

Black arrows symbolize mechanism of action through GPR10 and NPFFR2 and red arrows only through GPR10. ERK, extracellular-signal-regulated kinase; JNK, c-Jun N-terminal kinase; CREB, cAMP-responsive element binding protein; PIP2, phosphatidylinositol 4,5-bisphosphate; IP3, inositol 1,4,5-triphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PDK, phosphoinositide-dependent kinase 1; Akt, protein kinase B.

4.4 Metabolic Phenotyping of NPFFR2-Deficient and GPR10/NPFFR2-Deficient Mice

Because PrRP31 and its lipidized analogs exhibit strong dual agonist properties towards GPR10 and NPFFR2 receptors (Engstrom et al., 2003; Maletinska et al., 2015), we aimed to characterize the impact of NPFFR2 deletion and GPR10/NPFFR2 deletion in mice of both sexes fed either STD or HFD.

Both sexes of NPFFR2-deficient mice fed a STD revealed a lean phenotype. When given HFD, NPFFR2 KO male mice displayed reduced body weight, adipose tissue mass and liver weight, in addition to lower plasma leptin levels compared to WT controls. In contrary, study by Zhang *et al.* showed decreased body weight of male mice with NPFFR2 deletion fed STD, but when they fed HFD they revealed increased body weight, adipose tissue mass and leptin levels in serum (Zhang et al., 2018). It has been found that NPFF directly regulates glucose balance. NPFF deletion led to improved glucose tolerance and lower blood glucose levels. Moreover, Zhang *et al.* suggested that signaling of NPFF mediated by NPFFR2 in the central nervous system can modulate vagal output to peripheral tissues, including those crucial for controlling glucose metabolism (Zhang et al., 2022). This indicates that NPFFR2 could play a role in glucose homeostasis, and highlights the potential of NPFFR2 as a therapeutic target for metabolic disorders such as type 2 diabetes. Our study revealed a significant impaired glucose tolerance in NPFFR2 KO male and female mice of NPFFR2. Additionally, when given HFD, NPFFR2-deficient mice showed substantially greater glucose excursions compared to WT controls, without alterations in fasted glucose levels and plasma insulin levels between genotypes. The absence of NPFFR2 clearly resulted in a pronounced decrease in the ability to maintain glucose homeostasis, indicating the crucial role of this receptor in regulating blood glucose levels.

The outcomes of GPR10/NPFFR2-deficient mice correspond with the results obtained earlier from phenotyping studies with GPR10 KO (Prazienkova et al., 2021) and NPFFR2 KO mice. We previously showed that moderate obesity in GPR10 KO mice was more prominent in males than in females as a result of higher adiposity and increased plasma leptin level (Prazienkova et al., 2021). These results are in line with earlier studies (Bjursell et al., 2007; Gu et al., 2004). In this study, male dKO mice showed increased final body weight on STD, but when fed HFD the body and white adipose tissue weight was significantly decreased compared

to their WT controls. Moreover, deletion of both receptors resulted in late-onset obesity in female mice fed HFD. GPR10 KO mice on HFD exhibited significantly increased insulin plasma levels (Prazienkova et al., 2021). Hence, it is not unexpected that GPR10/NPFFR2-deficient mice showed impaired glucose tolerance on HFD along with elevated insulin levels. Increased insulin levels indicate compensatory mechanisms to overcome the reduced insulin sensitivity, which led to hyperinsulinemia.

5. CONCLUSIONS

PrRP represents one of the most promising candidates of anti-obesity neuropeptides. Our previous studies showed that peripheral administration of lipidized PrRP31 analogs led to a significant reduction in food intake and body weight in mice. Unfortunately, the mechanism of action of lipidized PrRP31 analogs remains unclear and here we aimed to further investigate it.

PrRP binds to its receptor GPR10 and with a high affinity also to the receptor NPFFR2. Palmitoylation increased the binding and agonist properties of two selected PrRP31 analogs, palm-PrRP31, and palm^{11}-PrRP, towards GPR10 and NPFFR2 receptors. Both analogs displayed a higher binding affinity for NPFFR1 than natural PrRP31, indicating NPFFR1 as a potential target for the lipidized analogs. Further studies are required to investigate the role of NPFFR1 in mediating the effects of the lipidized analogs. Palm^{11}-PrRP31 exhibited reduced off-target activity, indicating that it is a more suitable candidate for obesity treatment than palm-PrRP31. Moreover, we demonstrated that stimulation with these analogs led to significant phosphorylation of Akt at Thr308 and Ser473, as well as activation of MAPK family members ERK and JNK in cells expressing GPR10 and NPFFR2. We also observed significant activation of transcription factors c-Fos and c-Jun that form AP-1 complexes necessary for G1/S transition and cell cycle progression. These results provide insights into the intracellular mechanisms of palmitoylated PrRP31 analogs at GPR10 and NPFFR2 receptors.

To determine the effects on the particular receptors at targeting both GPR10 and NPFFR2 with lipidized PrRP31 analogs, we conducted the metabolic phenotyping experiments using NPFFR2-deficient and GPR10/NPFFR2-deficient mice fed either STD or HFD. The NPFFR2 deficiency resulted in glucose intolerance impaired on HFD. The prediabetic syndrome and sex-dependent late-on set obesity was observed in GPR10/NPFFR2 KO. These *in vivo* experiments demonstrated the importance of GPR10 and NPFFR2 receptors in regulating energy homeostasis and glucose metabolism and their targeting with lipidized PrRP31 analogs as a promising strategy for obesity treatment.

6. REFERENCES

Bechtold, D.A., Luckman, S.M., 2006. Prolactin-releasing Peptide mediates cholecystokinin-induced satiety in mice. Endocrinology. 147**,** 4723-9.

Bjursell, M., et al., 2007. GPR10 deficiency in mice results in altered energy expenditure and obesity. Biochem Biophys Res Commun. 363**,** 633-8.

Bonini, J.A., et al., 2000. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. J Biol Chem. 275**,** 39324-31.

Boyle, R.G., et al., 2005. Structure-activity studies on prolactin-releasing peptide (PrRP). Analogues of PrRP-(19-31)-peptide. J Pept Sci. 11**,** 161-5.

Cargnello, M., Roux, P.P., 2011. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. Microbiol Mol Biol Rev. 75**,** 50-83.

Desprat, C., Zajac, J.M., 1997. Differential modulation of mu- and delta-opioid antinociception by neuropeptide FF receptors in young mice. Neuropeptides. 31**,** 1-7.

Ellacott, K.L., et al., 2003. Repeated administration of the anorectic factor prolactin-releasing peptide leads to tolerance to its effects on energy homeostasis. Am J Physiol Regul Integr Comp Physiol. 285**,** R1005-10.

Engstrom, M., et al., 2003. Prolactin releasing peptide has high affinity and efficacy at neuropeptide FF2 receptors. J Pharmacol Exp Ther. 305**,** 825-32.

Fujii, R., et al., 1999. Tissue distribution of prolactin-releasing peptide (PrRP) and its receptor. Regul Pept. 83**,** 1-10.

Fukusumi, S., Fujii, R., Hinuma, S., 2006. Recent advances in mammalian RFamide peptides: the discovery and functional analyses of PrRP, RFRPs and QRFP. Peptides. 27**,** 1073-86.

Gicquel, S., et al., 1992. Analogues of F8Famide resistant to degradation, with high affinity and in vivo effects. Eur J Pharmacol. 222**,** 61-7.

Gouarderes, C., et al., 1993. Antinociceptive effects of intrathecally administered F8Famide and FMRFamide in the rat. Eur J Pharmacol. 237**,** 73-81.

Gu, W., et al., 2004. The prolactin-releasing peptide receptor (GPR10) regulates body weight homeostasis in mice. J Mol Neurosci. 22**,** 93-103.

Hayakawa, J., et al., 2002. Regulation of the PRL promoter by Akt through cAMP response element binding protein. Endocrinology. 143**,** 13-22.

Higo, S., Kanaya, M., Ozawa, H., 2021. Expression analysis of neuropeptide FF receptors on neuroendocrine-related neurons in the rat brain using highly sensitive in situ hybridization. Histochem Cell Biol. 155**,** 465-475.

Hinuma, S., et al., 1998. A prolactin-releasing peptide in the brain. Nature. 393**,** 272-6.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol. 22**,** 3099-108.

Iijima, N., et al., 1999. Cytochemical study of prolactin-releasing peptide (PrRP) in the rat brain. Neuroreport. 10**,** 1713-6.

Kalliomaki, M.L., et al., 2004. Prolactin-releasing peptide affects pain, allodynia and autonomic reflexes through medullary mechanisms. Neuropharmacology. 46**,** 412-24.

Karnosova, A., et al., 2021. Palmitoylation of Prolactin-Releasing Peptide Increased Affinity for and Activation of the GPR10, NPFF-R2 and NPFF-R1 Receptors: In Vitro Study. Int J Mol Sci. 22.

Kimura, A., et al., 2000. Prolactin-releasing peptide activation of the prolactin promoter is differentially mediated by extracellular signal-regulated protein kinase and c-Jun N-terminal protein kinase. J Biol Chem. 275**,** 3667-74.

Lawrence, C.B., et al., 2000. Alternative role for prolactin-releasing peptide in the regulation of food intake. Nat Neurosci. 3**,** 645-6.

Liu, Q., et al., 2001. Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception. J Biol Chem. 276**,** 36961-9.

Maixnerova, J., et al., 2011. Characterization of prolactin-releasing peptide: binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor. Peptides. 32**,** 811-7.

Maletinska, L., et al., 2015. Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. Int J Obes (Lond). 39**,** 986-93.
Marchese, A., et al., 1995. Cloning and chromosomal mapping of three novel genes, GPR9, GPR10, and GPR14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors. Genomics. 29**,** 335-44.
Morton, G.J., et al., 2006. Central nervous system control of food intake and body weight. Nature. 443**,** 289-95.
Motulsky, H., Neubig, R., 2002. Analyzing radioligand binding data. Curr Protoc Neurosci. Chapter 7**,** Unit 7 5.
Murase, T., et al., 1996. Neuropeptide FF reduces food intake in rats. Peptides. 17**,** 353-4.
Pirnik, Z., et al., 2015. Peripheral administration of palmitoylated prolactin-releasing peptide induces Fos expression in hypothalamic neurons involved in energy homeostasis in NMRI male mice. Brain Res. 1625**,** 151-8.
Pirnik, Z., et al., 2018. Repeated peripheral administration of lipidized prolactin-releasing peptide analog induces c-fos and FosB expression in neurons of dorsomedial hypothalamic nucleus in male C57 mice. Neurochem Int. 116**,** 77-84.
Prazienkova, V., et al., 2017. Impact of novel palmitoylated prolactin-releasing peptide analogs on metabolic changes in mice with diet-induced obesity. PLoS One. 12**,** e0183449.
Prazienkova, V., et al., 2021. GPR10 gene deletion in mice increases basal neuronal activity, disturbs insulin sensitivity and alters lipid homeostasis. Gene. 774**,** 145427.
Price, D.A., Greenberg, M.J., 1977. Structure of a molluscan cardioexcitatory neuropeptide. Science. 197**,** 670-1.
Roland, B.L., et al., 1999. Anatomical distribution of prolactin-releasing peptide and its receptor suggests additional functions in the central nervous system and periphery. Endocrinology. 140**,** 5736-45.
Spolcova, A., et al., 2015. Anorexigenic lipopeptides ameliorate central insulin signaling and attenuate tau phosphorylation in hippocampi of mice with monosodium glutamate-induced obesity. J Alzheimers Dis. 45**,** 823-35.
Sun, Y., et al., 2015. Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J Recept Signal Transduct Res. 35**,** 600-4.
Sunter, D., et al., 2001. Intracerebroventricular injection of neuropeptide FF, an opioid modulating neuropeptide, acutely reduces food intake and stimulates water intake in the rat. Neurosci Lett. 313**,** 145-8.
Takayanagi, Y., et al., 2008. Endogenous prolactin-releasing peptide regulates food intake in rodents. J Clin Invest. 118**,** 4014-24.
Yang, H.Y., et al., 1985. Isolation, sequencing, synthesis, and pharmacological characterization of two brain neuropeptides that modulate the action of morphine. Proc Natl Acad Sci U S A. 82**,** 7757-61.
Zhang, L., et al., 2018. Diet-induced adaptive thermogenesis requires neuropeptide FF receptor-2 signalling. Nat Commun. 9**,** 4722.
Zhang, L., et al., 2021. Lack of neuropeptide FF signalling in mice leads to reduced repetitive behavior, altered drinking behavior, and fuel type selection. FASEB J. 35**,** e21980.
Zhang, L., et al., 2022. Central NPFF signalling is critical in the regulation of glucose homeostasis. Mol Metab. 62**,** 101525.

Other references:

WHO European Regional Obesity Report 2022. Copenhagen: WHO Regional Office for Europe; 2022.

LIST OF MY PUBLICATIONS

Publications related to Ph.D. thesis:

1. Karnošová, A.; Strnadová, V.; Holá, L.; Železná, B.; Kuneš, J.; Maletínská, L. Palmitoylation of Prolactin-Releasing Peptide Increased Affinity for and Activation of the GPR10, NPFF-R2 and NPFF-R1 Receptors: In Vitro Study. *Int. J. Mol. Sci*. 2021, 22, 8904. https://doi.org/10.3390/ijms22168904. **IF_{2021} = 6.208**

2. Strnadová, V.; Karnošová, A.; Blechová, M.; Neprašová, B.; Holá, L.; Němcová, A.; Myšková, A.; Sýkora, D.; Železná, B.; Kuneš, J.; Maletínská, L. Search for lipidized PrRP analogs with strong anorexigenic effect: In vitro and in vivo studies. *Neuropeptides*. 2023, 98**,** 102319. https://doi.org/10.1016/j.npep.2022.102319. **IF_{2023} = 3.286**

Publications not related to Ph.D. thesis:

1. Holá, L.; Železná, B.; Karnošová, A.; Kuneš, J.; Fehrentz, JA.; Denoyelle, S.; Cantel, S.; Blechová, M.; Sýkora, D.; Myšková, A.; Maletínská, L. A Novel Truncated Liver Enriched Antimicrobial Peptide-2 Palmitoylated at its N-Terminal Antagonizes Effects of Ghrelin. *J Pharmacol Exp Ther.* 2022, 383(2), 129-136. https://doi.org/ 10.1124/jpet.122.001322. **IF_{2022} = 4.402**

2. 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, 774, 145427. https://doi.org/10.1016/j.gene.2021.145427.
   IF_{2021} = 3.913

3. 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.* 2020, 11, 597583. https://doi.org/10.3389/fendo.2020.597583. **IF_{2020} = 5.555**