**RFRP3 INCREASES FOOD INTAKE IN A SEX DEPENDENT MANNER IN THE SEASONAL HAMSTER
PHODOPUS SUNGORUS**

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Abstract (250 words max).

In addition to its regulatory role in luteinizing hormone secretion, RFamide related peptide 3 (RFRP3) has also been reported to modulate food intake in several mammalian species. Djungarian hamsters (*Phodopus sungorus*), like other seasonal mammals, display a remarkable inhibition of RFRP3 expression in winter short day conditions, associated with decreased food intake and bodyweight. This species is therefore a valuable model to assess whether RFRP3 might be involved in the seasonal control of feeding behavior and investigate its possible brain targets. We found that although both male and female animals exhibit the same robust reduction in *Rfrp* expression in short- (SD) as compared to long-day (LD) conditions, acute central administration of RFRP3 displays sex-dependent effects on food intake. RFRP3 increased food intake in female hamsters in SD or in LD-diestrus, but not in LD proestrus, indicating that the orexigenic effect of RFRP3 is observed in conditions of low circulating estradiol levels. In male hamsters, food intake was not changed by acute injections of RFRP3, whether animals were in SD or LD conditions. Analyzing the gene expression of various metabolic neuropeptides in the brain of RFRP3-injected Djungarian hamsters revealed that *Npy* expression was increased in female, but not in male animals. This study suggests that in Djungarian hamsters RFRP3 exhibits a sex dependent orexigenic effect possibly by inducing an increased *Npy* expression.

Introduction

Soon after the discovery by Tsutsui et al. (2000) of a hypothalamic neuropeptide with a potent inhibitory effect on the secretion of pituitary luteinizing hormone (LH) in quails, an orthologue peptide with a similar inhibitory effect on LH secretion was characterized in rodents by Kriegsfeld et al. (2006). Further studies confirmed the reproductive effect of this hypothalamic peptide, nowadays called (Arg)(Phe) related peptide-3 (RFRP3) (See for review Angelopoulou et al., 2019; Kriegsfeld et al., 2018; Tsutsui and Ubuka, 2016). Remarkably, the effect of RFRP3 on LH secretion seems to be sex-dependent, as it has been reported to be inhibitory in female rodents (Gibson et al., 2008; Henningsen et al., 2017), but either stimulatory or inhibitory in males (Ancel et al., 2012, 2017; Johnson et al., 2007; Kriegsfeld et al., 2006; Pineda et al., 2010; Simonneaux et al., 2013; Ubuka et al., 2012).

Increasing evidence now indicates that RFRP3 also affects food intake, exhibiting an orexigenic effect in rats (Clarke et al., 2012; Johnson et al., 2007), jerboas (Talbi et al., 2016a), mice, ewes and non-human primates (Clarke et al., 2012). In addition, RFRP3 projections and receptors (GPR147) have been described in the arcuate nucleus (Henningsen et al., 2016; Kriegsfeld et al., 2006), a hypothalamic region involved in the control of feeding behavior, mostly by gathering and integrating peripheral metabolic information (See for review Varela and Horvath, 2012). Moreover, it has been reported that RFRP3 affects the expression of both orexigenic and anorexigenic genes in the hypothalamus (Clarke et al., 2012; Talbi et al., 2016a).

In all seasonal mammals examined so far, RFRP3 expression was markedly inhibited during short day (SD) conditions, in response to the extended secretion of nocturnal melatonin (Angelopoulou et al., 2019; Dardente et al., 2017; Revel et al., 2008; Sáenz De Miera et al., 2014; Talbi et al., 2016b; Ubuka et al., 2012). Because most seasonal species display marked changes in both metabolic and

reproductive activities throughout the year, it is possible that in addition to reproductive activity, RFRP3 also affects food intake in a seasonal-dependent manner.

The Djungarian hamster (*Phodopus sungorus*) is an extraordinary seasonal model that displays a coordinated decrease in food intake and bodyweight together with an inhibition of reproductive activity when transferred from long day (LD) to SD conditions (Figala et al., 1973; Fine and Bartness, 1996; Warner et al., 2010). Therefore, the aims of this study were: i) to examine whether female Djungarian hamsters, like males (Klosen et al., 2013; Ubuka et al., 2012), display photoperiodic regulation of RFRP3 expression; ii) to assess the potential effect of RFRP3 on food intake in both male and female animals; iii) to determine whether the effect of RFRP3 depends on the animal's photoperiodic and reproductive status and iv) to identify the putative hypothalamic targets associated with the effect of RFRP3 on food intake.

Materials and methods

Animals

Adult male (n=24) and female (n=54) Djungarian hamsters born and raised in the Chronobiotron animal unit (UMS 3415, Strasbourg, France) were used for these experiments, after approval of the local ethical committee (CREMEAS) and the French National Ministry of Education and Research (authorization # 2015021010234017). From birth until puberty, animals were raised under long day conditions (LD: 16h light - 8h darkness, lights on at 4 am and off at 8 pm) at a temperature of 20 ± 2°C, with food (Altromin 720, GmbH & Co, Lage, Germany) and water available *ad libitum*. Experimental hamsters (three to four month-old) were housed individually with a cotton nest and a wooden bar to reduce isolation stress. Twelve male and 30 female hamsters were transferred for 10 weeks to short day conditions (SD: 8h light – 16 darkness, lights on at 10 am and off at 6 pm) in

order to evoke the well-known reproductive and metabolic inhibition (approximately 30% bodyweight loss), while 12 males and 24 females remained in LD conditions.

Cannula implantation and intracerebroventricular injection

In order to perform central peptide injections, an intracerebroventricular cannula was implanted in the lateral ventricle as described previously for the Djungarian hamster (Klosen et al., 2013). Briefly, animals were anesthetized with a mix of Rompun (Bayer Pharma, Puteaux, France) and Zoletil (Virbac, Carros, France) and a guide cannula was lowered reaching the lateral ventricle (coordinates: 1.5 mm lateral to bregma, 2.5 mm ventral to the dura) and a dummy cannula was used to block the canal of the guide cannula. Animals were allowed to recover for at least one week before any injection was performed. Intracerebroventricular (ICV) injections were performed under light isoflurane anesthesia (AErrane; Baxter, Maurepas, France). Animals were infused with 3 μ L of either RFRP3 (Djungarian hamster sequence TLSRVPSLPQRFa, Caslo laboratory, Lyngby, Denmark) or Vehicle (Saline 0.9%) at a constant flow rate of 1 μ L/min. The cannula was left in place for one more minute after infusion to allow correct product diffusion.

Estrous cycle determination

In order to establish the estrous stage of LD adapted female hamsters, vaginal smears were performed at midday as described previously (Henningsen et al., 2017). Briefly, the stages were defined based on the most abundant type of cells in the vaginal smear (Byers et al., 2012) and only females in proestrus (with the highest amount of nucleated rounded cells and very few to no leucocytes) or in diestrus (prominent leucocytes) were selected, considering these are opposite stages with the highest and the lowest levels of estrogen, respectively.

Food intake experiment

Studying food intake in *Phodopus sungorus* is complex due to their natural hoarding behavior (Bartness and Day, 2003). Therefore, we adapted our experimental procedure according to Schuler et al (Schuhler et al., 2003). Bedding was removed 24 h before each experiment with the cotton nest and wooden bar left in place in order to diminish animal's stress. Just before each injection, the few remaining hoarded pieces of food and feces were removed from the cages. Food content was measured manually just before and after each injection. In a first preliminary experiment, food intake was measured 3, 6, 12 and 24 hours after the injection, this allowed us to define that 3 hours after the injection provided sufficient time to establish an effect on food intake. In a second preliminary experiment performed on SD-adapted female hamsters, RFRP3 was injected at five different times of the day/night cycle (ZT1, ZT5, ZT9, ZT14, ZT19 with ZT0 being defined as light onset) in order to define whether there was a time of the day dependency of the RFRP3 effect (n=18). This second experiment allowed us to establish ZT5 as the most effective time of the day for the ICV injections in SD animals.

In the main experimental setup, hamsters were injected 3 hours before the dark onset in both photoperiodic conditions (at ZT5 in SD and ZT13 in LD) with vehicle or RFRP3. SD males and females, and LD males were tested with three different amounts of RFRP3 (0.5, 1.5 and 5 µg) or vehicle. LD females were injected with two amounts of RFRP3 (0.5 or 1.5 µg) or vehicle, in either diestrus or proestrus. A same individual received up to 5 independent ICV injections (all doses in a random manner) with at least one week of recovery in between each injection. At the end of the experiments, animals were sacrificed one hour and a half after the final ICV infusion by inhalation of increasing doses of CO₂ and then perfused transcardially with phosphate-buffered saline (PBS) followed by paraformaldehyde-lysine-periodate (PLP) fixative. The brains were removed and embedded in polyethylene glycol as described before (Klosen et al., 1993).

Non radioactive *in situ* hybridization

Polyethylene glycol-embedded brains were sectioned 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 (Klosen et al., 2013; Rasri et al., 2008). The *Phodopus sungorus Rfrp* probe (87-529 Genbank JF727837) and *orexin (Hcrt)* probe (1-411 of Genbank MG266908), the *Rattus norvegicus neuropeptide Y (Npy)* probe (87–522 of Genbank NM_012614.2) and *Proopiomelanocortin (Pomc)* probe (157–731 of Genbank NM_139326) 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 1 mg/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.

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 (Rasri-Klosen et al., 2017; Talbi et al., 2016a). The number of neurons expressing *Rfrp*, *Npy*, *Hcrt* or *Pomc* corresponds to the total amount of neurons, i.e., left and right, counted in the entire series of sections stained (10-15 sections per brain). The mean *in situ* hybridization signal intensity per neuron was analyzed with Image J software using a fixed sized circle for each analyzed neuron. For each animal analysis was performed in one anterior, medial and posterior section. Labeling intensity was measured in 30 to 40 neurons in each section (i.e., 90 - 120 neurons per animal), based on previous work demonstrating this number of cells provides a stable mean intensity (Klosen et al., 2013).

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 $p < 0.05$. Food intake treatments with RFRP3 were analyzed with One-way ANOVA and the Tukey post-hoc honestly significant difference (HSD) test. *In situ* hybridization results were analyzed with the student's *t* test.

Results

RFRP3 and POMC gene expression in male and female hamsters with a different photoperiodic and reproductive status

Quantification of *Rfrp* mRNA containing cell number and mean intensity of labeling per neuron showed that *Rfrp* mRNA expression was markedly reduced in SD as compared to LD conditions, both in male and female hamsters (Figure 1). Further, *Rfrp* expression was similar during diestrus and proestrus in LD-adapted females (Figure 1). Analysis of photoperiodic variation in *Npy*, *Hcrt* and *Pomc* gene expression in both female and hamsters showed that only *Pomc* was decreased in SD compared to LD hamsters, in both sexes, whereas *Npy* and *Hcrt* did not show significant differences when comparing SD vs LD (Figure 2).

RFRP3 effect on food intake in SD or LD-adapted male and female hamsters

The effect of a central injection of exogenous RFRP3 on food intake was first tested in SD-adapted hamsters as they exhibited the lowest levels of RFRP expression. Patency was verified during infusion by movement of a small air bubble. Correct placement was confirmed afterwards in Nissl stained brain sections. Firstly, an experiment was performed in SD-adapted female hamsters to investigate a putative time of the day effect of RFRP3. Administration of 1.5 ug of RFRP3 significantly increased food intake over the next 3 hours, at all time points investigated except just after lights on, at ZT1 (Treatment: $F(1, 80) = 7.503$, $P=0.0076$; ZT: $F(4, 80) = 5.502$ $P=0.0006$; Interaction: $F(4, 80) = 0.7794$, $P=0.5419$; Figure 3A). Accordingly, all other RFRP3 injections in SD were performed 3 hours before lights off, at ZT5. The orexigenic effect of RFRP3 in SD-adapted females appeared dose-dependent with only 1.5 μg , but not 0.5 μg or 5 μg , being able to significantly increase food intake as compared to vehicle ($F(3, 43) = 4.005$; $P=0.0134$; Figure 3B). On the other hand, in SD-adapted males, none of the RFRP3 doses tested significantly increased food intake ($F(3, 47) = 1.519$; $P=0.2219$; Figure 3B).

We next investigated the effect of an RFRP3 injection 3 hours before lights off (ZT13) on food intake in male and female hamsters adapted to LD, thus when they are sexually active and exhibit higher RFRP expression and larger food intake as compared to SD conditions. In females, 0.5 µg but not 1.5 µg increased food intake when applied on the day of diestrus ($F(2, 23) = 5.5$; $P=0.0112$; Figure 2C), but none of the two doses had an effect on the day of proestrus ($F(2, 23) = 0.03251$; $P=0.9681$; Figure 3C). None of the three doses of RFRP3 tested in LD-adapted male hamsters modified the amount of food intake ($F(3, 38) = 0.03049$; $P=0.9927$; Figure 3C).

Effect of RFRP3 on the hypothalamic genes involved in metabolism

In order to identify the putative targets associated with the orexigenic effect of RFRP3, the levels of mRNA coding for various metabolic peptides were analyzed in the hypothalamus of hamsters injected with either RFRP3 or vehicle and sacrificed 90 minutes after the injection. In the SD-adapted females, the dose of 1.5 µg, which exhibited a significant orexigenic effect, induced a significant increase in the number of *Npy* expressing neurons, but had no effect on *Pomc* or *Hcrt* expressing neurons (Figure 4A). In SD-adapted males, the dose of 1.5 µg RFRP3 did not alter the number of *Npy*, *Pomc* or *Hcrt* expressing neurons (Figure 4B) in agreement with the lack of effect on food intake. In LD-adapted male and female hamsters, none of investigated genes exhibited any significant change in expression (data not shown).

Discussion

The central mechanisms controlling food intake and reproduction are usually considered as separate systems. However, an increasing number of studies now points to tight interactions between the neuroendocrine circuits that regulate both functions (for review see Evans and Anderson, 2018). This interaction became especially clear when Kisspeptin (Kp) and RFRP3, two hypothalamic

neuropeptides acting upstream of the GnRH neurons, were suggested to also integrate metabolic information and to couple reproduction with energy requirements (see De Bond and Smith, 2014; Schneider et al., 2017; Tsutsui and Ubuka, 2016 for review). Seasonal species like the Djungarian hamster are a valuable model to investigate the central mechanisms coordinating changes in metabolic and reproductive activities as they display coordinated changes in food intake, bodyweight and reproduction according to the time of the year. In male hamsters, this seasonal physiology is associated with changes in the expression of the “reproductive” genes *Kiss1* (Rasri-Klosen et al., 2017) and *Rfrp* (Rasri-Klosen et al., 2017; Ubuka et al., 2012), but also “metabolic” genes such as *Somatostatin* (Herwig et al., 2012; Klosen et al., 2013) and *Pomc* (Helwig et al., 2006, 2013; Mercer et al., 2000), but surprisingly not *Npy* (Jethwa et al., 2010). In this study, we found a similar photoperiodic regulation of *Rfrp* and *Pomc*, in female and male Djungarian hamsters, but we report a sex dependent orexigenic effect of RFRP3, possibly through increased *Npy* expression.

The melatonin-driven SD-inhibition of *Rfrp* expression in the DMH/VMH area has been reported in the males of various species, including the Djungarian hamster (Rasri-Klosen et al., 2017; Revel et al., 2006). Here we found that female Djungarian hamsters, also displayed a marked reduction in *Rfrp* gene expression during SD, very similar to that observed in males (Rasri-Klosen et al., 2017). The Syrian hamster also displays a SD-induced inhibition of *Rfrp* expression in both sexes, but with a larger amplitude in females as compared to males (Henningsen et al., 2016). Here, in the sexually active LD-adapted female hamster, we further found that *Rfrp* expression is similar in diestrus and proestrus. The effect of sex steroids on *Rfrp* expression is a matter of debate with data indicating no effect (Henningsen et al., 2017; Quennell et al., 2010), a moderate (Poling et al., 2012) or an inhibitory effect (Molnár et al., 2011; Salehi et al., 2013; Soga et al., 2014) of estradiol. Our data do not support the regulation of *Rfrp* expression by estradiol in female Djungarian hamsters, which is in agreement with the absence of *Rfrp* regulation by testosterone in male Djungarian hamsters

(Rasri-Klosen et al., 2017). Thus, both male and female Djungarian hamsters exhibit a similar robust SD-dependent, sex steroid-independent, inhibition of *Rfrp* expression, probably driven by the melatonin rhythm as already demonstrated in the Syrian hamster (Revel et al., 2008).

GnIH/RFRP3 was first discovered by virtue of its inhibitory action on GnRH neuronal activity and gonadotropin secretion (Kriegsfeld et al., 2006; Tsutsui et al., 2000), however, later studies showed that these effects of RFRP3 were dose-, sex-, species- and photoperiod-dependent (Angelopoulou et al., 2019 for review). For instance, in the Syrian hamster and in the mouse, RFRP3 increases LH secretion in males, but inhibits elevated LH levels during the proestrus LH surge and in gonadectomized females (Ancel et al., 2012; Henningsen et al., 2017; Kriegsfeld et al., 2006). In the male Djungarian hamsters, RFRP3 increases LH secretion in SD-adapted animals, but decreases it in LD-adapted animals (Ubuka et al., 2012). These findings highlight the importance of investigating the biological effect of RFRP3 in various conditions, as attested in our current study where the acute effects of RFRP3 on food intake were actually found to depend on the sex and the photoperiodic status of Djungarian hamsters.

In females hamsters, RFRP3 increased food intake in a dose-dependent manner regardless of the photoperiod, but with different active doses (0.5 µg in LD and 1.5 µg in SD animals), suggesting a change in sensitivity according to the photoperiod. A photoperiod-dependent differential expression of the *GPR147*, the receptor for RFRP3, has indeed been reported for the female Syrian hamster (Henningsen et al., 2016),. An orexigenic effect of RFRP3 has also been reported in female jerboa in both, spring and autumn, although only one dose was tested in that study (Talbi et al., 2016a). In LD conditions, we observed that RFRP3 increased food intake in diestrus but not in proestrus, suggesting an estradiol-dependent orexigenic effect of the peptide during the estrous cycle. Studies have reported that daily food intake decreases during the peri-ovulatory period due to higher levels of estrogens (Asarian and Geary, 2002; Olofsson et al., 2009). Furthermore, a recent

study reported that RFRP3 neuronal activity in female Syrian hamsters correlated with low circulating sex steroid levels and higher food hoarding during most of the estrus cycle, except for the day of proestrus when estrogens are higher and animals eat less (Benton et al., 2018). Together, these studies indicate that mimicking higher RFRP3 neuronal activation and peptide release by an ICV infusion, promotes food intake in SD or in LD diestrus when estrogens are low, but not when estrogens are high in LD proestrus. Remarkably, in the SD-adapted female Djungarian hamster, the orexigenic effect of RFRP3 was associated with an increase in the number of NPY expressing neurons, as also reported in female jerboa (Talbi et al., 2016a). The delayed response after 3h would be in line with an indirect effect of RFRP3 via NPY. However, in contrast to what was reported in the female jerboa (Talbi et al., 2016a) and mice (Fu and van den Pol, 2010), RFRP3 does not appear to regulate *Pomc* gene expression. Our results show a marked sex difference in the acute effects of RFRP3 as various doses of RFRP3 given to male Djungarian hamsters had no effect on food intake and *Npy* gene expression. The reason for such sex a difference in RFRP3-elicited feeding activity is unclear, but probably independent of circulating testosterone since the same lack of effect was observed no matter if male hamsters were sexually active (in LD) or quiescent (in SD). Our finding is in contrast with earlier studies reporting an orexigenic effect of RFRP3 in the male rat (Clarke et al., 2012; Johnson et al., 2007).

Intriguingly, in our recent study in which we investigated the chronic (5 weeks) metabolic effect of a central infusion of RFRP3 in SD-adapted lean Djungarian hamsters, RFRP3 displayed an opposite sex-dependent effect as it was able to restore feeding activity, body weight and levels of leptin and insulin in the male but not the female animals (Cázarez-Márquez et al., 2019). This latter finding is in line with a recent report that long-term alteration of the RFRP3 signaling (through genetic mutation of its receptor) affects male, but not female mouse metabolic activity (Leon et al., 2018). Yet, when compared to the present study, these findings highlight another diversity aspect of the

biological effect of RFRP3 that is the acute *versus* long-term effects, indicating that the acute and chronic effects most probably involve different cellular mechanisms. Indeed, the acute orexigenic effect of RFRP3 in female Djungarian hamsters and jerboas is associated with an increase in *Npy* expression (this study, Talbi et al., 2016a), whereas the chronic orexigenic effect of RFRP3 in male Djungarian hamsters occurs with no apparent change in the expression of the metabolic genes *Pomc*, *Npy*, *Somatostatin* (Cázarez-Márquez et al., 2019).

In conclusion, this study confirms in the seasonal Djungarian hamster that RFRP3 exerts an acute orexigenic effect, probably acting through an increased *Npy* expression, however, with a marked sex-difference since females, but not males, increased their food intake after acute ICV injections of the peptide. Further, the orexigenic effect of RFRP3 depended on photoperiod and reproductive status, as it was observed in SD conditions, but in LD conditions only when circulating levels of estrogen were low (i.e., diestrus). Together, this study adds further evidence that various parameters, notably species, sex and reproductive status, but also duration of administration, must be taken into account when studying the central mechanisms responsible for the metabolic and reproductive effects of RFRP3.

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Legends.

Figure 1. Photoperiodic regulation of *Rfrp* gene expression in the hypothalamus of male and female Djungarian hamsters. A & B) Representative photos from *in situ* hybridization of *Rfrp* mRNA in female and male hamsters adapted to long days (LD) or short days (SD), scale bar correspond to 500

459 μM . C & D) Quantification of the number of mRNA positive neurons or labeling intensity per neuron;
460 white bars represent LD animals, gray bars represent SD animals, circles for females (closed circles
461 for diestrus and opened circles for proestrus) and squares for males. Paired testicles and uterus
462 weight were measured. Data are represented as mean $\pm$ SEM, n = 5 to 7 hamsters per photoperiod
463 group. **** is for $P < 0.0001$ after student t test when comparing SD vs LD groups.

464
465 Figure 2. Photoperiodic regulation of *neuropeptide Y* (*Npy*), *hypocretin* (*Hcrt*) and *pro-*
466 *opiomelanocortin* (*Pomc*) gene expression in the hypothalamus of male and female Djungarian
467 hamsters. A & B) Quantification of the number of mRNA positive neurons; white bars represent LD
468 animals, gray bars represent SD animals, circles for females (closed circles for diestrus and opened
469 circles for proestrus) and squares for males. C) Representative photos from *in situ* hybridization of
470 *Pomc* mRNA in female and male hamsters adapted to long days (LD) or short days (SD), scale bar
471 correspond to 200 μM . Data are represented as mean $\pm$ SEM, n = 5 to 7 hamsters per photoperiod
472 group *** is for $P < 0.001$ and **** is for $P < 0.0001$ after student t test when comparing SD vs LD
473 groups

474
475 Figure 3. Effect of acute intra-cerebroventricular injection of RFRP3 on food intake in female and
476 male Djungarian hamsters adapted to short (SD) or long day (LD) conditions. A) Food intake
477 measured 3 hours after the central injection of vehicle (white bars) or 1.5 μg RFRP3 (grey bars) at
478 different the time of the day in SD-adapted female hamsters. Data are presented as mean food
479 intake/100 g bodyweight (BW) $\pm$ SEM of n = 9 hamsters per experimental group). B & C) Food intake
480 measured 3h after the central injection of three different amounts of RFRP3 (0.5, 1.5 or 5 μg) in SD
481 (B) and LD (C) female or male hamsters. Food intake was measured during the first 3 hours after the

central injection. Data are presented as the mean food intake/100 g BW $\pm$ SEM of n= 6-12 for RFRP3 and 24 for vehicle (which was always performed in parallel to the various doses of RFRP3) animals tested per experimental group. White bars represent control animals and gray scale bars are for RFRP3. * P<0.05 after 1-W ANOVA vs vehicle.

Figure 4. RFRP3 effect on *neuropeptide Y (Npy)*, *hypocretin (Hcrt)* and *pro-opiomelanocortin (Pomc)* mRNA in the hypothalamus of SD-adapted female and male Djungarian hamsters. Female and male hamsters were injected centrally with vehicle or 1.5 μ g of RFRP3, and the number of neurons expressing the investigated genes were analyzed 90 minutes after injection. A & B) Number of neurons expressing *Npy*, *Hcrt* or *Pomc* after vehicle (white bar) or RFRP3 (grey bar) injection in female (A- circles) or male (B- squares). Data are represented as mean $\pm$ SEM, n = 6 hamsters per group *P<0.05 by students t test vs vehicle; C & D) Representative pictures from the *in situ* hybridization for *Npy*, *Hcrt* or *Pomc* after vehicle or RFRP3 injection in female (C) or male (D), F: fornix; 3V; third ventricle. Scale Bar = 200 μ M.