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Lipidized Prolactin-Releasing Peptide as a New Potential Tool to Treat Obesity and Neurodegeneration: Preclinical Studies in Rodent Models

Komise pro obhajoby doktorských disertací v oboru “Biomedicína”

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Summary

Obesity is an escalating epidemic, but an effective non-invasive therapy is still scarce. For obesity treatment, anorexigenic neuropeptides are promising tools, but their delivery from the periphery to the brain is complicated because peptides have a low stability and limited ability to cross the blood-brain barrier.

As a group leader of the senior research group at the Institute of Organic Chemistry and Biochemistry (IOCB) named Pathophysiological mechanisms of food intake regulation, I am leading several projects focused on peptides, both anorexigenic and orexigenic, involved in the regulation of food intake, and their synthetic analogs. We search for mechanism of action of these peptides and their impact on processes leading to obesity, diabetes and neurodegeneration. The methodology of our group combines biochemical, pharmacological and physiological methods and aims at clarifying the relationships among peptides involved in development of obesity-related diseases in order to design potential therapeutics. In this thesis, I have summarized more than 10 years of our work in one of our most successful projects, research on lipidized analogs of prolactin-releasing peptide and their biological properties.

Recently discovered anorexigenic neuropeptides, such as prolactin-releasing peptide (PrRP), represent new trends in development of anti-obesity agents. They are released and acting directly in brain areas regulating food intake, but generally do not cross the blood-brain barrier if administered peripherally. We succeeded to design stable lipidized analogs of PrRP with prolonged acute anorexigenic effect after peripheral administration as shown in mice and rats. Repeated peripheral administration of the lipidized PrRP analogs resulted in long-lasting anti-obesity and antidiabetic effects in rodent models of diet induced obesity and insulin resistance. We proved that lipidization of PrRP might be an effective way to transmit the desired effect to the central nervous system after peripheral administration, for a potential treatment of obesity and related complications such as type 2 diabetes (T2D).

Besides an old age, T2D and obesity were shown to be a risk factors for Alzheimer's disease (AD), thus compounds with glucose lowering and/or anorexigenic properties were proposed to be neuroprotective. In our group, we studied a possible crosstalk between obesity, T2D and AD-like pathology and investigated if our novel lipidized PrRP analogs could have beneficial effect on neurodegeneration in several mouse models of AD-like pathology and their age-matched wild type controls. We demonstrated that lipidized PrRP analogs are potentially neuroprotective substances improving spatial memory, neurogenesis, synaptogenesis and attenuating neuroinflammation and two hallmarks of AD, Tau hyper-phosphorylation and β-amyloid plaques in different mouse models of AD-like neurodegeneration.

Based on our results and granted patents describing anti-obesity and glucose lowering effects of novel lipidized PrRP analogs, the Research collaboration and licence agreement between IOCB, Institute of Physiology and Danish pharmaceutical company Novo Nordisk was signed in 2017.

1. Introduction

References in bold blue refer to our original publications as a basis of this thesis.

Obesity, together with type 2 diabetes (T2D) and hypertension, is reaching pandemic level worldwide, and they are a prerequisite for the development of metabolic syndrome (MetS), which culminates in an increased risk of metabolic and cardiovascular diseases (Engin, 2017; Said et al., 2016; Tune et al., 2017; **Vaneckova et al., 2014**). Despite the tremendous efforts, there is still a lack of weight-lowering pharmacotherapies that would be both efficacious and safe for the long-term treatment (Kumar, 2019; Rodgers et al., 2012; Rose et al., 2019; Williams et al., 2020). One of the promising tools for the treatment of obesity and other related metabolic complications is anorexigenic peptides that are synthetized endogenously in the brain or in the gastrointestinal tract and act centrally to decrease energy intake. As demonstrated in experimental models, these peptides have minimal side effects during long-term anti-obesity treatment (Arch, 2015; Bray et al., 2016; Patel, 2015).

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

Some lipidized peptide-based drugs for treatment of diabetes or obesity have been introduced into the market, such as liraglutide, a palmitoylated agonist of glucagon-like peptide 1 (GLP-1) (Gault et al., 2011) which has been approved also for anti-obesity treatment in the USA and Europe (Saxenda). Very recently, a once-weekly injection of the lipidized GLP-1 agonist semaglutide was approved by the FDA for treatment of obesity (Wegovy). Both drugs were developed by Novo Nordisk. Several other peptidomimetics, including multitargeted molecules – dual and triple agonists targeting GLP-1, glucagon and gastric inhibitory peptide receptors – are in clinical trials as possible future anti-obesity drugs (Williams et al., 2020).

Recently discovered anorexigenic neuropeptides, e.g. prolactin-releasing peptide (PrRP) and cocaine- and amphetamine-regulated transcript (CART) peptide, as well as antagonists of orexigenic peptide ghrelin (**Fig. 1**) represent new trends in development of anti-obesity agents **(Kunes et al., 2016; Mikulaskova et al., 2016a)**.

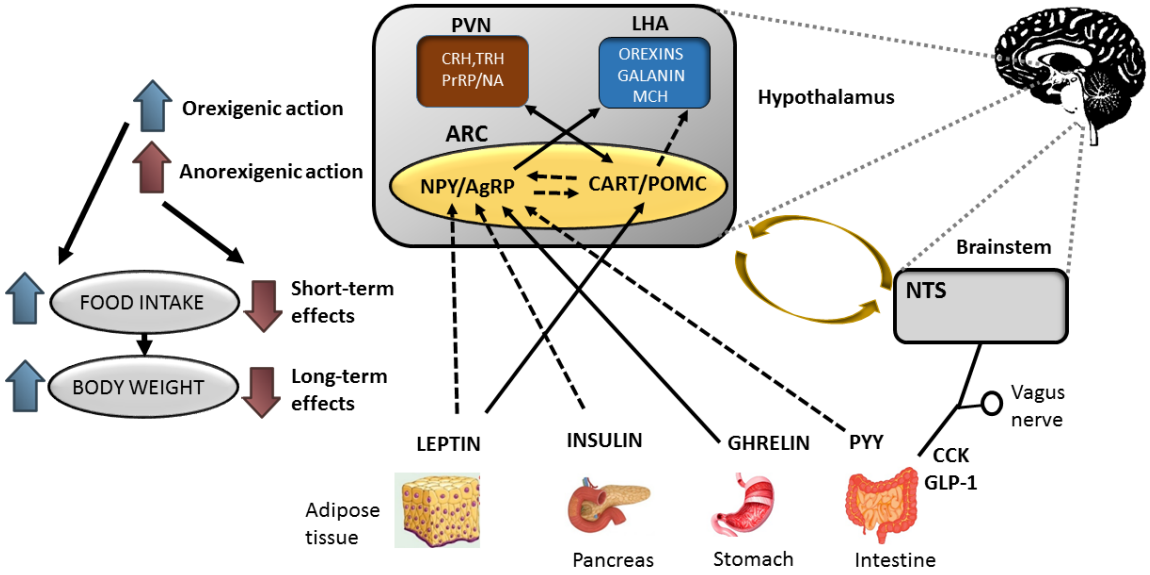

Fig. 1 The scheme of interrelationship between central and peripheral factors regulating food intake. The solid lines indicate a stimulatory effects and dotted lines indicate an inhibitory effects. PYY – peptide YY, PVN - paraventricular nucleus, LHA - lateral hypothalamic area, ARC - arcuate nucleus, NTS - solitary tract nucleus, CRH - corticotrophin-releasing hormone, TRH - thyrotropin-releasing hormone, PrRP - prolactin-releasing peptide, MCH - melanin-concentrating hormone, NPY - neuropeptide Y, AgRP - agouti-related peptide, CART -cocaine- and amphetamine-regulated transcript peptide, POMC - pro-opiomelanocortin, CCK - cholecystokinin, GLP-1 - glucagon-like peptide-1 **(Mikulaskova et al., 2016a)**.

1.1 Prolactin-releasing peptide and its role in food intake regulation

Prolactin-releasing peptide was initially isolated from the hypothalamus as a ligand for the human orphan G-protein coupled receptor (GPR10) (Hinuma et al., 1998) and a possible regulator of prolactin secretion from anterior pituitary cells. However, later findings suggested that prolactin release is not a primary function of PrRP (Jarry et al., 2000; Taylor and Samson, 2001). Shortly after its discovery, it was established that PrRP has other physiological functions, particularly it has been found to be involved in food intake, body weight (BW) and energy expenditure regulation (Atanes et al., 2021; Lawrence et al., 2000; Takayanagi et al., 2008). The fact that PrRP is

involved in food intake and BW regulation is supported by the detection of PrRP and its receptor GPR10 in brain areas involved in food intake regulation, such as several hypothalamic nuclei (e.g., nucleus arcuatus (Arc), paraventricular nucleus (PVN), dorsomedial nucleus (DMN)) and the brainstem (e.g., nucleus tractus solitarius (NTS)) **(Prazienkova et al., 2019a)**. PrRP was also found to have high affinity for the neuropeptide FF (NPFF)-2 receptor, resulting also in anorexigenic effects (Engström et al., 2003). It has also been shown in rodents that intracerebroventricular injection of natural PrRP decreased food intake and BW (Ellacott et al., 2003; Lawrence et al., 2000). Coadministration of PrRP and adipose tissue-born long-term acting regulator of energy balance leptin in rats resulted in additive reductions in nocturnal food intake and BW gain and an increase in energy expenditure (Ellacott et al., 2002).

There are two biologically active isoforms of PrRP, with either 20 (PrRP20) or 31 (PrRP31) amino acids. Both isoforms have a common sequence of 20 amino acids with C-terminal Arg-Phe-amide sequence, which is critical for their biological activity (Roland et al., 1999) (For sequences, see **Table 1**). It was recognized early that replacement of C-terminal amide with a carboxyl rendered PrRP inactive (Hinuma et al., 1998). An Ala scan showed the importance of the three Arg residues in positions 23, 26 and 30 and the critical importance of the C-terminal Phe amide for the biological activity of PrRP (Roland et al., 1999). C-terminal fragment of PrRP containing 13 amino acid (PrRP13) is sufficient for full binding potency to GPR10 receptor (Boyle et al., 2005). The results confirmed that the functionally important amino acids are located within the C-terminal heptapeptide, Ile-Arg-Pro-Val-Gly-Arg-Phe-NH_2, with Phe^{31} and Arg^{30} being particularly important. The aromatic ring of Phe^{31} may be modified, but not its spacing in relation to the backbone (Boyle et al., 2005).

Furthermore, both GPR10 knockout mice and PrRP-deficient mice developed late-onset obesity and exhibited a significant decrease in energy expenditure compared to wild-type mice (Bjursell et al., 2007). Moreover, PrRP-deficient mice also displayed increased food intake, lowering the cholecystokinin (CCK) and leptin signals (Takayanagi et al., 2008). Therefore, PrRP analogs and their receptor(s) might be new targets in obesity treatment.

2. Aims

The research work presented in this thesis is focused on understanding of a role of prolactin-releasing peptide and its analogs both at molecular level and in food intake and body weight maintenance and potential beneficial effects of lipidized PrRP analogs in rodent models of obesity, type 2 diabetes and neurodegeneration.

Specific aims:

- Design and structure-activity study of novel PrRP analogs both *in vitro* (binding to and signaling at specific receptors) and after acute administration into rodents (food intake and neuronal activation in lean mice and rats)
- Impact of lipidized PrRP analogs on metabolism of mice and rats with obesity and type 2 diabetes
- Effect of lipidized PrRP analogs on Alzheimer's disease-like pathology and neuroinflammation *in vitro* and in mouse models of neurodegeneration

3. Methods

All *in vitro* studies and *in vivo* studies with mouse models were carried out in my group at IOCB and animal facility of IOCB, respectively.

All methodologies are described in detail in the individual attached publications.

All peptide analogs used in our studies were synthesized at IOCB (M. Blechová) or in Apigenex, Prague. Palm^{11}-PrRP31 was in larger scale synthesized also in Polypeptide Laboratories (San Diego, CA, USA).

All studies with rat models as well as blood pressure measurement were performed in cooperation with Institute of Physiology AS CR (J. Kuneš, J. Zicha) or in Apigenex, Prague.

mRNA expressions of particular genes in tissues were determined in cooperation with Institute of Clinical and Experimental Medicine (M. Haluzík, Z. Lacinová) and Institute of Physiology (K. Bardová, J. Funda).

Measurement of neuronal brain activity in mice was performed in cooperation with Z. Pirník (Institute of Experimental Endocrinology, Slovak Academy of Sciences, Bratislava).

Studies in mouse models of neurodegeneration were performed in cooperation with University of Lancaster, UK (C. Holscher) and INSERM, Lille, France (M.-C. Galas).

Stability of peptides and pharmacokinetics were measured at the University of Chemistry and Technology, Prague (D. Sýkora, J. Zemenová).

Changes in brain lipids related to neurodegeneration were studied in cooperation with Mass spectrometry department at IOCB (V. Vrkoslav, Š. Strnad).

Metabolomics studies were performed at the Institute of Microbiology (H. Pelantová, M. Kuzma).

4. Results and Discussion

Presented results were obtained and published during about 10 last years under my leadership and represent the work of myself, my group and our collaborators. References in bold blue refer to our original publications as a basis of this thesis.

4.1 Structure-activity study of prolactin-releasing peptide analogs

Several publications describe a role of PrRP in food intake regulation and modulation of energy expenditure. Both PrRP and PrRP receptor knock-out mice show late-onset obese phenotype. Designing of PrRP analogs and their *in vitro* and *in vivo* evaluation in order to find potent and long-lasting analog(s) with selective anorexigenic properties could contribute to finding the mechanism and possible treatment of obesity and metabolic syndrome.

In our very first study with natural PrRP analogs **(Maixnerova et al., 2011)**, three rodent pituitary tumor-derived cell lines, RC-4B/C, GH3 and AtT20, were shown to possess high levels of the PrRP receptor, both by immunodetection and saturation specific binding with K_D in the nM range. PrRP31 and PrRP20 were equipotent in binding to these cells, while PrRP13 showed lower binding potency. All three PrRP analogs stimulated prolactin release and cAMP response element-binding protein (CREB) signaling very effectively and with comparable potency. However, only PrRP31 and PrRP20 had a significant central anorexigenic effect, as well as the effect on stimulation of mitogen-activated protein kinase/extracellular-regulated kinase (MAPK/ERK1/2). Thus, PrRP31 and PrRP20 rather than PrRP13 were approved as basic analogs for future structure-activity studies aimed at designing a stable PrRP analogs with high affinity to GPR10 receptor.

Based on our previous study and the structure-activity study of Boyle et al. (Boyle et al., 2005), PrRP20 analogs with modified C-terminal Phe were designed, synthesized and tested *in vitro* in RC-4B/C cells and *in vivo* in a feeding test with fasted mice after intracerebroventricular administration **(Maletinska et al., 2011)**. In this study, eight analogs of PrRP20 with C-terminal Phe amide modified with a bulky side chain or a halogenated aromatic ring revealed high binding potency, activation of

MAPK/ERK1/2 and CREB and prolactin release in RC-4B/C cells. In particular, [PheNO$_2^{31}$]PrRP20, [Tyr^{31}]PrRP20, [1-Nal^{31}]PrRP20, and [2-Nal^{31}]PrRP20 showed high binding affinity for the GPR10 receptor, activation of MAPK/ERK1/2 and CREB signaling, and a highly significant and long-lasting anorexigenic effect in fasted mice.

However, as a centrally released and centrally acting neuropeptide, natural PrRP has several limitations after peripheral administration: low stability in the organism to exert its central effect and inability to reach the target brain receptors.

To overcome these disadvantages, we designed analogs of PrRP lipidized at the N-terminal region, which is not essential for biological activity **(Kunes et al., 2016; Maletinska et al., 2015)**. Therefore, the N-terminus of both natural peptides, PrRP31 and PrRP20, was lipidized with fatty acids of different lengths in belief to preserve their full biological activity. Then, we have demonstrated that analogs of PrRP31 and PrRP20 lipidized by 8 to 18 carbon chain fatty acids showed high binding affinities with K_i in the nanomolar range to both GPR10 and NPFF2 receptors overexpressed in CHO cells **(Maletinska et al., 2015)**. Agonistic properties of the lipidized analogs of PrRP31 and PrRP20 were confirmed by an increased MAPK/EKR1/2 phosphorylation in CHO-K1 cells overexpressing GPR10. Structure of PrRP analogs and their binding affinities to GPR10 and NPFF2 receptors are summarized in **Table 1** (modified from **(Maletinska et al., 2015)**.

Table 1
Structures and binding affinities of PrRP analogs

Analog	Sequence	Human GPR10. ^{125}I-human PrRP31 binding K_i (nM)	Human NPFF2. ^{125}I-1DMe binding K_i (nM)	RC-4B/C cells. ^{125}I-rat PrRP31 binding K_i (nM)
PrRP31	SRAHQHSMETRTPDINPAWYTGRGIRPVGRF-NH_2	3.91 ± 0.21	42.21 ± 6.76	2.38 ± 0.11
oct-PrRP31	(N-oct)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2	1.49 ± 0.07	24.82 ± 13.2	0.98 ± 0.22
dec-PrRP31	(N-dec)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2	1.42 ± 0.55	14.73 ± 3.10	0.68 ± 0.12
dodec-PrRP31	(N-dodec)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2	1.15 ± 0.35	14.28 ± 6.40	0.38 ± 0.14
myr-PrRP31	(N-myr)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2	0.69 ± 0.09	1.59 ± 0.32	0.69 ± 0.09
palm-PrRP31	(N-palm)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2	2.94 ± 0.33	0.69 ± 0.36	0.51 ± 0.15
stear-PrRP31	(N-stear)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2	5.24 ± 0.57	15.92 ± 14.43	0.93 ± 0.08
PrRP20	TPDINPAWYTGRGIRPVGRF-NH_2	4.4 ± 0.77	21.80 ± 9.91	2.23 ± 0.19
oct-PrRP20	(N-oct)TPDINPAWYTGRGIRPVGRF-NH_2	1.88 ± 0.31	48.13 ±13.19	0.91 ± 0.23
dec-PrRP20	(N-dec)TPDINPAWYTGRGIRPVGRF-NH_2	2.94 ± 0.47	3.60 ± 2.57	0.41 ± 0.01
dodec-PrRP20	(N-dodec)TPDINPAWYTGRGIRPVGRF-NH_2	2.34 ± 0.25	9.97 ± 3.48	0.58 ± 0.22
myr-PrRP20	(N-myr)TPDINPAWYTGRGIRPVGRF-NH_2	4.21 ± 0.24	8.23 ± 1.97	1.02 ± 0.20

Nle – norleucine, oct – octanoyl, dec – decanoyl, dodec – dodecanoyl, myr – myristoyl, palm – palmitoyl, stear – stearoyl.
The means ± SEM of at least three separate experiments are shown. In competitive binding, K_i was calculated using the Cheng-Prusoff equation. The concentration of the radioligand was 0.1nM or 0.03nM, and the K_d that was calculated from saturation experiments was 4.21±0.66nM for RC-4B/C[26] or 0.95±0.20 nM for GPR10 receptor in CHO cells, respectively. K_d for NPFF2 receptor in CHO cells was 0.72±0.12nM.

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

Similarly, myristoylated and palmitoylated PrRP31 analogs with Phe^{31} replaced by aromatic non-coded amino acids or tyrosine revealed high binding affinity to rat pituitary RC-4B/C cells with endogenous both PrRP and NPFF2 receptors and to CHO-K1 cells overexpressing either GPR10 or NPFF2 receptors. The analogs also showed strong agonistic properties at the GPR10 receptor using the beta-lactamase reporter gene assay **(Prazienkova et al., 2016)**.

The anorexigenic potency of all lipidized PrRP analogs was tested *in vivo* in fasted and freely fed lean mice to determine whether the lipidization of PrRP could enable its central anorexigenic effects after peripheral administration. Only palm-PrRP31, stearoylated PrRP31 (stear-PrRP31) and myr-PrRP20 highly significantly and dose-dependently lowered food intake in lean overnight-fasted (**Fig. 2**) and freely fed mice after subcutaneous (SC) administration whereas analogs containing fatty acids with shorter carbon chains and the natural PrRP31 or PrRP20 had no effect on food intake **(Maletinska et al., 2015)**. These findings suggest that only palm- or stear-PrRP31 and myr-PrRP20 were able to overcome blood brain barried and exert their central effect on food intake. Similar efects followed intraperitoneal (IP) and intravenous (IV) administration of palm-PrRP31 in rats **(Mikulaskova et al., 2016b)**.

Analogously, lipidized PrRP31 analogs with modifications of the C-terminal amino acid showed significantly prolonged anorexigenic effects in fasted mice. Specifically, $PheCl_2^{31}$PrRP31 palmitoylated or myristoylated at N-terminus, showed strong long-lasting anorexigenic effect in fasted mice most probably owing a higher stabilization due to a non-coded C-terminal amino acid without a negative impact on their biological effect **(Prazienkova et al., 2016)**.

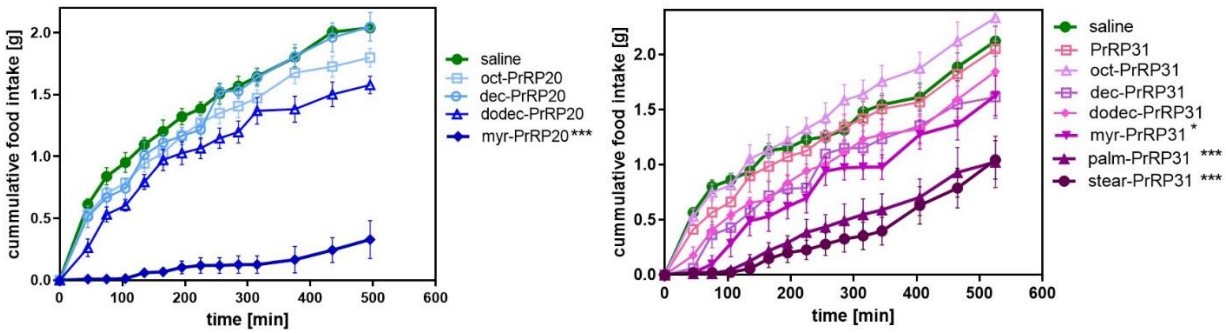

Fig. 2 Palmitoylated and stearoylated PrRP31 and myristoylated PrRP20 attenuate food intake after acute peripheral administration in fasted mice. Cumulative food intake of 17h fasted mice after acute SC administration of **A/** natural and lipidized PrRP31 analogs, **B/** lipidized PrRP20 analogs, at a dose of 5mg/kg. Food intake is expressed in grams of food consumed (n=6-8 mice per group). *** P<0.001 vs saline-treated group. The significance concerns the whole time course.
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In order to improve bioavailability of lipidized PrRP analogs, in our following study **(Prazienkova et al., 2017)**, two linkers, γ-glutamic acid at Lys^{11} ($palm^{11}$-PrRP31) and a short, modified polyethylene glycol ($palm^{11}$-PEG-PrRP31) at Lys^{11}, were applied for the palmitoylation of PrRP31 (structures in **Fig 3A**). These analogs had a high affinity to and activation ability of the GPR10 and the NPFF-2 receptor, as well as potent short-term anorexigenic effect similar to PrRP palmitoylated at the N-terminus.

In our recent study **(Karnosova et al., 2021)**, *in vitro* signaling after activation of GPR10, NPFF-2 and NPFF-1 receptors by natural PrRP31 and two palmitoylated PrRP31 analogs was tested. Palmitoylation of PrRP31 increased affinity for and activation of signaling pathways (MAPK/ERK1/2, protein kinase B - Akt, CREB) not only GPR10, but also NPFF-2 and NPFF-1 receptor. However, $palm^{11}$-PrRP31 exhibited fewer off-target activities than palm-PrRP31.

In order to determine the mininal dose of palm-PrRP31 necessary for significant effct on food intake, various routes of administration were imployed. Repeated administration of palm-PrRP31 to free-fed rats decreased food intake regardless the delivery route: SC, IP or IV **(Mikulaskova et al., 2016b)**. In all cases, food intake reduction was achieved; however, the degree of food intake decrease was partially dependent on the route of administration and on the dose of palm-PrRP31 and also differed after individual doses by particular administration routes. This may relate to the rate at which palm-PrRP31 is released from the subcutis, optionally with other unknown factors. Nevertheless, we have suggested that palmitoylation of the peptide probably enabled its central effect and stabilization in plasma. However, new specific conditions should be found for SC administration of palm-PrRP31 to decrease its dose for SC administration based on effective IV doses.

Fig. 3B shows anorexigenic effect of palmitoylated analogs after SC administration in fasted mice **(Prazienkova et al., 2017)** and **Fig. 3C** anorexigenic effect after IP administration in free fed rats **(Mikulaskova et al., 2016b)**.

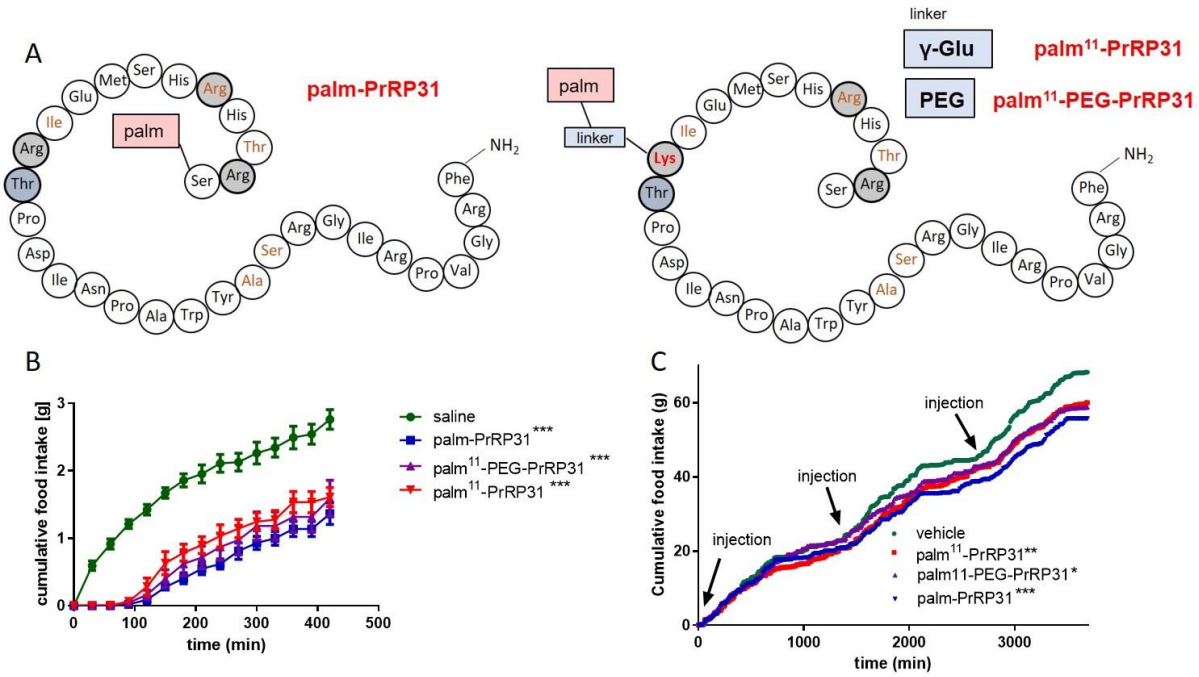

Fig. 3 A/ Structure of the most potent palmitoylated PrRP31 analogs and their anorexigenic effect in B/ fasted mice and C/ free-fed rats after peripheral administration of lipidized PrRP analogs **(Mikulaskova et al., 2016b; Prazienkova et al., 2017).** Palm palmitoylated, ɣ-Glu gamma glutamic acid, PEG 1,13-diamino-4,7,10-trioxatridecan-succinamic acid.

The idea about the central effect of lipidized PrRP was further supported by increase in c-Fos levels in the brain after SC administration to fasted mice **(Maletinska et al., 2015; Pirnik et al., 2015).** Both myr-PrRP20 and palm-PrRP31 significantly enhanced c-Fos immunoreactivity in hypothalamic (PVN) and brainstem (NTS) nuclei involved in food intake regulation that express both GPR10 and NPFF2 receptors (Roland et al., 1999), whereas natural and octanoylated PrRP31 did not **(Fig. 4)**. In addition, double c-Fos-GPR10 immunostaining in brainstem C1/A1 cells group indicated that the neurons containing GPR10 receptors were activated after palm-PrRP administration with intensity depending on the route of its peripheral administration – intravenous administration - compared to subcutaneous or intraperitoneal injection - caused the highest level of c-Fos activation in rats **(Mikulaskova et al., 2016b)**. Finally, both acute and repeated peripheral administration of palm^{11}-PrRP31 into lean C57 mice activated not only c-Fos but also FosB in dorsomedial hypothalamic nucleus suggesting association of this nucleus with long-term changes after the treatment **(Pirnik et al., 2018)**.

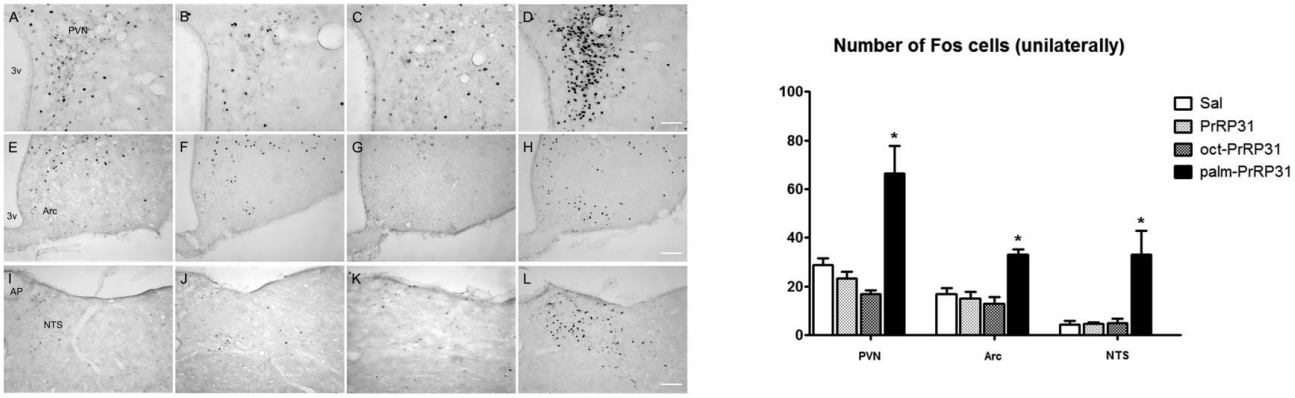

Fig. 4 Distribution of the c-Fos labeled cells in the paraventricular nucleus (PVN) (A-D), **nucleus arcuatus (Arc)** (E-H) and **nucleus tractus solitarii (NTS)** (I-L) 90 minutes after the saline (A,E,I), PrRP31 (B,F,J), oct-PrRP31 (C,G,K) and palm-PrRP31 (D,H,L) SC administration in mice. 3v – third brain ventricle, AP – area postrema.

Although we still have no direct proof for the palmitoylated PrRP analogs entrance to the brain, several pieces of indirect evidence strongly suggest eligibility for that view. First of all, the pattern of c-Fos distribution in the mentioned brain nuclei and areas detected after peripheral palm-PrRP31 administration was similar to data registered after ICV administered natural PrRP at a dose causing an anorexigenic effect (Lawrence et al., 2000). Second, peripherally administered lipidized PrRP had the anorexigenic effect but the non-lipidized PrRP molecule had not **(Maletinska et al., 2015)**. Finally, the anorexigenic activity of biologically active lipidized PrRP molecules (including palm-PrRP31) was associated with the presence of c-Fos immunostaining as a marker of neuronal activation in specific brain nuclei and areas (paraventricular nucleus, dorsomedial nucleus, nucleus arcuatus, lateral hypothalamic area, nucleus tractus solitarius) involved in food intake regulation and containing GPR10 and NPFF2 receptors **(Prazienkova et al., 2017)**. In addition, the central neuronal activation after peripheral palm-PrRP31 application was associated also with the selective activation of specific hypothalamic oxytocin and hypocretin neuronal subpopulations **(Pirnik et al., 2015)** both involved not only in food intake inhibition but also in energy expenditure.

In the hypothalamus, leptin receptor and PrRP are colocalized and leptin and PrRP have additive anorexigenic effects. Intracerebroventricular coadministration of PrRP and leptin in rats resulted in additive decrease in food intake and BW loss and an increase in energy expenditure (Ellacott et al., 2002). Furthermore, PrRP-expressing

neurons in brain regions involved in food intake regulation (ventromedial nucleus of hypothalamus and ventrolateral medulla and NTS of brainstem) also contain leptin receptors (Ellacott et al., 2002). An anorexigenic effect of PrRP independent of leptin but dependent on the peripheral short-term anorexigenic hormone CCK was suggested in the brainstem. CCK was shown to have no effect on food intake in PrRP receptor-knockout mice. This finding suggests that PrRP acting through its receptor may be a key mediator in the central satiating action of CCK (Bechtold and Luckman, 2006).

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

4.2 Role of prolactin-releasing peptide (PrPR) in etiopathology of obesity and potential of its lipidized analogs as anti-obesity compounds

Structure-activity study with lipidized PrRP analogs resulted in a selection of two most potent palmitoylated PrRP31 analogs: palm-PrRP31 and palm^{11}-PrRP31 (**Fig. 3A**). These analogs were tested in various mouse and rat models of obesity, glucose intolerance/ insulin resistance and T2D arising from high-fat (HF) diet feeding (diet-induced obesity – DIO models) or in rodents with nonfunctional leptin signaling due to a spontaneous mutation in the leptin receptor (summarized in **(Mrazikova et al., 2021)**).

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

intake regulation, such as the leptin system. Overview and the basic characterization of each model is shown in **Table 2**.

Table 2 Characterization of rodent models used in studies of interventions with palmitoylated PrRP31 analogs

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

C57BL/6 mice fed a HF diet containing 60% fat based on lard from 8 to 19 weeks of age developed severe obesity and prediabetes (Pelantová et al., 2016). In the studies of Maletínská **(Maletinska et al., 2015)** and Pražienkova **(Prazienkova et al., 2017)**, mice were treated SC with saline or palmitoylated PrRP31 analogs twice a day for two weeks. Treatment with palm^{11}-PrRP31 and palm^{11}-PEG-PrRP31 decreased food intake, body and liver weights, insulin, leptin, triglyceride, and free fatty acid plasma levels in obese mice (**Fig. 5**) **(Prazienkova et al., 2017)** together with decreased mRNA expression of enzymes involved in lipogenesis and increased mRNA expression of enzymes regulating lipolysis. Moreover, the expression of uncoupling protein-1 (UCP-1) was increased in brown fat (BAT) suggesting an increase in energy expenditure.

Taken together, these data suggested that newly designed palmitoylated analogs of PrRP31 hold promising features with respect to their possible use in the treatment of obesity and its related metabolic complications.

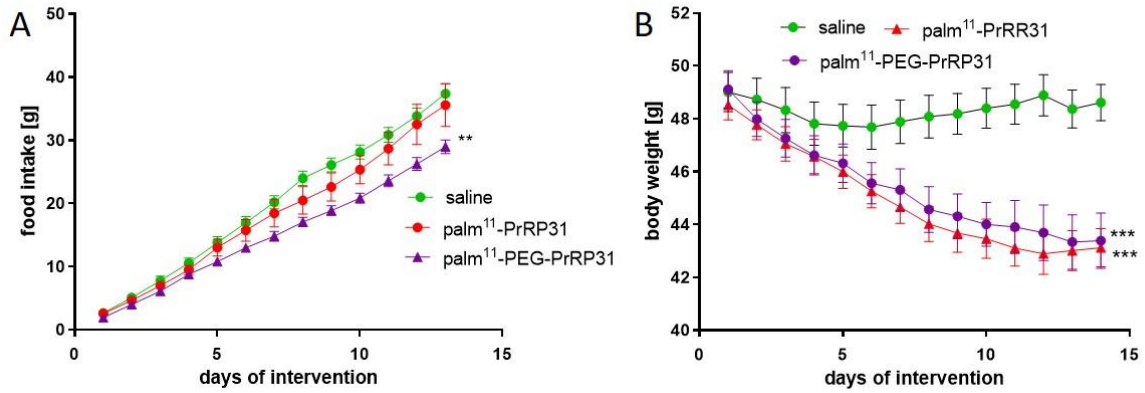

Fig. 5 Anti-obesity effect of palmitoylated PrRP31 analogs. A/ Food intake in and B/ Body weight of DIO mice after 14-days treatment with PrRP analogs palmitoylated in position 11 **(Pražienková et al., 2017)**.

Besides above mentioned studies with DIO mice, our additional study examined the impact of chronic peripheral administration of palm^{11}-PrRP31 in DIO mice after 28 days of treatment or after 14 days of peptide treatment followed by 14 days of discontinuation. Palm^{11}-PrRP31 induced a significant decrease in body weight and plasma leptin levels that continued even 14 days after termination of treatment and maintained increased neuronal activity in food intake-regulating neurons of the NTS and in the hypothalamus. Moreover, leptin-related signaling in the hypothalamus was ameliorated, and apoptotic signaling pathways were attenuated by the treatment with palm^{11}-PrRP31 (**Holubova et al., 2018**).

The chronic effect of palm-PrRP31 was studied in DIO Sprague-Dawley rats and leptin receptor-deficient Zucker diabetic fatty (ZDF) rats, where palm-PrRP31 was IP administered for two weeks. Palm-PrRP31 lowered food intake and body weight, improved glucose tolerance, and decreased leptin levels and adipose tissue weight in DIO rats (**Holubova et al., 2016**) (**Fig. 6 A,B**). In contrast, the administration of palm-PrRP31 did not significantly affect body weight or glucose tolerance in ZDF rats **(Holubova et al., 2016)**. In the following study, repeated administration of palm^{11}-PrRP31 improved glucose tolerance in Koletsky-spontaneously hypertensive obese

(SHROB) rats, which have mutations in their leptin receptor and, therefore, impaired leptin signaling **(Mikulaskova et al., 2018)** (**Fig. 6 C, D**). Treatment with palm^{11}-PrRP31 also decreased body weight in control spontaneously hypertensive rats (SHR), but not in SHROB rats. Moreover, in SHROB rats, palm^{11}-PrRP31 ameliorated the insulin/glucagon ratio and increased insulin receptor substrate mRNA expression in fat and insulin signaling in the hypothalamus, while it had no effect on blood pressure. An increase in all parameters mentioned pointed to a beneficial effect of palm^{11}-PrRP on the diabetic state. Additionally, in SHR and normotensive Wistar Kyoto (WKY) rats on a HF diet, treatment with palm^{11}-PrRP31 lowered body weight and improved biochemical and biometric parameters. Palm^{11}-PrRP31 also improved glucose tolerance in WKY rats on HF diet **(Čermáková et al., 2019)**.

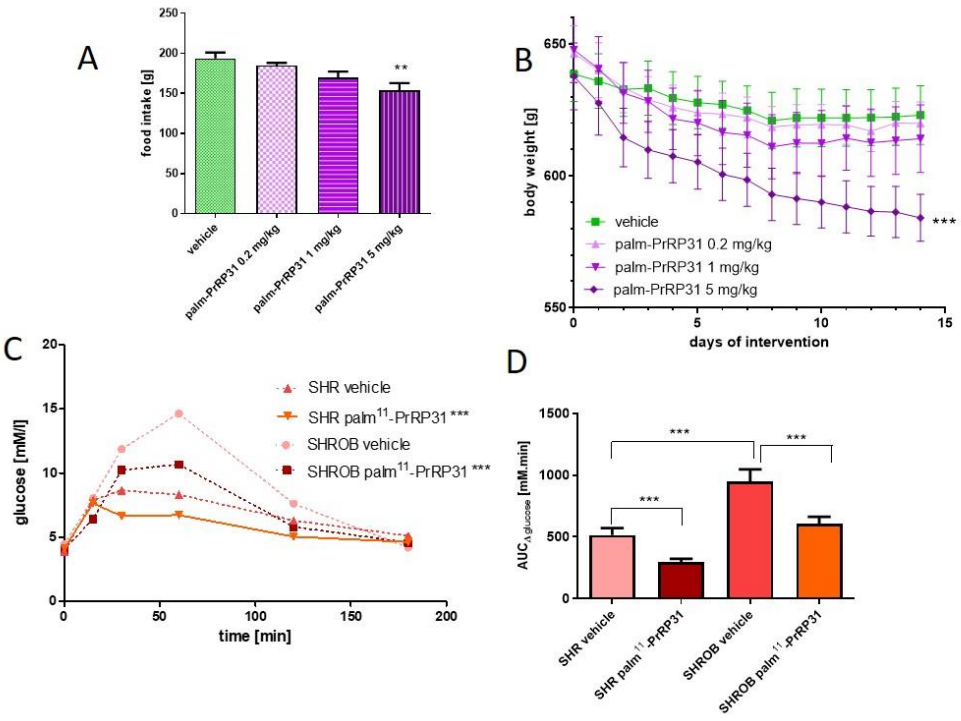

Fig 6 A, B/ Palm-PrRP31 lowered food intake and body weight in DIO rats. C, D/ Palm^{11}-PrRP31 ameliorated glucose intolerance in SHROB and SHR rats after chronic peripheral administration **(Holubova et al., 2016; Mikulaskova et al., 2018)**.

Finally, leptin and palm^{11}-PrRP31 synergistically lowered body weight and increased body temperature in leptin-deficient *ob/ob* mice with established morbid obesity **(Korinkova et al., 2020a)**. In this study, neither palm^{11}-PrRP31 nor leptin alone significantly decreased the BW, body fat or liver weight of *ob/ob* mice, but their combination significantly lowered all these parameters (**Fig. 7**). Moreover, an increase

in the rectal temperature in older *ob/ob* mice was detected after treatment with a combination of leptin and palm^{11}-PrRP. In the hypothalamus of *ob/ob* mice, two main leptin anorexigenic signaling pathways, namely, Janus kinase (JNK)/signal transducer and activator of transcription-3 (STAT3) activation and AMP-activated protein kinase (AMPK) deactivation, were induced by leptin, palm^{11}-PrRP31, and their combination (**Korinkova et al., 2020a**). Thus, palm^{11}-PrRP31 could partially compensate for leptin deficiency in *ob/ob* mice.

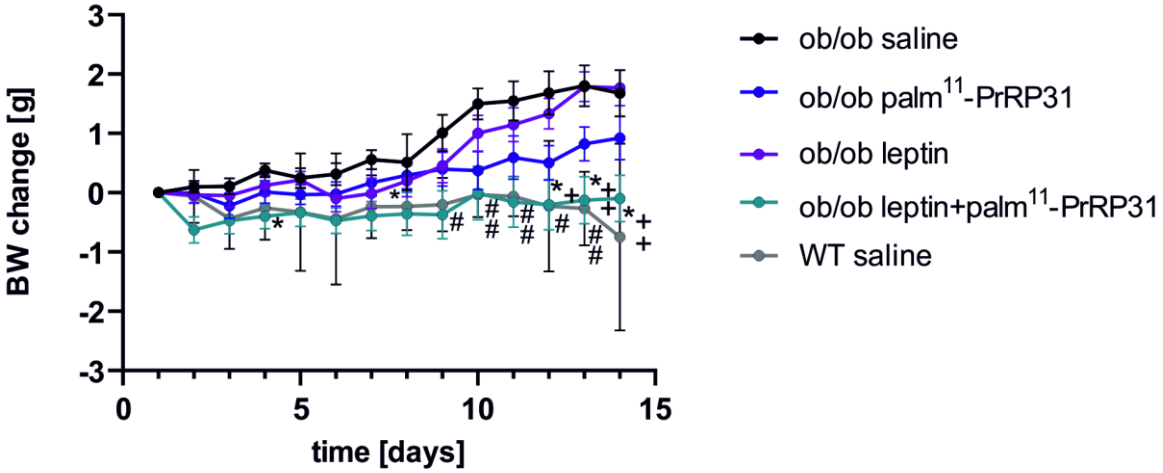

Fig. 7 Chronic effect of palm^{11}-PrRP31, leptin and their combination on BW change in *ob/ob* mice. Modified from **(Korinkova et al., 2020a).** Statistical analysis was performed by Two-way ANOVA with Tukey *post hoc* test, significance is $^{\#}$<0.05, $^{\#\#}$<0.01 *ob/ob* saline vs wild type (WT) saline, *<0.05, **<0.01 *ob/ob* leptin + palm^{11}-PrRP31 vs *ob/ob* saline, $^{+}$<0.05, $^{++}$<0.01 *ob/ob* leptin + palm^{11}-PrRP31 vs *ob/ob* leptin.

From the previous studies, we can summarize that palmitoylated PrRP analogs have anti-obesity and glucose-lowering effects in mouse and rat models of DIO, and these effects are accompanied by improved leptin and insulin signaling in brain. Anorexigenic effect of palmitoylated PrRP was shown to be dependent on the proper leptin signaling, but effect of palmitoylated PrRP31 on glucose metabolism seems to be independent of leptin signaling and body weight lowering. On the other hand, palmitoylated PrRP analogs had no influence on blood pressure.

Thus, from all above mentioned *in vivo* studies, we can conclude that dual GPR10 and NPFF-2R agonism was proven as a promising target for the treatment of obesity

and related complications, with palmitoylated PrRP31 analogs showing a high anorexigenic efficacy after peripheral administration.

4.2 Neuroprotective effect of lipidized PrRP analogs in neurodegeneration

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder in the elderly population. Numerous epidemiological and experimental studies have demonstrated that patients who suffer from obesity or T2D have a higher risk of cognitive dysfunction and of AD. It was demonstrated in epidemiological studies that patients with T2D have a higher incidence of AD (Ricci et al., 2017; Vagelatos and Eslick, 2013). Furthermore, it was discovered that T2D and AD share several molecular processes that underlie the degenerative developments that occur in these two conditions. One of the major risk factors for T2D development is obesity that contributes to the development of insulin resistance not only in periphery, but also in brain areas involved in memory formation (de la Monte, 2014; Li and Hölscher, 2007; Steen et al., 2005). Extracellular senile plaques formed by aggregated β-amyloid protein (Aβ) and intracellular neurofibrillary tangles formed by hyperphosphorylated Tau protein are two hallmarks of AD. However, also other pathological features are observed in AD patients, such as decreased synaptic plasticity and neurogenesis or increased neuroinflammation.

In our very first study with 12- and 33-week-old Zucker fa/fa rats, we showed that aging and obesity significantly contributed to increased peripheral insulin resistance (IR), which further worsened the activation of the hippocampal insulin signaling cascade. This effect resulted in decreased phosphorylation at the inhibitory epitope Ser 9 of glycogen-synthase kinase 3β (GSK-3β), one of the main kinases of Tau protein phosphorylation. Subsequently, an increase in the pathological hyperphosphorylation of Tau protein was observed in the hippocampi of fa/fa rats; thus, peripheral IR resulted in central insulin resistance and Tau hyperphosphorylation **(Spolcova et al., 2014)**.

Several recent studies demonstrated that anorexigenic peptides have the potential to improve metabolic disorders and that they may also potentially be useful in the treatment of neurodegenerative diseases (Holscher 2018; Mandal, et al. 2018). Thus, anorexigenic and/or antidiabetic substances began to be examined as

compounds with potential neuroprotective properties (reviewed in **(Maletinska et al., 2019)**). This potential is supported by the finding that receptors of anorexigenic peptides, such as GPR10 or GLP-1 receptor, are expressed in the hippocampus, which is the first brain region affected during AD. The major aim of this part of our research was to find out if novel anorexigenic peripherally administered and centrally acting lipidized analogs of PrRP have similar beneficial effect on the mentioned neurodegenerative changes both in cellular models and in several mouse models of neurodegeneration and their common aged wild type controls.

The effect of natural PrRP31 and its analog palm^{11}-PrRP31 on Tau hyperphosphorylation was examined *in vitro* using two cellular models: a model of hypothermia and a model of methylglyoxal (MG) – induced oxidative stress, in the human neuroblastoma cell line SH-SY5Y and on rat primary cortical neurons.

Hypothermic conditions resulted in increased Tau hyperphosphorylation at several epitopes in both cellular models. In both SH-SY5Y cells and primary cortical neurons, incubation with palm^{11}-PrRP31, as well as with PrRP31, attenuated Tau hyperphosphorylation at several pathological epitopes **(Prazienkova et al., 2019b)**. The potential neuroprotective properties of PrRP analogs were further studied through activation of anti-apoptotic pathways of PrRP31 and palm^{11}-PrRP31 using the SH-SY5Y cells and rat primary neuronal culture stressed with toxic MG. The results indicate increased viability of the cells treated with PrRP and palm^{11}-PrRP31 and a reduced degree of apoptosis induced by MG, suggesting their potential use in the treatment of neurodegeneration disorders **(Zmeskalova et al., 2020)**.

The effect of PrRP on Tau hyperphosphorylation was extensively studied *in vivo* using several mouse models of neurodegeneration. Mice with obesity induced by monosodium glutamate (MSG mice) develop increased Tau hyperphosphorylation due to central insulin resistance manifested by decreased activation of the insulin signaling cascade. In our first study, palm-PrRP31 ameliorated the activation of the insulin signaling cascade and subsequently decreased Tau phosphorylation at several epitopes, such as pThr231 and pSer396 **(Špolcová et al., 2015)**. A similar effect on Tau hyperphosphorylation was observed in the THY-Tau22 mouse model with overexpressed mutated human Tau protein, where the intervention with palm^{11}-PrRP31 improved short-term spatial memory in the Y-maze test and increased synaptic plasticity compared to the vehicle-treated group **(Popelova et al., 2018)** **(Fig.**

8). APP/PS1 mice are double transgenic mice expressing mutated amyloid precursor protein (APP) (Swedish mutation, K595N/M596L) and mutated presenilin (PS1) (deltaE9 PS1) exon deletion, are one of the most frequently used models to study Aβ pathology. Treatment with the lipidized analog palm^{11}-PrRP31 decreased the amount of senile Aβ plaques in APP/PS1 mice. Moreover, palm^{11}-PrRP31 lowered the markers of neuroinflammation colocalized with Aβ plaques—ionized calcium-binding adapter molecule 1 (Iba1), a marker of activated microglial cells, and glial fibrillary acidic protein (GFAP), a marker of reactive astrocytes. Potential neuroprotective properties were further manifested by increased levels of doublecortin, a marker of neurogenesis, in hippocampi **(Holubova et al., 2019)** **(Fig. 9)**.

Moreover, in our following study with APP/PS1 mice **(Mengr et al., 2021)**, treatment with palm^{11}-PrRP31 significantly reduced the Aβ plaque load and microgliosis in the cerebellum. Palm^{11}-PrRP31 also increased cortical astroglial water channel aquaporin 4, indicating improved clearance via glymphatic pathway, and the presynaptic markers synaptophysin and syntaxin1A and postsynaptic markers spinophilin and postsynaptic density protein 95, suggesting that palm^{11}-PrRP31 preserves synapses in the hippocampus. In addition, treatment with palm^{11}-PrRP31 tended to decrease CD68, a macrophage and monocyte marker, and the proinflammatory cytokines such as tumor necrosis factor α and interleukin-6. Furthermore, palm^{11}-PrRP31 decreased the Bax/BCl2 ratio (the ratio of the proapoptotic protein Bax to the antiapoptotic protein BCl2), a marker of cell defenselessness against apoptosis, indicating that it may exert anti-inflammatory and antiapoptotic effects **(Mengr et al., 2021)**. These results suggest palm^{11}-PrRP31 is a promising agent for the treatment of neurodegenerative disorders.

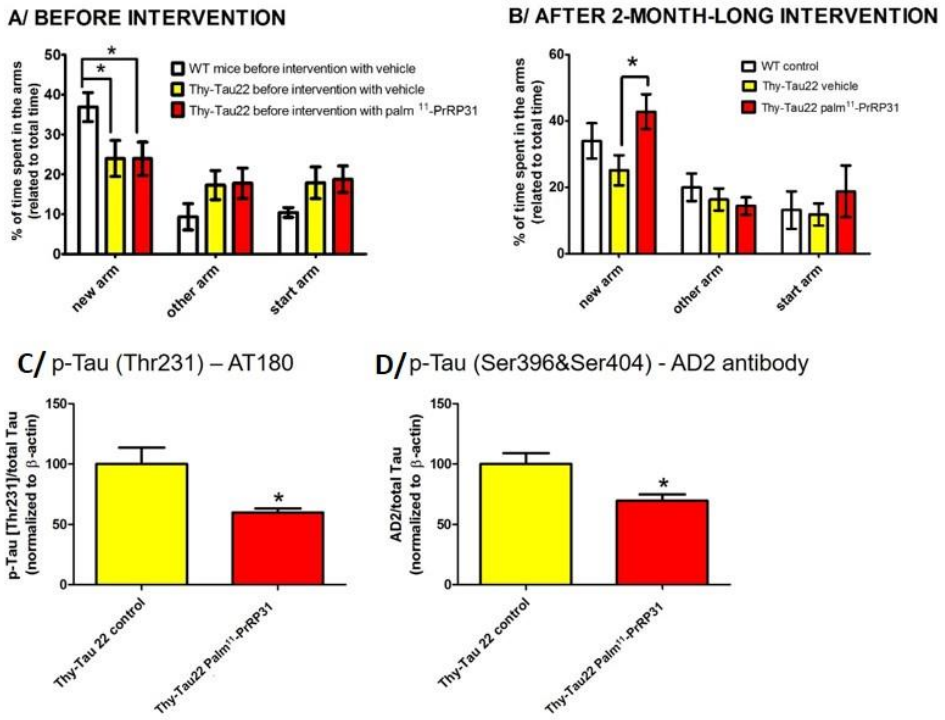

Fig. 8 Lipidized analog palm^{11}-PrRP31 A, B/ improves short-term memory and C, D/ decreases pathological Tau phosphorylation in THY-Tau22 mice. (C, D – quantification of Western blots in hippocampi of THY-Tau22 mice treated for one month with saline or palm^{11}-PrRP31) **(Popelova et al., 2018)**.

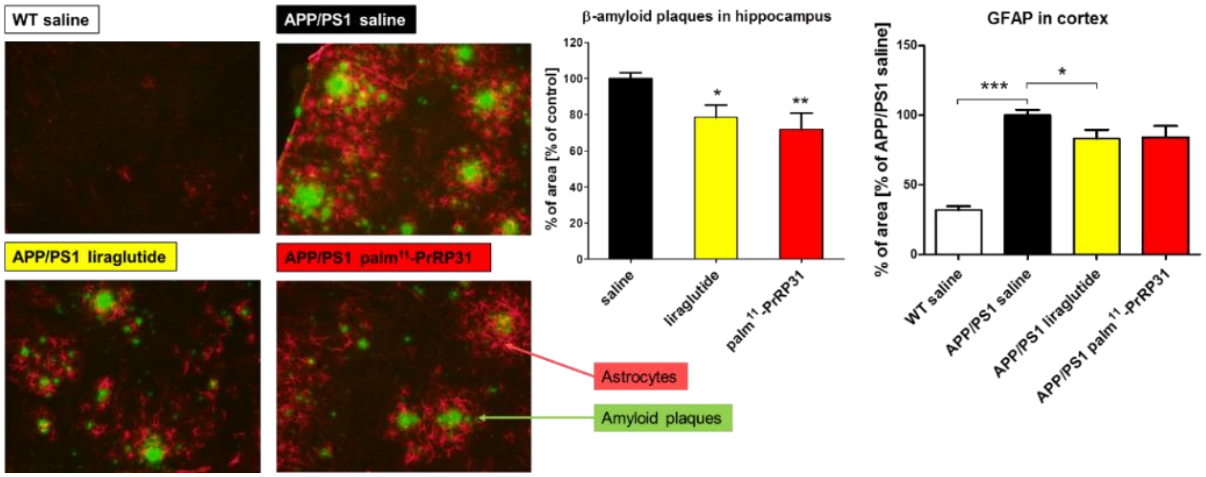

Fig. 9 Lipidized analog palm^{11}-PrRP31 and liraglutide decrease Aβ plaques and neuroinflammation in astrocytes of APP/PS1 mice. Immunohistochemistry in brain slices of WT control and in APP/PS1 mice treated with saline, palm^{11}-PrRP31 or liraglutide (GLP-1 agonist as a comparator) for two months **(Holubova et al., 2019)**.

Mass spectrometry imaging (MSI) is one of the emerging fields of MS with powerful capabilities to acquire chemical information directly from tissues sections (Strnad et

al., 2018). MSI is by principle label-free technique and this feature represents its significant advantage. From the point of view of neuroscience, frequently mapped compounds in central nervous system are lipids. In our recent study, we described the MALDI MSI method suitable for studying neurodegeneration based on spraying 1,5-diaminonaphtalene comparing lipid composition in brain of two mouse models of AD-like pathology (APP/PS1 and THY-Tau22 mice) (Strnad et al., 2019). In the following study, we demonstrated that paraformaldehyde-fixed free-floating brain sections can be successfully used for the MSI of lipids. MSI on the free-floating sections allows for the measurement of the samples already prepared for immunohistochemistry. In APP/PS1 mice treated with palm^{11}-PrRP31, we found a reduced extent of lipids that we linked to senile Aβ plaques colocalized with reactive astrocytes through the GFAP protein. Treatment with palmitoylated PrRP31 was able to decrease a level of pathological gangliosides in brain areas connected with AD-like pathology **(Fig. 10)** **(Strnad et al., 2020)**.

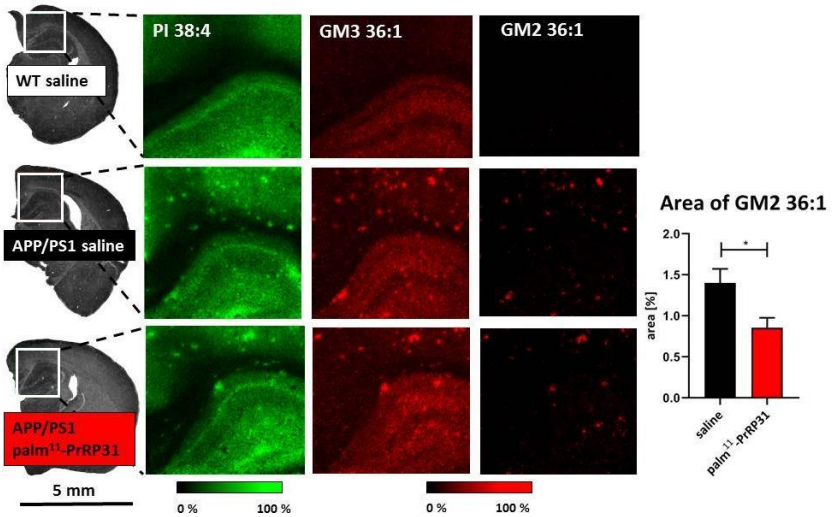

Fig. 10 Lipidized analog of PrRP decreases pathological lipids in hippocampi of APP/PS1 mice. MALDI MSI analysis in brains of WT control, and APP/PS1 mice treated with saline or palm^{11}-PrRP31**.** Optical images of the brain with measured regions and ion images of phosphatidylinositol (PI 38:4, m/z 885.6), gangliosides (GM3 36:1, m/z 1179.6; GM2 36:1, m/z 1382.6) **(Strnad et al., 2020)**.

5. Conclusions

In conclusion, we have proven in our studies, that our novel palmitoylated PrRP analogs have potent beneficial effects on obesity, glucose intolerance and neurodegenerative changes.

Fig. 11 shows the scheme of a potential role of PrRP and its lipidized analogs in food intake regulation and neurodegeneration.

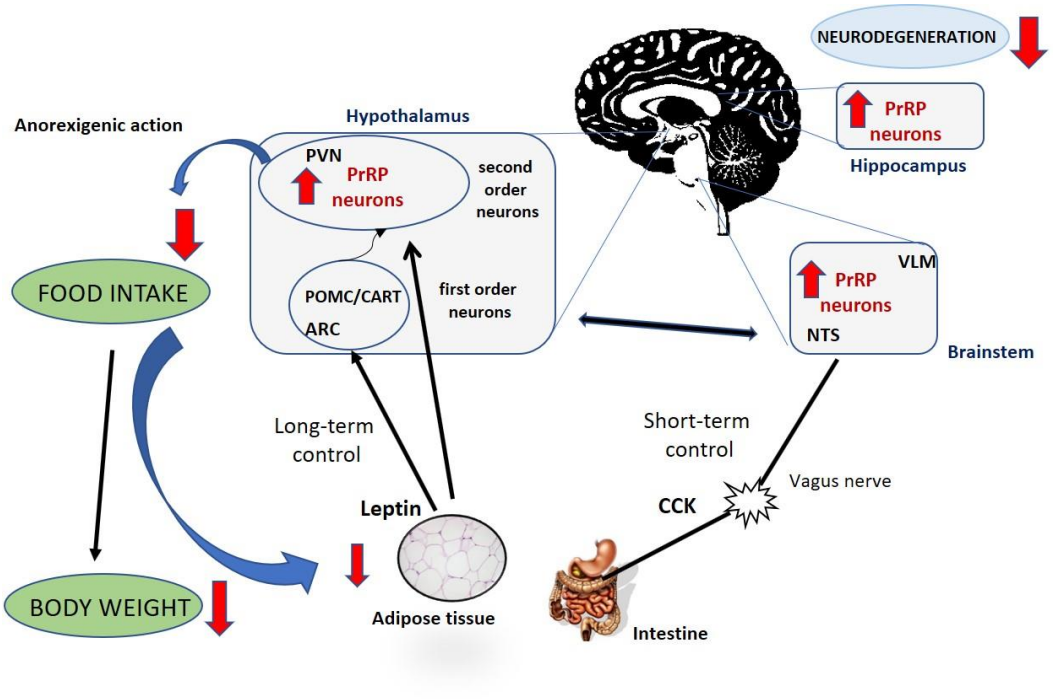

Fig. 11 The scheme of a potential role of PrRP and its analogs in food intake regulation and neurodegeneration and its interaction with leptin and cholecystokinin (CCK) (Kunes et al., 2016).
ARC – nucleus arcutatus, NTS – nucleus tractus solitarius, PVN – paraventricular nucleus, VLM – ventrolateral medulla. CART – cocaine- and amphetamine-regulated transcript, POMC – pro-opiomelanocortin.

Lipidized PrRP analogs showed binding affinity and signaling in GPR10-expressing cells similar to natural PrRP. Moreover, these analogs revealed high binding affinity also to anorexigenic NPFF-2 receptor. Acute peripheral administration of myristoylated and palmitoylated PrRP analogs to mice and rats induced strong and long-lasting anorexigenic effects and neuronal activation in the brain areas involved in food intake regulation.

Two most potent lipidized PrRP analogs, palm-PrRP31 and palm^{11}-PrRP31 were shown to ameliorate pathological features in several rodent models of diet-induced obesity and prediabetes. A decrease in food intake resulted in attenuated fat storage

and body and liver weight, accompanied by a decrease in leptin levels. Furthermore, palmitoylated analogs of PrRP affected lipid metabolism in adipose tissue and the liver by suppressing lipid synthesis and increasing lipid degradation. Moreover, increased mRNA expression of UCP-1 in BAT points to increased energy expenditure. A very interesting result was also demonstrated in the study when the treatment was discontinued: no yo-yo effect was observed after palm^{11}-PrRP31 treatment termination. Thus, palmitoylated PrRP analogs have the potential to be an attractive candidate for obesity therapy.

The rodent models with leptin deficiency or disturbances in leptin signaling develop obesity or morbid obesity. Treatment with palm-PrRP31 or palm^{11}-PrRP31 neither significantly decreased BW nor improved related metabolic parameters. In two rat strains with nonfunctional leptin signaling, ZDF diabetic rats and Koletsky rats, monotherapy with palm^{11}-PrRP31 or palm-PrRP did not have an anti-obesity effect, but there were significant glucose-lowering effects. These results suggest that to achieve the full anti-obesity effects of PrRP, intact leptin signaling is needed, but the effect on glucose tolerance could be independent of leptin signaling. On the other hand, treatment of *ob/ob* mice with a combination of leptin and palm^{11}-PrRP31 synergistically decreased BW. The central effect of both palmitoylated PrRP analogs was demonstrated by increased leptin and insulin signaling in the brain.

Finally, we have studied a possible relationship between obesity, T2D and AD-like pathology and investigated if our novel lipidized PrRP analogs could have beneficial effect on pathological changes related to AD in several *in vitro* cellular models and *in vivo* mouse models of neurodegeneration compared to their age-matched wild type controls. We demonstrated that palmitoylated PrRP31 analogs are a potentially neuroprotective substances improving spatial memory, neurogenesis, synaptogenesis and attenuating neuroinflammation and two hallmarks of AD, Tau hyper-phosphorylation and β-amyloid plaques in different mouse models of neurodegeneration resulting from obesity and central insulin resistance (MSG model), Tau pathology (THY-Tau22 model) or Aβ pathology (APP/PS1 model).

6. Future plans

During the next years, in collaboration with our scientific partners, we plan to continue our multidisciplinary research focused on search for mechanism of action of lipidized PrRP analogs involved in food intake regulation, and focus on their impact on different aspects of obesity, T2D and neurodegeneration.

Scheme in **Fig. 12** shows all substantial components of these processes studied in our group. Peripheral and central inflammation is proposed to underlie obesity, insulin resistance and neurodegeneration.

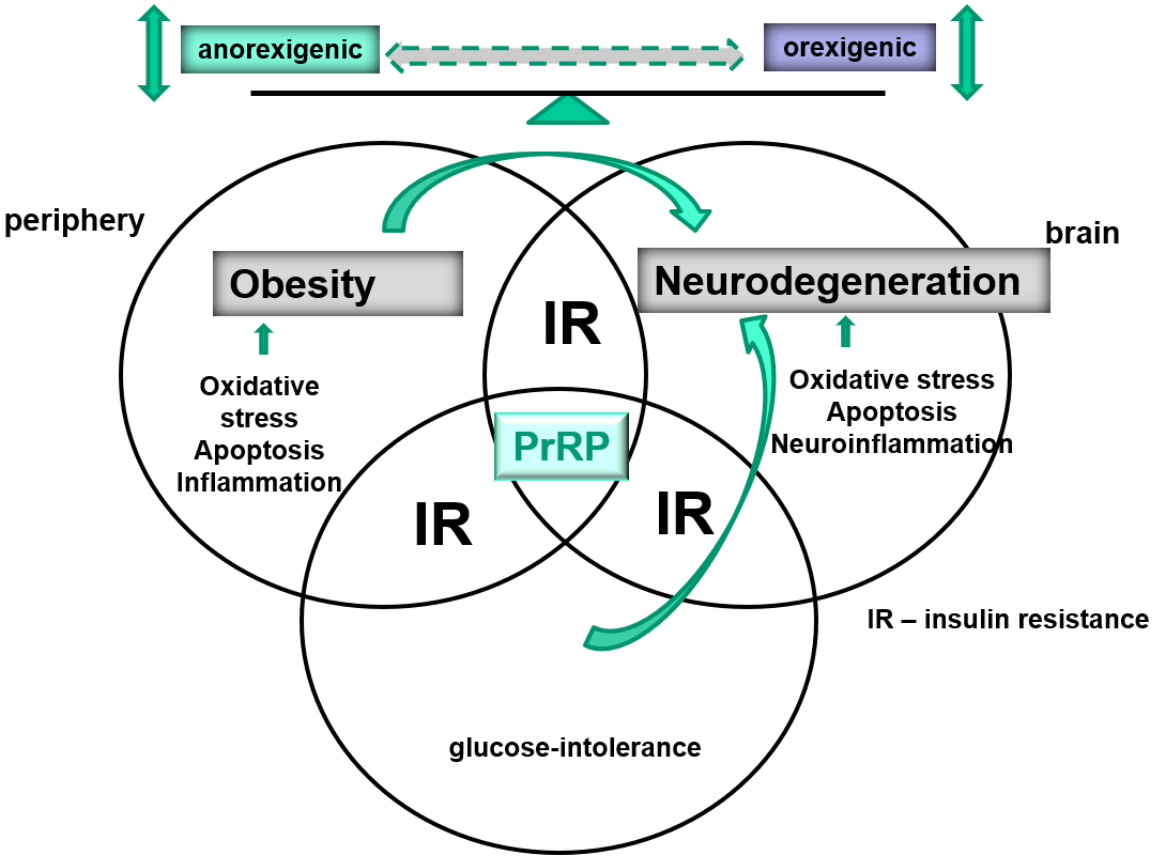

Fig. 12 Scheme of peripheral and central connectivity of obesity, insulin resistance, inflammation and neurodegeneration with underlying leptin resistance.

Based on our previous results, we would like to focus in PrRP - related projects on following topics:

- Palmitoylated PrRP analogs: search for mechanism of action in mouse models with deleted GPR10 and/or NPFF-2 receptor

- Impact of PrRP analogs on pathophysiology of non-alcoholic fatty liver disease (NAFDL) in mouse models
- Relationship between peripheral and central neuroinflammation as a possible link between obesity and neurodegeneration in mouse and rat models
- Relationship between neurodegeneration and oxidative stress: cardiovascular phenotyping in a model of small vessel disease and obesity/neurodegeneration

6.1 Palmitoylated PrRP analogs: search for mechanism of action

PrRP affinity both to GPR10 and NPFF-2R results in powerful anorexigenic effect. Moreover, NPFF-2R was reported to be involved in HF induced thermogenesis. As our lipidized PrRP analogs with anorexigenic effect have high affinity to and activate both GPR10 and NPFF-2R, we consider very important to distinguish their effects on the particular receptors. For this purpose, GPR10 and NPFF-2R knock-out (KO) mice, as well as double KO mice were prepared and bred.

In our recent study, GPR10 KO male mice revealed mild late-onset obesity and mild liver steatosis, but no changes in energy expenditure compared to WT controls as well as altered insulin sensitivity and lipid homeostasis **(Prazienkova et al., 2021)**. Deletion of GPR10 gene resulted in changes in lipid metabolism in mice of both sexes, however in different extent. GPR10 KO mice in our preliminary experiments still retained partial anorexigenic response to our lipidized PrRP analogs (unpublished results).

Therefore, we aim to explore NPFF-2R as another possible anorexigenic target of PrRP and NPFF analogs involved in food intake regulation. Moreover, we would like to test food intake in NPFF-2R KO mice where only GPR10 and not NPFF-2R should be activated by newly designed lipidized RF-amide peptides (NPFF, NPAF and PrRP) analogs. The double NPFF-2R/GPR10 KO mice of possibly obese phenotype could then point to or exclude possible off-target receptors (other than GPR10 and NPFF-2R) for our lipidized PrRP analogs.

Targeting NPFF-2R pathway resulting in regulation of food intake and energy expenditure could potentially aim to a new anti-obesity treatment.

In cooperation with D. Petřík, Cardiff University, UK, we will also explore a potential role of palmitoylated PrRP analogs in adult neurogenesis. We hypothesize that lipidized PrRP analogs exert their anti-obesity effects by a positive modulation of

adult neurogenesis and suppressing inflammatory milieu in the hypothalamus. The aim is to elucidate if PrRP analogs influence adult neurogenesis under HF diet, and how this process affects astrocytes and inflammation and to identify underlying mechanisms.

6.2 Impact of PrRP analogs on pathophysiology of non-alcoholic fatty liver disease in mouse models

6.3 Relationship between peripheral and central neuroinflammation as a possible link between obesity and neurodegeneration

Multiple lines of evidence highlight the importance of peripheral inflammation and its link to neuroinflammation, which can lead to neurodegenerative diseases such dementia, AD etc. In addition to the classical AD pathology in brain, activated microglia and reactive astrocytes are the main indicators of AD progression. Cytokines are key players in pro- and anti-inflammatory processes and are also produced by microglia and astrocytes. The interplay of these pathways between the periphery and the brain could have a common denominator, with inflammation being a key factor affecting neuronal processes in the brain. These processes could be also underlying obesity-related non-alcoholic fatty liver disease (NAFDL) further progressing to non-alcoholic steatohepatitis (see our reviews **(Kacirova et al., 2020; Korinkova et al., 2020b)**).

This project is based on following principal hypotheses:

- Obesity, type 2 diabetes and neurodegenerative diseases should have a common etiopathogenesis based on insulin resistance and subclinical inflammation, which seem to be the major players in the development of brain insulin resistance and neurodegeneration.
- Lipidized analogs of anorexigenic peptides can act in the brain in order to attenuate obesity and T2D by improving brain insulin resistance and modulating the energy balance. At the same time, they can improve neurodegenerative and neuroinflammatory changes in the brain acting by similar mechanism of action.
- Neurodegenerative changes in the brain of experimental models of obesity/T2D/neurodegeneration can be followed by complex metabolomic imprint in organs/blood/urine using NMR or LC/MS. These markers could be correlated with changes in brain metabolism as determined by immunohistochemistry and MSI.

Our first published results have shown that neuroinflammation in brain is worsened in THY-Tau22 mouse model fed HF diet **(Fig. 13)** as analysed by immunohistochemistry **(Kacířová et al., 2021)**.

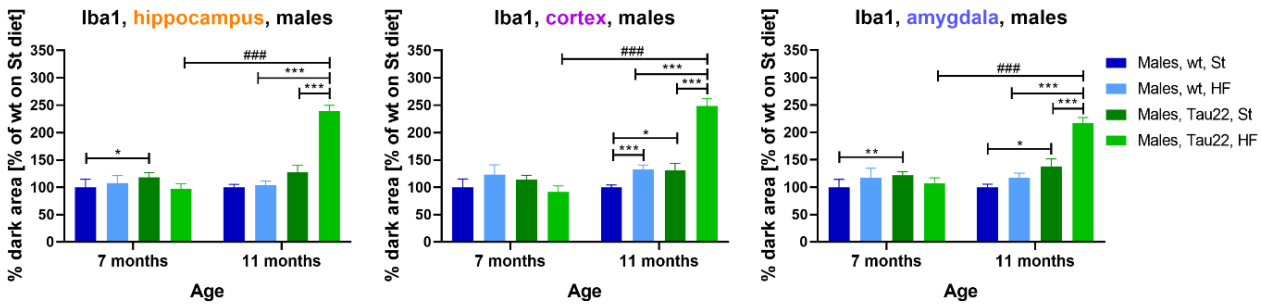

Fig. 13 Microgliosis marker Iba1-stained brain of THY-Tau22 male mice. Relative quantification of immunohistochemistry of 7- and 11-month-old THY-Tau22 and wt mice. Iba1: ionized calcium-binding adapter molecule 1.

6.4 Relationship between neurodegeneration and ischemia: cardiovascular phenotyping in a model(s) of small vessel disease and obesity / neurodegeneration

Cerebral small vessel disease (CSVD) leads to dementia and stroke-like symptoms and is characterized by a heterogeneous spectrum of histopathological features possibly initiated by an early endothelial dysfunction with subsequent BBB breakdown (Berry et al., 2019).

In cooperation with University of Glasgow, UK/ McGill University, Montreal, Canada (R. Touyz, A. Montezano), we started a new collaboration involving relationship of ischemia, oxidative stress and vascular dementia/ neurodegeneration. Tissues from our recently used models will be employed and all the studies will be performed in Glasgow with participation of students from both laboratories.

We hypothesize that wild type and APP/PS1 mice at HF diet exhibit altered peripheral vascular function and remodeling *via* redox-sensitive signaling pathways and increased interaction through receptors involved in food intake regulation such as GPR10 may reduce these alterations. Spontaneously hypertensive stroke-prone rats (SHR-SP) are a widely used model for CSVD which we plan also to employ in this project.

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8 List of publications as a basis of this thesis in chronological order

(IF from the year 2020, [number of citations] from Web of Sci, 15-02-2022)

34 publications (8 reviews and 26 original articles), from which Maletinska L: 3-times as a 1st author, 29-times as a corresponding author

Maixnerova, J., Spolcova, A., Pychova, M., Blechova, M., Elbert, T., Rezacova, M., Zelezna, B., and **Maletinska, L.** (2011) Characterization of prolactin-releasing peptide: Binding, signaling and hormone secretion in rodent pituitary cell lines endogenously expressing its receptor. *Peptides* **32**, 811-817 [18] 3.750

Maletinska, L., Spolcova, A., Maixnerova, J., Blechova, M., and Zelezna, B. (2011) Biological properties of prolactin-releasing peptide analogs with a modified aromatic ring of a C-terminal phenylalanine amide. *Peptides* **32**, 1887-1892 [11] 3.750

Spolcova, A., Mikulaskova, B., Krskova, K., Gajdosechova, L., Zorad, S., Olszanecki, R., Suski, M., Bujak-Gizycka, B., Zelezna, B., and **Maletinska, L.** (2014) Deficient hippocampal insulin signaling and augmented Tau phosphorylation is related to obesity- and age-induced peripheral insulin resistance: a study in Zucker rats. *BMC Neuroscience* **15** [24] 3.288

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Pirnik, Z., Zelezna, B., Kiss, A., and **Maletinska, L.** (2015) Peripheral administration of palmitoylated prolactin-releasing peptide induces Fos expression in hypothalamic neurons involved in energy homeostasis in NMRI male mice. *Brain Research* **1625**, 151-158 [11] 3.252

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Abbreviations

Aβ	amyloid beta
AD	Alzheimer's disease
Akt	protein kinase B
AMPK	AMP-activated protein kinase
Arc	nucleus arcuatus
BAT	brown adipose tissue
BBB	blood brain barrier
BW	body weight
CART	cocaine- and amphetamine- regulated transcript
CCK	cholecystokinin
CREB	cAMP response element-binding protein
CSVD	cerebral small vessel disease
DIO	diet induced obesity
DMN	dorsomedial nucleus
ERK	extracellular signal regulated kinase
GFAP	glial fibrillary acidic protein
GLP-1	glucagon-like peptide 1
GPR10	G-protein coupled receptor 10
GSK-3β	glycogen synthase kinase-3β
Iba-1	ionized calcium-binding adapter molecule 1
ICV	intracerebroventricular
IV	intravenous
HF	high fat
IP	intraperitoneal
IR	insulin resistance
JNK	c-Jun N-terminal kinase
K_d	dissociation constant
K_i	inhibition constant
LF	low fat
MAP	mitogen-activated protein kinase
MetS	metabolic syndrome
MG	methylglyoxal
MSG	monosodium glutamate
MSI	mass spectrometry imaging
Myr-PrRP20	myristoylated PrRP20
NAFLD	non-alcoholic fatty liver disease
NPFF	neuropeptide FF
NPY	neuropeptide Y
NTS	nucleus tractus solitarii
OGTT	oral glucose tolerance test
$Palm^{11}$-PrRP31	PrRP31 palmitoylated at position 11
Palm-PrRP31	palmitoylated PrRP31
PDK1	phosphoinositide-dependent protein kinase 1
PI3K	phosphatidylinositol-3-kinase
PrRP	prolactin-releasing peptide

PVN	paraventricular nucleus
SC	subcutaneous
SHR	spontaneously hypertensive rats
SHROB	spontaneously hypertensive obese rats
STAT3	signal transducer and activator of transcription-3
T2D	type 2 diabetes
UCP-1	uncoupling protein 1
WKY rats	Wistar Kyoto rats
WT	wild type
ZDF rats	Zucker diabetic fatty rats