Prolactin-releasing peptide: a new tool for obesity treatment

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Abstract

Obesity is an escalating epidemic, but an effective noninvasive 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. In this review, we summarize results of several studies with our newly designed lipidized analogs of prolactin-releasing peptide (PrRP). PrRP is involved in feeding and energy balance regulation as demonstrated by obesity phenotypes of both PrRP- and PrRP-receptor-knockout mice. Lipidized PrRP analogs showed binding affinity and signaling in PrRP receptor-expressing cells similar to natural PrRP. Moreover, these analogs showed high binding affinity also to anorexigenic neuropeptide FF (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-week-long subcutaneous administration of palmitoylated PrRP31 and myristoylated PrRP20 lowered food intake, body weight, improved metabolic parameters and attenuated lipogenesis in mice with diet-induced obesity. A strong anorexigenic, body weight-reducing and glucose tolerance-improving effect of palmitoylated-PrRP31 was shown also in diet-induced obese rats after its repeated 2-week-long peripheral administration. Thus, the strong anorexigenic and body weight-reducing effects of palmitoylated PrRP31 and myristoylated PrRP20 make these analogs attractive candidates for antiobesity treatment. Moreover, PrRP receptor might be a new target for obesity therapy.

Key Words
- prolactin-releasing peptide
- lipidization
- obesity
- GPR10
- anorexigenic
- mice

Journal of Endocrinology (2016) **230**, R51–R58

Introduction

Obesity is a frequent metabolic disorder with a steadily increasing prevalence. Because obesity triggers other life-threatening diseases, including type-2 diabetes mellitus (T2DM), hypertension, dyslipidemia and atherosclerosis (Simmons *et al*. 2010, Vaneckova *et al*. 2014), an effective noninvasive therapy would be beneficial.

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. The majority of current antiobesity drugs are analogs of anorexigenic neurotransmitters aiming to reduce food intake by either decreasing appetite or suppressing the

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DOI: 10.1530/JOE-16-0046

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craving for food. Unfortunately, their severe psychiatric or cardiovascular side effects have highlighted the need for alternative therapeutic strategies (for reviews, see Rodgers *et al*. 2012, Bray & Ryan 2014, Manning *et al*. 2014). The ideal antiobesity drug should produce sustained weight loss with minimal side effects. Recent progress in an understanding of peptidergic signaling of hunger and satiety, both from the gastrointestinal tract and its upstream pathways in the hypothalamus, has opened the possibility for the use of anorexigenic neuropeptides in obesity treatment (Arch 2015, Patel 2015).

In general, peptides are key regulators of physiological processes with low risk of toxicity and side effects. In spite of their clinical potential, native peptides have several limitations: poor bioavailability, low stability in the organism and dificulties to cross the blood–brain barrier (BBB) after peripheral application. Therefore, a considerable effort has been made to design new drugs based on peptides in order to produce stable analogs with a high effectiveness and potential to cross BBB.

One of the recently used strategies for design of peptidic drugs is based on lipidization of peptides, i.e. attachment of fatty acid to peptide through an ester or amide bond leading to an increased stability and half-life in an organism and possible delivery over the BBB (Brasnjevic *et al*. 2009, Bellmann-Sickert & Beck-Sickinger 2010, Malavolta & Cabral 2011). Usually, palmitoylation or myristoylation through amide bond at Lys have been used for this purpose. Recently, two peptide drugs for treatment of diabetes or obesity were introduced to the market: insulin analog detemir employing myristic acid attached through amide bond to insulin molecule (Havelund *et al*. 2004) and liraglutide, a palmitoylated analog of glucagon-like peptide 1 (GLP-1) (Gault *et al*. 2011). Both lipopeptides show strongly prolonged half-life due to their binding to serum albumin and slower proteolysis (liraglutide has plasma half-life 11–15 h compared with several minutes of natural GLP-1 (Agerso *et al*. 2002) and detemir 10 h compared with 4–6 min for natural insulin (Havelund *et al*. 2004)).

Therefore, lipidization of neuropeptides involved in food intake regulation might be a new way for the development of novel antiobesity drugs.

Prolactin-releasing peptide in food intake regulation

The anorexigenic neuropeptide prolactin-releasing peptide (PrRP) was initially isolated from the hypothalamus as a ligand for the human orphan G-protein-coupled receptor GPR10 (Hinuma *et al*. 1998) as a possible regulator of prolactin secretion from anterior pituitary cells. The PrRP receptor GPR10 was first reported to be involved in prolactin release from rat pituitary cells, both primary cells and rat cell line RC-4B/C (Hinuma *et al*. 1998). A direct prolactin-releasing effect of PrRP in mammals has, however, been questioned because no PrRP immunoreactive fibers were observed in the median eminence where classical hypophysiotropic hormones are released (Maruyama *et al*. 1999). This, together with the discovery that prolactin-positive cells in the human pituitary do not colocalize with the PrRP receptor (Abe *et al*. 2003) and the finding that PrRP increases prolactin responses to thyrotropin-releasing hormone (TRH) (Spuch *et al*. 2007), but itself does not significantly increase prolactin secretion in primary rat pituitary cells (Samson *et al*. 1998), has suggested that prolactin release is probably not a primary function of PrRP (Jarry *et al*. 2000, Taylor & Samson 2001).

PrRP was also found to have high affinity to the NPFF2 receptor, resulting in anorexigenic effect (Engström *et al*. 2003). The endogenous ligand of NPFF2 receptor, neuropeptide FF, also has hyperalgesic and antimorphine analgesic properties (for reviews, see Dockray 2004, Chartrel *et al*. 2006).

Shortly after its discovery, it was established that PrRP has other physiological functions, including the regulation of food intake (Lawrence *et al*. 2000) and energy expenditure (Takayanagi *et al*. 2008). PrRP and its receptor were detected in several hypothalamic nuclei as well as in brainstem suggesting an involvement of PrRP in the control of food intake and body weight regulation (Roland *et al*. 1999, Ibata *et al*. 2000).

The suggestion that PrRP may act as a homeostatic regulator of food intake was supported by the fact that PrRP mRNA is reduced in states of negative energy balance similarly to other anorexigenic peptides such as melanocyte-stimulating hormone and cocaine- and amphetamine-regulated transcript (Lawrence *et al*. 2000). Centrally administered PrRP was shown to inhibit food intake and body weight gain in rats, but did not cause conditioned taste aversion. Furthermore, Fos immunoreactivity was enhanced after PrRP administration in areas associated with food intake regulation, such as paraventricular nucleus (PVN) (Lawrence *et al*. 2002).

Intracerebroventricular (ICV) coadministration of PrRP and long-term acting regulator of energy balance leptin in rats resulted in additive reductions in nocturnal food intake and body weight gain and an increase in energy expenditure (Ellacott *et al*. 2002). Idea of connection

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between leptin and PrRP is further supported by the fact that PrRP neurons in brain regions involved in food intake regulation, where PrRP is expressed (ventromedial nucleus (VMH) of hypothalamus and ventrolateral medulla (VLM) and nucleus tractus solitarius (NTS) of brainstem), also contain leptin receptors (Ellacott *et al*. 2002).

Moreover, the anorexigenic peptide cholecystokinin (CCK) was shown to have no effect on food intake in PrRP-receptor-knockout mice. This finding suggested that PrRP acting through its receptor may be a key mediator in the central satiating action of CCK (Bechtold & Luckman 2006). The hypothetical anorexigenic interaction among PrRP, leptin and CCK is depicted in Fig. 1. This description of potential mechanism of action shows that peripheral signals (leptin, CCK) and central neuropeptide PrRP cooperate in the stimulation of food intake regulating pathways leading to decrease in food intake as well as leptin release.

Finally, GPR10 knockout mice had significantly higher body weight in males at 15 weeks of age compared with wild-type mice, and late-onset obesity was found significant in 11-week-old knockout female mice, which also exhibited a significant decrease in energy expenditure (Bjursell *et al*. 2007). Similarly, PrRP-deficient mice displayed late-onset obesity, increased food intake and attenuated responses to the anorexigenic signals cholecystokinin and leptin (Takayanagi *et al*. 2008).

Two biologically active isoforms of PrRP have either 31 (PrRP31) or 20 (PrRP20) amino acids, the shorter peptide is identical with C-terminal 20-peptide of the longer one. C-terminal Arg-Phe-amide sequence is critical for the preservation of biological activity of PrRP (Roland *et al*. 1999, Maletínská *et al*. 2011). While C-terminal fragment of PrRP containing 13 amino acid (PrRP13) is sufficient for full binding potency to GPR10 receptor (Boyle *et al*. 2005), PrRP analog with at least 20 amino acids is necessary for preservation of full biological activitiy *in vivo* (Maixnerová *et al*. 2011). In our previous study (Maletínská *et al*. 2011), we modified C-terminal Phe-amide with other bulky aromatic rings that showed not only high binding potency signaling in RC-4B/C cells comparable with or higher than those of PrRP20, but also a very significant and long-lasting anorexigenic effect after central administration in fasted mice (Maixnerová *et al*. 2011). In this way,

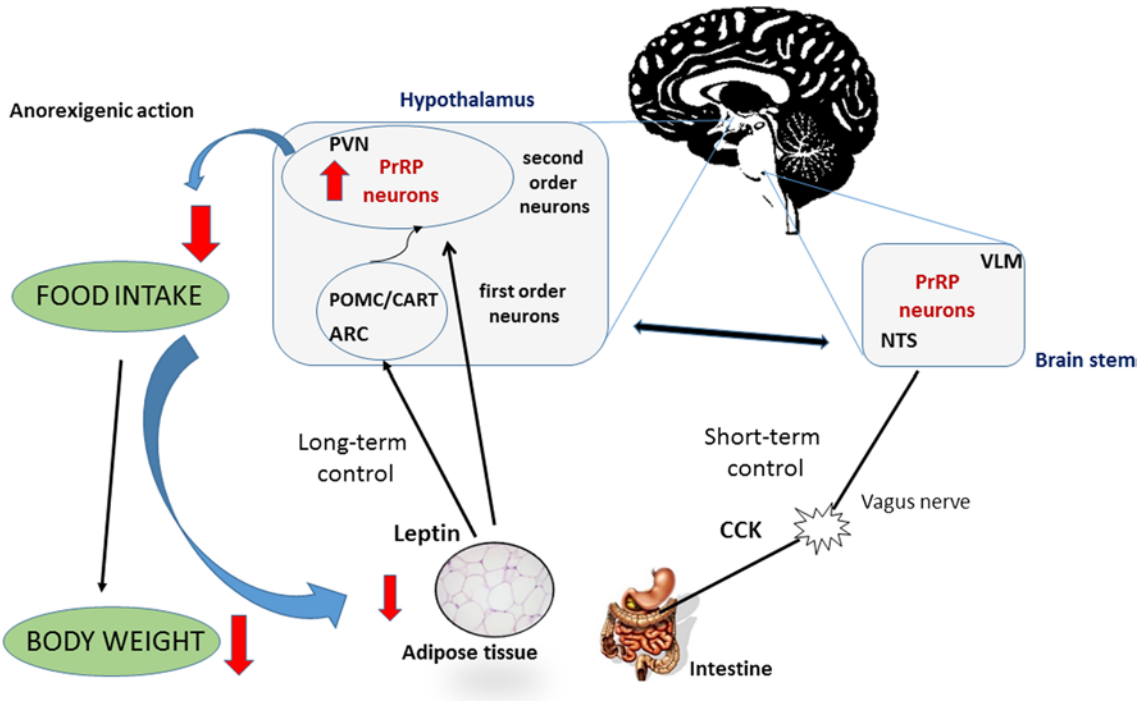

Figure 1
The scheme of a potential role of PrRP in food intake regulation and its interaction with leptin and cholecystokinin in food intake regulation. Peripherally released and centrally actin long-term satiety signal leptin stimulates anorexigenic neurons in arcuate nucleus (ARC) of hypothalamus. This activates PrRP release, which acts to decrease food intake and body weight. PrRP acts synergistically with peripherally released and centrally through nervus vagus acting short-term satiety signal cholecystokinin (CCK). CART, cocaine- and amphetamine-regulated transcript; NTS, nucleus tractus solitaries; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; VLM, ventrolateral medulla.

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we demonstrated that just a bulky aromatic ring, and not necessarily Phe-amide at the PrRP C-terminus, guarantee the biological activity of the peptide.

Lipidized PrRP analogs as potential antiobesity drugs

Because PrRP is a centrally acting neuropeptide and also food intake is regulated centrally, anorexigenic potential of PrRP after peripheral administration depends on its ability to exert its central effect and to reach the target brain receptors. On the other hand, stability of peptide in organism and extention of half-life is also important issue in potential drug development. Therefore, lipidization of PrRP seems to be a suitable way to modify its linear, one-chain peptidic structure.

In our first study, the N-terminus of both natural peptides, PrRP31 and PrRP20, was lipidized with fatty acids of different lengths in order to preserve their full biological activity. We have demonstrated that analogs of PrRP31 and PrRP20 lipidized by 8–18 carbon chain fatty acids showed high binding affinities with Ki 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).

Similarly, myristoylated and palmitoylated PrRP31 analogs with Phe^{31} replaced by aromatic noncoded 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 PrRP 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 action in the brain while retaining central PrRP anorexigenic effects after peripheral administration. Only palm- and stear-PrRP31 and myr-PrRP20 highly significantly and dose-dependently lowered food intake in lean overnight-fasted (Table 1) 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 probably able to exert their central effect on food intake.

Similarly, 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 noncoded amino acid at C-terminus without a negative effect on their biological effect (Prazienkova *et al.* 2016).

Moreover, short-lasted repeated (three injections) administration of palm-PrRP31 to free-fed rats decreased food intake using different routes of palm-PrRP31 delivery: SC, intraperitoneal (IP) or intravenous (IV) (Mikulášková *et al.* 2015). In all cases, food intake

Table 1 Structures and biological activities of rat PrRP analogs.

Analogs (sequences)	**Human GPR10** ^{125}I-human PrRP31 binding K_i (nM)	**Human NPFF2** ^{125}I-1DMe binding K_i (nM)	**Food intake in fasted mice** (5 mg/kg SC) % saline-treated group (45 min)
PrRP31 (SRAHQHSMETRTPDINPAWYTGRGIRPVGRF-NH_2)	3.91 ± 0.21	42.21 ± 6.76	96.3 ± 8.5
palm-PrRP31 ((N-palm)SRAHQHS Nle ETRTPDINPAWYTGRGIRPVGRF-NH_2)	2.94 ± 0.33	0.69 ± 0.36	3.8 ± 6.7
PrRP20 (TPDINPAWYTGRGIRPVGRF-NH_2)	4.4 ± 0.77	21.80 ± 9.91	102.5 ± 5.5
myr-PrRP20 ((N-myr)TPDINPAWYTGR GIRPVGRF-NH_2)	4.21 ± 0.24	8.23 ± 1.97	1.5 ± 3.4

1DMe, stable analog of neuropeptide FF (NPFF); Myr, myristoyl, palm-palmitoyl; SC, subcutaneous.
The mean ± S.E.M. 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 0.1 nM or 0.03 nM, and the K_d that was calculated from saturation experiments was 0.95 ± 0.20 nM for GPR10 receptor and 0.72 ± 0.12 nM for NPFF2 receptor in CHO cells, respectively.

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reduction was observed; however, the degree of food intake decrease was partially dependent on the route of administration and on the dose of palm-PrRP31 together with single or repeated administration. The route of administration was important for the minimal dose of palm-PrRP31 necessary to achieve a significant effect on food intake, especially after SC administration. This may relate to the speed 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 (Mikulášková *et al*. 2015). However, the new specific conditions should be found for SC administration of palm-PrRP31 to decrease its dose for SC administration based on effective IV doses.

The idea of the central effect of lipidized PrRP was further supported by changes of c-Fos levels in the brain after SC administration to fasted mice (Maletinska *et al*. 2015). Both myr-PrRP20 and palm-PrRP31 significantly enhanced c-Fos immunoreactivity in hypothalamic and brainstem nuclei involved in food intake regulation (PVN, NTS) and containing both GPR10 and NPFF2 receptors (Roland *et al*. 1999), whereas natural and octanoylated PrRP31 did not (Maletinska *et al*. 2015). In addition, double c-Fos-GPR10 immunostaining in brainstem C1/A1 cells group indicated that the neurons containing GPR10 receptors are activated after palm-PrRP31 administration with intensity depending on the route of its peripheral administration (intravenous administration, compared with subcutaneous or intraperitoneal injection) caused the highest level of c-Fos activation in rats (Mikulášková *et al*. 2015).

The long-lasting anorexigenic effect of palm- and stear-PrRP31 and myr-PrRP20 analogs could be explained by their prolonged stability due to binding to serum albumin similar to liraglutide. Our stability test confirmed that both palm-PrRP31 and myr-PrRP20 were stable for more than 24 h in rat plasma. *In vivo* pharmacokinetics in mice also showed very significantly longer stability and a higher area under the curve for palm-PrRP31 and myr-PrRP20 compared with natural, nonlipidized analogs (Maletinska *et al*. 2015).

From our experimental results, we can speculate that palm-PrRP31 and myr-PrRP20 food intake-lowering effect after peripheral administration are mainly central. However, any peripheral effects (e.g. on gastrointestinal tract) could not be excluded. Therefore, more detailed analyses of the interaction of lipidized PrRP analogs with other type of receptors and/or with other anorexigenic peptides both in periphery and the brain are needed. Although we still have no direct proof for the palm-PrRP entrance to the brain, several pieces of indirect evidence might 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 nonlipidized 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 (Maletinska *et al*. 2015). 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.

Anorexigenic effect of lipidized PrRP analogs was further supported by two studies with diet-induced rodent models. The 2-week-long, twice-daily administration of palm-PrRP31 and myr-PrRP20 to mice with high-fat diet-induced obesity (DIO) significantly decreased cumulative food intake and body weight (Fig. 2) (Maletinska *et al*. 2015). The decrease in body weight was due to a reduction in fat mass accompanied by a decrease in circulating leptin levels.

Decreased mRNA expressions of fatty acid synthase in both the adipose tissue and the liver along with a decreased expression of acetyl CoA carboxylase and sterol regulatory element-binding protein (SREBP) in the liver suggests that this reduction most likely resulted from a decreased *de novo* lipogenesis owing primarily to negative energy balance due to reduced food intake (Maletinska *et al*. 2015).

Finally, similar results were found in our following study demonstrating that a 2-week-long peripheral treatment of DIO Sprague-Dawley rats with a palm-PrRP31 analog significantly decreased food intake and body weight, with a tendency towards leptin and fat depot reduction (Holubova *et al*. 2016). This treatment was also associated with an improvement in glucose tolerance, and the effect was caused at least partially by an attenuating effect on lipogenesis. In contrast, despite a food intake-lowering effect, palm-PrRP31

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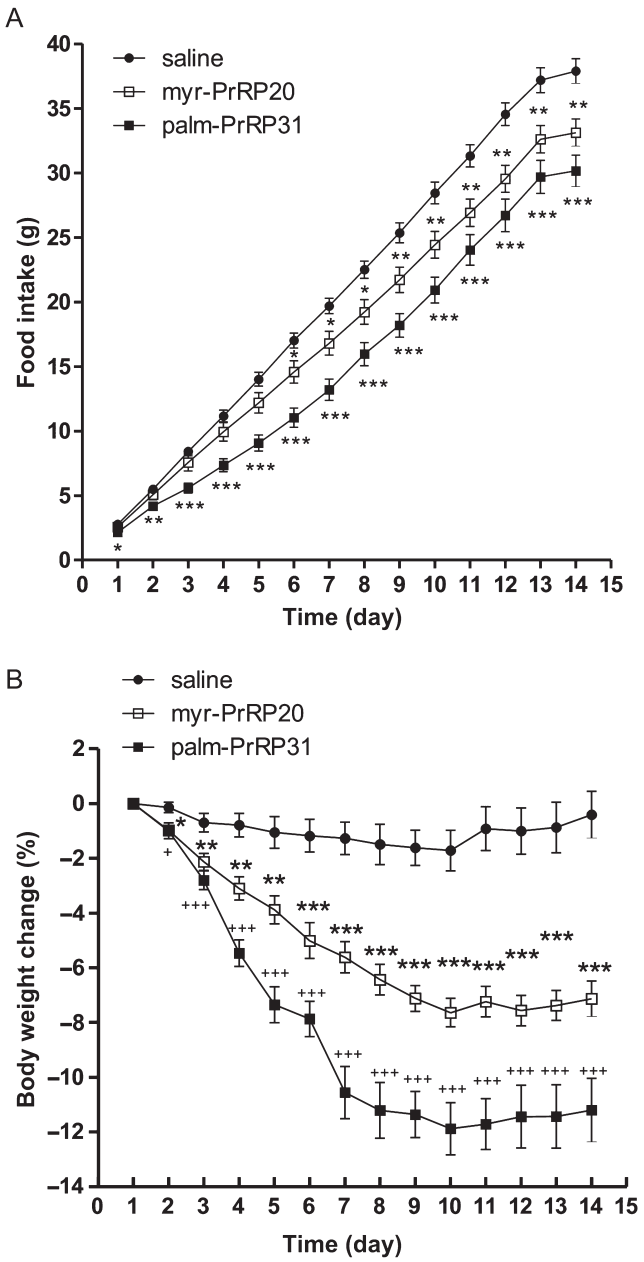

Figure 2
Palmitoylated PrRP31 and myristoylated PrRP20 reduce food intake and body weight of diet-induced obese (DIO) mice. Effect of 14-day administration of palm-PrRP31 and myr-PrRP20 on (A) food intake and (B) body weight of DIO mice. Mice were subcutaneously administered by saline or peptides at a dose of 5 mg/kg twice daily ($n = 10$). The data were analyzed by one-way ANOVA. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs saline-treated group.

failed to decrease body weight or improve glucose tolerance in Zucker diabetic fatty rats, probably due to a lack of functional leptin receptor leading to disrupted interaction of leptin and palm-PrRP31 in this rat model. Thus, GPR10 agonism was proven again to be a promising target for the treatment of obesity, with palm-PrRP31 showing a high anorexigenic efficacy after peripheral administration (Holubova *et al*. 2016).

In addition to above-mentioned biological effect of palmitoylated PrRP analogs, our recent study showed that subchronic peripheral administration of palm-PrRP31 and liraglutide ameliorated hippocampal insulin signaling, and attenuated the pathological hyperphosphorylation of Tau, a hallmark of Alzheimer disease, in monosodium glutamate-treated obese mice, which are a model of obesity and prediabetes. These findings support the potential use of the above-mentioned anorexigenic lipopeptides for the prevention and treatment of the Tau hyperphosphorylation that is connected with obesity-related T2DM. These data show, for the first time, the neuroprotective properties of PrRP (Spolcova *et al*. 2015).

Conclusions

Obesity is a serious medical problem reaching pandemic range. As obesity is a part of the metabolic syndrome, its prevention could also reduce other health problems such as T2DM, hyperinsulinemia or obesity-related hypertension. Despite the fact that many details about the development and maintenance of obesity have been identified, the exact mechanisms controlling food intake and energy expenditure need to be clarified. Recently, there is a boom of interest for several peptides participating in the regulation of appetite and feeding behavior.

In our studies, we have focused our attention on PrRP and its receptor which might be a new target in obesity treatment. We have demonstrated that the lipidization of PrRP enabled its central anorexigenic effect after peripheral administration in both acute and chronic settings by enhancing its stability in the blood and enabling its central action. Our data also confirmed that GPR10 and/or NPFF2 receptors are suitable targets for the treatment of obesity. Collectively, our data suggest that lipidized PrRP analogs have potential as possible future antiobesity drugs.

Nevertheless, the use of PrRP as an alternative to treat obesity still needs further investigation, particularly in regards to the mechanism of action and long-lasting effects of palm-PrRP31 analogs in treatment of the diet-induced obesity as well as in its prevention. Moreover, potential neuroprotective effect of palmitoylated PrRP analogs should be of interest.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding
This work was supported by GA CR grant no. 15-08679S, RVO:61388963 of AS CR and RVO:67985823 of AS CR.

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Received in final form 11 May 2016
Accepted 7 June 2016

http://joe.endocrinology-journals.org
DOI: 10.1530/JOE-16-0046

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