Kisspeptin and RFRP3 modulate body mass in the *Phodopus Sungorus* via two different neuroendocrine pathways

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Abstract

Many animals exhibit remarkable metabolic and reproductive adaptations to seasonal changes in their environment. When day length shortens, Djungarian hamsters (*Phodopus sungorus*) reduce their body weight and inhibit their reproductive activity, while the opposite occurs in springtime. These physiological adaptations are thought to depend on photoperiodic changes in hypothalamic genes encoding the peptides kisspeptin and RFRP3 for the control of reproduction, and pro-opiomelanocortin and somatostatin for metabolic regulation. The objective of this study was to investigate the effect of kisspeptin and RFRP3 on long-term body weight regulation in order to establish whether metabolic and reproductive hypothalamic networks may interact during adaptation to seasonal physiology. We found that chronic central administration of both kisspeptin or RFRP3 in short photoperiod-adapted male Djungarian hamsters increased body weight although through different pathways. The effect of kisspeptin was dependent on testicular activity, as castration prevented the body weight increase, and was associated with an increase in pro-opiomelanocortin and neuropeptide Y expression. On the other hand, the orexigenic effect of RFRP3 was associated with an increase in circulating insulin and leptin levels, but had no effect on any of the hypothalamic metabolic genes investigated, and did not change circulating levels of sex steroids. Notably, neither kisspeptin nor RFRP3 altered female hamster's metabolic parameters. Thus, using a rodent model exhibiting seasonal changes in reproduction and metabolism, this study demonstrates that in addition to their role in the central control of reproduction, kisspeptin and RFRP3 also participate in body weight control in a sex-dependent manner, kisspeptin's effects involving the anabolic action of testosterone and RFRP3 acting on adiposity.

22 **Introduction**

23 Mammals adapt their metabolic and reproductive activities to the seasonal changes in the environment
24 to ensure that offspring will be born in favorable conditions with enough resources for survival. Seasonal
25 rodents like Djungarian hamsters (*Phodopus sungorus*) increase their food intake and body weight and
26 activate their reproduction with increasing day length in spring and summer, whereas the opposite occurs
27 when days are shortening in autumn and winter ¹⁻³. These annual changes in metabolism and reproduction
28 are driven by the pineal hormone melatonin, whose nocturnal production depends on the length of the
29 night ⁴.

30 In the last ten years, major progress has been made to uncover the melatonin-regulated neuronal
31 networks that control seasonal reproduction and metabolism. Two hypothalamic RF-amide peptides,
32 kisspeptin (Kp) and RFamide related peptide 3 (RFRP3), both known to regulate GnRH neuronal activity
33 and gonadotropin secretion ^{5,6}, display marked melatonin-driven seasonal variation in a number of
34 seasonal species, including rodents ⁷⁻¹⁰. Notably, in all seasonal species investigated so far, RFRP3
35 expression is markedly inhibited in short day (SD) conditions, whereas the photoperiodic regulation of Kp
36 expression is modulated by the feedback of sex steroids (^{11,12} for reviews). Further, chronic central
37 infusions of Kp or RFRP3 in SD-adapted Syrian hamsters have been shown to restore reproductive activity
38 despite the photo-inhibitory conditions ^{7,13,14}. Also, changes in hypothalamic genes encoding peptides
39 related to energy homeostasis, such as *pro-opiomelanocortin (Pomc)* and *somatostatin (Sst)*, have been
40 associated with the SD-induced metabolic reduction observed in Djungarian hamsters ^{15,16}.

41 Previous observations in non-seasonal rodents, suggest that Kp and RFRP3 may as well regulate metabolic
42 activity. Disruption of the Kp receptor in mice not only interferes with reproductive activity, but also with
43 the control of body weight and *Pomc* mRNA expression ^{17,18}. Similarly, mutation of the receptor for RFRP3
44 alters the metabolic response to fasting conditions in mice ¹⁹ and a central injection of RFRP3 increased

food intake in rats as well as *Npy* mRNA^{20,21}. Therefore, the objective of this study was to investigate whether the seasonal changes in Kp and RFRP may impact the seasonal control of body weight. We assessed whether chronic central administration of Kp or RFRP3 in SD-adapted male and female Djungarian hamsters would restore the long day (LD)-related metabolic phenotype, and aimed to identify the putative hypothalamic targets involved.

Materials and methods

Animals

Post-pubertal (three to four months old at the beginning of experiments) male (n=72) and female (n=63) Djungarian hamsters were used. The animals were born and raised in the Chronobiotron animal unit (UMS 3415, Strasbourg, France). They were housed individually with an enriched environment known to reduce the depression like behavior caused by isolation, with food (Altromin 720, GmbH & Co, Lage, Germany) and water *ad libitum*, and under long day conditions (LD: 16h light - 8h darkness) unless otherwise stated. All the manipulations and experiments were done in accordance with the local ethical committee (CREMEAS) and the French National Ministry of Education and Research (authorization # 2015021010234017).

Experimental design

RFRP3 (TLSRVPSLPQRFa Caslo, Denmark) and Kp10, the shortest active form of Kp (YNWNSFGLRYa; Caslo, Denmark), were dissolved in artificial cerebral fluid (aCSF) and placed in osmotic minipumps (Alzet® Model 1004). The Kp dose of 170 pmol/hour (2 mg/mL) was selected from a previous experiment¹⁴ and maximal solubility reported by the manufacturer (ToCris Bioscience). RFRP3 is known to show clear dose-dependent effects^{8,13}. Here, a dose of 8.25 pmol/hour (105 µg/mL) was selected from a pilot experiment with doses

ranging from 2.75 pmol/hour to 250 pmol/hour in both male (n = 24) and female (n = 38) animals (Figure 1).

Experiment 1. The aim of this experiment was to test the effects of intracerebroventricular (ICV) applied Kp10 or RFRP3 on body weight and food intake in male and female Djungarian hamsters. Seventeen male and nineteen female animals were transferred for 10 weeks to short-day conditions (SD: 8h light - 16h darkness) in order to inhibit reproduction and cause a reduction in food intake and body weight. In the 10th week, animals, exhibiting the expected reproductive inhibition and body weight loss, were separated randomly into three groups (SD + Kp10, SD + RFRP3, SD + aCSF, n= 5 - 7 of each sex per group) and implanted with the corresponding osmotic minipumps for 5 weeks. In parallel, five male and six female animals, kept in LD during the whole experiment, were implanted with an aCSF filled osmotic pump (LD + aCSF) at the same time as the SD adapted animals to serve as control animals and reference for an active reproduction and high metabolic activity (no body weight loss).

Experiment 2. The objective of this experiment was to test the sex steroid dependency of the Kp10 effects observed in Experiment 1. Twenty-six male Djungarian hamsters were transferred to the SD conditions as described before. In the 10th week, the animals were gonadectomized (SD Gx, n=13) or sham operated (SD, n=13) and implanted with osmotic minipumps containing either Kp10 (170 pmol/hour) or aCSF for 5 weeks and assigned to either one of 4 groups (1: SD + Kp10; 2: SD + aCSF; 3: SD Gx + aCSF; 4: SD Gx + Kp10; n= 6 - 7 animals per group).

For both experiments, body weight and food intake were measured manually with a weighing machine once a week during 5 weeks. After 5 weeks, animals were sacrificed by CO₂ inhalation, blood was collected by a heart puncture and animals were perfused transcardially with phosphate-buffered saline (PBS) followed by paraformaldehyde-lysine-periodate (PLP) fixative and embedded in polyethylene glycol as described before ²². After 15 weeks of treatment none of SD + aCSF male or female hamsters showed

refractoriness that is known to occur spontaneously from the 16-20 week of SD-adaptation. Blood-heparin samples were centrifuged and plasma was stored at -20°C for hormone measurements. All the animals were sacrificed in *ad libitum* conditions during the light (sleep) phase.

Surgery

Minipump implantation was performed as described previously for the Djungarian hamster ¹⁴. Briefly, minipumps were prepared 72 hours before implantation and maintained at 37°C until surgery according to the manufacturer instructions. Animals were anesthetized with Rompun (Bayer Pharma, Puteaux, France) and Zoletil (Virbac, Carros, France) and the L-shaped cannula of the brain kit connected to the minipump was implanted in the lateral ventricle (coordinates: 1.5 mm lateral to bregma, 2.5 mm ventral to the dura), while the body of the pump was placed subcutaneously at the back of the animal.

For experiment 2, just before the minipump implantation and after anesthesia, thirteen hamsters were castrated by a one-centimeter laparotomy exposing the epididymal white adipose tissue (eWAT) to quickly localize and dissect the testes. The remaining animals were sham-operated meaning that eWAT was exposed but gonads were kept intact.

Non radioactive in situ hybridization

Polyethylene glycol-embedded brains were sectioned from the preoptic area to the mammillary bodies into serial 10 µm thick sections using a microtome. Series of sections were mounted selecting one out of every 12th section (the distance between two sections being 110 µm). Sections were mounted on Superfrost Plus™ slides and stored at -80°C until use. Non-radioactive *in situ* hybridization using digoxigenin labeled riboprobes was performed as reported before ^{23,24}. The rat *neuropeptide Y (Npy)* probe (87–522 of Genbank NM_012614.2; 91% of homology with the hamster's sequence), rat *Pomc* probe (157–731 of Genbank NM_139326, sequence verified by restriction mapping) and rat *Sst* probe (135–465

of Genbank NM_012659.2, 92-93% homology with the hamster's sequence) were transcribed from a linearized plasmid in the presence of digoxigenin-labeled nucleotides (Roche, Meylan France).

The sections were post-fixed with 4% formaldehyde, digested during 30 minutes at 37°C with 0.5 µg/mL proteinase K (Roche, Meylan France) and acetylated twice for 10 minutes in 100 mM triethanolamine and 0.25% acetic anhydride. The slides were hybridized with 400 ng/mL of labelled antisense probe in 50% formamide, 5X SSC, 5X Denhardt's solution and 1mg/mL Salmon sperm DNA for 38-40 h at 60°C. A high temperature stringency wash with 0.1x SSC at 72°C was applied 6 X 10 minutes to eliminate all the non-hybridized probes. The digoxigenin tag was detected using an alkaline phosphatase-coupled anti-digoxigenin antibody (1:5000, Roche, Meylan, France). Alkaline phosphatase activity was visualized with a mixture of Nitro Blue Tetrazolium/Bromo-Chloro-Indolyl Phosphate for one to two hours and stopped before the staining intensity reached saturation. Hybridization with corresponding sense probes gave no signal, indicating specificity of the antisense probes.

Hormone measurements

Leptin levels were measured in one time diluted plasma samples using the Multi Species RIA Leptin Kit (Millipore, # XL-85K, France) according to the manufacturer's protocol. Intra-assay variations was 3.4 to 3.6% and inter-assay variation 6.5 to 8.7%. The sensitivity of the assay was 0.8 ng/mL.

Insulin was measured in 20 times diluted plasma samples using the Hamster Insulin ELISA kit (Crystal Chem #90336, USA). Intra and inter-assay variations were less than 10%. The limit of sensitivity of the assay was 0.1 ng/mL.

Blood glucose levels were determined directly at the time of the sacrifice with the glucometer Accu-Check Performa Test strips® (Roche Diagnostic, France). The range of values for the measurements was from 1.81 to 600 mg/dL.

Testosterone was measured in 10 times diluted plasma samples using an ELISA kit from R&D Systems (#KGE010, Minneapolis, USA) according to the manufacturer protocol. The intra-assay variation was 2.9% to 4% and the inter-assay variation 5.6% to 6.8%. The sensitivity of the assay was 0.030 ng/mL.

Image Analysis

A person unaware of the animal's experimental set up quantified the *in situ* hybridization signals. A CCD camera (Sony Model 77CE) attached to a microscope (Zeiss Axios-kop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Germany) was used to take micrographs with an 10 X 0.63 objective. For each gene analyzed, pictures of the area of interest were taken at the same time with identical lighting conditions for all animals. For each animal, the total number of cells showing labeling of a given mRNA was counted manually in all sections of the area of interest, and given as number of mRNA positive neurons per animal, as reported previously^{14,25}. The number of neurons expressing *Npy* or *Pomc* are the total amount of neurons counted in the entire series of sections stained. SST neuron numbers were counted only in the caudal part of the arcuate nucleus (ARC) (Bregma -2.3 to -2.54) as SST expression shows photoperiodic variation only at this level^{16,23}. The mean *in situ* hybridization signal intensity per neuron was analyzed with Image J software using a fixed sized circle for each analyzed neuron. Analysis was performed in anterior, medial and posterior (*Npy* or *Pomc*) or only in posterior (*Sst*) ARC sections for each animal. Labeling intensity was measured in 30 to 40 neurons per section quantified (i.e., 90 or 120 neurons per animal), based on previous work demonstrating that this number of cells provides a stable mean intensity²³.

Statistical Analysis

All data are presented as mean $\pm$ SEM. All the graphs and statistical analyses were done using GraphPad Prism Software Inc. Statistical significance was set at $p < 0.05$. Body weight, food intake data after 5 weeks

treatments with RFamides, hormone assays and *in situ* hybridization were analyzed with One-way ANOVA and the Tukey post-hoc honestly significant difference (HSD) test. Body weight, food intake and seminal vesicles from experiment 2 have been analyzed by Two-way ANOVA and Bonferroni post-hoc HSD.

Results

1. Differential reproductive effects of chronic Kp10 or RFRP3 in SD-adapted hamsters

As expected, adaption to SD induced a marked decrease in the weight of male and female reproductive organs (Fig. 2). Five weeks ICV administration of Kp10 in SD-adapted hamsters increased the weight of the testes ($F(3, 18) = 74.7$; $P < 0.0001$), seminal vesicles ($F(3, 18) = 130.2$; $P < 0.0001$) and uteri ($F(3, 21) = 12.36$; $P < 0.0001$) up to the values observed in LD-adapted hamsters (Fig. 2). By contrast, administration of different doses of RFRP3 (from 2.75 to 250 pmol/hour) did not increase the size of the reproductive organs in either sex (Fig. 2). In male hamsters, the measure of circulating testosterone confirmed the differential reproductive effect of Kp10 (39.51 ± 7.8 ng/mL) and RFRP3 (0.64 ± 0.01 ng/mL, i.e., not different from aCSF treatment, 0.5 ± 0.03 ng/mL) in SD-adapted animals ($F(4, 19) = 21.34$, $P < 0.0001$).

2. Sex-dependent effects of chronic Kp10 or RFRP3 on body weight, food intake, and metabolic hormones in SD-adapted hamsters

Ten weeks after transfer to the SD photoperiod, hamsters displayed a lower body weight (33.30 ± 1.85 g for male; 29.55 ± 1.68 g for female) as compared to LD conditions (46.52 ± 1.85 g for male; 38.61 ± 1.45 g for female) (Fig. 3A, B). In SD-adapted male hamsters, chronic treatment with either 170 pmol/hour Kp or 8.25 pmol/hour RFRP3 for 5 weeks increased body weight as compared to aCSF control animals that showed no weight gain ($F(3, 18) = 13.03$; $P = 0.0001$) (Fig. 3A). The effect of RFRP3 treatment, but not that of Kp10, was associated with a significant increase in the weekly food intake when compared to aCSF

control animals (ICV Treatment: $F(3, 18) = 10.84$, $P=0.0003$; Time: $F(4, 72) = 3.821$, $P=0.0071$; Interaction $F(12, 72) = 1.385$, $P=0.1929$) as well as the cumulative food intake along the duration of the treatment when compared to aCSF control animals ($F(3, 18) = 7.3$; $P=0.020$) (Fig. 3A). Of note, none of the other doses of RFRP3 tested induced a significant change in the male's body weight (Fig. 1A). In SD-adapted female hamsters, none of the peptides altered body weight ($F(3, 21) = 1.898$; $P=0.1609$) or food intake (Treatment: $F(2, 15) = 1.044$; $P=0.3763$; Time: $F(4, 60) = 1.675$; $P = 0.1677$; Interaction: $F(8, 60) = 0.6686$; $P=0.7169$) (Fig. 3B).

In both male and female hamsters, circulating insulin and leptin, but not glucose, concentrations were significantly lower in SD- as compared to LD-adapted animals (Fig. 3). In SD-adapted male hamsters, RFRP3 treatment increased circulating insulin ($F(2, 10) = 7.977$; $P=0.0085$; Tukey HSD $P=0.0126$) and leptin ($F(2, 13) = 13.78$; $P=0.0002$; Tukey HSD $P=0.0014$) levels as compared to aCSF treated animals, but had no effect on glucose ($F(2, 14) = 0.02254$; $P=0.9778$; Tukey HSD $P= 0.9858$) (Fig. 3A). On the other hand, Kp10 treatment in SD male hamsters had no effect on glucose, insulin or leptin (Fig. 3A). In SD-adapted female hamsters, neither RFRP3 nor Kp10 altered the circulating levels of glucose ($F(2, 16) = 0.8513$; $P=0.5498$), insulin ($F(2, 16) = 0.7942$; $P=0.4690$) or leptin ($F(2, 14) = 4.196$; $P=0.0373$) as compared to the SD control group (Fig. 3B). All these measurements come from animals with *ad libitum* access to food and water.

3. Effects of chronic Kp10 or RFRP3 on expression of metabolic genes in the hypothalamus of SD-adapted hamsters

In order to find putative hypothalamic targets of the Kp10 and RFRP3 treatment, the number of neurons expressing the metabolic genes *Npy*, *Pomc* and *Sst* and the intensity of the mRNA labeling per neuron were quantified in the ARC at the end of the 5-week ICV treatments (Figs. 4-5).

In absence of treatment, the number of *Pomc* expressing neurons was higher in LD- as compared to SD-adapted male (Fig. 4A) and female (Fig. 5A) hamsters, while in contrast neuronal *Sst* mRNA intensity signal

was lower in LD- as compared to SD-adapted male (Fig. 4C) and female (Fig. 5C) hamsters. No photoperiodic variation was observed in the number of NPY neurons or in the neuronal *Npy* mRNA intensity signal in male (Fig. 4B) or female (Fig. 5B) hamsters.

In SD-adapted male hamsters, chronic Kp10 treatment increased the number of *Pomc* (Fig. 4A) ($F(3, 18) = 20.48$; $P < 0.0001$) and *Npy* (Fig. 4B) ($F(3, 17) = 4.25$; $P = 0.0206$) expressing neurons and did not change *Sst* expression (Fig. 4C). Chronic RFRP3 treatment by contrast had no effect on the expression of the investigated genes in the male hamsters (Fig. 4). In SD-adapted female hamsters, neither Kp10 nor RFRP3 treatment altered the number or intensity of neurons expressing the investigated genes (Fig. 5).

4. Sex steroid dependence of Kp10 effect in the male hamsters

In order to investigate whether the effect of Kp10 on body weight in male hamsters was sex steroid dependent, in Experiment 2 we repeated the Kp10 infusion in castrated (Gx) SD-adapted animals. As expected, Gx blocked the Kp10 induced increase in seminal vesicle weight (Castration: $F(1, 21) = 107.5$, $P < 0.0001$; ICV treatment: $F(1, 21) = 118.5$, $P < 0.0001$; Interaction: $F(1, 21) = 110.8$, $P < 0.0001$) (Fig. 6A) confirming the absence of circulating testosterone. Further, the Kp10-induced increase in body weight was totally abolished by Gx (Castration: $F(1, 21) = 4.884$, $P = 0.0383$; ICV treatment: $F(1, 21) = 18.05$; $P = 0.0004$; Interaction: $F(1, 21) = 7.666$; $P = 0.0115$) (Fig. 6B). Finally, Gx had no significant effect on the cumulative food intake and Kp10 did not modify food intake in either sham or Gx male hamsters (Castration: $F(1, 21) = 0.03778$, $P = 0.8477$; ICV treatment: $F(1, 21) = 4.232$; $P = 0.0523$; Interaction: $F(1, 21) = 0.1564$; $P = 0.6964$) (Fig. 6C).

Discussion

Different groups have investigated the hypothalamic mechanisms involved in seasonal metabolism and reproduction, highlighting the roles of POMC, SST, Kp, and RFRP3 neurons in either one or both of these processes^{14,26–28}. In the present study, we investigated putative central mechanisms responsible for the seasonal synchronization between body weight control and reproduction, including possible sex-differences. Notably, we observed sex-dependent and differential effects of central Kp and RFRP3 on body weight and metabolic hormones in the seasonal Djungarian hamster. Both Kp and RFRP3 increased body weight in SD-adapted male, but not female, hamsters. The effect of Kp, was testosterone-dependent and involved changes in *Npy* and *Pomc* gene expression, whereas the effect of RFRP3, but not Kp, was accompanied by an increase in food intake and circulating levels of insulin and leptin.

Sex-dependent central effect of RF-amides on body weight

Male and female Djungarian hamsters displayed the expected marked reduction in body weight and reproduction when exposed to SD photoperiodic conditions. The decrease in body weight correlated with a reduction in food intake and a down-regulation of plasma insulin and leptin levels as reported previously^{29,30}. In the hypothalamus, we confirmed the SD-induced down regulation of *Pomc* gene expression in the ARC of both sexes^{15,31} and found that the previously reported SD-induced up regulation of *Sst* gene expression in the posterior part of the male hamster ARC^{16,23} was also present in females. We also confirmed the lack of SD-induced changes in *Npy* expression as previously described^{15,31}.

Importantly, we demonstrated that a 5-week central administration of either Kp or RFRP3 increased body weight in SD-inhibited lean male Djungarian hamsters. However, neither Kp nor RFRP3 were observed to modulate female hamsters' body weight and food intake, although the Kp treatment did restore female reproductive activity as attested by uterus weight. These data indicate that the metabolic effects of central Kp and RFRP3 are sex-dependent, despite the seasonal changes in metabolic *Pomc* and *Sst*^{15,31} and

reproductive *Kiss1* and *Rfrp*^{14,32} gene expression being similar among male and female Djungarian hamsters, as also observed in the present study. The orexigenic effect of central RFRP3 has already been reported in several mammalian species^{20,21,25}, whereas for Kp an acute anorexigenic effect in fasted female jerboas²⁵ and a lack of effect in either ad libitum or fasted rats has been reported³³. Although there is no clear explanation as to why chronic Kp and RFRP3 do not alter female Djungarian hamster's body weight, the sex difference in energy metabolism and obesity susceptibility is well known, including previous studies reporting sex-dependent metabolic effects of the currently studied peptides. For instance, it was reported that Kp receptor mutation in mice affects body weight and glucose metabolism of female but not male mice¹⁷. The contrasting sex-dependent metabolic effect of Kp in mice and hamsters may rely on the different species and/or experimental paradigms used in both studies, with a life-long kisspeptin signaling *deficiency* in the knockout mice as compared to chronic kisspeptin *treatment* of several weeks in the seasonal hamsters. Sensitivity to Kp may also be different between sexes, as for instance, in adult hamsters³⁴ and prepubertal rats³⁵, acute kp10 injections produce smaller effect of gonadotropins secretion in females than in males. In line with this observation, hypothalamic kisspeptin receptor expression has been found to be lower in female than male striped hamsters³⁶.

Two RF-amides, two pathways

It has become clear that the hypothalamic Kp and RFRP3 neurons are a functional link between the seasonal changes in the pineal hormone melatonin and reproduction (³⁷ for review). Thus, melatonin acts on the *pars tuberalis* to control TSH production which, in turn, regulates deiodinase activity in tanycytes located in the wall of the third ventricle leading to seasonal changes in the content of T3 in the mediobasal hypothalamus. Remarkably, a central administration of TSH²³ or T3³⁸ can rescue the body weight reduction and the inhibited reproduction of SD-adapted male hamsters, as well as the LD phenotype of Kp and RFRP3 expression^{23,39}.

Chronic infusion of Kp⁷ or RFRP3¹³ has already been reported to rescue reproductive activity in SD-adapted Syrian hamsters. Here, both peptides are also found to rescue body weight loss in SD-adapted male Djungarian hamsters. Despite their similar effects on the body weight, the mode of action of Kp10 and RFRP3 differed in several aspects. First, the metabolic effect of Kp10 is probably mediated via an increased activity of the hypothalamo-pituitary-gonadal axis, since castration completely abolished the Kp10-induced increase in body weight. On the other hand, RFRP3 treatment increased animal's body weight without a restoration of reproductive activity. The lack of reproductive effect of chronic RFRP3 in the SD-inhibited Djungarian hamster contrasts with what has been observed in the Syrian hamster¹³. The acute effect of RFRP3 on LH secretion in male Djungarian hamster depends on its photoperiodic status, being stimulatory in the SD- and inhibitory in the LD-adapted animals⁸. Based on these findings, we expected increased reproductive activity after chronic RFRP3 in the male Djungarian hamsters, although there is also some evidence indicating that RFRP3 may inhibit reproduction in intermediate photoperiods (13.5 h Light)⁴⁰. Further, it is important to note that RFRP3 displays marked dose-dependent effects^{8,13} and in the present study only a dose of 8.25 pmol/hour was able to induce a metabolic effect among the different RFRP3 concentrations tested. It is likely that such a marked dose-dependent effect of RFRP3 is due to a change in receptor density, sensitivity or signaling. Previously it has been reported that the SD-induced decrease in Djungarian hamster's body weight is both independent and dependent of the gonads⁴¹, our data indicate that Kp10 may be involved in the testosterone-dependent and RFRP3 in the testosterone-independent seasonal regulation of the male hamster's body weight.

Strikingly, we found that the RFRP3- but not the Kp10-treated males increased their food intake and showed higher circulating leptin and insulin levels. Leptin provides an indirect measurement of the white adipose tissue (WAT) content in the body, indicating that in RFRP3-treated animals the increased body weight might be due primarily to increased adiposity. Although in our experiment we did not assess directly the fat mass content, our results are supported by recent findings reporting increased WAT

accumulation in mice injected intraperitoneally with RFRP3⁴². It is well known that increased adiposity results in insulin resistance⁴³, therefore, the increased plasma insulin levels found in the RFRP3-treated male hamsters might be due a compensatory mechanism to maintain stable glucose levels. Previously, leptin and insulin signaling have been reported to be differentially modulated in LD versus SD-adapted Djungarian hamsters^{44,45}, indicating the Djungarian hamster as an interesting animal model to study both, leptin and insulin sensitivity. Here our data indicate that photoperiodic changes in RFRP3 may possibly be implicated in this phenomenon.

The ARC is pivotal for metabolic regulation and because recent studies have shown that the Kp receptor Kiss1r⁴⁶ and the RFRP3 receptor GPR147⁴⁷ are both expressed in this brain area, we analyzed whether Kp10 or RFRP3 could target ARC metabolic genes. Chronic Kp10 increased *Pomc* and *Npy* in males, but not in females, which could explain why only male hamsters modified their metabolic phenotype. POMC neurons receive a dense Kp innervation and express Kiss1r in rats⁴⁸, they are directly activated by Kp in mice⁴⁹ and are regulated by a central Kp10 injection in jerboas²⁵, altogether supporting a direct effect of Kp on POMC neurons. On the other hand, castration completely abolished the effect of Kp10 on body weight and since testosterone is known to regulate *Pomc*^{50–52} and *Npy*^{53–55}, probably also part of the Kp effect on the metabolic genes is testosterone-dependent. The up-regulation of *Pomc* expressing neurons in animals with increased body weight may seem counterintuitive, but this observation matches the higher *Pomc* gene expression observed in the heavier LD-adapted hamsters¹⁵. In addition, even when the *Pomc* gene is down-regulated in SD, the levels of α -MSH, an anorexigenic peptide produced by the *Pomc* gene might be higher due to a reported increased activity of carboxipeptidase E^{56,57}. Therefore, in principle it is possible that the Kp10-induced increase in *Pomc* expression is associated with lower α -MSH. Further research on the production of either α -MSH or β -endorphin is necessary to conclude on the exact molecular targets of Kp for the photoperiodic regulation of metabolism. In contrast to POMC, NPY/AgrP neurons exhibit a very low level of Kiss1r⁴⁸ and Kp10 has been reported to exert an indirect local inhibitory

effect on these neurons^{49,58}. Further, when a metabolic effect of Kp10 or Kiss1r mutation was reported in female rodents, this effect was not associated with changes in *Npy* expression^{18,25}. Therefore, we assume that the increased number of NPY neurons in Kp10 treated male hamsters essentially depends on the increased testosterone secretion following testis reactivation^{53–55}.

The finding in males that the RFRP3-induced increase in body weight and circulating insulin and leptin levels was not associated with changes in metabolic genes was quite unexpected. The effect of chronic RFRP3 treatment on cumulative food intake in the seasonal hamster is in agreement with previous studies reporting that acute RFRP3 treatment increases food intake in mice, jerboas, rats, ewes and non-human primates^{20,21,25,42}. Some studies have proposed that RFRP3 may act directly on NPY and POMC neurons. Thus, in ewe RFRP3 fibers project to NPY and POMC neurons⁵⁹ and acute RFRP3 treatment increases their neuronal activity²¹. Further, in the female jerboas the acute orexigenic effect of RFRP3 is associated with increased *Npy* mRNA and decreased *Pomc* mRNA expression²⁵ and in male rats, RFRP3 treatment increased food intake and *Npy* mRNA levels in the ARC²¹. Interestingly, none of these short-term effects of RFRP3 on food intake and gene expression were associated with increased body weight supporting the idea that the chronic (photoperiodic) effects of RFRP3 on body weight may use different metabolic pathways and do not depend on changes in NPY and POMC. Actually, a study reported that Djungarian hamsters over-expressing the orexigenic AgRP, although exhibiting higher food intake and lower energy expenditure relative to the control hamsters, still show a significant, prolonged decrease in body weight when exposed to SD conditions⁶⁰. Alternatively, if leptin increases POMC and decreases NPY expression, as it does in other animals (⁶¹ for review) we cannot exclude that RFRP3 affects the neuronal activity of NPY and POMC neurons and counteracts the effects of leptin, resulting in no apparent changes. Finally, the chronic RFRP3 treatment may have caused changes in peptide content or release, which were not evaluated in our study.

Overall, our study shows that the central RF-amide peptides Kp and RFRP3, which are implicated in the control of reproduction, notably in seasonal species, may also participate in the seasonal regulation of body weight, at least in male Djungarian hamsters. Our results indicate that Kp modulates the seasonal changes in body weight in a sex-steroid dependent manner, and through an action on ARC POMC and NPY neurons. The long term effect of RFRP3 on body weight by contrast seems sex-steroid independent and does not appear to involve the ARC POMC and NPY neurons, but possibly a different neuro-endocrine pathway involving the control of food intake and adiposity. Further experiments are now necessary to dissect in more detail how Kp and RFRP3 separately affect lean (through the anabolic effect of testosterone) and fat mass. In conclusion, using a relevant seasonal model our study adds further support to the increasing evidence for an integrated regulation of both metabolism and reproduction.

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518

Figure Legends

Figure 1. Dose dependent effect of RFRP3 in male and female Djungarian hamsters. Male (A) or female (B) hamsters were raised in conditions of long days (LD, white bars) or short days (SD, grey bars) and treated with a 5 week-intra-cerebro-ventricular infusion of artificial cerebrospinal fluid (aCSF) or various concentrations (from) RFRP3. At the end of treatment, paired testicles and seminal vesicles weight in male and uterus weight in female were measured. The body weight change was calculated for both male and female during the 5 week treatment. Data presented are mean $\pm$ SEM of n= 6 to 7 per experimental group, and scattered dots represent individual data -. * $P < 0.05$; after One-way ANOVA and Tukey's post hoc honestly significance test, different vs SD aCSF group.

Figure 2. Effect of kisspeptin or RFRP3 on the reproductive status of photoinhibited male and female Djungarian hamsters. Male (A,B) or female (C) hamsters were raised in long day (LD) or short day (SD) conditions and treated with a 5 week intracerebroventricular (icv) infusion of artificial cerebrospinal fluid (aCSF), kisspeptin 10 (Kp10, 170 pmol/hour) or RFRP3 (8.25 pmol/hour). Testes and seminal vesicle values are given as paired weight. Data represented in bars are mean $\pm$ SEM of n= 5 to 7 per experimental group, and the dots represent individual data values. Different letters mean the groups are statistically different after One-way ANOVA and Tukey's post hoc honestly significance test.

Figure 3. Effect of kisspeptin or RFRP3 on the metabolic status of photoinhibited male and female Djungarian hamsters. At week 0, male (A) or female (B) hamsters raised in long days (LD) were either kept in LD (grey line) or placed in short day (SD) conditions (black lines). After 10 weeks (vertical dotted line) hamsters were treated with a 5 week intracerebroventricular (icv) infusion of artificial cerebrospinal fluid (aCSF), 0.17 nmol/hour kisspeptin 10 (Kp10) or 8.25 pmol/hour RFRP3. The body weight (BW) was followed weekly since the beginning of the experiment (week 0). Food intake was monitored once a week since the start of the ICV infusion (Week 10). The change (delta) in BW and cumulative food intake are summed for

the 5 weeks of treatment. Plasma levels of glucose, insulin and leptin were measured at the end of the 5 week treatment. Data are presented as mean $\pm$ SEM of n= 6 to 7 per experimental group and the dots represent individual data values. Different letters in histograms mean the groups are statistically different after One-way ANOVA and Tukey's post hoc honestly significance test.

Figure 4. Effect of kisspeptin or RFRP3 on metabolic gene expression in the arcuate nucleus of photoinhibited male Djungarian hamsters. Male hamsters were raised in of long day (LD) or short day (SD) conditions and treated with a 5 week intracerebroventricular infusion of artificial cerebrospinal fluid (aCSF), 170 pmol/hour kisspeptin 10 (Kp10) or 8.25 pmol/hour RFRP3. At the end of the treatment, *in situ* hybridization was performed for gene encoding A - POMC (pro-opiomelanocortin), B - NPY (neuropeptide Y) and C – SST (somatostatin). Pictures show representative images of the arcuate neurons expressing *Pomc*, *Npy* and *Sst* mRNA after the different treatments (scale bar=100 μ m). Bars show the semi-quantification of the mean number of labeled neurons per animal (left histogram) or the mean labeling intensity (in arbitrary units) per neuron (right histogram). Data are presented as mean $\pm$ SEM of n= 6 to 7 per experimental group, and the dots represent individual data values. Different letters mean the groups are statistically different after One-way ANOVA and Tukey's post hoc honestly significance test.

Figure 5. Effect of kisspeptin or RFRP3 on metabolic gene expression in the arcuate nucleus of photoinhibited female Djungarian hamsters. Female hamsters were raised in of long day (LD) or short day (SD) conditions and treated with a 5 week intracerebroventricular infusion of artificial cerebrospinal fluid (aCSF), 170 pmol/hour kisspeptin 10 (Kp10) or 8.25 pmol/hour RFRP3. At the end of the treatment, *in situ* hybridization was performed for gene encoding A - POMC (pro-opiomelanocortin), B - NPY (neuropeptide Y) and C – SST (somatostatin). Pictures show representative images of the arcuate neurons expressing *Pomc*, *Npy* and *Sst* mRNA after the different treatments (scale bar=100 μ m). Bars show the semi-quantification of the mean number of labeled neurons per animal (left histogram) or the mean labeling intensity (in arbitrary units) per neuron (right histogram). Data are presented as mean $\pm$ SEM of n= 6 to 7

per experimental group, and the dots represent individual data values. Different letters mean the groups are statistically different after One-way ANOVA and Tukey's post hoc honestly significance test.

Figure 6. Effect of castration on reproductive and metabolic physiology in kisspeptin-treated male Djungarian hamster. Hamsters adapted to the inhibitory short day (SD) conditions were gonadectomized (Gx, open symbols) or sham operated (sham, closed symbols), then were treated with a 5 week intracerebroventricular infusion of artificial cerebrospinal fluid (aCSF) or 170 pmol/hour kisspeptin. At the end of the treatment, A- the reproductive function was evaluated with the paired seminal vesicle weight, and the metabolism was measured by B - the change in body weight (BW) and C - the cumulative food intake. Data are presented as mean $\pm$ SEM. of n= 6 to 7 per experimental group and the points represent individual data values. * $P < 0.05$ after Two-way ANOVA and Tukey's post hoc honestly significance test vs Sham + aCSF.

A**Males****Testicles** **Seminal Vesicles** **BW change** **B****Females****Uterus** **BW change**

A**Testicles** **B****Seminal Vesicles** **C****Uterus**

A Males

B Females

A

LD + aCSF

SD + aCSF

SD + Kp10

SD + RFRP3

Pomc neurons*Pomc* intensity **B**

LD + aCSF

SD + aCSF

SD + Kp10

SD + RFRP3

Npy neurons*Npy* intensity **C**

LD + aCSF

SD + aCSF

SD + Kp10

SD + RFRP3

Sst neurons*Sst* intensity

A

LD + aCSF

SD + aCSF

SD + Kp10

SD + RFRP3

Pomc neurons*Pomc* intensity **B**

LD + aCSF

SD + aCSF

SD + Kp10

SD + RFRP3

Npy neurons*Npy* intensity **C**

LD + aCSF

SD + aCSF

SD + Kp10

SD + RFRP3

Sst neurons*Sst* intensity

(A)

Seminal vesicles

(B)

BW change

(C)

Cumulative Food intake

aCSF
Kp10