Integrating reproduction and energy metabolism

A round way trip via RF-amide peptides

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Publication date
2021

Document Version
Final published version

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Integrating reproduction and energy metabolism: a round way trip via RF-amide peptides
Academic thesis, University of Amsterdam, the Netherlands, University of Strasbourg, France

About the cover: Mexican traditions are an unvaluable heritage for its people. “Pecked or perforated paper” is commonly used for celebrations. We used a series of four colored papers to translate the four seasons typically known by western countries: spring (green), summer (pink), fall (orange) and winter (blue). A lot of shapes were used to fill with symbolisms the subjects studied in this book: hamsters, rats, male and female, reproduction and food, brain structures and the RF-amides. Finally, we included the life transitions made by the author during his scientific career from Mexico to Alsace and then Amsterdam, with allusive buildings to each place.

ISBN: 9789464193206
Author: Fernando Cázarez Márquez
Cover design: Emmanuel Flores Espinoza
Lay out: Fernando Cázarez Márquez

Printing: Gildeprint

Financial support for printing of this thesis was kindly provided by the Amsterdam Gastroenterology Endocrinology Metabolism (AGEM) research institute.

Université de Strasbourg

UvA

UNIVERSITÉ DE STRASBOURG
UNIVERSITÉ D'AMSTERDAM

ÉCOLE DOCTORALE Sciences de la Vie et de la Santé (ED 414)
Institut des Neurosciences Cellulaires et Intégratives (CNRS UPR 3212)

THÈSE EN COTUTELLE présentée par :

Fernando CÁZAREZ-MÁRQUEZ

soutenue le : **2 Novembre 2021**

Pour obtenir le grade de **: Docteur de l'université de Strasbourg**
&
Docteur de l'université d'Amsterdam

Discipline/ Spécialité : ***Sciences de la vie/Neurosciences***

Intégration de la reproduction et du métabolisme énergétique : un voyage aller-retour des peptides RF-amides

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INTEGRATING REPRODUCTION AND ENERGY METABOLISM: A ROUND WAY TRIP VIA RF-AMIDE PEPTIDES

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit van Amsterdam

op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op dinsdag 2 november 2021, te 12.00 uur

door Fernando Cazarez Marquez
geboren te Puebla

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Dit proefschrift is tot stand gekomen in het kader van het NeuroTime programma, een Erasmus Mundus Joint Doctorate, met als doel het behalen van een gezamenlijk doctoraat. Het proefschrift is voorbereid in: het Nederlands Herseninstituut en in het Academisch Medisch Centrum (AMC), Faculteit der Geneeskunde, van de Universiteit van Amsterdam; en in het Institut des Neurosciences Cellulaires et Intégratives van de Université de Strasbourg.

This thesis has been written within the framework of the NeuroTime program, an Erasmus Mundus Joint Doctorate, with the purpose of obtaining a joint doctorate degree. The thesis was prepared in: the Netherlands Institute for Neuroscience (NIN) and in the Academic Medical Centre (AMC), Faculty of Medicine at the University of Amsterdam; and in the Institut des Neurosciences Cellulaires et Intégratives of the Université de Strasbourg.

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based on

THE NEUROENDOCRINOLOGY OF SEASONALITY: RF-AMIDES LINKING REPRODUCTION AND METABOLISM

Fernando Cázarez-Márquez
Andries Kalsbeek
Valérie Simonneaux

In preparation

Contents of Chapter 1

I. SEASONAL METABOLISM

a) What is a season and how do organisms adapt to them?

The term "season" is used in different aspects of our lives, but most of the time it involves a long period of time that reflects a clear set of specific activities. Many places on earth know a raining season or monsoon season, but also crop seasons exist and even in sports, we have match seasons.

Many organisms on earth are exposed to cyclic changes in temperature, precipitation, and other environmental factors. Here, we will focus on the 4 cyclic seasons adopted by western societies: spring, summer, autumn (or fall) and winter. Astronomically, we find that the cause for the appearance of these main 4 established seasons is the tilted earth axis. The 23.5 degrees inclination of the axis causes sunlight to reach the surface of our planet with a different angle and intensity along the year, especially in the higher latitudes that are closer to the poles, as compared to the lower latitudes in between the tropics closer to the equator.

The 4 seasons each last for 3 months and were established taking the solstices (two periods of the year with the longest night or the longest day) and equinoxes (two periods of the year with equal duration of night and day) as a reference. Thus, the equinoxes mark the beginning of the spring and autumn, respectively, whereas the longest day and longest night solstices indicate the beginning of the summer and winter, respectively.

Organisms living at higher latitudes are submitted to large seasonal changes in their environment, such as light, temperature and food availability, to which they adapt either by escaping from them when being too adverse (i.e., migratory birds) or by modifying their physiology. Such seasonal changes in physiology and behavior not only requires an organism to sense, but also to anticipate these environmental changes.

b) The neuroendocrine basis for seasonal functions

A long series of studies in the previous century unraveled the basic neuroendocrine mechanisms responsible for the central control of seasonality, pointing out the important role of the pineal gland hormone melatonin and its action on the *pars tuberalis* (PT) of the pituitary gland. Melatonin is secreted at night with a duration proportional to the length of the night and thereby serves as a hormonal signal to convey information about the photoperiod and thus the time of the year to the rest of the body. The early studies from Hoffman[1,2] showed that the long winter-like melatonin peak not only inhibits reproduction in the Djungarian hamster (*Phodopus sungorus*), but also decreases its body weight and whitens its fur color. However, although these studies clearly evidenced the indispensable role of the melatonin rhythm to drive seasonal changes in reproduction, body weight and fur color, the targets in the central nervous system to accomplish these changes remained unknown at that time.

After many years of gathering additional evidence it was finally demonstrated that seasonal changes in the structure of the PT were accompanied by changes in the production of thyrotropin stimulating hormone subunit beta (TSHβ)[3]. Later on, it was found that the PT indeed expresses the type 1 melatonin receptor (MT1) in the non-seasonal Wistar rats[4] and the seasonal European hamster (*Cricetus cricetus*)[5].

As recently reviewed extensively[6], it is clear now that melatonin affects local TSH production

in the PT, but the PT-TSH released into the circulation has little activity on the thyroid gland. Actually, a detailed study from Ikegami *et al.*[7] revealed that there is a post-translational glycosylation differentiating TSH released from the anterior pituitary from TSH produced by the PT. PT-TSH binds to TSH receptors in the tanycytes located along the basal part of the neighboring third ventricle wall, where it activates deiodinase 2 (Dio2) and inhibits deiodinase 3 (Dio3) enzyme activity. This TSH-mediated switch in the Dio2/Dio3 ratio increases the levels of active thyroid hormone (T3) locally in the mediobasal hypothalamus (Figure 1)[8,9]. Importantly, the long winter-like melatonin peak inhibits TSH production from the PT, leading to an inverted Dio2/Dio3 ratio in the tanycytes and reduction in the hypothalamic content of T3. The melatonin-driven retrograde transport of TSH from the PT to the tanycytes in the third ventricle thus controls the conversion of thyroid hormones locally in the mediobasal hypothalamus, which ultimately is responsible for the seasonal changes in physiology.

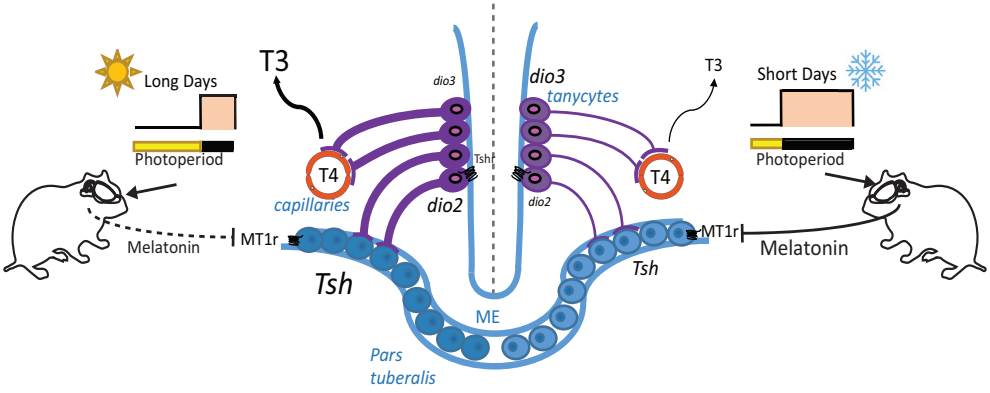

Figure1. Illustration of the central phototransduction pathway controlling seasonality in the Djungarian hamsters. On the right side, the hamster adapted to short days produces a long nocturnal peak of melatonin which inhibits thyroid stimulating hormone (TSH) production by the *pars tuberalis*. On the left side, the hamster adapted to long days produces a long nocturnal peak of melatonin which is no more inhibiting TSH production. TSH secreted under long days binds TSH receptors located on tanycytes to increase deiodinase 2 (dio2) and decrease dio3 expression leading to an increased conversion of circulating T4 into local T3. Therefore the seasonal change in the melatonin-controlled TSH production by the pars tuberalis drives seasonal changes in hypothalamic levels of T3.

Although the above studies have formally demonstrated the role of central PT-TSH and T3 in the seasonal regulation of reproduction, body weight and daily torpor[10–13], including recent experiments from our group demonstrating that the thyroid hormone receptor (Thr) is necessary for transmitting the melatonin cascade in the hypothalamus[14], the neuronal targets and mechanisms modulated by T3 in the hypothalamus of seasonal species remain a mystery.

c) The rationale of seasonal metabolism

In April 2020, the World Health Organization (WHO) reported that 650 million people, thus 13% of world's adult population, are obese[15]. It is critical to prevent and reduce the prevalence of obesity, as this condition leads to important cardio-metabolic, reproductive, and inflammatory co-morbidities. In this context, it is imperative to understand the environmental and physiological processes behind this pathology.

In natural conditions, many mammals exhibit physiological changes in food intake and body

weight according to seasons. Indeed, they increase their food intake and accumulate body fat to face the upcoming winter season characterized by low temperature and limited food availability. One of the best known (and famous) examples of coupling metabolic adaptations to environmental challenging conditions are bears who go into hibernation during the winter season and stop defecation and urination for a long period, while using mostly fat from their adipose tissue as an energy source for heat production[16]. Smaller mammals as the woodchuck (*Marmota monax*) or groundhog are other well-known and much studied hibernating animals that show a clear seasonal regulation of their food intake and body weight to prepare themselves for the hibernation time during winter[17]. Among the many animal species showing metabolic adaptations to cope with the cold temperatures in winter, we have decided to focus on an even smaller mammal, the seasonal hamsters. They enter torpor to reduce energy expenditure and cope with the decreasing temperatures during winter among other with metabolic adaptations that are discussed next.

d) Djungarian hamsters as a model for studying the interaction of reproduction and energy metabolism

The Djungarian hamster, also known as the Siberian hamster, was introduced as a seasonal animal model by Hoffman and Figala in 1973[1,2]. A special trait of the Djungarian hamsters is that when going from long day (LD) to short day (SD) conditions, i.e. mimicking the seasonal change from short summer nights towards longer autumn and winter nights, they exhibit marked metabolic changes, with the major reduction of their adipose tissue leading to a loss of body weight up to 30% as reviewed in[18–20]. Further, as many other mammalian species, these animals undergo a full inhibition of sexual activity in winter SD as demonstrated by a marked reduction in gonadal weight, circulating sex hormones and sexual behavior.

Whereas a number of other hamster species also exhibit an inhibition of their sexual activity in winter conditions, their seasonal metabolic phenotype varies. For instance, the Syrian hamster (*Mesocricetus auratus*) shows a moderate increase in body weight[21], and the Turkish hamster (*Mesocricetus brandti*) shows no changes[21]. The European hamster (*Cricetus cricetus*) shows a decrease in body weight that is strongly dependent on an annual clock, in addition to photoperiod[22]. Moreover, European, and Turkish hamsters are endangered species and therefore have a restricted availability. Altogether, this makes the Djungarian hamster an excellent animal model to investigate the functional interaction between reproduction and energy metabolism in the biological context of seasonality. Of note, such studies require the consideration of both male and female individuals, as there are obvious and important sex differences in the metabolic load of reproductive activity.

e) Seasonal variations of metabolic hypothalamic peptides

The neuronal circuits that regulate food intake and body weight mainly involve two neuronal populations with opposite functions, both located in the arcuate nucleus (ARC) of the hypothalamus, respectively the neuropeptide Y (NPY)/agouti related peptide (AgRP) and pro-opiomelanocortin (POMC)/cocaine-amphetamine-regulated transcript (CART) containing neurons[23]. Yet, several other hypothalamic neuropeptides have also been demonstrated to be involved in the regulation of metabolic activity, notably somatostatin (SST) (reviewed in[24]) and hypocretin (Hcrt, also known as orexin)[25], but also melanin-concentrating hormone (MCH) and VGF peptide (non-acronym). Although most studies investigating the role of

these neuropeptides in food intake, body weight regulation and energy balance have used the conventional non-seasonal rat and mice models, several of these neuropeptides exhibit striking annual variations in seasonal species (Figure 2). In the next paragraphs, we will briefly describe the general knowledge for some key hypothalamic neuropeptides involved in the control of energy metabolism. Each paragraph ends with a sum up of the known photoperiodic regulation in the Djungarian hamster.

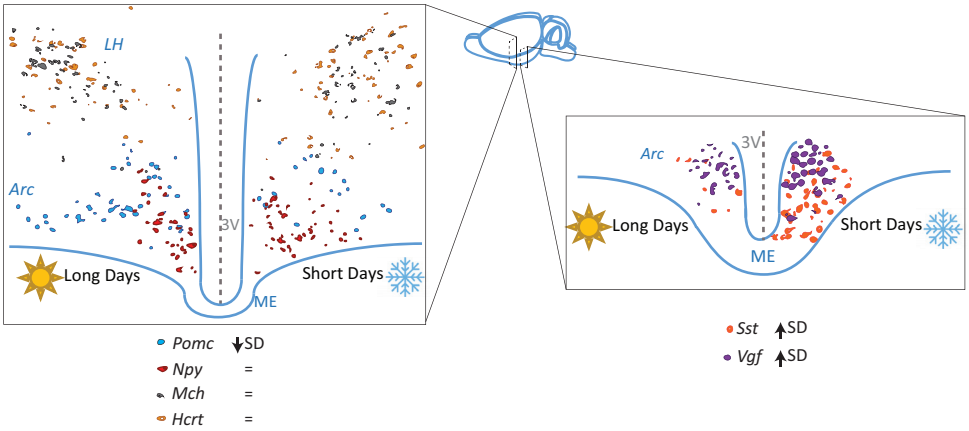

Figure 2. Seasonal variations of metabolic circuits in the hypothalamus of the Djungarian hamster. The left panel shows a diagram of a coronal section of the hypothalamus with the arcuate nucleus (Arc) containing the neuronal populations expressing pro-opiomelanocortin (Pomc, blue), Neuropeptide Y (Npy, red) and in the lateral hypothalamus (LH) neurons expressing melanin-concentrating hormone (Mch, gray) and hypocretins (Hcrt, mustard). The right panel shows a diagram of the posterior Arc showing neurons expressing of somatostatin (Sst, in orange) and Vgf (non-acronym, purple). In each panel the neuroanatomical distribution is illustrated under long days (left hemisphere) and short days (right hemisphere). Below each panel, the direction of change in mRNA expression of the neuropeptides in short days as compared to long days is indicated as "↑" for up-regulation, "↓" for down regulation, or "=" when there is no change. 3V, third ventricle; ME, median eminence.

Neuropeptide Y/Agouti related peptide

NPY was first described to be orexigenic in 1984 when it was injected intracerebroventricularly (ICV) in rats[26]. Later it was found that mainly NPY derived from neurons in the ARC and secreted in the paraventricular nucleus of the hypothalamus (PVN) was mediating this increase in food intake[27]. It was not until 10 years later that AgRP was discovered[28,29] to be co-expressed in the ARC NPY neurons and found to be also linked to food intake, body weight regulation and energy balance, and projecting towards the PVN. The NPY/AgRP neurons also may express GABA (reviewed in[30]). Surprisingly, $AgRP^{-/-}$ and $NPY^{-/-}$ mice do not have a strong metabolic phenotype, suggesting that there are compensatory mechanisms when ablating these peptides in early development[31,32].

In the Djungarian hamster no seasonal variations have been found for NPY/AgRP expression[33,34] although AgRP expression has been described to increase in the rostral ARC in SD, but with no data shown[34].

Proopiomelanocortin/Cocaine-amphetamine regulated peptide

The *POMC* gene was first described to be expressed in the brain by Gee *et al.*[35] and alpha-melanocyte-stimulating hormone (α-MSH) was proven to be the most potent anorexigenic POMC-derived peptide[36]. Later CART was found to be co-expressed in POMC neurons in the ARC and similar to POMC, to be decreased during fasting and increased after leptin

injections in obese animal models[37]. Second order neurons expressing the melanocortin receptor type 4 (MCR4) and recipient of the satiety signal from ARC-derived α-MSH are concentrated in the PVN (reviewed in[38]). Notably, POMC$^{-/-}$ mice show an obese phenotype and disruption in the melanocortin signaling pathway in general results in a dysregulation of energy metabolism (reviewed in[39]).

In the Djungarian hamster POMC neurons show a photoperiodic down-regulation in SD[33,34], the same is true for the MC3R, but not MC4R[33]. At the same time two peptides resulting from POMC processing, α-MSH and β-endorphin, are up regulated in SD in association with a higher expression and activity of two of the enzymes involved in the cleavage and maturation of pre-POMC, pro-convertase 2 (PC2) and carboxypeptidase E (CPE)[40,41]. The seasonal expression pattern of POMC follows the changes in plasma leptin and insulin, but the increase in the active peptides indicates that in SD the final anorexigenic drive from POMC neurons is enhanced despite the downregulation of the number of POMC mRNA expressing cells in the ARC.

Melanin Concentrating Hormone and Orexins/Hypocretin

An important set of second order neurons receiving inputs from the NPY/AgRP and POMC/CART neurons in the ARC are the MCH and Hcrt neurons in the lateral hypothalamus (LH). The LH became known as the hunger center of the hypothalamus in the 1950's when bilateral lesions of this brain area where shown to cause animals to die of starvation[42]. However, it was not until 1998, when two parallel studies by Sakurai *et al.* and de Lecea *et al.*[43,44] reported the discovery of orexin/Hcrt neurons in the LH and showed that ICV administration of orexin caused increased feeding behavior. Later, MCH was found to increase food intake as well[45,46].

In the Djungarian hamster, there is no clear evidence for seasonal variation in either of these two populations of second order neurons[34].

Somatostatin

Hypothalamic SST is best known for its inhibition of growth hormone production, either directly at the level of the somatotrophs in the anterior pituitary[47] or via inhibition of the growth hormone releasing hormone (GHRH) producing neurons in the hypothalamus by the SST neurons present in the periventricular area[48].

A clear SD-induced increase in SST gene expression has been described in the posterior ARC of the Djungarian hamster[12,49]. The role of SST in the control of seasonal metabolism is discussed later.

VGF and Histamine

VGF was discovered to be expressed in the brain by Van den Pol *et al.*[50,51]. VGF expression increases during fasting conditions and VGF knockout mice have a leaner phenotype as compared to their WT littermates due to an increased basal energy expenditure[52].

Particularly the posterior ARC (dmpARC) of Djungarian hamsters shows an up regulation[53] of VGF gene expression in SD conditions.

Histamine, a molecule synthesized since 1907[54] is also expressed in the hypothalamus, specifically in the tuberomammillar nuclei[55,56]. In the brain, histamine is best known for its role in the sleep/wake cycle and energy metabolism, but its expression is also upregulated in

hibernating rodents[57].

In Djungarian hamsters, histamine synthesis shows no photoperiodic regulation, but expression of the histamine receptor type 3 (H3R) is increased in SD hamsters[53].

f) Metabolic hormones with seasonal fluctuations

Leptin and insulin, the two most studied metabolic hormones because of their important effects on body weight control, have very evident seasonal variations.

Leptin

One of the major milestones in metabolic neurobiology was the discovery of the leptin protein[58] encoded by the *ob* gene in 1994, although without knowing the protein itself, its function had already been described since 1950[59]. The leptin peptide is produced by the adipocytes and signals to the rest of the body how much (white) adipose tissue has accumulated, and via signaling to the hypothalamus adapts food intake accordingly. In Djungarian hamsters too, leptin levels are directly correlated to the amount of adiposity, thus showing a marked decrease in SD animals[60].

Insulin

Insulin is probably the most studied metabolic hormone (>425.000 hits in PubMed, versus ~39.000 for leptin), with so far 3 Nobel prizes given to researchers involved in its discovery, from the pancreatic purification by Banting and Best in 1921 and the determination of the whole amino acid sequence by Sanger in 1955 till the development of its radioimmunoassay by Sussman-Yalow in 1973 (reviewed in[61]). Insulin was the first peptide known to function as a hormone. It is produced by the β-cells of the pancreatic islets and decreases plasma glucose levels by stimulating its uptake in peripheral tissues via the insulin receptor and glucose transporters.

Seasonal variations of insulin in the Djungarian hamsters follow the same pattern than leptin, with decreased levels in SD[62].

Glucagon and GLP-1

Glucagon is a hyperglycemic hormone produced by the α-cells in the pancreatic islets, discovered by Kimball and Murlin in 1923[63]. It increases glucose levels by increasing glycogenolysis and gluconeogenesis in the liver (reviewed in[64]). The pre-pro glucagon (PPG) sequence is also expressed in the central nervous system and its maturation results in additional peptides, such as glucagon like peptide 1 and 2 (GLP-1, GLP-2). Contrary to glucagon, GLP-1 has been described as a potent incretin with hypoglycemic, but also anorexigenic effects.

In the Djungarian hamsters, both PPG and GLP-1 have been described to be down-regulated in SD, particularly in the brainstem[65].

g) Reproductive effects of metabolic peptides

Regarding our current hypothesis that a temporal coordination of metabolic and reproductive activities is essential in seasonal species, it is interesting to observe that several

hormones and hypothalamic peptides involved in energy metabolism may also modulate reproduction.

Although it is understandable that a proper management of the energy balance is necessary for successful reproduction, this hypothesis was only clearly confirmed with the design of mutated mice. Indeed, the very first description of the *ob* mice by Ingalls *et al.*[59] reported compromised fertility in addition to the metabolic phenotype of the mutant *ob* homozygote animals. Then, 16 years later, the diabetic *db/db* mice, having their leptin receptor gene disrupted, were also reported to be infertile[66].

In support of a functional connection between the metabolic and reproductive brain circuits, it has been found that the canonical metabolic factors also impact reproductive activity. For instance, ICV injections of NPY not only increased food intake, but also reduced sexual behavior and gonadotropin release (reviewed in[27]). In contrast, *Npy* and *Agrp* knockout animals are fertile[31,32], suggesting that there are compensatory mechanisms or that NPY and AgRP are not essential, but modulate the short term regulation of reproduction. The anorexigenic peptide α–MSH increases lordosis in females next to the decreased hunger sensation[67,68]. Orexin A injections potentiate male sexual behavior in rats[69], although fertility is not compromised in pre-pro-orexin KO mice. Interestingly, *Vgf* knockout mice are infertile due to the reduced levels of LH and FSH in both sexes[52].

h) Seasonal body weight regulating molecules in Djungarian hamsters

With the premise of the melatonin-driven neural pathway controlling seasonal changes in reproductive physiology, we think it is relevant to review the different molecules that could be able to modulate seasonal changes in body weight.

The active thyroid hormone T3 has been shown to increase the body weight of hamsters kept in SD only when applied directly in the central nervous system[10], but not after peripheral administration[70]. Interestingly, both central and peripheral T3 administration resulted in gonadal reactivation. Upstream to T3 it has been shown that the ICV infusion of TSH also increases body weight[12], next to testes and seminal vesicles weight. So far, we are still missing the neural connection between the fluctuations in hypothalamic T3 and body weight control. Due to the photoperiodic changes in several hypothalamic areas nearby the *pars tuberalis* and the tanycytes (i.e. POMC, SST), it has been suggested that any of these or other non-identified neuronal populations could be the target for the thyroid hormone signal. Recently, a huge effort has been performed by Perry Barret *et al.*[71] in trying to understand the correlation between different hypothalamic changes and melatonin-driven phototransduction in natural-like conditions of temperature and photoperiod. However, no clear links were found with body weight (and energy metabolism), other than an increase in SST expression before body weight starts to drop following the reduction in natural photoperiod. In the same study, high levels of VGF expression were observed in short photoperiod, but only when animals were already in full SD and lighter in body weight. Further studies on the role of both neuropeptides in the seasonal control of Djungarian hamster's body weight reported that subcutaneous injections of pasiroitide, an analog of SST, decreased body weight of LD injected hamsters[72] and that adenoviral-driven overexpression of hypothalamic VGF decreased body weight and increased energy expenditure[73].

More recently, an extensive review by Morgan *et al.*[20] tried to find a consensus for the link

between reproduction and seasonal metabolism. They suggest that no matter whether the mammalian seasonal species studied is a long day (as Djungarian hamsters) or short-day breeding animal (i.e., sheep), there are fluctuations of T3 bioavailability in the mediobasal hypothalamus that then might influence the balance between differentiation and proliferation of cells (e.g., neurons).

Since there is only limited evidence of metabolic neuropeptides driving the seasonal changes in energy metabolism, and because seasonal reproduction goes along with seasonal changes in body weight, next we describe the hypothalamic circuits controlling the seasonal physiology of reproduction.

II. SEASONAL REPRODUCTION, THE ROLE OF KISSPEPTIN AND RFRP-3

In 1971, Schally *et al.* discovered a 10 amino acid hypothalamic peptide able to regulate the secretion of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland of several species[74]. This peptide, called gonadotropin releasing hormone (GnRH), was found to be synthesized in neurons in the preoptic area that project towards the median eminence to release GnRH in a pulsatile manner in the hypothalamo-pituitary portal system[75]. Interestingly, GnRH synthesis does not show seasonal changes, although GnRH release is reduced during photo-inhibitory conditions. Therefore, many studies have been performed to disclose the process operating up stream of the GnRH neurons to adjust GnRH release according to the time of the year. A number of pathways have been proposed to explain the photoperiodic synchronization of GnRH release, including sex steroid feedback (although GnRH neurons barely express sex steroid receptors[76]) and a physical barrier surrounding the GnRH nerve terminals at the median eminence in a photoperiod-dependent manner, built by the processes of ependymal tanycytes[77]. However, it was only after the discovery of the photoperiodic regulation of the (Arg)(Phe)related peptides, kisspeptin and RFRP-3, that the understanding of the neuroendocrine mechanisms regulating seasonal reproduction got a serious boost.

a) Kisspeptin is a potent stimulator of GnRH neuronal activity and gonadotropin release

It took more than 30 years after Schally's discovery of the GnRH neurons until De Roux *et al*.[78] and Seminara *et al.*[79] discovered the critical neurotransmitter stimulating GnRH neurons and the down-stream pituitary gonadal axis. Although using two different approaches, i.e. genomic-[78] and phenotypic-driven[79],both studies found that the integrity of the kisspeptin specific G protein-coupled receptor (GPR54) was necessary for puberty onset and normal reproductive activity. These pioneering findings triggered many studies demonstrating that in all mammalian species investigated so far, kisspeptin neurons not only potently activate the GnRH neurons that all express the GPR54 (now called Kiss1R and reviewed in[80]), but also mediate the positive and negative central feedback of sex steroids on the activity of the hypothalamic-pituitary-gonadal (HPG) axis[81,82]. The *Kiss1* gene, that encodes the kisspeptin peptide, was found to be expressed mainly in two hypothalamic nuclei, the anteroventral periventricular nucleus (AVPV) in rodents or preoptic area (POA) in large mammals, and the ARC (known as infundibular in the primate brain). In female mammals, AVPV/POA *Kiss1* neurons are critical in order to time the GnRH/LH surge that ultimately triggers ovulation, as they integrate both the circadian vasopressin signal and the positive feedback loop from

estrogens (E2) produced by the ovaries[83]. Arcuate *Kiss1* neurons co-express the stimulatory neurokinin B and inhibitory dynorphin neuropeptides that are both described to control the pulsatile release of kisspeptin, which in turn drives the pulsatile release of GnRH[84]. There is no doubt that regardless of the sex and the reproductive status (i.e. season), kisspeptin induces reproductive activity in all mammalian species investigated so far including rats[85,36], mice[83], hamsters[87,88], non-human-primates[89] and humans[90].

b) RFRP-3 as a versatile regulator of GnRH neuronal activity and gonadotropin release

In 2000, Tsutsui *et al*.[91] discovered another RF-amide that received a lot of attention with regard to its involvement in reproductive physiology. This peptide, initially discovered in the quail, was found to inhibit LH secretion, and was therefore named gonadotropin inhibitory hormone (GnIH). A few years later, Kriegsfeld *et al.*[92] found an ortholog gene (called *Rfrp*, *GnIH* or *Npvf*) expressed in the dorso- and ventromedial hypothalamus of various mammals, including the Syrian hamster. In mammals, the *Rfrp* gene encodes three peptides of which RFRP-3 and to a lesser extent RFRP-1, are relevant for the central control of reproduction[92]. In 2012, the *Rfrp* gene was cloned in the Djungarian hamster and both the RFRP-1 and -3 variant of the peptides were isolated[93]. RFRP-3 has a high affinity for GPR147, a receptor known to trigger an inhibitory effect on the substrate neuronal population[94]. Although initial studies indicated that RFRP-3, like GnIH in the quail, inhibits LH secretion in mammalian species[92], along the years it turned out to be challenging to understand its exact role in reproduction. Nevertheless, it is clear that RFRP-3 effects depend on sex, species and season[95].

c) Evidence for a role of kisspeptin and RFRP-3 in the seasonal reproduction

Due to the clear evidence for a role of both RF-amides in regulating GnRH neuronal activity, we and others investigated their potential seasonal regulation. We reported for the first time that in the Syrian hamster *Kiss1* exhibits a clear seasonal variation in the ARC, with higher expression in the photo-active long day conditions[96]. Then, Smith *et al*.[97] also found seasonal regulation of ARC Kiss1 in the ewe, with higher expression in short days, thus also photo-active conditions. These findings were followed by many other studies reporting up and down regulation of ARC *Kiss1* in different seasonal mammals, yet not always with a clear seasonal correlation between the highest values of Kiss1 and reproductive state, probably due to the strong negative feedback of sex steroids on these ARC neurons (Table 1 and Figure 3B). Regarding the AVPV/POA, *Kiss1* expression also shows seasonal variation, being up regulated in the sexually active season, and down regulated in non-sexually active mammals, regardless of whether the mammal is a long- (i.e., hamsters) or short- (i.e., sheep) day breeding animal (Table 1 and Figure 3A). Regarding RFRP expression, our lab also reported for the first time a clear seasonal regulation in the hypothalamus of Djungarian and Syrian hamsters[98]. Until now, whatever the seasonal mammalian species studied, and its seasonal phenotype, always a downregulation of RFRP-3 at both the mRNA and protein level was found in SD as compared to LD conditions (reviewed in[95]) (Table 1 and Figure 3B). The SD-induced down regulation of RFRP-3 was proven to be melatonin dependent[98], because pinealectomy completely abolished the SD-induced RFRP-3 inhibition, whereas nightly injections of melatonin in LD-adapted animals decreased RFRP-3 expression, at least in the Syrian hamster. In the Syrian and European hamster, ARC *Kiss1* has been shown to be also influenced by photoperiod and sex steroid hormones, increased melatonin levels in SD de-

crease *Kiss1* expression but this effect is overridden by the negative feedback of sex steroid hormones[99]. On the other side, AVPV/POA *Kiss1* expression seems to be fully dependent on the positive sex steroid feedback[99,100].

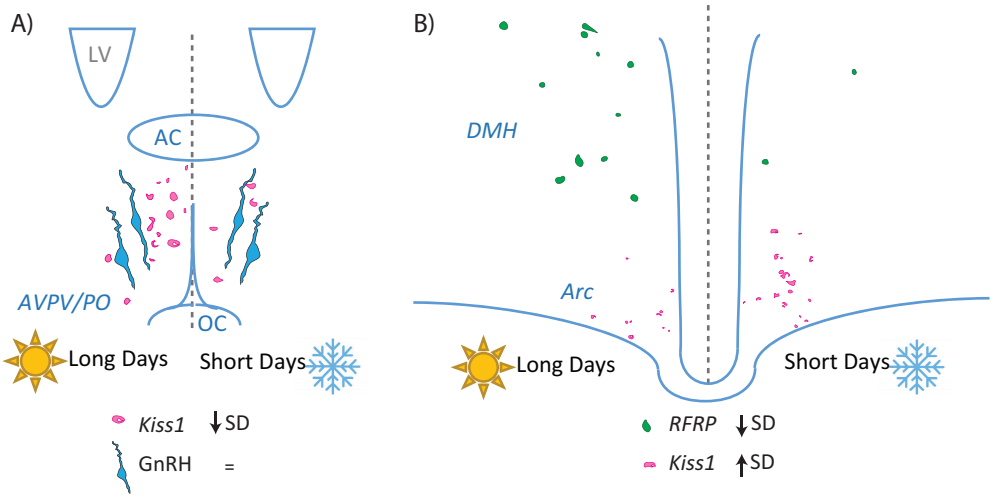

Figure 3. Seasonal variations of Kisspeptin and RFRP in the hypothalamus of Djungarian hamster. A) Schematic diagram of the seasonal variation in the Kisspeptin expressing neurons (Kiss1, pink) in the anterio-ventral periventricular nucleus (AVPV) in association with the gonadotropin releasing hormone (GnRH, clear blue) expressing neurons. OC, Optic Chiasm; AC, anterior commissure; LV, lateral ventricle. B) Seasonal variations of Kiss1 in the arcuate nucleus (Arc) and of (Arg)(Phe)-related peptide (RFRP, green) in the dorsomedial hypothalamus (DMH). In each panel the neuroanatomical distribution is illustrated under long days (left hemisphere) and short days (right hemisphere). Below each panel, the direction of change in neuropeptide expression in short days as compared to long days is indicated as "↑" for up-regulation, "↓" for down-regulation, or "=" when there is no change. 3V, third ventricle; ME, median eminence.

At this point, several players along the neuro-endocrine photoperiodic signaling pathway have been identified as potential seasonal regulators of kisspeptin and RFRP-3. As discussed in the foregoing, in mammals, the photoperiodic information starts with the nocturnal melatonin signaling to the PT, which then modulates the tanycytic expression of Dio2/Dio3 to change local levels of hypothalamic T3[10-13]. These hypothalamic changes in T3 are believed to synchronize reproduction with the time of the year, although via yet unknown mechanisms.

After the first demonstration of the seasonality of kisspeptin expression in the hypothalamus, Revel *et al*. succeeded in rescuing reproductive activity in Syrian hamsters by chronic ICV infusions of kisspeptin in photo-inhibited animals[96]. A further study reported that daily peripheral injections of kisspeptin could also restore the photo-inhibited reproductive axis in Syrian hamsters[101]. Although Grieves *et al*. reported that kisspeptin increases LH levels in the Djungarian hamster, depending on the photoperiod, sex and developmental moment, they were unable to rescue the reproductive function of SD-adapted hamsters by daily intraperitoneal injections of kisspeptin[102]. However, Rasri-Klosen *et al*. found that in SD-adapted Djungarian hamsters, ICV infusion of kisspeptin is able to rescue reproductive parameters to the same level as sexually active LD-adapted hamsters[100].

After the finding of the low levels of RFRP-3 in the hypothalamus of sexually inhibited Syrian and Djungarian hamsters, Ancel *et al*. demonstrated that a chronic ICV infusion of RFRP-3

in SD-adapted male hamsters was able to restore their reproductive activity[103]. A similar stimulatory effect of chronic RFRP-3 was further observed by Henningsen *et al*. in female Syrian hamsters[104]. In the male Djungarian hamster, Ubuka *et al*.[93] studied the effects of RFRP-3 on gonadotrophins and reported that it is clearly inhibitory in LD and stimulatory in SD male hamsters. Altogether, these data indicate that while the down regulation of RFRP-3 in SD conditions seems to be a conserved feature, its role in seasonal reproduction is not univocal among different species of seasonal breeders (Syrian and Djungarian hamsters, as well as sheep).

III. ROLE OF KISSPEPTIN AND RFRP-3 IN SEASONAL METABOLISM

a) Evidence for a role of kisspeptin and RFRP-3 in food intake and body weight regulation

With our premise that reproduction and energy metabolism are tightly interconnected, in part 2 we mentioned a few examples where obesity or anorexia resulted in clearly disrupted reproductive physiology, but is energy metabolism also affected by reproductive hormones?

By now it is well known that sex steroids can impact metabolic activity. For instance, estrogens have important roles in the control of energy expenditure (heat) and body weight, especially via their effect on fat accumulation[105]. During menopause, a dramatic drop in plasma estrogen levels occurs, causing an increase in fat depots and body weight. Estrogen was first isolated from pig ovaries by Allen *et al*.[106,107]. Later, it was shown that estradiol acts through two receptors, estrogen receptor alpha (ERα) and -beta (ERβ), with ERα being the most efficient activator of reproductive functions. Testosterone is considered as the "male" androgen hormone due to its high levels in males, and its role in masculinization of the body and in the acquisition of secondary sexual traits. With regard to metabolism, testosterone is best known for its clear anabolic role in lean mass (muscle and bone), and was recently reported to reduce mortality in men with metabolic syndrome[108,109]. Testosterone is produced by the Leydig cells in the testis, but before this was known, a collaboration by different laboratories isolated and synthesized testosterone for the first time[110–112] in 1935 (reviewed in[113]). To exert its action, testosterone binds to the androgen receptor (AR). It may be clear that if sex steroid hormones can modify energy metabolism, the hypothalamic RF-amides controlling the HPG axis are potentially also part of the connection between reproduction and metabolism.

Kisspeptin

Thompson *et al*.[85] were the first to evaluate the effect of a central injection of kisspeptin on food intake in Wistar rats. No changes in food consumption were found in *ad libitum* fed animals injected at the beginning of either the early light or early dark phase. Then, Tena-Sempere's lab[114] studied the effects of ICV kisspeptin injections in prepubertal rats with an energy deficit (i.e. fasting). They demonstrated that 72h fasting decreased kisspeptin expression and increased GPR54 (Kiss1r) expression. They suggested that fasted rats had a higher affinity to kisspeptin, since kisspeptin injections elicited increased LH secretion vs *ad libitum* fed animals. Interestingly, food intake was not affected by daily ICV injections of Kp, neither when rats were fed *ad libitum* nor in fasting conditions.

Later on, Stengel *et al*.[115] showed that ICV kisspeptin injections in fasted mice decreased cumulative food intake by increasing inter-meal intervals. Similarly, ICV kisspeptin injections

in fasted, but not *ad libitum* fed, jerboas decreased food intake and increased *POMC* gene expression, although with seasonal variations[116].

The first *in vivo* evidence for a long term effect of the kisspeptin system on energy metabolism was provided by Tolson *et al*.[117] who reported that male and female Kiss1r (Gpr54) KO mice showed increased adiposity, with female mice becoming overweight and glucose intolerant. Then, in a follow up study, Smith *et al*.[118] analyzed the kisspeptin targets in the hypothalamus and found that POMC neurons are upregulated in both female and male Kiss1r KO mice, but they concluded this effect was only reflecting the changed sex steroid levels.

Padilla *et al*. started studying the role of kisspeptin neurons in the ARC on heat production[119] and found that when activated they evoked hot flush-like responses. Then, in their next study in 2019[120], they showed that the kisspeptin neurons in the ARC are not only important for controlling fertility, but also for regulating the daily timing of food intake, locomotor activity, sleep and core body temperature. When synaptic transmission of the kisspeptin neurons in the ARC was blocked, the amount of food intake eaten during the light phase was increased almost 3 times without affecting the total amount of calories consumed. This disbalance in the daily rhythm of food intake, together with a reduction of locomotor activity and a reduced amplitude of the daily rhythm in core body temperature is probably the reason for the increase in body weight.

One of the latest studies on the metabolic actions of kisspeptin was published by Velasco *et al*.[121], who reported that rescuing Kiss1r expression only in the GnRH neurons of global Kiss1r KO mice, rescues the activity of the HPG axis and improves the dysregulation of body weight, fat mass and food intake in a sex dependent way; with males having a better rescue than females, in which food intake and glucose intolerance were only partially rescued.

RFRP-3

Several studies have reported that RFRP-3 exhibits an orexigenic effect. Chicken GnIH was the first RF-amide reported to have orexigenic effects[122], but in mammals Johnson *et al*.[123] were the first to show that RFRP-3 increased food intake in rats. Later on a similar effect was found in sheep, and non-human primates[124]. A study by Anjum *et al.*[125] showed that repeated intraperitoneal administration of RFRP has orexigenic and obesogenic effects in male mice. Most of the RFRP-3 effects on food intake have been found within 30 min to 4 hours after the injection. Due to the tight relationship between food intake and energy expenditure it has also been investigated whether RFRP-3 influences energy expenditure, but so far, no changes were found in rats and sheep. In the same study study, RFRP-3 increased neuronal activity of NPY and Orexin/Hcrt neurons, but also of α-MSH expressing neurons in ewes, as well as NPY mRNA expression in male rats[124].

Later, when characterization of the phenotype of RFRP-3 receptor (GPR147) knock-out animals was possible, it was shown that there are no changes in either reproduction or food intake in *ad libitum* fed animals; but KO mice showed no inhibition of gonadotropic functions in response to a 12h period of fasting to the same extent as their wildtype littermates[126]. When using the experimental fasting and refeeding paradigm, the reduced food intake in male RFRP-3 receptor KO mice correlated with higher levels of hypothalamic POMC gene expression[127], suggesting an endogenous orexigenic role of RFRP-3.

b) Efferents of Kp and RFRP-3 towards metabolic hypothalamic neurons

Evidence from female mice showed that kisspeptin neurons in the ARC project to various

hypothalamic nuclei involved in energy metabolism, such as the median preoptic nucleus (MnPO), the bed nucleus of the stria terminalis (BnST), the PVN, DMH and LH[128]. In mice, kisspeptin neurons in the ARC directly project and activate anorexigenic POMC neurons[129] and POMC neurons express GPR54 themselves. In sheep, NPY neurons also receive direct Kp projections[130], but in mice the inhibition of NPY by kisspeptin neurons *in vitro* was suggested to be rather indirect[129].

RFRP-3 neurons also project to NPY and POMC neurons in the ARC nucleus of the ewe[131], as well as to MCH and Hcrt/Orexins in the LH and corticotropin releasing hormone (CRH) and oxytocin neurons in the PVN. In mice, RFRP-3 inhibits POMC neurons and attenuates the kisspeptin-evoked POMC activation[129].

c) Are Kisspeptin and RFRP involved in seasonal metabolism?

Although the hypothalamic content of kisspeptin and RFRP-3 exhibits strong seasonal changes, and an increasing number of studies indicate that both peptides may regulate food intake and metabolic activity, very few studies have investigated the possible involvement of kisspeptin and RFRP-3 in the seasonal regulation of metabolism. In one study performed in our lab with the seasonal desert Jerboa (*Jaculus orientalis*), it was found that ICV injections of kisspeptin reduced food intake 1.5-fold in fasted, but not *ad libitum* fed animals[116]. Only in sexually active female Jerboas during spring, this effect was associated with an increase in *Pomc* and decrease in *Rfrp* mRNA levels. The same study found orexigenic effects of RFRP-3 on food intake of female jerboas in spring and autumn together with an increase in *Npy* and decrease in *Pomc* mRNA levels.

Table 1. Seasonal variations of peptide and mRNA of Kisspeptin and RFRP

	Species		Kisspeptin						RFRP					
			Male			Female			Male			Female		
			LD	SD	Ref	LD	SD	Ref	LD	SD	Ref	LD	SD	Ref
LD-breeders	Syrian hamster (*Mesocricetus aureatus*)	mRNA	↑ARC/AVPV	↓	96	↑AVPV/ARC	↓	99	↑	↓	98	↑	↓	132
		Peptide	↑ARC	↓	96	↑AVPV/ARC	↓	104	↑	↓	98	↑	↓	132
	Djungarian hamster (*Phodopus sungorus*)	mRNA	↑AVPV ↓ARC	↓ ↑	133				↑	↓	98			
		Peptide	↑AVPV ↓ARC	↓ ↑	88	↑AVPV ↓ARC	↓ ↑	87	↑	↓	98			
	European hamster (*Cricetus cricetus*)	mRNA	↓ARC	↑	134									
	Turkish hamster (*Mesocricetus brandtii*)	Peptide				↑AVPV	↓	135				↑	↓	135
	Gerbil (*Jaculus orientalis*)	mRNA	↑	↓	136							↑	↓	136
		Peptide	↑ARC	↓	137				↑	↓	137			
	Common vole (*Microtus arvalis*)	mRNA				↑AVPV/ ARC*	↓	138				↑	↓	138
	MSM mouse (*Mus musculus molossinus*)	mRNA							↑	↓	139	↑	↓	139
SD-breeders	Sheep (*Ovis aries*)	mRNA				↓ARC	↑	97				↑	↓	97
		Peptide				↓ARC	↑	140				↑ep cell layer	↓	141
	Dromedary camel (*Camelus dromedarius*)	Peptide	↓POA/ARC	↑	142	↓POA/ARC	↑	142	↑	↓	142	↑	↓	142

Notes. Ref, reference. LD, Long days. SD, Short days. ARC, Arcuate Nucleus. AVPV, Anteroventral periventricular nucleus. *determined by PCR from whole hypothalamus probably containing both AVPV and ARC.

SCOPE OF THE THESIS

The general aim of this thesis was to study whether the two hypothalamic RF-amides intimately involved in the central control of reproduction, i.e. kisspeptin and RFRP-3, are also involved in the central control of food intake, body weight, glucose and energy metabolism in the seasonal Djungarian hamster, but also in rats.

In the first part, we focused on the seasonal regulation of metabolism in the Djungarian hamster. As this species shows clear changes in body weight and adiposity together with fluctuations of reproduction along the year, traits that gave us a model for assessing and understanding how the hypothalamic circuitry modulating reproduction might interact with the hypothalamic nuclei modulating energy metabolism. Thus, in Chapters 2 and 3, our specific question was whether RF-amides would affect the metabolic phenotype as attested by food intake and body weight in SD- or LD-adapted hamsters; and if yes, which neuronal targets would be involved[116,124,143].

The discovery of the phenotype of body weight, adiposity and glucose intolerance in the kisspeptin receptor knock-out mice by Tolson *et al.*[117] raised our interest on hypothalamic metabolic control. In the same line, also RFRP-3 treatment was shown to modulate food intake[116,124,144] and more recently glucose metabolism[125]. Therefore, in the second part of this thesis, we studied the effects of central RF-amides on food intake, energy metabolism and endogenous glucose production in the male Wistar rat (Chapter 4).

Because SD conditions are associated with cold in the wild, in Chapter 5 we evaluated the effect of a 3h exposure to a cold environment on the reproductive and metabolic hypothalamic peptides at different time points along the 24h day/night cycle[145]. This allowed us to investigate: 1) the daily fluctuation in gene expression of a number of hypothalamic neuropeptides, and 2) whether there is a time-of-day difference in the response of these neuropeptides to cold.

Finally, in Chapter 6 we shared a technical note on the relevance of diet in our seasonal studies. The hamsters used in the seasonal studies described in Chapters 2 and 3 displayed a 30% loss in body weight as well as gonadal regression when the correct diet was used, but we encountered some problems when we had to change to a diet usually used for rats and mice in the animal facilities.

PART I

Chapter 2

KISSPEPTIN AND RFRP3 MODULATE BODY MASS IN *PHODOPUS SUNGORUS* VIA TWO DIFFERENT NEUROENDOCRINE PATHWAYS

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Journal of Neuroendocrinology 2019; 31(4):e12710

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, whereas the opposite occurs in springtime. These physiological adaptations are considered to depend on photoperiodic changes in hypothalamic genes encoding the peptides kisspeptin (Kp) and RF-amide-related peptide 3 (RFRP3) for the control of reproduction, as well as pro-opiomelanocortin and somatostatin for metabolic regulation. The present study investigates the effect of Kp and RFRP3 on long-term body weight regulation, aiming to establish whether metabolic and reproductive hypothalamic networks may interact during adaptation to seasonal physiology. We found that chronic central administration of both Kp and RFRP3 in short photoperiod-adapted male Djungarian hamsters increased body weight, although via different pathways. The effect of Kp was dependent on testicular activity because 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, although it had no effect on any of the hypothalamic metabolic genes investigated and did not change circulating levels of sex steroids. Notably, neither Kp, nor RFRP3 altered female hamster metabolic parameters. Thus, using a rodent model exhibiting seasonal changes in reproduction and metabolism, the present study demonstrates that, in addition to its role in the central control of reproduction, Kp also participates in body weight control in a sex-dependent manner via an anabolic action of testosterone. Conversely, RFRP3 affects body weight control in males mostly by acting on adiposity, with no overt effect on the reproductive system in both sexes.

Introduction

Mammals adapt their metabolic and reproductive activities to the seasonal changes in the environment to ensure that offspring will be born in favorable conditions with enough resources for survival. Seasonal rodents like Djungarian hamsters (*Phodopus sungorus*) increase their food intake and body weight and activate their reproduction with increasing day length in spring and summer, whereas the opposite occurs when days are shortening in autumn and winter[1,21,145]. These annual changes in metabolism and reproduction are driven by the pineal hormone melatonin, whose nocturnal production depends on the length of the night[147].

In the last ten years, major progress has been made to uncover the melatonin-regulated neuronal networks that control seasonal reproduction and metabolism. Two hypothalamic RF-amide peptides, kisspeptin (Kp) and RF-amide related peptide 3 (RFRP3), both known to regulate GnRH neuronal activity and gonadotropin secretion[83,92], display marked melatonin-driven seasonal variation in a number of seasonal species, including rodents[93,96,98,134]. Notably, in all seasonal species investigated so far, RFRP3 expression is driven by photoperiodic profile of night secretion of melatonin , whereas the photoperiodic regulation of Kp expression is modulated by the feedback of sex steroids[80,148]. Further, chronic central infusions of Kp or RFRP3 in SD-adapted Syrian hamsters have been shown to restore reproductive activity despite the photo-inhibitory conditions[96,100,103]. Also, changes in hypothalamic genes encoding peptides related to energy homeostasis, such as *pro-opiomelanocortin (Pomc)* and *somatostatin (Sst),* have been associated with the SD-induced metabolic reduction observed in Djungarian hamsters[34,49].

Previous observations in non-seasonal rodents, suggest that Kp and RFRP3 may as well regulate metabolic activity. Disruption of the Kp receptor in mice not only interferes with reproductive activity, but also with the control of body weight and *Pomc* mRNA expression [117,118]. Similarly, mutation of the receptor for RFRP3 alters the metabolic response to fasting conditions in mice[126] and a central injection of RFRP3 increased food intake in rats as well as *Npy* mRNA[123,124]. 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 cerebrospinal fluid (aCSF) and placed in osmotic minipumps (Alzet Model 1004; Curect Corp, Cupertino, CA USA). The Kp dose of 170 pmol/hour (2 mg/mL) was selected from a previous experiment[100] and maximal solubility reported by the manufacturer (ToCris Bioscience, Bristol, UK). RFRP3 is known to show clear dose-dependent effects[93,103]. 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) showing increased body weight after 5 weeks of treatment.

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: 8:16 hour light/dark photocycle) 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).

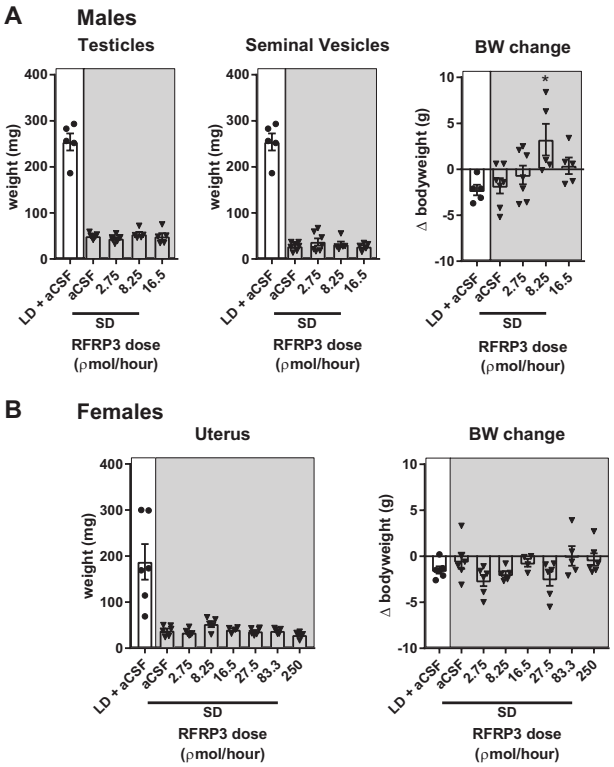

Figure 1. Dose dependent effect of RFRP3 in male and female Djungarian hamsters. Male (A) or female (B) hamsters were raised in conditions of long days (LD, white bars) or short days (SD, grey bars) and treated with a 5-week ICV infusion of artificial cerebrospinal fluid (aCSF) or various concentrations of 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 ± SEM of n= 5 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.

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 CO_2 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 previously[149]. 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[100]. 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 (4.5 mg/kg, Bayer Pharma, Puteaux, France) and Zoletil (50 mg/kg, 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 (Thermo Fischer Scientific, Waltham, MA, USA) and stored at -80°C until use. Non-radioactive *in situ* hybridization using digoxygenin labeled riboprobes was performed as reported before[12,150]. 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 glucome-

ter Accu-Check Performa Test strips (Roche). The range of values for the measurements was from 1.81 to 600 mg/dL.

Testosterone was measured in 10-fold 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

An individual who was 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 a 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[100,116]. 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[12,49]. 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 intensity[12].

Statistical Analysis

All data are presented as mean ± SEM. All the graphs and statistical analyses were done using Prism (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P<0.05$. Body weight, food intake data after 5-week treatments with RF-amides, hormone assays and *in situ* hybridization were analyzed with one-way ANOVA and the Tukey's 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 (Figure 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 (Figure 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 (Figure 1,2). 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 ± 0.01ng/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).

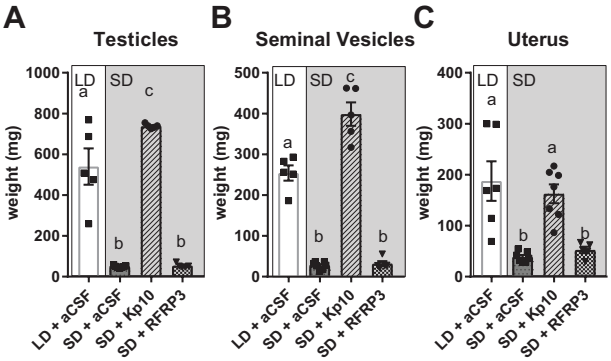

Figure 2. Effect of kisspeptin or RFRP3 on the reproductive status of photoinhibited male and female Djungarian hamsters. Male (A,B) or female (C) hamsters were raised in long day (LD) or short day (SD) conditions and treated with a 5-week ICV infusion of artificial cerebrospinal fluid (aCSF), kisspeptin 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 ± 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.

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) (Figure 3). 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) (Figure 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) (Figure 3A). Of note, none of the other doses of RFRP3 tested induced a significant change in the male's body weight (Figure 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) (Figure 3B).

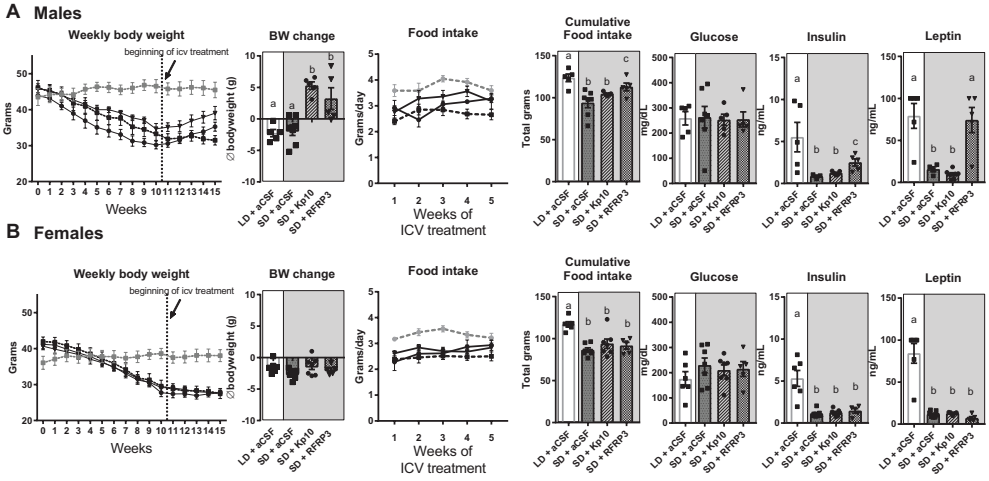

Figure3. Effect of kisspeptin or RFRP3 on the metabolic status of photoinhibited male and female Djungarian hamsters. At week 0, male (A) or female (B) hamsters raised in long days (LD) were either kept in LD (grey line) or placed in short day (SD) conditions (black lines). After 10 weeks (vertical dotted 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 ± 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.

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 (Figure 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) (Figure 3A). On the other hand, Kp10 treatment in SD male hamsters had no effect on glucose, insulin, or leptin (Figure 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 ($F_{2,1}$ = 4.196; *P*=0.0373) as compared to the SD control group (Figure 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

To identify 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 (Figures 4 and 5).

In absence of treatment, the number of *Pomc* expressing neurons was higher in LD- as compared to SD-adapted male (Figure 4A) and female (Figure 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 (Figure 4B) or fe-

male (Figure 5B) hamsters. In SD-adapted male hamsters, chronic Kp10 treatment increased the number of *Pomc* (Figure 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 (Figure 4C). Chronic RFRP3 treatment by contrast had no effect on the expression of the investigated genes in the male hamsters (Figure 4). In SD-adapted female hamsters, neither Kp10 nor RFRP3 treatment altered the number or intensity of neurons expressing the investigated genes (Figure 5).

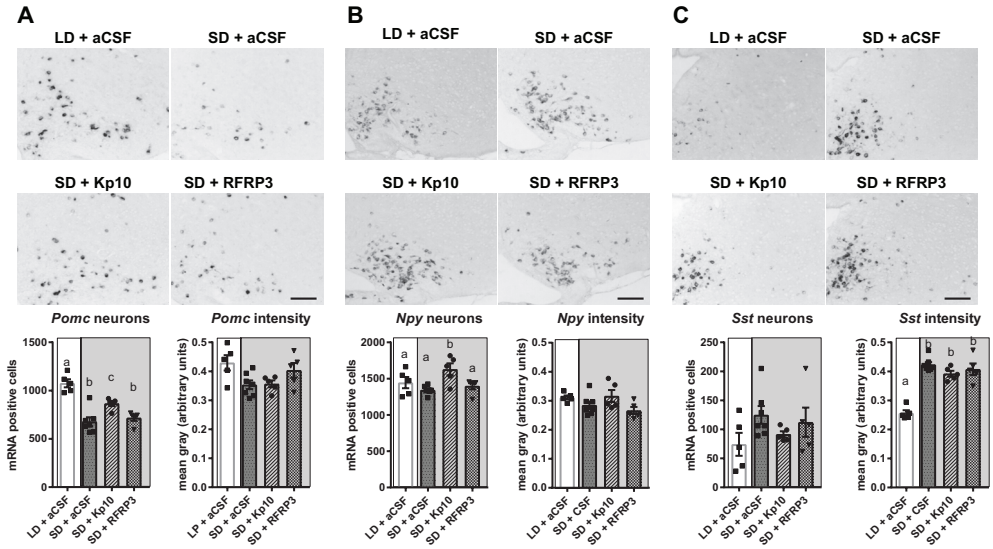

Figure 4. Effect of kisspeptin or RFRP3 on metabolic gene expression in the arcuate nucleus of photoinhibited male Djungarian hamsters. Male hamsters were raised in of long day (LD) or short day (SD) conditions and treated with a 5-week ICV infusion of artificial cerebrospinal fluid (aCSF), 170 pmol/hour 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 µm). 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 ± 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.

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

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) (Figure 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) (Figure 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) (Figure 6C).

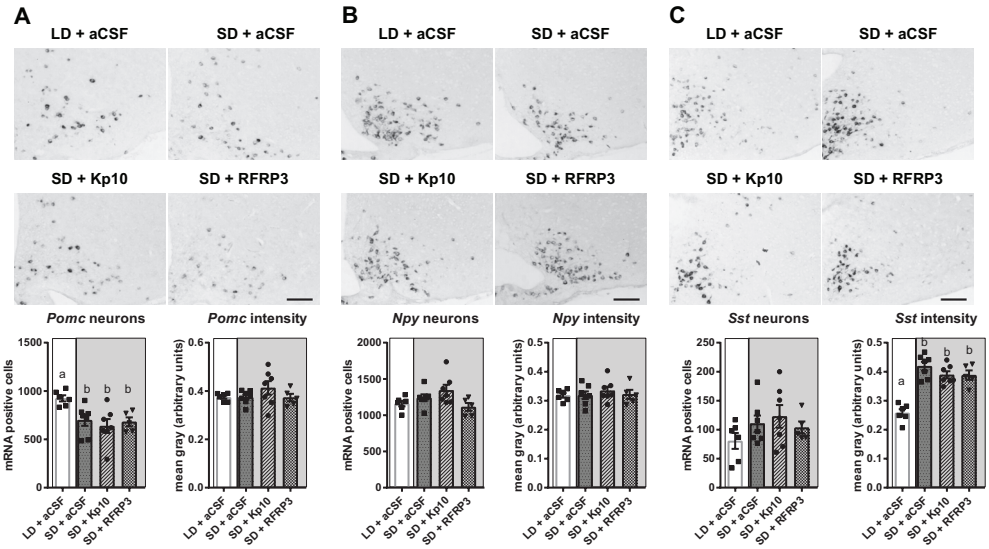

Figure 5. Effect of kisspeptin or RFRP3 on metabolic gene expression in the arcuate nucleus of photoinhibited female Djungarian hamsters. Female hamsters were raised in of long day (LD) or short day (SD) conditions and treated with a 5-week ICV infusion of artificial cerebrospinal fluid (aCSF), 170 pmol/hour 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 µm). 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 ± 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.

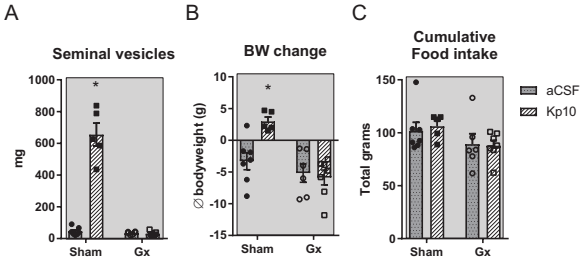

Figure 6. Effect of castration on reproductive and metabolic physiology in kisspeptin-treated male Djungarian hamster. Hamsters adapted to the inhibitory short day (SD) conditions were gonadectomized (Gx, open symbols) or sham operated (sham, closed symbols), then were treated with a 5-week ICV infusion 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 ± 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.

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[100,136,151,152]. 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[19,153]. In the hypothalamus, we confirmed the SD-induced down regulation of *Pomc* gene expression in the ARC of both sexes[33,34] and found that the previously reported SD-induced up regulation of *Sst* gene expression in the posterior part of the male hamster ARC[12,49] was also present in females. We also confirmed the lack of SD-induced changes in *Npy* expression as previously described[33,34].

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*[33,34] and reproductive *Kiss1* and *Rfrp*[87,100] 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[116,123,124] , whereas for Kp an acute anorexigenic effect in fasted female jerboas[116] and a lack of effect in either *ad libitum* or fasted rats has been reported[85]. 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 mice[117]. 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 hamsters[102] and prepubertal rats[154], 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 hamsters[155].

Two RF-amides, two pathways

It has become clear that the hypothalamic Kp and RFRP3 neurons are a functional link be-

tween the seasonal changes in the pineal hormone melatonin and reproduction[37]. 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 TSH[12] or T3[11] 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[12,157].

Chronic infusion of Kp[96] or RFRP3[103] 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 because 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 hamster[103]. 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 animals[93]. 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)[158]. Further, it is important to note that RFRP3 displays marked dose-dependent effects[93,103] 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 gonads[159], our data indicate that Kp10 may be involved in the testosterone-dependent 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 RFRP3[125]. It is well known that increased adiposity results in insulin resistance[160], 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[161,162], 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 Kiss1r[163] and the RFRP3 receptor GPR147[132] 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 rats[164], they are directly activated by Kp in mice[129] and are regu-

lated by a central Kp10 injection in jerboas,[116] 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*[165–167] and *Npy*[168–170], 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 hamsters[34]. In addition, even when the *Pomc* gene is down-regulated in SD, the levels of a-MSH, an anorexigenic peptide produced by the *Pomc* gene might be higher due to a reported increased activity of carboxipeptidase E[40,41]. 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 Kiss1r[164] and Kp10 has been reported to exert an indirect local inhibitory effect on these neurons[129,171]. 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[116,118]. 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[168–170].

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[116,123–125]. Some studies have proposed that RFRP3 may act directly on NPY and POMC neurons. Thus, in ewe RFRP3 fibers project to NPY and POMC neurons[131] and acute RFRP3 treatment increases their neuronal activity[124]. Further, in the female jerboas the acute orexigenic effect of RFRP3 is associated with increased *Npy* mRNA and decreased *Pomc* mRNA expression[116] and in male rats, RFRP3 treatment increased food intake and *Npy* mRNA levels in the ARC[124]. 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 conditions[172]. Alternatively, if leptin increases POMC and decreases NPY expression, as it does in other animals[23], 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, the results obtained in the present study show 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. By contrast, the long-term effect of RFRP3 on body weight appears to be 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.

Acknowledgements

We extend our special thanks to Dr. Dominic Ciocca, Aurore Senser and Nicolas Lethenet for their help with the caring of the animals used in the present study. We also thank Clarisse Quignon for providing technical support. This project was funded with support from the NeuroTime Erasmus+ program of the European Commission and the Repramide ANR-13-BSV1-001.

Chapter 3

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

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Journal of Neuroendocrinology 2020; 32(5)

Abstract

In addition to its regulatory role in luteinizing hormone secretion, RF-amide 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*. in 2000[91] 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*.[92]. Further studies confirmed the reproductive effect of this hypothalamic peptide, nowadays called (Arg)(Phe) related peptide-3 (RFRP3)[95,173]. Remarkably, the effect of RFRP3 on LH secretion seems to be sex-dependent, as it has been reported to be inhibitory in female rodents[104,174], but either stimulatory or inhibitory in males[92,93,103,123,175–177].

Increasing evidence now indicates that RFRP3 also affects food intake, exhibiting an orexigenic effect in rats[123,124], jerboas[116], mice, ewes and non-human primates[124]. In addition, RFRP3 projections and receptors (GPR147) have been described in the arcuate nucleus[92,132], a hypothalamic region involved in the control of feeding behavior, mostly by gathering and integrating peripheral metabolic information[23]. Moreover, it has been reported that RFRP3 affects the expression of both orexigenic and anorexigenic genes in the hypothalamus[116,124].

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 [93,95,98,134,136,178]. 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[1,146,179]. Therefore, the aims of this study were: i) to examine whether female Djungarian hamsters, like males[12,93], 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 (16:8 hour light/dark photocycle, lights on 4.00 am) at a temperature of 20 ± 2°C, with food (Altromin 720, Altromin Spezialfutter GmbH & Co, Lage, Germany) and water available *ad libitum*. Experimental hamsters (aged 3-4 months) were housed individually with a cotton nest and a wooden bar providing environmental enrichment. Twelve male and 30 female hamsters were transferred to short day conditions (6:8-hour light/dark photocycle, lights on 10.00 am) for 10 weeks to evoke the well-known reproductive and metabolic inhibition (approximately 30% bodyweight loss from 42.1 ± 2.7 g in LD to a 31.2 ± 4.9 g in SD), while 12 males and 24 females remained in LD conditions. In all experiment lights on is defined at Zeitgeber time 0 (ZT0).

Cannula implantation and intracerebroventricular injection

To perform central peptide injections, an intracerebroventricular (ICV) cannula was implanted in the lateral ventricle as described previously for the Djungarian hamster[12]. 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). In between experiments a dummy 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. ICV injections were performed under light isoflurane anesthesia (AErrane; Baxter, Maurepas, France). Animals were infused with 3 µL of either RFRP3 (Djungarian hamster sequence TLSRVPSLPQRFa, Caslo laboratory, Lyngby, Denmark) or Vehicle (Saline 0.9%) at a constant flow rate of 1 µL/min. The cannula was left in place for one more minute after injection to allow correct product diffusion. Patency of the cannula and effectiveness of the infusion was verified by movement of a small air bubble.

Estrous cycle determination

To establish the estrous stage of LD adapted female hamsters, daily vaginal smears were performed at midday as described previously[104], starting 3 days after the cannula placement and continuing all along the experiment. Briefly, the stages were defined based on the most abundant type of cells in the vaginal smear[180] 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

Investigating food intake in *Phodopus sungorus* is complex due to their natural hoarding

behavior[181]. Therefore, we adapted our experimental procedure according to Schuhler *et al*. [182]. 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. Two preliminary experiments were performed on 10-week SD-adapted female hamsters to evaluate the time-course and time-dependent effect of RFRP3 injection. First, RFRP3 was injected at ZT5 and food intake was measured 3, 6, 12 and 24 hours after the injection. Second, RFRP3 was injected at five different times of the day/night cycle (ZT1, ZT5, ZT9, ZT14, ZT19) to define whether there was a time of the day dependency of the RFRP3 effect (n=18).

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. Each individual received up to 5 independent ICV injections (doses in a random order) with at least one week of recovery in between each injection. At the end of the experiments, animals were killed 90 minutes after the final ICV injection by inhalation of increasing doses of CO_2 and then perfused transcardially with phosphate-buffered saline (PBS) followed by paraformaldehyde-lysine-periodate (PLP) fixative. This timing after peptide injection was previously found to be sufficient to induced significant effects on neuronal activity[116,124]. The brains were removed and embedded in polyethylene glycol as described before[149].

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) starting from the beginning of the *pars tuberalis*, approximately at Bregma -0.5 mm, and finishing after the mammillary recess at Bregma -2.7 mm. To ensure that the full extension of the brain regions of interest was sampled, additional sections more rostral and more caudal without positive gene expression were included in the series. Sections were mounted on Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80°C until use. Non-radioactive *in situ* hybridization using digoxygenin labeled riboprobes was performed as reported before[12,150]. 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, Basel, Switzerland). The sections were post-fixed with 4% formaldehyde, digested during 30 minutes at 37°C with 0.5 µg/mL proteinase K (Roche) 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

An investigator who was 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, Oberkochen, Germany) was used to take micrographs with a 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[100,116]. 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, labeling intensity was measured, in one anterior (bregma -0.50 to 0.74), one medial (bregma -1.80 to -2.04) and one posterior (bregma -2.30 to -2.54) section, 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[12].

Statistical Analysis

All data are presented as mean ± SEM. All the graphs and statistical analyses were performed with 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's post-hoc honestly significant difference (HSD) test. The results of the time-course experiment were analyzed with two-way ANOVA with repeated measures and Bonferroni's post-hoc HSD. For the experiment on the effects of time of day, two-way ANOVA and the Bonferroni post-hoc HSD test were used. *In situ* hybridization results were analyzed with one-way ANOVA and Tukey's post-hoc HSD.

Results

Hypothalamic 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 ($F_{1,11}$=118.9630, P<0.0001) and female hamsters ($F_{2,15}$=49.7600; P<0.0001 Figure 1). Further, *Rfrp* expression was similar during diestrus and proestrus in LD-adapted females (P=0.8654) (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 (females: $F_{2,15}$=32.44; P<0.0001 and males: $F_{1,11}$=38.3915, P=0.0001). Both, *Npy* and *Hcrt* did not show significant differences when comparing SD vs LD (males: *Npy* $F_{1,11}$=3.5817, P=0.0877 and *Hcrt* $F_{1,11}$=1.1714, P=0.3045; females: *Npy* $F_{2,15}$=0.2331, P=0.7949 & *Hcrt* $F_{2,15}$=2.145, P=0.1517) (Figure 2).

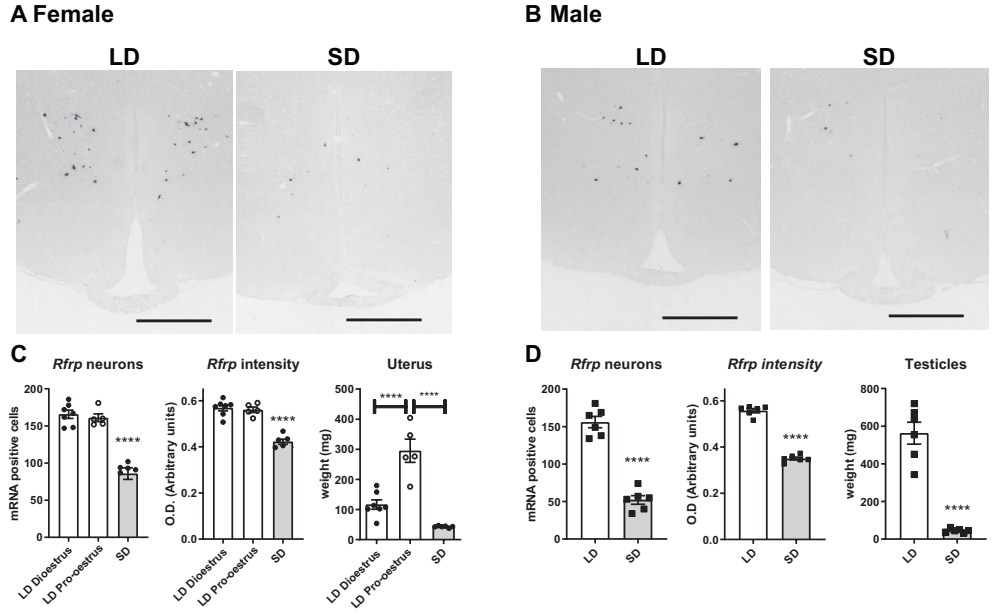


Figure 1. Photoperiodic regulation of *Rfrp* gene expression in the hypothalamus of male and female Djungarian hamsters. A & B) Representative photos from in situ hybridization of *Rfrp* mRNA in female and male hamsters adapted to long days (LD) or short days (SD), scale bar correspond to 500 µM. C & D) Quantification of the number of mRNA positive neurons or labeling intensity per neuron; white bars represent LD animals, gray bars represent SD animals, circles for females (closed for diestrus and opened for proestrus) and squares for males. Paired testicles and uterus weight were measured. Data are represented as mean ± SEM, n = 5 to 7 hamsters per photoperiod group. **** is for P<0.0001 after Student's t-test when comparing SD vs LD males, **** P<0.0001 after one-way ANOVA and Tukey's post-hoc honestly significance test when comparing female groups.

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

Pilot experiments were performed in SD-adapted female hamsters to investigate the time course and time dependent effect of an ICV injection of 1.5 ug RFRP3 on food intake. First, a delay of 3h after the peptide injection proved to be sufficient to observe an increase in

food intake (Treatment: $F_{1,20}$=5.045, P=0.0362, Time: $F_{3,60}$=296.7, P<0.0001; Interaction: $F_{3,60}$=1.301, P=0.2826)(Figure 3A). Second, the orexigenic effect of RFRP3 was observed at most time points investigated (ZT5, ZT9, ZT19, but not ZT1 and ZT14; Treatment: $F_{1,80}$=4.036, P=0.0479; ZT: $F_{4,80}$=4.761, P=0.0017; Interaction: $F_{4,80}$=0.8996, P=0.4683)(Figure 3B).

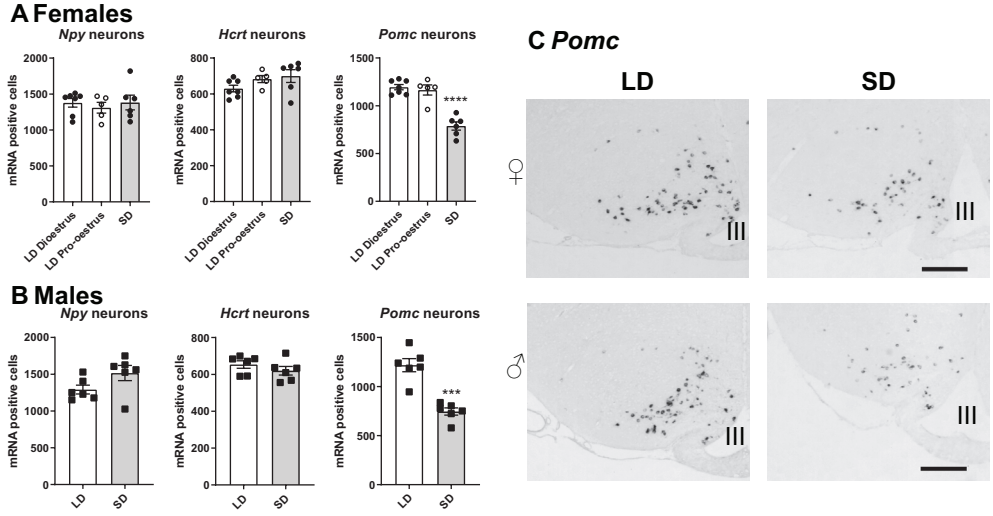


Figure 2. Photoperiodic regulation of *neuropeptide Y* (*Npy*), *hypocretin* (*Hcrt*) and *pro-opiomelanocortin* (*Pomc*) gene expression in the hypothalamus of male and female Djungarian hamsters. A & B) Quantification of the number of mRNA positive neurons; white bars represent LD animals, gray bars represent SD animals, circles for females (closed for diestrus and opened for proestrus) and squares for males. C) Representative photos from in situ hybridization of Pomc mRNA in female and male hamsters adapted to long days (LD) or short days (SD), scale bar correspond to 200 µM. Data are represented as mean ± SEM, n = 5 to 7 hamsters per photoperiod group. *** P=0.001 after Student's *t*-test when comparing SD vs LD males and **** P<0.0001 after one-way ANOVA and Tukey's post-hoc honestly significance test when comparing female groups.

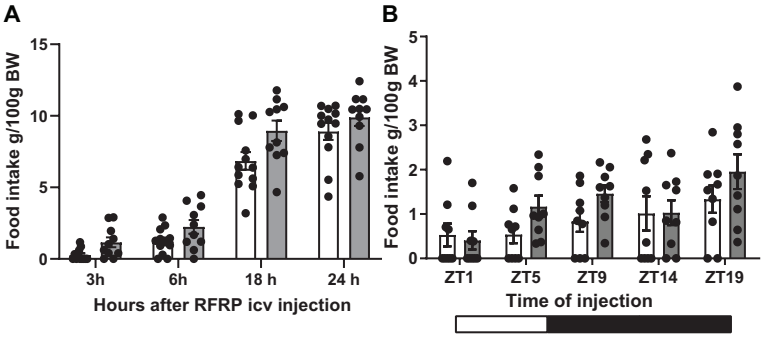


Figure 3. Duration and time of the day effect of acute ICV injection of RFRP3 on food intake in female Djungarian hamsters adapted to short day (SD) conditions. A, Food intake was measured 3, 6, 18 and 24 hours after the central injection of vehicle (white bars) or 1.5 µg RFRP3 (grey bars) at Zeitgeiber time (ZT) 5 (with ZT0 indicating lights on). B, Food intake was measured 3h after the central injection of vehicle (white bars) or 1.5 µg RFRP3 (grey bars) at different times of the day/night cycle. Data are presented as the mean ± SEM food intake/100 g BW of n=9 to 12 hamsters per experimental group.

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 was apparent with only 1.5 µg, but 0.5 µg or 5 µg did not significantly increase food intake as compared to vehicle ($F_{3,43}$=4.005, P=0.0134; Figure 4A). 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 4A).

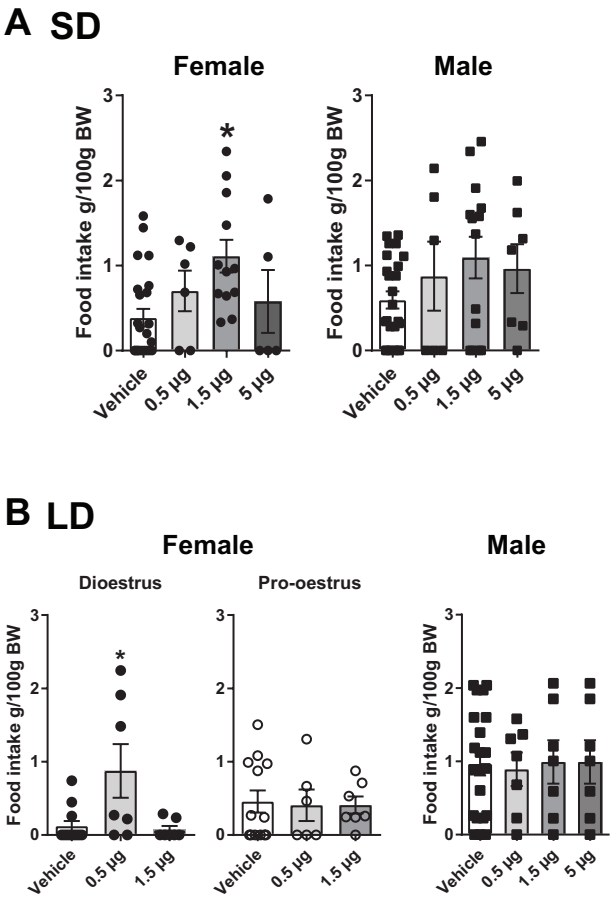


Figure 4. Effect of acute ICV injection of RFRP3 on food intake in female and male Djungarian hamsters adapted to short (SD) or long day (LD) conditions. Food intake was measured 3h after the central injection of vehicle (white bars) or three different amounts of RFRP3 (0.5, 1.5 or 5 µg; gray scale bars) in SD (A) and LD (B) female or male hamsters. Data are presented as the mean food intake/100 g BW ± 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. * P<0.05 after 1-W ANOVA vs vehicle.

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 4B), but none of the two doses had an effect on the day of proestrus ($F_{2,23}$=0.03251, P=0.9681; Figure 4B). 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 4B).

Effect of RFRP3 on the hypothalamic genes involved in metabolism

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 5A). In SD-adapted males, the dose of 1.5 µg RFRP3 did not alter the number of *Npy*, *Pomc* or *Hcrt* expressing neurons (Figure 5B) 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).

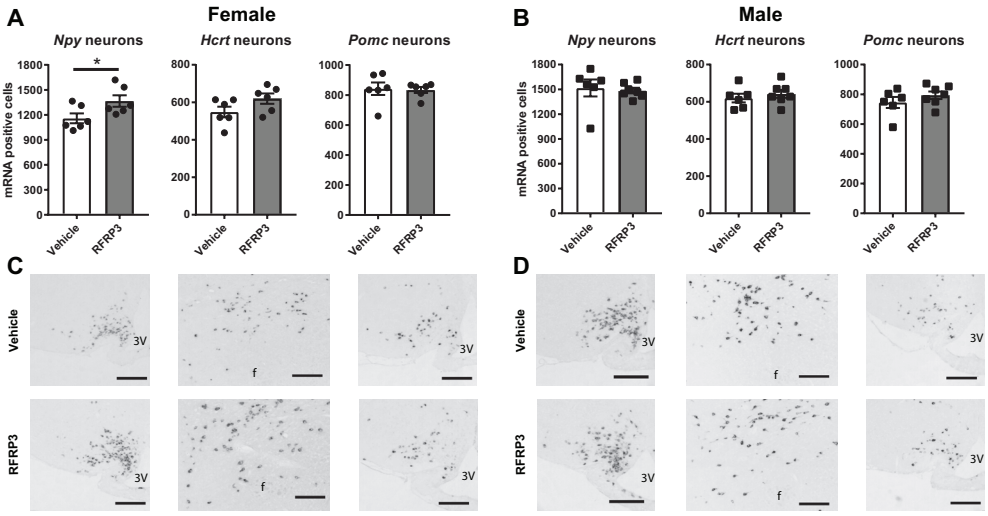


Figure 5. RFRP3 effect on neuropeptide Y (*Npy*), hypocretin (*Hcrt*) and pro-opiomelanocortin (*Pomc*) mRNA in the hypothalamus of SD-adapted female and male Djungarian hamsters. Female and male hamsters were injected centrally with vehicle or 1.5 µg of RFRP3, and the number of neurons expressing the 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 ± 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 µM.

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[183]. 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[152,173,184]. 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 "reproduc-

tive" genes *Kiss1*[100] and *Rfrp,*[93,100] but also "metabolic" genes such as *Somatostatin*[12,49] and *Pomc*[34,40,41], but surprisingly not *Npy*[172]. In the present 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. These observations are in line with the hypothesis that NPY is involved in the acute regulation of food intake, while by contrast *Pomc*-derived peptides and somatostatin are involved in the long-term (i.e., photoperiodic) regulation of energy balance in seasonal animals.

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[96,100]. In the present study we found that female Djungarian hamsters, also displayed a marked reduction in *Rfrp* gene expression during SD, very similar to that observed in males[100]. 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[132]. In the present study, 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[104,185], a moderate[186] or an inhibitory effect[187–189] 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[100]. 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[98].

GnIH/RFRP3 was first discovered by virtue of its inhibitory action on GnRH neuronal activity and gonadotropin secretion[91,92]; however, later studies showed that these effects of RFRP3 were dose-, sex-, species- and photoperiod-dependent[95]. For example, 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[92,103,104]. In the male Djungarian hamsters, RFRP3 increases LH secretion in SD-adapted animals, but decreases it in LD-adapted animals[93]. These findings highlight the importance of investigating the biological effect of RFRP3 in various conditions, as attested in our current study where the acute effect of RFRP3 on food intake was actually found to depend on the sex and the photoperiodic status of Djungarian hamsters.

In female hamsters, RFRP3 increased food intake 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 which may depend on a photoperiod-dependent differential expression of *GPR147*, the receptor for RFRP3, as reported earlier for the female Syrian hamster[132]. 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[116]. 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[190,191]. 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[192]. Taken together, these studies indicate that mimicking higher RFRP3 neuronal activation and peptide release by an ICV injection, promotes food intake, and that the RFRP3 orexigenic effect depends on low estrogen levels, in SD or in LD diestrus. Estrogens are able to modulate feeding behavior by changing the response to satiety hormones (e.g. ghrelin, cholecystokinin[193]), thus, it is not surprising that seasonal or oestral

fluctuations in estrogen may modulate the sensitivity of orexigenic hypothalamic circuits in response to RFRP3. The orexigenic effect of RFRP3 was associated to an increase in the number of NPY expressing neurons in SD females, as also reported in female jerboa[116], but not in LD diestrus female. In contrast to what was reported in the female jerboa[116] and mice[129], RFRP3 does not appear to regulate acutely *Pomc* gene expression. Altogether, these data indicate that the orexigenic effect of RFRP3 may use different indirect targets, which could also explain the 3-hour delay required to observe a significant orexigenic effect of RFRP3.

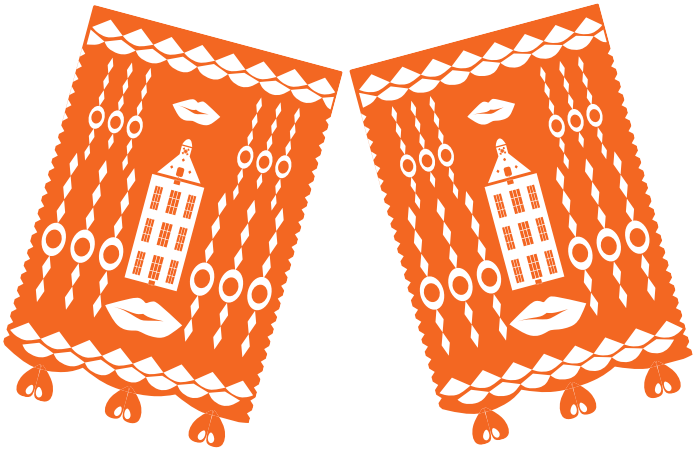
The results of the present study show a marked sex difference in the acute effects of RFRP3 as various doses of RFRP3 given to male Djungarian hamsters had no effect on food intake and *Npy* gene expression. The reason for such a sex difference in RFRP3-elicited feeding activity is unclear, but probably 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[123,124]. Intriguingly, in our recent study in which we investigated the chronic (5 weeks) metabolic effect of a central continuous infusion of RFRP3 in SD-adapted lean Djungarian hamsters, RFRP3 displayed an opposite sex-dependent effect as it was able to restore feeding activity, bodyweight and levels of leptin and insulin in the male but not the female animals[194]. 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[127]. Yet, when compared to the present study, these findings highlight another aspect of the biological effect of RFRP3 that is the acute vs long-term effects, indicating that the acute and chronic effects most probably involve different cellular mechanisms. The acute orexigenic effect of RFRP3 in female Djungarian hamsters and jerboas is associated with an increase in *Npy* expression (as shown in the present study as well as reported previously[116]), 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*[194].

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). Taken 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.

Acknowledgements

We thank Dr. Dominic Ciocca, Aurore Senser and Nicolas Lethenet for their help with the caring of the animals used in the present study. We also thank Eleni Angelopoulou and Sebastien Milesi for providing technical support. This project was funded with support from the NeuroTime Erasmus+ program of the European Commission and the Repramide ANR-13-BSV1-001.

PART II



Chapter 4

ROLE OF CENTRAL KISSPEPTIN AND RFRP-3 IN ENERGY METABOLISM IN THE MALE WISTAR RAT

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Journal of Neuroendocrinology 2021; 00:e12973

Abstract

Kisspeptin (Kp) and RFRP-3 are two RF-amides acting in the hypothalamus to control reproduction. In the past 10 years, it has become clear that apart from their role in reproductive physiology both neuropeptides are also involved in the control of food intake, as well as glucose and energy metabolism. To investigate further the neural mechanisms responsible for these metabolic actions, we assessed the effect of acute intracerebroventricular (ICV) administration of Kp or RFRP-3 in *ad libitum* fed male Wistar rats on feeding behavior, glucose and energy metabolism, circulating hormones (luteinizing hormone, testosterone, insulin and corticosterone) and hypothalamic neuronal activity. Kp increased plasma testosterone levels had an anorexigenic effect and increased lipid catabolism, as attested by a decreased respiratory exchange ratio (RER). RFRP-3 also increased plasma testosterone levels but did not modify food intake or energy metabolism. Both RF-amides increased endogenous glucose production, yet with no change in plasma glucose levels suggesting that these peptides not only provoke a release of hepatic glucose, but also a change in glucose utilization. Finally, plasma insulin and corticosterone levels did not change after the RF-amide treatment. The Kp effects were associated with an increased c-Fos expression in the median preoptic area and a reduction in pro-opiomelanocortin immunostaining in the arcuate nucleus. No effects on neuronal activation were found for RFRP-3. Our results provide further evidence that Kp is not only a very potent hypothalamic activator of reproduction, but is also part of the hypothalamic circuit controlling energy metabolism.

Introduction

The (Arg)(Phe)-amide peptides, Kisspeptin (Kp) and (Arg)(Phe) related peptide 3 (RFRP-3), are two hypothalamic peptides that are well known to modulate reproductive activity in mammals. Kp has been described as a potent activator of GnRH neuronal activity, leading to increased secretion of gonadotropins and sexual hormones in all mammalian species investigated, including humans[80,195]. By contrast, the role of RFRP-3 is still under debate as stimulatory, inhibitory or absence of effect have been reported according to species, sex and seasons[95,196].

Reproduction is a very expensive process in terms of energetic needs, which makes it essential for mammals to match the timing of reproduction with an optimal energetic and metabolic status. Thus it is not that surprising that recently Kp and RFRP-3 have also been linked to the control of food intake, body weight regulation and glucose homeostasis[117,127,194]. The scarce and scattered data so far point towards RFRP-3 having an orexigenic effect in different mammalian species[116,123,124,194,197] and Kp having an anorexigenic effect[115,116]. Regarding glucose homeostasis it has been shown that female mice with a KO for the Kp receptor Kiss1r are glucose intolerant[117], whereas intraperitoneal administration of RFRP-3 changed circulating glucose concentrations and insulin receptor and glucose transporter expression in testis and adipose tissue[123,125,194]. Interestingly, it has been found that 1 in 3 men with type 2 diabetes present detrimental effects on gonadal activity (hypogonadism)[198] and testosterone replacement has positive effects on metabolic syndrome survival rates[108,109].

Within the hypothalamus, the arcuate nucleus (ARC), a brain region well known to receive and integrate many metabolic signals from the periphery, shows a high expression of both Kp[164] and RFRP-3[93,132] receptors. The two main populations of neurons within the ARC that are responsible for the control of energy metabolism and glucose homeostasis are the orexigenic neuropeptide Y (NPY)/agouti-related peptide (AGRP)-expressing neurons and the anorexigenic pro-opiomelanocortin (POMC)/cocaine- and amphetamine regulated transcript (CART)-expressing neurons[28]. In our previous studies in the seasonal Djungarian hamster we showed that central administration of Kp increased body weight as well as NPY- and POMC-expression, whereas RFRP-3 increased food intake, body weight and circulating levels of leptin and insulin, without changing NPY and POMC expression in the ARC[194,197]. In this study, we tested the hypothesis that Kp and RFRP-3 would also affect energy metabolism in male Wistar rats. Therefore, we assessed the central effects of Kp and RFRP-3 on feeding behavior, energy metabolism and glucose homeostasis in this species and revealed possible hypothalamic pathways involved in the reported metabolic effects.

Materials and methods

Animals

Adult male Wistar rats (Charles River, Germany) weighing 250-280 g at the start of the experiment were used in all experiments. Animals were housed in individual cages in an enriched environment with a wooden stick under a 12:12 hour light/dark photocycle (lights on 7.00 am; =ZT0). Food (24% protein, 58% carbohydrate and 18% fat) (Teklad global diet 2918: Envigo, Indianapolis, IN, USA) and water were provided *ad libitum*. After arrival, rats could adapt to the animal facility with constant temperature (21 ± 2°C) and humidity (50 ± 5%) for at least one week. All experimental procedures performed were approved by the Animal Ethics Committee of the Royal Dutch Academy of Arts and Sciences (KNAW, Amsterdam)

and were in accordance with the guidelines on animal experimentation of the Netherlands Institute for Neuroscience (NIN).

Surgery

To infuse either RFRP-3 or Kp in the intracerebroventricular (ICV) space of the central nervous system, a unilateral brain cannula (Plastic One material, Dusseldorf, Germany) reaching the lateral ventricle was implanted. Surgery was done under anesthesia consisting of an intramuscular injection of a mix of Ketamine (80mg/kg; Eurovet Animal Health, Bladel, the Netherlands) and Xylazine (8mg/kg, Bayer health care, Mijdrecht, the Netherlands). The coordinates were defined using the rat brain atlas[199] as a reference: -0.8 mm AP, +2.0 mm lateral from bregma and -3.2 mm ventral from the dura. In part of the animals also silicon catheters were surgically implanted into the right jugular vein and the left carotid artery for intravenous infusion and blood sampling, respectively[200]. Brain cannula and catheters were fixed to the skull using dental cement. A cannula dummy was used to seal the guide cannula maintaining it open until the infusion. A metallic connector that could be attached to a chain swivel was added to the dental cement, which allowed us to execute the experiment without handling of the animals during the experiment. The animals received Carprofen as postoperative analgesic (2.5 mg/kg, Zoetis, Cappelle a/d IJssel, the Netherlands). The rats were allowed to recover for at least 10 days after the surgery, with experiments only being started after they had reached their initial pre-operative body weight again.

1.Experimental set-up for indirect calorimetry

Seven days after surgery, animals were single housed in metabolic cages (TSE, Bad Homburg, Germany) for three consecutive days. Day 1 was aimed for habituation, Day 2 for a baseline measurement and then on the morning of Day 3 animals were ICV injected (≈ZT5) and the automatized measurements continued for 24 h. Animals had *ad libitum* access to water and food from hanging bottles and baskets, respectively. Food and water intake, respiratory exchange ratio (RER), energy expenditure and locomotor activity were recorded continuously during these 3 days. On the afternoon of Day 4, animals were moved back to their regular housing conditions.

ICV peptide infusion

Every animal received a cross-treatment with vehicle (sterile NaCl 0.9%) and Kp (3nmol/5uL; Rat Kp10 sequence, ToCris Bioscience, Abingdon, UK, n=15) or RFRP-3 (50 or 250 pmol/5uL; Rat RFRP-3 sequence, Caslo Laboratory, Lyngby, Denmark, 50 pmol n=8, 250 pmol n=7). Brain injections were performed at a rate of 1uL/min and patency was corroborated by tracking the movement of a small air bubble. All animals were injected between ZT4.5 and ZT5.5. Animals were handled for 3 - 5 min/day for at least 4 days before each ICV injection in order to habituate them to the procedure. Animals were allowed to recover for 7 days after each ICV injection.

Perfusion and peripheral tissue sampling

At the end of the experiment, rats were given a third ICV injection with either vehicle or one of the RF-amides and sacrificed 1h after under an overdose of intraperitoneally injected pentobarbital. A blood sample was taken by heart puncture and then animals were perfused intracardially with 150 mL of NaCl 0.9%. Then, animals were perfused with 100 mL formaldehyde 4%, brains were removed and post-fixed overnight. Brains were then transferred to 30% sucrose in Tris Buffered Saline (TBS) for cryoprotection. Brains were sliced at 35 µm

thickness with a Thermo fisher Scientific Cryostar NX50 cryostat (Thermo Fischer Scientific, Waltham, MA, USA) and stored in cryoprotectant (30% ethylene glycol, 30% glycerol, 40% phosphate-buffered saline) for later immunostainings.

2.Experimental set-up for measuring endogenous glucose production.

We used [6,6-$^{2}H_{2}$]glucose (D2-glucose) in order to evaluate endogenous glucose production (EGP) during a 2-hour continuous ICV infusion of either Kp or RFRP-3. Experiments were performed using a crossover design with at least one week of recovery in between. On the evening prior to the EGP evaluation, animals were attached to a counterbalanced swivel that allows blood sampling without handling the animal. On the following morning, food was removed at ZT0 and the arterial and venous catheters were connected to a tubing line filled with heparinized (1%) saline (Figure 1). At ZT3, a blood sample was taken for basal measurements and the tubing for the ICV infusion was filled up, connected to the cannula injector and sealed to avoid leaking into the ventricular space prior to the start of the brain infusion. At ZT4, the D2 glucose infusion was started using a primed-continuous administration protocol, starting first with a 5 min infusion at a rate of 3000 µL/h and then continued with a rate of 500 µL/h till the end of the experiment. Ninety minutes later at ZT5.5, when a steady state was reached, 200 µL blood sample were taken every 10 min for a total of three samples to calculate the basal level of EGP before the start of the brain infusion. Finally, at ZT6 a primed ICV infusion was started. Either Kp (0.6 nmol/µL), RFRP-3 (50 pmol/µL) or vehicle (NaCl 0.9%) were infused at a rate of 1µL/min for the first 5 mins, which was then decreased to 5µL/hour for the remainder of the experiment. Blood samples were taken every 20 min during 2 hours to calculate the change in EGP during the brain infusion. After two hours, the ICV and D2-glucose infusions and blood sampling were stopped and all external tubing was removed. Animals were immediately returned to *ad libitum* water and food access. At the end of the experiment, animals were sacrificed, fresh brains were collected, frozen and sliced with a cryostat to verify the cannula placement.

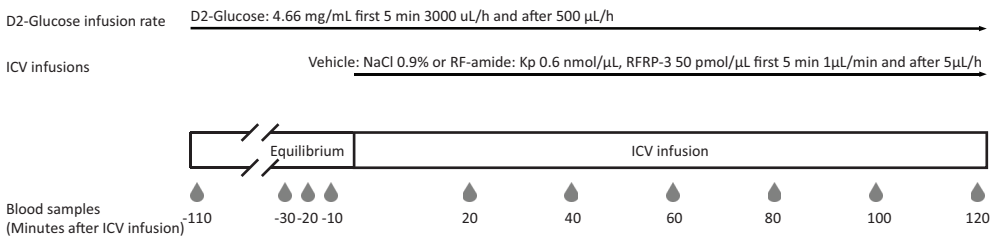


Figure 1. Experimental protocol of endogenous glucose production (EGP) during RF-amides or vehicle ICV treatment. The droplets and numbering in the bottom row indicate the timing of blood sampling in minutes.

Plasma hormonal measurements

Blood samples were centrifuged at 4000 rpm during 15 min at 4 °C and plasma was collected for hormone and labeled glucose measurements. Glucose concentrations were measured directly from blood samples with a glucometer (Abbott Laboratories, Chicago, IL, USA). Plasma [6,6-$^{2}H_{2}$] glucose enrichment was measured by GC-MS and EGP was calculated using the methods of Steele[201]. Corticosterone (MP Biomedicals, Santa Ana, CA, USA) and insulin (Millipore, Burlington, MA, USA) levels were determined by radioimmunoassays (RIA) according to the manufacturer's instructions, luteinizing hormone level was determined using and enzyme-linked immunosorbent assay (ELISA)[202] and testosterone levels were measured

using isotope dilution-liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS)[203].

Immunostainings

Brain slices containing hypothalamic nuclei were selected using bregma as reference according to the rat brain atlas from Paxinos and Watson (Table 1)[199]. Cellular activity was evaluated by c-Fos immunostaining. Sections were incubated with rabbit c-Fos antibody (1:1000, sc-52 Santa Cruz Biotechnology, CA, USA) 1h at room temperature followed by overnight at 4 °C, then sections were incubated with a goat biotinylated antibody anti-rabbit (1:500, BA1000, Vector labs, Burlingame, CA,USA) for 90 min. Signal was made visible by incubation with the avidin-biotin complex (1:500, Vector) for 1h and revealed with 3,3'-Diaminobenzidine (DAB, 0.5mg/mL), H_2O_2 (0.03%) and nickel ammonium sulfate (230 µg/mL). Finally, sections were mounted on slides, dehydrated in increasing concentrations of ethanol and xylene, and cover slipped with Entellan (Millipore).

Table 1. Brain nuclei stained for c-Fos

Distance from bregma (mm)	Abbreviation	Nucleus or Nuclei
0.48	OVLT	Organum vasculosum of the laminae Terminalis
0.24	aMnPO	Anterior part of the median preoptic nucleus (MnPO)
0.12	AVPV	Anteroventral periventricular nucleus
0	MPA	Medial preoptic area
-0.12	pMnPO	Posterior part of the MnPO
-0.48	aSCN	Anterior part of the suprachiasmatic nucleus (SCN)
-0.72	mSCN	Medial part of the SCN
-0.96	pSCN	Posterior part of the SCN
-1.8	aARC	Anterior part of arcuate nucleus (ARC)
-2.64	mARC	Medial part of the ARC
-3.84	pARC	Posterior part of the ARC

Double stainings for c-Fos and POMC were performed by staining one section at the anterior, medial and posterior part of the ARC (aARC, mARC and pARC) per animal. Sections were incubated with primary antibodies, rabbit anti POMC (1:4000, H-029-30, Phoenix Pharmaceuticals Inc., Belmont CA, USA) and sheep anti c-Fos (1:1000, PA1-18329, Thermofisher, Landsmeer, Netherlands) 1h at room temperature and overnight at 4 °C. Then, sections were incubated with a donkey anti-sheep (Alexa 594, A-11016, Invitrogen, Landsmeer, Netherlands) and a donkey anti-rabbit (Alexa 488, A32723, Invitrogen) coupled to fluorophore. Sections were mounted on slides and cover slipped with mounting media containing 4',6-diamidino-2-phenylindole (DAPI) (H-1200, Vectashield, Vector Labs).

For all experiments, the specificity of the first antibody was assessed by verifying that the removal of the primary antibody resulted in an absence of immunostaining. In addition, the specificity of the anti-POMC was verified by pre-absorption controls on ARC brain sections containing POMC neurons, where staining was abolished[204,205]. The specificity of the Santa Cruz rabbit c-Fos antibody (Sc-52) was demonstrated by Magno *et al.*[206] and that for the

Thermofisher sheep c-Fos antibody (PA1-18329) was done by Wang *et al.* [206].

Image analysis

Sections stained for c-Fos immunostaining were photographed with a CCD camera (Model 77CE; Sony, Tokyo, Japan) attached to a microscope (Zeiss Axios-kop with Plan-NEOFLUAR Zeiss objectives; Carl Zeiss GmbH, Oberkochen, Germany) using a 10 × 0.63 objective. Double immunofluorescence was pictured with a confocal microscope (Leica TCS SP8 SMD, Leica, Wetzlar, Germany) using a 40x objective. For each staining analyzed, images of the areas of interest were taken at the same time under identical lighting or laser setups for all animals. A person unaware of the treatments performed the quantification of either c-Fos expression, POMC staining and co-localization using the ImageJ software (NIH, Bethesda, MD, USA). Positive c-Fos staining was evaluated by setting a threshold to a mean size of the nuclear positive staining. Then the mean size was used to threshold the quantification for the rest of the images. A rectangle or circle of the size of the brain area of interest was drawn and superimposed accordingly. POMC immunoreactive (-ir) area was evaluated by using a set threshold and quantifying the same sampled area size for every level of the ARC. Double immunostaining was evaluated by counting exclusively POMC positive cells that contained both, c-Fos and DAPI in the nucleus.

Statistical analysis

Only data from animals with correct cannula placements and injections corroborated for patency were included in the data analysis. All data showed normal distribution and homogeneous variance according to the Saphiro-Wilk and Levene's tests and are represented as average ± SEM. Graphs and statistical analyzes were conducted with Prism, version 8 (GraphPad Software Inc., San Diego, CA, USA). EGP measurements were analyzed by repeated measures two-way ANOVA and Bonferroni or Tukey post-hoc honestly significant difference (HSD) test. Hormonal levels and neuro-anatomical results were compared by Student's *t*-test. TSE data (food and water consumption, RER, energy expenditure and locomotor activity) were analyzed by two-way repeated measures ANOVA and Bonferroni post-hoc honestly significant difference (HSD) test. Delta values were calculated as the difference compared to the mean value of the three pre-infusion samples. $P < 0.05$ was considered statistically significant.

Results

To investigate the effects of Kp and RFRP-3 on glucose and energy metabolism two different experimental techniques and two slightly different experimental set-ups were used. In Experiment-1 we used indirect calorimetry in so-called metabolic cages (TSE) to measure the effects of Kp and RFRP-3 on food and water intake, locomotor activity, energy expenditure and the respiratory exchange ratio (RER). In Experiment-2 we used a chronic intravenous infusion with D2-glucose to evaluate the effects on EGP. In Experiment-1 both RF-amides were administered intracerebroventricularly (ICV) as a bolus injection, whereas in Experiment-2 they were administered during a 2-hour continuous ICV infusion.

Experiment-1: Indirect calorimetry

Only the animals that successfully received both ICV injections (vehicle and RF-amide) were included in the analysis of food and water intake, RER, energy expenditure and locomotor activity; 9/15 for Kp and 9/15 for RFRP-3 (n=5 for 50 pmol, n=4 for 250 pmol). During the

third ICV injection, aimed for tissue sampling, one animal from the RFRP group had to be excluded due to a failure of the ICV injection.

RF-amide effects on the hypothalamus-pituitary-gonadal (HPG) axis, insulin and corticosterone

First, we verified the effect of both RF-amides on the activity of the HPG axis. One hour after ICV injection, both Kp and RFRP-3 increased systemic testosterone levels as compared to vehicle treated rats, however, with a less potent effect of RFRP-3 as compared to that of Kp (Kp: *P*=0.001, RFRP-3: *P*=0.045; Figure 2B). Plasma luteinizing hormone, insulin and corticosterone levels did not show statistically significant effects after the peptide treatment (Figure 2A,C,D).

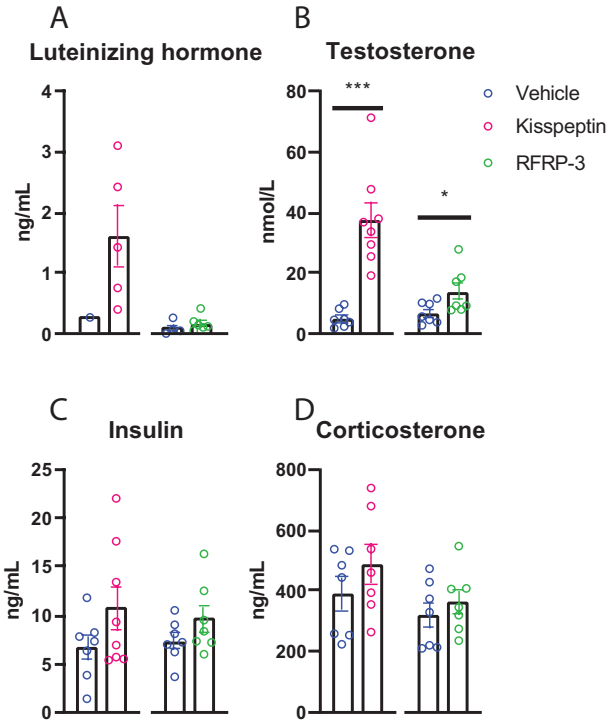


Figure 2. Effect of central Kisspeptin and RFRP-3 on circulating hormones in male Wistar rats. Luteinizing hormone (A) , testosterone (B), insulin (C) and corticosterone (D) plasma levels were measured 1 hour after the ICV injection of vehicle (NaCl 0.9%, blue circles), 3 nmol Kp (pink circles) or 250 pmol RFRP3 (green circles). Data are the mean ± SEM of n=7-8 animals per experimental group, with the scattered dots representing individual data values. For luteinizing hormone, 13 out of 29 samples fell in the non-detectable range as also reported previously for male rodents. ***P<0.001, *P<0.05 after Student's *t*-test when comparing the ICV peptide infusion vs its respective ICV vehicle control infusion.

Central kisspeptin injection decreases food intake

Rats injected with 3nmol Kp exhibited a decrease in 24h-food intake when compared to the previous baseline day as well as compared to their vehicle treatment (Figure 3A-C). Both the comparison "Kp ICV vs. vehicle ICV" and "Kp ICV vs. Kp baseline" revealed a significant effect of Treatment, respectively *P*=0.0006 and *P*=0.0059 (Table 2). Water intake was not changed

after Kp injection (Figure 3D-F and Table 2). By contrast, neither the 50 (Table S1; see also Supporting information Figure S1,) nor 250 pmol (Table S2; see also Supporting information, Figure S2) ICV injections of RFRP-3 significantly changed food or water intake. In addition, also when the two RFRP-3 experiments were combined (n=9) no significant effects on food or water intake were found (see Supporting information, Figure S3 and Table S3).

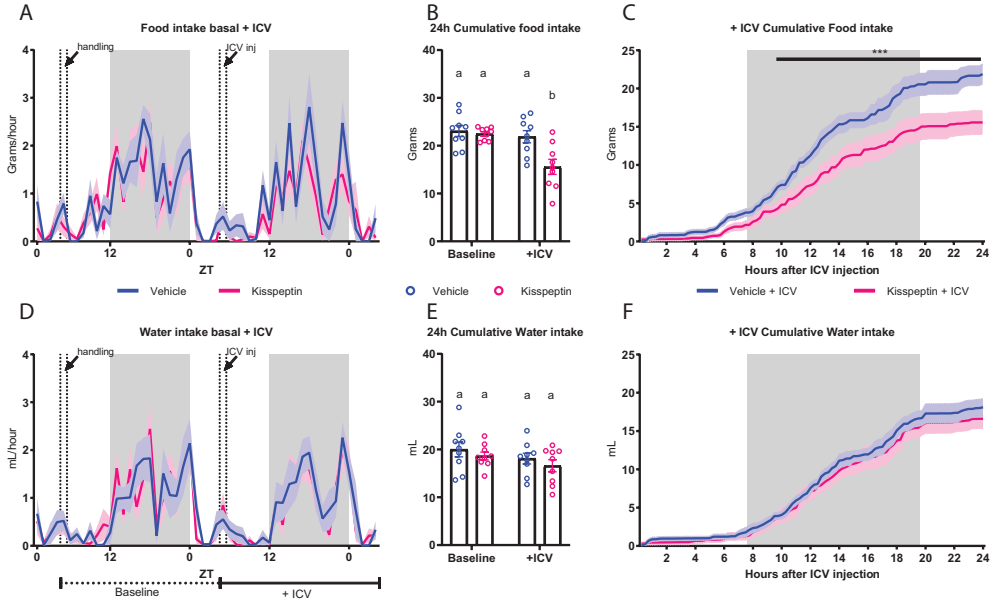


Figure 3. Effect of intracerebroventricular (ICV) injection of Kisspeptin (3 nmol) or vehicle (NaCl 0.9%) on food and water intake in male Wistar rats. Hourly food (A) and water (D) intakes are plotted for the baseline and experimental (+ICV) days, with data presented as the mean (solid line) ± SEM (shaded area) of n=9 animals. Total 24h food (B) and water (E) intake were calculated for the baseline and experimental day as indicated by the horizontal lines below figure D. Different lowercase letters in B and E indicate that the groups are statistically different after two-way ANOVA and Tukey's post hoc honestly significance test. Cumulative food (C) and water (F) intake during the experimental day with a 15-minute resolution during 24h and starting immediately after the Kp or vehicle injection. ***$P < 0.001$ statistical difference between Kp and vehicle after two-way ANOVA and Bonferroni's post-hoc honestly significance. ZT0 = lights ON, ZT12 = lights OFF. Gray background indicates the dark period. Handling or ICV injection occurred between the two vertical dashed lines.

Central Kisspeptin injection decreases the respiration exchange ratio (RER)

The RER value indicates the main fuel source utilized by the body for energy production. This ratio fluctuates over the daily cycle and is close to 1.0 during the dark (i.e. feeding) phase when mainly carbohydrates are oxidized, but decreases during the light (i.e. sleeping) period when animals are fasting and lipids become the preferential source of fuel. The ICV injection of Kp significantly decreased the RER (Figure 4B). Repeated masures-ANOVA showed significant Treatment effects for the "Kp ICV vs. vehicle ICV" and "Kp ICV vs. Kp baseline" comparison, P=0.0010 and P=0.0063, respectively (Table 2). In addition, the 24h mean RER level was decreased in the Kp – ICV group (Figure 4B). By contrast, energy expenditure was not significantly affected by Kp (Figure 4C). Locomotor activity showed a significant Treatment effect in the "Kp ICV vs. Kp baseline" comparison (P=0.0069) (Figure 4A and Table 2), but no differences in 24h total activity were detected (Figure 4A). None of the doses of RFRP-3, either separately or combined, significantly changed RER, locomotor activity or energy expenditure (see Supporting information, Figures S4 and S5 and Tables S1-S3).

Table 2. Effects of ICV Kisspeptin injection (3 nmol) on metabolic outcomes

Comparison	Parameter	Treatment	Time	Interaction
Baseline vs Vehicle ICV	Cumulative Food intake	$F_{1,8}$ = 0.9580 *P*=0.356	**$F_{95,760}$ = 206.5** ***P*<0.0001**	**$F_{95,760}$ = 1.337** ***P*=0.023**
	Cumulative Water intake	$F_{1,8}$ = 2.306 *P*=0.167	**$F_{95,760}$ = 132.6** ***P*<0.0001**	**$F_{95,760}$ = 3.595** ***P*<0.0001**
	Locomotor activity	$F_{1,8}$ = 4.798 *P*=0.060	**$F_{95,760}$ = 6.120** ***P*<0.0001**	*F* 95, 760) = 1.257 *P*=0.058
	RER	$F_{1,8}$ = 1.189 *P*=0.307	**$F_{95,760}$ = 13.00** ***P*<0.0001**	**$F_{95,760}$ = 1.571** ***P*=0.0008**
	Heat	$F_{1,8}$ = 0.5588 *P*=0.476	**$F_{95,760}$ = 6.942** ***P*<0.0001**	$F_{95,760}$ = 0.9260 *P*=0.675
Baseline vs Kp10 ICV	Cumulative Food intake	**$F_{1,8}$ = 13.84** ***P*=0.006**	**$F_{95,760}$ = 264.5** ***P*<0.0001**	**$F_{95,760}$ = 9.056** ***P*<0.0001**
	Cumulative Water intake	$F_{1,8}$ = 1.038 *P*=0.338	**$F_{95,760}$ = 259.7** ***P*<0.0001**	$F_{95,760}$ = 0.8811 *P*=0.779
	Locomotor activity	**$F_{1,8}$ = 13.04** ***P*=0.007**	**$F_{95,760}$ = 6.759** ***P*<0.0001**	**$F_{95,760}$ = 1.675** ***P*=0.0001**
	RER	**$F_{1,8}$ = 13.48** ***P*=0.006**	**$F_{95,760}$ = 12.87** ***P*<0.0001**	**$F_{95,760}$ = 3.121** ***P*<0.0001**
	Heat	$F_{1,8}$ = 4.479 *P*=0.067	**$F_{95,760}$ = 6.288** ***P*<0.0001**	**$F_{95,760}$= 1.779** ***P*<0.0001**
Vehicle ICV vs Kp10 ICV	Cumulative Food intake	**$F_{1,8}$ = 30.19** ***P*=0.0006**	**$F_{95,760}$ = 117.9** ***P*<0.0001**	**$F_{95,760}$ = 12.39** ***P*<0.0001**
	Cumulative Water intake	$F_{1,8}$ = 1.105 *P*=0.324	**F (95, 760) = 139.8** ***P*<0.0001**	$F_{95,760}$ = 0.5516 *P*=0.999
	Locomotor activity	$F_{1,8}$ = 0.8578 *P*=0.382	**$F_{95,760}$ = 6.409** ***P*<0.0001**	$F_{95,760}$ = 0.6796 *P*=0.991
	RER	**$F_{1,8}$ = 25.42** ***P*=0.001**	**$F_{95,760}$ = 12.78** ***P*<0.0001**	$F_{95,760}$ = 1.169 *P*=0.141
	Heat	$F_{1,8}$ = 0.04709 *P*=0.834	**$F_{95,760}$ = 5.061** ***P*<0.0001**	$F_{95,760}$ = 0.8237 *P*=0.883

Abbreviations: Kp, kisspeptin; RER, respiratory exchange ratio. Significant values are indicated in bold.

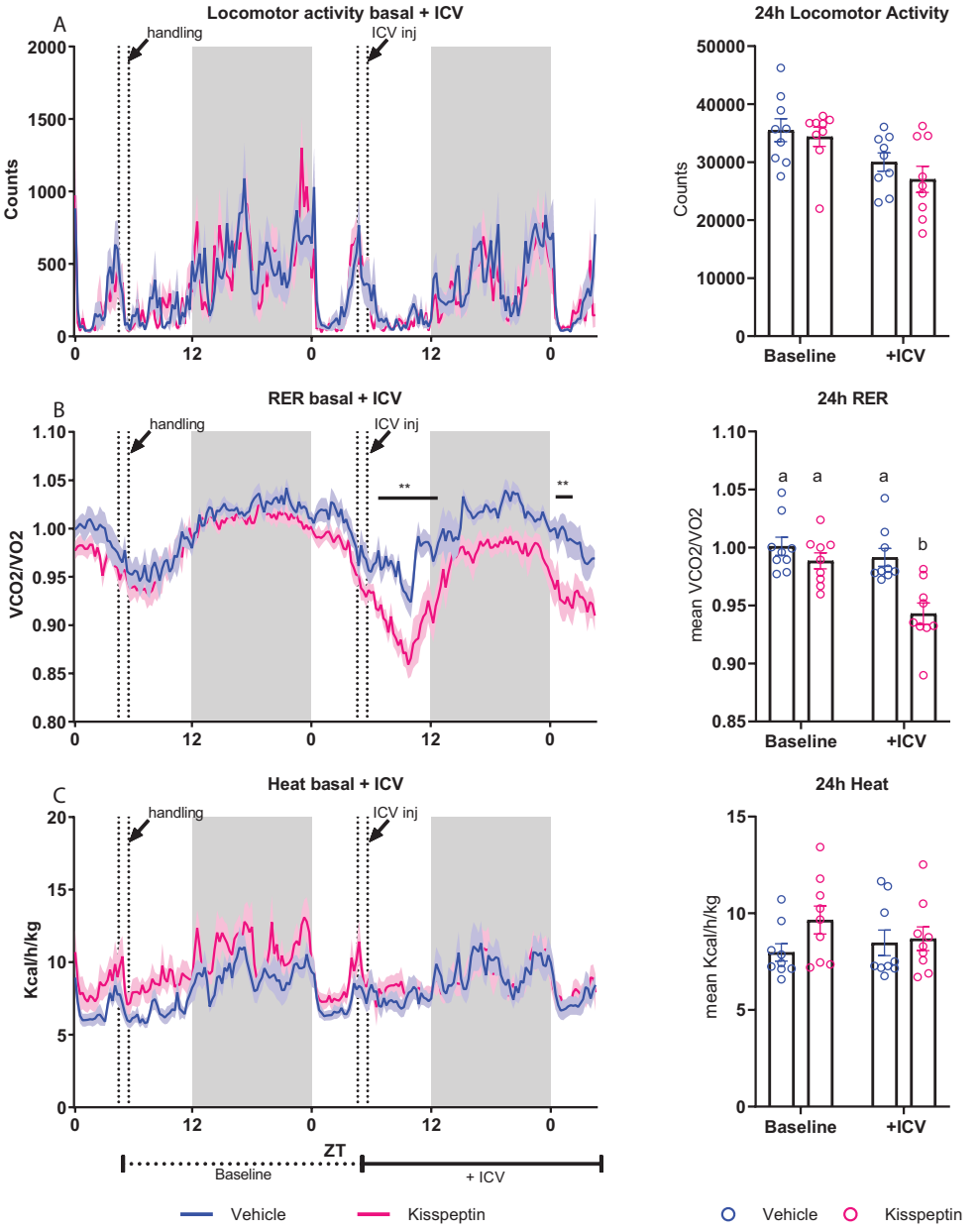


Figure 4. Effects of ICV Kisspeptin (3 nmol) or vehicle (NaCl 0.9%) on locomotor activity, respiratory exchange ratio (RER) and energy expenditure in male Wistar rats. Locomotor activity (A), RER (B) and energy expenditure or heat (C) are plotted for the baseline and experimental (+ICV) day, with data presented as the mean (solid line) ± SEM (shaded area) of n=9 animals; **P < 0.01 statistical difference between Kp and vehicle after two-way ANOVA and Bonferroni's post-hoc honestly significance test. Total locomotor activity, mean RER and energy expenditure were calculated over the 24h for the baseline and experimental day, as indicated by the horizontal lines below figure C. Different lowercase letters indicate that the groups are statistically different after two-way ANOVA and Tukey's post hoc honestly significance test. ZT0 = lights ON, ZT12 = lights OFF Gray shaded area indicates dark phase. Handling or ICV injection occurred between the two dashed lines.

Experiment-2: Endogenous glucose production

Ten animals from the Kp experiment (vehicle n=4, Kp n= 6) and 11 animals from the RFRP-3 experiment (vehicle n=5, RFRP-3 n=6) completed the experiment with correct infusions and complete blood samplings. None of the plasma measurements showed a statistically significant difference when comparing the RF-amide vs vehicle groups before the start of the ICV infusion (Table 3).

Table 3. Basal levels of plasma measurement before vehicle or RF-amide ICV infusion (mean ± SEM)

Experiment		Vehicle ICV	RF-amide	*P* value
Kisspeptin ICV infusion	Luteinizing hormone (ng mL^{-1})	−0.2479 ± 0.2593	0.03367 ± 0.08949	0.262
	Testosterone (nmol L^{-1})	3.475 ± 1.040	7.383 ± 1.985	0.173
	Corticosterone (ng mL^{-1})	65.60 ± 30.45	81.17 ± 40.82	0.863
	Insulin (ng mL^{-1})	2.110 ± 0.3548	1.437 ± 0.1749	0.095
	Glucose (mmol L^{-1})	3.883 ± 0.2327	3.933 ± 0.1805	0.868
	Endogenous glucose production(µmol kg·min^{-1})	73.56 ± 9.345	63.49 ± 2.600	0.248
RFRP-3 ICV infusion	Luteinizing hormone (ng mL^{-1})	−0.1300 ± 0.08961	−0.1767 ± 0.1818	0.834
	Testosterone (nmol L^{-1})	3.520 ± 0.5826	5.900 ± 2.452	0.411
	Corticosterone (ng mL^{-1})	34.80 ± 14.08	24.67 ± 7.575	0.522
	Insulin (ng mL^{-1})	1.288 ± 0.07186	1.475 ± 0.1245	0.250
	Glucose (mmol L^{-1})	3.953 ± 0.04295	4.100 ± 0.08735	0.192
	Endogenous glucose production (µmol kg·min^{-1})	61.09 ± 3.674	63.14 ± 7.244	0.8191

Effects of RF-amide infusion on LH, testosterone, insulin and corticosterone secretion

The ICV infusion of Kp increased plasma luteinizing hormone (LH) and testosterone concentrations, showing significant Treatment and Interaction effects (Figure 5A,C and Table 4). Post hoc analysis revealed that mean plasma levels of LH had already increased at 20 min, i.e., in the first sample after the start of the ICV infusion, but a statistically significant difference was only reached at 80 min (Figure 5A). Plasma testosterone levels showed statistically significant differences as compared to the vehicle group 60, 100 and 120 min after the start of the ICV infusion (Figure 5C). The ICV RFRP-3 infusions did not show any statistically significant difference in plasma LH or testosterone levels (Figure 5B,D and Table 4). Both RF-amides did not have any significant effects on either on plasma corticosterone or insulin levels (Table 4 and Figure 6).

Glycaemia and endogenous glucose production (EGP) after RF-amide ICV infusion

Both RF-amides did not result in any significant changes in blood glucose levels, but the ICV administration of Kp resulted in a significant increase of EGP as attested by a significant Interaction effect (*P*=0.0385). The post-hoc analysis showed that this increase started 20 min after the start of the ICV infusion (Figure 7C and Table 4). The ICV administration of RFRP-3 also tended to increase EGP as indicated by the borderline significant Interaction effect (*P*=0.050) (Figure 7D and Table 4).

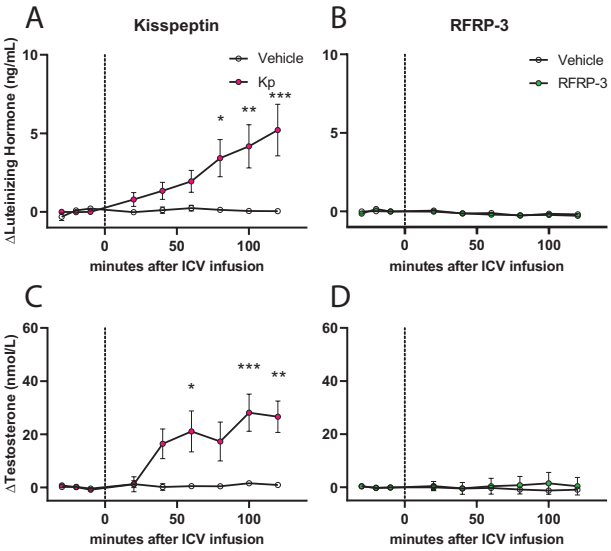


Figure 5. Effect of central Kisspeptin and RFRP-3 on HPG axis activity. Luteinizing Hormone (A,B) and testosterone (C,D) plasma levels were measured at -30, - 20, -10, 20, 40, 60, 80, 100 and 120 mins after the start of the ICV infusion of Kp (3 nmol/h, pink circles), RFRP-3 (50 pmol/h, green circles) or vehicle (NaCl 0.9%, open circles). Data are the mean ± SEM of n=4-6 for Kp and n=5-6 for RFRP-3 treated animals per experimental group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 statistical difference between Kp and vehicle after two-way RM ANOVA and Bonferroni's post-hoc honestly significance test.

Table 4. Effects of i.c.v. kisspeptin (3 nmol) and i.c.v. RFRP-3 (50 pmol) infusions on plasma hormone levels

Comparison	Parameter	Treatment	Time	Interaction
Vehicle vs kisspeptin	ΔLuteinizing hormone (ng mL-1)	**$F_{1,8}$ = 5.562 *P* = 0.046**	**$F_{8,64}$ = 5.538 *P* < 0.0001**	**$F_{8,64}$ = 5.209 *P* < 0.0001**
	ΔTestosterone (nmol L-1)	**$F_{1,8}$ = 6.928 P = 0.030**	**$F_{8,64}$ = 6.235 *P* < 0.0001**	**$F_{8,64}$ = 5.578 *P* < 0.0001**
	ΔCorticosterone (ng mL-1)	$F_{1,8}$ = 0.9052 *P* = 0.369	**$F_{8,64}$ = 3.845 *P* = 0.001**	$F_{8,64}$ = 0.5208 *P* = 0.836
	ΔInsulin (ng mL-1)	$F_{1,8}$ = 2.627 *P* = 0.144	**$F_{8,64}$ = 2.869 *P* = 0.009**	$F_{8,64}$ = 1.293 *P* = 0.263
	ΔGlucose (mmol L-1)	$F_{1,8}$ = 0.9598 *P* = 0.356	**$F_{8,64}$ = 3.048 *P* = 0.006**	$F_{8,64}$ = 0.7549 *P* = 0.643
	ΔEndogenous glucose production (µmol kg·min-1)	$F_{1,8}$ = 3.481 *P* = 0.099	$F_{8,64}$ = 1.090 *P* = 0.382	**$F_{8,64}$ = 2.204 *P* = 0.039**
Vehicle vs RFRP-3	ΔLuteinizing hormone (ng mL-1)	$F_{1,9}$ = 0.2578 *P* = 0.624	**$F_{8,72}$ = 4.828 *P* < 0.0001**	$F_{8,72}$ = 0.6371 *P* = 0.744
	ΔTestosterone (nmol L-1)	$F_{1,9}$ = 0.1266 *P* = 0.730	$F_{8,72}$ = 0.06769 *P* = 0.999	$F_{8,72}$ = 0.2284 *P* = 0.985
	ΔCorticosterone (ng mL-1)	$F_{1,9}$ = 2.667 *P* = 0.137	**$F_{8,72}$ = 3.698 *P* = 0.001**	$F_{8,72}$ = 0.9926 *P* = 0.449
	ΔInsulin (ng mL-1)	$F_{1,9}$ = 0.5046 *P* = 0.496	$F_{8,72}$ = 1.163 *P* = 0.334	$F_{8,72}$ = 0.9767 *P* = 0.461
	ΔGlucose (mmol L-1)	$F_{1,9}$ = 0.2453 *P* = 0.632	**$F_{8,72}$ = 4.245 *P* = 0.0003**	$F_{8,72}$ = 0.3165 *P* = 0.957
	ΔEndogenous glucose production (µmol kg·min-1)	$F_{1,9}$ = 2.746 *P* = 0.132	$F_{8,72}$ = 1.890 *P* = 0.075	**$F_{8,72}$ = 2.069 *P* = 0.050**

Significant values are indicated in bold.

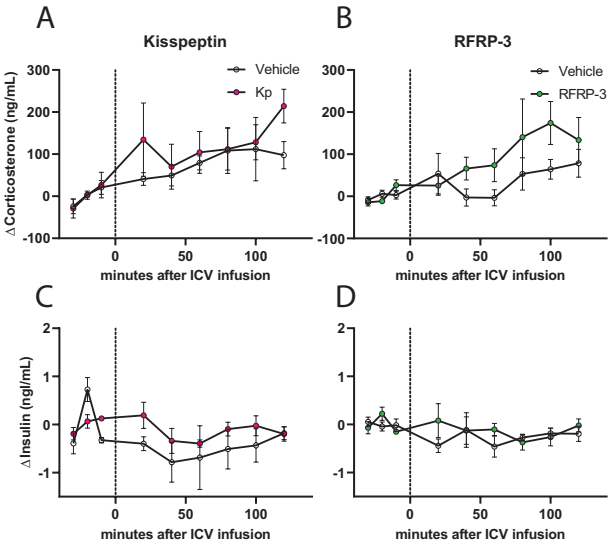


Figure 6. Effect of central Kisspeptin and RFRP-3 on corticosterone and insulin. Corticosterone (A,B) and testosterone (C,D) plasma levels were measured at -30, - 20, -10, 20, 40, 60, 80, 100 and 120 mins after the ICV infusion of Kp (3 nmol/h, pink circles), RFRP-3(50 pmol/h, green circles) or vehicle (NaCl 0.9%, open circles). Data are the mean ± SEM of n=4-6 for Kp and n=5-6 for RFRP-3 treated animals per experimental group.

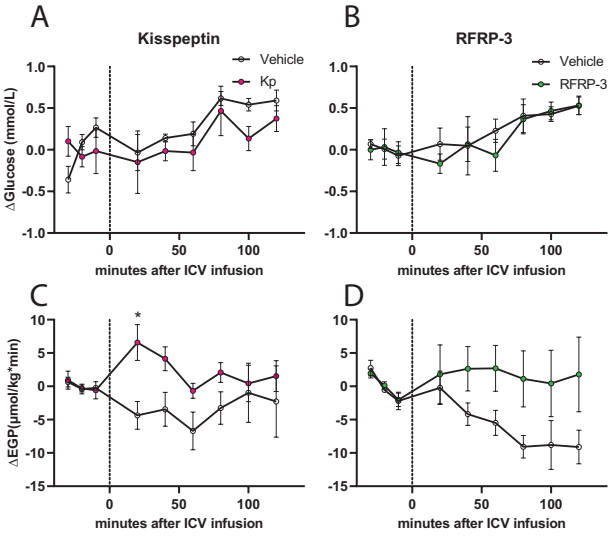


Figure 7. Effect of central Kisspeptin and RFRP-3 on blood glucose and endogenous glucose production (EGP). Blood glucose (A,B) and EGP (C,D) were measured at -30, - 20, -10, 20, 40, 60, 80, 100 and 120 mins after the ICV infusion of Kp (3 nmol/h, closed circles), RFRP-3(50 pmol/h, closed squares) or vehicle (NaCl 0.9%, open circles and open squares). Data are the mean ± SEM of n=4-6 for Kp and n=5-6 for RFRP3 treated animals per experimental group. *$P < 0.05$ statistical difference between Kp and vehicle after two-way RM ANOVA and Bonferroni's post-hoc honestly significance test.

Central targets of ICV Kp and RFRP-3

At the end of the Experiment-1, rats were given a third ICV injection with either vehicle or one of the RF-amides, and one hour later, animals were perfused and perfusion fixed brains processed for immunostainings.

Activation of median preoptic nucleus in response to kisspeptin

From all the brain regions analyzed for c-Fos immunoreactivity (Figure 8), only the posterior part of the median preoptic area (pMnPO) showed a significant increase in the number of c-Fos positive cells after the ICV Kp treatment (*P*=0.014) (Figure 8A). RFRP-3 injections did not result in any significant increase in c-Fos immunostaining in the investigated brain areas, in fact, in most brain areas the amount of c-Fos tended to decrease (Figure 8B,D), including the SCN in line with previously published results[208].

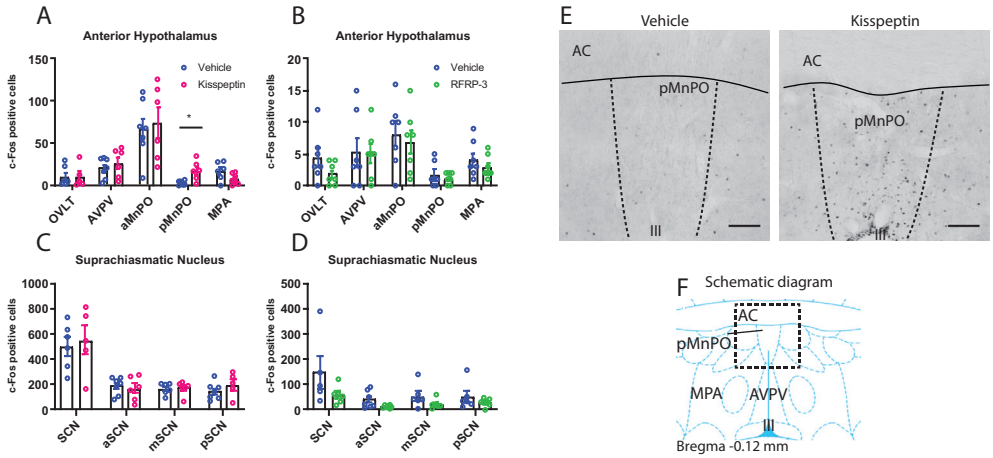


Figure 8. Effect of ICV Kisspeptin and RFRP3 on c-Fos expression in the male Wistar rat hypothalamus. The number of c-Fos expressing cells was evaluated in various brain areas, i.e. OVLT, AVPV, aMnPO, pMnPO, and MPA of the anterior hypothalamus (A, B); and anterior, medial and posterior parts of the suprachiasmatic nuclei (C, D), 60 minutes after the ICV injection of Kp (3 nmol), RFRP3 (250 pmol) or vehicle (NaCl 0.9%). E) Shows representative images of c-Fos staining in the median preoptic nucleus (pMnPO) of animals injected with vehicle (left) or Kp (right). F) Showing a schematic diagram obtained from the rat brain atlas (Paxinos and Watson) of the preoptic nuclei at a level equivalent to bregma -0.12 mm.[199] Data represent the mean ± SEM of n=5 - 7 animals, with scattered dots representing individual values per animal. * *P*<0.05 statistical difference between Kp and vehicle after Student's *t*-test. III, third ventricle; AC, anterior comissura; MPA, medial preoptic area; OVLT, organum vasculosum of the laminae terminalis; AVPV, anteroventral periventricular nucleus; aMnPO, anterior part of the median preoptic nucleus; pMnPO, posterior part of the median preoptic nucleus; SCN, suprachiasmatic nucleus. Scale bar = 200 µm.

POMC neurons respond to Kisspeptin

POMC expression was analyzed in the ARC of Kp- and RFRP-3-treated rats (Figure 9) because most of the POMC neurons have been reported to express RF-amide receptors[164]. Kp induced a significant decrease in the number of POMC-immunoreactive cells in the posterior part of ARC (*P*=0.024) (Figure 9C). Also, total POMC-immunoreactivity in the ARC was reduced, which was mainly due to a decrease in its posterior part (*P*=0.004) (Figure 9E). The number of POMC cells expressing c-Fos was not modified (Figure 9G). RFRP-3 injections had no significant effect on ARC c-Fos or POMC expression (Figure 9B,D,F,H).

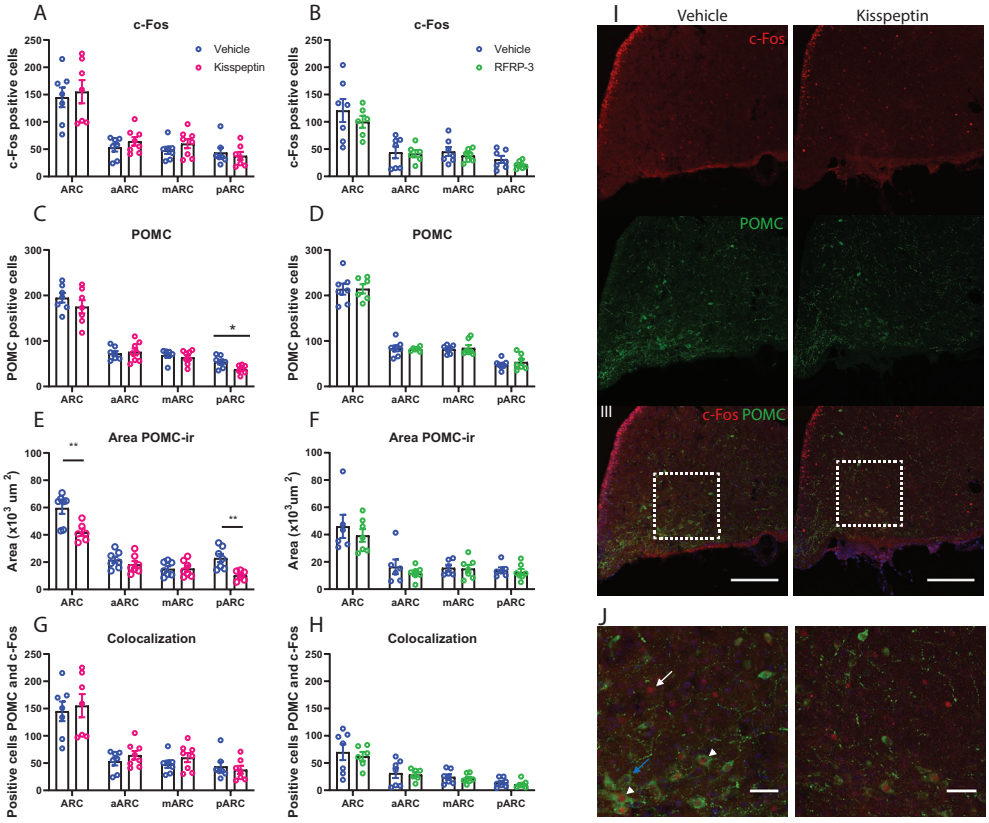


Figure 9. Effect of ICV Kisspeptin and RFRP3 on neuronal activity and POMC-ir in the arcuate nucleus. POMC neuronal activity was evaluated 1h after ICV administration of Kp (3 nmol), RFRP3 (250 pmol) or vehicle (NaCl 0.9%), as the number of c-Fos positive cells (A-B), number of POMC-ir cells (C-D), area of coverage of POMC immunoreactivity (E-F,) and number of POMC-ir neurons expressing c-Fos (G-H). I) Representative images of double immunofluorescence for c-Fos (red) and POMC (green) in the posterior arcuate nucleus (scale bar = 100 µm). J) Zoom in picture showing positive c-Fos (white arrow), POMC neurons (blue arrows), or co-localization (white arrowhead; Scale bar = 20 µm). Left panels: vehicle-treated animals; right pannels: Kp-injected animals. 4',6-Diamidino-2-phenylindole (blue) was used to stain nuclei. Data in A-H represent the mean ± SEM of n= 7 to 8 animals per experimental group and the scattered dots represent individual data values. ** P<0.01, * P<0.05 statistical difference between Kp and vehicle after Student's t-test.ARC, arcuate nucleus; aARC, anterior ARC; mARC, medial ARC; pARC, posterior ARC.

Discussion

The present study reports a clear anorexigenic effect of central Kp in *ad libitum* fed rats, which was associated with a significant decrease in RER. By contrast to Kp, RFRP-3 did not display any significant orexigenic effects. Regarding glucose metabolism, both RF-amides had a stimulatory effect on endogenous glucose production, although this effect was only significant for Kp. In agreement with these data, central administration of Kp, but not RFRP-3, changed neuronal activity in the preoptic nucleus of the hypothalamus. Together, these results provide further evidence that Kp is not only a very potent hypothalamic activator of reproduction but is also part of the hypothalamic circuit controlling glucose and energy metabolism, which makes the Kp system ideally suited to ensure an optimal match of energy

metabolism with the metabolic needs of reproduction. The metabolic effects of RFRP-3 on the other hand seem to be very much species dependent, just like its reproductive effects.

The hormone measurements showed that both the ICV administration of Kp and RFRP-3 was effective and reached their brain targets. The increased activity of the HPG axis upon ICV Kp, as shown by the marked increase in plasma testosterone concentrations, is in line with what has been reported before in rats[85], as well as after intravenous administration in humans[209]. ICV administration of RFRP-3 also increased circulating testosterone levels, but to a lesser extent. The different effect of RFRP-3 in Experiment-1 and -2 is most likely due to the different doses used, i.e., 250 pmol in Experiment-1 and 50 pmol in Experiment-2. Although RFRP-3 was initially discovered in birds and named gonadotropin-inhibitory hormone for its capacity to inhibit LH levels[91], recent studies reported an activation of the HPG axis in male mice and hamsters[103,177]. Therefore, our data confirm that RFRP-3 can induce activation of the HPG axis in male rodents.

Although the currently reported anorexic effect of central Kp in *ad libitum* fed rats confirms earlier observations in mice, rats and Jerboa's[115,136,210], in the very first studies no significant effects on feeding behavior were found[85,114], probably because the anorexic effect of Kp is only minor. In the present study the anorexigenic effect of Kp was associated with a decrease in RER. This may be explained by the fact that longer periods of fasting induce an increase in lipid oxidation, which results in lower RER values. However, when comparing figure 3A and 4B it seems that the RER starts going down already much earlier, i.e., immediately after the ICV injection of Kp. On the other hand, a closer look at figure 3C reveals that also the anorexigenic effect is apparent immediately after the ICV injection of Kp. This indicates that Kp simultaneously decreases food intake and stimulates lipid oxidation, causing a decrease of RER. The effect of Kp seems to be rather specific for food intake, as we found no changes in energy expenditure and locomotor activity, which is in line with the previous report of Thompson *et al*.[85]. Kiss1R KO mice not only showed a higher body weight, but also reduced energy expenditure, lower locomotor activity and reduced thermogenesis[117,121,127,211]. However, this difference of course may be due to the different experimental settings, i.e., acute ICV versus chronic and developmental KO effects. Notably, selective KO of the Kiss1R from brown adipose tissue caused a reduction in body weight and increased energy expenditure[211], clearly illustrating the tissue-specific functions of Kp.

To elucidate the neuronal targets that might be involved in the anorexigenic effect of Kp, we investigated the activity of POMC neurons, as they are the main neuronal population in the ARC expressing the Kiss1R[164] and are very well characterized for their inhibitory role on food intake and body weight[39]. We found that Kp significantly decreased POMC-immunoreactivity, demonstrating that ICV Kp was indeed affecting the activity of the POMC neurons. The decreased POMC-ir indicates that Kp increased the cleavage, transport and release of processed neuropeptides (i.e., alpha-Melanocortin Stimulating Hormone (α-MSH)). This hypothesis is supported by studies in mice, demonstrating a direct activation of POMC neurons by Kp[129,171] and our own studies in hamsters showing that Kp increases *Pomc* mRNA levels[194]. In line with the above physiological data, central administration of α-MSH has been shown to simultaneously decrease food intake and increase lipid oxidation[212]. In addition, reduction of brain-melanocortin signaling consistently results in an increased RER[213–216] indicative of reduced lipid utilization. Unfortunately, we found no increased c-Fos expression or POMC/c-Fos co-localization in the ARC, which would have provided further support for the Kp-induced activation of POMC neurons. The stimulatory effect of Kp on POMC neuronal activity has been clearly demonstrated with electrophysiological techniques, whereas Kp effects on NPY neuronal activity are more equivocal[129,171]. Surprisingly, as far as we know

increased Kp-induced expression of c-Fos has only been demonstrated for NPY neurons[144].

POMC- and NPY neurons in the ARC exhibit antagonistic effects on food intake[23,217]. Indeed, besides the above mentioned stimulatory effect of Kp on POMC neurons, Kp has been reported to inhibit NPY neuronal activity[129,171]. However, opposite effects were observed in ovariectomized sheep, i.e. increased NPY expression and reduced POMC expression[130], while in female Jerboas the anorexigenic effect of Kp was accompanied with an increased expression of POMC, but no changes in NPY expression[116]. Therefore, it will be interesting to analyze in future experiments whether central Kp also changes NPY expression. Notably, the central injection of Kp increased c-Fos expression in the MnPO, a region known to receive a direct POMC innervation from the ARC[218]. Pharmacological manipulations of α-MSH signaling to the MnPO changed both c-Fos expression and core body temperature. Recently, it has been shown that ARC Kp neurons are also part of the neural circuit that is modulating the circadian control of body temperature[120]. In addition, Kp is also known to change the expression of other (an)orexigenic peptides, such as brain-derived neurotropic factor (BDNF), melanin-concentrating hormone (MCH), nesfatin-1 and oxytocin[210,219,220]. Therefore, at present, no final conclusions can be made on the appetite-regulating pathways of Kp.

The currently observed effects of Kp on glucose metabolism seem to be in line with the glucose intolerance observed in Kiss1r KO mice[117,211]. In the present study, Kp increased glucose production, but plasma glucose levels were not changed indicating a concomitant increase in glucose tolerance. On the other hand, the increased glucose production does not seem to be in line with the decreased RER observed upon the central administration of Kp, i.e. increased lipid oxidation. However, experimental set-ups were different (ICV bolus injection plus *ad libitum* feeding in Experiment-1 and ICV continuous infusion plus fasting in Experiment-2) and the extra glucose produced does not necessarily have to be oxidized, but can also be stored in muscle or adipose tissue. Clearly, much remains still unknown regarding the central and peripheral glucoregulatory effects of Kp.

Surprisingly, RFRP-3 did not display any significant orexigenic effects. Although RFRP-3 did increase testosterone levels at the highest dose tested, no significant effects on food intake or energy metabolism were found. These findings are in clear contrast to what has been reported previously in other mammalian species[116,124,194,197], as well as in *ad libitum* fed Sprague Dawley rats[123,124]. However, except for the different species used, also the variance in doses, RFRP-3 preparation and experimental set-up used (e.g., ICV bolus injections vs 2-hour or 5-day infusions or fed versus fasted status) might be responsible for these apparent discrepancies. Regarding glucose metabolism, little is known about possible effects of RFRP-3. Intraperitoneal administration of RFRP-3 has been shown to change circulating glucose concentrations, as well as insulin receptor and glucose transporter expression in testis and adipose tissue[125,221] and lack of the RFRP-3 receptor in NPFF1R KO mice worsened the metabolic impact of a high-fat diet on glucose homeostasis in male but not female mice[127]. RFRP-3 neurons also project to the NPY- and POMC-containing neurons in the ARC[131]; thus in view of the reported opposite effects on POMC neuronal activity[129], the comparable effects on glucose metabolism may appear unexpected. Unfortunately, as a result of the lack of clear c-Fos activation, it remains unclear which of these neuronal populations could be the target for the metabolic effects of RFRP-3, although the absence of c-Fos activation could be related to the often reported inhibitory effect of RFRP-3[129,208,222,223].

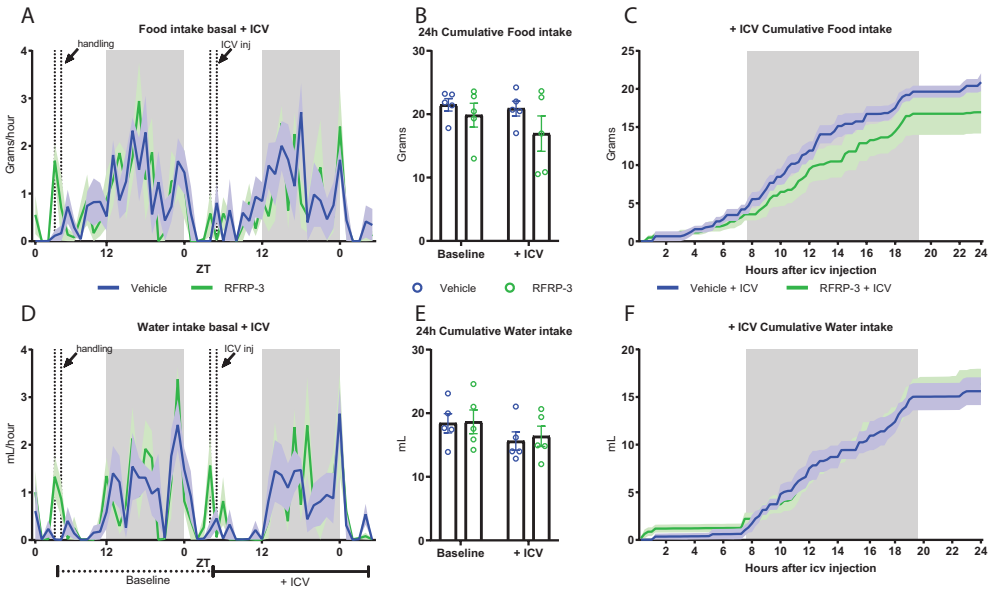
In conclusion, in male Wistar rats, central administration of Kp caused a clear activation of the HPG axis, reduced food intake, increased lipid oxidation and increased glucose production. The changes observed in ARC POMC-immunoreactivity and MnPO c-Fos expression,

support the idea that these metabolic changes are caused by a Kp-induced activation of POMC neurons. On the other hand, central administration of RFRP-3 caused a mild activation of the HPG axis, but did not result in any significant metabolic effects.

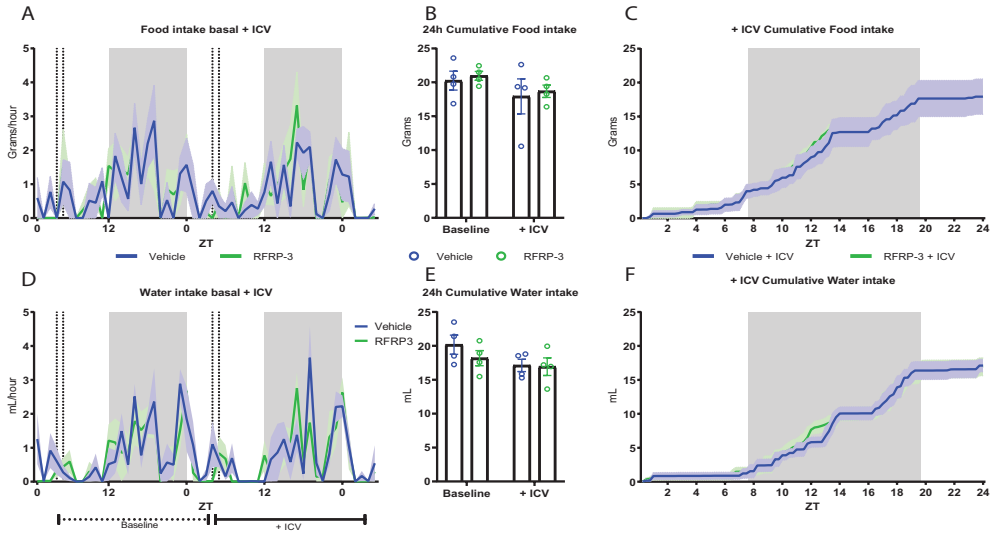
Acknowledgements

We thank Rainier Epping for husbandry of the experimental animals. This study was funded by the Agence National de la Recherche (ANR-13-BSV1-001) and the NeuroTime Erasmus+ program of the European Commission.

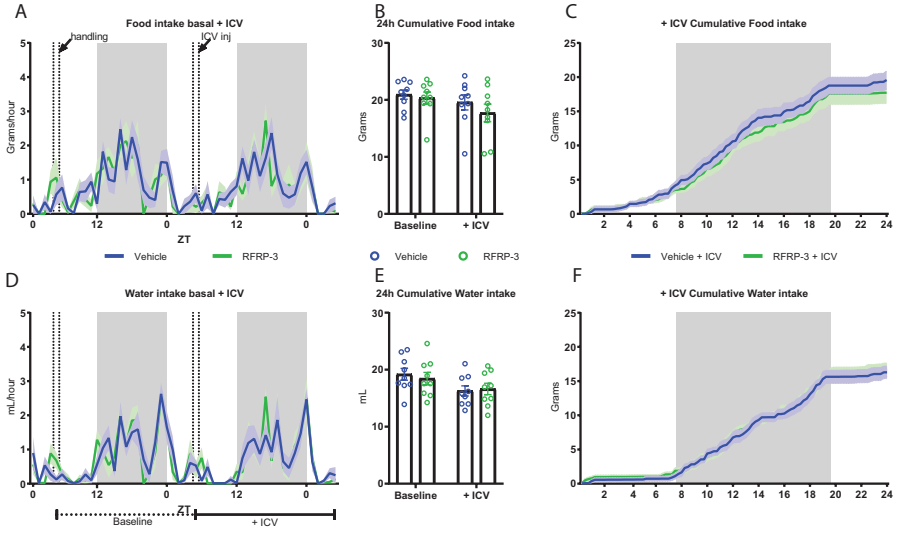
Supporting information



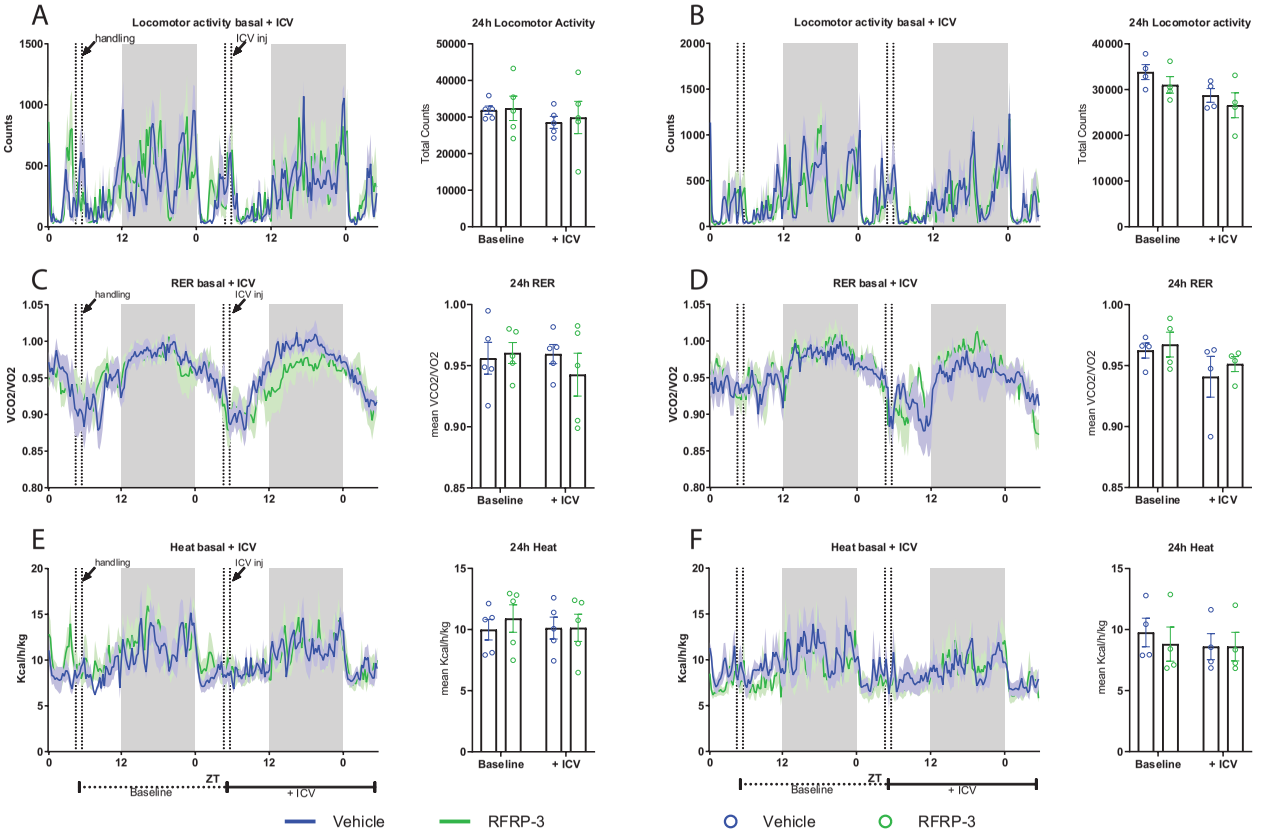
Suppl Fig 1. Effect of intracerebroventricular injection of RFRP-3 (50 pmol) or vehicle (NaCl 0.9%) on food and water intake in male Wistar rats. Hourly food (A) and water (D) intakes are plotted for the baseline and experimental (+ICV) days, with data presented as the mean (solid line) ± SEM (shaded area) of n=5 animals. Total 24h food (B) and water (E) intake were calculated for the baseline and experimental day. Cumulative food (C) and water (F) intake during the experimental day with a 15-minute resolution over 24h after Kp or vehicle injection. ZT0 = lights ON, ZT12 = lights OFF; gray background indicates the dark phase. Handling or ICV injection occurred between the two dashed vertical lines.



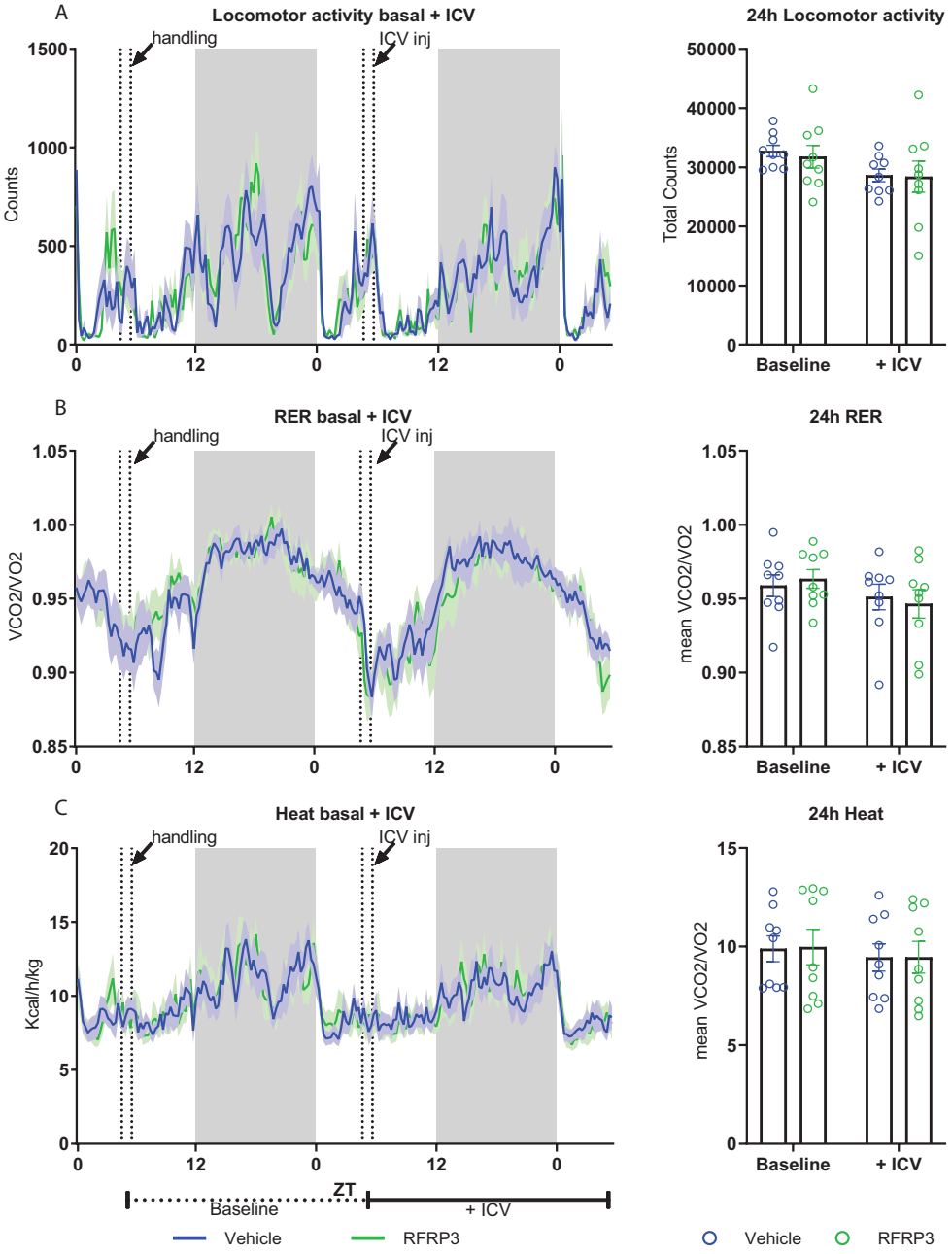
Suppl Fig 2. Effect of intracerebroventricular injection of RFRP-3 (250 pmol) or vehicle (NaCl 0.9%) on food and water intake in male Wistar rats. Hourly food (A) and water (D) intakes are plotted for the baseline and experimental (+ICV) days, with data presented as the mean (solid line) ± SEM (shaded area) of n=4 animals. Total 24h food (B) and water (E) intake were calculated for the baseline and experimental day. Cumulative food (C) and water (F) intake during the experimental day with a 15-minute resolution over 24h after Kp or vehicle injection. ZT0 = lights ON, ZT12 = lights OFF; gray background indicates the dark phase; Handling or ICV injection occurred between the two dashed vertical lines.



Suppl Fig 3. Effect of ICV injection of RFRP-3 (50 pmol and 250 pmol combined) or vehicle (NaCl 0.9%) on food and water intake in male Wistar rats. Hourly food (A) and water (D) intakes are plotted for the baseline and experimental (+ICV) days, with data presented as the mean (solid line) ± SEM (shaded area) of n=9 animals. Total 24h food (B) and water (E) intake were calculated for the baseline and experimental day as indicated by the horizontal lines below figure D. Cumulative food (C) and water (F) intake during the experimental day with a 15-minute resolution during 24h and starting immediately after the Kp or vehicle injection. ZT0 = lights ON, ZT12 = lights OFF. Gray background indicates the dark period. Handling or ICV injection occurred between the two dashed vertical lines.



Suppl Fig 4. Effects of RFRP-3 (50 or 250 pmol) or vehicle (NaCl 0.9%) on locomotor activity, respiratory exchange ratio (RER) and energy expenditure in male Wistar rats. Locomotor activity (A-B), RER (C-D) and energy expenditure or heat (E-F) are plotted for the baseline and experimental (+ICV) day, with data presented as the mean solid (line) ± SEM (shaded area) of n=5 animals for 50 pmol (left) and n=4 animals for 250pmol (right). Total locomotor activity, mean RER and energy expenditure were calculated over 24h for the baseline and experimental day. ZT0 = lights ON, ZT12 = lights OFF; gray shaded area indicates dark phase. Handling or ICV injection occurred between the two dashed vertical lines.



Suppl Fig 5. Effects of ICV RFRP-3 (50 pmol and 250 pmol combined) or vehicle (NaCl 0.9%) on locomotor activity, respiratory exchange ratio (RER) and energy expenditure in male Wistar rats. Locomotor activity (A), RER (B) and energy expenditure and heat (C) are plotted for the baseline and experimental (+ICV) day, with data presented as the mean (solid line) ± SEM (shaded area) of n=9 animals. Total locomotor activity, mean RER and energy expenditure were calculated over the 24h for the baseline and experimental day, as indicated by the horizontal lines below figure C. ZT0 = lights ON, ZT12 = lights OFF Gray shaded area indicates dark phase. Handling or ICV injection occurred between the two dashed vertical lines.

Table S1. Effects of ICV RFRP-3 injection (50 pmol) on metabolic outcomes

Comparison	Parameter	Treatment	Time	Interaction
Baseline vs Vehicle ICV	Cumulative food intake	$\mathbf{F_{1,4} = 1.509}$ **P=0.287**	$\mathbf{F_{95,380} = 318.6}$ **P<0.0001**	$\mathbf{F_{95,380} = 1.342}$ **P=0.029**
	Cumulative water intake	$\mathbf{F_{1,4} = 47.06}$ **P=0.002**	$\mathbf{F_{95,380} = 162.0}$ **P<0.0001**	$\mathbf{F_{95,380} = 2.262}$ **P<0.0001**
	Locomotor activity	$F_{1,4} = 1.831$ P=0.248	$\mathbf{F_{94,376} = 3.882}$ **P<0.0001**	$F_{94,376} = 1.248$ P=0.078
	RER	$F_{1,4} = 0.3141$ P=0.605	$\mathbf{F_{94,376} = 11.56}$ **P<0.0001**	$F_{94,376} = 1.037$ P=0.398
	Heat	$F_{1,4} = 0.1408$ P=0.727	$\mathbf{F_{94,376} = 3.893}$ **P<0.0001**	$F_{94,376} = 1.257$ P=0.071
Baseline vs RFRP-3 50 pmol ICV	Cumulative food intake	$F_{1,4} = 1.274$ P=0.322	$\mathbf{F_{95,380} = 99.65}$ **P<0.0001**	$\mathbf{F_{95,380} = 1.705}$ **P=0.0002**
	Cumulative water intake	$F_{1,4} = 0.04041$ P=0.851	$\mathbf{F_{95,380} = 93.83}$ **P<0.0001**	$\mathbf{F_{95,380} = 2.338}$ **P<0.0001**
	Locomotor activity	$F_{1,4} = 0.1634$ P=0.707	$\mathbf{F_{94,376} = 2.264}$ **P<0.0001**	$\mathbf{F_{94,376} = 1.474}$ **P=0.006**
	RER	$F_{1,4} = 1.436$ P=0.297	$\mathbf{F_{94,376} = 6.087}$ **P<0.0001**	$F_{94,376} = 0.5616$ P=0.999
	Heat	$F_{1,4} = 1.219$ P=0.332	$\mathbf{F_{94,376} = 3.131}$ **P<0.0001**	$F_{94,376} = 0.9944$ P=0.501
Vehicle ICV vs RFRP-3 50 pmol ICV	Cumulative food intake	$F_{1,4} = 1.810$ P=0.250	$\mathbf{F_{95,380} = 137.9}$ **P<0.0001**	$\mathbf{F_{95,380} = 1.781}$ **P<0.0001**
	Cumulative water intake	$F_{1,4} = 0.06417$ P=0.813	$\mathbf{F_{95,380} = 176.8}$ **P<0.0001**	$F_{95,380} = 0.4051$ P>0.999
	Locomotor activity	$F_{1,4} = 0.1245$ P=0.742	$\mathbf{F_{94,376} = 3.323}$ **P<0.0001**	$F_{94,376} = 1.093$ P=0.280
	RER	$F_{1,4} = 1.049$ P=0.364	$\mathbf{F_{94,376} = 9.408}$ **P<0.0001**	$\mathbf{F_{94,376} = 1.360}$ **P=0.024**
	Heat	$F_{1,4} = 0.001084$ P=0.975	$\mathbf{F_{94,376} = 4.278}$ **P<0.0001**	$F_{94,376} = 0.9422$ P=0.629

Table S2. Effects of ICV RFRP3 injection (250 pmol) on metabolic outcomes

Comparison	Parameter	Treatment	Time	Interaction
Baseline vs Vehicle ICV	Cumulative food intake	$F_{1,3}$ = 0.8773 *P*=0.418	**$F_{95,285}$ = 73.28 *P*<0.0001**	**$F_{95,285}$ = 2.251 *P*<0.0001**
	Cumulative water intake	$F_{1,3}$ = 4.311 *P*=0.130	**$F_{95,285}$ = 135.2 *P*<0.0001**	**$F_{95,285}$ = 2.009 *P*<0.0001**
	Locomotor activity	**$F_{1,3}$ = 16.25 P=0.027**	**$F_{95,285}$ = 3.786 *P*<0.0001**	**$F_{95,285}$ = 1.431 *P*=0.014**
	RER	$F_{1,3}$ = 3.866 *P*=0.144	**$F_{94,282}$ = 5.222 *P*<0.0001**	$F_{94,282}$ = 0.6330 *P*=0.995
	Heat	**$F_{1,3}$ = 17.02 *P*=0.026**	**$F_{94,282}$ = 3.003 *P*<0.0001**	$F_{94,282}$ = 0.7960 *P*=0.903
Baseline vs RFRP-3 250 pmol ICV	Cumulative food intake	$F_{1,3}$ = 2.953 *P*=0.184	**$F_{95,285}$ = 153.9 *P*<0.0001**	F95,285 = 1.303 *P*=0.051
	Cumulative water intake	$F_{1,3}$ = 0.9880 *P*=0.394	**$F_{95,285}$ = 242.7 *P*<0.0001**	F95,285 = 0.4465 *P*=0.792
	Locomotor activity	$F_{1,3}$ = 4.331 *P*=0.129	**$F_{94,282}$ = 4.507 *P*<0.0001**	$F_{94,282}$ = 1.431 *P*=0.013
	RER	$F_{1,3}$ = 3.944 *P*=0.141	**$F_{94,282}$= 7.620 *P*<0.0001**	$F_{94,282}$ = 0.9631 *P*=0.577
	Heat	$F_{1,3}$ = 0.8053 *P*=0.436	**$F_{94,282}$ = 4.301 *P*<0.0001**	$F_{94,282}$ = 1.076 *P*=0.320
Vehicle ICV vs RFRP-3 250 pmol ICV	Cumulative food intake	$F_{1,3}$ = 0.2429 *P*=0.656	**$F_{95,285}$ = 69.67 *P*<0.0001**	F95,285 = 0.7546 *P*=0.946
	Cumulative water intake	$F_{1,3}$ = 0.1799 *P*=0.700	**$F_{95,285}$ = 113.1 *P*<0.0001**	F95,285 = 1.232 *P*=0.098
	Locomotor activity	$F_{1,3}$ = 0.9357 *P*=0.405	**$F_{95,285}$ = 5.232 *P*<0.0001**	**$F_{95,285}$ = 0.7562 *P*=0.944**
	RER	$F_{1,3}$ = 0.3088 *P*=0.617	**$F_{94,282}$ = 4.248 *P*<0.0001**	**$F_{94,282}$ = 1.636 *P*=0.001**
	Heat	$F_{1,3}$ = 0.0003453 *P*=0.986	**$F_{94,282}$ = 4.726 *P*<0.0001**	F94,282 = 0.8825 *P*=0.756

Table S3. Effects of ICV RFRP3 injection (50and 250 pmol combined) on metabolic outcomes

Comparison	Parameter	Treatment	Time	Interaction
Baseline vs Vehicle ICV	Cumulative food intake	$F_{1,8}$ = 2.187 P=0.178	**$F_{95,760}$ = 300.8 P<0.0001**	**$F_{95,760}$ = 1.681 P=0.0001**
	Cumulative water intake	**$F_{1,8}$ = 26.84 P=0.0008**	**$F_{95,760}$ = 294.6 P<0.0001**	**$F_{95,760}$ = 3.745 P<0.0001**
	Locomotor activity	**$F_{1,8}$ = 8.525 P=0.019**	**$F_{94,752}$ = 5.711 P<0.0001**	$F_{94,752}$ = 1.005 P=0.471
	RER	$F_{1,8}$ = 1.187 P=0.308	**$F_{94,752}$ = 13.53 P<0.0001**	$F_{94,752}$ = 0.9255 P=0.675
	Heat	$F_{1,8}$ = 1.962 P=0.199	**$F_{94,752}$ = 5.702 P<0.0001**	$F_{94,752}$ = 1.035 P=0.396
Baseline vs RFRP-3 ICV	Cumulative food intake	$F_{1,8}$ = 2.949 P=0.124	**$F_{95,760}$ = 248.7 P<0.0001**	**$F_{95,760}$ = 2.499 P<0.0001**
	Cumulative water intake	$F_{1,8}$ = 0.8974 P=0.371	**$F_{95,760}$ = 262.1 P<0.0001**	**$F_{95,760}$ = 1.346 P=0.021**
	Locomotor activity	$F_{1,8}$ = 1.011 P=0.344	**$F_{94,752}$ = 5.306 P<0.0001**	**$F_{94,752}$= 1.623 P=0.0004**
	RER	$F_{1,8}$ = 4.012 P=0.080	**$F_{94,752}$ = 13.66 P<0.0001**	$F_{94,752}$ = 0.5677 P=0.999
	Heat	$F_{1,8}$ = 1.759 P=0.221	**$F_{94,752}$ = 6.548 P<0.0001**	$F_{94,752}$ = 1.178 P=0.1310
Vehicle ICV vs RFRP-3 ICV	Cumulative food intake	$F_{1,8}$ = 0.6190 P=0.454	**$F_{95,760}$ = 217.7 P<0.0001**	$F_{95,760}$ = 0.8117 P=0.899
	Cumulative water intake	$F_{1,8}$ = 0.1142 P=0.744	**$F_{95,760}$ = 294.5 P<0.0001**	$F_{95,760}$ = 0.2855 P>0.999
	Locomotor activity	$F_{1,8}$ = 0.004303 P=0.949	**$F_{94,752}$ = 6.274 P<0.0001**	$F_{94,752}$ = 0.5768 P=0.999
	RER	$F_{1,8}$ = 0.1417 P=0.716	**$F_{94,752}$ = 12.41 P<0.0001**	$F_{94,752}$ = 0.7319 P=0.971
	Heat	$F_{1,8}$ = 0.001425 P=0.971	**$F_{94,752}$ = 7.271 P<0.0001**	$F_{94,752}$ = 0.8236 P=0.881

Chapter 5

DAILY RHYTHMS IN REPRODUCTIVE AND METABOLIC NEUROCIRCUITS AND THE TIME-DEPENDENCE OF THEIR RESPONSES TO COLD

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In preparation

Abstract

Mammals confronted with daily and seasonal changes in environmental temperature trigger appropriate changes in physiology to maintain their internal temperature at a constant level. This homeostatic regulation in body temperature requires a tight regulation of hypothalamic signals controlling energy production and expenditure. The thyrotropin releasing hormone (TRH) producing neurons of the paraventricular nucleus (PVN) have been recognized as important regulators linking food intake and energy expenditure, but other hypothalamic systems may also be involved.

In the current study, we first evaluated the existence of a daily rhythm in the expression of a number of hypothalamic neuropeptides involved in reproduction and energy metabolism, i.e., neuropeptide Y (*Npy*), pro-opiomelanocortin (*Pomc*) and kisspeptin (*Kiss1*) in the arcuate nucleus (ARC), arginine-phenylalanine related peptide (*Rfrp*) in the dorsomedial hypothalamus (DMH) and *Trh* in PVN and medial pre-optic area (MPA). Next, we investigated how these neuronal populations respond to a 3h exposure to a cold environment (4 °C) at different times of the light/dark cycle.

The expression of these genes was analyzed by non-radioactive *in situ* hybridization. Among the investigated genes, only *Pomc* and *Npy* showed a significant daily rhythm in gene expression, with an acrophase in the light period. Three hours of cold exposition altered the expression level of most genes, although with specific differences. *Trh* expression in the PVN, but not in the MPA, was increased with the highest induction at ZT10. *Npy* expression was increased at dusk (ZT10 to ZT18), but *Pomc* expression was not changed. Both *Rfrp* and *Kiss1* expression showed a decrease, but at different time points, respectively ZT6 and ZT10.

Our data demonstrate that in response to low temperatures, the hypothalamus triggers various signals related to energy expenditure (TRH) and food intake (NPY) and to a lesser extend those related to reproduction (RFRP and kisspeptin), but this response differs according to the time of the day. These data provide a nice example of how the hypothalamus integrates different environmental and internal signals and sets priorities in energy challenging situations.

Introduction

Daily and seasonal fluctuations in temperature are among the many different environmental challenges that organisms will encounter and have to cope with. Virtually in every biotope, living organisms face variations in temperature, but the ones living far from the equator are exposed to more extreme yearly fluctuations of temperature. In mammals, thermoregulation is a complex adaptive process driven by the hypothalamus[224], particularly by a set of temperature sensitive GABAergic and glutamatergic neurons in the preoptic area. These neurons project to pre-autonomic neurons in neighboring hypothalamic nuclei that control different physiological processes necessary for the maintenance of normothermia. The hypothalamus-pituitary-thyroid (HPT) axis is well known for its involvement in linking the metabolic rate of different organs to the changes in environmental temperature[225]. The activity of the HPT axis is regulated by a set of thyrotropin releasing hormone (TRH) expressing neurons in the paraventricular nucleus of the hypothalamus (PVN) controlling the release of thyroid stimulating hormone (TSH) from the anterior pituitary. Additionally, also non-hypophysiotropic TRH neurons have been pointed out as regulators in the cold response[226].

The physiological response to cold is an energy demanding process as, for instance, long exposure to cold induces hyperphagia[227,228]. Thus, neuronal circuits in the arcuate nucleus (ARC) that control the energy balance are likely to be involved. Neuropeptide Y (NPY) neuronal activity has been reported to either increase, decrease or be without changes after cold exposure[228–231]. However, such an energy-demanding response may also affect other systems.

Evidence is accumulating that seasonal changes observed in the kisspeptin and RFRP systems are not only involved in the control of reproductive behavior[80,95], but may also be implicated in the control of (seasonal) changes in energy metabolism[221,232]. For instance, RFRP-3, a neuropeptide initially described to inhibit gonadotropin release[91], has repeatedly been found to have orexigenic properties as well[116,123,124,194,197]. Because seasonal rhythmicity not only involves changes in photoperiod and food availability, but often also environmental temperature, we were interested to assess how changes in environmental temperature would affect these neuropeptide systems. Indeed, recently it was shown in mice that RFRP expression was down-regulated after cold exposure, regardless of adiposity, leptin levels, food intake or functional brown fat[233]. In the same vein, kisspeptin has been linked to the circadian control of body temperature, locomotor activity and food intake[120]. In the current study we used the brains of cold exposed rats, collected at 6 different time points along the 24h light/dark cycle[145], to investigate the time-dependence of the cold response in several hypothalamic neuropeptide systems involved in reproduction and metabolism.

Materials and methods.

Brain samples.

Frozen brains (n=72) were obtained from the study of Machado *et al*.[145]. Briefly, animals had been exposed during 3h to a cold temperature (4°C) at 6 different time points along the 24h light/dark cycle and then sacrificed together with animals kept at room temperature (R.T., 20 ± 2 °C). Brains were dissected and frozen immediately on dry ice and stored at -80°C. Ten series of 20 µm thick sections were built when sectioning, starting from 0.6 mm anterior to Bregma up to -4.08 mm posterior to Bregma. Sections were sliced with a cryostat at -12°C with every second section collected and attached to Superfrost slides (Thermo Fisher Scientific, Waltham, MA, USA).

In situ hybridization.

Non-radioactive *in situ* hybridization using digoxygenin labeled riboprobes was performed. Plasmids containing the rat *neuropeptide Y* (*Npy*) probe (87–522 of Genbank NM_012614.2), the rat *Pomc* probe (157–731 of Genbank NM_139326), the hamster *Rfrp* (87-529 Genbank JF727837, 86% homology), the rat *Kiss1* (1-393 Genbank NM181692.1) and the rat *TRH* (probe kindly provided by Dr. Joseph Bravo and Dr Goodman's lab[234,235]) were used to transcribe the probes from PCR produced templates in the presence of digoxigenin-labeled nucleotides (Roche, Basel Switzerland).

To avoid background differences in the staining, for each gene the whole set of slides was always stained at once. The sections were post-fixed with 4% formaldehyde and acetylated for 10 min in 100 mM triethanolamine and 0.25% acetic anhydride, delipidated with 0.1% Triton X-100. The tissue was then hybridized with 100 ng/mL of labelled antisense probe in 50% formamide, 5X saline sodium citrate (SSC), 5X Denhardt's solution, 250ug/mL of baker's

yeast tRNA, and 200ug/mL herring sperm DNA for 14h at 55°C. Sequential stringency washes were performed at 55°C with 5x SSC (5 min), 2x SSC (1 min) and 0.2x SSC 50% formamide (30 min) and finally 0.2x SSC (5 min) at room temperature. 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 and stopped before the staining intensity reached saturation (*Trh:* 1h, *Pomc:* 1.5h, *Npy* and *Rfrp:* 3h, and *Kiss1:* 26h). Hybridization with corresponding sense probes gave no signal, indicating specificity of the antisense probes.

Image acquisition

Micrographs were acquired with a CCD camera (Sony Model 77CE) attached to a microscope (Zeiss Axios-kop with Plan-NEOFLUAR Zeiss objectives, Carl Zeiss GmbH, Germany), using a 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, optic density (O.D.) of the *in situ* hybridization signal per region was analyzed using the ImageProPlus (version 5.1) image analysis system (MediaCybernetics, Silver Spring, MD), with a set threshold of at least 2x the background. *Trh* expression in the PVN was measured in one anterior, one medial and one posterior section (Bregma levels -1.08, -1.44 and -1.8 mm, respectively). *Trh* expression in the medial preoptic area (MPA) was calculated as the average of the signal from slides at 0.48 and 0.12 mm posterior to Bregma, and *Kiss1* in the anteroventral periventricular (AVPV) area from 0.12 anterior to -0. 12 mm posterior to Bregma. For *Npy, Pomc* and *Kiss1* expression in the ARC, as well as *Rfrp* expression in the dorsomedial hypothalamus (DMH), all positive signal obtained from slides -1.56 to -4.08 mm posterior from Bregma was averaged.

Statistical analysis

All data are presented as mean ± SEM. Statistical significance was set at $P<0.05$. The results of the *in situ* hybridization were firstly analyzed with two-way ANOVA and Bonferroni honestly significant difference (HSD) test using Prism, version 8 (GraphPad Software Inc., San Diego, CA, USA). In addition, a possible differential time of the day expression was analyzed for R.T. and cold exposed groups independently by one-way ANOVA.

Then, to analyze the presence of a daily rhythm in the expression of the different genes, a cosinor regression analysis was performed with Sigma Plot 14 Software (Systat Inc. GmBH Germany). Optic density data were fitted to the following equation: $y=a+b\cos(2\pi (x-c)/24)$

In this formula y is the mean O.D. value for each animal, a the mesor (mean level of the samples used to determine the oscillation), b the amplitude of the calculated oscillation, and c the acrophase (peak time of the fitted curve). Differences between *a, b* and *c* for animals exposed to room temperature or cold were analyzed by Student's *t*-test.

Because often the effect of cold exposure on gene expression is only compared at one time point, we also compared the effects of 3h cold exposure with R.T. at the same time point, separately for each time point, by Student's *t*-test.

Results

Hypothalamic Trh gene expression

Trh gene expression showed an overall elevation after 3h of cold exposure in the PVN (Temperature effect, *P*=0.0129) (Figure 1B and Table 1), with the largest increase observed at ZT10. In the MPA only a trend towards a significant decrease was observed at ZT6 (Figure 1A and Table 4). Cosinor analysis revealed no significant daily rhythmicity in the PVN, but in agreement with the significant effect of Temperature the mesor was statistically increased in the PVN in the cold group (Table 2). In the MPA, cosinor analysis revealed no significant daily rhythm in *Trh* gene expression during cold exposure, despite the trend for a significant effect of ZT (*P*=0.0830) (Table 3).

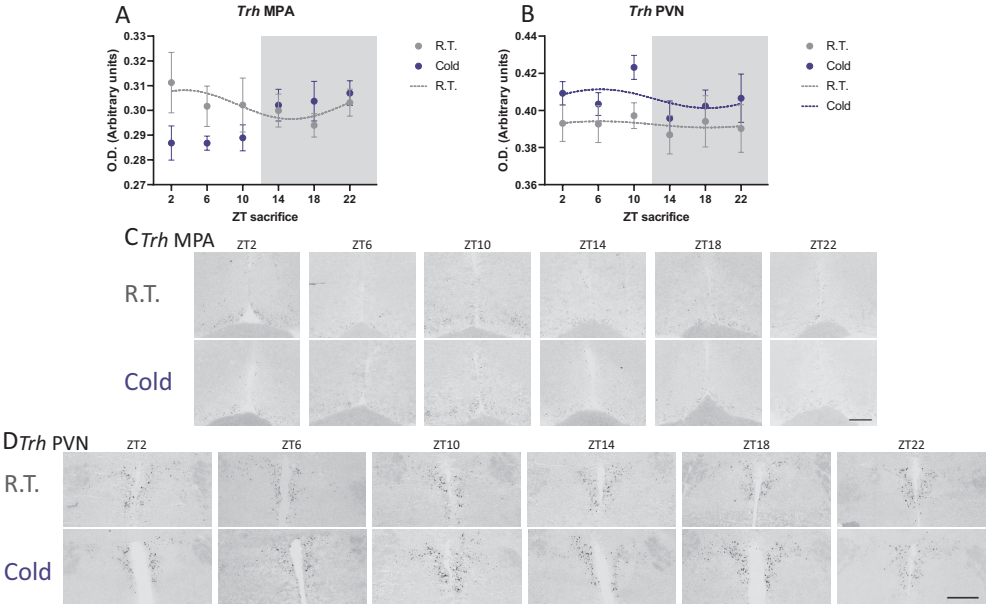

Figure 1. Time of the day effect of cold exposure on *Trh* expression in the median preoptic area (MPA) and the paraventricular nucleus (PVN) of the hypothalamus in male Wistar rat. Rats were exposed to 4°C (Cold, blue circles) for 3 hours at different times of the day, and sacrificed right after. *Trh* expression is compared to rats maintained at room temperature (R.T., gray circles). A-B) Graphs show the optic density (O.D.) of the gene signal (in arbitrary units) per region for each time point (ZT). Data are presented as mean ± SEM of n= 4 to 7 animals per experimental group. Solid lines represent a significant fit of the cosinor regression, dashed lines represent non-significant fits and no line means the cosinor analysis did not produce a fitted curve (R.T., gray.; cold, blue). ZT0 = lights ON, ZT12 = lights OFF; gray background indicates the dark phase. Pictures show representative images of the MPA (C) or PVN (D) neurons expressing Trh mRNA (scale bar=500 µm). Micrographs from MPA and PVN correspond to 0.12 and -1.44 mm from bregma as reference, respectively.

Npy and Pomc gene expression in the arcuate nucleus

Npy gene expression was increased in the cold group, as shown by the significant effect of Temperature (*P*=0.0041), with a more pronounced effect from ZT10 – ZT18 (Figure 2A and Table 4). *Npy* expression also significantly changed along the light/dark cycle as shown by the significant effect of Time (*P*=0.0475) (Figure 2A and Table 1,). The cosinor analysis revealed a significant daily rhythmicity in *Npy* expression in the room temperature (R.T.) (*P*=0.0142),

but not in the cold-exposed group (*P*=0.1874) (Table 2). The increased *Npy* expression in the cold group was confirmed by the higher mesor. The two-way ANOVA revealed no changes in *Pomc* expression due to time of day or cold exposure, although a near significant decrease was observed at ZT10. The one-way ANOVA indicated a nearly significant effect of Time in the R.T. group (*P*=0.0598; Table 3). In line with this, the cosinor analysis showed a statistically significant daily rhythmicity in the R.T. group with an acrophase at ZT7.1

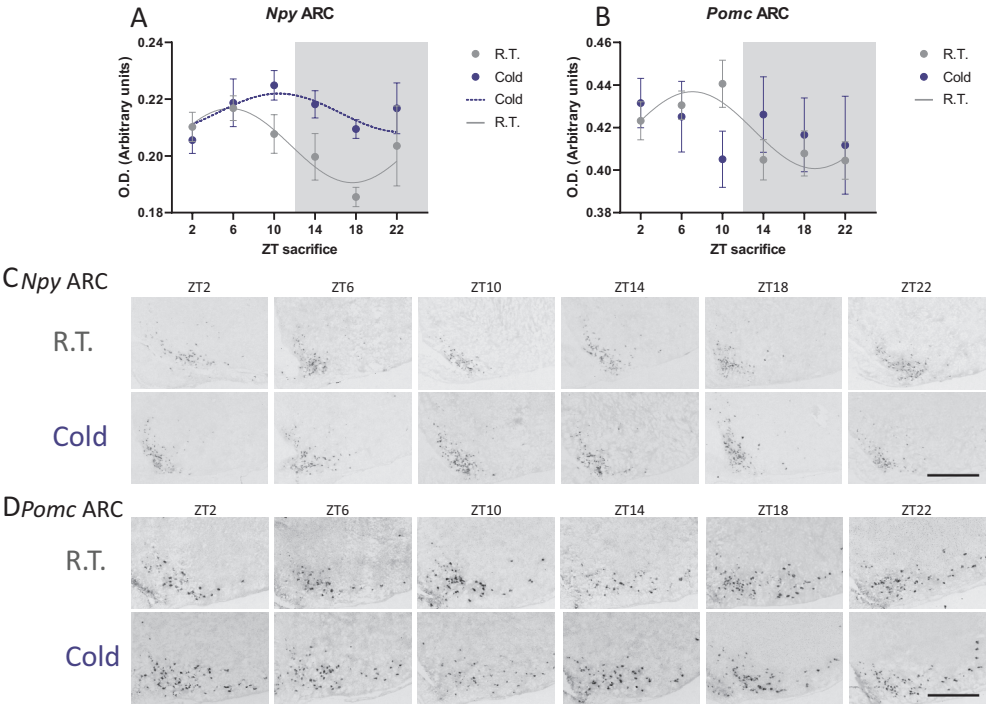

Figure 2. Time of the day effect of cold exposure on *Npy* and *Pomc* expression in the arcuate nucleus of the hypothalamus (ARC) in male Wistar rat. Rats were exposed to 4°C (Cold, blue circles) for 3 hours at different times of the day, and sacrificed right after. *Npy* and *Pomc* expression is compared to rats maintained at room temperature (R.T., gray circles). A-B) Graphs show the optic density (O.D.) of the gene signal (in arbitrary units) per region for each time point (ZT). Data are presented as mean ± SEM of n= 4 to 7 animals per experimental group. Solid lines represent a significant fit of the cosinor regression, dashed lines represent non-significant fits and no line means the cosinor analysis did not produce a fitted curve (R.T., gray.; cold, blue). ZT0 = lights ON, ZT12 = lights OFF; gray background indicates the dark phase. Pictures show representative images of the Npy (C) or Pomc (D) positive signal in the arcuate nucleus (scale bar= 500 µm). Micrographs correspond to -2.76 mm from bregma as reference.

Kiss1 and RFRP gene expression

Kiss1 and *Rfrp* gene expression did not show any significant effects of Time or Temperature according the 2-way ANOVA (Table 1 and Figure 3-4), only RFRP expression in the DMH showed a trend (*P*=0.0963) towards reduced gene expression due to the cold exposure. When comparing the single time points, *Kiss1* expression showed a significant decrease at ZT10, whereas *Rfrp* gene showed a significant decrease at ZT6 (Table 4). The cosinor analysis indicated no statistically significant daily rhythms in the expression of the two genes (Table 2).

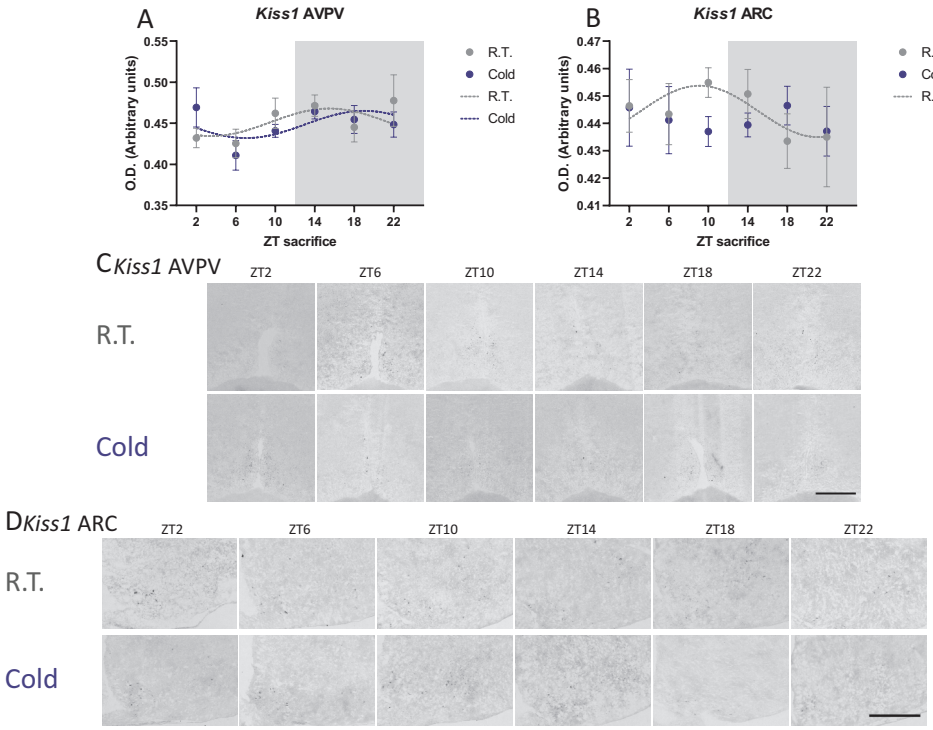

Figure 3. Time of the day effect of cold exposure on *Kiss1* expression in the anteroventral periventricular area (AVPV) and the arcuate nucleus (ARC) of the hypothalamus in male Wistar rat. Rats were exposed to 4°C (Cold, blue circles) for 3 hours at different times of the day and sacrificed right after. *Kiss1* expression is compared to rats maintained at room temperature (R.T., gray circles). A-B) Graphs show the optic density (O.D.) of the gene signal (in arbitrary units) per region for each time point (ZT). Data are presented as mean ± SEM of n= 4 to 7 animals per experimental group. Solid lines represent a significant fit of the cosinor regression, dashed lines represent non-significant fits and no line means the cosinor analysis did not produce a fitted curve (R.T., gray.; cold, blue). ZT0 = lights ON, ZT12 = lights OFF; gray background indicates the dark phase. Pictures show representative images of the AVPV (C) or ARC (D) neurons expressing Kiss1 mRNA (scale bar=500 µm). Micrographs from AVPV and ARC correspond to 0.12 and -1.44 mm from bregma as reference, respectively.

Table 1. Two-way ANOVA of 3h cold exposure in hypothalamic gene expression

Gene/Nucleus	Temperature	Time (ZT)	Interaction
Trh MPA	$F_{1,57}$ = 1.84 *P*=0.1803	$F_{5,57}$ = 0.4332 *P*=0.8235	$F_{5,57}$ = 1.559 *P*=0.1863
Trh PVN	**$F_{1,58}$ = 6.583** ***P*=0.0129**	$F_{5,58}$ = 0.7976 *P*=0.5559	$F_{5,58}$ = 0.2375 *P*=0.9444
Npy ARC	**$F_{1,58}$ = 8.916** ***P*=0.0041**	**$F_{5,58}$ = 2.404** ***P*=0.0475**	$F_{5,58}$ = 1.414 *P*=0.2329
Pomc ARC	$F_{1,57}$ = 0.00927 *P*=0.9236	$F_{5,57}$ = 0.6661 *P*=0.6507	$F_{5,57}$ = 1.083 *P*=0.3798
Kiss1 AVPV	$F_{1,56}$ = 0.1759 *P*=0.6765	$F_{5,56}$ = 1.946 *P*=0.1011	$F_{5,56}$ = 1.018 *P*=0.4158
Kiss1 ARC	$F_{1,56}$ = 0.2383 P=0.6274	$F_{5,56}$ = 0.2891 *P*=0.9171	$F_{5,56}$ = 0.5812 *P*=0.7142
Rfrp DMH	$F_{1,59}$ = 2.856 *P*=0.0963	$F_{5,59}$ = 0.7582 *P*=0.5836	$F_{5,59}$ = 0.1759 *P*=0.6765

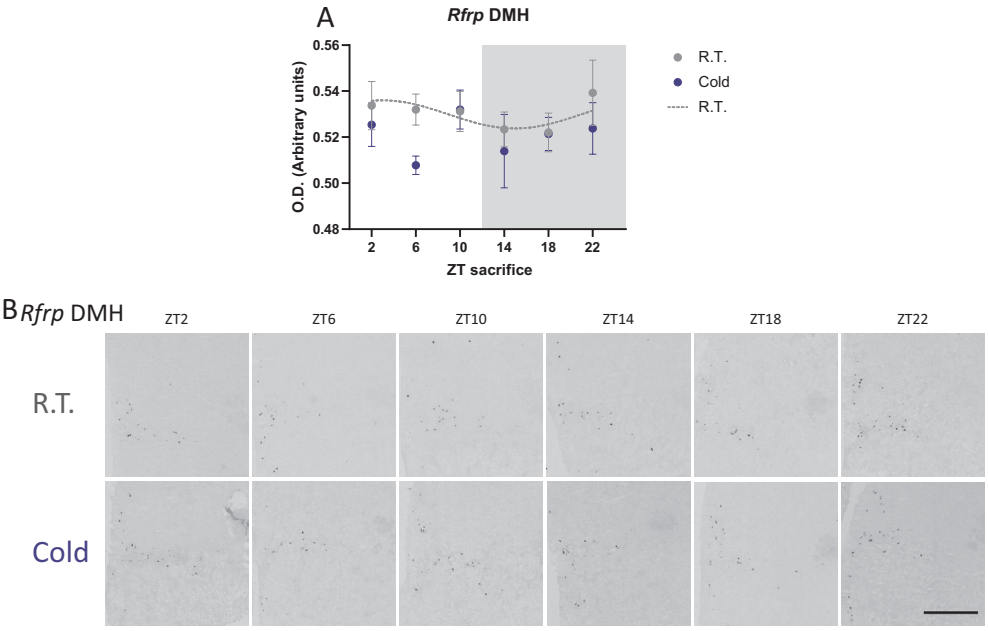

Figure 4. Time of the day effect of cold exposure on *Rfrp* expression in the dorsomedial hypothalamus (DMH) in male Wistar rat. Rats were exposed to 4°C (Cold, blue circles) 3 hours at different time points of the day, and sacrificed right after. *Rfrp* expression is compared to rats maintained at room temperature (R.T., gray circles). A) Graph shows the optic density (O.D.) of the gene signal (in arbitrary units) for each time point (ZT). Data are presented as mean ± SEM of n= 4 to 7 animals per experimental group. Solid lines represent a significant fit of the cosinor regression, dashed lines represent non-significant fits and no line means the cosinor analysis did not produce a fitted curve (R.T., gray.; cold, blue). ZT0 = lights ON, ZT12 = lights OFF; gray background indicates the dark phase. B) Pictures show representative images of the neurons expressing Rfrp mRNA (scale bar=500 µm). Micrographs from the DMH correspond to -3.12 mm with Bregma as reference.

Discussion

The present study clearly shows that cold exposure alters the expression of hypothalamic genes related to energy metabolism, and that this effect depends on the time of day. Our data confirmed that, in rats, cold exposure causes a significant increase of *Trh* expression in the PVN[236,237]. Furthermore, similar to the previous findings[238] we found a significant daily rhythmicity in ARC NPY and POMC mRNA. For both genes daily rhythmicity was blunted by the 3h cold exposure events. Cold increased *Npy* expression mainly at dusk and during the first half of the dark period but had no effect during the first half of the light period. *Pomc* expression on the other hand was inhibited by cold at the end of the light period. A similar time-dependent pattern was observed for *Trh* expression in the MPA, with a decrease during the light period and a stimulatory or no effect during the dark period. On the other hand, the cold-induced increase in *Trh* expression in the PVN was observed throughout the light/dark cycle. Finally, ARC *Kiss1* expression and DMH *Rfrp* expression were decreased by cold exposure during the light period, but not affected during the dark period. *Kiss1* expression in AVPV was not altered by cold exposure.

PVN *Trh* mRNA levels have been described to be transiently increased after 1h cold exposure and back to control levels after 2h in Wistar rats[237], whereas in Sprague Dawley rats they re-

Table 2. Cosinor analysis of 3h cold exposure on daily rhythms in hypothalamic gene expression

	R.T		**Cold**		*p*-value Student's *t*-test
Gene/Nucleus	Curve fit R^2		Curve fit R^2		R.T. vs cold
Trh MPA	0.0464 *P*=0.4790	a=0.3023 ± 0.0035 b=0.0058 ± 0.0047 c=3.4517 ± 3.5126	NR	- - -	- - -
Trh PVN	0.0025 *P*=0.9615	a=0.3925 ± 0.0042 b=0.0017 ± 0.0060 c=6.2562 ± 13.3375	0.0312 *P*=0.5922	a=0.4063 ± 0.0035 b=0.0050 ± 0.0049 C=6.2285 ± 3.8383	**0.0135** 0.6699 0.9984
Npy ARC	0.2335 ***P*=0.0142**	a=0.2036 ± 0.0029 b=0.0131 ± 0.0042 c=5.6581 ± 1.1649	0.0994 *P*=0.1874	a=0.2152 ± 0.0025 b=0.0068 ± 0.0036 c=10.4608 ± 1.8863	**0.0035** 0.2587 **0.0338**
Pomc ARC	0.2214 ***P*= 0.0161**	a=0.4188 ± 0.0040 b=0.0181 ± 0.0059 c=7.0637 ± 1.1629	NR	- - -	- - -
Kiss1 AVPV	0.0785 *P*=0.2704	a=0.4512 ± 0.0073 b=0.0167 ± 0.0101 c=15.4863 ± 2.4195	0.0810 *P*=0.2817	a=0.4485 ± 0.0071 b=0.0165 ± 0.0102 c=15.787 ± 2.2875	0.7924 0.9889 0.9286
Kiss1 ARC	0.0765 *P*=0.2800	a=0.4444 ± 0.0040 b=0.0094 ± 0.0058 c=9.1045 ± 2.2308	NR	- - -	- - -
Rfrp DMH	0.0426 *P*=0.4877	a=0.5299 ± 0.0037 b=0.0061 ± 0.0050 c=2.9662 ± 3.4015	NR	- - -	- - -

N.R. Non-rhythmic

Table 3. One-way ANOVA of 3h cold exposure or kept at room temperature in hypothalamic gene expression

Gene/Nucleus	**R.T.**	**Cold**
Trh MPA	$F_{5,28}$ = 0.4419 *P*=0.8154	$F_{5,29}$ = 2.180 *P*=0.0839
Trh PVN	$F_{5,28}$ = 0.1090 *P*=0.9894	$F_{5,30}$ = 1.088 *P*=0.3873
Npy ARC	$F_{5,29}$ = 2.093 *P*=0.0949	$F_{5,29}$ = 1.329 *P*=0.2799
Pomc ARC	$F_{5,30}$ = 2.408 *P*=0.0598	$F_{5,27}$ = 0.3162 *P*=0.8989
Kiss1 AVPV	$F_{5,29}$ = 1.330 *P*=0.2795	$F_{5,29}$ = 1.330 *P*=0.2795
Kiss1 ARC	$F_{5,29}$ = 0.7184 *P*=0.6149	$F_{5,27}$ = 0.1702 *P*=0.9714
Rfrp DMH	$F_{5,30}$ = 0.4408 *P*=0.8164	$F_{5,29}$ = 0.7156 *P*=0.6168

mained increased after 6h of cold exposure[236]. Similarly, in our experiment, animals exposed for 3h also showed an increased expression of PVN *Trh* mRNA. These data indicate that *Trh* mRNA levels may respond very quickly to a decrease in environmental temperature with this effect remaining as long as the low temperature persists. According to Cabral *et al.*[239]

Table 4. Student's *t*-test of 3h cold exposure vs room temperature in hypothalamic gene expression

Gene/Nucleus	ZT2	ZT6	ZT10	ZT14	ZT18	ZT22
Trh MPA	0.1253	0.0973	0.3055	0.8169	0.3399	0.6215
Trh PVN	0.1860	0.3628	**0.0247**	0.5448	0.6108	0.4087
Npy ARC	0.5236	0.8682	0.0927	0.0904	**0.0004**	0.4352
Pomc ARC	0.5764	0.7878	0.0663	0.2936	0.6910	0.7798
Kiss1 AVPV	0.1719	0.5804	0.3788	0.6703	0.7158	0.4011
Kiss1 ARC	0.9686	0.9001	**0.0467**	0.2832	0.3363	0.9153
Rfrp DMH	0.5728	**0.0107**	0.9527	0.5843	0.9527	0.4134

the cold-induced increase in TRH neurons activity is restricted to the PVN and the raphe pallidum in the brainstem and in line with it we found an effect of cold in the PVN, but not in the MPA.

It is known that cold exposure for several days results in hyperphagia in mice and rats[227,228]. Our previous analysis of the effect of cold on energy metabolism[145] showed that, no matter the time of day, cold exposure caused an increased energy expenditure, setting the animals in a negative energy balance. The ARC NPY/AgRP and POMC/CART neurons have an opposite effect on food intake, i.e., respectively orexigenic and anorexigenic. Thus, it is not so surprising that cold also has an opposite effect on ARC *Npy* (increase) and *Pomc* (decrease) expression, although the effects are observed at slightly different time points (late day and early night for NPY; late day for POMC). Interestingly, in our previous study[145] the time at which we observed the most pronounced cold-induced increase in food consumption nicely coincides with the time of increased *Npy* expression, i.e. ZT10, in the present work. A cold-induced increase in ARC *Npy* expression was also reported in mice[229] and Jerboas[240]. Surprisingly however, McCarthy *et al*.[230] and Park *et al.*[231] reported a decreased expression of ARC NPY after 2.5 or 1.5 hours of cold exposure, despite an increased NPY expression in many other hypothalamic areas, notably in the PVN. The PVN is an important target for the ARC NPY neurons to induce food intake, thus, these results were interpreted as reflecting an increased release of ARC-derived NPY in the PVN. Taken these results together, we hypothesize that upon cold exposure NPY synthesis and release is finely regulated with, on one hand, the release of ARC NPY resulting in reduced peptide levels and increased *Npy* mRNA synthesis in ARC neurons, and, on the other hand, increased PVN NPY content.

In addition to its orexigenic effect, NPY might also have thermoregulatory effects. Indeed, it has been found that central administration of NPY increases or decreases body temperature[241,242]. Low doses of NPY decrease temperature, while higher doses increase it[242]. The opposite regulation of ARC *Npy* and *Pomc* expression might suggest opposite roles in thermoregulation for these peptides as well. α-MSH (a product of POMC peptide cleavage) has been reported to have thermogenic effects[218,244]. Interestingly, to our knowledge, there is no evidence demonstrating that cold temperature regulates POMC neuronal activity or *Pomc* expression; however, impairing proper POMC signaling blocks the response to cold[245,246]. In view of the varying NPY effects, it is difficult to decide whether these are indeed opposite effects to POMC on the response to cold.

Expression of the two reproductive genes, *Rfrp* and *Kiss1*, did not show any pronounced daily rhythmicity. The absence of significant daily variation in *Rfrp* is in line with previous findings[98,134], but the absence of rhythmicity in *Kiss1* contrasts to previous reports. A daily expression rhythm of *Kiss1* has been described for both the AVPV[247] and the ARC[134]. It is not clear why such a daily rhythmicity was not found in the current study; but, both species and sex-differences might be involved, as the previous studies used either female mice or male hamsters, respectively.

Low temperatures inhibit reproduction in rodents[248–251]. Previous studies reported that cold exposure reduced *Kiss1* expression in voles[252] and *Rfrp* expression in mice[233]. Consistent with these observations we found that 3h cold decreased *Kiss1* expression at ZT10 and *Rfrp* expression at ZT6 (Table 4). The less pronounced effect of cold on *Rfrp* expression in our study as compared to that of Jaroslawska *et al*.[233] may be due to the fact that our exposure lasted only 3 h whereas in theirs, the cold exposure needed to last for at least 24h to induce a change in *Rfrp* expression. So *Rfrp* might be involved in more long-term adaptive functions.

Recent findings indicated that the activity of ARC kisspeptin neurons is necessary for maintaining rhythms of food intake and locomotion independent of temperature, as silencing these neurons blunted the daily control of food intake and body temperature leading to increased body weight[120]. These findings match to another study reporting that female mice with a global knock out of the Kiss1 receptor (Kiss1r) not only presented hypogonadism, but also obesity and a dysregulation of glucose metabolism[117]. Interestingly, the *Kiss1r* global KO animals also showed impaired heat production and lower core temperature as compared to the wild type animals when exposed to cold[211], suggesting that kisspeptin neurons may have a role in the cold response.

It is remarkable that no significant effects of cold exposure on gene expression were found at dawn (i.e., ZT22 or ZT2), which is like the lack of effect on body temperature at these time points[145]. We hypothesize that the endogenous decrease in body decrease before onset of the main sleep period, i.e., at dawn for nocturnal animals, removes the necessity of a cold-induced thermogenic response at this time of the day.

Conclusions

The current study stresses the importance of considering time of day when studying the hypothalamic responses to cold. It has been well established that cold exposure modifies *Trh* and *Npy* gene expression in the hypothalamus, so the differential *Npy* or *Trh* responses reported might depend not only on the physiological response itself but also on the time of day selected to evaluate such a change. Moreover, even within one brain area specific sub-populations may respond differently, such as for instance the hypophysiotropic and non-hypophysiotropic TRH neurons. Contrary to our expectations, we found only minor effects of cold exposure on *Kiss1* or *Rfrp* gene expression.

Acknowledgements

We would like to thank Dr. Patricia Joseph Bravo and Dr Rosa Maria Uribe Villegas for kindly sharing the plasmid containing the Trh cDNA created by Dr. Goodman with us, as well as Dr Paul Klosen and Marie-Pierre Laran-Chich for sharing the plasmids containing the probes for Npy, Pomc, Rfrp, Pomc and Kiss1. Finally we thank to Moqiu Jia for her technical support despite of the pandemic situations.

Chapter 6

DIET AFFECTS THE PHOTOPERIODIC ADAPTATION OF BODY WEIGHT IN *PHODOPUS SUNGORUS* IN A SEX DEPENDENT MANNER

- A technical note -

Introduction

Since the studies by Figala and Hoffman[1,2] in the early 70's, it is known that Djungarian hamsters (*Phodopus sungorus*) lose around 30% of its body weight (BW) when exposed to SD conditions.

In previous experimental work from the Simonneaux's lab performed on male Djungarian hamsters, it was reported that to obtain a similar drop in BW it is necessary to change the maintenance diet (MD, Safe 105, Augy, France) given to breeding couples and lactating mothers to a specific balanced diet (BD, Altromin 7020, GmBH & Co, Lage, Germany) after pup's weaning[12,98,100]. To study the effects of kisspeptin and RFRP-3 on seasonal changes in energy metabolism first we checked again this well-known drop in body weight induced by exposure to short days (SD) in male and female Djungarian hamsters. However, at the beginning of these studies we were lacking the BD, and therefore we decided to perform our photoperiodic investigation with feeding our hamsters with the MD for the whole experiment (instead of switching to the BD at weaning).

Materials and methods

Animals

In addition to the animals that remained on the MD for the whole experiment, for comparison, we are also reporting the food intake and body weight data from the animals of Chapter 3. These measurements comprehend from the moment of separation and photoperiodic transfer (at the age of 4 weeks), until the moment prior to the surgical procedure. Data on gonadal weight correspond to the same animals after finalizing the injection experiments; and because we know that RFRP3 may influence gonadal activity, we only used the vehicle treated animals. All animals used were born and raised in the Chronobiotron animal unit (UMS 3415, Strasbourg, France), after obtaining the approval of the local ethical committee (CREMEAS) and the French National Ministry of Education and Research (authorization #2015021010234017).

Diet composition.

The main macronutrients, mineral, vitaminic and aminoacidic composition of the two diets is showed in Table 1 and Table 2.

Table 1. Diet macronutrients composition

Macronutrient	Maintenance diet (%)	Balanced diet (%)
Carbohydrates	64.3	65
Fat	13.3	12
Protein	22.4	22
Total Kcal/Kg	3000	2868

Crude Nutrients	Maintenance diet (%)	Balanced diet (%)
Dry matter	88.8	89.0
Crude protein	19.4	19
Crude fat	4.6	4.2
Crude Fiber	4	5.8
Nitrogen free extracts	55.5	52.8

Table 2. Diets comparison in minerals, vitamins and aminoacids

	Maintenance diet	Balanced diet
Mineral		
Calcium	0.8 %	0.9 %
Phosphor	0.6 %	0.7 %
Magnesium	**1.33** %	**0.4** %
Sodium	**0.09 %**	**0.2** %
Potassium	0.75 %	1.0 %
Vitamins		
A	25000 IU	25000 IU
D3	**3000 IU**	**1000 IU**
B1	**7.5 mg**	**30 mg**
B2	**13 mg**	**20 mg**
B3	**37 mg**	**60 mg**
B6	**5.6 mg**	**15 mg**
B12	0.06 mg	0.04 mg
E	100 mg	125 mg
K3	5 mg	5 mg
Folic acid	**0.75 mg**	**3 mg**
Biotin	0.15 mg	0.1
Choline	**2100 mg**	**1000**
Meso-Inositol	1 mg	-
PAB Acid	0.5 mg	-
Amino acids	Maintenance diet (%)	Balanced diet (%)
Alanine	-	1
Arginine	1.27	1.3
Aspartic Acid	-	1.8
Glutamic Acid	-	3.7
Glycine	1.17	0.9
Histidine	-	0.4
Isoleucine	-	0.9
Leucine	-	1.4
Lysine	1.18	0.9
Methionine+Cysteine	0.77	0.6
Phenylalanine	-	0.8
Proline	-	1.1
Serine	-	0.9
Threonine	-	0.7
Tryptophan	0.24	0.2
Valine	-	0.9

Different values are indicated in bold

Statistical analysis

All data are presented as mean ± SEM. BW was converted to percentage BW change, 100% corresponding to the moment of single housing (Week 0). BW change was compared by paired Student's *t*-test. Food intake is presented as g/week or converted to Kcal or Kcal/100g BW. Cumulative food intake was calculated only for the last 8 weeks due to 2 weeks of missing data in one group. Gonads and seminal vesicles were weighted after sacrifice at the end of the brain injection protocol as presented in chapter 3 and analyzed by two-way ANOVA post-hoc honestly significant difference (HSD) test. As stated above, only data from animals with vehicle injections prior to the sacrifice are included in this analysis. $P<0.05$ was considered statistically significant.

Results

The photoperiodic adaptation of body weight is affected by the diet.

Female hamsters maintained in long days (LD) had a stable body weight when fed with the BD (0.97 ± 1.06%), but showed an increased body weight when fed with MD (13.65 ±

0.93%). When transferred to short days (SD) female hamsters displayed a decreased body weight on both diets, although the reduction of BW was smaller (10.58 ± 3.38) with the MD as compared to hamsters fed with BD (18.78 ± 1.9%) (Figure 1A, B). Male hamsters kept in LD and fed the MD, gained BW (25.55 ± 3.03%), whereas with the BD they had a stable BW (4.51 ± 2.08%). Upon transfer to SD, male hamsters only lost BW when fed the BD (22.49 ± 1.39%), but not when fed the MD (1.55 ± 3.58%; Figure 1C, D).

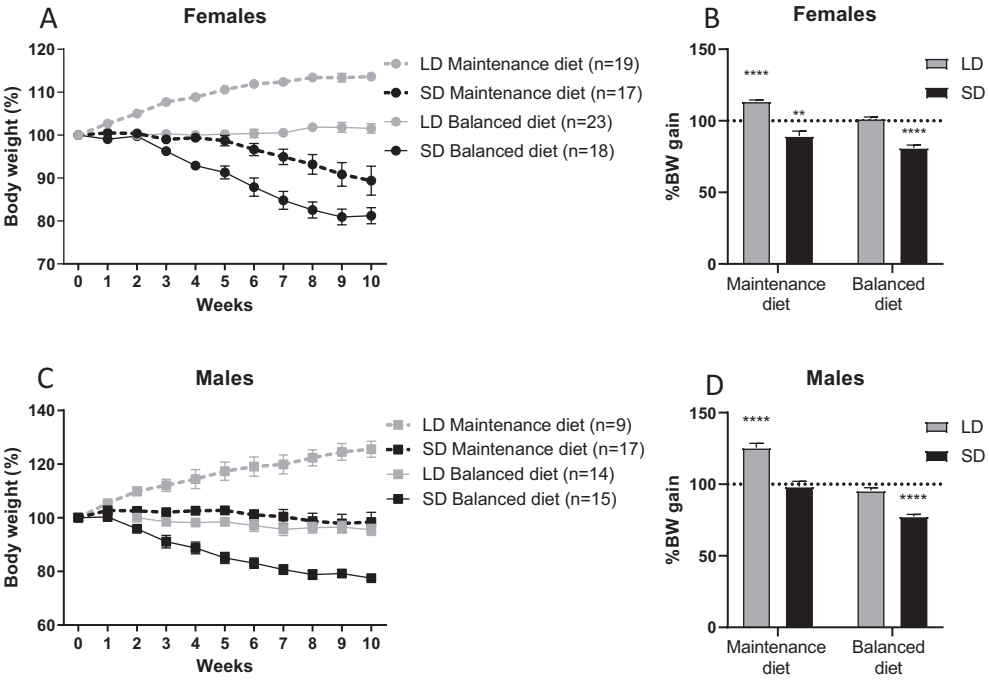

Figure 1. Diet affects bodyweight loss response to photoperiod in a sex dependent manner. A,C) Body weight was followed in post-pubertal female (circles) and male (squares) hamsters for 10 weeks starting after the transfer (week 0) to short (SD-black) or kept in long (LD-gray) day conditions. In panels A & C continuous lines correspond to Balanced diet and dotted line to Maintenance diet fed hamsters. Panels B & D show BW gain at week 10 after SD transfer or LD maintained hamsters vs their own body weight considered as 100% at week 0. Data presented are average ± SEM. **** $P<0.001$, ** $P>0.01$ after paired Student's *t*-test vs 100% BW at Week 0 .

Food intake.

Food intake was followed for 10 weeks except for the LD animals fed with BD, due to missing measurements for the first two weeks. For that reason, cumulative food intake for all the groups was only compared for the remaining 8 weeks. We evaluated net grams, kcal and kcal corrected for 100g BW. We found that in both, female and male hamsters, photoperiod, and diet affected food intake in grams and kcal (Table 2). Interestingly, when normalizing food intake by the BW, only a Diet effect was found but no effect of Photoperiod anymore (Table 2). The Tukey post-hoc HSD showed that indeed only BD fed SD females decreased food intake in grams and kcal vs LD BD fed (Figure 2A, 2C).

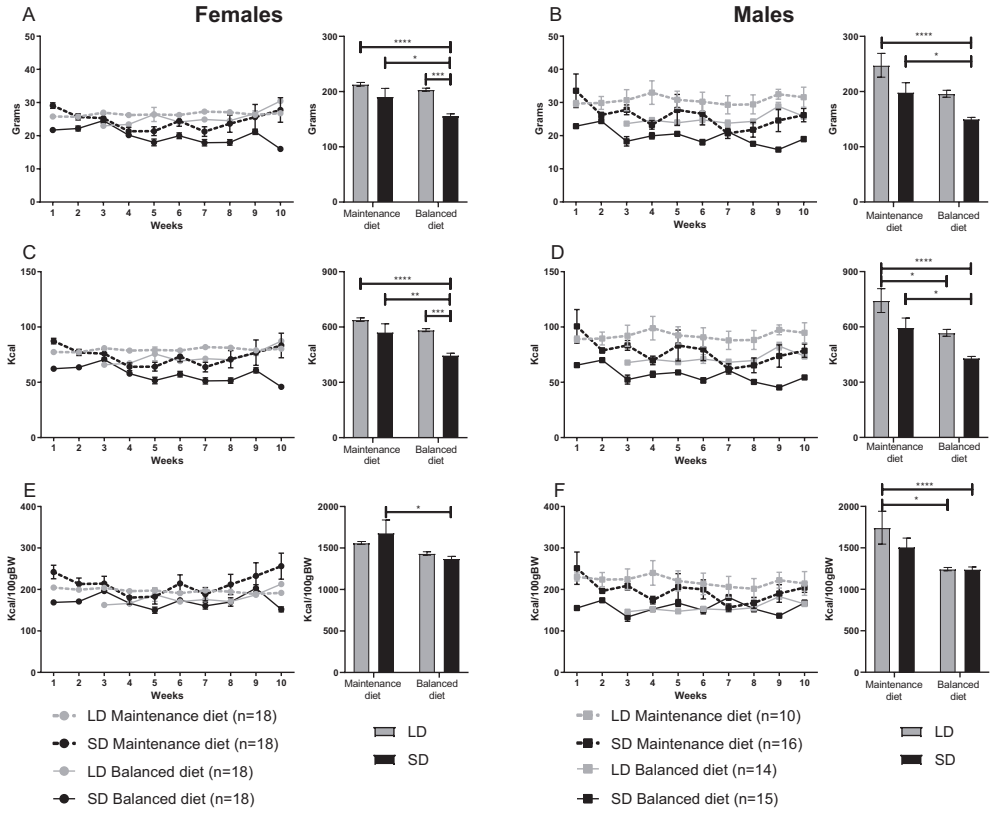

Figure 2. Diet affects food intake response to photoperiod in sex dependent manner. Food intake was followed in post-pubertal female (circles) and male (squares) hamsters for 10 weeks after the transfer (week 0) to short (black) or kept in long (gray) day conditions. Continuous lines correspond to Balanced diet- and dotted line to Maintenance diet-fed hamsters. Food intake is presented as grams (A-B), Kcal (C-D) or Kcal/100g of BW (E-F). Histograms on the right side of each time line graph corresponds to the last 8 weeks cumulative food intake. Gray bars and lines are LD- and black bars and lines SD-adapted animals. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$ after Two-way ANOVA and Tukey's post hoc honestly significance difference test. Data presented are average ± SEM.

Gonadal regression after SD transfer

The two diets did not result in differential effects on the SD-induced regression of either uterus-, testes or seminal vesicle weight, although testes and seminal vesicles weight of the SD hamsters on the MD were not as low as those on the BD (Figure 3). Moreover, according to the 2-way ANOVA there was not only an effect of photoperiod, but also an effect of diet (Table 3).

Discussion

These data show that a change in diet may have profound effects on seasonal adaptation of metabolic activity. Indeed, the MD diet strongly impaired the SD-induced BW loss in both male and female Djungarian hamsters. It seems unlikely that the minor changes in the macronutrients of diet composition were responsible for this major effect, therefore we went on checking in further detail the mineral, vitamin, and amino acid composition of the two

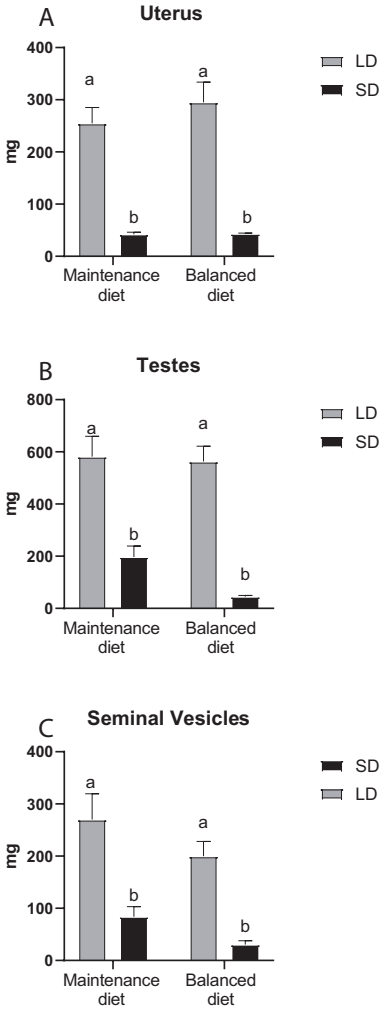

Figure 3. Diet effect on photoperiodic regulation of gonadal weight in male and female hamsters. Gonads were measured after the sacrifice of animals kept in long days (LD, grey bars) or adapted to short days (SD, black bar). Different case letters mean that the groups are statistically different after Bonferroni post hoc HSD. Data presented are average ± SEM.

Table 3. Two-way ANOVA of food intake.

Parameter	Sex	Photoperiod	Diet	Interaction
Grams	Female	**$F_{1,68}$ = 18.6 P<0.0001**	**$F_{1,68}$ = 7.565 P=0.0076**	$F_{1,68}$ = 2.375 P=0.1279
	Male	**$F_{1,51}$ = 12.3 P=0.0009**	**$F_{1,51}$ = 13.78 P=0.0005**	$F_{1,51}$ = 0.01407 P=0.9060
Kcal	Female	**$F_{1,68}$ = 17.6 P<0.0001**	**$F_{1,68}$ = 13.95 P=0.0004**	$F_{1,68}$ = 2.009 P=0.1609
	Male	**$F_{1,51}$ = 12.36 P=0.0009**	**$F_{1,51}$ = 17.79 P=0.0001**	$F_{1,51}$ = 0.01556 P=0.9012
Kcal/100g BW	Female	$F_{1,68}$ = 0.125 P=0.7245	**$F_{1,68}$ = 7.238 P=0.0090**	$F_{1,68}$ = 1.25 P=0.2665
	Male	$F_{1,51}$ = 1.43 P=0.2365	**$F_{1,51}$ = 14.88 P=0.0003**	$F_{1,51}$ = 1.36 P=0.2482

Table 4. Two-way ANOVA of reproductive organs.

	Photoperiod	Diet	Interaction
Uterus	$\boldsymbol{F_{1,23} = 97.50}$ **P<0.0001**	$F_{1,23}$ = 0.7716 *P*=0.3888	$F_{1,23}$ = 0.7000 *P*=0.4114
Testes	$\boldsymbol{F_{1,22} = 73.80}$ **P<0.0001**	$F_{1,22}$ = 2.632 *P*=0.1190	$F_{1,22}$ = 1.609 *P*=0.2179
Seminal Vesicles	$\boldsymbol{F_{1,22} = 37.13}$ **P<0.0001**	$\boldsymbol{F_{1,22} = 4.474}$ **P=0.0460**	$F_{1,22}$ = 0.08293 *P*=0.7761

diets as available by the manufacturer. Even though we are unable to perform statistics on the data collected, we saw clear differences in the mineral content. Magnesium is more than 3-fold higher in the MD vs the BD, whereas Sodium is half the amount in the MD vs the BD. Regarding the vitamin content, we found higher levels in the BD for Vitamin B1, B2, B3, B6, and folic acid, but lower amounts of Vitamin D3 and choline. The amino acid content is difficult to analyze since the BD diet provided information on this for 17 amino acids, whereas the MD only provided information for 5 amino acids. For the 5 amino acids that we can compare (Arginine, Glycine, Lysine, Methionine + Cysteine and Tryptophan), we did not fine major differences.

At present, it is not clear which differences in the production process could be responsible for the important metabolic differences we observed and the impairment of the SD-induced body weight adaptation. On the other hand, we should keep in mind that a switch in diet after weaning was absent in these animals. We are not the first ones to describe that a change in diet may affect the SD-induced body weight response and sometimes even induce resistance to the SD. Ruf *et al.*[253] showed that hamsters fed with a "cafeteria" diet not only increased their body weight, but also exhibited less torpors in SD conditions, meaning that diet is not only influencing BW, but also the thermoregulatory capacity or adaptations necessary for winter like conditions.

Therefore, because our goal was to evaluate metabolic changes between different photoperiods and the diet induced an extra confounder, for future research done with seasonal animals, in this cases Djungarian hamsters, we strongly advice to be careful in the diet chosen otherwise it could impair or block any possible result.

Chapter 7 General Discussion

The objective of this thesis was to study the metabolic effects of two RF-amides reported be involved in the central control of reproduction, and to decipher the putative role of these neuropeptides in the interaction between energy metabolism and reproduction. Experimental studies are often performed in one species, one sex, and in fixed conditions of light, temperature, and diet; but in this thesis, we have studied the RF-amides kisspeptin and RFRP-3, in two species, the Djungarian hamster and the Wistar rat. The two species providing different biological interests, hamsters exhibit photoperiod dependent up- and down-regulation of reproduction and metabolism, whereas rats are widely used in biomedical research particularly in neuroscience and endocrinology and by consequence more is known about their physiology. The metabolic effects of both RF-amides were compared between male and female Djungarian hamsters. In addition, we analyzed the central effects of both RF-amides on food intake and energy metabolism in the male Wistar rats. Finally, we analyzed how external factors such as environmental temperature and food intake modified the hypothalamic expression of RFRP-3 and kisspeptin in the male Wistar rat or affected the photoperiodic response of Djungarian hamsters, respectively.

1. Sex differences in the RF-amide regulation of seasonal metabolism in the Djungarian hamsters

For many years, studies in neuroscience have been done with clear sex biases[254]. Up to 2009, 6-times more studies were performed in male than in female rodents[255]. In 2014, the meta-analysis of Prendergast *et al*.[256] reported that several biological parameters did no vary differentially in single housed female more than single housed male mice.

In the first two experimental chapters (Chapters 2 and 3), we studied the effects of central RF-amide treatments in both male and female Djungarian hamsters kept in either long or short photoperiod. Therefore, we will now first compare the seasonal adaptations in males and females and then next describe the metabolic effects of the central treatments with kisspeptin and RFRP-3, administered either acutely or chronically.

Both male and female Djungarian hamsters adapted to the short-day conditions displayed a clear inhibition of reproduction and metabolism, with a decrease in gonadal weight and hormone production, and reduced food intake and body weight, as compared to animals adapted to long-days. The seasonal change in metabolism was associated with a clear seasonal variation of POMC and STT expression in male and female hamsters. By contrast NPY and orexin expression did not show seasonal variations in either sex. Regarding the humoral metabolic signals, leptin and insulin, both displayed seasonal variations, with leptin following the changes in the body weight and insulin levels reduced similarly in male and female hamsters.

As described in the General Introduction, the inhibitory role of melatonin on reproductive functions was suggested already many decades ago (Reviewed in[257]) and its downstream signaling implicating TSH production from the pars tuberalis, is becoming better understood. However, so far there is no clear mechanism explaining how TSH from the pars tuberalis and the subsequent changes in local T3 availability in the mediobasal hypothalamus are triggering the seasonal control of reproduction and metabolism. Indeed, short-day habituated male hamsters that received ICV TSH, increased their gonad mass and body weight at the same time as the expression of kisspeptin and RFRP-3[12]. T3, directly applied within the mediobasal hypothalamus, increased testes mass and body weight[10], but it is yet unknown whether central T3 administration also regulates RF-amide expression in short-day Djungar-

ian hamsters.

Role of RFRP-3 in seasonal metabolism

We have evidence that the long melatonin peak during short days inhibits RFRP-3 expression in all seasonal species investigated so far[98–100]. Furthermore, exogenous administration of RFRP-3 reactivated seasonal reproductive functions in male and female SD-adapted Syrian hamsters[96,101,103,104]. However, Syrian hamsters do not show clear seasonal variations in body weight[21] therefore it is not an appropriate species to evaluate the mechanisms regulating seasonal metabolism. Thus, thanks to their natural characteristics, we used the Djungarian hamster to study the role of RF-amides on the seasonal control of reproduction and body weight. Ubuka *et al*.[93] showed that an acute injection of RFRP-3 exhibited different effects on LH release in SD and long days (LD) male hamsters, being inhibitory in LD and stimulatory in SD.

In agreement with the above, we found that acute ICV injections of RFRP-3 not only increased LH release in SD males[93], but also in SD females (non-published data Figure 1). By contrast, the chronic infusions of various doses of RFRP-3 (from 2.75 to 250 pmol/h) did not restore reproduction (gonadal mass) in either sex[194]. Therefore, it appears that the reproductive effect of chronically infused RFRP-3 exhibits a clear species-dependent effect despite the stimulatory effects observed in SD male and female Syrian hamsters, RFRP-3 did not increase gonadal activity in either sex of the Djungarian hamster.

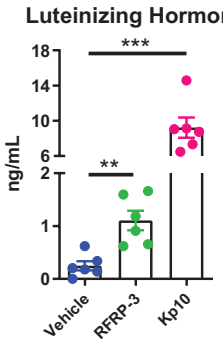

Figure 1. Effects of RFRP-3 and kisspeptin (Kp10) on luteinizing hormone (LH) levels in short-day (SD)-adapted female Djungarian hamsters. Female hamsters were kept in SD conditions for 10 weeks and treated with an intracerebroventricular (ICV) injection of vehicle or 1 nmol of RFRP-3 or Kp10. Thirty minutes after the ICV injection animals were sacrificed and blood was obtained by hearth puncture for LH determination in plasma. ***P*<0.01, ****P*<0.001 after Student's *t*-test vs vehicle injected hamsters.

It has been demonstrated that RFRP increases food intake in several non-seasonal mammals and birds. In male mice, daily peripheral treatment with RFRP-3 increased food intake, body weight, and white adipose tissue (WAT). Therefore, we also studied the acute effects of RFRP-3 on food intake in both male and female Djungarian hamsters, adapted to either long or short photoperiods[197]. In females, acute RFRP-3 injections increased food intake, but only when in SD or in LD in diestrus. This orexigenic effect was associated with an increased NPY expression. In males, by contrast, acute RFRP-3 injections had no effect on food intake or NPY and POMC expression.

We also found a clear sex-dependent effect of the chronic administration of RFRP-3 in short-day adapted hamsters, with only males, but not the females, showing an increased body weight, food intake, leptin and insulin levels[194].

Altogether, these data confirm the orexigenic effect of central RFRP-3 in the seasonal Djungarian hamster, although with major sex-dependent effects. Currently, we have no data to explain why the acute injections increased food intake in females, whereas the chronic infusions increased food intake in males.

Role of kisspeptin in seasonal metabolism

As already published for other mammalian species, also in the current study we found that kisspeptin administration reactivated gonadal activity, as attested by the gonad mass, in both male[12] and female SD Djungarian hamsters. We also observed that a single ICV injection of kisspeptin increases LH release in female hamsters (Figure 1).

Regarding the metabolic effect of chronic kisspeptin, we observed a major sex difference. Indeed, in males, kisspeptin increased body weight together with NPY and POMC expression. However, in females, kisspeptin had no effect on body weight nor on any of the hypothalamic genes analyzed[194]. Interestingly, kisspeptin-induced changes in NPY expression turned out to be sex steroid independent since they were still present in castrated male hamsters (non-published data Figure 2) (Treatment effect*:* $F_{1,21}$ = 5.347, *P*=0.031; Castration effect: $F_{1,21}$ = 0.3784, *P*=0.545; *Interaction effect*: $F_{1,21}$ = 0.158, *P*=0.695). In mice, kisspeptin has been proposed to act directly on POMC and indirectly on NPY neurons; however, kisspeptin effects on NPY are most likely not a consequence of the direct POMC activation because of two reasons: 1) POMC expression did not change after kisspeptin infusion in castrated animals (data not shown) and 2) there is no POMC to AgRP/NPY neuronal communication[258].

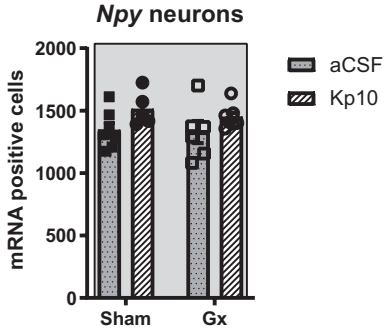

Figure 2. Effects of castration and kisspeptin (Kp10) treatment on neuropeptide Y (NPY) expression in the arcuate nucleus of male Djungarian hamsters adapted to short day (SD) conditions. Hamsters adapted to inhibitory SD conditions were castrated (Gx, open symbols) or sham operated (sham, closed symbols) and then were treated with a 5 week intracerebroventricular (ICV) infusion of artificial cerebrospinal fluid (aCSF) or 170 pmol h-1 Kp10. Bars show the semi-quantification of the mean number of NPY labelled neurons per animal. Data are presented as the mean ± SEM of n= 5-7 animals per experimental group and the symbols represent individual data points. Two-way ANOVA statistical output is indicated in the text.

Concluding remarks on the sex dependent effects of RF-amides on the seasonal control of body weight and hypothalamic networks controlling energy metabolism

Based on the data presented in this thesis we propose that the RF-amides act downstream of the melatonin-TSH-dio2-T3 pathway not only to control seasonal reproduction, but also to modulate seasonal changes in metabolism. Indeed, the LD associated increase in hypothalamic T3 levels is thought to be upstream of the neuronal populations that control reproductive behavior and metabolic activity, since central administration of T3 or TSH has been shown to increase gonad size and body weight, including epididymal fat[5,10].

Central TSH also increased Kiss1 and RFRP expression, likely though an increase in hypothalamic T3 levels. Thus, we hypothesize that either TSH or T3 may be causing an increase in body weight through the increased expression of RF-amides, with subsequently kisspeptin affecting lean mass by increasing NPY and POMC expression and RFRP-3 affecting fat mass through a still unknown pathway associated with an increase in leptin and insulin. According to this hypothesis both RF-amides would need to be produced to accomplish an increase of both lean and fat mass and restore gonadal activity in male hamsters.

Somatostatin (SST) producing neurons in the ARC have been identified as another neuronal population regulated by the TSH-T3 pathway. However, it is not clear yet whether activity of these SST neurons is regulated directly by T3. Our current data suggest that this SST neuron

regulation might be partially downstream of the RF-amides, as the chronic administration of kisspeptin also induced a mild effect on the intensity of SST expression (Figure 3).

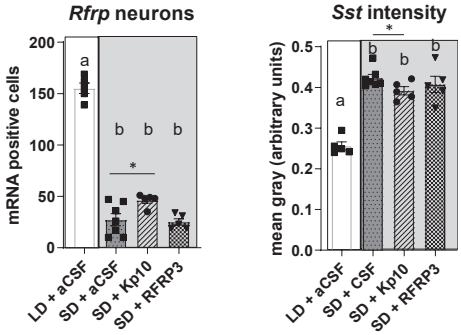

Figure 3. Effect of kisspeptin (Kp10) and RFRP-3 on somatostatin (Stt) and Rfrp gene expression in male Djungarian hamsters. Hamsters were raised in long-day (LD) or short-day (SD) conditions and SD animals were treated with a 5 week intra-cerebro-ventricular (ICV) infusion of artificial cerebrospinal fluid (aCSF), 170 pmol h-1 Kp10 or 8.24 pmol h-1 RFRP-3. Different lower case letters indicate that the groups are statistically different after one-way ANOVA and Tukey's post hoc honestly significance test. *P< 0.05 after Student-s t-test when comparing ICV peptide infusion vs SD+aCSF.

It cannot be excluded that the RF-amides also regulate each other's expression. For example, we found that the ICV administration of kisspeptin stimulated RFRP expression in the DMH (non-published data, Figure 3), probably independently of a change in the sex steroid hormones as testosterone does not increase RFRP expression[100]. Such influence of kisspeptin on RFRP-3 expression has already been reported for the seasonal jerboa, where kisspeptin increased food intake and decreased the levels of RFRP-3[116]. Besides, Kiss1r was found to be expressed in the DMH in the rat brain[259], supporting the possibility of an indirect communication, although in mice *Kiss1r* is not expressed in RFRP neurons[260]. On the other hand, NPY fibers have been found to contact RFRP neurons in the Syrian hamster, possibly coming from neurons in the ARC. Therefore, in Figure 4 we propose two hypothetical models to explain the kisspeptin-induced RFRP increase in a sex steroid hormone independent manner.

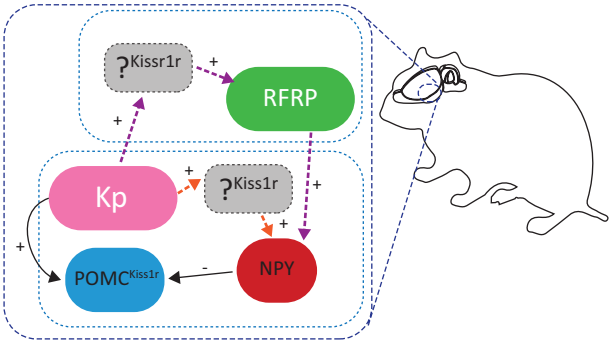

Figure 4. Proposed models explaining the stimulatory effect of kisspeptin (Kp) on neuropeptide (NPY) expression in the arcuate nucleus (ARC) and on RFRP in the dorsomedial hypothalamus (DMH) in the seasonal Djungarian hamster. In the first proposed circuit (purple arrows), Kp neurons (pink) project to unknown neurons expressing its receptor Kiss1R in the DMH (gray), which increases RFRP (green) expression and subsequently NPY (red) expression in a sex steroid independent manner. At the same time, an increase in proopiomelanocortin (POMC) expression is induced via Kiss1r. In a second possible pathway (orange arrows) Kp increases NPY expression in the through an unknow neuronal population expressing Kiss1R in the ARC.

The chronic infusion of RFRP-3 increased circulating plasma leptin and insulin levels, which are both known to stimulate POMC gene expression[23]. Thus, it was surprising that RFRP-3 did not clearly increase POMC expression. We only found a tendency for an increased staining intensity in a few neurons. Recently, Quarta *et al*. demonstrated in mice that the ARC neuronal POMC population is quite heterogenous[261], so it cannot be excluded that also in the hamster ARC only a few neurons might be responding to leptin and insulin, without a significant change in our analysis in the overall ARC.

Another missing point in the current set of experiments is that we did not really look at a full reactivation of sexual capabilities, i.e., in terms of sexual behavior and fertility, as this was not the main scope of this thesis. Recent studies, using a rescue of kisspeptin receptors only in the GnRH neurons of a whole body KO Kiss1r mice, not only found a partial rescue of HPG axis activity[121], but also an incomplete/partial improvement of the metabolic disturbances[117]. Therefore, future studies should not only consider hypothalamic changes, sex steroid and gonad mass, but also investigate oocyte and sperm development and sexual behavior.

2. Differential effects of the two RF-amides on rat energy metabolism

Food intake and energy metabolism

In our experiments in Chapter 4, we investigated the metabolic effect of kisspeptin and RFRP-3 in male Wistar rats.

We first showed that central kisspeptin treatment also increases LH and testosterone levels in the male rat. Our analysis of the effects of both RF-amides on food intake in an automated manner and with a higher resolution, reported an anorexigenic effect of kisspeptin, not a full inhibition but a significant decrease of the cumulative 24h intake. We also found decreased levels of RER, probably a consequence of the diminished food consumption. Kisspeptin decreased POMC immunoreactivity which might be explained by an increased processing of the pro-peptide POMC to the anorexigenic peptide α-MSH. However, up till now it cannot be excluded that the observed metabolic effects of kisspeptin are a consequence of changes in circulating levels of testosterone as we have not performed castrations with or without testosterone replacement to prove or disprove the dependency of the kisspeptin effects in the male Wistar rats on sex steroid hormones.

Interestingly, central kisspeptin activated neurons in the median preoptic nucleus (MnPO), an area known to be thermosensitive. However, we did not find any change, in body temperature after kisspeptin infusion (data not shown). Next to the anterior hypothalamus, Kiss1r is expressed in many more brain regions[259], such as the PVN, DMH and parabrachial nucleus, which remain to be analyzed.

In our experiments with Wistar rats, also RFRP-3 brain infusions affected HPG axis activity, as attested by the increased testosterone levels. By contrast, we did not observe the expected orexigenic effect of RFRP-3, nor any other metabolic parameter investigated. As Johnson *et al*. and Clarke *et al*.[123,124] used Sprague Dawley rats to show the orexigenic effect of RFRP-3, the discrepancy may be related to the different rat strains used in these studies.

On the other hand, despite the small increase of testosterone after RFRP-3 in the current study, we found no changes in POMC-ir in agreement with the absence of changes in food intake. We suspect that the amount of RFRP-3 used in our experimental set up in the Wistar rat (50 ρmol and 250 ρmol), was too low as compared to what has been used in other stud-

ies when investigating the effects of central RFRP-3 on luteinizing hormone (LH)[175,262]. This clearly accentuates the possible strain difference in sensitivity to central RFRP-3 in Wistar vs Sprague Dawley rats. Furthermore, our increased levels of testosterone are in contrast with the inhibitory role of ICV RFRP-3 on LH in the male Sprague Dawley rat[123].

In rats and ewes, RFRP-3[123] and kisspeptin[144] have been reported to control SST and growth hormone releasing hormone (GHRH). In Sprague Dawley rats, RFRP-3 increases growth hormone release, whereas in sheep the stimulatory effect of kisspeptin on growth hormone is dependent on the presence of ghrelin[144]. It would be interesting to verify whether neuronal SST and GHRH activity are regulated by RFRP-3 and kisspeptin in our experimental set up.

Glucose metabolism

Central administration of kisspeptin in rats caused a clear acute increase of endogenous glucose production, but without significant changes in systemic glucose levels. The latter observation indicates that probably also glucose uptake was changed by the central RF-amide treatment. Since insulin levels were not increased, this would indicate an increase in insulin sensitivity. Interestingly, glucose uptake in the gonads is possible without insulin[263], this would suggest that in addition to the earlier demonstrated peripheral effects on the expression of glucose transporters (GLUT) in the gonads after daily injections of RFRP-3 in mice[125], both, central kisspeptin and in a lesser extent, RFRP-3, might be influencing glucose uptake in the gonads, something that has not been tested so far.

Even though, peripheral effects of both RF-amides are beyond the scope of this thesis, we should recall that the Kiss1r KO animal model with the obese and glucose intolerant phenotype lacks the receptor expression not only in the brain, but in the whole body[116]. Kiss1 itself and Kiss1R are expressed in a number of metabolically active tissues, such as pancreas, liver, and white and brown adipose tissue[264], and mice lacking the Kiss1R in pancreatic β-cells or the liver also show changes in glucose metabolism[265,266]. Furthermore, both *in vitro* and *in vivo* studies have shown a stimulatory effect of kisspeptin on glucose-stimulated insulin secretion[267–274]. Finally, although the glucose intolerance resulting from the Kiss1R KO might also partly be due to the hypogonadal status as hypothesized previously[275], re-expression of the Kiss1R selectively in GnRH neurons only partly rescued the phenotype[121]. Clearly a lot is still unknown regarding the central and peripheral glucoregulatory effects of kisspeptin.

In the previous studies in hamsters (this work), jerboas[116] and mice[127], clear sex dependent metabolic effect of both RF-amides have been reported. For instance, female hamsters acutely treated with RFRP-3 and jerboa females treated with kisspeptin appeared more sensitive than males. In contrast, the long term effects of knocking out (KO) the receptors of RF-amides[117,127], impacted female body weight and glucose tolerance in the case of Kiss1r KO, whereas GR147 KO (RFRP-3 receptor) caused male mice to have an anorectic phenotype when they were fed a chow diet. On a high fat diet (HFD) glucose homeostasis was impaired in males, but not in females. On the other hand, in males, bodyweight and adiposity was not worsened as compared to their WT littermates, whereas in females body weight was exaggerated after HFD. In our current study, we have investigated the RF-amide effect on glucose and energy metabolism only in the male Wistar rat but based on the above-mentioned sex dependent effects these experiment should clearly be repeated in female animals too.

Taken together the kisspeptin effects on food intake, RER and glucose production we would like to propose a hypothetical model of the direct and indirect possible ways of action in the Wistar rat (Figure 5).

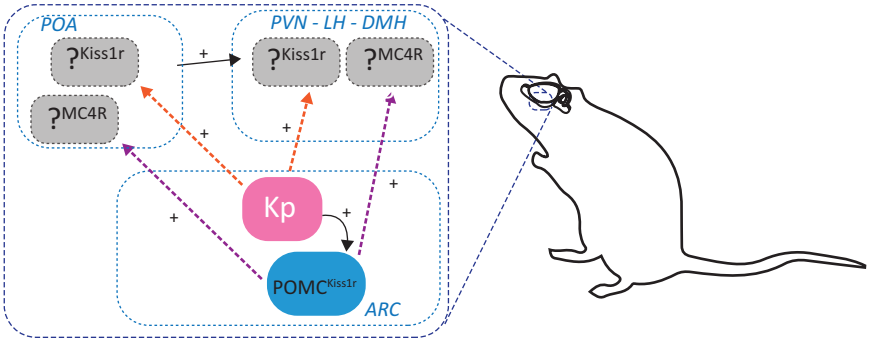

Figure 5. Proposed models explaining the kisspeptin (Kp) effects on satiety, RER, and endogenous glucose production in Wistar rat. One way is that directly (orange arrows) Kp neurons (pink) project to an unknown neurons expressing its receptor Kiss1R in the second order neruons in the PVN, LH or DMH and preoptic area (POA) (gray). A second pathways (purple) is that Kp first activates POMC and then POMC acts through MC4r on the targeted areas involved in food intake and glucose production.

3. Impact of cold temperature on RF-amide and energy metabolism gene expression in the male rat hypothalamus

So far, we discussed if and how RF-amides could affect glucose and energy metabolism in seasonal hamsters and laboratory rats. However, we were also interested if external signals other than photoperiod could impact on the hypothalamic expression of the RF-amides.

Effects of a cold environment on the RF-amide and energy metabolism controlling neurons in the hypothalamus

The capacity of mammals to adapt to environmental temperature variations is tightly regulated, and temperature fluctuations is a major environmental signal that influences body's physiology. Animals in the wild are not only sensitive to the seasonal change in photoperiod, but also to those in the environmental temperature. For example, at lower temperature Djungarian hamsters show accelerated changes to the short photoperiod[276], indicating synergetic effects of photoperiod and temperature variations in the wild life. Ruf *et al*.[277] showed that animals in SD and at 5 °C display more incidences of torpor compared to animals in SD kept at room temperature. Even non seasonal species like Wistar rats, known to be strictly homeothermic, present adaptations to cold including shivering and non-shivering thermogenesis[278] to cope with the cold environmental conditions.

In the final experimental chapter, we analyzed whether a 3h cold exposure at different time points along the L/D cycle would modulate the hypothalamic expression of both RF-amides. We found that such a short stimulus resulted in changes in gene expression only during the light phase. The lack of effect of cold temperature on kisspeptin and RFRP-3 expression at other time points of the day may be due to a too short duration of the cold exposure. Indeed, in the seasonal Brandt's vole cold exposition (4±1°C degree) diminished Kiss1 levels after day 26 followed by a decrease in testosterone levels at 40 days[252]. RFRP gene expression (or NPVF as it has also been called) was reduced after 24h of exposure to low temperatures[233]. Thus, it would be interesting to analyze the effect of cold when associated to short photoperiod in the hypothalamus of seasonal species.

4. Diet importance for photoperiodic metabolic response

Diet is another external factor that critically influences the central regulation of both metabolic and reproductive status. Our technical note provides a clear example that small variations in the vitamin and/or mineral content of the food may have a major impact on the endogenous short day-induced reduction in body weight, in our case mainly in male Djungarian hamsters.

The different diet evoked a diet-induced obese (DIO) phenotype, with increases in body weight and food intake, although we do not know whether it also caused a diabetic like phenotype[279]. In view of this obese phenotype, it is not surprising that the different diet also compromised the short day induced body weight loss. We have evaluated the effects of acute RFRP-3 injections also in these DIO-like hamsters (data not shown). We found similar effects than with the balanced diet, with the orexigenic effect being associated with an upregulation of NPY in short-day adapted female hamsters, similar to the animals fed the regular diet. However, in addition, the DIO-like female hamsters injected with RFRP-3 also showed an upregulation of *Hcrt* in the lateral hypothalamus.

Conclusions

Overall, we have found central RF-amide effects on energy metabolism in both, Djungarian hamsters and Wistar rats. Despite that almost 20 years ago these two peptides were described for their role in controlling reproduction, now it is clear that they not only control the functioning of GnRH neurons, but they also use their integrated information to affect the central control of energy metabolism.

We provided clear evidence that it is important to study both male and female animal models, as at several instances the effects of the same RF-amide greatly differed between male and female hamsters, even when circulating sex steroid hormones are low as in short photoperiod.

Regarding food intake, we found clear indications for species differences in the effects of the RF-amides. In hamsters, RFRP-3 increased food intake in females only after acute administration, but in males only after chronic administration. In the Wistar rat we found no effect of RFRP-3 on food consumption. In male hamsters, kisspeptin did not influence food intake, but it increased body weight together with an increased expression of arcuate POMC and NPY. On the other hand, in male rats, kisspeptin decreased food intake, which was associated with reduced arcuate POMC expression.

Regarding the effects on adipose tissue, we observed that chronic infusion of RFRP-3 in male hamsters increased body weight and fat mass as attested by increased circulating leptin, whereas in rats, kisspeptin increased fat oxidation when injected acutely.

We also found evidence for effects of the RF-amides on glucose metabolism with species differences. On one hand, RFRP-3 increased insulin levels in the male hamsters and on the other hand, endogenous glucose production was increased by both RF-amides in rats, but insulin levels were not changed.

Finally, we found that lowering the environmental temperature altered the expression of genes controlling reproduction and energy metabolism in the male Wistar rat and this effect was dependent of the time of day. Expression of both RF-amides was reduced in the light

period, but only at one time point. NPY in the ARC and TRH in the PVN were up-regulated, whereas POMC gene expression was down-regulated. The response of all three genes was time of day dependent. Thus, during cold TRH and NPY expression are upregulated to stimulate metabolism, while reproductive activity (kisspeptin and RFRP) is reduced.

To conclude, even though neuropeptides are sometimes referred to as being strongly implicated in a specific function, such as reproduction (kisspeptin/RFRP) or energy metabolism (NPY/POMC), the current work provides clear examples that typical "reproductive neuropeptides" also have metabolic effects. The other way around, others have already shown that signals often described as purely "metabolic", strikingly leptin[232], also alter reproductive physiology.

Perspectives

There is always more to do, as with every new result (positive or negative), new questions arise.

In both parts of this thesis, we studied how RFRP-3 and kisspeptin affected the gene expression of the so called first order neurons in the arcuate nucleus (NPY/POMC), and second order neurons in the lateral hypothalamus (orexin/HCRT). For the chronic experiments in hamsters, it would be interesting to investigate how the kisspeptin-induced increase in the number of positive POMC/NPY neurons is induced.

Unfortunately, we have not been able to perform the chronic RFRP-3 infusions in gonadectomized animals with or without sex steroids supplementation. Although in the male Djungarian hamsters RFRP expression does not seem to be affected by sex steroids, we do not know whether this is also true for the female hamster.

For future research, it would also be interesting to further assess the effects of RFRP-3 on adiposity. We analyzed a few possible brain regions that could be affected by RFRP-3 and seemed the most logic regarding the regulation of food intake (ARC). However, GRP147 has been reported to be expressed in many more brain regions, such as the POA, PVN, SCN, the ventromedial hypothalamus (VMH) and the habenula in the thalamus[132], so more areas should be investigated.

In addition, we have been thinking of the brain as mainly a neuron-based organ, but clearly the whole brain contains a number of other cell types. Particularly, glial cells such as astrocytes and microglia could also be involved in many of the centrally driven seasonal adaptations. Noteworthy, the ependymal tanycytes are key regulators of the phototransduction signals, which clearly indicates the importance of including cell types other than only neurons in the analysis to understand the hypothalamic regulation of energy metabolism and reproduction. In fact, more recently, our group has found that microglial cells in the infundibular nucleus of the hypothalamus in humans (the equivalent of the ARC in rodents) show seasonal variation[280], indicating that indeed hypothalamic microglia might have a role in seasonal reproductive and metabolic adaptations.

In our neuro-anatomical study on the effects of cold in the hypothalamus of the rat, we have looked at the changes of a few neuropeptides involved in either the control of reproduction or energy metabolism. Since the original question of this study was whether the response to cold would depend on the time of day[145], it will also be important to assess whether hypothalamic clock gene expression is affected by cold and whether this also depends on the

time of the day. For example, it has been shown that the expression of Period 2 (Per 2), one of the genes of the core circadian clock machinery, is necessary for the correct response to cold[281]. Although the processes that control homeothermic physiology in the rat and torpor of the hamsters are not fully comparable or might be even in opposite directions sometimes, it would be interesting to analyze in more detail two molecules that are known to change during torpid episodes in Djungarian hamsters, i.e. orexin/Hcrt and the histamine H3 receptor[282]. Besides, recently, a set of neurons denominated as QPLOT have been discovered in the preoptic area of the hypothalamus and pointed out as pivotal in the integration of ascending temperature stimuli (e.g. cold) in the thermoregulatory circuit[283].

For the translational value of our work, we would like to mention two main aspects:

1) The long time discussed presence or absence of seasonality in human biology and physiology and thus, the possible extrapolation of our findings in rodents to humans, notably the RFRP-3 effects on food intake, body weight and fat mass. Recently, it has been shown that people with a higher content of adipose tissue content (and reduced adiponectin levels) had a higher incidence of seasonal affective disorder (SAD)[284]. Because RFRP-3 increases fat mass, we think it would be interesting to assess whether these animals, or even the animals from our technical note with the impaired response of body weight, might have more SAD-like symptoms or any other resemblance to people with SAD. We hypothesize that in humans, even though not so well known or understood, seasonal mechanisms may have been affected by our modern lifestyle (i.e., food intake and light pollution) and impair whole body homeostasis, which finally may lead to long term affections as SAD, but possibly also metabolic disturbances such as the metabolic syndrome. 2) The possible role of the RF-amides in the human control of any type of (seasonal) metabolism, particularly glucose metabolism, as it has been described that in humans kisspeptin is able to affect glucose homeostasis[285]. As for the possible hypothalamic regulation of human seasonality, it is known that some neuropeptides have a seasonal pattern of expression, for instance, vasopressin in the SCN[286]. Interestingly, the central master clock in the SCN is known to be involved in reproductive physiology in several animal models[287]. Therefore, it would be of interest to assess whether there might be also a seasonal pattern of expression in kisspeptin and RFRP in the human hypothalamus, especially when considering the above mentioned finding of seasonal variation in microglial cells in the human infundibular nucleus.

Appendices

SUMMARY

SAMMENVATTING

RÉSUMÉ

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AUTHORS AFFILIATIONS

PhD PORTFOLIO

PUBLICATIONS

ACKNOWLEDGEMENTS

ABOUT THE AUTHOR

SUMMARY

The general aim of this thesis was to evaluate the possible role of RF-amides as a link between the hypothalamic control of reproduction and energy metabolism.

With the compilation of the literature as shown in **Chapter 1**, we described to the best of our knowledge the current status of understanding of the seasonal control of metabolism, with a special focus on the control of body weight and food intake in the Djungarian hamsters, a species that shows clear seasonal changes in reproduction and energy metabolism.

In humans, it is still debatable whether seasonal rhythms are present, and clear evidence of seasonal changes in the hypothalamic circuits controlling reproduction and metabolism are lacking. However, in a recent review by Yoshimura *et al.*[288] it was mentioned that many physiological parameters do show seasonal changes, notably, the immune response[289].

The discovery of the RF-amide system about 20 years ago has been a breakthrough for understanding the neuroendocrine circuit that controls reproductive activity in different physiological and environmental conditions. Notably hypothalamic neurons expressing the neuropeptides kisspeptin and RFRP-3 have proven to be pivotal for the regulation of the hypothalamo-pituitary-gonadal axis in mammals. The use of seasonal animals provides a unique way to understand how reproduction is controlled in nature, as it allows to study animals that are sexually active or inactive at different times of the year without the necessity of genetic manipulations to induce what already happens endogenously.

We described how the phototransduction mechanism starts with the daily melatonin signal and ends with changes in the local concentrations of T3 in the mediobasal hypothalamus. Even though we do not know the exact targets for the T3 molecule, neuroanatomical and physiological studies point for an essential role of both kisspeptin and RFRP-3 in seasonal reproduction. In addition, an increasing number of studies indicates that both peptides can regulate food intake, body weight and the energy homeostasis. This chapter ends with the hypothesis that RF-amides are involved in both the control of reproduction and metabolic activity, and as such they could be part of the link between environmental signals and the changes in reproductive and metabolic physiology along the year.

In **Chapter 2** we found that chronic (5 weeks) central administration of either kisspeptin or RFRP-3 increased the body weight of hamsters that were sexually and metabolically photo-inhibited. Of note, RFRP-3 showed a small concentration range of efficacy for body weight regulation. This effect was sex dependent, with only males increasing body weight. We further demonstrated that the kisspeptin effect on body weight in male hamsters was dependent on the increased levels of circulating sex steroids produced by the reactivated testis. This was not the case for the RFRP-3 effect, which had no stimulatory effect on testis activity.

Looking at the expression of metabolic genes in the hypothalamus, we found that the kisspeptin-induced increased body weight in male hamsters was accompanied by an increased *Npy* and *Pomc* expression in the arcuate nucleus after 5 weeks of central infusion. By contrast, RFRP-3 did not change the expression of any of the hypothalamic genes studied, but it did increase food intake and circulating levels of leptin and insulin.

After these studies reporting increased body weight and food intake in short-day adapted hamsters with chronic application of RF-amides, but without striking changes in hypothalamic gene expression for RFRP-3, we investigated the possible acute effects of RFRP-3 on

food intake in **Chapter 3**. We found that RFRP3 elicited orexigenic effects in a sex-dependent manner, with females increasing food intake in both short- and long-photoperiod, but only in the diestrus stage of the estrous cycle under long photoperiod. The increased food intake was accompanied by an increase in NPY expression in short photoperiod. We hypothesized that the orexigenic effect of RFRP-3 is dependent on sex steroid hormones, as the increase in food intake was only found in animals that had low levels of E2 (i.e., in short photoperiod and during diestrus).

In **Chapter 4** we studied the metabolic effects of kisspeptin and RFRP-3 in male Wistar rats. We found that central injections of kisspeptin decreased food intake in ad libitum fed rats. Contrary to our expectation RFRP-3 did not increase food intake as previously reported for the Sprague Dawley rats but it did increase testosterone levels althouth in a lesser extent compared to the kisspeptin effects. The decrease in food intake after kisspeptin was accompanied by a decrease in the respiratory exchange ratio, indicating an increased fat oxidation. In the hypothalamus, POMC immunostaining in the posterior arcuate nucleus was reduced after kisspeptin injections and further analysis showed that kisspeptin activated the median preoptic nucleus, a brain area well known to receive POMC neuronal fibers and to control core body temperature. We also investigated whether the central treatments with kisspeptin or RFRP-3 influenced glucose metabolism, specifically endogenous glucose production. We found that a 2-hour central infusion of kisspeptin activated the HPG axis as indicated by increased circulating levels of luteinizing hormone and testosterone. RFRP-3 did not cause an activation of the reproductive axis. Both RF-amides increased endogenous glucose production without modifying systemic glucose levels, although the RFRP-3 effects were milder than those of kisspeptin. Circulating levels of insulin and corticosterone were not affected. We concluded that kisspeptin and RFRP-3 affect food intake and glucose metabolism, with the more potent effect of kisspeptin possibly due to the increased production of sex steroids.

Next to photoperiod, environmental temperature is another factor that shows clear seasonal fluctuations. In **Chapter 5** we investigated whether 3h of cold exposure (4 C degrees) was able to modify the gene expression of hypothalamic RF-amides and a few “metabolic” neuropeptides and whether these effects would be dependent on the time of day. Even though RFRP and kisspeptin showed no daily rhythmicity, cold exposure decreased their mRNA expression only during the light phase. On the other hand, NPY and POMC gene expression in the arcuate nucleus showed significant daily rhythmicity, and NPY expression was increased after 3h of cold exposure depending on the time of the day. Finally, cold exposure also increased TRH expression in the paraventricular nucleus, but not in the medial preoptic area.

Conclusions

In conclusion, our work has shown that RF-amides regulate energy metabolism in both seasonal and non-seasonal animal models. Central treatments in the Djungarian hamster caused an increase in energy metabolism mostly towards a spring-summer phenotype, despite of living under inhibitory photoperiodic conditions. In the non-seasonal male Wistar rat, RF-amides also modified food intake and glucose metabolism. Thereby, RF-amides are not only capable of regulating metabolic and reproductive activity, but also of integrating external information such as changes in photoperiod and changes in environmental temperature. These results reinforce our hypothesis of an essential role for the RF-amides in coordinating the control of reproduction and metabolism in response to environmental changes induced by the change of seasons.

SAMENVATTING

Het uiteindelijke doel van dit proefschrift was om de mogelijke rol van RF-amiden als link tussen de hypothalame controle van reproductie en energie metabolisme te onderzoeken.

Met de compilatie van de in **Hoofdstuk 1** beschreven literatuur hebben we naar ons beste vermogen, de huidige status van de kennis met betrekking tot de seizoensgebonden controle van energiemetabolisme beschreven, met een speciale focus op de controle van het lichaamsgewicht en de voedselinname bij de Djungarian hamsters, een soort die duidelijke seizoensveranderingen in reproductie en energiemetabolisme laat zien.

Bij mensen is het nog steeds de vraag of seizoensritmes aanwezig zijn, een duidelijk bewijs voor seizoensveranderingen in de hypothalame circuits die reproductie en metabolisme regelen, ontbreken nog steeds. In een recent review door Yoshimura *et al*.[288] werd echter beschreven dat veel fysiologische parameters seizoens afhankelijke veranderingen vertonen, met name de immuunrespons[289].

De ontdekking van het RF-amide systeem, nu ongeveer 20 jaar geleden, was een grote doorbraak in het begrip van het neuro-endocriene circuit dat de reproductieve activiteit in verschillende fysiologische en omgevingscondities regelt. Met name de hypothalame neuronen die de neuropeptiden kisspeptide en RFRP-3 tot expressie brengen, zijn cruciaal gebleken voor de regulatie van de hypothalamus-hypofyse-gonade as bij zoogdieren. Het gebruik van dieren met een duidelijk seizoens ritme biedt een unieke mogelijkheid om te begrijpen hoe voortplanting in de natuur wordt gecontroleerd, omdat het het mogelijk maakt om dieren te bestuderen die op verschillende tijdstippen van het jaar seksueel actief of inactief zijn zonder de noodzaak van genetische manipulaties om te induceren wat al endogeen gebeurt.

We hebben beschreven hoe het fototransductie mechanisme begint met het dagelijkse melatoninesignaal en eindigt met veranderingen in de lokale concentraties van T3 in de mediobasale hypothalamus. Hoewel we de exacte doelwitten van het T3 molecuul niet kennen, wijzen neuro-anatomische en fysiologische studies op een essentiële rol van zowel het kisspeptide als RFRP-3 bij seizoensgebonden reproductie. Bovendien blijkt inmiddels uit meerdere studies dat beide peptiden de voedselinname, het lichaamsgewicht en de energiehomeostase kunnen reguleren. Dit hoofdstuk eindigt met de hypothese dat RF-amiden betrokken zijn bij zowel de controle van de reproductie als de metabole activiteit, ze zouden daarom deel kunnen uitmaken van het mechanisme dat omgevingssignalen verbind met veranderingen in de reproductieve en metabole fysiologie gedurende het jaar.

In **Hoofdstuk 2** vonden we dat chronische (5 weken) centrale toediening van ofwel kisspeptide ofwel RFRP-3 het lichaamsgewicht van hamsters verhoogde die seksueel en metabool geremd waren door de korte een korte daglengte. RFRP-3 vertoonde hierbij een opmerkelijk klein concentratiebereik van werkzaamheid waar het de regulering van het lichaamsgewicht betreft. Dit effect was geslachtsafhankelijk, waarbij alleen mannen het lichaamsgewicht verhoogden. Verder lieten we zien dat het kisspeptide-effect op het lichaamsgewicht van de mannelijke hamster afhankelijk was van de verhoogde circulerende spiegels van de geslachtssteroïden geproduceerd door de gereactiveerde testis. Dit was niet het geval voor het RFRP-3 dat geen stimulerend effect had op de testisactiviteit.

Kijkend naar de expressie van metabole genen in de hypothalamus, ontdekten we dat het door kisspeptide geïnduceerde verhoogde lichaamsgewicht bij de mannelijke hamsters gepaard ging met een verhoogde Npy- en Pomc-expressie in de nucleus arcuatus na 5 weken centrale infusie. Daarentegen veranderde RFRP-3 de expressie van geen van de onderzochte

hypothalamische genen, maar het verhoogde wel de voedselinname en de circulerende niveaus van leptine en insuline.

Na deze studies waarin we rapporteerden dat chronische toediening van RF-amiden bij kortedag-aangepaste hamsters het lichaamsgewicht en de voedselinname verhoogden, maar zonder opvallende veranderingen in hypothalame genexpressie door RFRP-3, bestudeerden we in **Hoofdstuk 3** de mogelijke acute effecten van RFRP- 3 op voedselinname. We ontdekten dat RFRP-3 op een geslachtsafhankelijke manier orexigene effecten opwekte, waarbij het in vrouwlijke hamsters de voedselinname verhoogde tijdens zowel korte- als lange dag condities, maar in de lange dag condities alleen in het diestrus-stadium van de oestrus cyclus. De verhoogde voedselinname ging gepaard met een toename van de NPY-expressie in de korte dag condities. We stelden voor dat het orexigene effect van RFRP-3 afhankelijk is van geslachtssteroïd hormonen, aangezien de toename van de voedselinname alleen werd gevonden bij dieren met lage niveaus van E2 (d.w.z. in korte dag condities en tijdens diestrus).

In **Hoofdstuk 4** bestudeerden we de metabole effecten van kisspeptide en RFRP-3 in mannelijke Wistar ratten. We ontdekten dat centrale injecties van kisspeptide de voedselinname verlaagden bij ad libitum gevoede ratten. In tegenstelling tot onze verwachting verhoogde RFRP-3 de voedselinname niet zoals eerder gemeld voor de Sprague Dawley-ratten, maar het verhoogde de testosteronniveaus weliswaar in mindere mate in vergelijking met de kisspeptide-effecten. De afname van de voedselinname na kisspeptine ging gepaard met een afname van de respiratoire uitwisselingsratio, wat wijst op een verhoogde vetoxidatie. In de hypothalamus was POMC-immunokleuring in de achterste boogvormige kern verminderd na kisspeptide-injecties en verdere analyse toonde aan dat kisspeptine de mediane preoptische kern activeerde, een hersengebied waarvan bekend is dat het POMC-neuronale vezels ontvangt en de kernlichaamstemperatuur regelt. We hebben ook onderzocht of de centrale behandelingen met kisspeptide of RFRP-3 het glucosemetabolisme beïnvloedden, met name de endogene glucoseproductie. We ontdekten dat een 2 uur durende centrale infusie van kisspeptine de HPG-as activeerde, zoals blijkt uit verhoogde circulerende niveaus van luteïniserend hormoon en testosteron. RFRP-3 veroorzaakte geen activering van de voortplantingsas. Beide RF-amiden verhoogden de endogene glucoseproductie zonder de systemische glucosespiegels te wijzigen, hoewel de RFRP-3-effecten milder waren dan die van kisspeptine. De circulerende niveaus van insuline en corticosteron werden niet beïnvloed. We concludeerden dat kisspeptide en RFRP-3 de voedselinname en het glucosemetabolisme beïnvloeden, waarbij het krachtigere effect van kisspeptide mogelijk te wijten is aan de verhoogde productie van geslachtssteroïden.

Naast daglengte is ook de omgevingstemperatuur een factor die duidelijke seizoens fluctuaties laat zien. In **Hoofdstuk 5** hebben we onderzocht of 3 uur blootstelling aan een koude (4 ° C) omgeving de genexpressie van de hypothalame RF-amiden en enkele “metabole” neuropeptiden kon wijzigen en of deze effecten afhankelijk zouden zijn van het tijdstip van de dag. Hoewel RFRP en kisspeptide geen dagelijkse ritmiek vertoonden, verminderde blootstelling aan koude hun mRNA-expressie alleen tijdens de lichtfase. NPY- en POMC-genexpressie in de nucleus arcuatus vertoonden een significante dagelijkse ritmiek, en NPY-expressie was verhoogd na 3 uur blootstelling aan koude, afhankelijk van het tijdstip van de dag. Ten slotte verhoogde blootstelling aan koude ook de TRH-expressie in de paraventriculaire kern, maar niet in het mediale pre-optische gebied.

Conclusies

Concluderend heeft ons werk aangetoond dat RF-amiden het energiemetabolisme kunnen reguleren in zowel seizoensgebonden als niet-seizoensgebonden diermodellen. Centrale behandelingen bij de Djungarian-hamster veroorzaakten een toename van het energiemetabolisme, vooral in de richting van een lente-zomer achtig fenotype, ondanks dat de dieren gehuisvest waren onder remmende korte dag condities. Ook in de niet-seizoensgebonden mannelijke Wistar-rat, beïnvloedden de RF-amiden de voedselinname en het glucosemetabolisme. RF-amiden zijn dus niet alleen in staat om de metabole en reproductieve activiteit te moduleren, maar ook om externe informatie zoals als veranderingen in de daglengte en de omgevingstemperatuur te integreren. Deze resultaten ondersteunen onze hypothese dat RF-amiden een essentiële rol spelen bij het coördineren van veranderingen in reproductie en metabolisme als reactie op seizoensafhankelijke veranderingen in de omgeving.

RÉSUMÉ

L'objectif général de cette thèse était d'évaluer le rôle possible des RF-amides comme lien entre le contrôle hypothalamique de la reproduction et du métabolisme.

L'analyse de la littérature présentée au **chapitre 1**, nous a permis de faire un état des lieux de nos connaissances sur le contrôle saisonnier du métabolisme, avec un accent particulier sur le poids corporel et le contrôle de la prise alimentaire chez les hamsters Djungarien, une espèce qui montre des changements saisonniers clairs dans la reproduction et le métabolisme énergétique.

Chez l'homme, l'existence de rythmes saisonniers est encore discutée, et des preuves de changements saisonniers dans les circuits hypothalamiques contrôlant la reproduction et le métabolisme font défaut. Pourtant, une étude récente de Yoshimura *et al.*[288] a décrit que de nombreux paramètres physiologiques présentent des changements saisonniers, notamment la réponse immunitaire[289].

La découverte du système RF-amide il y a environ 20 ans a été une avancée majeure dans la compréhension du circuit neuroendocrinien, contrôlant l'activité de reproduction dans différentes conditions physiologiques et environnementales. Notamment, les neurones hypothalamiques exprimant les neuropeptides kisspeptine et RFRP-3 se sont avérés essentiels pour la régulation de l'axe hypothalamo-hypophyso-gonadique chez les mammifères. L'utilisation d'animaux saisonniers offre un moyen unique de comprendre comment la reproduction est contrôlée dans la nature, car elle permet d'étudier des animaux sexuellement actifs ou inactifs à différents moments de l'année sans avoir besoin de manipulations génétiques pour induire ce qui se passe déjà de manière endogène.

Nous avons décrit comment la phototransduction commence avec le signal journalier de mélatonine et se termine par des changements dans les concentrations locales de T3 dans l'hypothalamus médiobasal. Même si nous ne connaissons pas les cibles précises pour cette molécule, des études neuroanatomiques et physiologiques indiquent que la kisspeptine et le RFRP-3 jouent un rôle essentiel dans la reproduction saisonnière. De plus, un nombre croissant d'études indique que ces deux peptides peuvent réguler la prise alimentaire, le poids corporel et l'homéostasie énergétique. Ce chapitre se termine par l'hypothèse que les RF-amides, étant impliqués à la fois dans le contrôle de la reproduction et de l'activité métabolique, pourraient faire partie du lien entre les signaux environnementaux et les changements de la physiologie reproductive et métabolique au cours de l'année.

Dans le **chapitre 2**, nous avons constaté que l'administration centrale chronique (5 semaines) de kisspeptine ou de RFRP-3 augmentait le poids corporel des hamsters photo-inhibés sexuellement et métaboliquement. Il est à noter que RFRP-3 a montré une petite plage de concentration efficace sur la régulation du poids corporel. Cet effet était dépendant du sexe, seuls les mâles augmentant leur poids corporel. Nous avons en outre démontré que l'effet kisspeptine sur le poids corporel du hamster mâle dépendait de l'augmentation des niveaux de stéroïdes sexuels circulants produits par les testicules réactivés. Alors que ce n'était pas le cas pour l'effet RFRP-3 qui lui, n'avait aucun effet stimulant sur l'activité des testicules.

En examinant l'expression des gènes métaboliques dans l'hypothalamus, nous avons constaté que l'augmentation du poids corporel induite par la kisspeptine chez les hamsters mâles s'accompagnait également d'une augmentation de l'expression de *Npy* et *Pomc* dans le noyau arqué après 5 semaines de perfusion centrale. En revanche, RFRP-3 n'a modifié l'ex-

pression d'aucun des gènes hypothalamiques étudiés, mais il a augmenté la prise alimentaire et les niveaux circulants de leptine et d'insuline.

Après ces études rapportant que l'application chronique de RF-amides chez des hamsters adaptés aux jours courts augmentait le poids corporel et l'apport alimentaire, mais sans changements notables dans l'expression du gène hypothalamique pour RFRP-3, dans le **chapitre 3**, nous avons étudié plus en détail les effets aigus possibles de RFRP- 3 sur la prise alimentaire. Nous avons constaté que RFRP-3 provoquait des effets orexigènes de manière dépendante au sexe : les femelles augmentant leur prise alimentaire à la fois en photopériode courte et longue, mais seulement au stade diestrus du cycle oestral sous photopériode longue. L'augmentation de la prise alimentaire s'accompagnait d'une augmentation de l'expression de NPY en photopériode courte. Nous avons émis l'hypothèse que l'effet orexigène de RFRP-3 dépend des hormones stéroïdes sexuelles, car l'augmentation de la prise alimentaire n'a été trouvée que chez les animaux qui avaient de faibles niveaux d'E2 (c'est-à-dire en photopériode courte et pendant le diestrus).

Dans le **chapitre 4**, nous avons étudié les effets métaboliques de la kisspeptine et du RFRP-3 chez des rats Wistar mâles. Nous avons constaté que les injections centrales de kisspeptine diminuaient la prise alimentaire chez les rats nourris à volonté. Contrairement à nos attentes, le RFRP-3 n'a pas augmenté l'apport alimentaire comme indiqué précédemment pour les rats Sprague Dawley, mais il a augmenté les niveaux de testostérone dans une moindre mesure par rapport aux effets de la kisspeptine. La diminution de la prise alimentaire après la kisspeptine s'accompagnait d'une diminution du rapport d'échange respiratoire, indiquant une augmentation de l'oxydation des graisses. Dans l'hypothalamus, l'immunocoloration POMC dans le noyau arqué postérieur a été réduite après des injections de kisspeptine et une analyse plus approfondie a montré que la kisspeptine activait le noyau préoptique médian, une zone cérébrale bien connue pour recevoir les fibres neuronales POMC et pour contrôler la température corporelle centrale. Nous avons également étudié si les traitements centraux avec la kisspeptine ou le RFRP-3 influençaient le métabolisme du glucose, en particulier la production endogène de glucose. Nous avons alors constaté qu'une perfusion centrale de kisspeptine de 2 heures activait l'axe HPG, comme indiqué par l'augmentation des taux circulants d'hormone lutéinisante et de testostérone. En rechance, RFRP-3 n'a pas provoqué d'activation de l'axe reproducteur. Les deux RF-amides ont augmenté la production de glucose endogène sans modifier les taux de glucose systémiques, bien que les effets du RFRP-3 aient été plus légers que ceux de la kisspeptine. Les taux circulants d'insuline et de corticostérone n'ont, quant à eux, pas été affectés. Nous avons conclu que la kisspeptine et le RFRP-3 affectent la prise alimentaire et le métabolisme du glucose, l'effet plus puissant de la kisspeptine étant probablement dû à la production accrue de stéroïdes sexuels.

À côté de la photopériode, la température environnementale est un autre facteur qui montre des fluctuations saisonnières claires. Dans le **chapitre 5**, nous avons examiné si une exposition au froid (4 degrés Celsius) pendant 3 heures était capable de modifier l›expression des gènes des RF-amides hypothalamiques et de quelques neuropeptides et, le cas échéant, si ces effets dépendent de l'heure de la journée. Même si l'expression de RFRP et de kisspeptine n'ont montré aucune rythmicité quotidienne, l'exposition au froid a diminué leur expression d'ARNm pendant la phase lumineuse. En outre, l'expression des gènes NPY et POMC dans le noyau arqué a montré une rythmicité quotidienne significative, et l'expression du NPY a été augmentée après 3h d'exposition au froid en fonction du moment de la journée. Enfin, l'exposition au froid a également augmenté l'expression de la TRH dans le noyau paraventriculaire, mais pas dans l'aire pré-optique médiale.

Conclusions

En conclusion, nos travaux ont démontré que les RF-amides régulent le métabolisme énergétique dans les modèles animaux saisonniers et non saisonniers. Les traitements centraux chez le hamster Djungarien ont provoqué une augmentation du métabolisme énergétique davantage vers un phénotype printemps-été, malgré le fait de vivre sous le signal photopériodique inhibiteur. Concernant le rat Wistar mâle non saisonnier, les RF-amides ont également modifié la prise alimentaire et le métabolisme du glucose. Ainsi, les RF-amides sont capables non seulement de réguler les activités métabolique et reproductrice, mais aussi d'intégrer des informations externes comme des changements de photopériode et une baisse de la température ambiante. Ces résultats renforcent notre hypothèse d'un rôle essentiel des RF-amides dans la coordination des contrôles reproducteurs et métaboliques, en réponse aux changements environnementaux.

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PhD PORTFOLIO

Fernando Cázarez Márquez

PhD Period: November 2014 - November 2021

PhD supervisors: dr. Valérie Simonneaux, prof. dr. Andries Kalsbeek

Courses	Year
ImageJ and Fiji use for histological image analysis, Unistra, Strasbourg France	2015
Animal experimentation course, Unistra, Strasbourg France	2016
Surgery of rodents, Unistra, Strasbourg France	2016
Seahorse training, AMC, Amsterdam, the Netherlands	2019

Oral presentations	Year
• 3rd Neurotime meeting, Basel, Switzerland	2015
"Interactions of reproduction and energy metabolism: a round way trip via RF-amide peptides"	
• 4th Neurotime meeting, Strasbourg, France	2016
"RFRP-3 metabolic effects, say hello to seasonal reproduction from the other side"	
• 45th SFC, Strasbourg, France	2016
"Kisspeptin and RFRP3 neuropeptides: from reproduction to metabolism"	
• 5th Neurotime meeting, Amsterdam, the Netherlands	2017
"From Reproduction to Metabolism: Kisspeptin and RFRP3"	
• EBRS meeting, Amsterdam, the Netherlands	2017
"Kisspeptin and RFRP3 neuropeptides: from reproduction to metabolism"	
• 42nd SNE meeting, Dijon, France	2017
"Kisspeptin and RFRP3 modulate lean and fat mass in the *Phodopus Sungorus* via two different hypothalamic neuronal pathway"	
• Annual Dutch Diabetes Research Meeting, Oosterbeek, the Netherlands	2017
"Metabolic effects of Kisspeptin and RFRP3"	

• Dutch Endocrinology meeting 2018, Noordwijk, the Netherlands	2018
"Kisspeptin/RFRP and ON/OFF switch for reproduction and metabolism"	
• Dutch Neuroscience meeting 2019, Lunteren, the Netherlands	2019
"Hypothalamic Kisspeptin and RFRP3: Reproduction, Seasonality and wait… metabolism?"	
• AGEM 2020 PhD retreat, webinar	2020
"Central kisspeptin and not RFRP-3 affect energy metabolism in the male Wistar rat"	
• Kisspeptin meeting 2021, webinar	2021
"Role of central Kisspeptin and RFRP-3 in energy metabolism in the male Wistar rat"	
• AGEM 2021 PhD retreat blitz, webinar	2021
"Cold around the clock and how it affects the hypothalamus"	

Poster presentation	**Year**
• EBRS meeting, Manchester, UK	2015
"The effect of RFRP-3 on food intake in Siberian hamster in two different photoperiodic conditions in male and female"	
• 40th SNE/BSN meeting, Lille France	2015
"The effect of RFRP-3 on food intake in Siberian hamster in two different photoperiodic conditions in male and female"	
• International Chronobiology Summer School, Beijing, China	2016
"Kisspeptin displays sex-dependent metabolic and reproductive effects in a seasonal rodent"	
• SNE/CSN meeting Corte, France	2016
"Kisspeptin and RFRP3 neuropeptides: from reproduction to metabolism"	
• NeuroFrance, Bordeaux, France	2017
"Kisspeptin and RFRP3 neuropeptides: from reproduction to metabolism"	
• SRBR, Amelia Island plantation USA	2018
"Hypothalamic reproduction circuits also regulate body mass in the Djungarian hamster *Phodopus sungorus*"	
• International Congress of Neuroendocrinology, Toronto Canada	2018
"Kisspeptin and RFRP3 modulate body mass in the *Phodopus sungorus* via different hypothalamic pathways"	

• Muscle clocks and diabetes, Amsterdam, the Netherlands	2019
"Hypothalamic RFamide peptides affect Energy metabolism and Endogenous Glucose Production"	
• EBRS meeting, Lyon, France	2019
"RFRP3 increases food intake in a sex dependent manner in the seasonal *Phodopus sungorus*"	
• Annual Dutch Diabetes Research Meeting, webinar	2020
"PPARdelta in hypothalamic microglial controls glucose metabolism and insulin sensitivity"	

Tutoring	**Year**
• Technical highschool internship - Maja Meskovic (3 months)	2017
• Erasmus master internship - Simone Pelizzari (7 months)	2019
• Master Internship - Moqiu Jia (2 months)	2020

Parameters of esteem

Grants	**Year**
• Travel Grant: 40th SNE meeting 2015, Lille France. Fondation Obelisque	2015
• Travel Grant: 41st SNE meeting, Corte, France Fondation Obeslisque	2016
• Travel Grant: International Chronobiology Summer School, Beijing China. Provided by the SRBR	2016
• Travel Grant 42nd SNE meeting 2017, Dijon, France, Foundation Obelisque	2017
• Travel Grant Summer school "Basic and clinical aspects of neurobiology of rhythms", Strasbourg France	2017
• Global Diversity fellowship, SRBR	2018
• Travel Grant ICN, Toronto Canada, SNE and Fondation Obelisque	2018

Others	**Year**
Scientific theater play – Science communication "*Neurones et Chatiments*"	2015
French Foreigner Language Advanced level, Unistra, France	2015

PUBLICATIONS

Peer reviewed in this thesis

Cázarez-Márquez F, Milesi S, Laran-Chich M-P, Klosen P, Kalsbeek A, Simonneaux V. Kisspeptin and RFRP3 modulate body mass in *Phodopus sungorus* via two different neuroendocrine pathways. *J Neuroendocrinol.* 31(4):e12710 (2019)

Cázarez-Márquez F, Laran-Chich M-P, Klosen P, Kalsbeek A, Simonneaux V. RFRP3 increases food intake in a sex-dependent manner in the seasonal hamster *Phodopus sungorus*. *J Neuroendocrinol.* 32, 1–9 (2020)

Cázarez-Márquez F, Eliveld J, Ritsema WIGR, et al. Role of central kisspeptin and RFRP-3 in energy metabolism in the male Wistar rat. *J Neuroendocrinol*. 00:e12973 (2021)

Peer reviewed (not in this thesis)

N. Saderi, **F. Cázarez-Márquez,** f. N. Buijs, R. C. Salgado-Delgado, m. A. Guzman-Ruiz, m. Del Carmen Basualdo, C. Escobar and R. M. Buijs. The NPY intergeniculate leaflet projections to the suprachiasmatic nucleus transmit metabolic conditions. *Neuroscience*. 246:291-300 (2013)

Guzmán-Ruiz M., Saderi N., **Cázarez-Márquez F**, Guerrero-Vargas NN, Basualdo CM, Acosta-Galván G and Buijs RM. The Suprachiasmatic Nucleus changes the daily activity of the Arcuate Nucleus <alpha>-MSH neurons in male rats. *Endocrinology*. 155(2):525-235 (2014)

Buijs FN, **Cázarez F**, Basualdo MC, Scheer FAJL, Perusquia M, Centurion D and Buijs RM. The suprachiasmatic nucleus is part of a neural feedback circuit adapting blood pressure response. *Neuroscience*. 266: 197-207 (2014)

Báez-Ruiz A, Guerrero-Vargas NN1, **Cázarez-Márquez F**, Sabath E, Basualdo MDC, Salgado-Delgado R, Escobar C, Buijs RM. Food in synchrony with melatonin and corticosterone relieves constant light disturbed metabolism. *J Endocrinol.* 235(3):167-178 (2017)

ACKNOWLEDGEMENTS

First, thank you very much to my supervisors Dr Valerie Simonneaux and Dr Andries Kalsbeek, without you, this thesis would have not been possible.

Chère **Valérie**, je suis tellement ravi d'avoir eu l'opportunité de travailler chez vous. Grâce à vous que je me suis ressenti très bien accueilli, pas seulement à l'INCI dans votre équipe, mais aussi en France, même quand j'étais Pedro pour vous pendant quelques mois. Sans le savoir, j'ai rencontré la passion et l'intérêt pour la physiologie saisonnière de la reproduction, les hormones sexuelles et ses effets sur le métabolisme. Merci pour tout le support que vous m'avez apporté pendant ces 7 ans.

Beste **Dries**, hartelijk dank u wel. I still remember the first talk we had at the SfN in San Diego, when you explained to me a student's poster using the model with 6 meals during the day. Since then, but probably already before, I knew that no matter what, I should come to your lab, to learn from you as much as I could about circadian rhythms and their importance and connections with energy metabolism. Thanks as well for all the support you have given to me, not only in my professional career but in personal aspects. Het was erg mooi en gezellig! Thank you especially for the help and support in the last period in Amsterdam with the pandemics.

To all the committee members, thank you very much for reading our thesis, **Dr Chun-Xia Yi**, **Dr Marie Paule Felder-Schmittbuhl, Dr Julie Bakker, Dr Frederic Simonin, Dr Eric Fliers** and **Dr Roelof Hut**.

Thanks a lot, to **Dr Paul Pevet** and **Dr Domitille Boudard**, for creating and taking care of the Neurotime Erasmus+ program, with most of us coming from developing countries. Along this program I was not only living in two labs in two European countries, but also making friends (neurotimers and non-neurotimers) from all around the world. Chronobiology rules!

Thanks to the NBR for all the fruitful discussions we had and the daily life, especially to **Dr Paul Klosen**, **Dr Jorge Mendoza**, **Dr Dominque Ciocca**, **Dr Béatrice Bothorel, Dr Nadia Mazzaro**, and **Dr Etienne Challet**. Thanks to **Dr Frederic Simonin** and **Dr Patrick Vuillez** for taking part in the Thesis Follow-Up-Committee.

To all the former Valerie's (or NBR) students and now Doctors: **Rajae, Jo, Cristina, Thibault, Sebastien, Nora, Hassan, Eleni, and Clarisse**. And to the current doctors in the making: **Marie-Azélie, Marine, Guillaume**. Merci beaucoup **Perrine**, la pépette de mon cœur !

To my Neurotime siblings: **Aurea, Jacob, Mili, Shery, Seb, Meltem, Nemanja, Udita, Anayanci, Jiahao, XiaoLan, Seb, Satish, Orkan, Jim, Pawan, Muzeyen, Lamis.**

Para la mafia Latina del INCI: **Cathy, Nuria, Álvaro, Dani y Aurea**. Fue un fantástico placer coincidir con todos ustedes dentro y fuera del laboratorio, son maravillosos chicos ¡los quiero!

Pour les travailleurs du Chronobiotron : **Aurore, Nico, Olivier et Bruno.** Merci beaucoup pour votre aide, c'était très amusent de travailler avec vous tous, merci beaucoup pour votre responsabilité et professionnalisme.

To Dries' team in Amsterdam: **Anneloes, Paul, Martin, Nikita**. It was a lot of fun knowing not only the lab life but also Dutch lifestyle together with you. To my three students in there: **Maja, Simone and Moqiu**. I enjoyed and appreciated very much your engagement and com-

promise with the projects we ran together. I hope I was able to teach you some of the good things my mentors have taught me as well.

To the animal facility staff at IWO: **Natasha, Rainier, Jacques,** thanks a lot for the daily help, thanks for your kindness and efficiency on caring the animals, and it was always fun to talk with you.

To the people at the Endo lab: **Dr Susanne LaFleur**, **Dr Anita Boelen**, and **Dr Annemieke Heijboer, Khalid, Charlene, Joram, Myrtille, Leslie, Cecilia, Mariette** and **Yvonne. Unga**, even when we did not interact too much, we did not need to, your unprecedent efficiency clearly was enough for us to do what we wanted to do; also, thanks for the suggested places to visit around Amsterdam, I loved them all! **Li**, Oh my god Madame Li, Gaat goed?! I am going to miss you so much, but I wish you the best of the best for your retirement, you are the smartest and sweetest person ever. To **Lin** and **Rawien** from Dick Swaab's lab, it was a pleasure sharing time with you, it was always fun. Thanks, you saved my experiments several times. Thank you very much **Joop**, you were extra helpful with all the microscopy and automatized analysis.

To the whole Yi group, thanks for receiving me in the microglia team: **Irina** hold in there m8, you are almost there. **Shanshan** you are a fantastic colleague and an amazing person to work with, thanks for all your teaching and the nice children's poetry in Chinese you shared with me. Pra o **Felipe**, muito obrigado, it was a real pleasure meeting you! Please continue being as you are, I am sure we will stay in touch for long time in either science or in our personal lives. **Chun-xia**, thanks for trusting me scientifically, it meant a lot to me; and thank you for the poinsettias that made me think of my grandma's yard.

Dany y **Ewout** and **Glia**, thanks a lot! especially for the last months when we experienced together in Amsterdam most of the pandemic's situation, I was so happy that we were sharing time in the lab and delicious dinners with you. Ewout you are a better Mexican cook than many of us.

To **Dr Ruud Buijs** and **Dr Carolina Escobar**. Your passion for neuroscience and chronobiology is just contagious. You are the professional and scientific reason of why I started and now ended up following a scientific career. Thanks a lot for seeding curiosity and teaching us how to have a critical mind, to trust our eyes and data and to always ask ourselves why things are happening. To all the friends in the chronobiology lab from UNAM I made along the years: **Liz, Luis, Estefa, Roberto, Adrián, Sofía, Arlen, Melina, Eva, Betty**. **Mara**, mi querida Dra. Mara, eres una doctora fregonsísima, gracias por todo el apoyo, por tu amistad y la inspiración que has sido para mí durante esto años. **Nat**, mil gracias por todo, trabajar juntos, sufrir con el LPS y las ratitas, pero también por las fiestas y los buenos momentos. **Nadia**… (momento de silencio con una sonrisa dibujada en mi cara… seguro se activó algo en el sistema autónomo) nos conocimos en 2009 y hoy aquí estamos. Te mando un fuerte abrazo y un beso querida Nadia, sé que nos veremos pronto, molto grazzie. **MariCarmen**$^{+}$, con mucho cariño te dedico esta tesis y no te preocupes, nunca salgo del laboratorio sin lavar mi taza antes.

To **Freddy Buijs**, my brother from another mother! More than thank words, we should rather meet in real life and toast, we made it Freddy! Saca las chelas, o los vinos, up to you!

Extiendo también un sincero agradecimiento a las Dras: P**atricia Joseph Bravo, Rosa María Uribe Villegas, Mariana Gutiérrez Mariscal** y **Edith Sánchez Jaramillo**, por su eterna paciencia e incondicional disposición para hacer ciencia. Gracias a que tuvieron la paciencia y

dedicación para enseñarme *in situs* radioactivas, es que años después se ha logrado gran parte de esta tesis doctoral.

To both of my paranymphen: Merci beaucoup **Marie-Pierre**, tu t'es devenu pas seulement une collègue mais je garde notre amitié très précieusement. Tu m'as appris à travailler d'une manière incroyable. Tu étais très stricte depuis le début, et je suis sûr que c'est grâce à ça qu'on a réussi dans cette thèse. Ensemble, nous avons pu faire des manips les jours, les soirs, même les WEs. En plus, tu m'as aidé au niveau personnel dans tout genre des situations, avec la CAF, la CPAM, des autres assurances, et j'ai gardé mon genou et toujours ma vie sportif grâce à toi, je ne saurais comme te remercier. **Wayne**, thank you very much hermano. We started to work almost at the same time in Amsterdam at Dries' group, learning new things together and close… sometimes too close, but still fun of course, of course. I appreciate the time we spent when doing all the experiments! It was easier with you around to have a gezellige time! Always nice to learn from you and your exceptional time-wise organizational skills and the nice vibes you carry with you. Salamat Wayne!

Outside of the lab I cannot miss thanking to the other part of my life, my taekwondo people. Profesores: **Mario Juárez Santiago**, **Mario Eduardo Sánchez Acencio**, **Carlos Escobar Noriega** y **José Sámano Hernández**, todos ustedes fueron parte esencial de mi aprendizaje marcial, en Angelopolis TKD, TOM Morelos y PUMAS TKD. Pour tous dans le taekwondo Koryo et maintenant ESTKD, merci beaucoup spécialement aux maîtres **Frederic Schmidt** et **Clément Rauscher**. In Amsterdam, thanks to all my friends from Black Bear Taekwondo, particularly to the trainers: **Bas** and **Tim Wind**, **Kama Wlodzimirow**, **Herry Tsui**, **Nick Verboom** and **Marvin Blagrove**.

A mis amigo de congales y travesías en Ámsterdam, **Ale**, **Alan** y **Kari**, los quiero mucho y salucita. **Rory**, one of my few NIN friends in and out of the lab, thank you for your honest friendship.

Finalmente, pero no por eso menos importante, a doña **Tere**, a don **Pablito**, mis hermanos **Adry** y **Rolo**, **Dany** y **Samu** y a **Chris**, los quiero mucho y los extraño cada día. Ya organicemos otro viajecito, ¿no? También muchas gracias a mis tíos y a mis abuelitos en paz descansen, a mi **Chanita** y a don **Carlitos**$^{+}$, mamita **Ana**$^{+}$ y papito **Lute**$^{+}$ quien estoy seguro está muy orgulloso de todos nosotros.

A mi familia García Sanchéz, **Inés**, **Manuel** y **Alicia**, muchas gracias por todo, los quiero. **Manu**, 5 años de estar para acá y para allá, de alegrías y tristezas y de abajo para arriba, una y otra vez, de Alsacia a Ámsterdam y de regreso, qu'est ce que je peux te dire d'autre, Je t'aime !

ABOUT THE AUTHOR

Fernando was born and raised in Zacatlán, Puebla. He has a technical high school degree in electricity and electronics. Somehow, he studied biology at the faculty of Science in the Universidad Nacional Autónoma de México (UNAM). He obtained a Master's deegree in Biochemistry at the Faculty of Chemistry at UNAM. He moved to Europe to pursuit a PhD degree at the UniStra in France and the UvA in the Netherlands.

He enjoys very much physical activities outside and after many years of practicing martial arts, he has become a taekwondo trainer in Amsterdam and now he might be doing the same activity while back in Strasbourg.

Fernando just moved with Manu to an apartment in Illkirch-Graffenstaden were they both enjoy very much of the biking routes and the nature from the area.

Université de Strasbourg

Fernando CÁZAREZ-MÁRQUEZ

École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

Intégration de la reproduction et du métabolisme énergétique : un voyage aller-retour des peptides RF-amides

Résumé

La reproduction est un processus qui nécessite une état métabolique approprié pour assurer le développement et la survie correcte de la progéniture. Dans la nature, les animaux adaptent et anticipent les conditions environnementales afin de coordonner leurs approvisionnements énergétiques, avec le moment de la gestation et la naissance des petits. Nous savons maintenant que le contrôle de la reproduction, de la prise alimentaire et du poids corporel est situé dans l'hypothalamus. Deux populations spécifiques de neurones exprimant les peptides RF-amides, kisspeptine et RFRP-3, sont en amont du contrôle de l'axe hypothalamo-hypophysaire gonadique. Au cours des 16 dernières années, des travaux ont montré que ces RF-amides sont non seulement impliqués dans la reproduction mais aussi dans le contrôle de la prise alimentaire et du poids corporel. Nous avons étudié les effets de ces deux peptides sur le métabolisme énergétique dans deux modèles animaux : le hamster Djungarian dont la reproduction est saisonnière et le rat Wistar. Dans l'ensemble, nous avons constaté que l'administration des RF-amides dans le système nerveux central impacte le métabolisme énergétique des deux espèces. Les hamsters Djungarian présentent des changements dans la prise alimentaire et le poids corporel, principalement avec un effet majeur dans le tissu adipeux, alors que les rats Wistar modifient leur prise alimentaire, leur métabolisme lipidique et leur homéostasie du glucose. Par ailleurs, nous avons observé chez le rat que l'expression des RF-amides est influencé par l'environnement froid et les adaptations saisonnières métaboliques sont atténuées par un régime obésogène chez les hamsters. Avec cette thèse, nous confirmons que la kisspeptine et le RFRP-3, originalement décrits pour leur implication dans le contrôle de la reproduction, sont également impliqués dans la régulation centrale du métabolisme énergétique, ce qui renforce notre hypothèse que ces peptides jouent un rôle dans la coordination centrale des fonction s reproductives et métaboliques.

Mots clés : RF-amides, métabolisme énergétique, saisonnalité, homéostasie du glucose, poids corporel, apport alimentaire

Summary

Reproduction is a process that requires a tight and appropriate metabolic status to ensure the correct development and survival of the offspring. In nature, animals match and anticipate the environmental conditions with their energy supplies and the timing of gestation and delivery. We now know that the control of reproduction, food intake and body weight is centrally located in the hypothalamus. Two specific populations of RF-amide peptide-producing neurons, Kisspeptin and RFRP-3, are upstream to the control of the hypothalamic pituitary gonadal axis. In the last 16 years, these RF-amides have also been implicated in the control of food intake and body weight regulation. We studied the effects of these two peptides on energy metabolism in two animal models: the seasonal reproductive Djungarian hamster and the Wistar rat. Overall, we found clear effects of the RF-amides in the central nervous system on energy metabolism in both species. Djungarian hamsters showed changes in food intake and body weight, mainly in the adipose tissue, whereas the Wistar rats changed their food intake, lipid metabolism and glucose homeostasis. In addition, we showed that the hypothalamic expression of both RF-amides is also influenced by cold environment in rats and that metabolic seasonal adaptations are dampened by an obesogenic diet in the hamsters. With this thesis we confirm that kisspeptin and RFRP-3, originally described for their implication in the control of reproduction, are also involved in the central regulation of energy metabolism, which reinforces our hypothesis that these peptides play a role in the central coordination of reproductive and metabolic functions.

Key words: RF-amides, energy metabolism, seasonality, glucose homeostasis, body weight, food intake