



The roles of RFRP in the central control of reproduction : photoperiodic and sex-specific differences

Jo Beldring Henningsen

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Jo Henningsen

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Rôle du RFRP dans le contrôle central de la reproduction
saisonnière en fonction du sexe et de la photopériode.

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PhD Thesis

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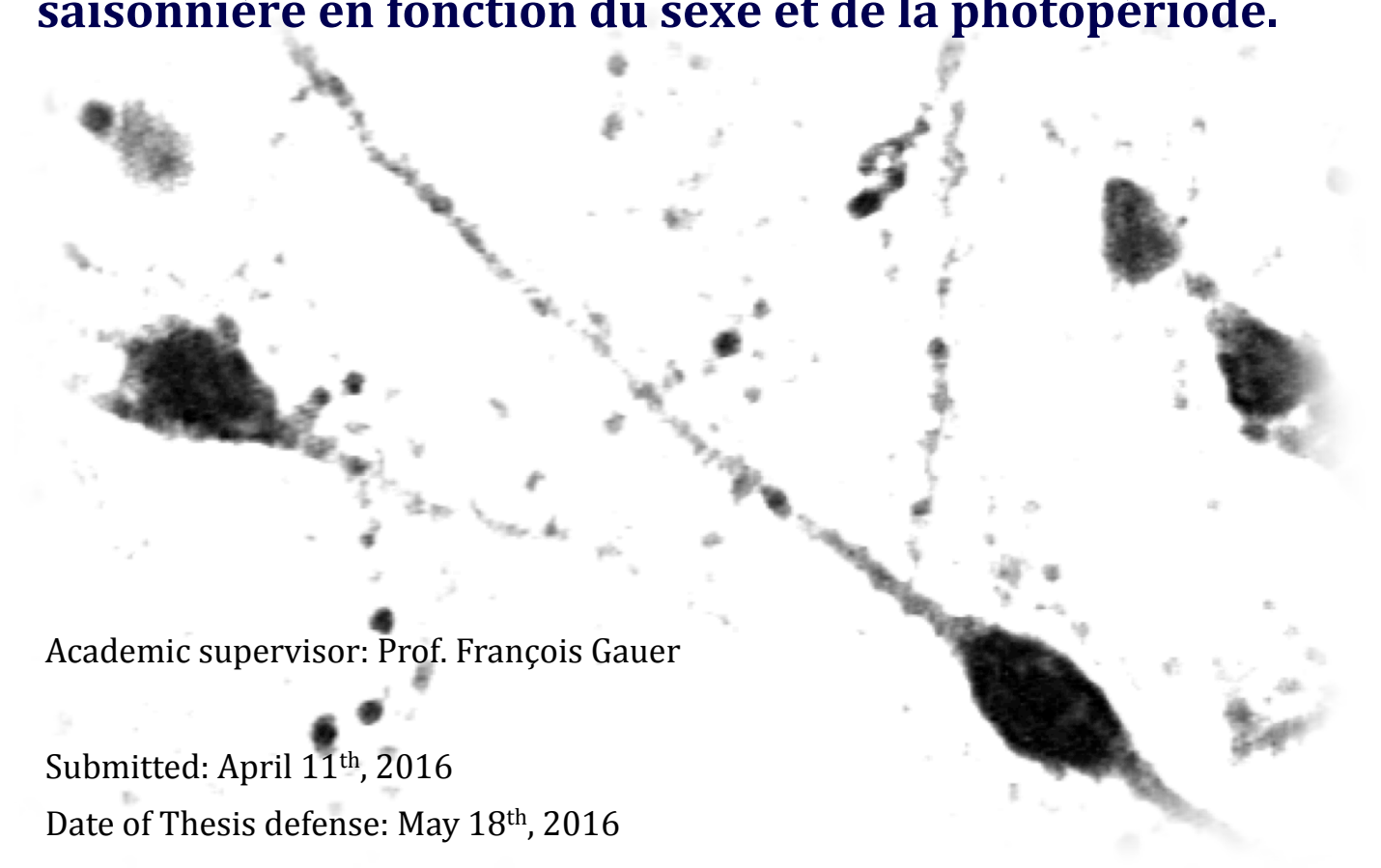
The roles of RFRP in the central control of reproduction: photoperiodic and sex-specific differences.

Rôle du RFRP dans le contrôle central de la reproduction saisonnière en fonction du sexe et de la photopériode.

Academic supervisor: Prof. François Gauer

Submitted: April 11th, 2016

Date of Thesis defense: May 18th, 2016



PREFACE

The present thesis is based on experimental work, conducted as part of the Neuroscience PhD program, at the Faculty of Life Sciences, University of Strasbourg, France, in the period October 2012 – May 2016. The work was primarily performed in the group of Melatonin and Seasonal Rhythms, at the Institute of Cellular and Integrative Neurosciences, University of Strasbourg, under the supervision of Prof. François Gauer and with daily guidance from Dr. Valérie Simonneaux. Parts of the project have been carried out in the laboratory of Prof. Jens Damgaard Mikkelsen at the Neurobiological Research Unit, Rigshospitalet, University of Copenhagen, Denmark.

The thesis has mainly been financed by a three-year grant from the Initiative d'excellence (IdEx). I have in this period been a member of the European Doctoral College of the University of Strasbourg, as part of the Nelson Mandela cohort from year 2012, and have therefore followed additional specific courses, concerned with topics of general European interests, presented by experts on the topics. The European Doctoral College has moreover financially supported me during the last part of my studies, by accepting me as a candidate for receiving a three-month prolongation grant.

The work has lead to the preparation and publication of the following manuscripts;

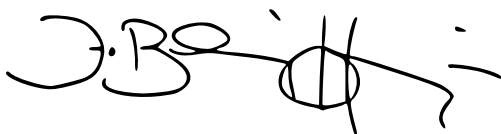
Simonneaux V, Bahougne T, **Henningsen JB**, Beymer M (2016). Role of kisspeptin and RFRP on the circadian control of female reproduction. Review in preparation for submission to Elsevier; special issue: Signaling in the hypothalamus- new concepts for Molecular and Cellular Endocrinology.

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ABBREVIATIONS

aa:	amino acid	ipRGC:	intrinsically photoreceptive RGC
AAAD:	aromatic amino acid decarboxylase	ir:	immunoreactive
AA-NAT:	aralkylamine N-acetyltransferase	ISH:	in situ hybridization
aCSF:	artificial cerebrospinal fluid	iv:	intravenous
ACTH:	adrenocorticotrophic hormone	KO:	knock out
AH:	anterior hypothalamus	Kp:	kisspeptin
ANOVA:	analysis of variance	LH:	luteinizing hormone
AR:	androgen receptor	LHRH:	luteinizing hormone-releasing hormone
ARC:	arcuate nucleus	LP:	long photoperiod
AVP:	arginine vasopressin	MAPK:	mitogen activated protein kinase
AVPV:	anteroventral periventricular nucleus	MBH:	mediobasal hypothalamus
BBB:	blood brain barrier	ME:	median eminence
BNST:	bed nucleus of the stria terminalis	MEL:	melatonin
BSA:	bovine serum albumin	MPN:	medial preoptic nucleus
cAMP:	cyclic adenosine monophosphate	mRNA:	messenger RNA
CNS:	central nervous system	MS:	medial septum
CRH:	corticotropin-releasing hormone	NA:	noradrenaline
DAB:	diaminobenzidine	NAS:	N-acetyl serotonin
DAG:	diacylglycerol	NE:	norepinephrine
Dio:	diodinase	NKB:	neurokinin B
Dyn:	dynorphin	NPFF:	neuropeptide FF
DMH:	dorsomedial hypothalamus	NPY:	neuropeptide Y
E2:	estradiol	OVLT:	organum vasculosum of the lamina terminalis
ER:	estrogen receptor	OVX:	ovariectomized
ERK:	extracellular signal-regulated kinase	PACAP:	pituitary adenylate cyclase-activating polypeptide
FSH:	follicle stimulating hormone	PAF:	paraformaldehyde
GABA:	γ -aminobutyric acid	PBS:	phosphate-buffered saline
GDX:	gonadectomized	PG:	pineal gland
GnIH:	gonadotrophin releasing hormone	PKA:	protein kinase A
GnRH:	gonadotropin-releasing hormone	PKC:	protein kinase C
GPCR:	G-protein coupled receptor	PLC:	phospholipase C
Hb:	habenula	POA:	preoptic area
HIOMT:	hydroxyindole O-methyltransferase	POMC:	proopiomelanocortin
HPA:	hypothalamo-pituitary-adrenal	PT:	pars tuberalis
HPG:	hypothalamo-pituitary-gonadal	PVN:	paraventricular hypothalamic nucleus
(5)-HT:	(5)-hydroxytryptamine, serotonin	PVT:	paraventricular thalamic nucleus
(5)-HTP:	(5)-hydroxytryptophan	RF:	Arginine (Arg)-Phenylalanine (Phe)
icv:	intracerebroventricular	RFRP:	RFamide-related peptide
IHC:	immunohistochemistry	RGC:	retinal ganglion cells
IML:	intermediolateral nucleus		
IP3:	phosphatidylinositol-triphosphate		

RHT: retino-hypothalamic tract
SCG: superior cervical ganglia
SCN: suprachiasmatic nucleus
SP: short photoperiod
SSC: standard saline citrate
T3: triiodothyronine
T4: thyroxine
TBS: tris-buffered saline
TBS-T: TBS with triton-x 100

TH: thyroid hormone
TPOH: tryptophane hydroxylase
Trp: thryptophan
TSH: thyroid stimulating hormone
TSHR: TSH receptor
VIP: vasoactive intestinal peptide
VMH: ventromedial hypothalamus
Wt: wild type

SUMMARY (ENGLISH)

Mammals are facing strong daily and annual changes of their living environment to which they need to adapt their biological functions. This is particularly true for reproductive activity, which requires a precise temporal adjustment to insure species perpetuation. Seasonal information is translated into a rhythmic production of the pineal hormone melatonin, via a well-described photo-neuroendocrine system (Simonneaux and Ribelayga, 2003), which ultimately regulates the reproductive axis. In our team “Melatonin and Seasonal Rhythms” at the Institute of Cellular and Integrative Neurosciences, we have recently made major steps towards understanding the melatonin-driven seasonal control of reproduction. Indeed, we have demonstrated that seasonal variation in melatonin production regulates the synthesis of the hypothalamic peptides kisspeptin and RFamide-related peptide (RFRP), which are critical for the regulation of reproductive activity, by a specific action upstream of the gonadotropin-releasing hormone (GnRH) neurons. Our current analyses point to the RFRP-expressing neurons as a downstream target for melatonin and therefore critical gatekeeper of seasonal reproduction. RFRP neurons are mainly expressed in the dorso- and ventro-medial hypothalamus (VMH/DMH), and its expression is strongly down regulated by melatonin in short photoperiod (winter like condition), in all seasonal species investigated so far. Our recent observations show, that acute intracerebroventricular (icv) injections of RFRP-3 induce expression of the immediate early gene c-Fos (used as indicator of neuronal activation) in a subset of GnRH neurons in male Syrian hamsters, leading to a release of luteinizing hormone (LH) from the pituitary. Moreover, we have demonstrated that chronic infusion of RFRP-3 in SP-adapted male Syrian hamsters, with an endogenous low level of RFRP, reactivated the reproductive axis despite photoinhibitory conditions. In contrast to these findings, RFRP-3, and the avian variant gonadotropin-inhibiting hormone (GnIH), has been shown to inhibit reproductive activity in several other species, as well as in ovariectomized (OVX) female Syrian hamsters, suggesting that there are both species- and sex-specific differences in the effects of RFRP-3.

The present work was dedicated to study the main three questions; 1) what are the cellular and molecular sites through which RFRP regulates the reproductive axis, 2) what is the relevance for the reported sex-differences in the effects of RFRP-3 on the Syrian hamster reproductive axis and 3) what is the role of RFRP in the melatonin-dependent regulation of reproduction in seasonal rodents.

In the first part of the studies, we aimed at providing a better characterization of RFRP neurons and their modes of action in the Syrian hamster, as well as investigating potential seasonal and sex-specific differences in the RFRP system. In brains of female and male Syrian hamsters, kept in long (LP) and short (SP) photoperiod, we used an immunohistochemical (IHC) approach to examine the properties of RFRP-expressing neurons. To investigate their sides of action, we analyzed projections of RFRP-immunoreactive (ir) fibers, whereas an *In Situ* Hybridization (ISH) approach was used to study the

distribution and expression of the RFRP receptor, GPR147. First, we performed a thorough characterization of RFRP antisera available, in order to find the appropriate antibody for detecting both RFRP-ir neurons and fibers in the Syrian hamster and to further investigate the specificity these RFRP antibodies. Based on this characterization, we selected a quail-derived RFRP antiserum that was found to specifically bind both RFRP-3 and RFRP-1, and to potently label RFRP cell bodies as well as fibers in Syrian hamster brain sections. This antiserum was then used in the IHC setup to study the possible seasonal and sex-specific differences in the distribution and levels of RFRP immunoreactivity. We found that the neuroanatomical distribution of RFRP-ir neurons and fibers was similar in brains of male and female Syrian hamster, however, when analyzing levels of RFRP immunoreactivity, we found a strong photoperiodic variation in the expression of RFRP neurons, which was significantly reduced in SP as compared LP in both male and female Syrian hamsters. Interestingly, we moreover found a strong sex-difference in the expression of RFRP, since both number of neurons as well as the intensity of the neuronal labeling was significantly higher in females than in males. Next, we examined the prevalence of RFRP-ir fibers e.g. in areas known to be involved in the central regulation of the reproductive axis, the preoptic area (POA), where the GnRH neurons and one of the two clusters of kisspeptin neurons are located, and the arcuate nucleus (ARC), where the other of the two populations of kisspeptin neurons is found. While we found RFRP-ir fibers in each of these areas, our quantitative analysis revealed a specific increase in the number of RFRP-ir fibers projecting to the anteroventral periventricular (AVPV) and the medial preoptic (MPN) nuclei of the POA in females that were kept in SP as compared to LP conditions, whereas in males, the amount of fibers were at a constant low level. This region is known to be particularly important for proper regulation of the female reproductive cycle, since kisspeptin neurons in the AVPV/MPN generates the stimulatory signal that drives a surge of LH to induce ovulation. The presence of RFRP-ir fibers in this area, which were specifically regulated in females and not in males, suggests that RFRP neurons might also be involved in regulating reproductive features specific to females. When analyzing the expression of GPR147 mRNA, in the brain of the Syrian hamster, we found that the receptor is expressed in various intra- and extra-hypothalamic structures, including brain regions that have been functionally linked to the control of reproduction, feeding, sexual behavior and stress. The distribution of GPR147 was similar between males and females, and correlated well with our mapping of RFRP-ir projections. Interestingly, levels of GPR147 mRNA were also found to vary according to seasonal changes, particularly in females that showed a strong and consistent down-regulation in GPR147 mRNA levels in SP as compared to LP. When comparing mRNA levels between male and female Syrian hamsters, we moreover found strong sex-specific differences, with much higher levels of GPR147 mRNA detected in females than in males. Altogether, these data have provided a thorough characterization of the RFRP system in a seasonal rodent, which has revealed strong photoperiod and sex-dependent differences in the expression and regulation of RFRP neurons and the cognate receptor for RFRP peptides. The findings have recently been published in the *Journal of Comparative Neurology* (Henningsen et al., 2015).

Keeping in mind the sex-dependent differences in the RFRP system and in the effects of RFRP-3 on reproductive activity, the aim of the second part of the work was to determine the physiological role of RFRP-3 in the control of female reproductive activity. The kisspeptin-mediated preovulatory LH-surge is precisely timed by ovarian-derived estradiol (E2) and suprachiasmatic nuclei (SCN)-dependent circadian signals, to take place in the afternoon of proestrus (Simonneaux and Bahougne, 2015). Intriguingly, recent findings have made a link between daily changes in RFRP neuronal activity and the occurrence of the LH-surge (Russo et al., 2015)(Gibson et al., 2008). We have, using the Syrian hamster as a seasonal model for female reproduction, investigated the expression and activation (c-Fos co-expression) of RFRP neurons, along different time points of the estrous cycle. Similarly to previous findings, we found that the activity of RFRP neurons is specifically decreased in the afternoon of proestrus, coinciding with the dramatic increase in AVPV/MPN kisspeptin activation and LH release from the pituitary. Interestingly, we found that this down-regulation of RFRP activity also occurred at a similar time point on the day of diestrus, indicating that there is a circadian rhythm in RFRP activation, that potentially is involved in regulating the kisspeptin-mediated LH-surge. Because RFRP (GnIH) has been found to inhibit LH release in the female Syrian hamster, we speculated whether RFRP neurons exerts a tonic inhibition on central sites regulating reproductive activity that, via a circadian control of the RFRP neurons, is lifted specifically in the afternoon to allow the LH-surge to take place. In line with this hypothesis, we found that acute administration of RFRP-3, just prior to or in the beginning of the LH-surge, where we had observed a decrease in RFRP activity, caused a significant but not full inhibition of LH released at the surge. Analysis so far shows that this effect of RFRP-3 is not mediated via AVPV/MPN kisspeptin neurons, but is probably, or at least partly, mediated through a modest decrease in GnRH neuron activity. To further explore the role of RFRP-3 in the seasonal control of female reproductive activity, we continuously administered RFRP-3 in SP-adapted and LP-adapted female Syrian hamsters in a period of 5½ weeks. Interestingly, chronic infusion of RFRP-3 in SP females significantly increased the activity of the reproductive axis, to a level comparable to what was found in LP animals. These findings clearly describe an essential role of RFRP in the central regulation of seasonal reproduction, and point towards RFRP neurons being a key intermediate through which the melatonin-dependent seasonal signal reaches and thereby regulates the reproductive axis.

Altogether, this work has provided a much better characterization of the RFRP system and its role in regulating reproductive activity in a seasonal model, the Syrian hamsters. We show that besides being strongly regulated by annual changes in day/night cycles, the RFRP system is differently regulated in males and females, and is more strongly expressed in females than in males. In line with these observations we here unveil that RFRP has multiple and distinct roles in regulating female reproductive activity, by acting as a key component in the seasonal control of reproduction and at the same time being an important regulator of cyclic events controlling the pre-ovulatory LH-surge.

RESUME (FRANÇAIS)

Chez les mammifères, l'activité de l'axe reproducteur est synchronisée avec les variations saisonnières de la photopériode. Notre équipe travaille sur les systèmes neurobiologiques impliqués dans l'intégration du message véhiculé par la mélatonine, relais endocrine des variations annuelles de la photopériode. Le lien entre la régulation saisonnière des profils circulants de la mélatonine et le rythme saisonnier de la sécrétion de GnRH est encore l'objet de grandes interrogations. Notre équipe travaille en particulier sur des neuropeptides hypothalamiques: le kisspeptine et le RFamide-related peptide (RFRP), des peptides considérés depuis peu comme des pivots du contrôle central de la fonction de reproduction. Les neurones à kisspeptine intègrent plusieurs paramètres environnementaux, humoraux et métaboliques et le kisspeptine est toujours un stimulateur puissant de l'axe gonadotrope via une libération du GnRH. L'expression de RFRP est également contrôlée par la photopériode, et en particulier inhibée par la mélatonine en photopériode courte. Mais à l'inverse de kisspeptine, son action sur l'axe gonadotrope varie avec les espèces et le sexe, et est tantôt activatrice, tantôt inhibitrice. Nos travaux récents ont permis de montrer qu'une injection intra-cérébro-ventriculaire de RFRP-3 active l'expression de c-Fos dans une sous population de neurones à GnRH et que le RFRP-3 augmente l'expression de kisspeptine. Ces résultats confortent l'hypothèse selon laquelle RFRP-3 agirait en amont de kisspeptine, qui serait le régulateur ultime (toujours activateur) de l'expression du GnRH quand RFRP-3 serait le pivot central synchronisant la physiologie de la reproduction avec les paramètres de l'environnement. Il a été précédemment montré dans notre équipe que des injections chroniques de RFRP-3 sont capables de réactiver le fonctionnement de l'axe reproducteur chez des hamsters mâles gardés en photopériode courte et sexuellement inactifs, encore démontrant un rôle stimulateur du RFRP-3 chez les mâles.

Dans le cadre de mon projet de thèse, je me suis concentrée sur la caractérisation du rôle des neurones à RFRP-3 dans le contrôle de l'activité de l'axe reproducteur d'une espèce photopériodique, le hamster doré.

L'objectif de la première partie de mon travail de thèse était d'étudier plus en détails les modes et les sites d'action des neurones à RFRP en analysant la distribution des fibres à RFRP par immunohistochimie dans le cerveau du hamster doré, mais également en comparant la distribution et la densité des fibres de projections dans des cerveaux de hamster mâles et femelles placés en photopériode courte (SP) et en photopériode longue (LP). Un autre objectif était de dresser une cartographie de la distribution des récepteurs du RFRP (GPR147) par hybridation in situ dans le cerveau du hamster doré, et de comparer les niveaux d'expression des ARNm codant pour GPR147 chez des hamsters mâles et femelles placés en LP et en SP. Dans un premier temps, j'ai réalisé une caractérisation complète des différents anticorps anti RFRP à notre disposition pour déterminer le

meilleur anticorps possible pour identifier à la fois les corps cellulaires et les fibres contenant du RFRP. En comparant ensuite les résultats obtenus chez les animaux des deux sexes, j'ai pu montrer que la distribution neuroanatomique des fibres RFRP sont similaires chez les hamsters mâles et femelles. Les résultats obtenus à partir des cerveaux de hamsters placés en photopériodes courte et longue montre que dans la majorité des structures, les niveaux d'expression des fibres ne changent pas à l'exception notoire des noyaux antéroventro-périventriculaires (AVPV), dans lesquelles la densité des fibres projetées est fortement augmentée chez des femelles placées en photopériode courte. Les noyaux AVPV sont le lieu de l'exercice du rétrocontrôle activateur de l'estradiol sur la sécrétion du kisspeptine qui stimule la sécrétion de GnRH qui, à son tour, induit le pic de LH précédant l'ovulation. J'ai ensuite réalisé une cartographie complète de la distribution des ARNm GPR147 dans des cerveaux de hamsters dorés. Cette étude a révélé que ces récepteurs sont exprimés dans de nombreuses structures hypothalamiques et thalamiques qui sont impliquées dans le contrôle de l'axe reproducteur. En particulier, les ARNm codant pour le récepteur des RFRP-3 (GPR147) sont exprimés dans l'aire pré optique, les noyaux AVPV, les noyaux paraventriculaires, l'hypothalamus ventromédian, les noyaux suprachiasmatiques, les noyaux habénulaires et les noyaux arqués. La cartographie de la distribution des sites exprimant les récepteurs au RFRP correspond très bien avec le patron de projection des fibres révélées. Par contre, en comparant les niveaux d'expression mesurés chez des individus mâles et femelles placés dans des conditions photopériodiques différentes, j'ai pu ensuite démontrer que l'expression des ARNm codant pour GPR147 et de RFRP-3 est beaucoup plus élevée chez les femelles que chez les mâles, que chez les deux sexes, les niveaux d'expression sont fortement diminués en photopériode courte, mais que cette inhibition par la photopériode courte est néanmoins plus forte chez les femelles que chez les mâles. Ces résultats suggèrent que les neurones du RFRP ont sans doute un rôle régulateur différentiel sur l'activité de l'axe gonadotrope des mâles et des femelles. Ces travaux ont été publiés dans *The Journal of Comparative Neurology* (Henningsen et al., 2015).

L'objectif de cette partie de mon travail de thèse était de déterminer le rôle précis de RFRP-3 dans le contrôle du pic pré-ovulatoire de LH chez le hamster doré femelle. Pour cela, j'ai étudié le patron d'expression du RFRP dans la période pré et post-ovulatoire. Nous voulions en effet savoir si les événements neuroendocrines associés à la survenue du pic de LH étaient corrélés à des modifications de l'activation des neurones à RFRP et de l'expression de ce peptide. Nous avons observé que l'activité des neurones à RFRP (mesurée par l'activation de c-Fos) est en effet à la fois corrélée à l'augmentation de l'activation des neurones à kisspeptine et à l'augmentation de la sécrétion de LH pré ovulatoire. En fait, l'activité des neurones à RFRP est fortement diminuée dans les heures qui précèdent le pic de LH. Comme par ailleurs, il a été montré que RFRP est un inhibiteur de l'axe hypothalamo-hypophyso-gonadique chez le hamster doré femelle, nous suggérons que RFRP exerce cet effet inhibiteur en diminuant l'activité des neurones à kisspeptine. De plus cet effet régulateur serait, via un contrôle circadien de l'activité des neurones à RFRP, à la base de l'expression temporelle du pic de LH. Pour tester cette hypothèse nous avons testé les effets d'injections intra-cérébro-ventriculaires aigues de

RFRP-3 à différents points horaires sur la modulation de l'amplitude et le décours temporel de la survenue du pic de sécrétion de la LH. J'ai obtenu des résultats qui montrent que des injections aiguës dans l'heure qui précède le pic pré-ovulatoire de LH induisent une diminution significative de l'amplitude de la sécrétion de LH, un effet qui semble être effectué par une diminution marginale d'activité des neurones GnRH et sans effet sur l'activité du kisspeptine. Dans cette partie j'ai aussi étudié les effets d'administrations chroniques de RFRP-3 chez des hamsters dorés femelles adaptés soit à la photopériode longue (animaux sexuellement actifs), soit à la photopériode courte (animaux sexuellement inactifs). Des canules intra-cérébrales connectées à des mini-pompes osmotiques ont été implantées dans les ventricules latéraux, qui permettent une perfusion chronique du peptide d'intérêt pendant une période de 6 semaines. Les résultats montrent que l'injection chronique de RFRP-3 induit, chez des femelles placées en photopériode courte, une augmentation significative du poids des gonades et d'hormones circulantes qui atteignent les valeurs mesurées chez des animaux placés en photopériode longue. Actuellement, il est très clair que le RFRP a un rôle régulateur essentiel dans le contrôle saisonnier de l'activité de l'axe reproducteur chez le hamster doré. Ces résultats constituent une avancée majeure dans notre compréhension des mécanismes qui relient la perception de la photopériode et le contrôle de l'axe reproducteur chez les mammifères saisonniers.

L'ensemble de ces résultats nous ont permis d'affiner considérablement notre compréhension des différentes actions du RFRP sur l'axe reproducteur de hamster doré. En effet, nous avons montré que la synthèse et la sécrétion du RFRP est un élément clé de la régulation saisonnière de l'axe reproducteur par la mélatonine. Pendant la phase d'inactivité sexuelle, la synthèse et la sécrétion du peptide sont très fortement inhibées, et nous avons montré que cela constitue un pré-requis à la mise en place de cette phase de repos de l'axe gonadique. Comme l'expression du RFRP est inhibée en SP par la mélatonine chez la plupart des mammifères à reproduction saisonnière, il est hautement probable que le rôle du RFRP ait été conservé au cours de l'évolution chez les espèces saisonnières. Cette hypothèse demandera cependant à être spécifiquement vérifiée. Par ailleurs, les neurones à RFRP sont également impliqués dans la régulation du pic pré-ovulatoire de LH, une étape clé du cycle ovarien de tous les mammifères, qu'ils présentent ou non une reproduction saisonnière.

L'activité des neurones à RFRP est significativement diminuée en diestrus et en proestrus dans la deuxième partie de la phase lumineuse du cycle jour/nuit. Elle semble donc être contrôlée selon une base circadienne qui pourrait participer en synergie avec le rétrocontrôle des stéroïdes gonadiques à la survenue du pic de LH le jour du proestrus. Dans une étude récente, il a été montré que des neurones à VIP des noyaux suprachiasmatiques, inhibe l'activité des neurones à RFRP, mais que cette inhibition n'est observée que pendant l'après-midi. Ces observations sont tout à fait en accord avec nos interprétations. Cependant, il semble peu probable que l'effet du RFRP sur le pic pré-ovulatoire de LH soit médié par une action directe du RFRP sur les neurones à kisspeptine de l'AVPV. En effet, au moment où cette inhibition est observée, ni l'activité ni le nombre des neurones à kisspeptine ne sont

modifiés. Il est donc beaucoup plus probable que cet effet inhibiteur résulte d'une action directe du RFRP sur les neurones à GnRH, ou qu'il soit médié par un autre interneurone non identifié à ce jour.

Les différents rôles joués par le RFRP sur la physiologie de la reproduction du hamster doré nous permettent aujourd'hui de mieux comprendre certains résultats publiés dans la littérature et qui apparaissaient contradictoires à première vue. Ils nous encouragent à prolonger les travaux sur le RFRP et en particulier à mieux caractériser les spécificités liées au sexe, aux différences inter spécifiques et à l'état reproductif des animaux.

INTRODUCTION

Reproduction is an event that requires synchronization of peripheral organs with the central nervous system (CNS) to allow internal and external inputs to create the optimal conditions for successful reproduction and thus perpetuation of species. How reproduction is and can be regulated along the entire length of the axis has been widely studied. In particular, the involvement of the CNS has, with the first discoveries of the hypothalamic control in the production of sex hormones in the middle of the 20th century, created the basis for research in the field of neuroendocrinology, as we know it.

Regulation of reproduction has nowadays become a major societal and economic challenge. Dysregulation of the reproductive axis can lead to infertility, and in healthy women, oral contraception, based on estrogen and progesterone derivatives, is widely used to control reproductive activity despite well-characterized side effects. The reasons for infertility can be many, including lack of ovulation, sperm deficiencies etc., some of which can be treated by gonadotropin stimulation of gonadal activity. Although there in many cases is a genetic basis for infertility (Shah et al., 2003), one widely discussed factor can be exposure to substances that interfere with our endogenous hormonal homeostasis or functions, known as endocrine disruptors (Buck Louis et al., 2008)(Crain et al., 2008)(Euling et al., 2008)(Mouritsen et al., 2010)(Wohlfahrt-Veje et al., 2012)(Dean et al., 2016).

In the attempt to increase the availability of food and dairy products, effort is made to control reproduction in livestock animal (i.e. seasonally controlled breeders). This is mainly done by use of hormonal treatments although these methods might have a negative impact on the environment and the public health. Estrogen derivatives are thought to induce feminization of male fishes found in fresh water (Kidd et al., 2007), which might also compromise the health of the consumers. Tendencies towards prohibiting such treatments are rapidly emerging, but in order to find alternatives to overcome these issues, a much better understanding of the central mechanisms acting on the reproductive axis is needed.

Early studies on the neural pathways involved in the control of reproduction have highlighted the importance of a population of neurons in the anterior hypothalamus that synthesize GnRH (gonadotropin releasing hormone). Among all species, these so-called GnRH neurons constitute the final neural output that regulates the reproductive axis. However, a complete characterization of neural pathways operating upstream of GnRH neurons is lacking, which could potentially help to evolve alternative approaches to control reproductive activity and ways to treat infertility with lower negative impacts on the environment and our health.

Recent years, studies have revealed that hypothalamic neuropeptides of the RF (Arg-Phe)amide family, in particular kisspeptins and RFamide-related peptide (RFRP), are central regulators of reproductive activity. By acting upstream of GnRH neurons, kisspeptin is now known as a very potent stimulator of GnRH release. RFRP, on the other hand, was initially thought to be an inhibitor of GnRH release,

however, recent studies have revealed a much more complex role of RFRP in regulating reproductive activity, and points toward critical roles of RFRP in regulating certain important physiological events of female reproduction, as well as in the seasonal regulation of reproduction, which in seasonal species is a prerequisite for survival of the species.

To elaborate on our understanding of how RFRP functions to regulate reproduction, the primary focus of this work has been to characterize the RFRP system and its role in a seasonal breeder, the Syrian hamster. In the following chapter, I will give a theoretic introduction to the reproductive axis and the concepts behind its regulation.

INTRODUCTION (FRANÇAIS)

La reproduction est un événement qui nécessite une synchronisation des organes périphériques avec le système nerveux central (SNC) pour permettre afin de créer les conditions optimales pour une reproduction réussie et donc la perpétuation des espèces. La régulation de la reproduction sur l'ensemble de cet axe fonctionnel a été largement étudiée. En effet, la participation du SNC a été reconnue dans le milieu du 20^e siècle avec les premières découvertes du contrôle hypothalamique de la production des hormones sexuelles, ce qui a été la base de la recherche dans le domaine de la neuroendocrinologie.

La régulation de la reproduction est devenue aujourd'hui un défi économique et sociétal majeur. Une mauvaise régulation de l'axe de la reproduction peut mener à l'infertilité, et chez les femmes en bonne santé, la contraception orale, dérivée d'estrogène et de progestérone, est largement utilisée pour contrôler l'activité de reproduction en dépit des effets secondaires bien caractérisés. Les causes de stérilité peuvent être nombreuses, y compris l'absence d'ovulation, des déficiences de sperme, etc., dont certaines peuvent être traitées par stimulation de l'activité de l'axe gonadotrope. Bien qu'il y ait dans de nombreux cas une base génétique pour l'infertilité (Shah et al., 2003), un facteur largement discuté peut être l'exposition à des substances qui interfèrent avec notre homéostasie ou fonction hormonale endogène, un phénomène connu sous le nom de perturbateurs endocriniens (Buck Louis et al., 2008)(Crain et al., 2008)(Euling et al., 2008)(Mouritsen et al., 2010)(Wohlfahrt-Veje et al., 2012)(Dean et al., 2016).

Dans le but d'augmenter la disponibilité des produits alimentaires et laitiers animaux, des efforts sont faits pour contrôler la reproduction saisonnière des animaux d'élevage. Ceci est principalement fait par l'utilisation de traitements hormonaux, bien que ces méthodes ont un impact négatif sur l'environnement et la santé publique. Les dérivés d'estrogène induisent une féminisation des poissons mâles trouvés dans l'eau douce (Kidd et al., 2007), un effet qui pourrait aussi compromettre la santé des consommateurs. Les tendances actuelles poussent à l'interdiction de tels traitements, aussi il est important de trouver des alternatives pour surmonter ces problèmes.

L'ensemble de ces questions sanitaires, environnementales et économiques nécessite une meilleure compréhension des mécanismes centraux agissant sur l'axe reproducteur. Les premières études sur les voies neuronales impliquées dans le contrôle de la reproduction ont mis en évidence l'importance d'une population de neurones dans l'hypothalamus antérieur qui synthétisent le GnRH (gonadotropin-releasing hormone). Chez toutes les espèces de Vertébrés, ces neurones à GnRH constituent la sortie neuronale finale qui régule l'axe reproducteur. C'est pourquoi, une caractérisation complète des voies neuronales et hormonales qui régulent l'activité des neurones à GnRH pourrait aider à développer des approches alternatives pour contrôler l'activité de reproduction et traiter l'infertilité avec des impacts négatifs réduits sur l'environnement et notre santé.

Ces dernières années, des études ont révélé que les neuropeptides hypothalamiques, kisspeptine et RFamide-related peptide (RFRP), sont des régulateurs centraux de l'activité de reproduction. En amont des neurones à GnRH, kisspeptin est maintenant reconnu comme un stimulateur très puissant de la libération de GnRH. RFRP a d'abord été décrit comme un inhibiteur de la libération de GnRH, cependant des études récentes ont révélé un rôle beaucoup plus complexe de RFRP dans la régulation de l'activité de gonadotrope. Il semble jouer un rôle critique dans la régulation de certains aspects spécifiques de la reproduction des mammifères femelles, ainsi que dans le contrôle saisonnier la reproduction de nombreuses espèces.

Pour mieux comprendre les fonctions de RFRP dans la régulation centrale de la reproduction, l'objectif principal de ce travail a été de caractériser le système de RFRP et son rôle chez un rongeur saisonnier, le hamster syrien.

THE HYPOTHALAMO-PITUITARY-GONADAL (HPG) AXIS

The hypothalamo-pituitary-gonadal (HPG) axis describes a pathway of signaling cascades that takes place in the hypothalamus, the pituitary gland and the gonads, whose primary role is to regulate reproductive activity (figure 1). Although specific mechanisms regulating reproductive activity might vary between species, the central control of reproduction involves the hypothalamic GnRH synthesizing neurons projecting to the median eminence (ME), where they release GnRH to the portal circulation (Hahn and Coen, 2006). The anterior lobe of the pituitary expresses five different hormone-producing cell types; gonadotrophic, corticotrophic, thyrotrophic, somatotrophic, and lactotrophic cells. GnRH acts on gonadotrophs to stimulate the synthesis and release of gonadotropins luteinising hormone (LH) and follicle-stimulating hormone (FSH) (McArdle and Roberson, 2015). The effects of LH and FSH differ between sexes. In females, LH stimulates sex steroid (estrogen and progesterone) release from ovaries and FSH, as the names indicates, stimulates follicular growth through mechanisms that will be described in more detail in the section “Physiology of the female ovarian cycle”. In males, LH regulates the synthesis and release of testosterone from leydig cells in the testis, whereas FSH stimulates sertoli cells to regulate spermatogenesis.

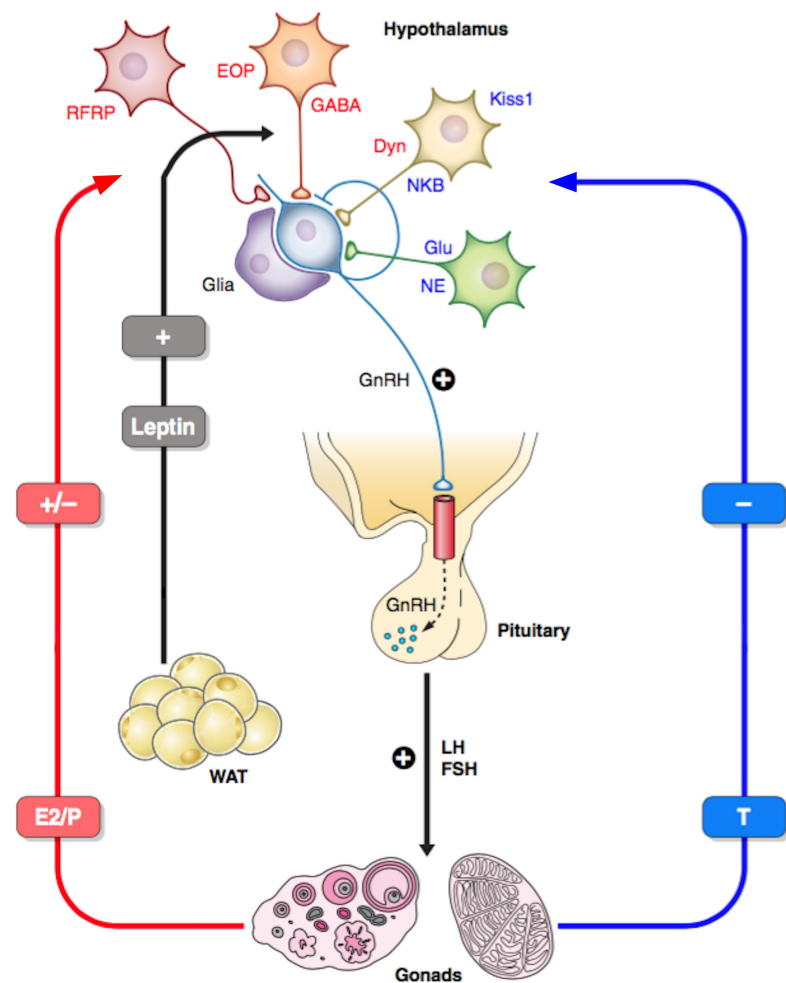


Figure 1. Schematic presentation of the hypothalamo-pituitary-gonadal (HPG) axis. The hypothalamo-pituitary-gonadal (HPG) axis is regulated by hypothalamic gonadatropin-releasing hormone (GnRH) neurons, which projects to the median eminence, where they release GnRH into the portal blood. GnRH regulates the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the pituitary that via the blood reaches the gonads, where they regulate sex steroids (estrogen, E2; progesterone, P; testosterone, T). GnRH activity is regulated by positive and negative feedback from sex steroids as well as several other factors, including the two RFamide peptides RFRP and kisspeptin, dynorphin (Dyn) and neurokinin B (NKB) as well as Glutmate (Glu), γ -aminobutyric acid (GABA), endogenous opioid peptides (EOP) and norepinephrine (NE), through the involvement of multiple internal and external mechanisms, such as lactation, stress, season and metabolism. One such example is the stimulation of GnRH neurons by leptin, which is released from white adipose tissue (WAT). Modified from Pinilla *et al.*, 2012.

At times, the axis might need to be up- or down-regulated according to internal and external factors, such as stressors, environmental (e.g. seasonal) and metabolic factors and much effort have in the past decades been put into identifying the underlying mechanisms of how the activity of GnRH neurons is controlled.

GONADOTROPIN RELEASING HORMONE (GnRH)

GnRH is a 10-amino acid peptide (decapeptide), which was first isolated and established as a potent stimulator of LH in 1970 by Schally and Guillemin and colleagues (Schally et al., 1971)(Amoss et al., 1971). Because of its effect on LH, it was first named luteinizing hormone-releasing hormone (LHRH), but is nowadays almost exclusively referred to as GnRH. The *GnRH* gene was characterized and cloned by Seeburg and co-workers in the mid 1980s (Seeburg and Adelman, 1984)(Adelman et al., 1986). Transcription of the gene gives rise to a 92 amino-acid (aa) long GnRH prepropeptide that after posttranslational modifications results in expression of two to three forms of GnRH peptide variations in most vertebrates (King and Anthony, 1983)(Rubin et al., 1987)(Wetsel and Srinivasan, 2002). Although, there is a high similarity in aa-sequences among GnRH variants, sub-characterization according to origin, localization and morphology of the expressing neurons, suggests the existence of more GnRH isoforms and that GnRH neurons might be divided into different functional subgroups, as reviewed by (Urbanski, 2012) and (Lee et al., 2008). Besides the classical form of GnRH (GnRH-I), another GnRH peptide, GnRH-II (or chicken GnRH-II), has also been shown to stimulate gonadotropin release, despite variations in their sequences (Lescheid et al., 1997)(Jimenez-Liñan et al., 1997)(Densmore and Urbanski, 2003)(Lethimonier et al., 2004). There is relatively little knowledge about the role of other GnRH variants e.g. GnRH-II and thus in this context, the focus will be kept on the role of GnRH-I, which is referred to as GnRH.

GnRH development and morphology

GnRH neurons are derived from the nasal placode that gives rise to the olfactory epithelium, and migrate into the brain along nasal axons during embryogenesis (Wray, 2002a)(Wray, 2002b). Interestingly, the morphology of GnRH neurons have been shown to change dramatically during postnatal development and goes from having complex dendritic structures to becoming uni- and bi-polar cells in the adult brain (Cottrell et al., 2006). Due to the migration pattern of GnRH during development, the distribution of GnRH neurons in the developed brain is scattered throughout areas extending all the way from the olfactory bulb to the mediobasal hypothalamus (MBH) (depending on species), with the majority of GnRH neurons located in the medial septum (MS) and the preoptic area (POA) (Krey and Silverman, 1978)(Goldsmith et al., 1990)(King and Anthony, 1984). Despite the dispersed localization of GnRH neurons, their long dendritic outgrowths extend to the median eminence, where GnRH is released in a synchronized manner. Significant advances in understanding GnRH morphology and function has been made through the impressive work of Herbison, Campbell

and colleagues, who for example have used biotin-filling tracing approaches to map the expression and interacting properties of GnRH neurons. By doing so, they have shown that GnRH dendrites form multiple close dendritic bundling interactions with other GnRH neuron dendrites, which can help to explain how GnRH neuron activity is synchronized (Campbell et al., 2009)(Campbell et al., 2005). Moreover, it has been established that GnRH neuronal projections function simultaneously as an axon and dendrite, so-called dendrons, which allows GnRH neurons to integrate synaptic inputs along their entire length (figure 2), thereby providing a dynamic control of GnRH peptide secretion from the ME (Roberts et al., 2008)(Herde et al., 2013)(Herde and Herbison, 2015).

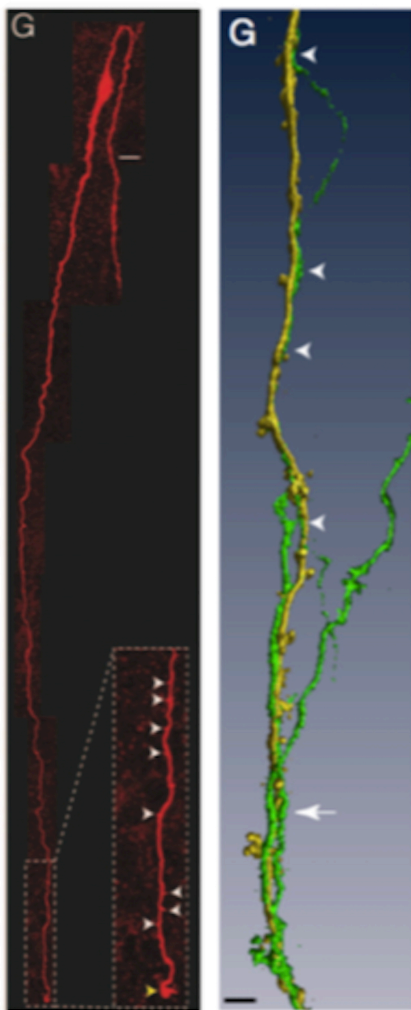


Figure 2. GnRH morphology, dendritic spining and GnRH dendritic bundling. Left panel: Confocal image of a biotin-filled GnRH neuron in its full extent that runs app. 538 μm in the vertical plane. Arrowheads in the inset indicate spines. Scale bar = 10 μm . Right panel: Bundling of three juxtaposed GnRH neuron dendrites as indicated by arrowheads. Scale bar = 5 μm . Modified from Campbell *et al.*, 2005 and 2009.

GnRH sites of action and biological effects

When released in the portal blood system, GnRH binds to and activates the G-protein coupled receptor (GPCR), GnRHR, expressed in gonadotrophs, as reviewed by Naor et al., 2009 (Naor, 2009). Three types of the receptor have been identified; type -I, -II and -III GnRH receptor of which GnRHR-I is predominantly expressed, also in gonadotrophs (Millar, 2005)(Cheung and Wong, 2008). Once activated, GnRHR couples to the $G\alpha_q$ protein, which leads to the activation of phospholipase C (PLC)- β and subsequent activation of phosphatidylinositol-triphosphate (IP3) and diacylglycerol (DAG), causing

the release of intracellular Ca^{2+} stores. DAG moreover activates protein kinase C (PKC) leading to opening of L-type voltage gated channels and influx in extracellular Ca^{2+} and thereby, activation of the extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway, which drives the transcription of genes encoding LH and FSH subunits (Bliss et al., 2010).

Goodman and Karsch first described the concept of a “hypothalamic pulse generator” in 1981 (Goodman et al., 1981) that causes GnRH to be released in the ME in a pulsatile manner. Electrical recordings of GnRH neurons revealed specific firing patterns (bursting) in their activity and in another study, GnRH promoter activity has been shown to oscillate spontaneously even in single neurons (Choe et al., 2013). This endogenous rhythm in GnRH activity is dependent on a dynamic of excitatory and inhibitory inputs, provided by a network of e.g. γ -aminobutyric acid (GABA) and glutamate signals, opioids, catecholamines and various neuropeptides (Maeda et al., 2010).

GnRH neuron activity is, as before mentioned, tightly controlled by sex steroid feedback despite the fact that most GnRH neurons themselves do not express sex steroid receptors. In contrast, two hypothalamic neuronal populations, one found in the anteroventral periventricular (AVPV) and medial preoptic (MPN) nuclei and the other in the arcuate nucleus (ARC), express the RFamide peptide, kisspeptin, as well as sex steroid receptors and are strongly regulated by the feedback of sex steroid (Lehman et al., 2010b). The ARC kisspeptin population is inhibited by estradiol (E2) and testosterone released from the gonads upon stimulation of the reproductive axis, whereas the sexually dimorphic AVPV/MPN population of kisspeptin neurons is stimulated by E2. Indeed, kisspeptin has been shown to be a potent stimulator of GnRH activity, and the ARC kisspeptin population is thus considered to be important for suppressing GnRH activity in basal conditions through the negative regulation of E2, while the AVPV/MPN kisspeptin neurons are mainly thought to be involved in generating the pre-ovulatory surge of LH in females, as will be described in more detail in the following section. GnRH synthesis and release is further regulated upstream by another RFamide peptide, RFRP (figure 3). While there is evidence for a direct effect of RFRP on GnRH neurons, the output of RFRP signaling varies between species and even seems to be sex- and season-dependent, as will be discussed in more detail later in this chapter.

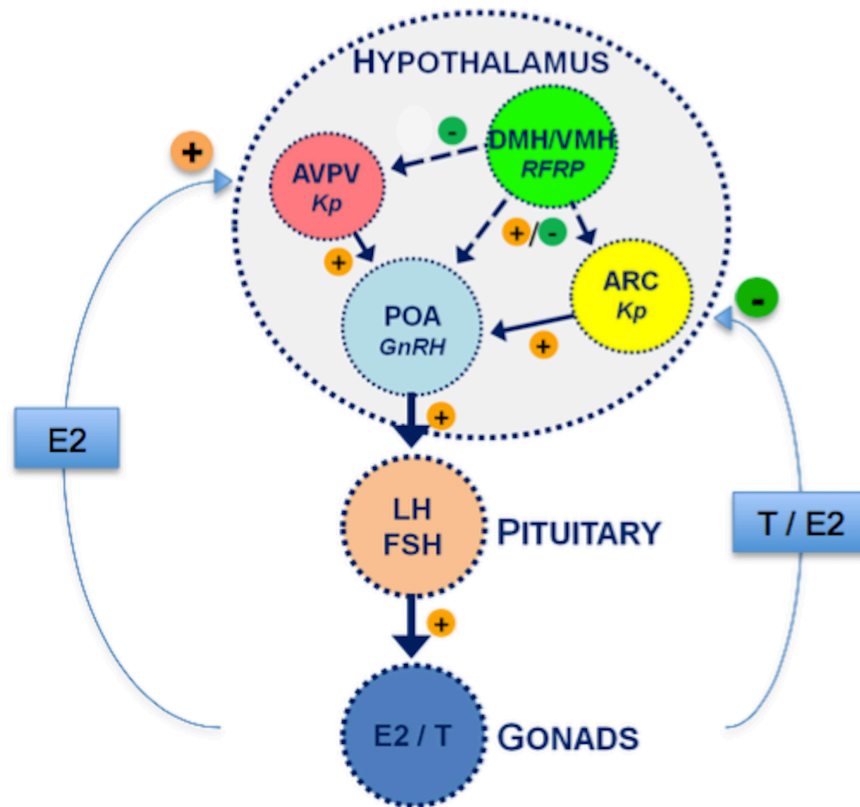


Figure 3. Hypothalamic RFamide peptides, kisspeptin and RFRF, are regulators of GnRH activity. Schematic diagram presenting the current model of the central control of reproductive activity by the RFamide peptides. Kisspeptin (Kp) neurons of the arcuate (ARC) and anteroventral periventricular (AVPV) nuclei project to the GnRH neurons, where kisspeptin binds Kiss1 receptors expressed on GnRH neurons, which triggers GnRH release into the portal blood. GnRH in turn stimulates the synthesis and release of the pituitary luteinizing (LH) and follicle-stimulating (FSH) hormones. Gonadotropins stimulate sex steroid (estrogens, E2 and testosterone, T) production that in turn provides positive (AVPV) and negative (ARC) feedback on Kp neurons. RFRP-3 neurons located in the dorso/ventromedial hypothalamus (DMH/VMH) are proposed to project to GnRH and kisspeptin neurons and either inhibit or stimulate GnRH neuron activity, dependent on species and sex.

In females, reproductive activity is subjected to a more complex regulation and is controlled by cyclic events of sexual activation and inactivation to control follicle development and ovulation.

PHYSIOLOGY OF THE FEMALE OVARIAN CYCLE

Reproductive activity in female mammals displays a regular cycle, driven by a complex interaction of the circadian system, hypothalamic neuropeptides, gonadotropins and sex steroids produced by the ovaries, known as the menstrual cycle in human and estrous or ovarian cycle in rodents (Knobil, 1980). In the interest of the focus of this work, the section will focus entirely on the mechanisms controlling the rodent estrous cycle.

The cycle lasts approximately 4 days and is composed of 4 distinct stages; metestrus, diestrus, proestrus and estrus that are characterized by changing levels of pituitary and ovarian hormones (figure 4). Throughout diestrus and until mid-day of proestrus (also known as the follicular phase), FSH stimulates the growth of ovarian follicles that while maturing produce increasing amounts of E2.

In proestrus, the high levels of E2 reach a certain threshold or time point that via positive feedback stimulates the kisspeptin-mediated pre-ovulatory surge in LH, triggering ovulation. If conception does not take place, the cycle enters estrus and thereafter metestrus (collectively known as the luteal phase), a phase of degeneration of the corpus luteum, which produces E2 and progesterone, and thereby re-establishes the maintenance of the negative feedback on gonadotropin release (Levine, 2015).

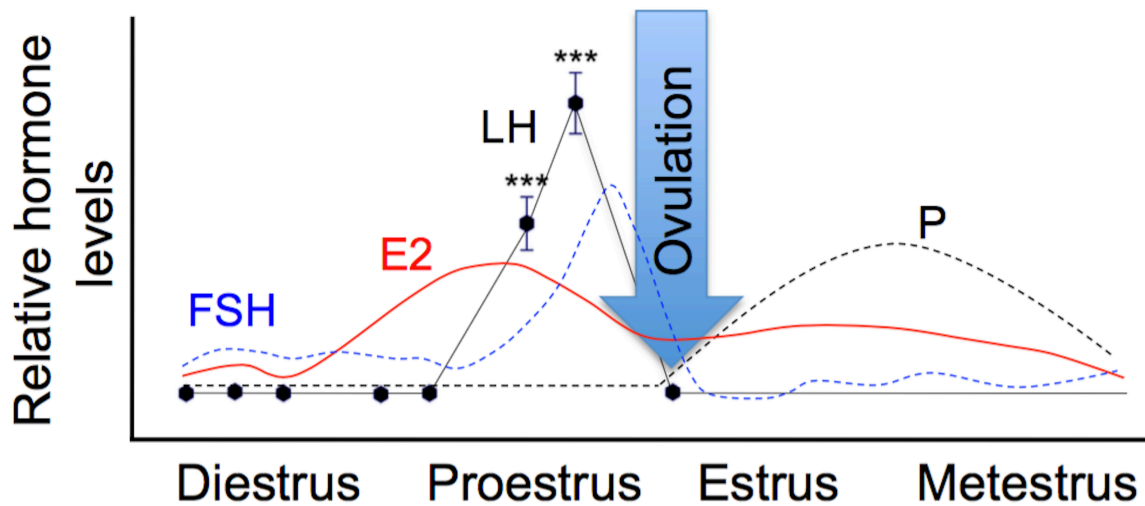


Figure 4. Schematic overview of the circulating hormonal levels during the rodent estrous cycle. In female rodents, the estrous cycle lasts approximately 4 days and is composed of four stages; diestrus, proestrus, estrus and metestrus. In diestrus, follicle-stimulating hormone (FSH) stimulates follicle maturation and growth, which produces increasing levels of estrogen (E2). In proestrus, high levels of E2 trigger the kisspeptin-mediated surge in luteinizing hormone (LH, full line) and subsequent ovulation. If fertilization does not take place, the corpus luteum is degraded leading to the release of progesterone (P, dotted line) and E2, which in turn, by negative feedback, normalizes the axis thus ending the first cycle.

Besides the tightly controlled release of gonadotropins and sex hormones through feedback mechanisms, the timing and generation of the LH-surge relies also on circadian signals arising in the suprachiasmatic nuclei (SCN) of the hypothalamus, adding a novel degree of complexity in the control of female reproduction (Fitzgerald and Zucker, 1976)(de la Iglesia and Schwartz, 2006)(Williams and Kriegsfeld, 2012). In example, AVP released from the SCN in the afternoon, projects to kisspeptin neurons in AVPV/MPN and regulate their daily activity, which in combination with high levels of E2, causes the dramatic increase in kisspeptin activity leading to GnRH release and the surge in LH (Williams et al., 2011a)(Kalsbeek et al., 2010)(Piet et al., 2015b). Moreover, SCN-derived vasoactive intestinal peptide (VIP) directly modulates GnRH neuron activity (Van Der Beek et al., 1997)(Horvath et al., 1998)(Smith et al., 2000) and recent studies indicate that RFRP neurons in the dorsomedial hypothalamus (DMH) are regulated by VIP to add an extra dimension of the control of the LH-surge, which will be touched upon later on.

The discovery of kisspeptin

Kiss1, the gene encoding kisspeptin was discovered in 1996 by Lee and co-workers (Lee et al., 1996). Initially, kisspeptin was known for its anti-metastatic properties and thus termed metastatin up until 2003, where mutations in the *Kiss1* gene were identified as one of the main causes of reproductive dysfunctions in hypogonadotropic hypogonadism. The *Kiss1* gene is translated into a 145 aa long prepropeptide that is cleaved into kisspeptin peptides with sequences varying from 10 aa to 52 (rodents) or 54 (human) aa (Kotani et al., 2001)(Ohtaki et al., 2001). Common to all kisspeptin peptides is the 10 aa of the carboxy (C)-terminus as well as a C-terminal RF amidation that makes kisspeptins part of the large family of RFamide peptides.

Kisspeptins (referring to all kisspeptin variants) are throughout species expressed by neurons clustered in two distinct hypothalamic areas, one in the preoptic area that in rodents is confined to the AVPV/MPN, and one in the ARC (Gottsch et al., 2004)(Smith et al., 2005a)(Smith et al., 2005b)(Mason et al., 2007)(Revel et al., 2006a). The AVPV/MPN population is sexually dimorphic, with a higher level of expression in this area in females than in males, which is important for the previously described involvement in regulation of female cyclicity. Interestingly, comparative studies of the kisspeptin system e.g. between rats and mice, reveal species differences that are most likely due to differences in the dynamics of peptide processing (Overgaard et al., 2013). ARC kisspeptin cells can, depending on species, be hard to detect due to a very high density of kisspeptin fiber innervations. Despite this fact, sex-differences are also found in the ARC kisspeptin population, with a higher level of kisspeptin immunoreactivity in both female rats and mice than in males, as demonstrated in figure 5.

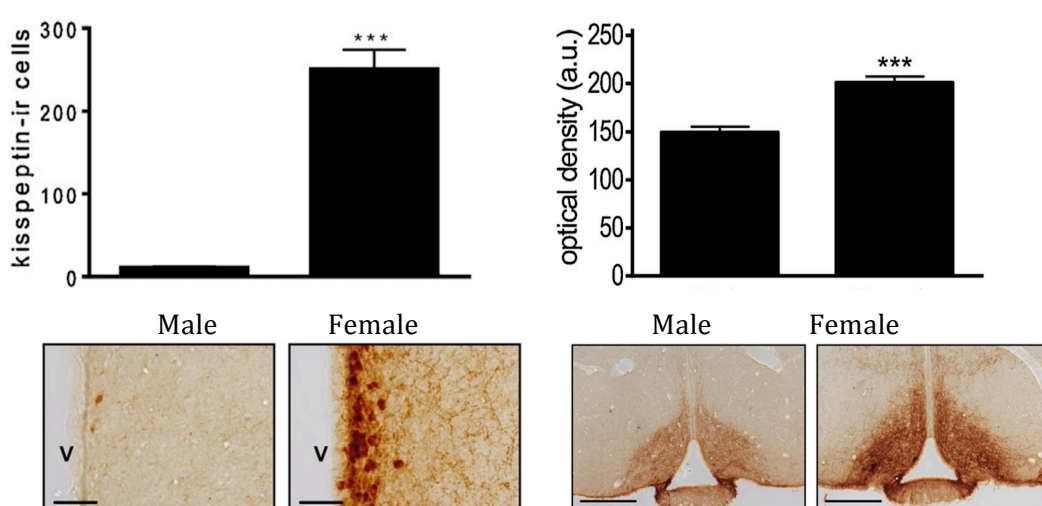


Figure 5. Kisspeptin expression in the AVPV/MPN and the ARC in female and male mice. Number of kisspeptin-immunoreactive cells in the AVPV/MPN of female and male mice (left panels), scale bar = 20µm, V = 3rd ventricle. Optical density measures of kisspeptin-immunoreactive neurons in the ARC of female and male mice (right panels). Scale bars = 250µm. Modified from Overgaard *et al.*, 2013.

Kisspeptin modes and sites of actions

Despite differences, all kisspeptins binds to the same GPCR, GPR54, also known as Kiss1r (Kotani et al., 2001)(Mikkelsen et al., 2009), and potently stimulate the reproductive axis through the activation of GPR54 specifically expressed on GnRH neurons (Clarkson and Herbison, 2006). Downstream, GPR54 couples to $G_{\alpha q}$ protein leading to an activation of PLC and IP3-mediated Ca^{2+} mobilization, followed by an activation of PKC and MAPK pathways (Kotani et al., 2001)(Castaño et al., 2009)(Liu et al., 2008). Kisspeptin release is in all species manifested by a subsequent increase in GnRH release and thereby LH released from the pituitary (Gottsch et al., 2004)(Mikkelsen et al., 2009)(Matsui et al., 2004)(Thompson et al., 2004)(Navarro et al., 2005). Interestingly, however, a recent tracing study describes how kisspeptin inputs originating from either the AVPV/MPN or ARC neurons are integrated differently on GnRH neurons. GnRH somas and proximal dendrites receives input from AVPV/MPN kisspeptinergic projections only, whereas fibers derived from both populations are found in close contact to distal GnRH processes at the level of the ARC and the ME (Yip et al., 2015a). Indeed, action potentials can be generated along the entire GnRH Dendron, and the fact that kisspeptin stimulation of mediobasal hypothalamic explants containing only GnRH nerve terminals and not cell bodies still induce GnRH release (d'Anglemont de Tassigny et al 2008), further supports that kisspeptin neurons regulate GnRH activity at multiple sites.

Kisspeptin and the reproductive axis

The understanding of the role of the kisspeptin system in regulating reproductive activity advanced significantly in 2003, when it became clear that a loss-of-function mutation in the gene encoding GPR54, *Kiss1*, were the major cause of hypogonadotropic hypogonadism in human and mice (de Roux et al., 2003)(Seminara et al., 2003). Subsequent studies in a GPR54 knock out (KO) mouse model showed severe impaired development of reproductive organs, as demonstrated in figure 6 (Funes et al., 2003).

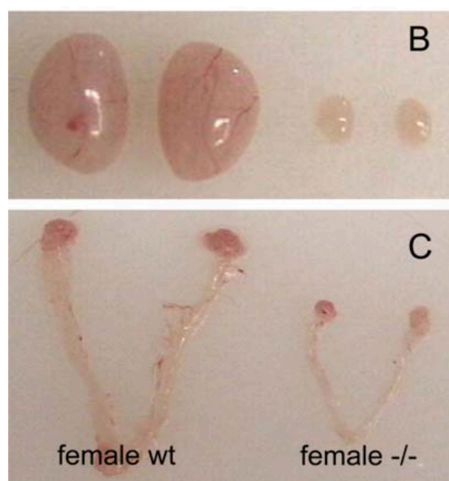


Figure 6. Reproductive organs in 30 days old wild type and GPR54 deficient female and male mice. B) Testis and C) ovaries from 30-day old female and male wild-type (wt) or GPR54 knock out mice (-/-). Funes et al., 2003.

As previously mentioned, it is well established that feedback mechanisms regulating GnRH activity is in part mediated via kisspeptin. Kisspeptin neurons in the ARC express estrogen receptors (ER) and androgen receptors (AR) and are inhibited by E2 and testosterone. As a consequence, removal of the negative feedback by gonadectomy, increases ARC kisspeptin expression (Smith et al., 2005b)(Navarro et al., 2004)(Revel et al., 2006a)(Ansel et al., 2010a). In contrast, AVPV/MPN kisspeptin neurons are stimulated by E2 (Kauffman et al., 2007)(Quennell et al., 2010)(Smith et al., 2005b) and interestingly, both the positive and negative feedbacks of E2 are mediated through the estrogen receptor ER α (Xu et al., 2012)(Clarkson et al., 2008)(Mayer et al., 2010).

Kisspeptin neurons co-express a high number of other neuropeptides, i.e. ARC kisspeptin neurons co-express neurokinin B (NKB) and dynorphin (dyn), and are therefore increasingly referred to as KNDy neurons (Goodman et al., 2007). KNDy neurons themselves express the NKB receptor, NK3R, and NKB is found to stimulate kisspeptin release (Amstalden et al., 2010)(Navarro et al., 2011), while Dyn on the other has been shown to inhibit kisspeptin expressing neurons (Goodman et al., 2012). Since increasing evidence couples GnRH transcription to secretion of kisspeptin, like the fact that inhibition of kisspeptin suppresses pulsatile GnRH/LH secretion (Choe et al., 2013), one current hypothesis is that NKB and Dyn in concert act to control a pulsatile release of kisspeptin that subsequently regulates GnRH pulsativity (Goodman et al., 2007)(Lehman et al., 2010a)(Navarro et al., 2009).

Reproducing is highly energy demanding and therefore, metabolic signals also regulate reproductive activity, one example being energy insufficiency, which can cause a delay in pubertal development. Kisspeptin has been shown to be particularly affected by metabolic stress (fasting) around the time of puberty, manifested by a decrease in expression of ARC kisspeptin (Castellano et al., 2005), and have therefore been linked to how metabolic cues reaches the reproductive axis, as reviewed by Pinilla *et al.*, (Pinilla et al., 2012). Indeed, kisspeptin neurons in the ARC express receptors for leptin, a hormone derived from the white adipose tissue, that plays an important role in metabolic homeostasis (Smith et al., 2006a). Despite contradictory results in the actual effects of leptin on kisspeptin functions, it has been proposed that leptin regulate GnRH and thus, reproductive activity via kisspeptin. Besides its effects on reproductive and metabolic homeostasis, kisspeptin has been shown to be involved in many different processes, such as inhibition of metastasis and regulation of proliferation.

RF (ARG-PHE)AMIDE-RELATED PEPTIDE (RFRP)

Another hypothalamic RFamide peptide, the RF amide-related peptide (RFRP), has been extensively studied over the past decade for a putative role in regulating the reproductive axis. RFRP was initially thought to inhibit reproductive activity throughout species, and is despite well-documented stimulatory effects in some species, still often referred to as GnIH as named after the inhibitory ortholog, the avian gonadotropin-inhibiting hormone (GnIH).

Discovery of RFRP

The *RFRP* gene was discovered in birds and mammals in 2000 (Hinuma et al., 2000). The gene encodes a prepropeptide, which is processed into two or three functional peptides, depending on species. All peptides are characterized by a C-terminal LPXRFamide motif, where X is substituted either by leucine (L) or glutamine (Q). The RF amidation categorizes RFRP peptides as member of the RFamide family of peptides, along with e.g. kisspeptins. The avian GnIH precursor encodes one GnIH and two GnIH related peptides (GnIH-RP-1 and GnIH-RP-2). GnIH and GnIH-RP-1 contain an LPLRFa motif in the C-terminal, whereas GnIH-RP-2 contains an LPQRFa motif. The mammalian gene encodes RFRP-1, -2 and -3 peptides, of which RFRP-1 (containing a LPLRFa motif and also known as NPSF) and RFRP-3 (containing a LPQRFa motif and also known as NPVF) are functional peptides (table 1), (Fukusumi et al., 2001a)(Hinuma et al., 2000)(Ukena et al., 2002).

Species	Amino acid sequence	Reference
Human RFRP-1	MPHSFANLPLRFa	Ubuka et al., 2009
Human RFRP-3	VPNLPQRFa	Ubuka et al., 2009
Monkey RFRP-3	SGRNMEVSLVRQVLNLPQRFa	Ubuka et al., 2009
Bovine RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLPLRFa	Fukusumi et al., 2001
Bovine RFRP-3	AMAHPLRLGKNREDSLSRWVVPNLPQRFa	Yoshida et al., 2003
Rat RFRP-1	SVTFQELKDWGAKKDIKMSPAPANKVPHSAANLPLRFa *	Hinuma et al., 2000
Rat RFRP-3	ANMEAGTMSHFPSLPQRFa	Ukena et al., 2002
Siberian hamster RFRP-1	SPAPANKVPHSAANLPLRFa	Ubuka et al., 2012
Siberian hamster RFRP-3	TLSRVPSLPQRFa	Ubuka et al., 2012
Syrian hamster RFRP-1	SPAPANKVPHSAANLPLRFa *	Kriegsfeld et al., 2006
Syrian hamster RFRP-3	ILSRVPSLPQRFa *	Kriegsfeld et al., 2006
Quail GnIH	SIKPSAYLPLRFa	Tsutsui et al., 2000
Quail GnIH-RP-1	SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVANLPLRFa *	Satake et al., 2001
Quail GnIH-RP-2	SSIQSLNLPQRFa	Satake et al., 2001

Table 1. Mammalian and quail amino acid sequences of RFamide peptides containing a LPXRFa (X = L or Q) motif. * Putative peptide sequences are hypothesized from their precursor mRNA sequences.

RFRP and GnIH neurons were upon their discovery found to be primarily expressed in neurons located in the paraventricular nucleus (PVN) and in between the DMH and ventromedial hypothalamus (VMH) in birds and rats, respectively (Tsutsui et al., 2000)(Hinuma et al., 2000). Subsequent studies in mammalian species show that the majority of RFRP neurons are located in the DMH, in between the

DMH and VMH, with some neurons located within the boundaries of VMH (Henningesen et al., 2015)(Kriegsfeld et al., 2006). Their expression can however be detected along the third ventricle from the caudal part of the medial anterior hypothalamus throughout the medial tuberal hypothalamus (figure 7). In sheep, the pattern of RFRP expression seems to be slightly different, being highly expressed also in the PVN (as also observed in avian species), in addition to the DMH (Clarke et al., 2008)(Dardente et al., 2008)(Smith et al., 2008).

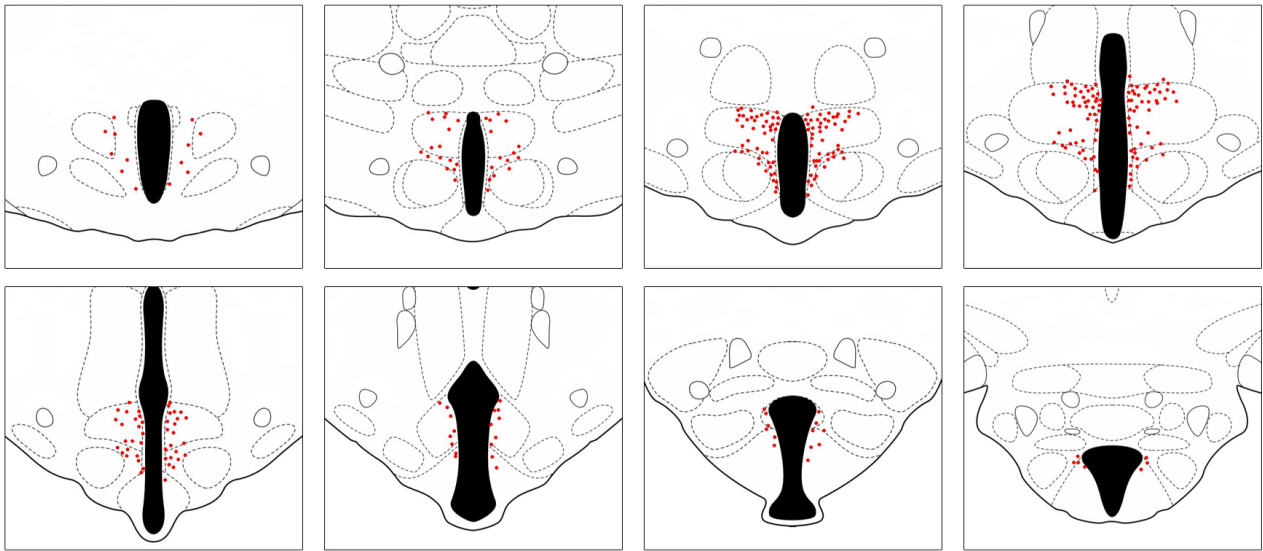


Figure 7. Distribution of RFRP neurons in the Syrian hamster brain. RFRP-immunoreactivity can be detected along the third ventricle from the caudal part of the medial anterior hypothalamus throughout the medial tuberal hypothalamus in the Syrian hamster. Every red dot represents one RFRP-immunoreactive neuron.

RFRP during development

The RFRP system changes during development and in female rats RFRP-1 immunoreactive (ir) expression has been found to be significantly higher in adulthood as compared to pre-pubertal developmental stages. Interestingly, RFRP mRNA levels have been shown to increase during development in both male and female rats and mice to peak around the time of puberty. Hereafter, RFRP mRNA levels decrease in males, whereas in females the expression remains at high levels (Quennell et al., 2010)(Iwasa et al., 2012)(Poling et al., 2012). In line with these observations, we recently reported sex-differences in the expression of RFRP in adult Syrian hamsters, with a higher expression in females than in males (Henningesen et al., 2015). Altogether, these findings indicate a role for RFRP in regulating reproduction in adulthood rather than pre-pubertal, but also suggest that development of the RFRP system and differences between males and females could be programmed by sex-steroids during the pre-pubertal stages of development. Moreover, while the peri-pubertal peak in RFRP mRNA levels might be a consequence of pubertal changes, it could also reflect that RFRP is involved in regulating puberty. However, studies in a RFRP receptor, GPR147, deficient mouse model,

shows that although pre-pubertal KO males displayed elevated LH levels, which normalized after puberty, pubertal timing was not altered in GPR147 deficient mice (Leon et al., 2014).

RFRP modes and sites of action

RFRP-ir fiber projections are widely distributed in the brain, but are mainly concentrated in and around the organum vasculosum of the lamina terminalis (OVLT), the AVPV/MPN, the anterior part of the SCN, PVN, anterior hypothalamus (AH), VMH and ARC, as well as in the bed nucleus of the stria terminalis (BNST), habenular nuclei (Hb), and paraventricular nucleus of the thalamus (PVT) (Henningsen et al., 2015)(Kriegsfeld et al., 2006)(Ubuka et al., 2012). Interestingly, RFRP terminals have been found to make apparent contact to 20-40% of GnRH somas in rodents and sheep (Kriegsfeld et al., 2006)(Ubuka et al., 2012)(Rizwan et al., 2012)(Smith et al., 2008) and in female mice around 20% of AVPV/MPN kisspeptin neurons and 35% of ARC kisspeptin neurons receive RFRP-fiber contacts (Poling et al., 2013)(Rizwan et al., 2012). Although of controversy, some studies report the presence of RFRP fibers in the ME and therefore propose a hypophysiotropic effect of RFRP (Kriegsfeld et al., 2006)(Clarke et al., 2008).

RFRP-1 and -3 peptides bind two GPCRs, GPR147 (also known as NPFF1) and GPR74 (also known as NPFF2) (Hinuma et al., 2000). Although RFRP peptides bind with nanomolar (nM) affinities to both receptors, the affinity towards GPR147 is higher, making it the preferential target of downstream signaling of RFRP peptides (Yoshida et al., 2003)(Elhabazi et al., 2013). Intracellularly, GPR147 has been found to couple to both stimulatory and inhibitory G protein subunits *in vitro* (Gouarderes et al., 2007)(Bonini et al., 2000), whereas in GPR147 transfected CHO cells, human RFRP-1 induces a maximal inhibition of a forskolin-induced cAMP accumulation, indicating that RFRP-1 inhibit adenylate cyclase through a $G_{\alpha i}$ -bound receptor complex (Mollereau et al., 2002). However, the exact signaling events occurring downstream of GPR147 in its natural cellular environment still remain unknown. GPR147-encoding mRNA is widely distributed in the brain, but particular strong expression is observed in hypothalamic regions as the OVLT, MPN/AVPV, SCN, PVN, AH, VMH and ARC, and outside the hypothalamus in the posterior part of BNST, Hb, and the pyramidal cell layer of the hippocampus (Henningsen et al., 2015)(Gouarderes et al., 2004)(Dardente et al., 2008). GPR147 has also been found in moderate amounts in eye, testis, and medulla. Interestingly, GPR147 has been shown to be expressed in 15-33% of mice GnRH neurons and a subpopulation of kisspeptin neurons in the AVPV (5-16%) and the ARC (25%) (Poling et al., 2012)(Poling et al., 2013)(Rizwan et al., 2012), which together with what is known about RFRP fiber distribution, strongly indicates that RFRP peptides have multiple central targets through which they regulate reproduction.

RFRP and its effects on the reproductive axis

In the first reports of the effects of RFRP peptides on reproductive activity, the avian GnIH was found to inhibit gonadotropin secretion from cultured quail pituitaries (Tsutsui et al., 2000). In contrast to

RFRP-3, Hinuma *et al.* reported no effect of RFRP-1 on the reproductive axis, and subsequent studies have therefore mainly focused on the effects of RFRP-3 rather than RFRP-1. RFRP-3 and GnIH have since their discovery been shown to inhibit GnRH neuron activity and gonadotropin release in several other non-avian species i.e. mice, rats and sheep (Anderson *et al.*, 2009)(Clarke *et al.*, 2008)(Ducret *et al.*, 2009)(Johnson *et al.*, 2007)(Kriegsfeld *et al.*, 2006)(Tsutsui *et al.*, 2000)(Pineda *et al.*, 2010a)(Leon *et al.*, 2014). In agreement with this, RFRP-3 application to mice brain slices inhibits the firing rate of 41% GnRH neurons, but intriguingly, a small populations (app. 12%) of the GnRH neurons are in contrast stimulated by RFRP-3 (Ducret *et al.*, 2009). There is evidence for a hypophysiotropic effect of GnIH and RFRP-3 in birds and ewes, respectively, although in ewes the effect is still very much debated. One study finds that intravenous (iv) infusion of RFRP-3 inhibits pulsatile LH secretion in ovariectomized (OVX) ewes (Clarke *et al.*, 2008), whereas two recent studies show no variation in LH plasma concentrations in neither ovariectomized nor intact ewes injected either icv or iv with RFRP-3 (Caraty *et al.*, 2012)(Decourt *et al.*, 2016a). Recent findings from our group have revealed that the effect of RFRP-3 is species-dependent. In male Syrian hamsters, RFRP-3 stimulates the reproductive axis and concomitantly induces c-Fos expression in 20-30% GnRH neurons (Ancel *et al.*, 2012), (figure 8).

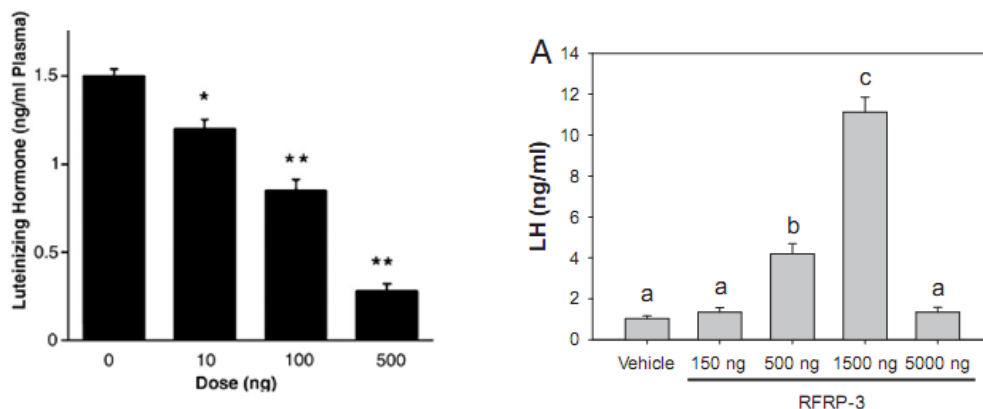


Figure 8. The effect of RFRP-3 on LH release is species dependent. Central administration of RFRP-3 dose-dependently inhibits LH release in male rats (Left panel, Johnson *et al.*, 2007), whereas in male Syrian hamsters, increasing doses of RFRP-3 gradually increase the release of LH from the pituitary (right panel, Ancel *et al.*, 2012).

A similar stimulatory effect has been found in male Siberian hamsters. However in this seasonal species, the effect varies with the breeding state and stimulates LH release in sexually inactive animals (winter-like conditions), while inhibiting LH release in sexually active animals (summer-like condition) (Ubuka *et al.*, 2012), thus adding a supplementary season-dependent difference in the effects of the peptide, at least in this species. To make matters even more complicated, RFRP function seems also to be sex-dependent. While RFRP-3 have a stimulatory effect in male Syrian hamsters, the avian GnIH inhibits LH secretion in OVX females (Kriegsfeld *et al.*, 2006). To summarize on the many reported findings so far, the effects of RFRP-3 in mammalian reproductive activity has been listed in table 2.

Species	Sex and Status	Effects of RFRP-3	Reference
Mice	Female	Inhibits GnRH firing in brain slices.	Ducret <i>et al.</i> , 2009
	Male	No peripheral effects. Inhibits LH release from cultured pituitaries. Inhibits GnRH firing in brain slices.	Mikkelsen <i>et al.</i> , 2009 Leon <i>et al.</i> , 2014 Ducret <i>et al.</i> , 2009
	Female OVX	Central administration (icv) inhibits LH release.	Leon <i>et al.</i> , 2014
Rat	Male	Central administration (icv) inhibits LH release. Central administration (icv) inhibits LH release.	Johnson <i>et al.</i> , 2007 Pineda <i>et al.</i> , 2010
	Female OVX	Central administration (icv) inhibits LH release. Peripheral administration inhibits LH release, no effect of central administration (icv). Central administration (icv) inhibits during an E2-induced LH-surge. No peripheral effect.	Pineda <i>et al.</i> , 2010 Murakami <i>et al.</i> , 2008 Anderson <i>et al.</i> , 2009
	Male GNX	Central administration (icv) inhibits LH release. Inhibits LH release from cultured pituitaries.	Pineda <i>et al.</i> , 2010
Siberian hamster	Male	Central administration (icv) inhibits LH release in LP, while stimulating LH release in SP.	Ubuka <i>et al.</i> , 2012
Syrian hamster	Female	Central administration inhibits LH release in proestrus, but has no effect in diestrus.	Henningesen <i>et al.</i> , Unpublished
	Male	Central acute and chronic administration stimulates LH release in LP and SP. No peripheral effect.	Ancel <i>et al.</i> , 2012
	Female OVX	Central and peripheral administration (GnIH) inhibits LH release in LP.	Kriegsfeld <i>et al.</i> , 2006
Sheep	Female OVX	Repeated peripheral injection has no effect on pulsatile LH release in LP. 24-hour perfusion has no effect on E2-induced LH surge in SP. Central and peripheral administration has no effect on LH release. Peripheral administration inhibits E2-induced LH surge in SP. Peripheral administration inhibits pulsatile LH release in SP. Peripheral administration inhibits GnRH-induced LH release. RFRP-3 is detected in the portal blood in SP and LP with higher conc. detected in LP.	Decourt <i>et al.</i> , 2016 Caraty <i>et al.</i> , 2012 Clarke <i>et al.</i> , 2008 Clarke <i>et al.</i> , 2012 Smith <i>et al.</i> , 2012
	Female	Peripheral injection or a 4-hour perfusion has no effect on kisspeptin-mediated increase in LH. Peripheral administration inhibits LH release.	Decourt <i>et al.</i> , 2016 Clarke <i>et al.</i> , 2012
Cattle	Male GNX	Peripheral administration inhibits LH release. Inhibits LH release from cultured pituitaries in the presence of GnRH.	Kadokawa <i>et al.</i> , 2009

Table 2. Overview of the reported effects of RFRP-3 on mammalian reproduction.

RFRP and sex steroids

As previously mentioned, sex differences are found in the expression of RFRP in rats and Syrian hamsters, which a higher expression detected in females as compared to males (Jorgensen et al., 2014)(Henningsen et al., 2015). These differences could potentially be driven by differences in gonadal steroid hormone feedback. To support this hypothesis, some studies report the presence of ER α in RFRP neurons in female Syrian hamsters and mice (Kriegsfeld et al., 2006; Molnar et al., 2011; Poling et al., 2012), and in lines with these observations, E2 has been found to stimulate RFRP expression in female Syrian hamsters and rats, whereas in males, testosterone and castration seem to have no effect on RFRP expression (Kriegsfeld et al., 2006; Mason et al., 2010; Revel et al., 2008)(Iwasa et al., 2012). In contrast, both E2 and testosterone have been shown to decrease *Rfrp* expression in mice (Molnar et al., 2011; Poling et al., 2012). Due to the conflicting findings and the relatively limited information available, further studies are needed in order to fully determine whether RFRP neurons express sex steroid receptors and whether the above-mentioned differences could reflect actual differences between species.

RFRP and female reproductive activity

RFRP neurons project to the AVPV/MPN where the kisspeptin neurons, responsible for initiating the pre-ovulatory LH-surge, are expressed (Henningsen et al., 2015)(Kriegsfeld et al., 2006)(Rizwan et al., 2012)(Smith et al., 2006c). Besides the high levels of E2, as an indicator of ovarian maturation, timing of the LH-surge is also gated by circadian signals (Chassard et al., 2015)(Simonneaux and Bahougne, 2015), and recently, it has been suggested that RFRP neurons mediate a SCN-generated circadian output onto the AVPV/MPN kisspeptin neurons, thereby modulating the timing and generation of the surge. RFRP-ir cell numbers and their activational state were found to be decreased at the time of the LH-surge on the day of proestrus, as compared to other time points on the day of proestrus and in diestrus, in the Syrian hamster (Gibson et al., 2008), and similarly, in rats and ewes, RFRP expression is reduced during the pre-ovulatory period (Clarke et al., 2012)(Jorgensen et al., 2014). Interestingly, another study further demonstrated that SCN-derived VIP-ergic terminal fiber projections are found in close apposition to RFRP neurons in female Syrian hamsters and more importantly, they found that central VIP administration markedly suppressed RFRP cellular activity, when injected in the afternoon/evening, but not in the morning (Russo et al., 2015). Russo *et al.* found however, no co-localization between RFRP positive labeled cells and the VIP receptor, suggesting that the effect is indirect (Russo et al., 2015). Altogether, these data point towards a specific circadian rhythm in RFRP expression and release in females, adding a supplementary role of RFRP in regulating female reproductive activity, at least in this seasonal species. To date, the effect of RFRP-3 administration on the female rodent reproductive axis have primarily been investigated in OVX animals, in order to bypass the potential interference of varying levels of circulating sex steroids. In OVX rats (Pineda et al., 2010a)(Murakami et al., 2008) and Syrian hamsters (Kriegsfeld et al., 2006), central administration of

GnIH inhibits artificially-elevated plasma LH levels. Considering the inhibitory effect of GnIH found in female Syrian hamsters, along with the possibly VIP-mediated decrease in RFRP expression at the time of the LH-surge, it seems that RFRP neurons play an important role in regulating the timing and/or generation of the LH-surge. However, it is important to keep in mind that the effects of RFRP-3, rather than GnIH, should ideally be investigated in the intact female to further test this hypothesis, which is presented in figure 9.

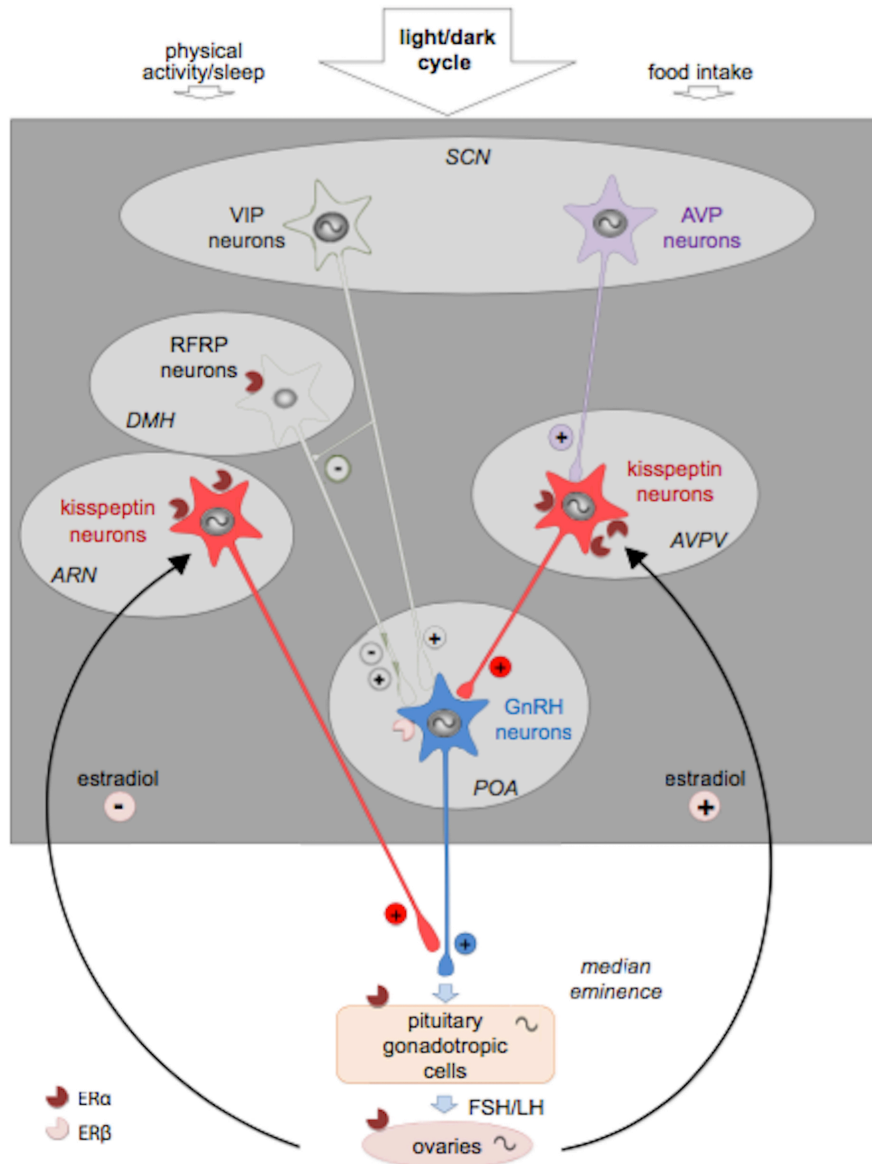


Figure 9. Schematic representation of neuroendocrine pathways timing female reproduction. The master circadian clock located in the suprachiasmatic nucleus (SCN) is synchronized to 24h primarily by changes in the light/dark cycle and to a less extent by changes in food intake and the sleep/wake cycle. Two SCN-derived peptides forward the daily information to the reproductive axis. Arginine vasopressin (AVP) projects to and activates kisspeptin (kp) neurons in the anteroventral periventricular nuclei (AVPV), leading to activation of GnRH neurons. Vasoactive intestinal peptide (VIP) directly modulates GnRH neuron activity and possibly indirectly via RFRP neurons of the dorso/ventromedial hypothalamus (DMH). GnRH release activates the synthesis and release of the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn regulate follicle maturation, estradiol synthesis, and finally ovulation triggered by the LH surge. Kp neurons are the main target of estradiol, with an inhibitory feedback of low estradiol levels on kp neurons in the arcuate nucleus (ARN) and a stimulatory feedback of high estradiol on kp neurons in the AVPV. Modified from Simonneaux and Bahougne, 2015.

Dissecting the roles of RFRP with pharmacological tools

RFRP peptides are, as already briefly mentioned, members of the large RFamide family of peptides, consisting of five main members; PrRP, NPFF, QRFP/26RFa, kisspeptin and RFRP (Fukusumi et al., 2006) that all share a common LPXRFamide (X=L or Q) C-terminal motif. Each peptide, in the given order, binds to its cognate receptor; Gpr10, Gpr74, Gpr103, Gpr54 and Gpr147, but also has the potential to cross react with other receptors, to an extent that is yet unknown. RFamide peptides and their receptors are implicated in various functions, however, one other RFamide peptide, 26RFa, has been shown to potentially play a role in regulating reproductive activity (Chartrel et al., 2011). These findings underline the need for additional studies, in order to understand the contribution of each RFamide peptide and receptor in regulating reproductive activity, but advances have, until recently, been limited due to the lack of appropriate pharmacological tools.

In 2014, Tena-Sempere and colleagues published their first observations made in a mouse KO model lacking a functional RFRP receptor, GPR147, (Leon et al., 2014). Surprisingly, the GPR147 deficient mice did not display strong reproductive phenotypic alterations as compared to wild type (wt) mice. However, during pubertal transition, male KO mice exhibit increased LH levels and in adulthood, FSH levels were higher in both female and male KO mice, as compared to wt mice. Moreover, litter sizes from KO mice were slightly increased as compared to wild type litter sizes. Interestingly, the male KO mice moreover showed an increased level of kisspeptin expression in the ARC, but not in the AVPV/MPN, suggesting that in male mice RFRP neurons provide a tonic inhibition on ARC kisspeptin neurons. Besides the observed effects on reproductive activity, the GPR147 KO mice also showed some metabolic deficits, thereby linking RFRP peptides to pathways regulating metabolism.

In the attempt to gain more insight into the underlying mechanisms of how RFRP functions to regulate reproductive activity, tools like receptor modulators (agonists and antagonist) are very useful. One such example is the GPR147 receptor antagonist, RF9, which has been extensively studied for its antagonistic effect of RFRP signaling, through its capability to non-selectively block GRP147 and GPR74. RF9 was the first small molecule GPR147 antagonist that could be administered systemically and was found to successfully block the inhibitory effect of RFRP observed in several species (Simonin et al., 2006). Recent findings, however, show that RF9 also binds to GPR54, through which it agonizes the effect of kisspeptin (Min et al., 2015)(Leon and Tena-Sempere, 2015), making it a less fit candidate for studying the role of RFRP in reproduction. Not long ago, another study presented a novel GPR147 antagonist, named GJ14, which was found to successfully block the anxiogenic effect of RFRP (Kim et al., 2015), however, the potential of this compound to antagonize reproductive-related effects of RFRP have not yet been reported.

RFRP and its effects outside the HPG axis

At times, the reproductive might have to be up- or down-regulated to overcome sudden or adaptive changes to environmental factors e.g. changing seasons, metabolic challenges and stress. Proper regulation of reproductive activity thus also relies on the interplay between the different endocrine axes, and since RFRP peptides have been shown to have effects related to feeding, nociception and stress, they have been proposed as a likely candidate for how these axes can signal to influence one another.

Energy demanding reproductive events, such as pubertal development, are highly sensitive to disruptions in the normal energy homeostasis and RFRP might play an important role in how the reproductive axis is regulated, after experiences of metabolic stress. The ARC nucleus is an important area for maintaining energy homeostasis and contains neurons, which express i.e. neuropeptide Y (NPY), proopiomelanocortin (POMC) and orexin, all known to be necessary for proper regulation of energy uptake and expenditure. As previously mentioned, RFRP fibers are found in this area, but more importantly, RFRP-3 administration has been shown to increase food intake in several species (Murakami et al., 2008)(Johnson et al., 2007)(Clarke et al., 2012). In contrast, RFRP-1 administration is followed by a decrease in food intake in rat and chicken (Kovacs et al., 2012)(Newmyer and Cline, 2009), indicating that the two peptides might have opposite effect on food intake. Interestingly, studies in GPR147 deficient mice show that GPR147 KO mice are less effective in dampening LH release in response to metabolic stressors, such as short-term fasting and high-fat diet (Leon et al., 2014), further implying a function of RFRP in the maintenance of metabolic homeostasis.

Long-term use of opioids for treatment of pain has been linked to changes in endocrine functions (Brennan, 2013). A recent and worrying finding describes how analgesic exposure to pregnant rats, causes severe fetal germ cell development in either sex. Female offspring (F1) showed reduced ovarian and litter sizes, and follicle number, deficits that were detectable also in the second (F2) generation of offspring (Dean et al., 2016). Hormonal treatments, and other potential endocrine disrupters, have received a lot of attention for their damaging effects on normal endocrine functions, but findings like the one above clearly highlights the importance of examining potential endocrine effects of other pharmaceutical compounds e.g. analgesics, which are generally considered to be a safe drug also during pregnancy. Another RFamide peptide, Neuropeptide FF (NPFF), was originally discovered for its ability to induce hyperalgesia and modulate opioid analgesia, and was thought to be the endogenous ligand of GPR147 (or neuropeptide FF receptor 1, NPFF1), which like RFRP-1 and -3 binds to both GPR147 and GPR174 (NPFF2) with high affinity. Now, it is clear that RFRP-1 and -3 bind with higher affinities towards GPR147 and thus preferentially bind to this receptor, while NPFF has higher affinity towards GPR74, and is considered as the endogenous ligand for GPR74. Many studies are dedicated to search for new drugs and targets to find alternatives to the use of opioids, like morphine, in the treatment of pain, which is known to have several side effects (Kieffer and Evans,

2002). GPR147, GPR74 and their respective RFamide peptidergic ligands have been extensively studied for their involvement in pain responses and analgesia (Yang et al., 1985)(Roumy and Zajac, 1998)(Liu et al., 2001)(Elhabazi et al., 2012)(Lameh et al., 2010), as reviewed by Ayachi and Simonin 2014 (Ayachi and Simonin, 2014) and recent work has led to the development of several GPR47 antagonists, of which the RF9 compound have shown great clinical potential to block hyperalgesia induced by chronic opiate treatment and preventing opiate tolerance (Simonin et al., 2006)(Bihel et al., 2015).

As a normal response to stressful events, the hypothalamo-pituitary-adrenal (HPA) axis is activated through the release of corticotropin-releasing hormone (CRH) from the PVN. CRH stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary, leading to increased release of cortocosterone (cortisol in human) from the adrenal gland that via negative feedback in the hypothalamus modulates the reaction to stressful stimuli. The HPA and HPG axes affect one another to a great extent and proper interplay between the two is a prerequisite for reproductive success. Stress stimuli and stress hormones inhibit GnRH and gonadatropin release and stress-induced high levels of norepinephrine (NE) are known to act in ovaries, leading to disrupted cyclic regulations and development of cysts (Toufexis et al., 2014)(Chrousos et al., 1998)(Aaron et al., 1993)(Lara et al., 1990)(Mayerhofer et al., 1997). RFRP-3 has been shown to induce stress and anxiety-like behavior in animal models, and is thus thought to be a possible intermediate for the communication between the HPA and the HPG axes. To support this hypothesis, RFRP fiber projections are found in the PVN and have been shown to project directly to CRH neurons (Qi et al., 2009). Moreover, administration of both RFRP-1 and -3 induce c-Fos expression in the PVN and a subsequent activation of the HPA axis, manifested by an increase in ACTH release (Kaewwongse et al., 2011). Similarly, a recent published study shows that RFRP-3 induces CRH activation and corticosterone release, an effect that could be blocked completely by co-treatment with the selective antagonist of GPR147, GJ14 (Kim et al., 2015).

SEASONAL CONTROL OF THE REPRODUCTIVE AXIS

Mammals are facing strong daily and annual changes of their living environment to which they need to adapt their biological functions. This is particularly true for reproductive activity, which requires a precise temporal adjustment to ensure species perpetuity. Seasonal information is translated into a rhythmic production of the pineal hormone melatonin via a well described photo-neuroendocrine system (Simonneaux and Ribelayga, 2003)(Hazlerigg and Simonneaux, 2015). We and other groups working in the field of seasonal reproduction have recently made major steps towards understanding the melatonin-driven seasonal control of reproduction. Indeed, we have demonstrated how seasonal variation in melatonin production regulates the synthesis of both kisspeptin and RFRP, which are critical for proper synchronization between GnRH activity and season. We have, in a recent review, summarized on the role of RFRP in regulation of seasonal reproduction, which can be viewed in

appendix 1. However, in order to introduce the readers with a more detailed description of the basis behind such regulation, I have in the following sections elaborated on the concepts presented in the review.

THE CONCEPT OF SEASONAL RHYTHMS

We are under the influence of two constant movements of our planet. First, the earth rotates around its own axis in a period of app. 24 hours, which causes the transitions between night and day. Second, the earth revolves in an elliptic axis around the sun, with a period of 365 days (or one year), leading to an annual cycle in changes of solar exposure. Because the earth is tilted at an angle of $23,5^{\circ}$ degrees relative to its plane, changes in solar exposure becomes more pronounced at higher latitudes and as a consequence the seasonal impact increases dramatically the further one gets from equator (figure 10). When the axis is tilted away from the sun, the northern hemisphere experiences winter, whereas the southern hemisphere experiences summer and when the axis is tilted towards the sun, the northern hemisphere experiences summer, while the southern hemisphere experiences winter.

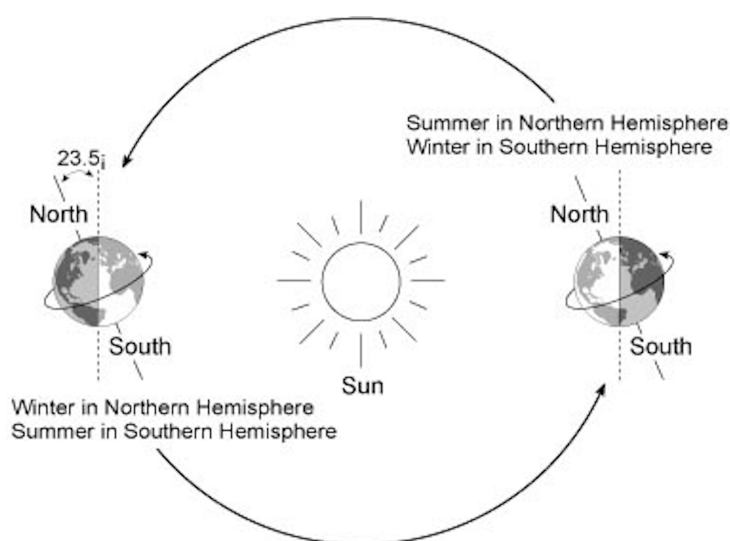


Figure 10. Geophysical properties of the earth's rotation and seasonal changes.

The earth is tilted $23,5^{\circ}$ degrees relative to its own plane, while rotating around its own axis with a period of 24 hours (one day) and on an axis around the sun with a period of 365 days (one year), thereby creating the seasonal changes in solar exposure, as we know it. Picture by Prof. Kenneth R. Lang, tufts University, MA, USA. Source: www.ase.tufts.edu/cosmos/index.asp

As a consequence, many species show seasonal changes in several aspects of their physiology e.g. reproduction, metabolism, and behavior, in order to overcome the challenging environmental changes and increase their chances of survival. In seasonal animals, reproduction is limited to a certain time of year to ensure that birth of the newborn occurs, when environmental conditions favor weaning and survival of the offspring, typically in spring or summer. In animals with a short period of gestation (e.g. hamsters and rabbits), the breeding season is limited to spring or summer and these animals are thus referred to as long day breeder. In contrast, larger mammals with a longer gestation time (e.g. sheep and deer) are sexually active in autumn, so-called short day breeders.

The annual variations in day length are transduced into a neuroendocrine message, namely the nocturnal secretion of the pineal hormone melatonin, which through mechanisms described in more

detail below, acts as a potent seasonal synchronizer of physiological changes in reproduction (Carter and Goldman, 1983a)(Hoffman and Reiter, 1965)(Pevet, 1988)(Malpaux et al., 2001)(Hazlerigg and Simonneaux, 2015).

THE PINEAL HORMONE MELATONIN SYNCHRONIZES REPRODUCTION WITH SEASONS

Early studies have demonstrated that synchronization of reproductive activity with season is driven by the pineal hormone melatonin (Hoffman and Reiter, 1965). Photoc information reaches the pineal gland via the retino-hypothalamo-pineal pathway that during nighttime generates a release of NE, which acts as a potent and reliable regulator of the rhythmic release of melatonin from the pineal gland. As a consequence, melatonin is synthesized and secreted in a diurnal fashion with a dramatic increase during nighttime that returns to nearly undetectable levels at daytime, with the duration of elevated melatonin depending on night length (Karsch et al., 1984)(Carter and Goldman, 1983b)(Hazlerigg and Simonneaux, 2015)(Hoffman and Reiter, 1965)(Malpaux et al., 2001)(Pévet, 1988). In the LP-breeding Syrian hamster, which is widely used as a rodent model to study seasonal reproduction, short day lengths and increased nocturnal release of melatonin, inhibits the reproductive axis, and removal of the melatonin signal by pinealectomy, prevents the short day-mediated inhibition of reproductive activity (Hoffman and Reiter, 1965)(Revel et al., 2007), as demonstrated in figure 11. In contrast, SP-breeding sheep becomes sexually quiescent after transfer to LP conditions (Bittman and Karsch, 1984)(Malpaux et al., 1997). Despite the fact that the reproductive timing is opposite in hamsters and sheep, in both cases the photoperiodic changes in circulating levels of melatonin synchronize reproduction with season.

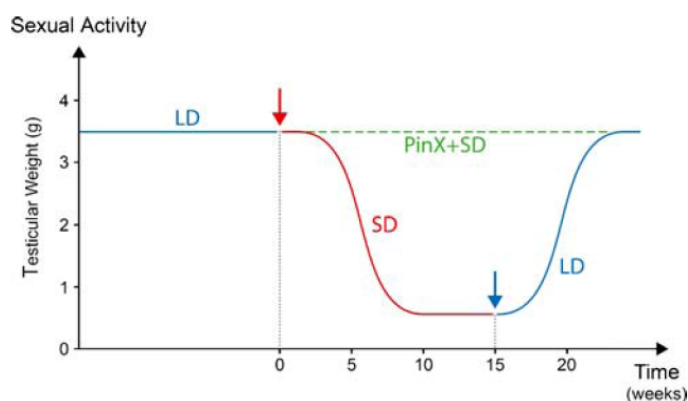


Figure 11. Photoperiod and the pineal hormone melatonin modulate reproductive activity in Syrian hamsters. In the LP-breeding Syrian hamster, sexually activity is promoted by the long hours of light, whereas exposure to SP inhibits the reproductive axis within 8-10 weeks. Removal of the melatonin input by pinealectomy, prevents synchronization of reproductive activity with changes in the light/dark cycle and abolishes SP-induced testicular regression in male Syrian hamsters. Revel *et al.*, 2007.

Although the focus in this work will be on the melatonin-dependent regulation of reproductive activity, it is worth mentioning that studies of pinealectomized Siberian hamsters and sheep have revealed a free-running endogenous rhythm of sexual activity (Bittman et al., 1983)(Woodfill et al., 1994)(Saenz de Miera et al., 2014), indicating that in some cases melatonin seems to synchronize an endogenous circannual rhythm by which reproduction is synchronized, rather than being directly

responsible for the circannual changes in the reproductive status (Malpaux et al., 1998)(Dahl et al., 1994a).

The retino-hypothalamo-pineal pathway

Changes in the light/dark cycle (photoperiod) is captured by the retina and transduced to the pineal gland via a well-characterized photoneuroendocrine pathway named the retino-hypothalamo-pineal pathway (figure 12).

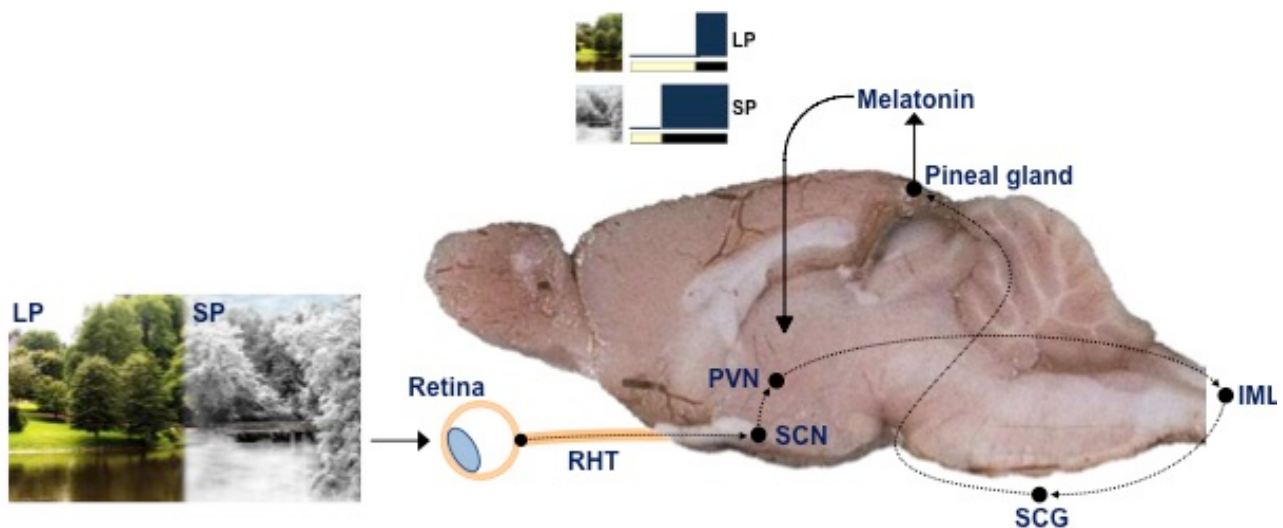


Figure 12. The retino-hypothalamo-pineal pathway. Light/dark information is perceived by the retina and converted into a rhythmic release of melatonin from the pineal gland, via a multisynaptic neuroendocrine pathway; retinal ganglion cells transduce the photic signal to the suprachiasmatic nucleus (SCN) via the retino-hypothalamic tract (RHT). The SCN projects to the paraventricular hypothalamic nucleus (PVN), which in turn projects to the intermediolateral cells (IML) of the spinal cord. IML neurons innervate the rostral pole of the superior cervical ganglion (SCG) from where noradrenergic neurons project to the pineal gland, where they release norepinephrine, which promotes the synthesis and release of melatonin into the bloodstream. The SCN restricts norepinephrine release to nighttime and as a result, melatonin plasma levels are increased at night. In summer, long photoperiod (LP) and shorter nights are associated with short melatonin peaks, whereas in winter, short photoperiod (SP) and longer nights are associated with longer melatonin peaks.

Photic information mainly reaches the brain via the retino-hypothalamic tract (RHT), which receives input from rod and cone photoreceptors through retinal ganglion cells (RGCs), whose axons bundle together in the optic nerve along the RHT (Moore and Lenn, 1972). Other intrinsically photoreceptive retinal ganglion cells (ipRGCs) have the ability to sense light, by expressing the photopigment melanopsin and can engage in the non-image forming visual system (Lucas et al., 1999). From the RHT, the signal reaches the SCN as a release in glutamate and pituitary adenylate cyclase activating peptide (PACAP) upon light stimulation, which entrains a circadian molecular clockwork to the light/dark cycle (Hannibal et al., 1997)(Ebling, 1996)(Hannibal, 2002).

The SCN, located in the anterior hypothalamus, has through extensive studies been identified as the seat of an endogenous circadian oscillator, also referred to as the master circadian clock. The nuclei are subdivided into a ventrolateral and a dorsomedial part, which are characterized by a nocturnal

peak of VIP and a diurnal peak of AVP, respectively (Tominaga et al., 1992)(Abrahamson and Moore, 2001). This rhythmic expression of neuropeptides is synchronized by a molecular clock that is tightly controlled through positive and negative feedback loops regulating the transcription of so-called clock genes (e.g. *Clock*, *Bmal1*, *Per* and *Cry*). Importantly, daily rhythms in clock gene expression in the SCN respond to annual changes in photoperiod, demonstrated by an extended peak of diurnally expressed clock genes in LP as compared to SP (Tournier et al., 2007)(Johnston et al., 2005). Among the many functions of the master clock in regulating daily synchronization of several physiological features, the primary focus herein will be on the regulation of the rhythmic secretion of melatonin.

From the SCN, the photic message is forwarded to the PVN (Klein et al., 1983)(Hermes et al., 1996), which continuously stimulates pineal activity unless inhibited by GABA released from the SCN during daytime (Kalsbeek et al., 2000). From the PVN, the message is further conveyed via the intermediolateral cells of the upper three segments of the spinal cord (IML) (Tecemariam-Mesbah et al., 1997) and the superior cervical ganglia (SCG) (Strack et al., 1988) from where noradrenergic neurons project to the pineal gland. Here, the photic message finally reaches the pineal gland as a nocturnal release of NE that potently regulates melatonin synthesis and release (Larsen et al., 1998)(Larsen, 1999)(Drijfhout et al., 1996b)(Drijfhout et al., 1996a).

Melatonin synthesis

When released into the pineal gland, NE binds to $\beta 1$ - and $\alpha 1$ -adrenergic receptors (Deguchi and Axelrod, 1972), which triggers a large increase in intracellular levels of cyclic adenosine monophosphat (cAMP) and Ca^{2+} leading to the downstream activation of cAMP dependent protein kinase A (PKA) (Sugden et al., 1987)(Strada et al., 1972), which in turn regulates the activity of enzymes involved in the biosynthesis of melatonin (figure 13).

Melatonin is synthesized from the amino acid tryptophan (Trp), taken up from the blood and converted into 5-hydroxytryptophan (5-HTP) and serotonin (5-HT) by tryptophan hydroxylase (TPOH) and aromatic amino acid decarboxylase (AAAD). Serotonin is then acetylated by arylalkylamine-N-acetyltransferase (AA-NAT) into N-acetyl serotonin (NAS). Interestingly, with the onset of darkness, NE causes a dramatic increase in AA-NAT activity, and subsequent reduction in activity at light onset, thereby regulating the rhythm or duration of melatonin synthesis (Klein and Weller, 1970)(Gastel et al., 1998)(Illnerova et al., 1978). NAS is upon methylation converted into N-acetyl 5-methoxytryptamine, commonly known as melatonin, by hydroxyindole-O-methyltransferase (HIOMT). In contrast to AANAT, HIOMT activity does not show any daily variation. Instead, HIOMT activity has been found to be higher in SP as compared to LP, resulting in an elevated peak of melatonin and thus, seems to control the amplitude of the melatonin peak according to changes in season (Ribelayga et al., 1999b)(Ribelayga et al., 1999a). AA-NAT and HIOMT thus act in concert to control the duration and amplitude of the nocturnal peak of melatonin to reflect the daily and seasonal changes.

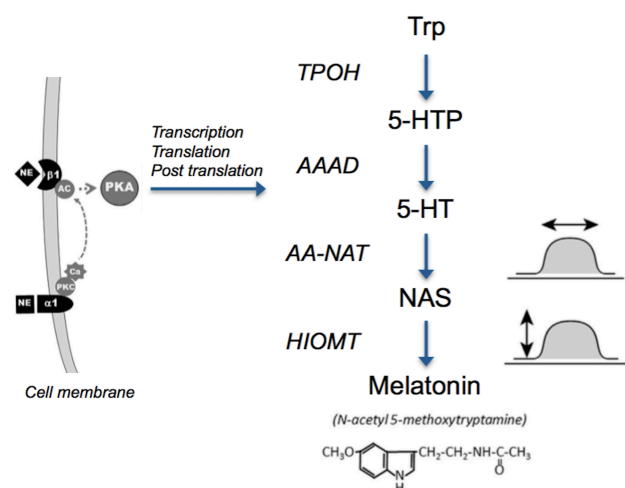


Figure 13. Melatonin biosynthesis pathway. Norepinephrine (NE) binds to β_1 - and α_1 -adrenergic receptors in the pineal gland and dramatically increases arylalkylamine-N-acetyltransferase (AA-NAT) activity. Tryptophan (Trp) is taken up from the blood and converted into 5-HTP and serotonin (5-HT) by tryptophan hydroxylase (TPOH) and aromatic amino acid decarboxylase (AAAD). Serotonin is then acetylated by arylalkylamine-N-acetyltransferase (AA-NAT) into N-acetyl serotonin (NAS) that upon methylation by hydroxyindole-O-methyltransferase (HIOMT) is converted into N-acetyl 5-methoxytryptamine, commonly known as melatonin. Modified from Hazlerigg and Simonneaux, 2015.

The pineal gland is a highly vascularised structure and melatonin enters the blood by simple diffusion. Although melatonin has been shown to be synthesized in other structures, such as the retina, hardierian gland and gut, levels of melatonin in the circulation and in the cerebrospinal fluid (CSF) are dependent entirely upon synthesis and release of pineal melatonin, which suggests that melatonin in other structures than the pineal glands might serve for autocrine and/or paracrine signaling.

Melatonin modes and sites of action

Three melatonin receptor subtypes have been characterized so far; MT1 (Mel1a), MT2 (Mel1b) and Mel1c, with its mammalian ortholog GPR50 (Dufourny et al., 2008)(Reppert et al., 1996). Melatonin receptors are distinct pharmacological subtypes that couples to various intracellular signaling pathways, however, the best characterized effect of melatonin is an acute inhibitory and chronic sensitizing effect on adenylyl cyclase/cAMP signaling (Carlson et al., 1989)(Hazlerigg et al., 1993)(Vaněček and Vollrath, 1989)(Morgan et al., 1991b)(Morgan et al., 1991a). Using the highly specific 2-[125 I]-iodomelatonin, high affinity melatonin binding sites have been found in the hypothalamus and the *pars tuberalis* (PT) of mammals. In more details, melatonin binding sites have been found in the SCN, DMH, PVT and the VMH of the Syrian hamster hypothalamus (Morgan et al., 1994). Amongst species, the highest concentration of melatonin receptors is found in the PT (Weaver et al., 1989)(Vaněček et al., 1987)(Vaněček, 1988)(Williams et al., 1989). The MT1 subtype seems to be dominantly expressed throughout species and is known to be responsible for the neuroendocrine integration of season (Poirel et al., 2003)(Cogé et al., 2009). Siberian hamsters (also known as the Djungarian hamster or *Phodopus sungorus*), lack a functional MT2 receptor and are therefore referred to as a natural KO for the MT2 receptor subtype. Despite the circumstances, Siberian hamsters show seasonal reproductive responses to melatonin, further supporting that MT1, and not MT2, is responsible for transmitting the melatonin-dependent seasonal input (Weaver et al., 1996).

Maywood and Hastings have shown that site specific lesions of iodomelatonin-binding sites in the MBH prevent testicular regression in Syrian hamsters exposed to SP (Maywood et al., 1996), and in

Siberian hamsters, melatonin infusion into or lesions of the SCN, alter the reproductive response to seasonal changes (Bartness et al., 1991)(Badura and Goldman, 1992). Finally, in sheep, the premammillary region of the hypothalamus contains melatonin binding sites (de Reviere et al., 1989)(Malpaux et al., 1998), and melatonin implantations in the area of this structure, but not in the PT, prevent synchronization of reproduction with photoperiod (Lincoln and Maeda, 1992)(Malpaux et al., 1998)(Malpaux et al., 1993)(Malpaux et al., 1995). Altogether, these data have pointed towards the potential importance of these hypothalamic regions, for proper integration of the melatonin-dependent photoperiodic signal onto the reproductive axis. However, it has not been possible to determine whether and how melatonin can act directly on these hypothalamic sites, and accumulating evidence now point towards the PT as the major site for the hypothalamic integration of the melatonin signal in seasonal breeders (Clarke et al., 1983)(Johnston et al., 2005)(Hanon et al., 2008a)(Dardente et al., 2014)(Hazlerigg and Simonneaux, 2015).

TSH, THYROID HORMONES AND THE MELATONIN-DRIVEN REPRODUCTIVE ACTIVITY

In 2003, Yoshimura and colleagues made a remarkable finding, which unveiled a link between the thyroid stimulating hormone (TSH) pathway and seasonal reproduction. They showed that light-induced type 2 thyroid hormone deiodinase (Dio2) expression, and subsequent hormone conversion of thyroxine (T4) into the bioactive triiodothyronine (T3), regulates the photoperiodic response of gonads in birds (Yoshimura et al., 2003). Since this discovery, it has been shown that seasonal variations in TSH and Dio2 in seasonal mammals are also melatonin-dependent (Revel et al., 2006b)(Hanon et al., 2008a). MT1-expressing cells in the PT synthesize TSH, and its production in the PT is strongly inhibited by the SP pattern of melatonin (Dardente et al., 2003)(Wittkowski et al., 1988)(Böckers et al., 1995). Recent work moreover disclosed that melatonin regulates the transcription of transcriptional factor EYA3 and clock proteins to drive the photoperiodic changes in TSH expression (Dardente et al., 2010)(Masumoto et al., 2010). Another primary response to photoperiodic changes in melatonin is the opposite regulation of Dio2 and type 3 thyroid hormone deiodinase (Dio3) expression in the MBH (Revel et al., 2006b)(Yasuo et al., 2007)(Yoshimura et al., 2003)(Köhrle, 1999)(Watanabe et al., 2004)(Hanon et al., 2010)(Hanon et al., 2008a). While Dio2 catalyzes the conversion of T4 to T3, Dio3 catalyzes both the conversion of T4 to the biological inactive reverse-T3 (rT3) and the conversion of T3 to the also inert diiodothyronine (T2). Thus, in concert Dio2 and Dio3 regulate the hypothalamic T4/T3 balance according to photoperiod with a higher production of T3 in LP as compared to SP (Barrett et al., 2007)(Hanon et al., 2010)(Saenz de Miera et al., 2013)(Klosen et al., 2013). Thyroid hormones have long been known to be important for the transitions between the breeding and non-breeding state. In 1940, thyroidectomy in starlings was shown to result in persistent breeding (Woitkewitsch, 1940). Similarly, thyroidectomized sheep remain in the breeding state, when changing from spring to anestrus, which can be reversed by T4 replacement, but interestingly, thyroidectomy displays no effect in the transition from the anestrus

state to the breeding state (Moenter et al., 1991)(Dahl et al., 1994b)(Webster et al., 1991)(Billings et al., 2002). Lastly, T3 administration in LP-breeding Siberian hamsters has been shown to block the SP-induced gonadal regression (Barrett et al., 2007)(Freeman et al., 2007)(Murphy et al., 2012).

Both deiodinases are highly expressed in a population of specialized glial cells in the ependymal cell layer lining the third ventricle, named tanycytes (Rodríguez et al., 2005). Interestingly, tanycytes co-express the TSH receptor (TSHR) and recent data clearly show that activation of these receptors increases *Dio2* expression, thereby increasing levels of T3 in the MBH in a number of seasonal mammals (Hanon et al., 2008a)(Klosen et al., 2013)(Bolborea et al., 2015)(Ono et al., 2008a)(Hanon et al., 2010)(Nakao et al., 2008c)(Revel et al., 2006b)(Dardente, 2012). In line with these observations, a recent study shows that PT-derived TSH, in contrast to *pars distalis*-derived TSH, does not stimulate the thyroid gland, but rather acts via TSHR on the tanycytes (Ikegami et al., 2014). Altogether, these studies have unveiled a conserved photoperiodic transduction pathway, which explains how the melatonin signal is integrated in the PT and transduced into a local thyroid message in the MBH (Hazlerigg and Simonneaux, 2015). Thyroid hormones mediate their neuroendocrine effects through still undefined hypothalamic sites, but intriguingly, a recent study from our group describes how chronic TSH administration in SP-adapted Siberian and Syrian male hamsters, reactivates the reproductive axis, while at the same time increasing the expression of RFRP and kisspeptin, suggesting that the melatonin/TSH/thyroid hormone pathway reaches the reproductive axis via these neurons (Klosen et al., 2013), (figure 14). PT-derived TSH seems to be the key between photoperiod and deiodinases in both birds and mammals, although birds are thought to be able to synchronize their reproduction to season in a melatonin-independent manner, via deep brain photoreceptors with opsin-like features in the PVN (Nakane and Yoshimura, 2010)(Halford et al., 2009).

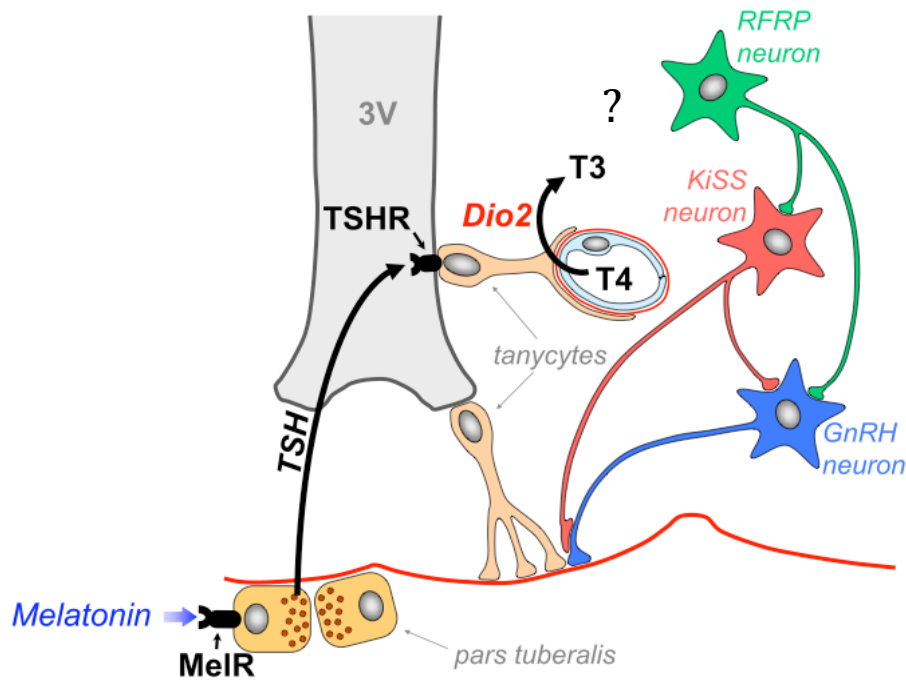


Figure 14. Melatonin-TSH-thyroid hormone signaling in the seasonal control of reproduction. In SP, the large production of melatonin inhibits TSH synthesis in the PT, whereas the lower production of melatonin in LP allows the synthesis and release of TSH. TSH is transmitted via TSH receptors expressed in tanycytes surrounding the 3rd ventricle and activates the enzyme deiodinase 2 (Dio2). Dio2 ultimately controls and increases the local availability of the active form of the thyroid hormone, T3, in the mediobasal hypothalamus, to regulate the neuroendocrine components of the reproductive axis. Modified from Klosien *et al.*, 2013.

HYPOTHALAMIC REGULATION OF SEASONAL REPRODUCTION

Hypothalamic control of the reproductive axis is commonly regulated amongst species through the release of GnRH from GnRH fiber terminals projecting to the ME. Despite the marked decrease of GnRH release during sexual quiescence, most seasonal species display an unchanged number and level of GnRH neurons and GnRH immunoreactivity in the different photoperiods (Urbanski *et al.*, 1991)(Barrell *et al.*, 1992). Much effort have been put into studying upstream regulators of GnRH activity, that could be responsible for transmitting the melatonin-dependent seasonal input further onto the GnRH neurons. As previously mentioned, site-specific lesions of the MBH prevent testicular regression in Syrian hamsters exposed to SP (Maywood and Hastings 1995 and 1996), and in Siberian but not Syrian hamster, melatonin infusion into the SCN, as well as SCN lesions, alter the reproductive response to seasonal changes (Bartness TJ, 1991) (Badura LL, 1992). In the sheep, implantation of melatonin in the VMH and the premammillary arcuate, prevent synchronization of reproduction with photoperiodic changes (Lincoln GA, 1992)(Malpoux B, 1993) (Malpoux B, 1995)(Malpoux B, 1998). Altogether, these findings suggest that melatonin might still act specifically on yet unidentified targets in these regions, but also, that there might differences in the areas involved in the downstream signaling of melatonin amongst species.

KISSPEPTIN AND SEASONAL REPRODUCTION

Kisspeptin neurons are regulated by photoperiod and are significantly down-regulated in their expression by melatonin in SP in both the AVPV/MPN and in the ARC (Ansel et al., 2010b)(Revel et al., 2006a), as demonstrated in figure 15. Central infusion of melatonin decreases the overall kisspeptin expression in both males and females adapted to LP, but interestingly, removal of sex steroid feedback in males by castration, leads to only the ARC population being down-regulated by melatonin. These findings have revealed that in Syrian hamsters, the two kisspeptin populations are regulated by melatonin via different mechanisms, and that the down-regulation of kisspeptin in the AVPV/MPN is a secondary effect caused by a decrease in the positive feedback of sex steroid.

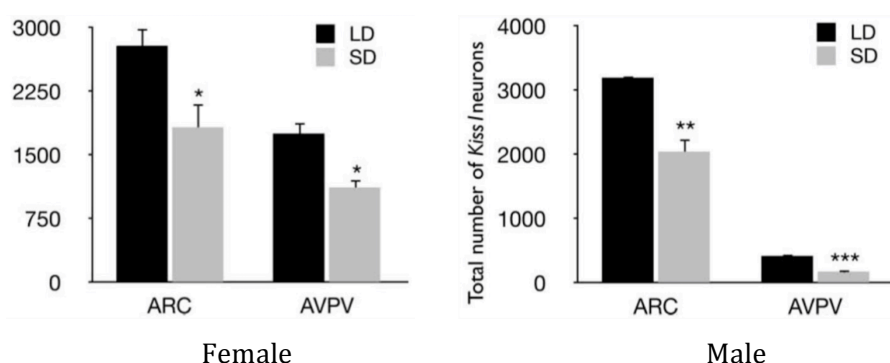


Figure 15. Seasonal variations in kisspeptin expression in the Syrian hamster. The number of immunoreactive kisspeptin neurons in both the AVPV and the ARC, is significantly decreased in SP in both female (left panel) and male (right panel) Syrian hamsters. Ansel *et al.*, 2010.

In the SP-breeding sheep, kisspeptin expression is oppositely up-regulated in SP, as compared to LP (Wagner et al., 2008)(Smith et al., 2008)(Chalivoix et al., 2010), which suggests that kisspeptin expression reflects the breeding state rather than the seasonal state of the animal. In both hamsters and sheep, continuous infusion of kisspeptin during sexual quiescence have been shown to fully restore reproductive activity (Revel et al., 2006a)(Ansel et al., 2011a)(Caraty et al., 2007)(Sébert et al., 2010), and with these findings, it seemed clear that kisspeptin neurons are an essential component between the photoperiodic signal and seasonal activation of GnRH neurons. This statement is however challenged by findings in two other seasonal hamster models, the Siberian and European hamster, in which the ARC kisspeptin expression is decreased in the breeding season (LP), as compared to the non-breeding season (Mason et al., 2007)(Greives et al., 2008)(Saenz et al., 2014). Whether these differences arise from differences in sex-steroid feedback or maybe differences in how kisspeptin neurons are implicated in the photoperiodic response, remains to be investigated. The inconsistencies, however, indicate that kisspeptin are unlikely to be solely responsible for mediating the melatonin-dependent seasonal signal onto the reproductive axis.

RFRP AND SEASONAL REPRODUCTION

Despite the fact that melatonin receptors have been found in the MBH and that electrolytic lesion of this area prevents SP-induced gonadal regression in the Syrian hamster (Maywood et al., 1996), it has not been possible to specifically link the presence of these binding sites to a direct effect of melatonin in this area. Accumulating evidence does, however, point towards an essential role of RFRP neurons, expressed in this area, in mediating the effects of photoperiod on the reproductive axis. RFRP expression is strongly regulated by photoperiod and is down-regulated in SP in all seasonal mammals investigated so far (Janati et al., 2013)(Ancel et al., 2012)(Revel et al., 2008)(Saenz de Miera et al., 2014)(Simonneaux and Ancel, 2012)(Smith et al., 2008). Studies in male Syrian and Siberian hamsters have shown that this SP-induced down-regulation is melatonin-dependent and can be prevented by removal of the pineal gland (Ubuka et al., 2012)(Revel et al., 2008). Our recent findings have revealed that in female Syrian hamsters as well, RFRP expression is down-regulated in SP, probably driven by the same mechanisms (Henningesen et al., 2015). Interestingly, RFRP expression is similarly down-regulated in SP in the SP-breeding sheep (Dardente et al., 2008)(Smith et al., 2008), underlines that the SP pattern of circulating melatonin displays a conserved inhibition on RFRP expression independent of whether mammals are long or short day breeders. In contrast to kisspeptin, the photoperiodic variations in RFRP expression is not modulated by the gonadal hormone feedback, since neither gonadectomy nor sex-hormone implants alter RFRP expression, at least in male Syrian hamster (Revel et al., 2008). In Siberian hamsters and sheep, the amount of GnRH neurons receiving RFRP fiber contacts has been found to be decreased in SP conditions, consistent with the decrease in RFRP expression in SP (Clarke et al., 2008)(Ubuka et al., 2012), but additional work is required in order to determine the physiological relevance of a possible decreased interaction in SP.

Species- and sex-specific differences in the effects of RFRP on seasonal reproduction

RFRP-3 stimulates the reproductive axis in male Syrian hamsters regardless of the photoperiodic state of the animal (Ancel et al., 2012). Because the expression of RFRP is low in SP, it was in the same study evaluated, whether an increase in RFRP, through a chronic delivery of RFRP-3 in SP-adapted male Syrian hamsters, would affect its reproductive status. Indeed, RFRP-3 was shown to reactivate reproductive activity, despite the SP-inhibitory conditions, via an increase in ARC kisspeptin expression, as shown in figure 16. The stimulatory effect of RFRP-3, in male Syrian hamsters, fits well with the high expression of RFRP observed in sexually active LP animals, and the data furthermore indicates that the stimulation of reproductive activity could be mediated via the ARC kisspeptin neurons. Thus, in the male Syrian hamster, RFRP neurons appear to integrate and transfer the seasonal input towards kisspeptin neurons. In male Siberian hamsters, RFRP-3 displays reverse effects depending on the photoperiodic condition, and stimulates LH release in SP, while inhibiting LH levels in LP conditions (Ubuka et al., 2012). The underlying mechanisms controlling such photoperiod-dependent effect of RFRP-3 in this species are unknown, but the stimulating effect of RFRP-3 in SP

might help to explain why there in this species is an up-regulation in ARC kisspeptin expression in SP-adapted Siberian hamsters (Simonneaux et al., 2009)(Greives et al., 2007)(Mason et al., 2007). Taken together, these findings indicate that in the two hamster species, RFRP-3 either has opposite sites of action in LP-adapted animals or is integrated differently along the hypothalamo-pituitary-gonadal axis.

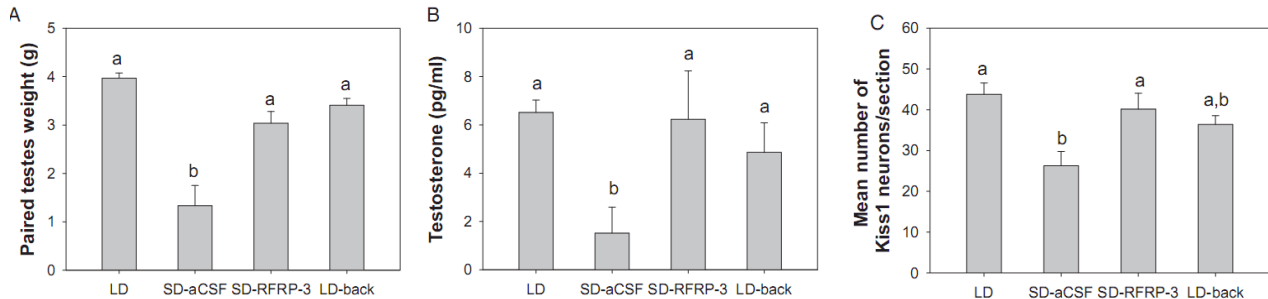


Figure 16. Chronic infusion of RFRP-3 reactivates the reproductive axis in SP-adapted sexually inactive male Syrian hamsters. Chronic administration of RFRP-3 (1mM) in SP-adapted males (SD+RFRP-3) significantly increases A) gonadal weight, B) testosterone levels and C) kisspeptin expression levels in the ARC as compared to treatment with vehicle (SD+aCSF) to levels comparable to those found in intact LD-adapted sexually active animals (LD) and hamsters with a re-activated reproductive axis after transfer from SD to LD (LD-back). Ancel *et al.*, 2012.

In ewes, peripheral injections of RFRP-3, inhibits LH release from the pituitary. Furthermore, RFRP-3 has been detected in the portal blood, which altogether indicate that RFRP-3 has a hypophysiotropic effect in ovine species (Clarke et al., 2012)(Sari et al 2009)(Smith et al 2012). However, the effect of RFRP-3 in sheep has been strongly questioned with recent reports showing that neither central nor peripheral administration of RFRP-3 has an effect on levels of circulating LH (Decourt et al., 2016b). Nevertheless, the fact that RFRP is regulated by photoperiod in a similar manner in sheep and hamsters supports our hypothesis that a similar neuroendocrine pathway is conserved between LP- and SP-breeders, with RFRP neurons playing a pivotal role in adapting reproductive activity to the environment (figure 17). Further analyses are required to test this hypothesis, in particular whether a possible inhibitory effect of RFRP-3, observed in some studies of the ewe, could account for the lower expression of kisspeptin in LP-adapted sexually inactive sheep.

In quail, studies have revealed that GnIH expression and release is directly regulated by melatonin acting on Mel1c receptors, specifically expressed in GnIH neurons (Ubuka et al., 2005). In contrast to mammalian seasonal species, GnIH expression is increased by melatonin and consequently GnIH-ir expression is increased in SP, as compared to LP (Ubuka et al., 2005). *In vitro* studies furthermore show that GnIH release has a diurnal rhythm and is increased during nighttime in quail hypothalamic explants (Chowdhury et al., 2010). In house and song sparrows GnIH-ir neurons are reported to be bigger towards the end of the breeding season (Bentley et al., 2003), whereas in wild Australia zebra finches, there is no variation in neither GnIH-ir nor *GnIH* expression between the breeding and non-breeding state (Perfito et al., 2011).

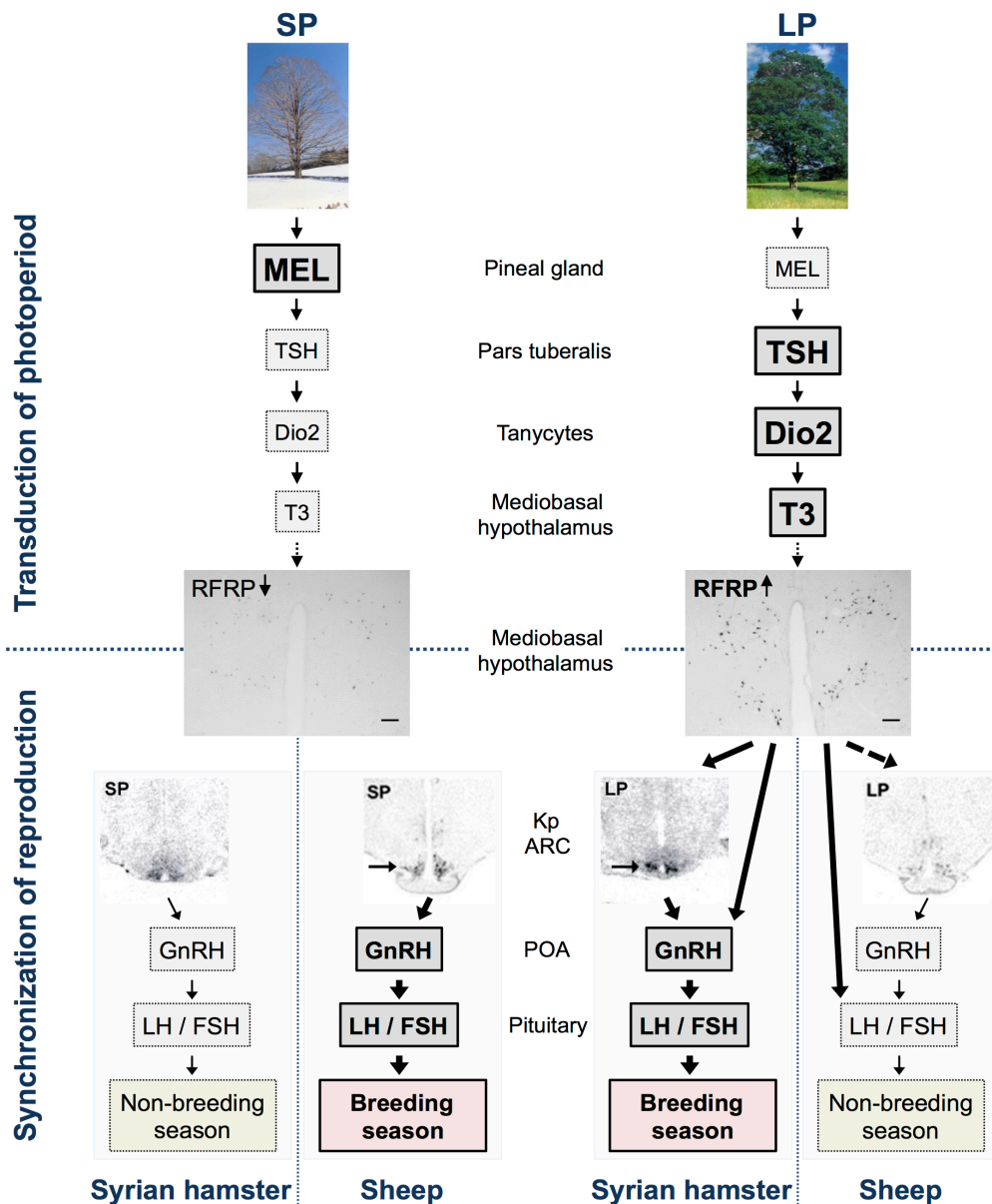


Figure 17. Model of the transduction of photoperiod and seasonal regulation of the reproductive axis in long (Syrian hamsters) and short day (sheep) breeders. In short photoperiod (SP), the large production of melatonin from the pineal gland inhibits TSH synthesis in the *pars tuberalis*, whereas the lower production of melatonin in long photoperiod (LP) allows the synthesis and release of TSH. TSH is transmitted via TSH receptors expressed in tanycytes surrounding the 3rd ventricle and activates the enzyme deiodinase 2 (Dio2). Dio2 ultimately controls and increases the local availability of the active form of the thyroid hormone, T3, in the mediobasal hypothalamus. T3 subsequently regulates the expression of RFRP also in the mediobasal hypothalamus so that there is a high expression in LP and a low expression in SP in both LP and SP breeders, as demonstrated with pictures of RFRP-ir neurons in brains from Syrian hamster kept in LP and SP (scale bar 100µm, taken from Henningsen *et al.*, 2015). In Syrian hamsters, RFRP subsequently acts either directly on GnRH neurons or indirectly *via* kisspeptin (kp) neurons (indicated by arrows) or other interneurons in the arcuate nucleus (ARC) to synchronize reproduction with season. In sheep, RFRP regulates the reproductive axis directly at the level of the pituitary (indicated by arrow) and possibly also directly or indirectly via kp neurons (indicated by dotted arrow) and/or GnRH neurons. Expression of the gene encoding kp in the ARC displays an opposite photoperiodic regulation in the two species being elevated in both LP-adapted sexually active Syrian hamsters (see arrow in picture taken from Revel *et al.*, 2006) and SP-adapted sexually active sheep (see arrow in picture taken from Wagner *et al.*, 2008). High levels of expression are highlighted using bold letters and arrows, as opposed to low levels of expression that are indicated by narrow letter and arrows.

In contrast to the stimulatory effect of RFRP-3 we have observed in the male Syrian hamsters (Ancel et al., 2012), central injections of the avian RFRP peptide GnIH inhibits LH release in ovariectomized females (Kriegsfeld et al., 2006). It should be stressed, however, that GnIH contains a LPLRFamide motif similar to that of the mammalian RFRP-1 and not RFRP-3. A similar sex-specific difference in RFRP expression is also reported in the non-seasonal rat, where RFRP-1 expression is found to be higher in females as compared to males (Jorgensen et al., 2014). Altogether, these findings point towards a particular importance of the RFRP system in regulating female reproductive activity, in seasonal and possibly also non-seasonal species.

The discovery of GnIH and RFRP, which in quail and rats were found to inhibit gonadotropins, led to a widespread belief that neurons expressing these peptides could act as counterpart to the stimulating effect of kisspeptin. Recent advances have made it clear, that the role of RFRP in regulating the reproductive axis is far more complicated than initially thought, and even varies according to species and sex. Moreover, RFRP neurons have been shown to somehow be involved in regulating reproduction in response to photoperiodic changes in seasonal species. Major steps have been taken towards understanding the melatonin-driven seasonal control of reproduction and current analyses point at RFRP neurons as a likely candidate for conveying the melatonin-dependent seasonal input onto the reproductive axis.

Increasing knowledge about the many effects of RFRP has at the same time left us with many unanswered questions such as, how and why RFRP can have opposite effects in species, sex and photoperiods.

In the attempt to answer some of these questions, the overall aim of this thesis has been to characterize the RFRP system and its effects on the reproductive axis in a seasonal mammal, the Syrian hamster. The main objectives of these studies have been to carry out a thorough characterization of the RFRP system in Syrian hamsters, taking into consideration both potential sex-differences as well as photoperiodic variations. To complete this, we aimed at investigating the cellular and molecular sites through which RFRP regulates the reproductive axis, as well as at comparing sites of actions and the downstream effects of RFRP between male and female Syrian hamsters. In order to evaluate the actual role of the RFRP system in the central regulation of seasonal reproduction, the effects of RFRP were analyzed in animals adapted to either long or short photoperiod. Our initial findings lead us to believe that there are fundamental differences in the RFRP system between male and female Syrian hamsters and therefore, we carried out a more detailed characterization of the RFRP system and its effects in female Syrian hamsters, where both cyclic changes, known as the estrous cycle, as well as seasonal changes, were taken into consideration.

OBJECTIFS (FRANÇAIS)

La découverte que les neuropeptides GnIH et RFRP inhibent la sécrétion des gonadotrophines chez la caille et le rat a conduit à généraliser leur action inhibitrice en tant que contrepartie de l'effet stimulateur du kisspeptine. Mais des données récentes ont clairement montré que le rôle du RFRP dans la régulation de l'axe reproducteur est beaucoup plus complexe qu'on ne le pensait au départ, et même varie selon l'espèce et le sexe. De plus, les neurones à RFRP se sont révélés spécifiquement impliqués dans la régulation de la reproduction en réponse aux changements photopériodiques de la production de mélatonine chez les espèces saisonnières.

De nombreuses questions se posent actuellement pour comprendre les mécanismes sous-jacents aux divers effets du RFRP, en particulier comment et pourquoi le RFRP peut avoir des effets opposés selon les espèces, le sexe et la photopériode.

Dans la tentative de répondre à certaines de ces questions, le projet général de cette thèse a été de caractériser le système RFRP et ses effets sur l'axe reproducteur chez un mammifère saisonnier, le hamster syrien. Les principaux objectifs ont été de caractériser le système RFRP (distribution des projections des neurones à RFRP et de ses récepteurs) chez les hamsters syriens, en tenant compte à la fois des différences sexuelles potentielles ainsi que des variations photopériodiques. Pour compléter cela, nous avons cherché à étudier les sites cellulaires et moléculaires par lesquels RFRP régule l'axe de la reproduction, et à comparer les sites d'actions et les effets de RFRP entre les hamsters syriens mâles et les femelles. Afin d'évaluer le rôle du système de RFRP dans la reproduction saisonnière, les effets de RFRP ont été analysés chez les animaux adaptés en photopériode longue ou courte. Finalement, nos premières résultats suggérant des régulations spécifiques chez hamsters syriens femelles, nous avons procédé à une caractérisation plus détaillée des effets du RFRP sur leur axe reproducteur en tenant compte de leurs cycles journaliers, oestral et saisonniers.

EXPERIMENTAL APPROACHES

This section describes some general concepts of the experimental approaches used in the present work. For a more detailed description of specific circumstances concerning individual experiments, the reader is referred to the materials & methods section of the given manuscript.

ANIMAL EXPERIMENTS

For all experiments we used the LP-breeding Syrian hamster (*Mesocricetus auratus* or the golden hamster), as a model for seasonal reproduction. This model has been extensively used in the study of both seasonal and non-seasonal features of the neuroendocrine control of reproduction, primarily due to its very short gestation period of approximately 16 days. In the wild, the Syrian hamster's natural habitats are found in the north of Syria and south of Turkey. Although often considered as nocturnal animals, meaning that they are active at night, in the wild, Syrian hamsters have a behavioral pattern that is more crepuscular rather than nocturnal. This means that they are primarily active at twilight, the periods before dawn and after dusk, which amongst other factors keeps the threat from predators at a minimum.

DETERMINATION OF THE ESTROUS CYCLE IN FEMALE SYRIAN HAMSTERS

The rodent estrous cycle is (most often) a 4-day cycle, which is characterized by four distinct stages called the proestrus, estrus, metestrus and diestrus. When evaluating the stages of the estrous cycle, there are different experimental approaches that can be applied. In example, the four stages can be determined by regularly, most often daily, collecting vaginal smears that allow one to monitor the cellular composition of the uterine epithelial lining that changes over the cycle (figure 18). Proestrus is characterized by a sparse distribution of non-nucleated squamous cells that are large, often polygonal and cornified epithelial cells and round nucleated or cuboidal cells. Following proestrus, estrus is dominated by squamous cells, many of them being cornified. The estrus is considered terminated with the characteristic appearance of a large number of elongated or spindle-shaped squamous cells in metestrus. The diestrus (the longest stage of the cycle), can be subdivided in early, mid and late diestrus, as indicated in the figure 18. At first, diestrus is determined by a dramatic increase in numbers of leukocytes and cuboidal or round nucleated cells. Along diestrus, the number of cells becomes sparse and towards the end of diestrus many of the round or cuboidal nucleated cells have undergone transition to become squamous cells, resulting in a smear containing a sparse but somewhat equal presentation of the different epithelial cell types.

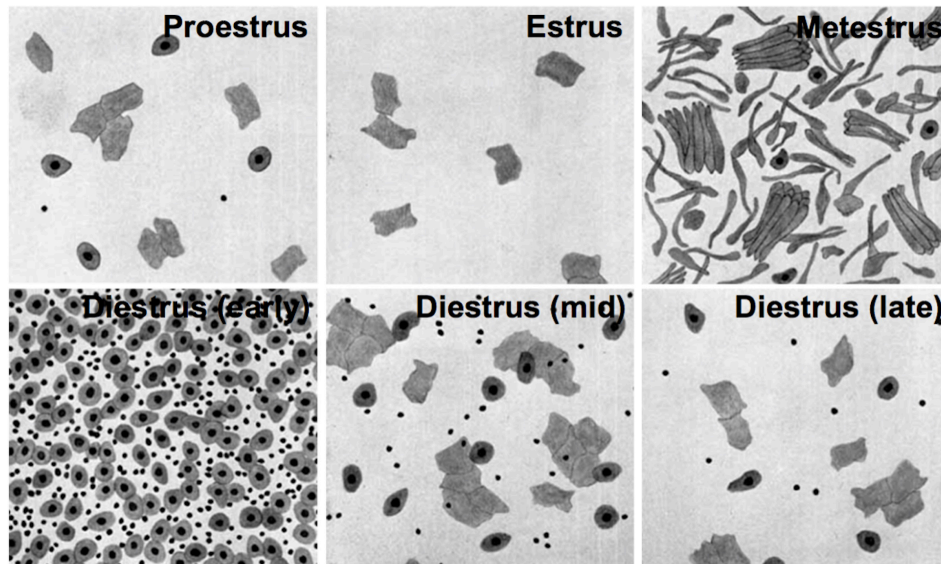


Figure 18. Schematic drawings of the epithelial cell compositions, which characterizes the four stages of the Syrian hamster estrous cycle. Proestrus is characterized by sparse distribution of both non-nucleated and often polygonal and cornified squamous cells and round nucleated or cuboidal cells. Cornified squamous cells dominate estrus, which comes to an end with a characteristic appearance of a large number of elongated squamous cells in metestrus. The diestrus is subdivided in early, mid and late diestrus, as indicated. Early diestrus is dominated by a dramatic increase in numbers of leukocytes and cuboidal and round nucleated cells. In mid diestrus the numbers of cells become sparse and towards late diestrus, many of the round and cuboidal nucleated cells have undergone transition to become squamous cells. Modified from Kent *et al.*, 1945.

Along staging the estrous cycle on vaginal smears, as mentioned above, we moreover tested the eligibility of determining the stages by measuring the electrical impedance of the epithelial cell layer (fig. 19). The impedance changes over the cycle, and peaks in proestrus, which in some cases can serve as a reliable and easy way to determine this stage of the cycle. In our setup, the peaks in impedance were not necessarily correlated with proestrus, as determined by vaginal smearing and LH measurements (figure 19), and showed a high degree of variation from one animal to another and from one cycle to another, and was therefore not considered to be a reliable method to determine the estrous cycle in our animals.

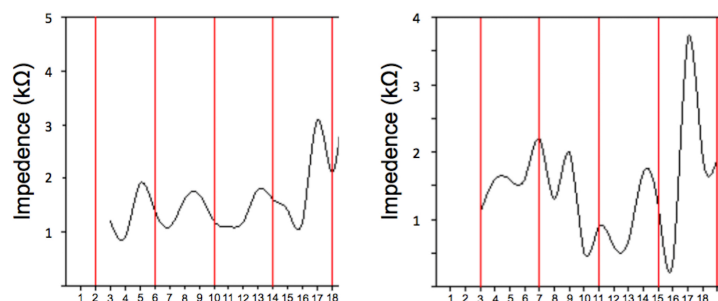


Figure 19. Electrical impedance measured in two individual animals over a period of 18 days. The epithelial impedance was measured daily for app. 4,5 cycles in female Syrian hamsters. Red lines indicate the day of metestrus (day 2, the day after proestrus day 1) as determined by smears.

INTRACEREBROVENTRICULAR (ICV) INFUSION

Implantation of a canula e.g. into the lateral ventricle, allows the delivery and diffusion of a compound of interest, which might not under normal conditions cross the blood brain barrier (BBB), and was in this work used in order to perform both acute and chronic icv injections. Syrian hamsters were anaesthetized using a mixture of Zoletil 20 (40 mg/kg, Virbac, Carros, France) and Rompun (10 mg/kg, Bayer Pharma, Puteaux, France) and positioned in a stereotaxic apparatus. The head of the animal was shaved and prepared for aseptic surgery and a single incision was made on the midline of the scalp. Once the area had been prepped, a stainless steel canula was placed in the lateral ventricle at 2 mm lateral to the midline, 0.8 mm anterior to the Bregma and 3 mm inferior of the dura mater. The canula was kept in place on the skull, by first placing two bone screws and then covering the area with dental cement. For acute icv injections, the cannula (PlasticsOne, Roanoke, VA, USA) was after implantation blocked with a metallic wire, protected with a plastic cap after which the animals were left one week to recover from the surgery. Injections (4 μ L/animal; flow rate 1 μ L/min) were performed under light anesthesia with isoflurane vapour (AErrane; Baxter, Maurepas, France), using a stainless steel internal canula (PlasticsOne), attached to polyethylene tubing prefilled with the solution of interest, and a 50 μ L Hamilton syringe (Hamilton Inc., Reno, NV, USA).

Chronic infusion

For chronic icv infusions, the canula (Alzet brain infusion kit1, Durect, Cupertino, CA, USA) was connected via subcutaneous polyvinylchloride tubing to an osmotic minipump (Alzet minipump model 2006, flow rate 0.15 μ L/hr, duration: 6 weeks, Durect, Cupertino, CA, USA), pre-filled with the solution of interest and placed under the skin of the back of the hamsters.

In the present work, we wanted to evaluate the effects on seasonal reproduction, animals were adjusted to long and short photoperiod, respectively, after which the canulas and minipumps were implanted. For injections in SP-adapted animals, the animals were adjusted to SP for app. 9 weeks, to allow regression of the photoperiod. The injections were performed over a period of app. 5,5 weeks, thus leading to a final exposure of SP conditions to app. 14,5-15 weeks, as demonstrated in figure 20. When keeping the animals in a constant short photoperiod, the reproductive axis will, after app. 16-17 weeks, spontaneously reactivate, a phenomena known as photorefractoriness, which should be taken into consideration when setting up the protocol for long term infusions.

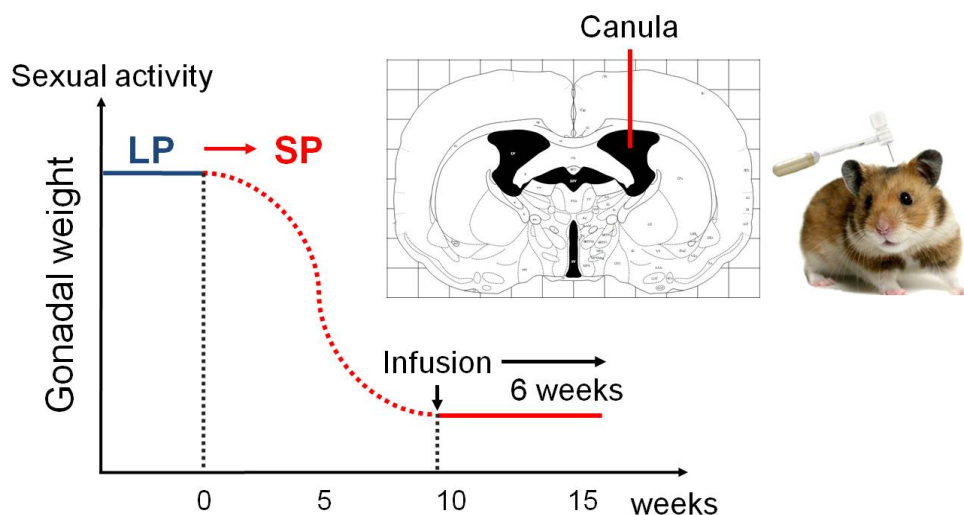


Figure 20. Principle behind canula implantation and chronic infusion in Syrian hamsters adapted to short photoperiod (SP). Animals are raised in long photoperiod (LP) until sexually matured. Animals are transferred to SP conditions that in sexually active animals cause a full regression of reproductive activity within 8-10 weeks. In the present work, animals were implanted with a canula into the lateral ventricle (0,8mm anterior to the bregma, 2mm lateral to the midline), connected to an osmotic minipump allowing central continuous delivery of a compound of interest for up to 6 weeks.

The impact of surgery and chronic delivery was evaluated on the gonadal weight, by comparing intact animals with aCSF treated animals (figure 21).

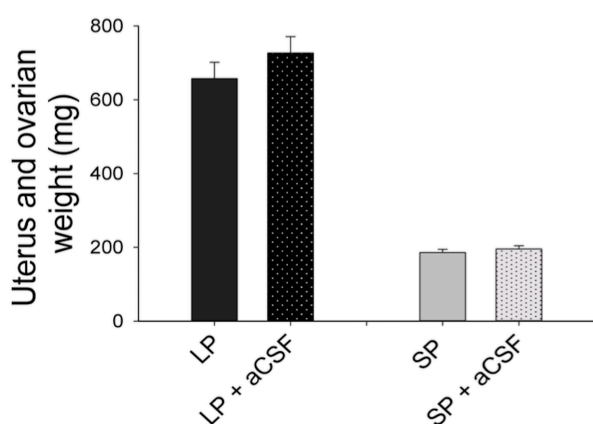


Figure 21. Validation of the model of chronic infusions. The procedure of canula implantation and chronic infusion has in itself no effect on reproductive features, which is validated by the lack of impact of chronic infusion of vehicle (a-CSF) in LP and SP animals on gonadal weight, as compared to intact LP and SP animals.

IN SITU HYBRIDIZATION (ISH)

In this work we used a radioactive *in situ* Hybridization (ISH) to detect GPR147 mRNA in the brain of Syrian hamsters. For this, a pSTBlue-1 vector containing a 1028bp Syrian hamster GPR147 (NPFF-1) insert, generated by Vincent-Joseph Poirel (Melatonin and seasonal rhythms, university of Strasbourg) was used for amplification of the insert. Sense and antisense GPR147 riboprobes were transcribed from linearized plasmids with either T7 (antisense promoter) or SP6 (sense promoter) RNA polymerase, according to the protocol provided with the MAXIscrip[®] kit (Ambion, USA). Transcription was done in the presence of radiolabeled [35S]UTP (1250 Ci/mmol, PerkinElmer, Courtaboeuf, France) allowing detection of the complementary riboprobe.

Brains were immediately after isolation frozen at app. -50°C and series of 20µm coronal sections were cutted on a cryostat (MICROM HM 560, Thermo Scientific, Waltham, MA), thaw-mounted on SuperFrost® ultraplus sections and stored at -80°C before proceeding with the hybridization protocol, as described in the following.

Sections were treated with 4% paraformaldehyde in 0.05 M phosphate-buffered saline (1× PBS, pH 7.4) for 15 min at RT, and then rinsed in 1× PBS for 2 min. The sections were acetylated in 0.75% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min and then rinsed for 2 min in 1× PBS. Next, sections were dehydrated by immersing the slides into increasing concentrations of ethanol (70, 90, 95 and 100%) for 1 min each after which sections were allowed to dry at RT before hybridization. The sections were hybridized in a humid chamber with 400 pM of the [³⁵S]UTP-labeled GPR147 cRNA sense or the antisense riboprobe, in a solution containing 50% deionized formamide, 10% dextran sulfate, 50 mM dithiothreitol, 1× Denhardt's solution, 2× standard saline citrate (SSC), 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast RNA, at 54°C for 16 h. After hybridization, the sections were rinsed for 5 min at RT in 2× SSC before being treated with ribonuclease A (0.02 µg/ml, Sigma, Lyon, France) in 10 mM Tris, pH 7.4, 0.5 M NaCl, 10 mM EDTA buffer (30 minutes at 37°C). Slides were then rinsed at RT in 1× SSC and 0.05x SSC at 52°C for 5 and 30 minutes, respectively, in order to reduce non-specific labeling. Finally, sections were dehydrated in a graded ethanol series (70, 90, 95 and 100%, 1 min each), left to dry at RT and subsequently exposed to X-ray films (HyperfilmMP, Amersham, UK) for 15 days concomitantly with [¹⁴C] microscale standards.

For semi-quantitative analysis and comparisons, all groups were processed using the same procedure and were blinded throughout processing, quantification, and evaluation. The location of the bound probe was visualized by autoradiography and captured as a digital image for quantifications. Semi-quantitative analysis of the autoradiogram was performed with a picture analysis system using ImageJ software (US National Institutes of Health, Bethesda, MD), which allows each structure of interest to be equally defined on all sections. Like that, semi-quantitative measurements were conducted in the same fixed measuring area, which was kept constant across all sections and animals and in accordance to a stereotaxic atlas of the Syrian hamster brain. Measures of the mean gray value in the region of interest were measured along with two background measurements after which the mean background was subtracted from that of the region of interest. Gray values were standardized to the [¹⁴C] microscale standards of known radioactivity placed on each film and as a negative control we quantified mean gray levels in areas with nonspecific binding. Lastly, specific hybridization was determined as the difference between the signals obtained with the antisense and the sense probe, respectively.

IMMUNOHISTOCHEMISTRY (IHC)

Immunoreactive neurons were visualized using an avidin-biotin immunohistochemical approach optimized for use on free-floating brain section, by Jens Damgaard Mikkelsen (Neurobiological research Unit, Copenhagen).

Prior to tissue collection, animals were transcardially perfused with 0.05M phosphate-buffered saline (PBS) immediately followed by perfusion with a periodate-lysine-paraformaldehyde fixative (4% paraformaldehyde, pH 7.4). The brains were removed and post-fixed over night at 4°C in the same fixative, transferred to PBS + 0.02% NaN₃ and stored at 4°C until further use. Brains were then dehydrated in a 30% sucrose-PBS solution for 2-3 days, frozen on the stage of a sliding microtome (Bright Series 8000, Bright Instruments Co Ltd., England), and sliced into 4 serial sets of 40µm thick coronal sections.

Brain sections were rinsed in PBS 3× 10 min and incubated in 1% hydrogen peroxide (H₂O₂) diluted in PBS for 10 min in order to block endogenous peroxidase activity. This was followed by 20 min incubation in a blocking buffer of 5% swine serum, 0.3% BSA and 0.3% Triton® X-100 in PBS (PBS TX) to block binding sites block unspecific binding. Sections were then incubated overnight (app. 18 h) at 4°C in a primary antiserum directed against the substrate of interest and diluted in blocking buffer. After the primary antiserum incubation sections were washed for 3× 10 min in PBS TX and then incubated for 60 min in biotin-conjugated secondary antibody directed against the Fc part of the primary antibody (normally used at a 1:1000 dilution; Jackson ImmunoResearch, USA). The procedure was followed by a 3× 10 min wash PBS TX, after which the sections were left to incubate for 60 min in avidin-biotin-peroxidase complexes (Vectastain ABC kit, Vector laboratories, USA) diluted in PBS TX that facilitates the connection between the biotin-conjugated secondary antibody and the biotin-conjugated peroxidase enzyme. Brain sections were then washed for 10 min in PBS TX, 10 min in PBS and 10 min in Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; pH 7.6). Finally, sections were treated with 0.1% diaminobenzidine (DAB; Sigma-Aldrich, MO, USA) and 0.03% H₂O₂ in Tris-HCl, allowing DAB to be oxidized by the peroxidase enzyme in the presence of H₂O₂, creating a brown precipitate. The sections were then washed for 5× 6 min in 1x PBS, mounted on glass slides using a mounting medium composed of 0.5% gelatine and 0.05% chromalun in distilled water, dried and coverslipped in Pertex® (Histolab, Sweden).

DUAL IMMUNOHISTOCHEMICAL LABELING

Co-visualization of two substrates of interest was facilitated using an avidin-biotin immunohistochemical approach, optimized from the above-mentioned procedure, to allow concomitant immunolabeling with primary antibodies derived from the same host species.

In the present work, this procedure was applied to label c-Fos positive nuclei along with neurons of interest, described in detail for each experiment in the method section of the respective publication. As a first step for all c-Fos dual labelings, sections were incubated overnight at 4°C with a primary rabbit polyclonal antiserum directed against c-Fos diluted 1:12000 (Mikkelsen et al., 1998). After following the procedure as described above, sections were developed in a nickel-enhanced 0.05% diaminobenzidine (Sigma-Aldrich, St Louis, MO) with 0.05% H₂O₂ in 0.05 M Tris-HCl buffer (pH 7.6) for 6 min, which instead of the brown precipitate generated with DAB only, generates a black precipitate within the nucleus. Towards the end of the procedure, the sections were developed with 0.1% DAB (as described in the single IHC procedure), resulting in a brown (cytoplasmic) precipitate, thus allowing the distinction of c-Fos (black nuclear labeling) positive and negative immunoreactive neurons.

RESULTS

The result section is divided in two parts and is presented as scientific articles, which have been published or submitted for publication, as indicated in each part.

Some additional and unpublished results are consulted in the discussion section.

Part I: Sex differences in the photoperiodic regulation of RF-amide related peptide (RFRP) and its receptor GPR147 in the Syrian hamster

- Neuroanatomical characterization of RFRP and its receptor, GPR147, in the Syrian hamster.
- Characterization of the photoperiodic regulation of the RFRP system in the Syrian hamster.
- Characterization of sex-dependent differences in the RFRP system in the Syrian hamster.

Part II: Multiple roles of the RFRP system in regulating reproductive activity in female Syrian hamsters

- RFRP expression and activation at different stages of the estrous cycle; diestrus and proestrus.
- Effects of acute central administration of RFRP-3 on the generation of the pre-ovulatory surge in LH on the day of proestrus.
- Effects of chronic central administration of RFRP-3 on the female Syrian hamster reproductive axis: photoperiod-dependent differences in the effects.

PART— SEX DIFFERENCES IN THE PHOTOPERIODIC REGULATION OF RF-AMIDE RELATED PEPTIDE (RFRP) AND ITS RECEPTOR GPR147 IN THE SYRIAN HAMSTER.

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ABSTRACT

RF-(Arg-Phe) related peptides (RFRP-1 and -3) are considered to play a role in the seasonal regulation of reproduction however the peptides effect depends on species and gender. This study aimed at comparing the RFRP system in male and female Syrian hamsters in long and short photoperiod to investigate the neuroanatomical basis of these differential effects.

The neuroanatomical distribution of RFRP neurons and fibers, revealed using an antiserum recognizing RFRP-1 and -3, as well as GPR147 mRNA, are similar in male and female Syrian hamsters. RFRP neurons are mainly found in the medial hypothalamus while RFRP projections and GPR147 mRNA are observed in the preoptic area, anteroventral-periventricular nucleus, suprachiasmatic nucleus, paraventricular nucleus, bed nucleus of stria terminalis, ventromedial hypothalamus, habenular nucleus and the arcuate nucleus. Number of RFRP neurons is higher in females than in males, and in both sexes, the number of RFRP neurons is reduced in short photoperiod. GPR147 mRNA levels are higher in females than in males and is down-regulated in short photoperiod, particularly in females. Interestingly, the number of RFRP-positive fibers in the anteroventral-periventricular nucleus is higher only in females adjusted to short photoperiod.

Our results suggest that the RFRP system, which is strongly regulated by photoperiod in both male and female Syrian hamsters, is particularly important in females with a distinct role in the anteroventral-periventricular nucleus, possibly in the regulation of the pre-ovulatory LH-surge via kisspeptin neurons.

INTRODUCTION

The central control of reproduction involves the gonadotropin-releasing hormone (GnRH) synthesizing neurons projecting to the median eminence where they release GnRH to the portal circulation. This neurohormone in turn activates the synthesis and release of pituitary gonadotropins luteinising hormone (LH) and follicle-stimulating hormone (FSH) (McArdle and Roberson, 2015). In many species reproductive activity is synchronized with seasons so that birth and weaning of the newborn occurs at the most favourable time of the year. Annual variations in day length are transduced into an endocrine message, namely the nocturnal secretion of the pineal hormone melatonin, which acts as a potent seasonal synchroniser of reproductive activity (Carter and Goldman, 1983b)(Hoffman and Reiter, 1965)(Pevet, 1988)(Malpaux et al., 2001)(Hazlerigg and Simonneaux, 2015). The exact mechanism through which melatonin regulates GnRH activity is not fully understood. However, recent studies point to RFamide-related peptide (RFRP) neurons in the medial hypothalamus as candidates for integrating and transmitting the photoperiodic melatonin signal onto the reproductive axis to synchronize reproduction with season (Ancel et al., 2012)(Klosen et al., 2013)(Revel et al., 2008)(Ubuka et al., 2012).

RFRP was discovered in birds and mammals in 2000 (Hinuma et al., 2000)(Tsutsui et al., 2000). In birds, the peptide was shown to inhibit gonadotropin secretion from cultured quail pituitaries and thus termed gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000). The mammalian gene named *RFamide-related peptide (rfrp)* produces two functionally peptides; RFRP-1 and RFRP-3 (Hinuma et al., 2000)(Fukusumi et al., 2001b)(Ukena et al., 2002). RFRP-3 has been shown to inhibit GnRH neuron activity and gonadotropin release in several species (Anderson et al., 2009)(Clarke et al., 2008)(Ducret et al., 2009)(Johnson et al., 2007)(Kriegsfeld et al., 2006)(Pineda et al., 2010a), however two recent studies reported that RFRP-3 administration stimulates reproductive activity in male Syrian and Siberian hamster, indicating that the effect of the peptide is species-dependent (Ancel et al., 2012)(Ubuka et al., 2012). Strikingly, in the Syrian hamster, gonadotropins are stimulated by central RFRP-3 administration in male (Ancel et al., 2012) but inhibited in female (Kriegsfeld et al., 2006), adding a supplementary sex-difference in the effect of the peptide on reproductive activity and possibly on its seasonal control. Identification of the downstream targets of RFRP neurons, as well as their modulation by environmental cues and sex steroids, may help to understand such variability in RFRP-3 effects.

RFRP fiber projections have been found in multiple regions of the rodent brain (Kriegsfeld et al., 2006)(Ubuka et al., 2012). In particular, RFRP terminals make apparent contact to 20-40% of GnRH neurons in rodents and sheep (Kriegsfeld et al., 2006)(Ubuka et al., 2012)(Rizwan et al., 2012)(Smith et al., 2008). In female mice, 20% of kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) and 35% of kisspeptin neurons in the arcuate nucleus (ARC) receives RFRP-fiber contacts (Poling et al., 2013)(Rizwan et al., 2012). RFRP-3 application to mouse brain slices inhibits the firing rate of 41%

GnRH neurons and stimulates the firing rate of 12% of the GnRH neurons (Ducret et al., 2009), whereas intracerebroventricular (icv) infusion of RFRP-3 in male hamsters induces c-Fos expression in 20-30% GnRH neurons but also in non-kisspeptinergic neurons of the arcuate nucleus (Ancel et al., 2012). RFRP peptides bind preferentially to GPR147 (also known as NPFF1), which has been reported to be expressed in 15-33% of mice GnRH neurons and a subpopulation of kisspeptin neurons in the AVPV (5-16%) and the ARC (25%) (Poling et al., 2012)(Poling et al., 2013)(Rizwan et al., 2012).

The sex-dependent regulation and seasonal modulation of the RFRP neuron projections and sites of action has never been fully investigated and functional differences amongst species, sexes and seasons have emphasized the need for a thorough characterization of the RFRP system. Therefore, in the present study we aimed at providing a better neuroanatomical description of the RFRP system in brain areas involved in the control of reproduction in Syrian hamsters, with a specific focus on potential sex- and photoperiod-dependent differences.

MATERIAL AND METHODS

ANIMALS

Adult sexually developed male and female Syrian hamster, *Mesocricetus aureatus*, bred in-house were used in this study. From birth, they were maintained in a long photoperiod (LP) consisting of 14h light and 10h dark (lights on at 05h00), at $22\pm 2^{\circ}\text{C}$ with *ad libitum* access to food and water. When required, some animals were transferred to short photoperiod (SP) consisting of 10h light and 14h dark (lights on at 09h00) for 10 weeks to induce full gonadal regression. A total of 28 male and female hamsters were used in this study. All protocols were submitted to the Comité Régional d’Ethique en Matière d’Expérimentation Animale (CREMEAS). All experiments were conducted in accordance with the French National Law (license n° 67-32 and n° 01663.01) and with the rules of the European Committee Council Directive of September 22, 2010 (2010/63/UE).

TISSUE PROCESSING

Both LP and SP animals ($n = 7$ per group) were killed in a CO_2 chamber at mid day and the gonads were weighted to verify the SP-induced regression of gonadal axis activity. For *in situ* hybridization analysis, brains were rapidly removed from the skull, snap frozen at approximately -40°C using liquid nitrogen, and stored at -80°C until sectioning. 6 serial sets of coronal brain sections ($20\mu\text{m}$) were cut throughout the forebrain at -20°C in a cryostat (MICROM HM 560, Thermo Scientific, USA). The sections were thaw mounted and stored at -80°C until use. For immunohistochemistry, animals ($n = 7$ per group) were transcardially perfused with 0.05M phosphate-buffered saline (PBS) followed by perfusion with a periodate-lysine-paraformaldehyde fixative (4% paraformaldehyde, pH 7.4). The brains were removed and post-fixated over night at 4°C in the same fixative, transferred to PBS + 0.02% NaN_3 and stored at 4°C until further use. Brains were then dehydrated in a 30% sucrose-PBS solution for 2-3 days, frozen on the stage of a sliding microtome (Bright Series 8000, Bright Instruments Co Ltd., England), and sliced into 4 serial sets of $30\mu\text{m}$ thick coronal sections throughout the anterior, dorsomedial and caudal hypothalamic regions. The sections were stored as free-floating sections in a cryoprotectant antifreeze solution (30% ethylene glycol, 30% glycerol in 0,2M sodium phosphate buffer) at -20°C , until immunohistochemical processing. All brains were randomized and blinded during sectioning and throughout processing, quantifications and evaluations.

IN SITU HYBRIDIZATION

GPR147 mRNA expression was analyzed by *in situ* hybridization using a Syrian hamster GPR147 (NPFF-R1) antisense riboprobe (1028bp) transcribed with T7 RNA polymerase according to the protocol provided with the MAXIscript® kit (Ambion, USA). Transcription was done in the presence of radio-labeled $[^{35}\text{S}]\text{UTP}$ (1250 Ci/mmol, NEN, France) allowing detection of the complementary riboprobe. Specificity of the riboprobe labeling was tested with the radio-labeled sense probe

complementary to the antisense probe, which showed no specific labeling compared to the antisense probe. Moreover, specificity was tested by applying increasing concentrations of the riboprobe, from 75pM to 600pM. In all structures of interest the labeling was saturated with the highest concentrations of riboprobe as compared to background areas, where the intensity increased linearly with increasing concentrations of the riboprobe. Figure 22 shows sections at the SCN level labeled with the GPR147 antisense probe and sense probe, respectively, as well as a graph showing the saturation of the binding of the riboprobe in the SCN (specific labeling) and the non-saturated binding to background i.e. Striatum (non specific labeling).

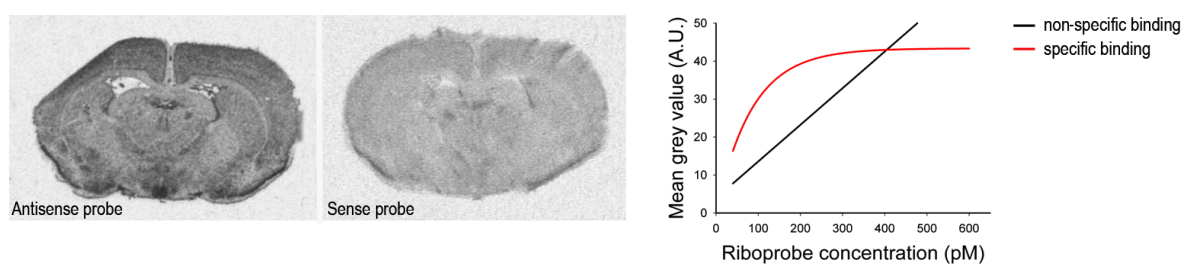


Figure 22. Characterization of GPR147 mRNA labeling in the Syrian hamster. A) In situ hybridization of the ^{35}S -labeled GPR147 antisense probe (left) and sense probe (right) at the level of SCN in female Syrian hamster brain sections. B) Mean grey value of GPR147 antisense probe labeling in the SCN (red line, specific labeling) and on background (black line, non-specific labeling).

The optimal concentration of the labeled riboprobe was set to 400pM. Frozen brain sections were treated with 4% paraformaldehyde in 1× PBS, pH 7.4 for 15 min at room temperature, and then rinsed in 1× PBS for 2 min. The slides were acetylated in 0.75% acetic anhydride in 0.1M triethanolamine (pH 8.0) for 10 min and rinsed 2 min in 1× PBS. The slides were then dehydrated in a graded ethanol serie (70, 90, 95 and 100%, 1 min each) and dried at room temperature before hybridization. Sections were hybridized in a humid chamber with 400pM of the ^{35}S UTP-labeled GPR147 cRNA riboprobe in a solution containing 50% deionized formamide, 10% dextran sulfate, 50 mM dithiothreitol, 1× Denhardt's solution, 2× SSC, 0.5mg/ml salmon sperm DNA, 0.25mg/ml yeast RNA, at 54°C for 16 h. After hybridization, the sections were rinsed for 5 min at room temperature in 2× SSC before being treated with ribonuclease A (0.02μg/ml, Sigma, France) in 10 mM Tris pH 7.4, 0.5 M NaCl, 10 mM EDTA buffer (30 min at 37°C). Slides were then rinsed at room temperature in 1× SSC and in 0.05× SSC at 52°C for 5 and 30 min, respectively, to reduce non-specific labeling. Finally, sections were dehydrated in a graded ethanol serie (70, 90, 95 and 100%, 1 min each), dried at room temperature and subsequently exposed to X-ray films (HyperfilmMP, Amersham) for 15 days with ^{14}C microscale standards. All groups were processed in the same procedure and blinded throughout processing, quantifications and evaluations. The location of the bound probe was visualized by autoradiography and captured as a digital image for quantifications. Semi-quantitative analysis of the autoradiogram was performed with a picture analysis system using ImageJ software (National Institute of Health, USA). Each structure of interest was equally defined on all sections according to a stereotaxic atlas of

the Syrian hamster brain and semi-quantitative measurements were conducted in the same fixed measuring area, which was kept constant across all sections and animals. Three sections per animal were quantified for each of the structures of interest. For each section the mean grey value in the region of interest was measured along with two background measurements. The mean of these two background measurements was subtracted from that of the region of interest. Grey values were standardized to the [^{14}C] microscale standards of known radioactivity placed on each film. As a negative control we quantified mean grey levels in areas with nonspecific binding and where RFRP is thought to have no functional relation; the corpus callosum and the striatum. Levels quantified in these structures showed no photoperiodic or sex specific variation (not shown).

IMMUNOHISTOCHEMISTRY

Antibody characterization

In order to specifically characterize and quantify RFRP immunoreactivity in the Syrian hamster we tested the specificity of the RFRP/GnIH antiserum (Table 1), kindly provided by Prof. Kazuyoshi Tsutsui (Tsutsui et al., 2000), by dot blot and adsorption tests according to protocols set in previous studies (Larsen et al., 1989). Dot blot papers were prepared by spotting 5 μl of concentrations of different peptides diluted in water at 10 μM , 100 μM or 1 mM (rat RFRP-1 (*VPHSAANLPLRF-NH₂*) and five C-terminal amino acids of RFRP-1 *YLPRF* from Phoenix Pharmaceuticals Inc., USA; rat RFRP-3 (*ANMEAGTMSHFPSLPQRF-NH₂*) from Abgent, USA; Syrian hamster RFRP-3 (*ILSRVPSLPQRF-NH₂*), Siberian hamster RFRP-3 (*TLSRVPSLPQRF-NH₂*) and human RFRP-3 (*VPNLPQRF-NH₂*) from Caslo Laboratory, Denmark), or bovine serum albumin (BSA, Sigma-Aldrich, USA) diluted at 0.3% in PBS as negative control. The papers were allowed to dry, followed by incubation for 90 min at 75°C in a sealed box containing powdered paraformaldehyde. After fixation, the papers were washed 3 $\times$ 10 min and immunoreacted identical to the single labeling protocol for free floating sections using the GnIH antiserum as described below at a dilution of 1:2500. Pre-adsorption tests were carried out on hamster brain sections. The RFRP antiserum used at an optimal dilution of 1:2500 was preincubated with rat RFRP-1, Syrian hamster RFRP-3 or Kisspeptin-10 (*YNWNSFGLRY-NH₂*) in concentrations of 20 μM , 4 μM or 0.8 μM for one hour at room temperature. The solution was then used for immunostaining of brain sections as described below.

Immunohistochemistry single labeling

Brain sections were rinsed in PBS 3 $\times$ 10 min and incubated in 1% hydrogen peroxide (H_2O_2) diluted in PBS for 10 min, followed by 20 min incubation in a blocking buffer of 5% swine serum, 0.3% BSA and 0.3% Triton® X-100 in PBS (PBS TX). The sections were then incubated overnight at 4°C in primary antiserum diluted 1:2500 in the blocking buffer without swine serum. After the primary antiserum incubation sections were washed for 3 $\times$ 10 min in PBS TX and then incubated for 60 min in biotinylated donkey anti rabbit secondary antibody (1:1000; Jackson ImmunoResearch, USA). The

procedure was followed by a 3× 10 min wash PBS TX, after which the sections were left to incubate for 60 min in avidin-biotin-peroxidase complexes (Vectastain ABC kit, Vector laboratories, USA) diluted in PBS TX. Brain sections were then washed for 10 min in PBS TX, 10 min in PBS and 10 min in Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; pH 7.6) before developing the sections for 10 min in 0.1% diaminobenzidine (DAB; Sigma-Aldrich, MO, USA) and 0.03% H₂O₂ in Tris-HCl, which resulted in a brown precipitate within the cytoplasm of the labeled cell. Lastly the sections were washed for 5× 6 min in PBS, mounted on glass slides using a mounting medium composed of 0.5% gelatine and 0.05% chromalun in distilled water, dried and coverslipped in Pertex® (Histolab, Sweden).

QUANTIFICATIONS

The number of RFRP-ir neurons, the grey level intensity of RFRP-ir labeling per neuron and the number of RFRP-ir fibers were quantified for each male and female Syrian hamster in different photoperiodic conditions. Identities of the animals were blinded to the observer throughout processing and quantification by manual counts was checked by independent researcher. Moreover, only groups immunoreacted in the same immunohistochemical (IHC) procedure were compared. Quantifications of single immunohistochemical labeling were done using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy). The total number of RFRP-ir neurons was counted manually on 11 sections from each animal, from the caudal part of medial anterior hypothalamus throughout the medial hypothalamus to include the entire dorsomedial hypothalamic region. Semi-quantitative analysis of the level of RFRP immunoreactivity in positive labeled neurons were done by measuring the mean grey value in individual cells using the ImageJ software (National Institute of Health, USA). Photomicrographs (10× magnification) were taken on either side of the third ventricle of 4 sections per animal. A fixed-size circle was applied over labeled cells and to allow quantification of the same number of cells between groups, the mean grey value of 12 cells was systematically measured in each image. The 12 most prominent stained cells were measured to reflect the majority of the labeled cells. For each image, four background measurements were conducted and the mean of these four background measurements was subtracted from the mean grey level of each cell. Data are presented as the mean grey level per RFRP-ir neuron. RFRP fiber immunoreactivity was counted using Visiopharm Integrator System software (VIS, Visiopharm, Hoersholm, Denmark) on respectively 2 (POA, OVLT), 3 (AVPV, MPN) and 2 (caudal ARC) sections per animal. For each structure a region of interest was defined and applied equally from one section to another. Horizontal lines were applied and quantifications were done by counting number of fiber crossing at 40× magnification. Data are presented as the mean count of RFRP-ir fibers per section for animal group.

DIGITAL IMAGES

Digitally captured images were converted to 8-bit grey scale images using ImageJ software (National Institute of Health, USA). Scale bars were added and brightness/contrast was equally adjusted on all images if compared to one another.

STATISTICAL ANALYSIS

Results are given as mean $\pm$ SEM (n=7) for each of the four groups. Data were analyzed by unpaired students *t*-test and multiple group comparisons were analyzed using one-way analysis of variance (ANOVA), followed by Holm-Sidak test, as appropriate. Statistical significance was set at $P_{value} < 0.05$.

RESULTS

CHARACTERIZATION OF THE QUAIL-DERIVED RFRP ANTISERUM IN THE SYRIAN HAMSTER.

In order to test the specificity of the RFRP antiserum, we carried out a number of pre-adsorption tests on Syrian hamster brain sections. Pre-adsorption of the quail-derived RFRP antiserum with either RFRP-3 or RFRP-1 abolished all staining throughout the brain sections (figure 23). By contrast, the immunostaining was not affected by pre-incubation with kisspeptin-10. Thus the RFRP antiserum recognizes both RFRP-1 and RFRP-3 peptides but does not cross-react with kisspeptin in our studies performed on Syrian hamster brain sections. Moreover, the specificity of the antiserum was tested on dot blots spotted with rat RFRP-1, the five C-terminal amino acids of RFRP-1 (YLPRF), rat RFRP-3, Syrian hamster RFRP-3, Siberian hamster RFRP-3, human RFRP-3 or bovine serum albumin. The RFRP antiserum binds spots of RFRP-1, RFRP-3 and YLPRF, but not BSA (data not shown).

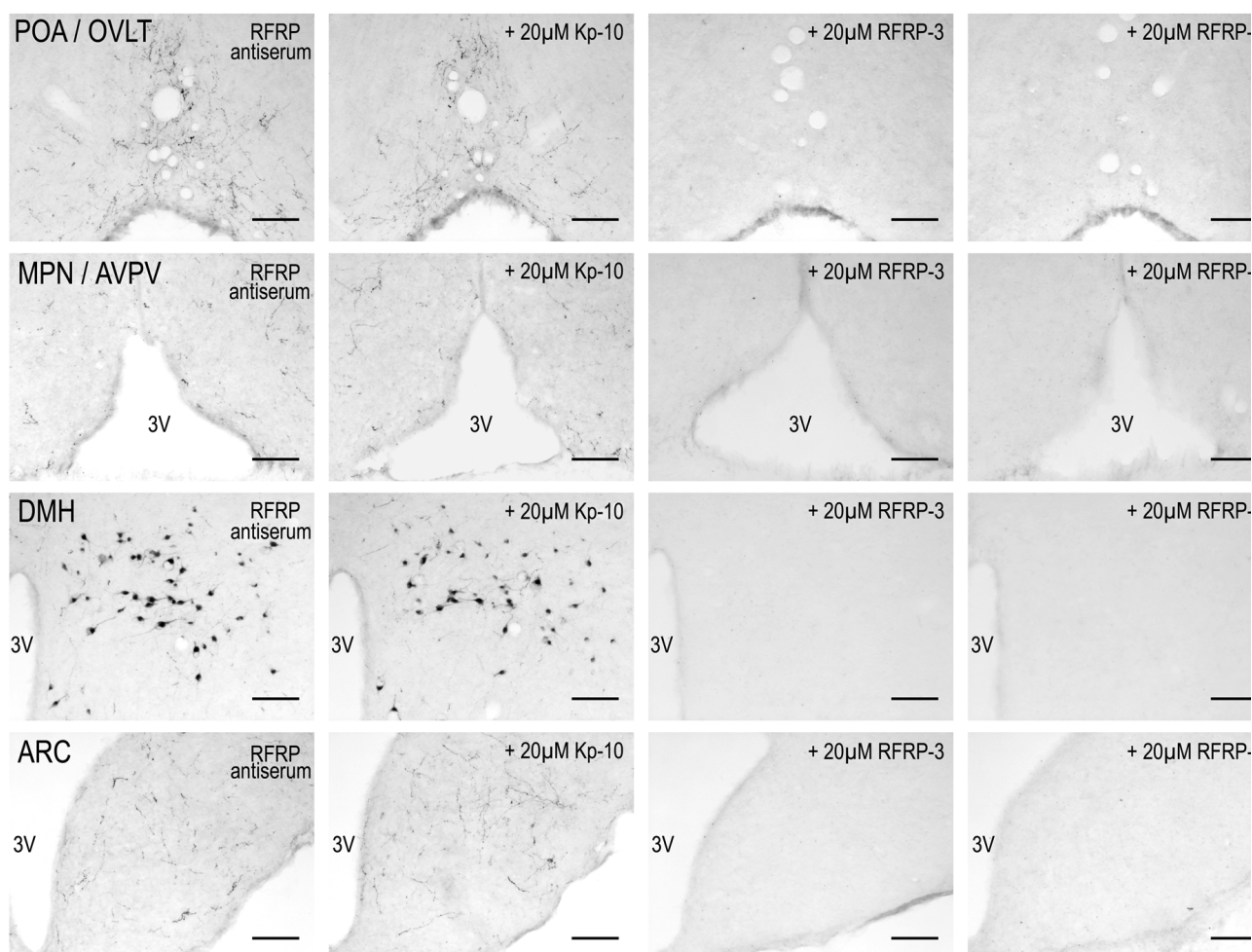


Figure 23. Characterization of the quail-derived RFRP antiserum in the Syrian hamster. Immunolabeling was observed in the preoptic area and organum vasculosum of lamina terminalis (POA/OVLT), the median preoptic nucleus and anteroventral periventricular nucleus (MPN/AVPV), dorsomedial hypothalamus and ventromedial hypothalamus (DMH/VMH), as well as the arcuate nucleus (ARC) after incubation with the quail-derived RFRP antiserum at a dilution of 1:2500. The immunolabelling was preserved with a pre-incubation with 20µM rat kisspeptin-10, and completely diminished after pre-incubation with 20µM Syrian hamster RFRP-3 or rat RFRP-1. 3V: third ventricle; scale bar: 100µm.

SEX- AND PHOTOPERIOD-DEPENDENT VARIATIONS IN RFRP IMMUNOREACTIVE NEURONS AND FIBERS IN THE SYRIAN HAMSTER HYPOTHALAMUS.

RFRP-ir neurons were found along the third ventricle from the caudal part of the medial anterior hypothalamus throughout the medial tuberal hypothalamus, with the majority of the RFRP-ir cell bodies being located within the dorsomedial hypothalamus (DMH), in-between the DMH and ventromedial hypothalamus (VMH) and few labeled neurons within the boundaries of VMH (figure 24A). The number of RFRP-ir neurons (figure 24B; $P < 0.05$) as well as the intensity of RFRP neuronal grey level (figure 24C; $P < 0.001$) was significantly higher in females as compared to males in sexually active LP-adapted animals. Moreover, the number of RFRP-ir neurons was significantly decreased in both sex in SP exposed animals when compared to LP animals (figure 24B; $P < 0.001$ for females; $P < 0.01$ for males). The grey level intensity of the cell bodies was significantly decreased in SP in females ($P < 0.001$) but not in males ($P = 0.0521$) (figure 24C).

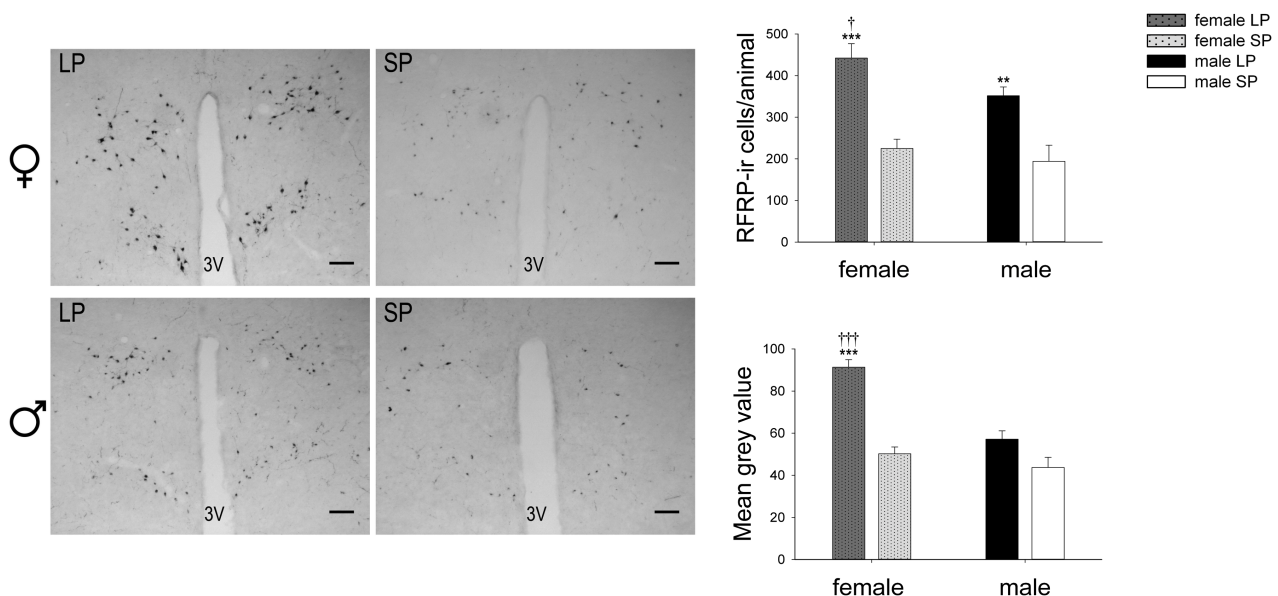


Figure 24. Sex- and photoperiod-dependent regulation of RFRP-ir neurons in the Syrian hamster. A) RFRP neuronal expression in the dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH) in female and male Syrian hamster kept in long (LP) or short (SP) photoperiod; 3V: third ventricle, scale bar: 100 μ m. B) Number of RFRP-immunoreactive (ir) neurons in the DMH/VMH of female and male Syrian hamster kept in LP or SP condition; data are presented as the mean number of RFRP-ir neurons counted per animal $\pm$ SEM ($n = 7$ /group); female values are significantly higher than male values in LP ($\dagger P < 0.05$); LP values are significantly higher than SP values in female ($*** P < 0.001$) and male ($** P < 0.05$). C) RFRP-ir neuronal intensity in the DMH/VMH of female and male Syrian hamster kept in LP or SP condition; data represent the mean grey intensity value per cell $\pm$ SEM ($n=7$ /group); labeling intensity is significantly higher in female than in male kept in LP ($\dagger\dagger\dagger P < 0.001$); in female labeling intensity is significantly higher in LP than in SP ($*** P < 0.001$) as compared to all other groups.

RFRP-ir fibers were found in the POA/OVLT, MPN/MPN and ARC (figure 25A) as well as in other hypothalamic areas: anterior part of the suprachiasmatic nucleus (SCN), paraventricular hypothalamic nucleus (PVN), anterior hypothalamus (AH) and VMH. In addition, RFRP-ir fibers were found in the

bed nucleus of the stria terminalis (BNST), habenular nuclei (Hb) and paraventricular nucleus of the thalamus. The overall anatomical distribution of RFRP-ir fibers was similar in female and male brains.

The amount of RFRP-ir fibers was counted in the specific areas considered to be relevant for the control of reproductive activity in male and female hamsters adapted to LP and SP. In the AVPV/MPN the number of RFRP-ir fibers was significantly higher in female Syrian hamster adapted to SP (figures 25B and 25C), but not in males. No sex- or photoperiod-specific differences were observed in any of the other areas analyzed (figure 25B).

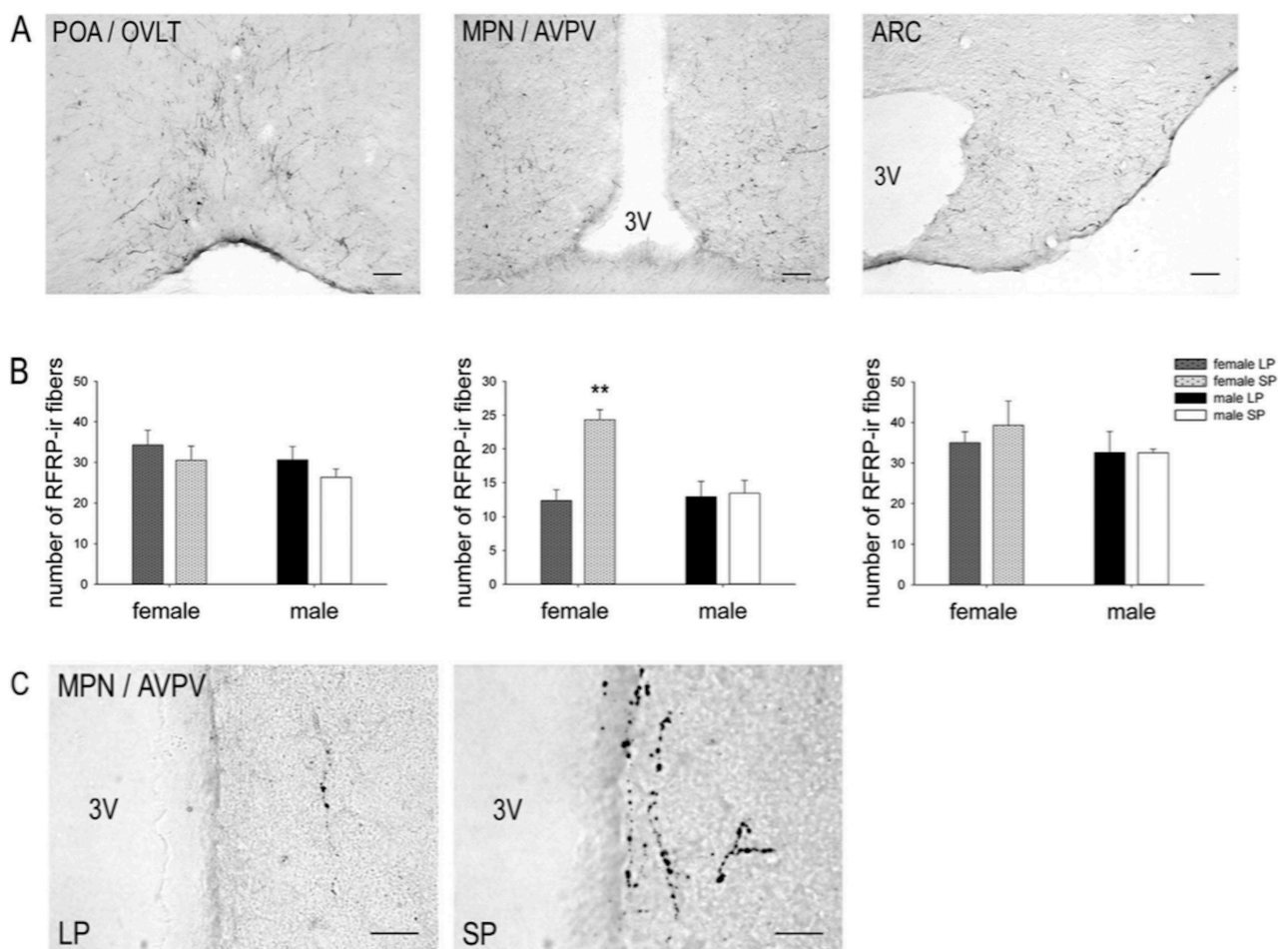


Figure 25. Sex- and photoperiod-dependent regulation of RFRP-ir fibers in the Syrian hamster. A) RFRP-ir fibers in the preoptic area, in and around the organum vasculosum of lamina terminalis (POA/OVLT), the median preoptic nucleus and anteroventral periventricular nucleus (AVPV/MPN) and arcuate nucleus (ARC) in brains of female Syrian hamster kept in LP condition; 3V: third ventricle, scale bar 100µm. B) Quantification of RFRP-ir fibers in the POA/OVLT (left panel), AVPV/MPN (middle panel) and ARC (right panel) in brains of male and female Syrian hamsters kept in LP or SP; data represent the mean number of RFRP-ir fibers $\pm$ SEM, (n=7/group); the number of RFRP-ir fibers in AVPV/MPN is significantly increased in females in SP as compared to LP (** $P < 0.01$). C) RFRP-ir fibers in the AVPV/MPN of female Syrian hamster kept in LP (upper panel) or SP (lower panel); scale bar: 20µm.

BRAIN MAPPING AND SEX- AND PHOTOPERIOD-DEPENDENT REGULATION OF GPR147 mRNA IN THE SYRIAN HAMSTER.

GPR147 mRNA expression was revealed using *in situ* hybridization in various areas of the forebrain. In the hypothalamus a high level of expression was observed in the POA/OVLT, AVPV/MPN, SCN, PVN, AH, VMH, and ARC (figure 26).

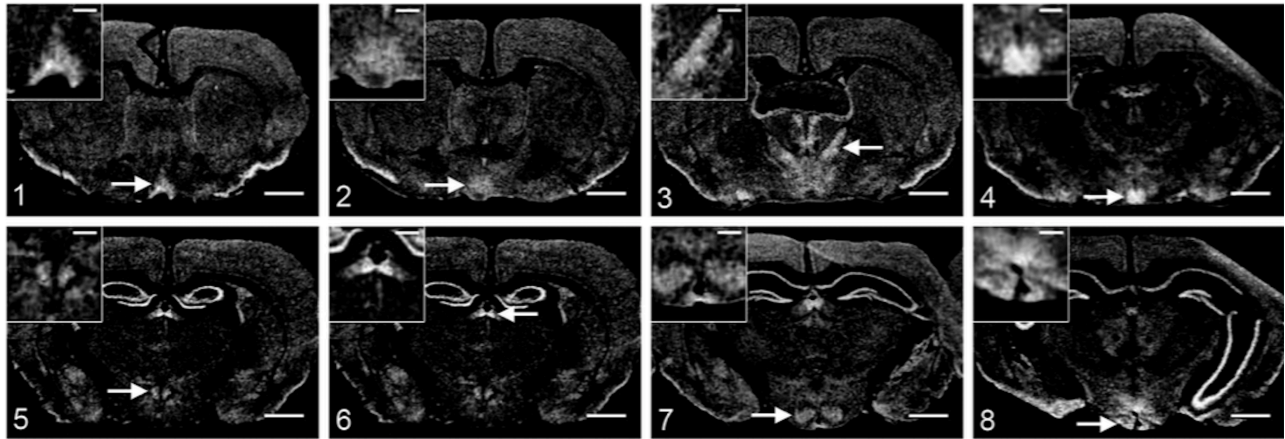


Figure 26. Mapping of the RFRP receptor, GPR147, mRNA in the Syrian hamster. In situ hybridization of the ³⁵S-labeled antisense GPR147 riboprobe reveals the expression of GPR147 mRNA in the preoptic area and organum vasculosum of lamina terminalis (POA/OVLT, arrowhead in 1), the medial preoptic nucleus and anteroventral periventricular nucleus (AVPV/MPN, arrowhead in 2), bed nucleus of the stria terminalis (BST, arrowhead in 3), suprachiasmatic nucleus (SCN, arrowhead in 4), paraventricular hypothalamic nucleus (PVN, arrowhead in 5), habenular nuclei (Hb, arrowhead in 6), ventromedial hypothalamic nucleus (VMH, arrowhead in 7), and arcuate nucleus (ARC, arrowhead in 8). Scale bar whole section images: 1,32mm, scale bar enlargements: 396µm.

Outside of the hypothalamus a high level of expression was observed in the posterior part of the BST, Hb and the pyramidal cell layer of the hippocampus (figure 26). There was no difference in the overall distribution of GPR147 between female and male Syrian hamsters. In nearly all structures investigated GPR147 mRNA levels were significantly higher in females as compared to males, independent of photoperiod (figures 27A and 27B). Furthermore, in females GPR147 mRNA levels were significantly decreased in SP as compared to LP, in all structures investigated (figure 27C). In males, a similar trend was observed in some structures, but only in the Hb was the expression found to be significantly lower in SP than in LP (figure 27D). Figure 27E shows the photoperiod- and sex-dependent differences in levels of GPR147 mRNA at the level of SCN in sections from female and male Syrian hamsters kept in LP and SP. The level of unspecific background labeling in the corpus callosum and the striatum showed no photoperiodic or sex specific variation (data not shown).

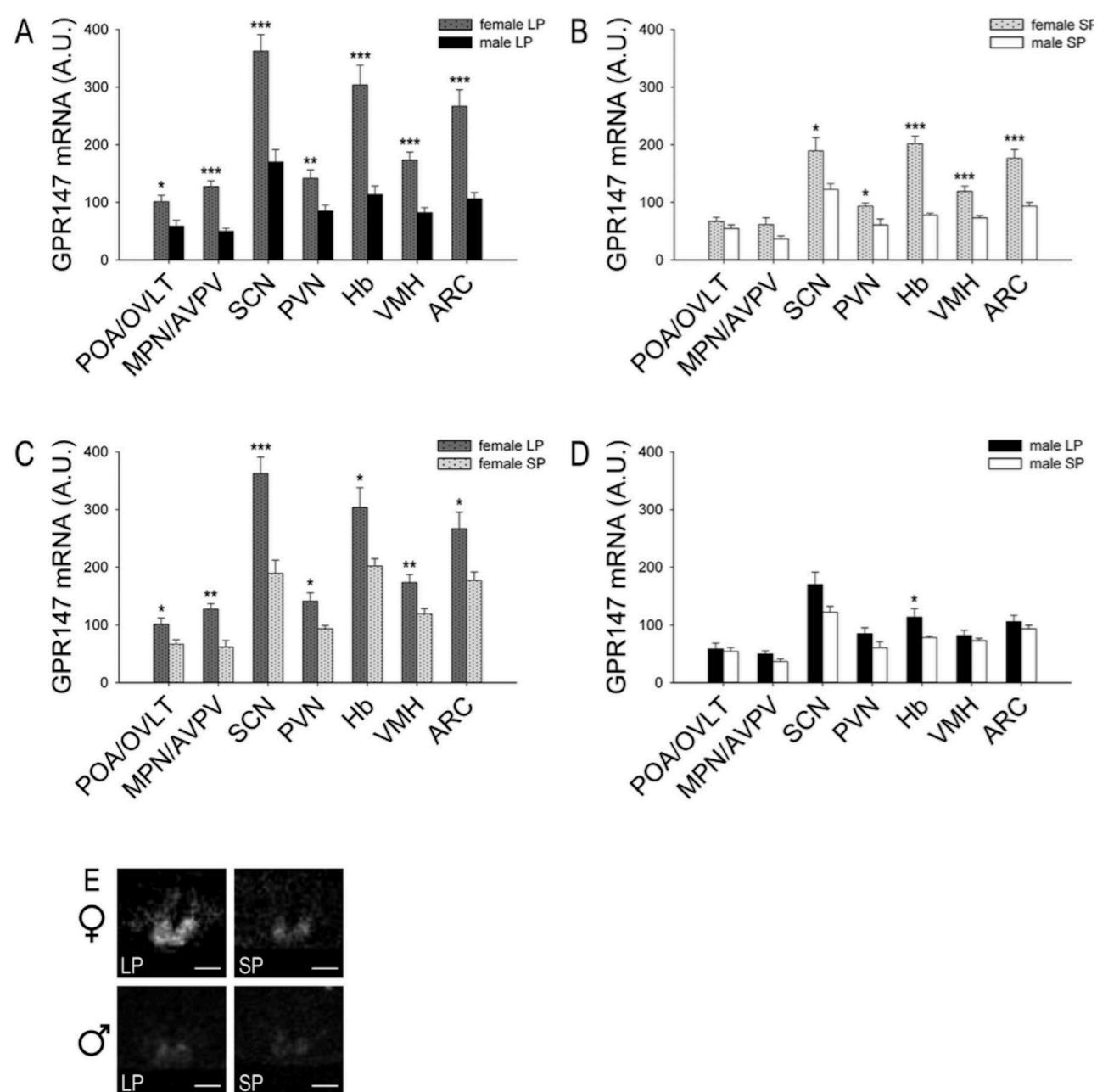


Figure 27. Sex- and photoperiod-dependent variation in the encoding RFRP receptor GPR147 mRNA in the Syrian hamster. A, B, C, D) Semi-quantitative analysis of GPR147 mRNA expression in the POA/OVLT, MPN/AVPV, SCN, PVN, Hb, VMH and ARC in female *versus* male hamsters kept in LP (A) or SP (B) condition; and in LP *versus* SP conditions in female (C) or male (D) hamsters; Data represent the mean grey value intensity $\pm$ SEM (n=7/group); differences are considered significant for $P < 0.05$ (*); $P < 0.01$ (**) and $P < 0.001$ (***) by one way ANOVA followed by Holm Sidak analysis. E) Comparative images of GPR147 mRNA labeling at the level of SCN in females and males kept in LP and SP as indicated on the figures, scale bar 396 μ m.

DISCUSSION

This study is the first to report insight into the photoperiodic regulation of the RFRP system in a comparative study between female and male Syrian hamsters. Strikingly, the number and intensity of RFRP-ir neurons, as well as the level of GPR147 mRNA, were significantly higher in the sexually active LP-adapted females as compared to males. Furthermore, both RFRP-ir expression and GPR147 mRNA levels were found to be seasonally regulated. RFRP-ir expression was significantly decreased in SP in both sexes, whereas GPR147 mRNA levels were strongly decreased in SP only in females. Importantly, the number of RFRP-ir fibers in the AVPV/MPN was found to be significantly increased in female Syrian hamsters adapted to SP as compared to LP. All together these findings revealed strong sex-dependent differences in the RFRP system in the Syrian hamster pointing towards a critical role of RFRP in the central control of (seasonal) reproduction particularly in female Syrian hamsters.

The RFRP system in the Syrian hamster brain was investigated using a quail-derived RFRP antiserum that was shown to label both RFRP-1 and -3 in hamster brain sections and showed no cross reactivity to kisspeptin. We found RFRP-ir neurons distributed from the caudal part of the medial anterior hypothalamus throughout the medial tuberal hypothalamus with the majority of the RFRP-ir neurons expressed within the dorsomedial hypothalamus (DMH) and in-between the DMH and ventromedial hypothalamus (VMH), which is in accordance with what has previously been reported with other antibodies in the Syrian hamster (Kriegsfeld et al., 2006)(Revel et al., 2008). Few labeled neurons were however found within the boundaries of VMH, but whether these are indeed functional VMH neurons or displaced neurons remains to be established. We found no difference in the distribution of RFRP-ir neurons between females and males, however when we quantified the number and grey level intensity of RFRP-ir neurons we found strong sex-specific differences. RFRP expression was markedly higher in the sexually active females as compared to males, in particular the mean neuronal intensity of RFRP-ir labeling was found to be almost twice as high in females than in males. Similar sex-specific differences were found for the expression of RFRP-1 in rats, with a higher expression in females than in males (Jorgensen et al., 2014), suggesting that these sex-differences could be driven by gonadal steroid hormones in seasonal as well as non-seasonal species. A portion of the RFRP neurons have been shown to co-express estrogen receptor ER α in female Syrian hamsters and mice (Kriegsfeld et al., 2006)(Molnar et al., 2011)(Poling et al., 2012). Interestingly, estradiol has been found to induce c-Fos expression in RFRP neurons in female Syrian hamsters whereas in males, treatment with testosterone has no effect on RFRP expression (Kriegsfeld et al., 2006)(Revel et al., 2008)(Mason et al., 2010). In line with these observations, Revel *et al.* 2008 found no changes in RFRP expression levels in castrated males as compared to intact males (Revel et al., 2008). These data suggest that the stimulatory effect of estradiol on RFRP expression could explain the increased level of RFRP expression observed in female rats and Syrian hamsters as compared to males. Similarly in rats, estradiol stimulates *Rfrp* expression in females, in contrast to testosterone that has no effect on *Rfrp* expression in males (Iwasa et al.,

2012). In contrast, both estradiol and testosterone have been shown to decrease *Rfrp* expression in adult mice indicating that there are differences in how RFRP expression is regulated by sex steroids amongst species (Poling et al., 2012).

RFRP expression has been shown to be down-regulated by melatonin in SP in male Syrian and Siberian hamsters (Ancel et al., 2012)(Revel et al., 2008)(Ubuka et al., 2012). Our findings showed that in female Syrian hamsters as well, RFRP expression is down-regulated in SP, probably driven by the same mechanisms. Recent studies demonstrated that the mechanisms underlying the photoperiodic regulation of RFRP expression involve an indirect action of melatonin onto thyroid stimulating hormone (TSH) synthesizing cells of the *Pars tuberalis* (Klosen et al., 2013). In SP, the large production of melatonin inhibits *Pars tuberalis* TSH synthesis, whereas the lower production of melatonin in LP allows the synthesis and release of TSH, which activates deiodinase 2 in tanycytes surrounding the 3rd ventricle (Revel et al., 2006b)(Ono et al., 2008b)(Nakao et al., 2008a)(Hanon et al., 2008b)(Dardente, 2012). Activation of this local enzyme leads to an increased level of triiodothyronine (T3) in the mediobasal hypothalamus, which was found to increase both neuronal RFRP expression and gonadal activity (Klosen et al., 2013). Furthermore, we recently demonstrated that a chronic central administration of RFRP-3 in SP-adapted sexually inactive male Syrian hamsters was able to rescue testicular activity to a level similar to LP-adapted littermates (Ancel et al., 2012). Altogether these recent findings point to hypothalamic RFRP neurons as a critical node between the photoperiodic melatonin message and reproduction (Hazlerigg and Simonneaux, 2015). In SP-breeding sheep RFRP expression is also down-regulated by melatonin in SP (Dardente et al., 2008)(Smith et al., 2008), thus it would be interesting to study whether continuous administration of RFRP in sexual active sheep would show similar yet reverse effects than that in hamsters and de-activate reproductive activity. Strikingly, we found that the SP-induced decrease in RFRP expression was stronger in females, in which both the number and grey level intensity of RFRP-ir labeling were significantly reduced in SP, as compared to males, in which only the number of RFRP-ir neurons was reduced. This sex-difference is likely a result of the combination of the inhibitory effect of melatonin together with the decreased stimulatory effect of estradiol in sexually inactive females. In SP conditions, we found a comparable low level of RFRP-ir expression between males and females.

In agreement with previous studies, RFRP-ir fibers were found in intra- and extra hypothalamic areas (Ubuka et al., 2012)(Kriegsfeld et al., 2006)(Rizwan et al., 2012)(Poling et al., 2013). We found that the amount of RFRP-ir fibers was significantly and specifically increased in the AVPV/MPN of females exposed to SP. This area is of particular importance for female reproductive activity since it contains kisspeptin neurons, which are positively regulated by the pre-ovulatory rise in estrogen. Activation of these kisspeptin neurons provides a stimulatory signal onto the GnRH neurons causing the LH surge and thereby ovulation to take place (Smith et al., 2006b). It has been suggested that RFRP neurons are mediating a SCN-generated circadian output onto the kisspeptin neurons of the AVPV/MPN, thereby

modulating the timing and generation of the LH surge (Gibson et al., 2008). Indeed, Gibson *et al.* (2008) reported a decrease in RFRP expression around the time of the LH-surge in the Syrian hamster (Gibson et al., 2008). Similarly in rats the c-Fos activated number of RFRP neurons has been shown to be specifically decreased on the day of proestrus (Jorgensen et al., 2014) and in ewes RFRP expression is reduced during the pre-ovulatory period (Clarke et al., 2012). Furthermore, chronic icv infusion of RFRP-3 inhibits the activity of GnRH neurons as well as unidentified AVPV neurons during an estradiol-progesterone induced LH-surge in ovariectomized rats (Anderson et al., 2009). Altogether these observations suggest that RFRP neurons are important regulators for the proper generation and/or timing of the LH-surge in females. The observed increased amount of RFRP peptide in the AVPV/MPN of SP female hamsters could be explained by an increased RFRP release in the sexually inactive females and occurs concomitant with the decrease in RFRP-ir expression. Altogether these data points towards a specific regulation of RFRP function and release in females that signals independent of the melatonin-dependent seasonal regulation, possibly via outputs from the SCN as suggested by Gibson *et al.* Although it seems less likely, we can however not exclude that the increase in RFRP-ir fiber in the AVPV/MPN reflects a decreased release and accumulation of peptide in the fiber terminals. No sex- or photoperiod-dependent differences were observed in any of the other regions analyzed. Interestingly however, RFRP-ir fibers in the POA were found to specifically project to the highly vascularised region of the OVLT, suggesting that RFRP neurons might have access to structures outside the blood brain barrier.

RFRP terminals make apparent contact to 20-40% of GnRH neurons in rodents and sheep (Ubuka et al., 2012)(Kriegsfeld et al., 2006)(Rizwan et al., 2012)(Smith et al., 2008) and in Siberian hamsters and sheep the amount of GnRH neurons receiving RFRP fiber contacts is decreased in SP conditions (Ubuka et al., 2012)(Clarke et al., 2008). However, recent studies have revealed that GnRH neuronal projections, dendrons, functions simultaneously as an axon and dendrite that allows GnRH neurons to integrate synaptic inputs along their entire length, thereby providing a dynamic control of GnRH peptide secretion from the median eminence (Campbell et al., 2005)(Herde et al., 2013)(Roberts et al., 2008). Thus, future studies should aim to study in detail RFRP-GnRH connections along the entire dendron to fully establish how and where these interact.

In this study we also report the first mapping of GPR147 mRNA in the brain of the Syrian hamster. High level of GPR147 gene expression was observed primarily in hypothalamic areas (POA/OVLT, AVPV/MPN, SCN, PVN, AH, VMH and ARC) as well as the BST and Hb. The distribution of GPR147 mRNA was similar between females and males. We found a strong correlation between the distribution of RFRP-ir fibers and GPR147 mRNA, which underlines the functional significance of these areas for RFRP downstream signaling both in male and female hamsters. In a previously published study, NPFF1 (another name for GPR147) was mapped by autoradiography in several mammalian species, amongst them the guinea pig (Gouarderes et al., 2004). The findings in the guinea pig and the

Syrian hamster show a similar pattern of expression for GPR147. In sheep, *rfr-2* (another name for GPR147) was mapped by autoradiography and found to be similarly expressed in the SCN and PVN, but also in the supraoptic nucleus and the pars tuberalis (Dardente et al., 2008). In line with these observations RFRP neurons have been found to project to the external layer of the median eminence in ovine and directly inhibit GnRH-stimulated gonadotropin secretion from ovine pituitaries (Qi et al., 2009)(Sari et al., 2009), which is in contrast to what was found in Syrian hamsters where RFRP-3 has no effect on gonadotropin release from cultured pituitaries (Ancel et al., 2012). Interestingly, we observed strong sex-dependent differences in the level of GPR147 mRNA when performing semi-quantitative analysis of GPR147 expression in areas involved in the regulation of reproductive function. Females had significantly higher expression of GPR147 mRNA as compared to males, a difference that was consistent in all areas quantified and in both photoperiodic conditions. At this point it is not known whether this sex difference is a direct consequence of sex differences in the amounts of RFRP peptide that binds to and signals via the receptor, or whether the differences could arise from a differential regulation of the gene by gonadal steroids. The photoperiodic regulations of GPR147 mRNA levels were found to be different between females and males, with a stronger SP-induced decrease in GPR147 mRNA levels in females as compared to males.

Our findings suggest that RFRP regulates reproduction via several central sites known to be involved in the control of the reproductive axis, i.e. the GnRH neurons (POA) and kisspeptin neurons (AVPV/MPN and ARC). Indeed, RFRP peptides have been shown to regulate reproductive activity however with differential effects according to sex and species. In the male Syrian hamster we found that RFRP-3 stimulates the hypothalamic-pituitary-gonadal (HPG) axis both in LP and SP conditions; although to a lesser extent in SP animals (Ancel et al., 2012), whereas in male Siberian hamsters RFRP-3 displays reverse effects according to the photoperiodic condition, inhibitory in LP and stimulatory in SP (Ubuka et al., 2012). The avian RFRP peptide, GnIH, inhibits LH release in ovariectomized female Syrian hamsters (Kriegsfeld et al., 2006) and similarly, the ovine RFRP-3 peptide, GnIH3, inhibits the release of LH in ovariectomized ewe (Clarke et al., 2012). Therefore, the RFRP system seems to have a specific role in seasonal species possibly playing a key role in the integration of the photoperiodic input onto the reproductive axis. Our findings have shown large variation in level of GPR147 mRNA between male and female Syrian hamsters, but this is not sufficient to explain the sex-specific difference in the effect of RFRP. Further pharmacological investigations are necessary to clarify how and why RFRP peptides have opposite effects between sexes. Thus future studies should aim at distinguishing RFRP-1 and RFRP-3 signaling as well as at investigating whether RFRP peptides could act through another G-protein coupled receptor GPR74 (NPFF2) known to interact with RFRP peptides although with lower affinity than GPR147. In contrast to males, female Syrian hamsters display a dramatic reduction in GPR147 mRNA when adapted to SP conditions. It might be interesting to investigate whether RFRP-3 exerts differential photoperiodic effects in female seasonal rodents.

Our neuroanatomical findings suggest that RFRP plays a role not only in structures related to reproductive function but possibly also in other structures related to anxiety and stress (PVN, BST and Hb), metabolism (ARC and VMH) and circadian activity (PVT, SCN). Kirby *et al.* (2009) have shown the expression of glucocorticoids receptors in RFRP neurons and hypothesized that RFRP neurons contribute to hypothalamic suppression of reproductive function in response to stress (Kirby *et al.*, 2009). Furthermore, GPR147 KO-mice show impaired decrease in LH release from the pituitary due to metabolic stressors, further indicating that RFRP neurons might mediate the signal of (metabolic) stress onto the reproductive axis (Leon *et al.*, 2014). RFRP peptides have also been shown to regulate food intake in rats (Johnson *et al.*, 2007)(Kovacs *et al.*, 2014)(Kovacs *et al.*, 2012). In mice, RFRP-3 and GnIH have been shown to directly inhibit the firing rate of POMC- and NPY-neurons, two neuronal populations known to regulate food intake (Fu and van den Pol, 2010)(Jacobi *et al.*, 2013). Altogether these studies suggest that RFRP neurons might be an important intermediate between metabolic, stress-related and time (day and season) cues towards the central control of reproduction, a hypothesis in agreement with the wide distribution of both GPR147 and RFRP projections observed in the rodent brain.

In conclusion, our findings indicate that the critical role played by RFRP in the seasonal regulation of male reproduction is not only conserved in female rodents but may even be a key component in regulating female reproductive activity due to a stronger expression and SP inhibition of both the peptide synthesis and GPR147 receptor. However, not much is known about the actual role that RFRP-3 plays in regulation of seasonal reproduction in females. Furthermore, the reproductive physiology in females is obviously more complicated than that in males as females undergo regular cycles of sexual activation and inactivation and their reproductive activity is inhibited during gestation and lactation, underlining the need for more complex regulatory mechanisms in females than in males (de la Iglesia and Schwartz, 2006)(Fitzgerald and Zucker, 1976)(Williams and Kriegsfeld, 2012). Following our findings of significant sex differences in the neuronal RFRP system, it seems crucial now to investigate to what extent RFRP-3 is involved in the regulation of photoperiodic and daily oestral changes in female reproductive activity in order to fully understand the role of RFRP in reproduction.

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PART II – MULTIPLE ROLES OF THE RFRP SYSTEM IN REGULATING REPRODUCTIVE ACTIVITY IN FEMALE SYRIAN HAMSTERS

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ABSTRACT

Hypothalamic RF (Arg-Phe) amide-related peptides (RFRP-1 and -3) are considered to play a role in the (seasonal) regulation of reproduction. In females, reproductive activity relies on proper integration of daily and environmental changes as well as cyclic sex-steroid feedback and recent findings indicate that RFRP neurons take part in regulating the pre-ovulatory LH-surge. This study aimed at investigating the role of RFRP in daily and seasonal control of reproductive activity in female Syrian hamsters, by studying the RFRP system along the oestrus cycle and the effects of acute and chronic administration of RFRP-3 at different reproductive stages.

We found that the effect of RFRP-3 depends on seasonal and oestral changes in female Syrian hamsters. Number of c-Fos activated RFRP-ir neurons was specifically reduced in the afternoon of both diestrus and proestrus, the latter being correlated with a dramatic increase in kisspeptin activity in the AVPV/MPN and the LH-surge. Moreover, we show that acute RFRP-3 administration prior to the LH-surge decreases the surge peak. Interestingly, we found that prolonged exposure to RFRP-3 in SP-adapted females, despite photoinhibitory conditions, completely reactivates the reproductive axis by means of increased gonadotropin and estradiol levels, kisspeptin-ir expression and gonadal weight.

Our findings reveals a critical role of RFRP in the control of the female reproductive axis and suggest that RFRP neurons not only are essential for proper synchronization of reproductive activity with season, but also in the cyclic regulation of reproductive features, particularly the kisspeptin-mediated LH-surge, possibly via a SCN-generated circadian control of their activity.

INTRODUCTION

The hypothalamic-pituitary-gonadal axis is controlled by gonadotrophin-releasing hormone (GnRH) neurons in the hypothalamus, which are responsible for the regulation of gonadotrophin secretion from the pituitary and subsequent downstream effects on the gonads. Reproductive success depends on environmental and metabolic factors, which in species living in temperate climates is overcome through synchronization of reproductive activity with seasonal changes in day length i.e. photoperiod (Hoffmann, 1979). Identification of GnRH neuron activity regulators has been the focus of many studies, and various neurotransmitters, neuromodulators and hormones have been shown to modulate the activity of GnRH neurons.

RF (Arg-Phe) amide-related peptides (RFRPs) were discovered in the brain of birds and mammals in 2000 (Tsutsui et al., 2000)(Hinuma et al., 2000). The mammalian *Rfrp* gene encodes RFRP-1, -2 and -3 peptides, of which only RFRP-1 and RFRP-3 are functional peptides (Fukusumi et al., 2001a)(Hinuma et al., 2000)(Ukena et al., 2002) that are expressed in neurons primarily located in the dorsomedial hypothalamus (DMH) and in between the DMH and ventromedial hypothalamus (VMH), (Hinuma et al., 2000)(Kriegsfeld et al., 2006)(Revel et al., 2008)(Ubuka et al., 2012)(Henningsen et al., 2015). RFRP-3 inhibits GnRH neuron activity and gonadotropin release in several mammalian species (Anderson et al., 2009)(Clarke et al., 2008)(Ducret et al., 2009)(Johnson et al., 2007)(Kriegsfeld et al., 2006)(Pineda et al., 2010a)(Leon et al., 2014)(Rizwan et al., 2012), however two recent studies reported that central administration of RFRP-3 stimulates reproductive activity in male Syrian and Siberian hamster, indicating that the effect of the peptide is species-dependent (Ancel et al., 2012)(Ubuka et al., 2012). Strikingly, in the Syrian hamster, gonadotropins are stimulated by RFRP-3 in males (Ancel et al., 2012) but inhibited in ovariectomised (OVX) females (Kriegsfeld, 2006), adding a supplementary sex-difference in the effect of the peptide.

In seasonal species RFRP expression is markedly down-regulated by melatonin in short photoperiod (SP), (Janati et al., 2013)(Revel et al., 2008)(Dardente et al., 2008)(Saenz de Miera et al., 2014)(Smith et al., 2008)(Ubuka et al., 2012)(Henningsen et al., 2015) and chronic infusion of RFRP-3 in SP-adapted male Syrian hamsters restores reproductive activity despite photoinhibitory conditions (Ancel et al., 2012). Therefore, increasing evidence now points towards RFRP neurons as a critical intermediate between the melatonin-dependent photoperiodic signal and central control of the reproductive axis (Simonneaux and Ancel, 2012)(Klosen et al., 2013) (Hazlerigg and Simonneaux, 2015).

Regulation of reproductive activity has rarely been investigated in intact female seasonal rodents because along with seasonal regulation, the reproductive state depends on ovarian-derived estradiol (E2) and suprachiasmatic nuclei (SCN)-dependent circadian signals, that in combination constitutes the complex control of female reproduction (Fitzgerald and Zucker, 1976)(de la Iglesia and Schwartz, 2006)(Williams and Kriegsfeld, 2012). The estrous cycle is characterized by a surge in LH in the late

afternoon on the day of proestrus, responsible for subsequent ovulation. Neurons located in the anteroventral periventricular (AVPV) and medial preoptic (MPN) nuclei, expressing another RF-amide peptide, kisspeptin, are critical for integrating both SCN-derived vasopressin and E2 signals to time the surge (Smith et al., 2006b)(Dungan et al., 2007)(Robertson et al., 2009)(Vida et al., 2010)(Williams et al., 2011b)(Chassard et al., 2015). Recently, it was hypothesized that RFRP neurons too are involved in modulating the LH-surge, since they are regulated by a SCN-generated output and display a daily rhythm in neuronal activity, that in proestrus correlates with the time of the surge (Gibson et al., 2008)(Russo et al., 2015). Indeed, RFRP neurons project specifically to a subset of GnRH and AVPV/MPN kisspeptin neurons (Kriegsfeld et al., 2006)(Rizwan et al., 2012), however the underlying mechanisms through which RFRP-3 modulates the regulation of female reproductive activity are yet unknown.

Using the female Syrian hamster as a seasonal rodent model of female reproduction, the aim of this study was to investigate if the RFRP system is subjected to estrous-dependent regulations and more importantly to explore whether RFRP-3 displays differential reproductive effects according to the time of the day, the stage of the estrous cycle and season.

MATERIAL AND METHODS

ANIMALS

Adult sexually mature female Syrian hamster, *Mesocritus aureatus*, bred in-house were used in this study. From birth, they were maintained in a long photoperiod (LP) consisting of 14h light and 10h dark (lights on at 05h00), at 22±2°C with *ad libitum* access to food and water. When required, some animals were transferred to short photoperiod (SP) consisting of 10h light and 14h dark (lights on at 09h00) for 10 weeks to induce full gonadal regression. All protocols were approved by the Comité Régional d’Ethique en Matière d’Expérimentation Animale (CREMEAS). All experiments were conducted in accordance with the French National Law (license n° 67-32 and n° 01663.01) and the rules of the European Committee Council Directive of September 2, 2010 (2010/63/UE).

SURGICAL PROCEDURES AND ANIMAL MANIPULATION

Determination of estrous cycle and timing of the LH surge

In the Syrian hamsters, the estrous cycle is, like in most other rodent species, a four-day cycle characterized by four distinct stages named proestrus, estrous, metestrus and diestrus. In the present study, the stage of the cycle was determined by daily examination of vaginal smears during three weeks prior to sacrifices, where the appearance of a large number of densely packed elongated squamous cells in the uterine epithelial lining indicates the day of metestrus and thus the day subsequent to the pre-ovulatory LH-surge in proestrus. The production of circulating LH was analyzed at different time points of the day of proestrus (08h, 11h, 15h, 16h, 20h; n=6/group) and diestrus (08h, 15h, 20h; n=5/group).

Intracerebroventricular (icv) infusion

Acute or chronic central administration of RFRP-3 (Caslo Laboratory, Lyngby, Denmark) was performed according to the protocol described previously in male Syrian hamster (Ancel et al., 2012), with only slight modifications. Female Syrian hamsters adapted to LP or SP were anaesthetized using a mixture of Zoletil 20 (40 mg/kg, Virbac, Carros, France) and Rompun (10 mg/kg, Bayer Pharma, Puteaux, France) and positioned in a stereotaxic apparatus. The head of the animal was shaved and prepared for aseptic surgery and a single incision was made on the midline of the scalp. Once the area had been prepped, a stainless steel cannula was placed in the lateral ventricle at 2 mm lateral to the midline, 0.8 mm anterior to the Bregma and 3 mm inferior of the dura mater. The cannula was kept in place on the skull by dental cement and bone screws. For acute icv injections, the cannula (PlasticsOne, Roanoke, VA, USA) was blocked with a metallic wire, protected with a plastic cap and the animals were left one week to recover from the surgery before injection under light anaesthetized with isoflurane vapour (AErrane; Baxter, Maurepas, France). Injections (4µL/animal; flow rate 1µL/min) were done

using a stainless steel internal canula (PlasticsOne) attached to polyethylene tubing prefilled with either vehicle (Ringer's solution, B. Braun Medical, Boulogne, France) or RFRP-3 (0.375 µg/µl diluted in Ringer's solution) and a 50 µl Hamilton syringe (Hamilton Inc., Reno, NV, USA). LP-adapted female hamsters were injected at 10h30, 12h30 or 14h30 (n = 6-7 per group) and all sacrificed at 15h. For chronic icv infusions, the cannula (Alzet brain infusion kit1, Durect, Cupertino, CA, USA) was connected via subcutaneous polyvinylchloride tubing to an osmotic minipump (Alzet minipump model 2006, flow rate 0.15 µl/hr, duration: 6 weeks, Durect, Cupertino, CA, USA) filled with artificial cerebrospinal fluid (aCSF) or Syrian hamster RFRP-3 diluted in aCSF (0.25 nmol/hour, 12 µg/day) that was placed under the skin of the back of hamsters adapted to LP- or SP-conditions (n = 8 per group). The animals were kept in the respective photoperiods during the 5½ weeks of infusion.

Ovariectomy and estradiol capsule implantation

Female Syrian hamsters were anesthetized using a mixture of Zoletil 20 (40 mg/kg, Virbac, Carros, France) and Rompun (10 mg/kg, Bayer Pharma, Puteaux, France). SP-adapted animals were implanted subcutaneously with an estradiol [1,3,5[10]-estratriene-3,17β-estradiol; E2, Sigma, St. Quentin Fallavier, France]-filled silastic capsules (i.d. 1.47 mm; o.d. 1.95 mm; length: 13 mm). LP-adapted or SP-adapted animals were subjected to bilateral ovariectomy (OVX). Animals were sacrificed four weeks after surgical treatment.

TISSUE PROCESSING

All animals were killed in a CO₂ chamber. Blood was taken by intracardial puncture and inverted with heparin (250 IU per animal; Lique mine®, Roche, Meylan, France) for subsequent hormonal analysis. To evaluate the effects of chronic RFRP-3 icv infusion on the reproductive axis, gonads (uterus and ovaries) were isolated and weighted. For immunohistochemistry analysis, animals were transcardially perfused with 0.05M phosphate-buffered saline (PBS) followed by perfusion with a periodate-lysine-paraformaldehyde fixative (4% paraformaldehyde, pH 7.4). The brains were removed and post-fixed over night at 4°C in the same fixative, transferred to PBS + 0.02% NaN₃ and stored at 4°C until further use. Brains were then dehydrated in a 30% sucrose-PBS solution for 2-3 days, frozen on the stage of a sliding microtome (Bright Series 8000, Bright Instruments Co Ltd., England), and sliced into 4 serial sets of 40 µm thick coronal sections throughout the anterior, dorsomedial and caudal hypothalamic regions. The sections were stored as free-floating sections in a cryoprotectant antifreeze solution (30% ethylene glycol, 30% glycerol in 0.2M sodium phosphate buffer) at -20°C, until immunohistochemical processing. For in situ hybridization analysis, brains were rapidly removed from the skull, snap-frozen at approximately -40°C using liquid nitrogen, and stored at -80°C until sectioning. Six serial sets of coronal brain sections (20 µm) were cut throughout the forebrain at -20°C in a cryostat (MICROM HM 560, Thermo Scientific, Waltham, MA). The sections were thaw-mounted

and stored at -80°C until use. All brains were randomized and double-blinded: first during sectioning and again throughout processing, quantification and evaluation.

HORMONE MEASUREMENTS

ELISA (enzyme-linked immunosorbent assay) for LH measurement

LH levels presented in this study were all determined in a volume of 50µl sample plasma (diluted 1:20) using a sandwich ELISA method developed by Steyn et al. (Steyn et al., 2013) with slight modifications adapted with the help from Jenny Clarkson (Herbison lab, Department of Physiology, University of Otago, NZ). Briefly, each well of a 96-well high bind polystyrene microplate (Corning) was coated with 50µl of bovine LHβ 518B7 monoclonal antibody (obtained from Lillian E Sibley, University of California) diluted 1:1000 in 0.05 M phosphate-buffered saline (PBS), covered and incubated overnight at 4°C. Next day, wells were blocked with 200µL blocking buffer containing 5% skim milk powder, w/v, in PBS-T (PBS with 0.05% Tween-20) for 1 hour at room temperature (RT) on an orbital mixer at 40-50 rpm. A standard curve was generated using rLH (reference preparation, rat LH-RP-3, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program [NIDDK- NHPP]) in a 2-fold serial dilution in 0.2% BSA (w/v) in PBS-T. 50µl plasma sample (diluted 1:20 in PBS) and standards were then added to wells in duplicates and incubated at RT for 2 hours at 40-50 rpm after which 50µL detection antibody (polyclonal rabbit LH antibody AFP240580Rb, NIDDK-NHPP) diluted 1:10.000 in blocking buffer were added and left to incubate 1½ hour at RT at 40-50 rpm. 50µL Polyclonal goat anti-rabbit IgG/HRP antibody (DAKO Cytomation; cat # P0448), diluted 1:2000 in a 1:1 mix of blocking buffer and PBS was added per well and left at 40-50 rpm at RT for 60 min. Finally, 100µl of o-Phenylenediamine (OPD tablets, cat # 00-2003; Invitrogen) substrate containing 0.1% H₂O₂ was added to each well and left at RT for 30 minutes after which the reaction was stopped with the addition of 50µl 3M HCL. Absorbance of each well was read at a wavelength of 490nm and 655nm using a standard absorbance plate reader. Final concentration of LH in plasma samples was determined by subtracting 655nm values and zero standards from 490nm values and interpolating the OD values from the rat LH standard curve. The intraassay variations were for most experiments <6%, but the highest detected 13%. The interassay variations were <5-10% and the sensitivity of the assay was 3.9 pg/ml.

RIA (radioimmunoassay) for estradiol measurement

Serum estradiol (E2) levels were determined using an estradiol (CT, for coated tube) RIA kit (RIA-4381, DRG Diagnostics GmbH, Marburg, Germany) that takes advantage of a competition reaction between ¹²⁵Iodine-labeled E2 and E2 in the plasma sample for a fixed amount of E2 antibody sites immobilized on tubes provided with the kit. 50µl plasma or standard dilution (provided with kit) was added to the E2-antibody coated tubes along with 500µl of ¹²⁵I labeled E2 and incubated at 37°C for 3 hours. The content was discarded and the tubes were washed with 3ml Tris-HCL buffer and left at

room temperature overnight. The following day, ^{125}I labeled E2 was counted in a gamma counter and E2 concentrations of the samples were determined by dose interpolation from the calibration curve. The intra- and inter-assay variations are <8% and <14% respectively and the sensitivity of the assay is from the provider specified to 6.4 pg/ml.

IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry single labeling for kisspeptin

Kisspeptin immunoreactive neurons were visualized by an avidin-biotin immunohistochemical procedure approach similar to what has been previously described (Mikkelsen and Simonneaux, 2009)(Desroziers et al., 2010) with slight modifications. Brain sections were rinsed in 0.05 M phosphate-buffered saline (PBS) 3×10 min and incubated in 1% hydrogen peroxide (H_2O_2) diluted in PBS for 10 min, followed by 20 min incubation in a blocking buffer of 5% swine serum, 0.3% BSA and 0.3% Triton® X-100 in PBS (PBS-TX). The sections were then incubated overnight (app. 18 h) at 4°C in a primary antiserum raised against full-length rat kisspeptin-52 (JLV-1) diluted 1:200 in blocking buffer without swine serum. After the primary antiserum incubation sections were washed for 3×10 min in PBS TX and then incubated for 60 min in biotinylated donkey anti rabbit secondary antibody (1:1000; Jackson ImmunoResearch, USA). The procedure was followed by a 3×10 min wash PBS TX, after which the sections were left to incubate for 60 min in avidin-biotin-peroxidase complexes (Vectastain ABC kit, Vector laboratories, USA) diluted in PBS TX. Brain sections were then washed for 10 min in PBS TX, 10 min in PBS and 10 min in Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl; pH 7.6) before developing the sections for 10 min in 0.1% diaminobenzidine (DAB; Sigma-Aldrich, MO, USA) and 0.03% H_2O_2 in Tris-HCl, which resulted in a brown precipitate within the cytoplasm of the labeled cells. Lastly the sections were washed for 5×6 min in PBS, mounted on glass slides using a mounting medium composed of 0.5% gelatine and 0.05% chromalun in distilled water, dried and coverslipped in Pertex® (Histolab, Sweden).

Dual immunohistochemical labeling

Co-visualization of c-Fos positive labeled and RFRP-, kisspeptin- or GnRH-immunoreactive neurons, respectively, were facilitated using the avidin-biotin immunohistochemical procedure as describes above with some modifications to enable detection of two primary antisera derived from the same host species. First, sections were immunoreacted for c-Fos by incubating them overnight at 4°C with a primary rabbit polyclonal antiserum directed against c-Fos diluted 1:12000 (Mikkelsen et al., 1998). After following the procedure as described above, sections were developed in a nickel-enhanced 0.05% diaminobenzidine (Sigma-Aldrich, St Louis, MO) with 0.05% H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6) for 6 min, which generates a black precipitate within the nucleus. For the second immunolabeling, the procedure was repeated by incubating sections overnight at 4°C with primary kisspeptin (JLV-1) antiserum diluted 1:200, RFRP/GnIH antiserum (quail GnIH antiserum, RRID:

AB_2531898) kindly provided by Prof. Kazuyoshi Tsutsui (Tsutsui et al., 2000) diluted 1:5000 or a polyclonal rabbit anti-GnRH antibody (AB1567, Merck MilliPore, Darmstadt, Germany) diluted 1:2500. When staining for GnRH, the biotinylated donkey anti rabbit secondary antibody was used at a dilution of 1:1500 rather than 1:1000. Development was done using 0.1% diaminobenzidine (DAB; Sigma-Aldrich, MO, USA) and 0.03% H₂O₂ in Tris-HCl alone, for 10 min (JLV-1 and RFRP) and 6 min (GnRH), respectively, resulting in a brown cytoplasmic precipitate. Finally, sections were rinsed and mounted on glass slides as described for the single immunolabeling.

Quantification of immunolabelled cells

The total number of kisspeptin-ir, GnRH-ir and RFRP-ir labeled cells was counted manually while blinded for animal's identities. Only groups reacted in the same IHC setup were compared. Quantifications of single IHC labeling were done using a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy, Göttingen, Germany). For each animal, the total number of RFRP-ir neurons was counted on 11 serial sections (one every 160 µM) from the caudal part of medial anterior hypothalamus throughout the medial hypothalamus thus including the entire DMH/VMH region. The total number of kisspeptin-ir was counted in the AVPV and the arcuate nucleus (ARC) separately. For each animal, kisspeptin positive labeled neurons were counted on 7 serial sections (one every 160 µM) at the level of the AVPV/MPN and on 11 serial (one every 160 µM) sections at the level of the ARC. Total number of GnRH-ir neurons was counted at the level of the preoptic area on 9 serial sections (one every 160 µM). The relative number of kisspeptin, RFRP, and GnRH neurons containing c-Fos immunoreactivity was counted manually in each animal and presented as percentage of the total number of the respective labeled neurons.

IN SITU HYBRIDIZATION (ISH)

In one experiment, *Rfrp* mRNA was analyzed using non-radioactive *in situ* hybridization performed according to a previously described protocol (Klosen et al., 2013). The sections were fixed in 4% paraformaldehyde, digested for 30 min at 37°C with 1µg/ml proteinase K (Roche, Meylan, France) in 0.05 M phosphate-buffered saline (PBS), postfixed in cold 2% paraformaldehyde and acetylated in triethanolamine buffer. After the riboprobe was denatured and mixed with hybridization medium (200 ng/mL) it was applied to the slides and incubated for 40 h at 60°C. Six stringency rinses were performed at 72°C. Digoxigenin-labeled bound probes were detected with an alkaline phosphatase-labelled antidigoxigenin antibody (Roche, Meylan, France). Alkaline phosphatase activity was detected with bromochloroindolyl phosphate and nitroblue tetrazolium in the presence of 5% polyvinyl alcohol (70.000-100.000 MW; Sigma, St. Quentin Fallavier, France).

Quantification of labeled cells.

Identities of the animals were blinded to the observer throughout processing and quantification. Semi-quantitative analysis were done by measuring total integrated density of *Rfrp* mRNA per animal and only groups reacted in the same hybridization procedure were compared.

DIGITAL IMAGES

Digitally captured photomicrographs (10x, 20x or 40x magnification) were taken with a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy, Göttingen, Germany). Scale bars were added using ImageJ software (National Institute of Health, USA). When needed photomicrographs were converted to 8-bit grey scale images and if necessary brightness/contrast was equally adjusted on all images when compared to one another.

STATISTICAL ANALYSES

Results are shown as mean $\pm$ SEM (n = 6-8 per group). Data were analyzed by unpaired Student's t-test, and multiple group comparisons were analyzed using one-way analysis of variance (ANOVA), followed by post-hoc analysis; the Holm-Sidak test or Tukey's Honestly Significant Difference test, as appropriate. Statistical significance was set at $P_{value} < 0.05$.

RESULTS

RFRP- AND KISSPEPTIN-IR EXPRESSION AND NEURONAL ACTIVITY AT DIFFERENT STAGES OF THE ESTROUS CYCLE IN THE FEMALE SYRIAN HAMSTER.

In one set of experiments, we investigated the immunoreactivity and activation of RFRP- and kisspeptin neurons at different stages of the estrous cycle in the female Syrian hamster. The estrous cycle of female hamsters adapted to LP conditions was monitored and LH levels were measured at different time-points on the day of diestrus and proestrus, including two points in the late afternoon at the time of the preovulatory surge (figure 28A). Next, we evaluated the number of neurons immunoreactive for kisspeptin (in the AVPV/MPN) or RFRP-3 as well as their neuronal activation assessed by the relative number of c-Fos positive labeled neurons. We saw no changes in the number of kisspeptin-ir and RFRP-ir neurons at the different time points of proestrus and diestrus (figures 28B and 28D, left panels). On the day of proestrus, we observed an opposite regulation of the two neuronal populations in that the number of c-Fos positive kisspeptin neurons in the AVPV/MPN was significantly increased around the time of the LH-surge in proestrus (figures 28B, right panel, and 28C), whereas the number of c-Fos positive RFRP-3 neurons was significantly reduced (figures 28D, right panel, and 28E) at the time of the LH surge. On the day of diestrus, kisspeptin neuronal activity was at an unchanged low level (figures 28B, right panel, and 28C) whereas RFRP neuronal activity displayed a significant decrease at late afternoon (figures 28D, right panel, and 28E), despite low stable levels of LH.

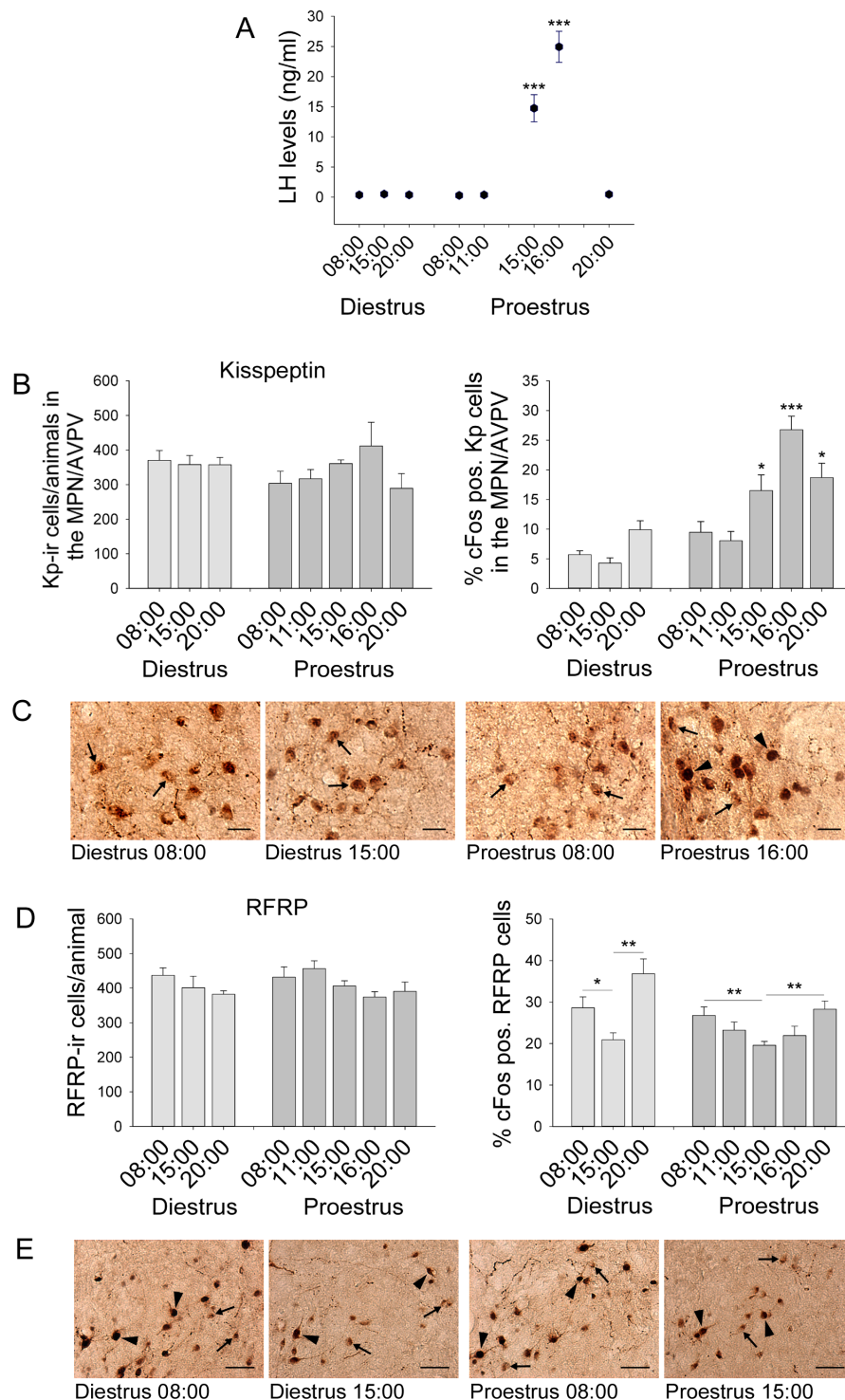


Figure 28. RFRP- and kisspeptin-ir expression and neuronal activity at different stages of the estrous cycle in the long photoperiod-adapted female Syrian hamster. A) LH plasma concentrations in female Syrian hamsters at various time points during the day of diestrus and proestrus; B) Number (left panel) and percentage of c-Fos co-expressing (right panel) kisspeptin-ir neurons in the anteroventral periventricular area (AVPV) at various time points during the day of diestrus or proestrus; C) Photographs showing c-Fos positive Kp-ir neurons (indicated by arrowheads) and c-Fos negative kisspeptin-ir neurons (indicated by arrows) in the AVPV of female in diestrus (time points 08:00 and 15:00) and proestrus (time points 08:00 and 16:00), scale bars = 20 μ m; D) Number (left panel) and percentage of c-Fos co-expressing (right panel) RFRP-3-ir neurons at different time points during the day of diestrus or proestrus; E) Photographs showing c-Fos positive RFRP-ir neurons (indicated by arrowheads) and c-Fos negative RFRP-ir neurons (indicated by arrows) of female in diestrus (time points 08:00 and 15:00) or proestrus (time points 08:00 and 16:00), scale bars = 50 μ m). Data are presented as mean $\pm$ SEM of $n = 5$ for diestrus and $n = 6$ for proestrus; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ for significant differences when compared to all other groups (1A, 1B) or when compared between groups (indicated with lines on 1D).

THE ACUTE INHIBITORY EFFECT OF RFRP-3 DEPENDS ON THE TIME AND REPRODUCTIVE STATE IN THE INTACT FEMALE SYRIAN HAMSTER.

Investigation of the central effect of RFRP-3 on the female Syrian hamster gonadotropic axis was carried out in physiologically intact female hamsters raised in either LP or SP conditions and evaluated at different estrous stages and time points of the day. In SP-adapted female hamsters, central injection RFRP-3 (1500 ng) had no effect on the basal level of LH in contrast to kisspeptin-10 (700 ng) that potently stimulated a release of LH (vehicle: 0.0407ng/ml; RFRP-3: 0.0695ng/ml and kisspeptin-10: 1.3485ng/ml, $P < 0.0001$), (data not shown). In LP-adapted female hamsters, the estrous cycle was monitored and an icv injection of vehicle (4 μ l Ringer's solution) or RFRP-3 (1500 ng diluted in 4 μ l Ringer's solution) was given at three different time points in proestrus prior to the pre-ovulatory surge of LH (morning, 10:30; mid-day, 12:30; and just before or in the very beginning of the surge in LH, 14:30) as well as in diestrus. On the day of proestrus, RFRP-3 significantly reduced the LH surge amplitude measured at 15:00 when injected 30 minutes prior to the surge, but not when injected earlier in the morning or mid-day (figure 29B). RFRP-3 had no effect on basal LH level when injected at diestrus (figure 29A). Subsequently, we analyzed the number of kisspeptin-ir neurons (in the AVPV/MPN) and GnRH-ir neurons as well as their neuronal activation (c-Fos co-expression) in the brains of the animals showing a reduced LH-surge after RFRP-3 administration as compared to vehicle (time point 14:30). RFRP-3 central injection displayed no effect on either the number of kisspeptin-ir neurons (figure 29C, left panel), or the activation of kisspeptin neurons (figure 29C, right panel). No changes were observed in the number of GnRH neurons after injection of RFRP-3 (figure 29D, left panel) and despite GnRH neuronal activity being decreased by 33% in animals injected with RFRP-3 as compared to vehicle, the difference between groups were not significant ($P = 0.0539$), (figure 29D, right panel).

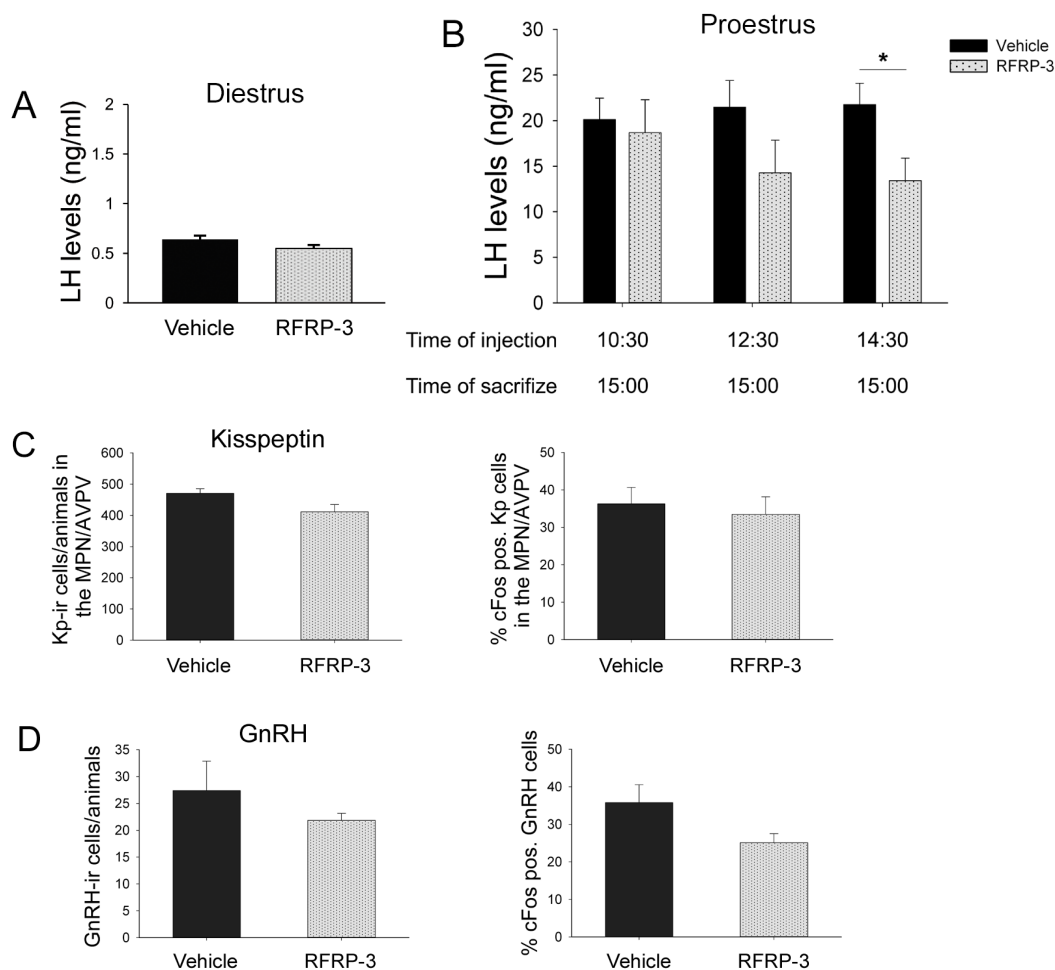


Figure 29. Effects of intracerebroventricular (icv) RFRP-3 administration at different time and reproductive stages in the long photoperiod-adapted female Syrian hamster. A) In LP-adapted female hamsters, an icv injection of vehicle (4 μ l Ringer or 0.09% NaCl?) or RFRP-3 (1500 ng in 4 μ l Ringer) was given at three different time points on the day of proestrus (morning, 10:30; mid-day, 12:30; and just before or in the very beginning of the surge in LH, 14:30) in as well as in diestrus (time of the day); and circulating LH was measured at 15:00 (time of the putative LH surge) in proestrus hamsters or at time of the day+30min in diestrus hamsters;; data represent the mean level of LH $\pm$ SEM ($n = 7$ in proestrus, $n = 6$ in diestrus), * $P < 0.05$ indicates a significant effect of RFRP-3 when compared to vehicle; B/C) Number (left panel) and percentage of c-Fos co-expressing (right panel) kisspeptin-ir neurons (B) or GnRH-ir neurons (C) in hamsters injected with vehicle or RFRP-3 at 14:30 and sacrificed at 15:00 on the day of proestrus; Kisspeptin neurons were measured in the anteroventral periventricular area (AVPV) and the medial preoptic nucleus (MPN) and GnRH neurons in the preoptic area (POA); data represent mean value $\pm$ SEM ($n = 7$).

CHRONIC INTRACEREBROVENTRICULAR (icv) OF RFRP-3 RESTORES REPRODUCTIVE ACTIVITY IN SP-PHOTOINHIBITED AND INHIBITS REPRODUCTIVE ACTIVITY IN LP-PHOTOACTIVATED FEMALE SYRIAN HAMSTERS.

Given that in female Syrian hamster RFRP expression is strongly down-regulated in sexually quiescent SP-adapted animals (Henningsen et al., 2015) and RFRP-3 inhibits LH secretion in LP-adapted sexually active animals (this study), we questioned whether a chronic infusion of RFRP-3 to SP-inhibited or LP-activated female Syrian hamsters could alter their reproductive status. For this, female Syrian hamsters were kept in LP or adapted to SP conditions for 9-10 weeks in order to obtain full regression

of the reproductive axis. Animals were then infused icv with either vehicle (artificial (a)-CSF) or RFRP-3 (0.25nmol/hour, 12µg/day) and kept in their respective photoperiods. After 5^{1/2} weeks of treatment the female hamster reproductive status was evaluated by assaying circulating LH and E2, measuring uterus and ovarian weight and counting the number of kisspeptin-ir neurons in the AVPV/MPN and ARC. As expected, the SP-aCSF hamsters had undergone a complete sexual regression manifested by lower LH and E2 circulating values (figure 30A and B), lower uterus and ovarian weight (figure 30C) and a lower number of kisspeptin-ir neurons (figure 30D and E) as compared to LP-aCSF group. Interestingly, the SP-adapted female hamsters receiving a chronic administration of RFRP-3 (SP-RFRP-3) underwent a marked increase in circulating LH and E2 values (figure 30A and B), gonadal weight (figure 30C) and number of kisspeptin-ir neurons in both AVPV/MPN and ARC (figure 30D and E) as compared to the SP-aCSF group. The levels of LH and E2, as well as the number of AVPV/MPN and ARC kisspeptin-ir neurons in SP-RFRP-3 animals were similar to those in LP-aCSF animals, indicating that RFRP-3 was able to fully restore reproductive activity in SP-photoinhibited female hamsters. Interestingly, chronic infusion of RFRP-3 in LP-adapted hamsters displayed the opposite effect than that observed in SP-adapted hamsters, as it significantly reduced LH levels (figure 30A), gonadal weight (figure 30C) and the number of ARC kisspeptin-ir neurons (figure 30E) as compared to LP-aCSF animals. Circulating E2 (figure 30B) and the number of AVPV/MPN kisspeptin neurons (figure 30D) were also slightly reduced by chronic RFRP-3, but with no significant difference to vehicle-treated hamsters. These chronic experiments were repeated twice with the same outcomes. Moreover, in one setup LP and SP intact animals were included to test the impact of surgery and chronic infusion and no differences were found between vehicle-treated and intact LP or SP animals.

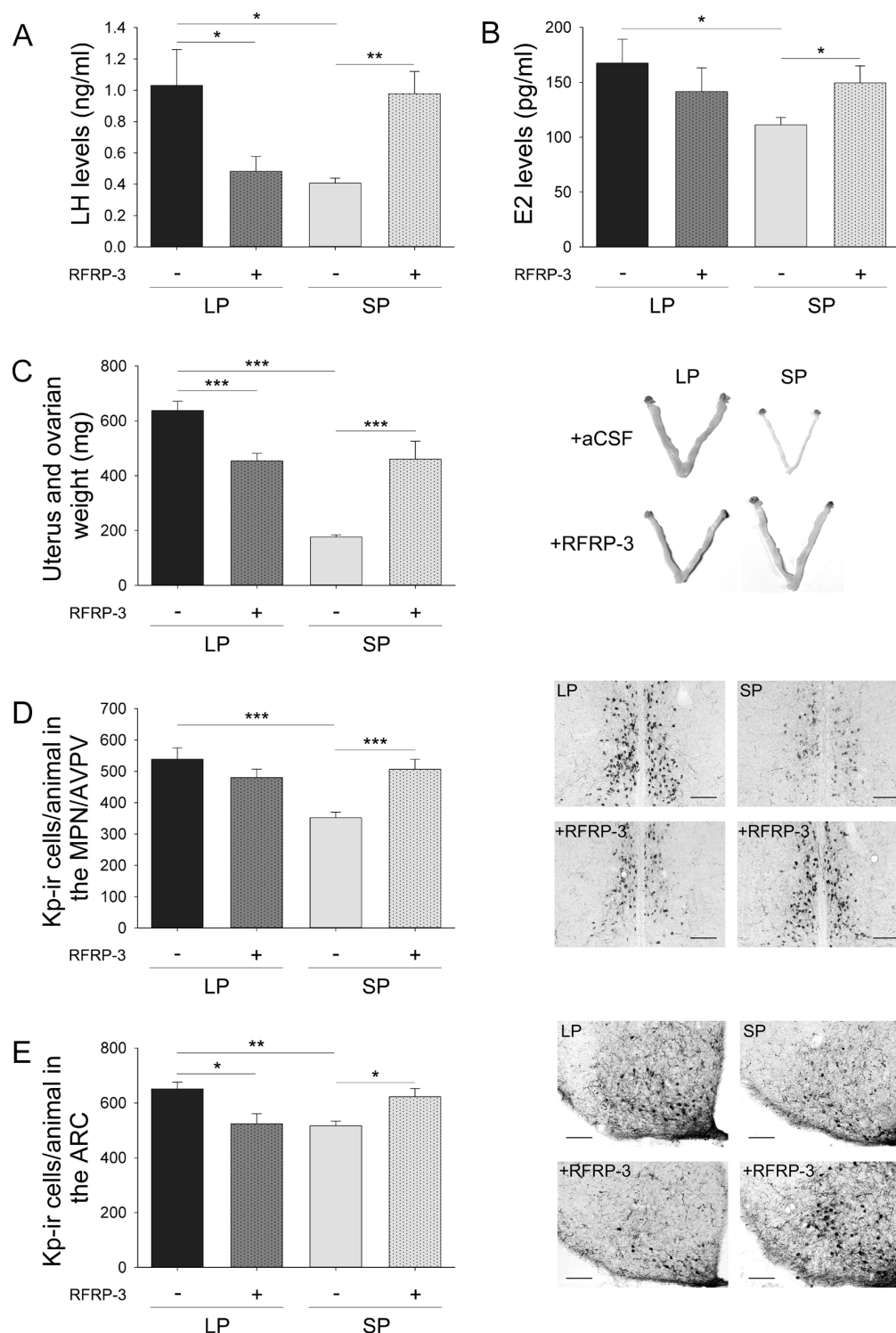


Figure 30. Effects of chronic intracerebroventricular (icv) infusion of RFRP-3 on the reproductive status of female Syrian hamsters kept in short (SP) or long (LP) photoperiod. Female Syrian hamsters were kept in LP or placed in SP conditions for 9 weeks in order to obtain complete regression of reproductive activity. Animals were implanted with an icv cannula linked to an osmotic minipump (flow rate: 0.15 μ l/hour) filled with either aCSF (SP-aCSF and LP-aCSF) or hamster RFRP-3 (SP-RFRP-3 and LP-RFRP-3, 0.25nmol/hour, 12 μ g/day) and kept in their respective photoperiod throughout infusion. After 5^{1/2} weeks of treatment the reproductive status was determined by measuring circulating LH (A), and E2 (B), uterus and ovarian weight (C, D) and the number of kisspeptin-ir neurons in the anteroventricular periventricular (AVPV) and medial preoptic nucleus (MPN) area (E) and the arcuate nucleus (F); Data represent mean values $\pm$ SEM (n = 8). with * P < 0.05, ** P < 0.01, *** P < 0.001 indicating significant differences between group comparisons as indicated with lines; Representative photographs of reproductive organs (D) and kisspeptin immunolabeling in the AVPV/MPN (E, right panel, scale bars = 100 μ m) and ARC (F, right panel, scale bars = 100 μ m) are shown for each treatment group as indicated on the figures.

GONADAL HORMONES DO NOT ALTER *RFRP* EXPRESSION IN THE HYPOTHALAMUS OF LP- AND SP-ADAPTED FEMALE SYRIAN HAMSTER

In one set of experiments, female Syrian hamsters were placed for 10 weeks in LP or SP conditions. Subgroups of female hamsters were OVX prior to LP or SP exposure, or implanted with E2 capsules prior to SP exposure in order to analyze the putative involvement of gonadal steroids in the photoperiodic variation in female hamster *Rfrp* gene expression. Our results confirmed that *Rfrp* mRNA levels are strongly down-regulated in SP as compared to LP conditions. Furthermore, we observed that OVX in LP- and SP-adapted hamsters and E2 implants in SP-adapted hamsters did not alter *Rfrp* mRNA levels compared to control animals (figure 31).

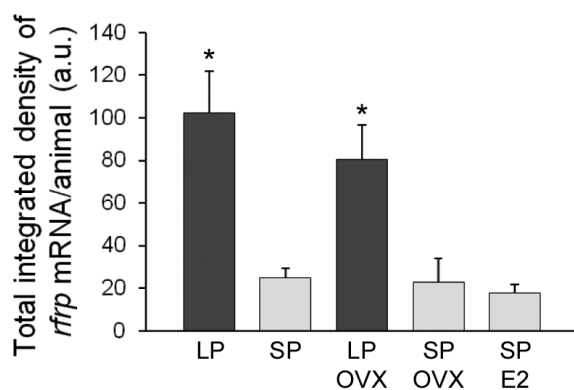


Figure 31. Effect of gonadal hormones on *rfrp* expression in LP- and SP-adapted female Syrian hamsters. Quantification of *Rfrp* mRNA levels in the dorso/ventro medial hypothalamus of sham or ovariectomised (OVX) female hamsters raised in LP, and of sham, OVX or estradiol implanted (E2) female hamsters maintained in SP. Data represent mean *Rfrp* levels $\pm$ SEM ($n = 6$ per group), with * $P < 0.05$ indicating significant differences between bars marked with stars as compared to bars without stars.

DISCUSSION

Reproduction in females undergoes regular phases of activation and inactivation throughout the lifetime and in seasonal species, sexual activity is furthermore submitted to a strong annual regulation to ensure optimal timing of birth and thus species survival. In this study we have examined how the hypothalamic RFRP system may be involved in the various daily, oestrian and seasonal cycles of female reproduction. Using the female Syrian hamster as a seasonal model we here provide evidence for a role of RFRP neurons in the regulation of two distinct features of female reproductive activity; daily changes necessary for the timing of the pre-ovulatory LH surge and synchronization of the reproductive axis with the seasonal changes in photoperiod.

A previous study showed that RFRP-ir cell numbers and their activational state is decreased at the time of the LH-surge on the day of proestrus as compared to other time points on the day of proestrus and to diestrus (Gibson et al., 2008). In rat and ewes, RFRP expression is similarly reduced during the pre-ovulatory period (Clarke et al., 2012)(Jorgensen et al., 2014). Our analysis of c-Fos expression in RFRP neurons supports that RFRP neuronal activation is specifically decreased in the afternoon of proestrus, concomitantly to a dramatic increase in kisspeptin activation, known to stimulate GnRH neurons and the surge of LH (Khan and Kauffman, 2012). Interestingly, by including more time points to our analysis in diestrus, we saw that the activity of RFRP neurons, in contrast to kisspeptin neurons, is similarly regulated in diestrus and was decreased in late afternoon, which indicates that the daily rhythm in RFRP neuronal activity is independent of the stage of the estrous cycle. A recent study demonstrated that SCN-derived vasoactive intestinal peptide (VIP) neuronal projections form close appositions to RFRP neurons in female Syrian hamsters and that central VIP administration markedly suppressed RFRP cellular activity in the afternoon/evening, but not in the morning (Russo et al., 2015). Therefore, it seems likely that a SCN-derived VIP-ergic signal is responsible for the daily decrease in RFRP activity occurring in the late afternoon of any estrous stage. Russo *et al.*, found however no co-localization between RFRP cells and VIP receptor and therefore suggested that VIP regulation of RFRP neurons is indirect (Russo et al., 2015). We did not see any changes in number of RFRP-ir neurons, nor did we see change in the levels of RFRP mRNA (data not shown) along the daily cycle in proestrus. This is in contrast to a previous report (Gibson et al., 2008) although in both studies RFRP neurons were immunostained using the same avian GnIH antiserum. However, the fact that our immunohistochemical results are confirmed with our mRNA level analysis strengthens the conclusion that the RFRP peptide content displays no significant daily and estrous-dependent variation in the female Syrian hamster. In the same manner, and as already reported in female mice (Chassard et al., 2015), the number of kisspeptin-ir neurons remained unchanged throughout the daily time points analyzed in proestrus and diestrus.

To date, the effect of RFRP-3 administration on the female rodent reproductive axis have exclusively been investigated in OVX animals in order to bypass the potential interference of varying levels of

circulating sex steroids (Pineda et al., 2010a)(Murakami et al., 2008)(Kriegsfeld et al., 2006). In OVX rats (Pineda et al., 2010a)(Murakami et al., 2008) and Syrian hamsters (Kriegsfeld et al., 2006), central administration of RFRP-3 inhibits the artificially-elevated plasma LH levels. The present study is the first to investigate the effects of RFRP-3 at two different stages of the estrous cycle in a female rodent kept in physiological conditions. Our data show that central administration of RFRP-3 significantly decreases the LH-surge peak when injected just before/in the beginning of the surge, whereas injections of RFRP-3 earlier in the morning has no effect on the afternoon peak of LH. Considering the inhibitory effect of RFRP-3 along with the endogenous decrease in RFRP activation observed in the afternoon of proestrus, it appears that the RFRP neuronal network is exerting a tonic inhibition on the hypothalamo-pituitary gonadal axis in female Syrian hamsters, which is specifically lifted through a SCN-generated circadian and possibly VIP-ergic output, allowing an intact peak of LH to drive ovulation. In our attempt to study the underlying targets through which this inhibitory effect of RFRP-3 is mediated, we looked at the expression and activation of AVPV/MPN kisspeptin neurons and GnRH neurons, respectively. RFRP neurons project to the AVPV/MPN (Henningsen et al., 2015)(Kriegsfeld et al., 2006) and in rats, a subpopulation of these kisspeptin neurons (app. 20%) expresses the RFRP receptor, GPR147 (Rizwan et al., 2012). Several studies also report that RFRP neurons projects to GnRH neurons and that RFRP-3 directly modulates the activity of a subpopulation of GnRH cells (Kriegsfeld et al., 2006)(Rizwan et al., 2012)(Poling et al., 2012)(Anderson et al., 2009)(Ubuka et al., 2012)(Ancel et al., 2012). We found no changes in the number or activation of AVPV/MPN kisspeptin neurons, indicating that the inhibitory effect of RFRP-3 on the surge of LH is not mediated at the level of kisspeptin in the AVPV/MPN. GnRH neurons in and around the OVLTL express c-Fos around the time of the LH-surge (Herde et al., 2011) and one might expect to see an effect on GnRH neurons through the level of their activation i.e. nuclear expression of c-Fos. We observed that RFRP-3 injection leads to a marginal decrease in c-Fos expressing GnRH neurons. Actually using a cell-attached electrophysiological approach, Ducret et al. (2009) reported that RFRP-3 inhibits the firing rate of 41% GnRH neurons while activating the firing rate in 12% of GnRH neurons, the remainder being unaffected. Therefore it might be difficult to detect a significant RFRP-3-induced decrease when analyzing the overall population of c-Fos expressing GnRH neurons. Some studies report the presence of RFRP-ir fibers in the median eminence (ME), although our previous work shows no hypophysiotropic effect of RFRP in the Syrian hamster (Ancel et al., 2012) and we can not rule out RFRP-3 interactions with GnRH neurons at this level, as has been described for kisspeptin (Yip et al., 2015b).

The present data strengthens the evidence for sex-specific differences in the acute effects of RFRP-3 in the Syrian hamster, stimulating in males (Ancel et al., 2012) while inhibiting in females (this study) the GnRH-induced gonadotropin secretion. A possible explanation for this sex-related discrepancy may be a difference in sites of action. The effect of RFRP-3 may as well be mediated through intermediate neurons, and these neurons could be sexually different. Indeed, acute administration of RFRP-3 in

male Syrian hamster induces c-Fos expression in a yet unidentified but non-kisspeptinergic neuronal population in the ARC (Ancel et al., 2012), but whether this population is also a target in females remains unknown. In spite of the fact that we did not see any effect on preoptic kisspeptin expression, we did however observe a strong induction in c-Fos positive neurons in the preoptic area at the level of the MPN. ER α expressing GABA-ergic neurons in this area have recently been shown to be essential for proper positive feedback of E2 (Cheong et al., 2015) and although highly speculative, it is suggested to investigate whether the effect of RFRP-3 could be mediated via these neurons. It should however be stressed that further investigations are necessary in order to determine first the identity of the activated neurons as well as the physiological relevance of their activation.

The breeding season is characterized by an increased amount of circulating sex steroids as compared to sexual quiescence and during sexual activity high circulating E2 levels, as an indicator of ovarian maturation, stimulate kisspeptin release from the AVPV/MPN and downstream the surge of LH that subsequently triggers ovulation (Hoffman and Reiter, 1966)(Smith et al., 2005a)(Simonneaux and Bahougne, 2015). A portion of RFRP neurons have been found to co-express estrogen receptor ER α (Kriegsfeld et al., 2006)(Molnar et al., 2011)(Poling et al., 2012) and in rats and Syrian hamsters, E2 stimulates RFRP neurons in female Syrian hamsters whereas in males, testosterone has no effect on RFRP expression (Kriegsfeld et al., 2006)(Mason et al., 2010)(Revel et al., 2008)(Iwasa et al., 2012). RFRP-ir expression is higher in female rats and Syrian hamsters as compared to males (Jorgensen et al., 2014)(Henningsen et al., 2015) and it has been suggested that gonadal steroid hormones could drive these sex-differences. In our study, however, we found that neither OVX in LP-adapted females nor E2 implants in SP-adapted females alters *Rfrp* expression. Therefore, it seems evident that seasonal and cyclic changes in RFRP expression occur independently of sex steroid feedback. To further support this hypothesis, a study in rats showed no difference in RFRP mRNA levels of OVX females, whatever treated with estrogen or progesterone (Quennell et al., 2010).

Our data reveal a previously uncharacterized role of RFRP-3 in females as an important relay for proper melatonin-dependent seasonal regulation of reproduction. We show that chronic infusion of RFRP-3 in female Syrian hamsters maintained in photoinhibitory SP conditions, where RFRP expression is normally low, reactivates the reproductive axis to levels comparable with LP animals, attested by an increase in LH and E2 levels and gonadal weight. We observed that this reproductive reactivation is accompanied by a marked increase in kisspeptin-ir expression in both the ARC and MPN/AVPV. It is likely that the RFRP-3 driving force for the stimulatory effect on gonadal activity results from an increased ARC kisspeptin neurotransmission towards GnRH neurons. In MPN/AVPV, the increase in kisspeptin-ir could result from a similar RFRP-3 stimulatory action or from an indirect positive E2 feedback due to an increased ovarian activity. The restoration of ARC kisspeptin expression and reproductive activity by chronic RFRP-3 infusion in the SP-inhibited female hamsters is similar to our previous findings in SP-adapted male Syrian hamsters (Ancel et al., 2012). Taken

together, these findings suggest that the chronic release of RFRP-3 increases ARC kisspeptin expression and further down the hypothalamo-pituitary gonadal axis, an hypothesis consistent with a conserved pathway through which RFRP-3 mediates the photoperiod-driven melatonin signal toward ARC kisspeptin neurons to drive the seasonal control of reproduction. Indeed, continuous central administration of thyroid stimulating hormone (TSH), a pars tuberalis hormone whose synthesis is markedly inhibited by melatonin (Hanon et al., 2008b), reactivates reproductive activity in SP-adapted hamsters via a full restoration of LP-like RFRP expression (Klosen et al., 2013). Altogether, our experimental findings of a stimulatory effect of chronic TSH and RFRP-3 in seasonal rodents, together with the conserved up regulation of RFRP expression in all seasonal species investigated so far (Revel et al., 2008)(Ubuka et al., 2012)(Henningsen et al., 2015)(Janati et al., 2013)(Ancel et al., 2012)(Saenz de Miera et al., 2014)(Smith et al., 2008)(Simonneaux and Ancel, 2012)(Talbi et al., 2015) supports the hypothesis of a conserved pathway through which melatonin, via the RFRP neurons, regulates reproductive activity. In contrast to SP-adapted animals, we find that continuous administration of RFRP-3 in LP-adapted females causes a significant decrease in LH levels, gonadal weight as well as in the number of kisspeptin-ir neurons in the ARC, thus inhibiting the reproductive axis. These data fit well with the inhibitory effect we observe in LP females following acute administration of RFRP-3, although this effect was specifically linked to the LH-surge and injections at other time points had no effect on LH levels. One possibility would be that chronic RFRP-3 in LP-hamsters induces a repeated reduced LH surge finally leading to a reduction in reproductive capacity. This hypothesis is strengthened by the observation that chronic RFRP-3 treatment, like the acute RFRP-3 injection, in LP-adapted female hamster does not significantly alter the MPN/AVPV kisspeptin expression. Alternatively, one could speculate whether inhibition occurs via ARC kisspeptin neurons, since we see a decrease in this population after continuous infusion of RFRP-3 in females kept in LP, although the levels of circulating E2 were not significantly reduced, thus excluding an indirect negative feedback on ARC kisspeptin.

It remains to be answered how in the same species and sex RFRP-3 displays opposite effects according to the seasonal environment as also reported for male Siberian hamster, where RFRP-3 stimulates LH release in animals kept in SP, while inhibiting LH release in LP (Ubuka et al., 2012).

In a recent study we show that GPR147 mRNA expression is regulated by photoperiod, but interestingly we observed a higher expression of GPR147 in females as compared to males and moreover, we saw a more profound photoperiodic regulation of GPR47 mRNA levels with a marked reduction in SP as compared to LP conditions in female Syrian hamsters, that was not similarly observed in males. Since in the Syrian hamster, photoperiod-dependent differences in the effects of RFRP-3 is observed only in female and not in male, it is possible that the strong variations in GPR147 expression specific to the female might help to explain the diverging effects of RFRP-3. At this point it is not known whether this difference is a direct consequence of sex differences in the amounts of RFRP

peptides that binds to and signals via the receptor, or whether the differences could arise from a differential regulation of the GPR147 gene by i.e. gonadal steroids. Although RFRP peptides binds with highest affinity to GPR147, it might be relevant to investigate whether the higher expression of GPR147 in the female as compared to male as well as the strong down-regulation of GPR147 in females kept in SP is somehow being compensated for through an increased expression of and signaling through GPR74.

Altogether, the present data demonstrate for the first time how the RFRP system is involved in both daily and seasonal regulation of female reproductive activity, pointing to a critical role of this neuropeptide along with that of kisspeptin for the neuroendocrine control of reproduction. However, although kisspeptin has always been reported as a strong activator of the reproductive axis, whatever the species, sex and environmental conditions (Pinilla et al., 2012 for review), the effect of RFRP-3 is far more complicated than initially expected. For future experiments it will be essential to keep in mind that there might be sex and photoperiod-dependent differences in the effects of RFRP-3 in other species than the Syrian hamster and that there might also be fundamental differences in the RFRP system between seasonal and non-seasonal species.

DISCUSSION

Over the past nearly two decades RFRP peptides have been extensively studied for their putative involvement in the regulation of the reproductive axis. Despite the well-documented stimulatory effect of RFRP-3 in Siberian and Syrian hamster species (Ancel et al., 2012)(Ubuka et al., 2012), RFRP peptides are still widely referred to as GnIH, referring to its inhibitory effect initially observed in quails (Tsutsui et al., 2000). However, recent advances made in the field of RFRP research, have challenged the conception of a conserved inhibitory role of RFRP amongst species, and underline the necessity to delineate its effects in one species, sex and physiological condition.

The present work provides a thorough characterization of the hypothalamic RFRP system, and its role in regulating reproductive activity in a seasonal model, the Syrian hamster. We demonstrate that there are strong seasonal differences in the expression of RFRP neurons and its receptor, GPR147, and when comparing the RFRP system between males and females, we moreover find strong sex-specific differences; RFRP neuronal expression and GPR147 mRNA levels being significantly higher in females than in males. Intrigued by these findings, we further aimed at understanding why the RFRP system is more strongly expressed and regulated in females, and therefore investigated the RFRP system and its involvement in daily changes of the female estrous cycle. Interestingly, our findings demonstrate that the expression and activity of RFRP neurons vary according to the estrous cycle, and that central injections of RFRP-3 can decrease the pre-ovulatory luteinizing hormone surge. Lastly, the work describes how chronic central infusion of RFRP-3 in SP-adapted female Syrian hamsters, with a normally low endogenous expression in RFRP, reactivates their reproductive activity. Strikingly, together these data unveil multiple and distinct roles of RFRP in regulating female reproduction, by acting as a key regulator of the seasonal synchronization of reproduction, while at the same time being an important regulator of cyclic events controlling the pre-ovulatory LH surge in females.

THE RFRP SYSTEM AND MODES OF ACTION

The RFRP system has in this work been characterized using a quail-derived RFRP antiserum that potently recognizes both RFRP-1 and -3 in Syrian hamster brain sections. We found RFRP-ir neurons distributed from the caudal part of the medial anterior hypothalamus, throughout the medial tuberal hypothalamus, with the majority of the RFRP-ir neurons being expressed in the DMH and in-between the DMH and VMH, and with few labeled neurons detected in the dorsomedial VMH. RFRP-ir fibers project to various hypothalamic areas including the POA/OVLT, MPN/AVPV, ARC, the anterior part of the SCN, PVN, AH, and the VMH, as well as outside of the hypothalamus in the BNST, Hb, and the PVT. These findings are in overall accordance with what has previously been reported (Kriegsfeld et al., 2006)(Poling et al., 2013)(Rizwan et al., 2012)(Ubuka et al., 2012)(Revel et al., 2008). Importantly, the

anatomical distribution of RFRP-ir neurons and their projections were overall similar between females and males. In the POA, it is striking how RFRP-ir fibers are densely and specifically organized into the highly vascularised region of the OVLT. GnRH processes are similarly found in the OVLT, and has been shown to be mostly dendritic rather than axonal outgrowths, which respond to stimuli applied outside the BBB (Herde et al., 2011)(Prevot, 2011). GnRH neurons, which project to the OVLT are thus in contact with the periphery and it would be interesting to investigate whether RFRP neurons, in a similar manner, are sensitive to the peripheral environment.

Our current findings report the first mapping of GPR147 mRNA in the brain of the Syrian hamster. We find high levels of GPR147 gene expression primarily in hypothalamic areas (POA/OVLT, MPN/AVPV, SCN, PVN, AH, VMH and ARC) as well as the BST and Hb, and so the distribution of GPR147 is strongly correlated with the distribution of RFRP-ir projections. The pattern of GPR147 mRNA expression is overall similar between females and males, although the degree of expression is very different, as will be discussed later. In previous studies, NPFF1/GPR147 has been mapped by autoradiography in other mammalian species, amongst them the guinea pig and sheep (Gouarderes et al., 2004)(Dardente et al., 2008). In guinea pig, the distribution is quite similar to what we find in the Syrian hamster, and in sheep, the receptor distribution has been mapped to the SCN and the PVN, but also to the supraoptic nucleus and the PT. Although there are controversies in the effects of RFRP in sheep, RFRP neurons have been found to project to the external layer of the ME in ovine and directly inhibit GnRH-stimulated gonadotropin secretion from ovine pituitaries (Qi et al., 2009)(Sari et al., 2009). Whether GPR147 is expressed in the PT of Syrian hamster remains to be investigated, but nevertheless, RFRP-3 has no direct effect on gonadotropin release from cultured pituitaries (Ancel et al., 2012).

RFRP-1 AND RFRP-3 DIFFERENCES

RFRP-1 and -3 peptides are often considered as being secreted from the same neurons, since studies using antibodies generated against either RFRP-1 or -3 show that the two peptides co-localize to the same neurons. However, antibodies directed against RFamide peptides often show a high degree of cross-reactivity with other RFamide peptides. Therefore, we tested the specificity of a range of RFRP antibodies generated against RFRP-1 and RFRP-3 peptides of different species, as shown on dot blots below (figure 32). The quail GnIH antiserum (developed by Prof. Kazuyoshi Tsutsui), potentially recognizes both RFRP-1 and RFRP-3 peptides. In agreement, pre-adsorption with both RFRP-1 and RFRP-3 abolished all staining in hamster brain sections. Another antibody, raised in sheep against human RFRP-3 (generated by Alain Caraty), recognizes both RFRP-1 and RFRP-3 peptides on the dot blot, whereas an antibody raised in guinea pig against rat RFRP-1 (generated by Jens D. Mikkelsen), was shown to specifically bind to RFRP-1, and not RFRP-3 peptides. At high concentrations (1mM), however, a small fraction of the antibody bound to human RFRP-3 peptide, and in even smaller amounts to Syrian hamster RFRP-3 peptide, but further testing its specificity in rat brain sections have

shown that this antibody is indeed RFRP-1 specific (Jorgensen et al., 2014). Lastly, we tested an antibody raised in rat against white crowned sparrow GnIH (provided by Lance Kriegsfeld). Despite recognizing both RFRP-1 and RFRP-3, this antibody more potently binds RFRP-3 peptides than RFRP-1, but further tests are necessary to confirm this observation.

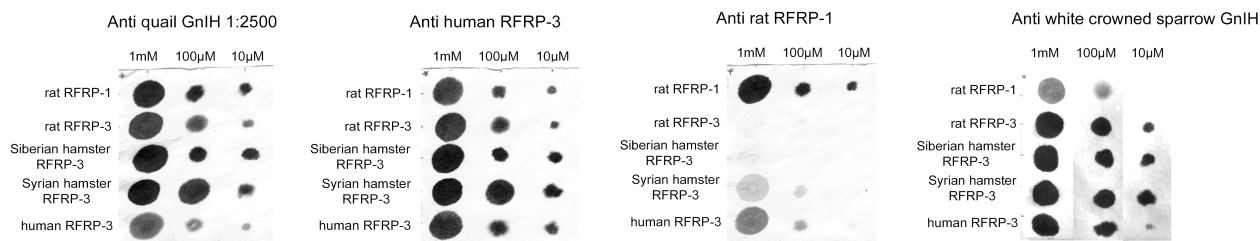


Figure 32. Characterization of different RFRP antiserum using the dot-blot technology. Rat RFRP-1 and different RFRP-3 peptides in concentrations of 10µM, 100 µM and 1 mM (with highest concentrations to the left) are spotted on the blot and immunoreacted similar to tissue sections, as described in the method section. Henningsen *et al.*, unpublished.

Although, dot blots are not final proof of antibody specificity, our findings highlight the importance of evaluating the specificity of antibodies in the tissue of interest, and to keep in mind potential species-specific differences in the binding properties of an antibody. Moreover, it underlines the need of antibodies specific for one and not the other RFRP variant, in order to better understand the properties of RFRP expressing neurons. Jens D. Mikkelsen and colleagues have succeeded in addressing some of these challenges with the antisera directed against rat RFRP-1, which is proven to specifically recognize RFRP-1. This antibody has been used successfully to characterize RFRP-1 expression and distribution during development and in the adult rat (Jorgensen et al., 2014), however, a similar detailed description of RFRP-3 is missing due to the lack of RFRP-3 specific antisera. The distinction between RFRP-1 and RFRP-3 is highly relevant, because the two peptides are very different in terms of potency and effects and therefore, it seems very likely that there are differences not only in the expression of the two peptides, but also in sites of action. To fully understand the pathway through which RFRP-3 specifically regulates the reproductive axis, future investigations should aim at generating new tools or antibodies, to overcome the challenges of distinguishing RFRP-1 and -3 expression and signaling in the brain.

SPECIES AND SEX-SPECIFIC DIFFERENCES IN RFRP AND ITS EFFECTS

SPECIES DIFFERENCES IN RFRP AND ITS EFFECTS

Until recently, only an inhibitory effect had been reported for RFRP in regulating reproductive activity, which is the case for several species including birds, rats, mice and sheep (Tsutsui et al., 2000)(Pineda et al., 2010a)(Murakami et al., 2008)(Kriegsfeld et al., 2006)(Clarke et al., 2012)(Leon et al., 2014)(Anderson et al., 2009). Characterization of the first mouse line with a genetically impaired

GPR147 receptor revealed increased litter size, increased gonadotropin levels, although dependent on sex and developmental stage, and increased ARC kisspeptin expression, which overall fits well with the inhibiting effect of RFRP-3 reported in mice (Leon and Tena-Sempere, 2015). Worth mentioning is however, that the effects are moderate and that the mice experience a normal pubertal development and are fertile. The perception of a conserved inhibitory effect of RFRP-3 among species is challenged by recent findings in male Syrian and Siberian hamsters, where central administration of RFRP-3, stimulates the HPG axis in LP and SP adapted male Syrian hamsters, although to a lesser extend in SP animals, as well as in SP-adapted male Siberian hamsters (Ancel et al., 2012)(Ubuka et al., 2012). The effect of RFRP-3 in sheep is questioned, by recent extensive work from Beltramo and colleagues, who show that RFRP-3 has no effect on LH secretion in ewe, no matter the mode of application (Decourt et al., 2016a). How come there are opposite effects of RFRP between species remains unanswered. The fact that the stimulatory effect of RFRP-3 only has been observed in seasonal hamster species, might reflect a fundamental difference in how reproduction is regulated between seasonal and non-seasonal species. To support this hypothesis, we here demonstrate that RFRP-3, amongst other functions, relays the melatonin-dependent seasonal input onto the reproductive axis in female Syrian hamsters, similar to our previous findings in male Syrian hamsters (Ancel et al., 2012). Taken together, these findings clearly show that RFRP-3 plays a specific role in regulating seasonal reproduction, which could explain discrepancies in the effects between seasonal and non-seasonal species. However, additional studies are required in order to find out whether RFRP-3 plays a similar important role in all seasonal mammals or whether it is specific to these hamster species. Furthermore, future work should aim at investigating whether the different roles of RFRP-3 are coupled to different downstream targets of RFRP-3 and therefore future studies should aim at identifying and comparing the neuronal targets, through which RFRP-3 exerts its effects, from one species to another.

SEX-SPECIFIC DIFFERENCES IN RFRP IN THE SYRIAN HAMSTER

Until now, the effect of RFRP administration on the female reproductive axis has mainly been investigated in OVX animals, in order to bypass the potential interference of varying levels of circulating sex steroids (Pineda et al., 2010a)(Murakami et al., 2008)(Kriegsfeld et al., 2006)(Clarke et al., 2012). In OVX female Syrian hamsters, avian GnIH (ortholog to RFRP-1) inhibits LH release (Kriegsfeld et al., 2006), and herein we describe how central injections of Syrian hamster RFRP-3, similarly inhibits LH release in intact female Syrian hamsters. These findings further strengthen the evidence for sex-specific differences in the effects of RFRP-3 in the Syrian hamster, stimulating reproductive activity in males, while inhibiting in females (Ancel et al., 2012)(Kriegsfeld et al., 2006). A relevant matter is to unveil how RFRP peptides can have opposite sex- and species-dependent effects and the answer might very well lie in different downstream signaling of RFRP peptides. As previously mentioned, GPR147 is capable of binding both stimulatory and inhibitory G proteins *in vitro* (Gouarderes et al., 2007)(Bonini et al., 2000), although in CHO cells, activation of the receptors inhibit

forskolin-stimulated cAMP accumulation. Nevertheless, it remains to be established if there could be fundamental species or sex-specific differences in the downstream signaling cascades after activation of GPR147, in physiological surroundings. Alternatively, it is likely that the cellular response to RFRP is conserved among species, but mediated via different targets or intermediate neurons, and in this case one can speculate whether the observed stimulatory effect of RFRP-3 in male Syrian and Siberian hamsters, could arise through inhibition of inhibitory interneurons, thus resulting in a stimulatory outcome. Indeed, acute administration of RFRP-3 in male Syrian hamsters induces c-Fos expression in a population of neurons in the ARC, which was found not to co-localize with kisspeptin positive labeled cells (Ancel et al., 2012). Identifying the phenotype of these neurons should be addressed in future studies, and furthermore, it would be interesting to investigate, if central administration of RFRP-3 in another sex or species evokes a similar response in c-Fos expression in this area of the brain.

In our work, we moreover find strong sex-specific differences in the RFRP system, manifested by a markedly higher expression in the number of RFRP-ir neurons and in the intensity of the RFRP-ir neuronal labeling in sexually active females, as compared to males. Similar sex differences have been found in the expression of RFRP-1 in rats, with a higher expression in females than in males (Jorgensen et al., 2014), and might indicate that the RFRP system is more important in females than in males.

The first obvious hypothesis to test would be to suggest that these differences could be driven by gonadal steroid feedback. Actually, the effect of sex steroids on RFRP neurons have been subject to several studies, however, the results are not consistent. Some studies describe that a portion of RFRP neurons co-express the estrogen receptor ER α in female mice and Syrian hamsters, whereas no androgen receptor co-expression is found in males (Kriegsfeld et al., 2006)(Molnar et al., 2011)(Poling et al., 2012). As for the effects of gonadal steroid treatment, E2 induces c-Fos expression in RFRP neurons in OVX female Syrian hamsters, whereas in males, treatment with testosterone has no effect on RFRP expression (Kriegsfeld et al., 2006)(Mason et al., 2010)(Revel et al., 2008). In rats, one study found that E2 stimulates *Rfrp* expression in OVX females, and that testosterone has no effect on *Rfrp* expression in GNX males (Iwasa et al., 2012), whereas one other study found no effect of E2 on RFRP expression in OVX females (Quennell et al., 2010). Lastly, in mice, both E2 and testosterone have been shown to decrease *Rfrp* expression in adult GDX mice (Molnar et al., 2011)(Poling et al., 2012). In terms of the impact of gonadectomy, no differences are found in the expression of RFRP after GDX in male and female rats (Iwasa et al., 2012)(Quennell et al., 2010), or after castration in male Syrian hamsters (Revel et al., 2008). Despite the indications of a direct effect of E2 on RFRP neurons in female Syrian hamsters, we herein show that removal of the sex steroid input by OVX, has no effect on levels of RFRP mRNA measured in the brains of female Syrian hamsters, kept in both LP and SP. Therefore, we can exclude that a positive regulation from E2 in females leads to sex-differences in RFRP expression in the Syrian hamster.

Another possibility could be that sex-differences in the RFRP system in adulthood, results from developmental differences between the two sexes. Looking at the developmental profile of RFRP neurons, it seems that the adult level of RFRP expression in males and females, is primed around the peri-pubertal period (Jorgensen et al., 2014)(Iwasa et al., 2012)(Quennell et al., 2010). In both sexes, RFRP expression increases during development, until puberty, where the level of expression seems to drop in males, but not in females. Therefore, a likely explanation for the sex-differences observed in adult Syrian hamsters is, that the RFRP system is sexually differentiated around puberty to obtain the higher levels of RFRP in females as compared to males, rather than be being differently regulated in adulthood. Another important point to mention is that the sex-differences might arise from differences in RFRP-1 and RFRP-3 levels between males and females. Indeed, Jorgensen *et al.*, (2014) finds a higher expression of RFRP-1 in female than in male rats, but unfortunately, in our study, the use of the non-selective avian GnIH-derived antibody, prevents us from exploring whether the sex-differences observed in the Syrian hamster are caused by a higher expression of both peptides or just e.g. RFRP-1. Thus, future studies should aim at providing a more detailed characterization of the RFRP system, and how it is regulated around puberty, as well as investigating if these sex-differences result from a higher expression of both RFRP-1 and -3 in females than in males, or i.e. only RFRP-1.

In the current study, we also observe a strong sex-dependent difference in levels of GPR147 mRNA, with significantly higher levels of GPR147 mRNA expression in females than in males. The difference was consistent in all areas investigated, independent of photoperiod, and further suggests that the RFRP system plays a more important role in females than in males. At this point, it is not known whether this difference is a direct consequence of sex-differences in the amount of RFRP peptides, which bind to and signals via the receptor, or whether differences could arise from a differential regulation of the GPR147 gene by e.g. gonadal steroids, an aspect that needs to be examined in further detail.

RFRP AND THE FEMALE CYCLE

The reproductive physiology in females is obviously more complicated than that in males, as females undergo regular cycles of sexual activation and inactivation (menstrual cycle or estrous cycle), throughout their lifespan. In addition, sexual activity is inhibited during gestation and lactation, underlining the need for more complex regulatory mechanisms in females than in males (de la Iglesia and Schwartz, 2006)(Fitzgerald and Zucker, 1976)(Williams and Kriegsfeld, 2012). In this study, we have examined changes in the hypothalamic RFRP system along the estrous cycle as well as the gonadotrophic effect of RFRP-3, at different reproductive stages in a female seasonal rodent, the Syrian hamster. Gibson *et al.*, have previously shown, that RFRP-ir cell numbers and their activational state is decreased at the time of the LH-surge on the day of proestrus, as compared to other time points on the day of proestrus and to diestrus (Gibson et al., 2008). In rats and ewes, RFRP expression

has similarly been shown to be reduced during the pre-ovulatory period (Clarke et al., 2012)(Jorgensen et al., 2014). Our present findings confirm that RFRP neuronal activation is specifically decreased in the afternoon of proestrus, concomitantly to the strong increase in kisspeptin activation and the surge of LH. Interestingly, by including more time points to our analysis in diestrus, we show that the activation of RFRP is similarly decreased in diestrus, at the same time point as the observed down-regulation in proestrus. A recent study demonstrates, that SCN-derived VIP-ergic terminal projections are found in close apposition to RFRP neurons in female Syrian hamsters, and that central VIP administration markedly suppresses RFRP cellular activity in the afternoon/evening, but not in the morning (Russo et al., 2015). Therefore, it seems legitimate to suggest that it is an SCN-derived VIP-ergic signal, which decreases RFRP activity in the afternoon of diestrus and proestrus. Russo *et al.*, found, however, no co-localization between RFRP positive labeled cells and the VIP receptor, suggesting that the effect could be indirect (Russo et al., 2015). In contrast to Gibson and colleagues (Gibson et al., 2008), we did not see any change in the number of RFRP-ir neurons along the cycle time points, and neither did we see any change in the levels of RFRP mRNA (data not shown). In our, as well as Gibson's study, RFRP neurons were detected using an avian GnIH antiserum and can therefore not explain the diverging results in number of RFRP neurons. Nonetheless, the fact that the lack of changes in number of immunoreacted cells in our study is confirmed with our mRNA level analysis, strengthen our conclusion that the peptide content is not changing in RFRP neurons during proestrus and diestrus, at least in our setup.

The present study is the first to investigate the effect of RFRP-3 at two different stages of the estrous cycle in female rodents kept in physiological conditions. Our data show, that central administration of RFRP-3 significantly decreases the LH-surge peak when injected just before/or in the beginning of the surge, whereas injections of RFRP-3 in the morning or mid-day has no effect on the afternoon peak of LH. Considering the inhibitory effect of RFRP-3, along with the endogenous decrease in RFRP activation observed in the afternoon of diestrus and proestrus, it appears that the RFRP neuronal network is exerting a tonic inhibition on the HPG axis in LP-adapted female Syrian hamsters, which is specifically lifted through a SCN-generated circadian signal, allowing an intact peak of LH to drive ovulation. In our attempt to study the underlying targets through which this inhibitory effect of RFRP-3 is mediated, we looked at the expression and activation of AVPV/MPN kisspeptin neurons and GnRH neurons, respectively. We found no change in the number or activation of AVPV/MPN kisspeptin neurons, indicating that the inhibitory effect of RFRP-3 on the LH-surge is not mediated through this population of kisspeptin neurons. GnRH neurons in and around the OVLT express c-Fos around the time of the LH-surge (Herde et al., 2011), and because RFRP-3 has no hypophysiotrophic effect in the Syrian hamster (Ancel et al., 2012), we would expect to see the RFRP-3 induced inhibition in LH through a decrease in GnRH activation. Although, we observe a tendency towards a decrease in GnRH activation, with approximately 10% lower activation after injections of RFRP-3, the differences are not significant ($P = 0,0539$). However, these results does not finally exclude that the effect of RFRP-3 is

mediated via GnRH neurons. Electrophysiological investigations of the activity pattern of GnRH neurons show, that RFRP-3 inhibits a subset (app. 40 %) of GnRH neurons in mice, while at the same time stimulating another small fraction (app. 12%) (Ducret et al., 2009). In line with these observations, RFRP-3 causes a decrease in LH release, but not a complete inhibition of the surge and therefore, it might be difficult to detect a significant RFRP-3-induced decrease, when analyzing the overall population of c-Fos expressing GnRH neurons. Despite the fact, that we did not see any effect on preoptic kisspeptin expression, we noticed a strong induction in c-Fos positive neurons in the preoptic area, more specifically in the MPN, that were not kisspeptin positive neurons, as presented in figure 33. Unfortunately, the identity of these neurons has not been clarified so far.

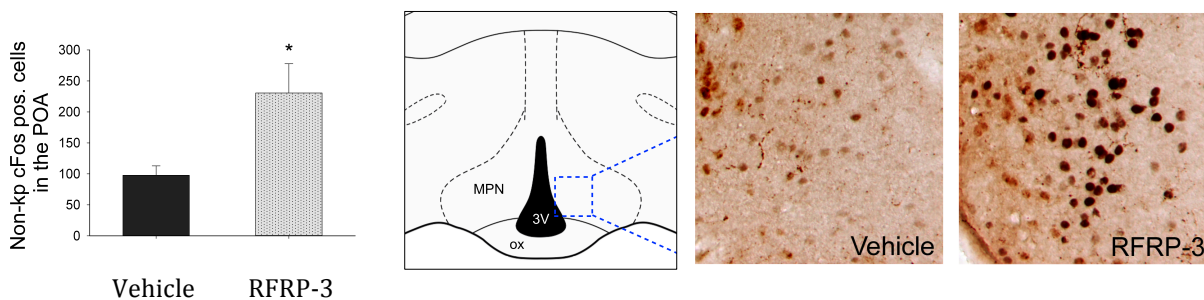


Figure 33. RFRP-3 induces c-Fos activation in non-kisspeptinergic cells in the preoptic area. Acute administration of RFRP-3 (0.75 nM) in female Syrian hamsters significantly increases the number of c-Fos cells in the MPN (left panel), in cells that are not co-localized with kisspeptin (right panel, kisspeptin; brown staining, c-Fos; dark nuclear staining). Henningsen *et al.*, unpublished.

Herbison and colleagues have recently investigated the involvement of GABA- and glutamatergic neurons in the estrogen feedback in female mice, by specifically deleting $ER\alpha$ receptors in GABA and glutamatergic neurons (Cheong et al., 2015). While both strains are infertile, they found important differences in deficits; deletion of $ER\alpha$ in GABA-ergic neurons leads to an abnormal estrous cycle and complete failure in the positive negative feedback of E2, while these mice experience a normal puberty onset and negative feedback of E2. On the other hand, deletion of $ER\alpha$ in glutamatergic neurons, leads to a wider range of deficits including impaired E2 negative and positive feedback and advanced puberty onset. Importantly, around 70% of the $ER\alpha$ expressing glutamatergic neurons were found to be co-localized with kisspeptin neurons in the ARC, which can help to explain the glutamate-specific impairment in puberty onset. Taken together, it seems that $ER\alpha$ expressing GABA-ergic neurons are essential for the positive feedback of E2 that drives the pre-ovulatory LH-surge. Interestingly, deleting $ER\alpha$ in these GABA-ergic neurons causes a dramatic decrease in the $ER\alpha$ expression in the AVPV/MPN as shown in figure 34.

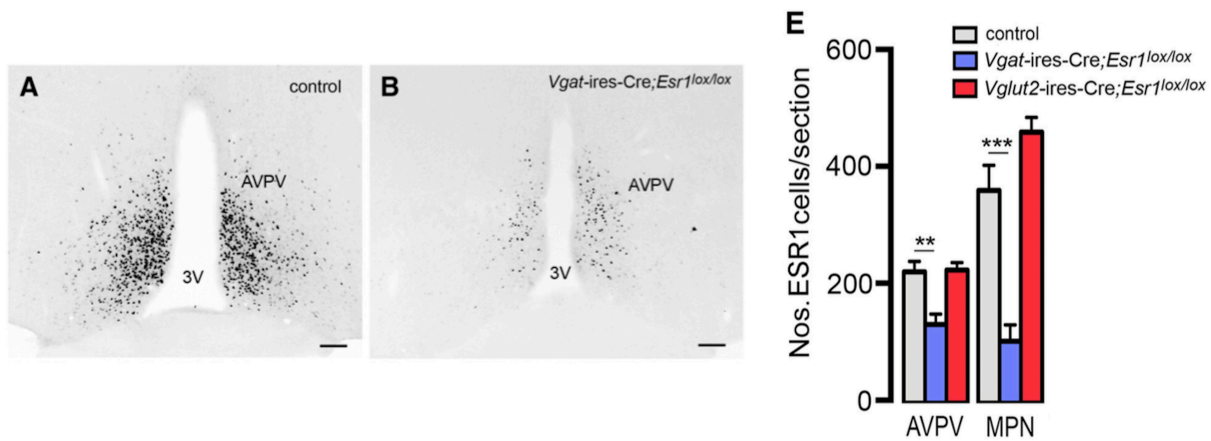


Figure 34. Deletion of ER α in GABA- and glutamatergic neurons in the mouse. ER α (Esr1) immunoreactive nuclei in the AVPV of a female A) control and B) mutant Vgat-ires-Cre;Esr1^{lox/lox} mouse. E) Histograms represent the mean number of ER α positive cells per section, in control or mutant mice, in areas of the AVPV and the MPN. Cheong *et al.*, 2015.

Comparing the pattern of expression demonstrated by Cheong *et al.*, with the increased c-Fos expression we observed in this region after injecting RFRP-3, as demonstrated above (figure 33), it is tempting to hypothesize, that these GABA-ergic neurons could be the target of RFRP-3, however, such a statement requires additional investigations. Nevertheless, in future work it would be interesting to test this hypothesis by examining the identity of these neurons, stimulated by RFRP-3, and in addition, it would be interesting to investigate whether injections of RFRP-3 in male Syrian hamsters evoke a similar response in c-Fos activation as observed here in females.

RFRP AND THE SEASONAL REGULATION OF REPRODUCTION

SEASONAL VARIATIONS IN THE RFRP SYSTEM

As reported in this work and elsewhere, RFRP neurons are in seasonal animals strongly regulated with season and are submitted to a melatonin-dependent down-regulation in SP (Revel *et al.*, 2008)(Ubuka *et al.*, 2012)(Henningsen *et al.*, 2015)(Janati *et al.*, 2013)(Ancel *et al.*, 2012)(Saenz de Miera *et al.*, 2014)(Smith *et al.*, 2008)(Simonneaux and Ancel, 2012). Interestingly, we here show that the SP-induced decrease in RFRP expression is stronger in females, in which both the number and grey level intensity of RFRP-ir labeling is significantly reduced in SP, as compared to males, where only the number of RFRP-ir neurons decreases. In SP conditions, we found a comparable low level of RFRP-ir expression between males and females, indicating that sex-differences, in terms of the expression of RFRP, is relevant only during the breeding season. The marked down-regulation of RFRP expression observed in seasonal mammals, independently of sex, species and breeding behavior, provides evidence for both distinct and conserved roles of this peptide in the integration of the photoperiodic melatonin signal, which is now known to be mediated via a TSH-dependent regulation of thyroid

hormone locally in the MBH (Simonneaux and Ancel, 2012)(Klosen et al., 2013)(Hazlerigg and Simonneaux, 2015).

In this work, we describe that the RFRP receptor, GPR147, is also regulated by photoperiod and is down-regulated in SP conditions. Strikingly, this down-regulation was much stronger and consistent in females than in males, in which only levels in the Hb were significantly different between SP and LP (Henningsen et al., 2015). In terms of RFRP-ir fiber projections, we found no photoperiodic or sex-specific variations in the number of fibers projecting to the OVLT or ARC, whereas in the AVPV/MPN, the amount of RFRP-ir fibers is specifically and significantly increased only in females, which are exposed to SP. The fact that this increase occurs in SP, concomitantly with RFRP neuronal expression being at its lowest, raises some interesting questions, however, at this point, it is unclear whether the increase is explained by an increased RFRP release, or if the increase reflects a decreased release and thereby accumulation of peptide in the fiber terminals, and further studies are required in order to establish the functional relevance of this female specific SP-induced increase in the number of RFRP fibers projecting to the AVPV/MPN.

In Siberian hamsters and sheep, the amount of GnRH neurons receiving RFRP-ir fiber contacts is decreased in SP conditions (Clarke et al., 2008)(Ubuka et al., 2012). We have as well investigated RFRP fibers and their appositions to GnRH neurons in Syrian hamsters, and although we see a tendency of a decrease in GnRH-RFRP appositions in SP in both female and male Syrian hamsters, the differences are not significant ($P = 0.188$ for females and $P = 0.407$ for males), (figure 35). We find however, that the percentages of RFRP-ir fibers in close proximity to GnRH neurons range between 25%-37%, which corresponds to what has previously been reported (Kriegsfeld et al., 2006)(Rizwan et al., 2012)(Smith et al., 2008)(Ubuka et al., 2012). The differences in seasonal impact on fiber appositions might arise from differences in technical approaches. For example, we have in this study used a conventional microscopic approach to study RFRP-GnRH interactions, and therefore these data should be considered as potential sites of contacts rather than actual contacts. Despite differences, it is highly relevant to bear in mind the complexity of GnRH neuron morphology that allows integration of synaptic inputs along their entire dendron length (Campbell et al., 2005)(Herde et al., 2013)(Roberts et al., 2008), and therefore the reported findings are not necessarily sufficient to explain how RFRP and GnRH neurons communicate with one another.

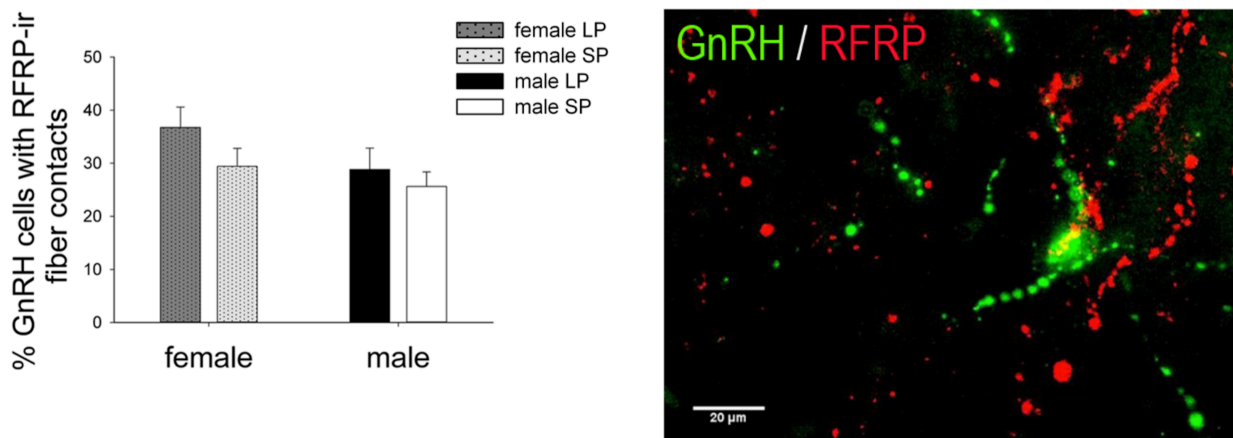


Figure 35. RFRP-ir fibers form close apposition to GnRH neurons in the Syrian hamster. Left panel) Analysis of RFRP-ir fibers in close apposition to GnRH-ir neurons in the POA of female and male Syrian hamsters kept in LP or SP condition; data represent the mean percentage of GnRH cells in close proximity with one or more RFRP-ir fiber $\pm$ SEM ($n=7$ /group). Right panel: merged fluorescence microscopic image showing a GnRH-ir neuron (green) in close proximity to a RFRP-ir fiber (red); scale bar: 20 μ m. Henningsen *et al.*, unpublished.

THE EFFECT OF RFRP ON SEASONAL REPRODUCTION

Our group was the first to show that RFRP-3 stimulates LH release in male Syrian hamsters, regardless of the photoperiodic condition (Ancel *et al.*, 2012). Intriguingly, chronic infusion of RFRP-3 in SP-adapted male Syrian hamsters, which under normal conditions have an endogenous low RFRP expression, even reactivates the reproductive axis despite being kept in winter-like photoinhibitory conditions. Here, we show that acute central administration of RFRP-3 in SP-adapted female Syrian hamsters has no measurable effect on LH plasma levels, which is in sharp contrast to the very potent stimulatory effect of kisspeptin-10, as seen in figure 36, as well as to findings obtained in male Syrian hamsters.

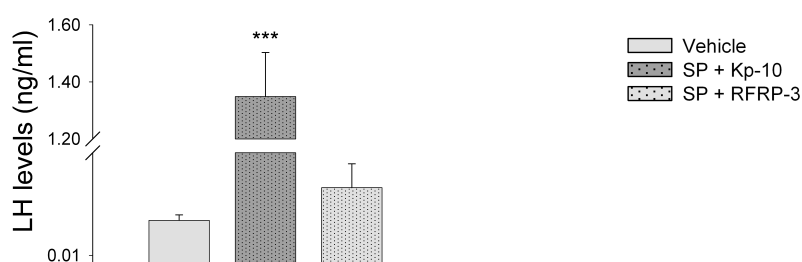


Figure 36. Acute effects of central administration of RFRP-3 in female Syrian hamsters kept in SP. The effect of RFRP-3 on LH levels in SP-adapted female hamsters evaluated within 30 minutes of administration. Icv injection of RFRP-3 (0.75 nM) has no effect on the basal levels of LH, whereas kisspeptin-10 (700 ng), significantly stimulates LH release from the pituitary, as compared to vehicle. Henningsen *et al.*, unpublished.

Our present data, however, reveal so far uncharacterized effects of chronic administration of RFRP-3 in the female Syrian hamsters. Indeed, we show that chronic infusion of RFRP-3, leads to a reactivation of the reproductive axis in female Syrian hamsters maintained in photoinhibitory SP conditions, to

levels comparable to the ones obtained in LP animals, manifested by an increase in LH and E2 levels, gonadal weight, as well as in kisspeptin-ir expression in both the AVPV/MPN and ARC. Considering the potent stimulatory effect of kisspeptin on the reproductive axis, increased gonadal activity is likely a result of the increase in kisspeptin levels, but since the ARC kisspeptin population is negatively regulated by sex steroid feedback, we can rule out the possibility, that the increased kisspeptin-ir expression in the ARC occurs as a result of gonadal reactivation. Therefore, we propose that the stimulating effect of RFRP-3 is mediated via ARC kisspeptin. In AVPV/MPN however, kisspeptin neurons are stimulated by E2 feedback and it is therefore highly likely that the increased immunoreactivity in this region is stimulated by higher ovarian activity and E2 release. In males, levels of ARC *Kiss1* expression are similarly increased after chronic infusion of RFRP-3 in SP, and this is despite the increased level of testicular activity and testosterone, and therefore negative feedback on ARC kisspeptin neurons. Based on these findings, we hypothesize that, at least in this species, RFRP-3 mediates the photoperiod-driven melatonin signal toward ARC kisspeptin neurons, to drive the seasonal control of reproduction. We, and other groups, have previously shown that long-term treatment with kisspeptin, reverses sexual quiescence in both sheep and male hamsters (Revel et al., 2006a)(Ansel et al., 2011a)(Caraty et al., 2007)(Sébert et al., 2010)(Ansel et al., 2011b). In an additional experiment, we evaluated for the first time the effects of central chronic infusion of kisspeptin-10 in SP-adapted female Syrian hamsters, and further compared it to that of RFRP-3 (figure 37). As expected, kisspeptin-10 significantly stimulated gonadal weight in SP-females, as compared to vehicle, but interestingly, it seems that RFRP-3 had a more potent, although not significant different, stimulatory effect on gonadal weight, which further supports a more important role of RFRP-3 in regulating the seasonal control of reproduction.

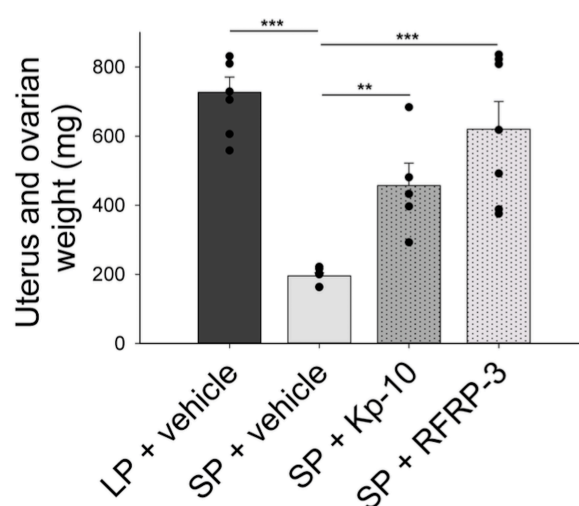


Figure 37. Effects of chronic icv infusion of RFRP-3 and kisspeptin-10 in female Syrian hamsters kept in SP. Female Syrian hamsters ($n = 8$ per group) were placed in either SP conditions for 8 weeks in order to obtain complete regression of reproductive activity, or LP. Animals were implanted with an icv cannula linked to an osmotic minipump (flow rate: $0.15 \mu\text{l}/\text{hour}$) filled with vehicle (aCSF), kisspeptin-10 ($0.25\text{nmol}/\text{hour}$) or hamster RFRP-3 ($0.25\text{nmol}/\text{hour}$) and kept in their respective photoperiod throughout infusion. After 5,5 weeks of treatment the level of reproductive activity was determined by means of uterus and ovarian weight. RFRP-3 and kisspeptin-10 infusion for 5,5 weeks in SP females was sufficient to restore reproductive activity by means of increased gonadal weight, despite SP-photoinhibitory conditions. Henningsen *et al.*, unpublished.

Contrastingly, we here show that continuous administration of RFRP-3 in LP-adapted females causes a significant decrease in LH levels, gonadal weight as well as in the number of kisspeptin-ir neurons in the ARC, thus inhibiting the reproductive axis. These data fit well with the inhibitory effect we observed in LP females following acute administration of RFRP-3. In this case, it could be speculated that the inhibition occurs via ARC kisspeptin neurons, since we only see a decrease in this kisspeptin population after continuous infusion of RFRP-3. The fact that there was no change in AVPV/MPN kisspeptin expression, is likely due to an also unchanged level of circulating E2, and further supports that the inhibiting effect of RFRP-3 in female Syrian hamsters, is not mediated via the AVPV/MPN kisspeptin population.

The acute effect of RFRP-3 in male Siberian hamsters is photoperiod-dependent and stimulates LH release when injected in SP, while inhibiting gonadotropin release in LP (Ubuka et al., 2012). Opposite to Syrian hamsters, the ARC kisspeptin expression in Siberian hamsters is significantly increased during the non-breeding season, as compared to a low expression during the breeding season. These observations indicate that in the two hamster species, RFRP-3 either has opposite sites of action in LP-adapted animals or is integrated differently along the HPG axis, which could also help to explain the opposite regulation of ARC kisspeptin between male Syrian and Siberian hamsters (Simonneaux et al., 2009)(Greives et al., 2007)(Mason et al., 2007). Worth mentioning is that continuous administration of TSH reactivates reproductive activity in both SP-adapted male Syrian and Siberian hamsters and more importantly that this reactivation is mediated via an increase in RFRP expression (Klosen et al., 2013), thus indicating that the species-specific differences in the role of RFRP in regulation of the reproductive axis lies in the downstream signaling of RFRP.

In SP-breeding sheep, RFRP expression is down-regulated in SP, when this species is sexual active (Dardente et al., 2008)(Smith et al., 2008), and further proves that the melatonin-driven inhibition of RFRP expression occurs independently of species, sex, breeding behavior and even discrepancies in the effect of RFRP peptides. This, however, also means that while the melatonin-dependent regulation of RFRP is likely conserved amongst seasonal species, the RFRP signal in the SP-breeding sheep would have to be interpreted or integrated differently than in the LP-breeding Syrian hamsters. Interestingly, kisspeptin expression both in the AVPV/MPN and ARC is significantly down-regulated in SP-adapted Syrian hamsters (Ansel et al., 2010b)(Revel et al., 2006a), whereas in sheep, ARC kisspeptin expression is oppositely up-regulated in SP (Wagner et al., 2008)(Smith et al., 2008)(Chalivoix et al., 2010). These discrepancies indicate that kisspeptin expression in the ARC reflects the breeding state, rather than the seasonal state, of the animal and therefore also must be differently regulated downstream of the melatonin-TSH pathway. Despite the controversies of RFRP's effect in sheep, in some studies, RFRP-3 is shown to inhibit reproductive activity (Clarke et al., 2008)(Clarke et al., 2012)(Smith et al., 2012). Although, more work is required to test this hypothesis, it is possible that the lower expression of kisspeptin in LP-adapted sexually inactive sheep is caused by the high endogenous expression of the

inhibitory RFRP-3. In this case, one could imagine that increasing RFRP concentrations by chronically injecting RFRP-3 in SP-adapted sexual active sheep, which have a normal low endogenous expression of RFRP, could lead to regression of the reproductive axis.

RFRP – PHARMACOLOGICAL TOOLS

RFamide peptides and their receptors are implicated in various functions; however, more detailed knowledge about their exact roles is limited due to the lack of pharmacological tools. In the past few years, development of GPR147 and GPR74 receptor antagonists have provided the first steps towards the possibility of studying in more detail the localization of the receptors, how they are regulated, as well as the intracellular downstream signaling from the receptors. To better understand the role of RFRPs in regulating the HPG axis and to study whether the effects of RFRP-3 on the Syrian hamster gonadotrophic axis are in fact mediated via GPR147, we have investigated the effects of RFRP-3 administration, with or without the presence of three selective and non-selective GPR47 antagonists; RF9, RF313 and RF3286, all developed in the group of Dr. Frédéric Simonin.

RF9 AND ITS ANTAGONISTIC EFFECT OF RFRP-3 SIGNALING?

In 2006, Frédéric Simonin and colleagues discovered a new compound with a potent antagonistic activity and similar binding affinity for GPR147 and GPR74, named RF9. This dipeptide was initially used to block the effects of NPFF on heart rate and blood pressure and was shown to also block opioid-induced hyperalgesia and tolerance in rats (Simonin et al., 2006)(Bourguignon et al., 2006). RF9 has, since its discovery, also been widely used to study RFRP modes of action in the control of reproductive functions. In rats and mice, icv administration of RF9 increases levels of gonadotropins, which was initially thought to result from RF9 antagonizing the inhibitory effect of RFRP-3 (Pineda et al., 2010b). Subsequent studies have, however, shown that RF9 binds KISS1R receptor and agonize the effect of kisspeptin, thus stimulating LH release in a RFRP-independent manner (Liu and Herbison, 2014)(Sahin et al., 2015). A similar stimulating effect of RF9 has been observed also *in vitro* as well as *in vivo* in both wt and GPR147 KO mice, whereas in GPR54 deficient mice, the stimulatory effect is markedly reduced (Min et al., 2015). In male Syrian hamsters, RF9 administration similarly stimulates LH secretion and does not abolish the stimulatory effect of RFRP-3 on gonadotrophin secretion (figure 38). These results further support that RF9 is not a suitable tool for specifically studying the function of RFRP-3.

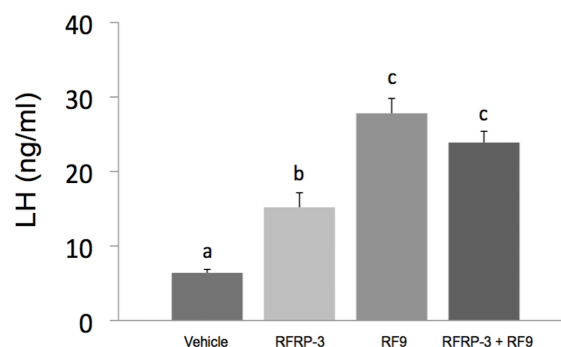


Figure 38. RF9 stimulates LH release in male Syrian hamsters in a RFRP-3 independent manner. Icv injections of RFRP-3 (0.75 nM) induce a significant increase in LH secretion as compared to vehicle. RF9 (8 nM) injected alone, potently stimulates LH secretion to levels significantly higher than those following RFRP-3 administration and when injected simultaneously with RFRP-3 (0.75 nM), RF9 (2 nM) induce an increase in LH secretion comparable to the levels observed with RF9 administration alone. Unpublished.

RF313 AND RF3286 - SELECTIVE ANTAGONISTS FOR THE EFFECTS OF RFRP-3 ON REPRODUCTIVE ACTIVITY?

Recently, Frédéric Simonin and co-workers have discovered two novel GPR147 antagonists and termed them RF313 and RF3286. RF313, despite having highest affinity for GPR147, also has a low antagonistic activity for GPR74, whereas RF3286 has a strong selectivity towards GPR147, almost 4 fold higher than the affinity for GPR74. Both antagonists have nanomolar affinities towards GPR147, which are in the range of affinities found for endogenous GPR147 ligands, and therefore appear as valuable tools to study RFRP-3 modes of action for the control of the gonadotrophic axis.

Interestingly, we find that RF313 administration abolishes the stimulatory effect of RFRP-3 on gonadotrophin secretion in male Syrian hamsters. When injected alone, RF313 has no effect on LH release, whereas concomitantly administration of RFRP-3 and RF313 block the RFRP-3-induced increase in LH released from the pituitary (figure 39), which shows that the effect of RFRP-3 on gonadotrophin secretion in Syrian hamsters is indeed mediated via GPR147. Because it is hypothesized that RFRP neurons specifically suppress the reproductive activity in females other than the time of the LH surge, where this inhibition is lifted, we wondered whether injections of RF313 in the morning of diestrus could stimulate the reproductive axis. However, no such effect of RF313 was found in levels of circulating LH or E2. Considering our present results, which show that changes in RFRP-activity only has a relevant effect around the time of the LH-surge in proestrus and not in diestrus, this likely explains the lack of effect of RF313 in diestrus.

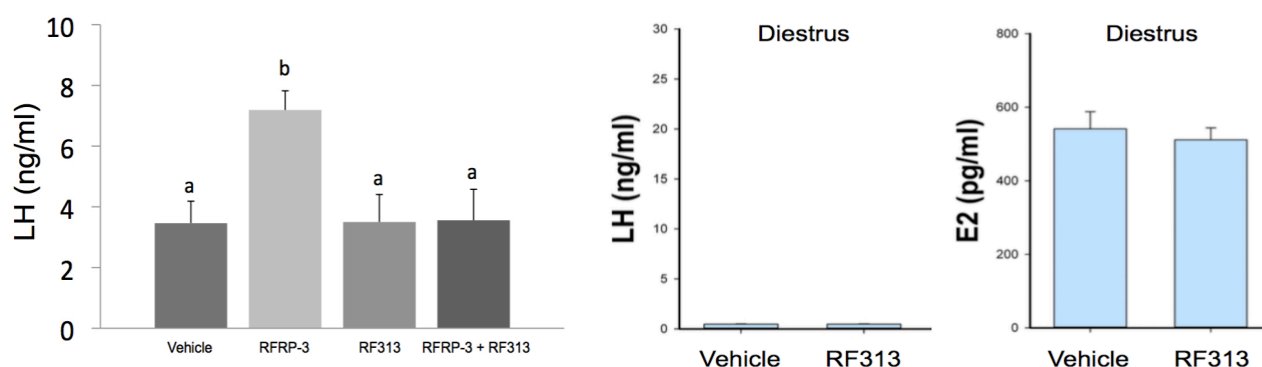


Figure 39. Effects of the GPR147 receptor antagonist, RF313, in the Syrian hamster. Left panel) Icv injections of RFRP-3 (0.75 nM) induce a significant increase in LH secretion as compared to vehicle in the male Syrian hamster. When injected alone, RF313 (10 nM) has no effect on LH secretion, concurrent injections of RF313 (10 nM) and RFRP-3 (0.75 nM) potently blocks the stimulatory effect of RFRP-3. Right panel) In female Syrian hamsters, RF313 (10 nM) has no effect on LH levels or E2 levels when injected in diestrus. Unpublished.

When studying the effect of RF3286, we found that this antagonist also potently blocks the stimulating effect of RFRP-3 on gonadotropin release, in male Syrian hamsters (figure 40). RF3286, injected alone, has no measurable effect on LH levels, although, we observe a tendency towards RF3286 decreasing endogenous levels of circulating LH, as compared to vehicle treated animals. When simultaneously injecting RFRP-3 and RF3286, RF3286 blocks the ability of RFRP-3 to stimulate LH release from the pituitary, and results in very low levels of LH, comparable to those measured after injecting RF3286 alone. These findings further strengthen that the role of RFRP-3 in regulating reproductive activity, at least in male Syrian hamsters, is mediated specifically via GPR147. Interestingly, we observed a tendency towards lower levels of LH in animals treated or co-treated with RF3286, as compared to vehicle. Because these sexually active male hamsters have an endogenous high expression of RFRP, any further decrease of LH after treatment with RF3286, could be explained by RF3286 antagonizing the signaling of endogenous RFRP-3. Further studies are however needed in order to establish if this RF3286-induced decrease in endogenous LH is physiologically relevant, as well as if it arises from regulation of RFRP-3/GPR147 signaling only. Nevertheless, RF3286 seems to be a promising tool to study RFRP-3 modes of action for the (seasonal) control of gonadotropin secretion. To better understand the specific roles of RFRP-3 and the potential of RF3286 to antagonize these effects, it would be interesting to investigate if prolonged treatment with RF3286, hence blocking endogenous changes in RFRP-3 signaling, disrupts synchronization of reproductive activity with photoperiod in a seasonal model, and if treatment with RF3286 in female Syrian hamsters, has any effect on the RFRP-3-induced decrease in LH released at the surge that we report here.

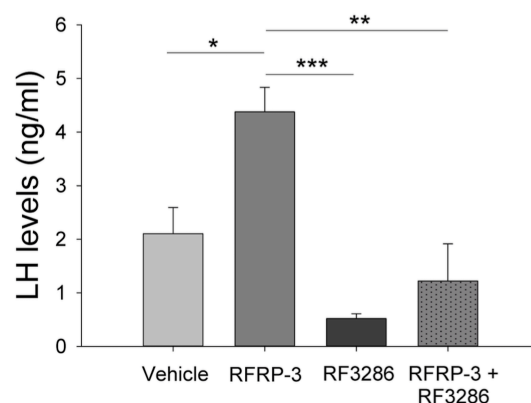


Figure 40. Effects of the GPR147 receptor antagonist, RF3286, in the male Syrian hamster. Icv injections of RFRP-3 (0.75 nM) induce a significant increase in LH secretion as compared to vehicle in the male Syrian hamster. RF3286 (10 nM), injected alone, has no significant effect on LH secretion, whereas concurrent injections of RF313 (10 nM) and RFRP-3 (0.75 nM), completely blocks the stimulatory effect of RFRP-3. Unpublished.

OTHER ROLES OF RFRP

As proven from the extensive work with GPR147 and GPR74 receptor antagonists and knockout models, RFamide peptides and their receptors are implicated in various pathways involved in nociception, stress and metabolic inputs. Our neuroanatomical findings indeed support the roles of RFRP in various structures that have been functionally related to e.g. anxiety, stress, sexual behavior (PVN, BST and Hb), metabolism (ARC and VMH) and circadian activity (PVT, SCN). Kirby *et al.* (2009) have shown that glucocorticoids receptors are present in RFRP neurons and hypothesize that RFRP neurons contribute to hypothalamic suppression of reproductive function in response to stress (Kirby *et al.*, 2009). Interestingly, GPR147 KO-mice show an impaired decrease in LH release from the pituitary in response to metabolic stress, and further indicates that RFRP neurons might mediate the signal of (metabolic) stress onto the reproductive axis (Leon *et al.*, 2014), as will be discussed in more detail below.

RFRP AND METABOLIC EFFECTS

RFRP has been shown to regulate food intake and, despite some contradictions, RFRP seems to have a predominant orexigenic effect. Indeed, central administration of RFRP-3 has been shown to increase food intake in rats and sheep (Johnson *et al.*, 2007), with a concomitant increase in the activity of orexigenic neurons (Clarke *et al.*, 2012). In mice, RFRP-3 and GnIH have been shown to directly inhibit the firing rate of anorexigenic POMC neurons, which fits with the previously described stimulatory effect of RFRP-3 on food intake, but paradoxically the firing rate of the orexigenic NPY neurons were also inhibited (Fu and van den Pol, 2010)(Jacobi *et al.*, 2013). Worth mentioning is that, in contrast to RFRP-3, RFRP-1 decreases food intake when specifically injected into the amygdala (Kovacs *et al.*, 2014). Here, we have measured the food intake in female Syrian hamsters in the hours after

administration of RFRP-3, and show that RFRP-3, as compared to vehicle, causes a significantly increase in food intake (figure 41).

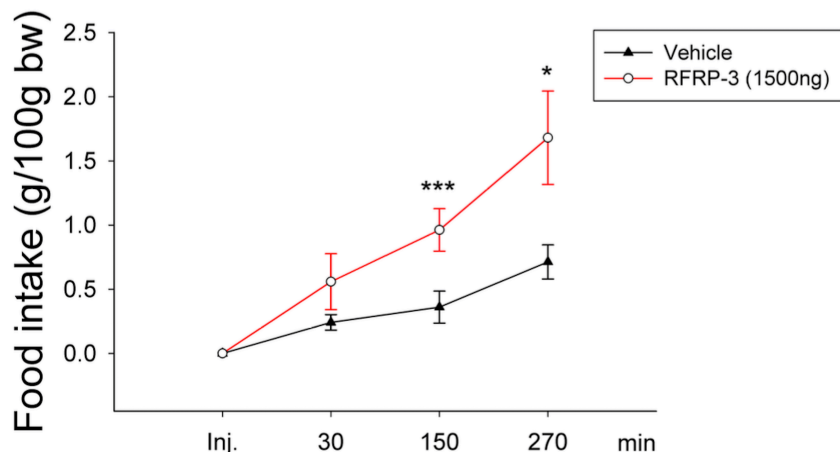


Figure 41. Acute injections of RFRP-3 increase food intake in female Syrian hamsters. Icv injections of RFRP-3 (0.75 nM) significantly increase food intake (measured in g/100g bodyweight) as compared to vehicle when evaluated 150 and 270 minutes post injection in female Syrian hamsters. Henningsen *et al.*, unpublished.

We furthermore examined the feeding behavior and body weight in female Syrian hamsters during chronic treatment with RFRP-3 (figure 42). Here, we found no differences in neither food intake nor bodyweight between RFRP-3- and vehicle-treated animals.

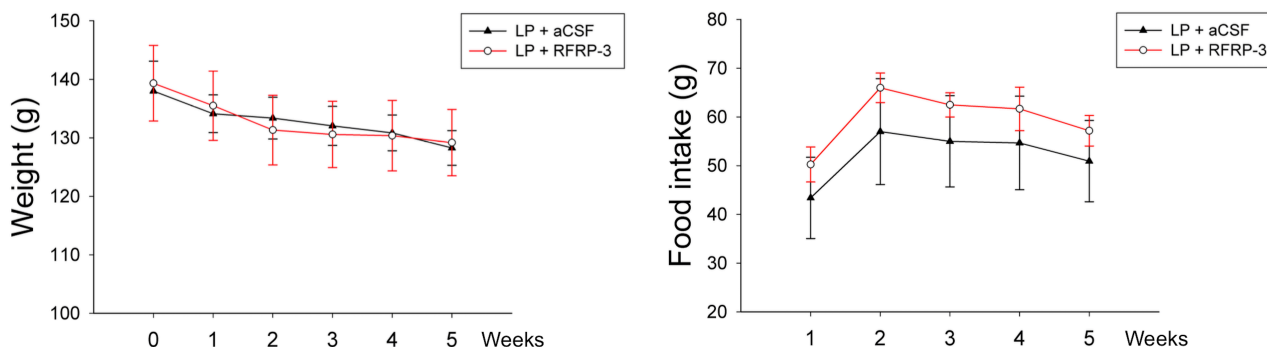


Figure 42. RFRP-3 has no long-term effects on food intake or bodyweight in LP-adapted female Syrian hamster. Female Syrian hamsters (n = 8 per group) were implanted with an icv cannula connected to an osmotic minipump (flow rate: 0.15 μ l/hour) filled with vehicle (aCSF) or hamster RFRP-3 (0.25nmol/hour). Bodyweight (left panel) and food consumption (right panel) was measured once per week during an infusion time of app. 5,5 weeks. Henningsen *et al.*, unpublished.

It is well established that there is a close relationship between energy balance and the reproductive axis. Reproduction is highly energy demanding and therefore, in conditions of negative energy balance, the reproductive axis is suppressed. It has been suggested that RFRP neurons are the missing link that bind the two axes together, but that they primarily play a role in cases of extreme metabolic conditions, adjusting reproduction accordingly (Clarke et al., 2012), which fits well with the lack of long-term effects of RFRP-3, observed here. To further strengthen this hypothesis, studies in GPR147

deficient mice show an impaired down-regulation of reproductive activity after metabolic stress in terms of both fasting and high fat dieting, as compared to wt mice that experiences a significant decrease in LH release (Leon et al., 2014)(Leon and Tena-Sempere, 2015). During other highly energy demanding reproductive events, such as lactation, reproductive activity, as well as response to stressful stimuli, is similarly repressed. In lactating rats, suckling, besides stimulating the release of prolactin, also stimulates RFRP mRNA expression (Noroozi et al., 2015), and therefore the authors suggest that RFRP neurons might be involved in the prolactin-mediated suppression of the reproductive axis. Despite the need for investigating the underlying mechanisms, through which RFRP might be involved in such events, these findings strongly demonstrates the link between energy homeostasis and the HPG axis and point towards RFRP neurons as a possible candidate for this interaction.

The many recent advances made to understand the roles of RFRP, have unveiled that RFRP neurons are implicated in several neuroendocrine responses to internal as well as external cues. Therefore, it seems likely to propose that RFRP neurons might collect all information regarding e.g. season, metabolic status and stress, and properly distribute this information onto i.e. the reproductive axis.

CONCLUDING REMARKS AND PERSPECTIVES

The data presented in this thesis have elucidated important aspects of the role of RFRP neurons in regulating reproductive activity, but also raise many new questions to be answered in future studies. Our findings so far strongly suggest that RFRP neurons play a critical role in female reproduction in regards to the timing of both daily and seasonal synchronization of their reproductive activity and at the same time describes the underlying mechanism on how melatonin acts on the HPG-axis. Over the past nearly two decades RF-amide related peptides have been extensively studied for their putative involvement in the regulation of the reproductive axis (Tsutsui et al., 2015) is still widely referred to as GnIH, named after its inhibitory effect in avian species, despite its now well-documented stimulatory effect in Siberian and Syrian hamster species (Ancel et al., 2012)(Ubuka et al., 2012) and in the present work.

The present study is the first to report insights into the photoperiodic regulation of the RFRP system in a comparative study between female and male Syrian hamsters. We show that RFRP neurons as well as levels of GPR147 mRNA are significantly higher in the sexually active LP-adapted females as compared to males. Interestingly, both RFRP-ir expression and GPR147 mRNA levels are regulated by the seasonal input and RFRP-ir expression is significantly decreased in SP in both sexes, whereas GPR147 mRNA levels are strongly decreased in SP only in females. Importantly, the number of RFRP-ir fibers in the MPN/AVPV is significantly increased only in female Syrian hamsters adapted to SP as compared to LP. All together these findings reveal strong sex-dependent differences in the RFRP system in the Syrian hamster. We moreover show that RFRP-3 has opposite effects on reproductive activity in female and male Syrian hamsters, and stimulates LH release in males, while inhibiting it in females. The effect in females is, however, dependent on the stage of the estrous cycle. We show that RFRP neuronal activity is specifically down-regulated in the afternoon of diestrus and proestrus, which is correlated with kisspeptin activation, as well as the LH-surge in proestrus, and that injections of RFRP-3, just before or in the beginning of the surge, causes a moderate but significant decrease in levels of LH released at the surge. We furthermore describe a critical role of RFRP-3 in mediating the melatonin-dependent seasonal input onto the reproductive axis in female Syrian hamsters. Based on these findings we suggest that this feature could be a conserved role of RFRP amongst seasonal species, although the specific mechanisms of how the seasonal RFRP output is integrated on the reproductive axis might vary from one species to another. Taken together, we here unveil that RFRP neurons play multiple roles in the regulation of the female reproductive axis, by being an important regulator of cyclic events controlling the pre-ovulatory surge in LH, and at the same time being a key component in the melatonin-dependent synchronization of reproductive activity with season.

Altogether, the present data show that the involvement of the RFRP network in the regulation of reproductive functions is far more complicated than initially expected. Based on our findings, we can

conclude that the critical role played by RFRP in the seasonal regulation of male reproduction is conserved between sexes in the Syrian hamster. Following our findings of significant sex differences in the neuronal RFRP system, we show that in Syrian hamsters, RFRP neurons indeed plays distinct roles, specific to the female, since they are involved in both daily and seasonal regulation of reproductive activity. For future experiments it will be essential to keep in mind the sex-specific differences in the effects of RFRP-3, and that there might be fundamental differences in the RFRP system between non-seasonal and seasonal species, in which there might be additional photoperiod-dependent differences in the effects of RFRP-3. Future studies should moreover aim at distinguishing RFRP-1 and RFRP-3 signaling, as well as at investigating whether RFRP peptides could act through another G-protein coupled receptor, GPR74 (NPFF2), known to interact with RFRP peptides although with lower affinity than GPR147. Finally, given the many reported effects of RFRP in regulating e.g. metabolic, HPA and HPG pathways, it is relevant to propose that RFRP neurons might indeed be a relay, acting as a homeostatic switch between the many neuroendocrine functions of the hypothalamus.

CONCLUSION ET PERSPECTIVES (FRANÇAIS)

Les données présentées dans cette thèse ont permis d'élucider des aspects importants du rôle des neurones à RFRP dans la régulation de l'activité de la reproduction, mais soulèvent également de nouvelles questions. Jusqu'à présent nos résultats suggèrent fortement que les neurones à RFRP jouent un rôle essentiel dans la reproduction des mammifères femelles en ce qui concerne la synchronisation journalière et saisonnière de leur activité de reproduction. Au cours des deux dernières décennies les peptides RFRP ont été largement étudiés pour leur implication dans la régulation de l'axe de la reproduction (Tsutsui et al., 2015). Le peptide est encore largement dénommé GnIH, d'après son effet inhibiteur chez les espèces aviaires, en dépit de son effet stimulateur maintenant bien documenté chez les espèces de hamster de Sibérie et Syriens (Ancel et al., 2012 ; Ubuka et al., 2012 ; Henningsen et al., soumis pour publication).

Les travaux de cette thèse sont les premiers à rapporter la une étude comparative de la régulation photopériodique du système RFRP entre les hamsters Syriens femelles et mâles. Nous montrons que les neurones à RFRP, ainsi que les niveaux d'ARNm GPR147 sont significativement plus élevés chez les femelles sexuellement actives par rapport aux mâles. De façon intéressante, à la fois l'expression du peptide RFRP et de son récepteur GPR147 sont régulés par les saisons ainsi, RFRP-ir est significativement diminuée en SP chez les deux sexes, alors que les niveaux d'ARNm GPR147 ne sont fortement diminués en SP que chez les femelles. De plus, le nombre de fibres RFRP-ir dans le AVPV est significativement augmenté seulement chez les hamsters Syriens femelles adaptées à la SP par rapport à LP. Ensemble, ces résultats révèlent de fortes différences liées au sexe du système RFRP du hamster Syrien. Nous montrons par ailleurs que le RFRP-3 a des effets opposés sur l'activité de reproduction chez les hamsters Syriens femelles et mâles, il stimule la libération de LH chez les mâles, et l'inhibe chez les femelles. Cependant, l'effet chez les femelles dépend du stade du cycle œstral. Nous montrons que l'activité neuronale RFRP est spécifiquement inhibée dans l'après-midi du diestrus et proestrus, ce qui est en corrélation avec l'activation des neurones à kisspeptine et l'arrivée du pic pré-ovulatoire de LH l'après-midi du proestrus. Nous montrons également que les injections de RFRP-3 juste avant ou au début du pic de LH, provoque une diminution modérée mais significative des niveaux de LH. L'activité des neurones à RFRP semble donc être contrôlée selon un rythme circadien qui pourrait participer en synergie avec le rétrocontrôle des stéroïdes gonadiques à la survenue du pic de LH le jour du proestrus. Nous décrivons en outre un rôle critique de RFRP-3 dans la médiation de l'entrée saisonnière de la mélatonine sur l'axe de la reproduction chez les hamsters syriens femelles. Sur la base de ces résultats, nous suggérons que cette fonctionnalité pourrait être un rôle conservé de RFRP parmi les espèces saisonnières, bien que les mécanismes spécifiques de la façon dont la sortie RFRP saisonnière est intégrée sur l'axe de la reproduction peut varier d'une espèce à l'autre. Dans l'ensemble, nous dévoilons ici que les neurones à RFRP jouent plusieurs rôles dans la régulation de

l'axe reproducteur femelle, en étant un régulateur important des événements cycliques contrôlant le pic pré-ovulatoire de LH, et en même temps un élément clé dans la synchronisation de l'activité de reproduction avec la saison.

Au total, les données actuelles montrent que l'implication du réseau RFRP dans la régulation des fonctions de reproduction est beaucoup plus compliquée que prévu initialement. Sur la base de nos résultats, nous pouvons conclure que le rôle crucial joué par RFRP dans la régulation saisonnière de la reproduction est conservé entre les deux sexes chez le hamster Syrien. De plus,, nous montrons que chez les hamsters Syriens, les neurones à RFRP joue également un rôle spécifique chez la femelle outils sont impliqués dans la régulation journalière et estrienne de l'activité de reproduction. Pour les expériences à venir, il sera essentiel de garder à l'esprit qu'il existe des différences spécifiques au sexe dans les effets de RFRP-3, et qu'il pourrait y avoir des différences fondamentales dans le système de RFRP entre les espèces saisonnières et non saisonnières. . Les études futures devraient par ailleurs viser à distinguer RFRP-1 et RFRP-3 de signalisation, ainsi qu'à examiner si les peptides RFRP pourraient agir par un autre récepteur GPR74 (NPFF2), connu pour interagir avec des peptides RFRP bien qu'avec une affinité plus faible que GPR147. Enfin, compte tenu des autres effets du RFRP sur la régulation du métabolisme et du stress, il serait pertinent d'étudier l'hypothèse que les neurones à RFRP pourrait être un relai, agissant comme un commutateur homéostatique entre les nombreuses fonctions neuroendocrines de l'hypothalamus.

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APPENDIX I- RFRP NEURONS - THE DOORWAY TO UNDERSTANDING SEASONAL REPRODUCTION IN MAMMALS

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ABSTRACT

Seasonal control of reproduction is critical for the perpetuation of species living in temperate zones that display major changes in climatic environment and availability of food resources. In mammals, seasonal cues are mainly provided by the annual change in the 24h light/dark ratio (i.e. photoperiod), which is translated into the nocturnal production of the pineal hormone melatonin. The annual rhythm in this melatonin signal acts as a synchronizer ensuring that breeding occurs when environmental conditions favor survival of the offspring. Although specific mechanisms might vary among seasonal species, the hypothalamic RF (Arg-Phe) amide-related peptides (RFRP-1 and -3) are believed to play a critical role in the central control of seasonal reproduction and in all seasonal species investigated, the RFRP system is persistently inhibited in short photoperiod. Central chronic administration of RFRP-3 in short day-adapted male Syrian hamsters fully reactivates the reproductive axis despite photoinhibitory conditions, which highlights the importance of the seasonal changes in RFRP expression for proper regulation of the reproductive axis. The acute effects of RFRP peptides, however, depend on species, photoperiod and recent studies point towards a different role of RFRP in regulating female reproductive activity. In this review we summarize the recent advances made to understand the role and underlying mechanisms of RFRP in the seasonal control of reproduction, primarily focusing on mammalian species.

INTRODUCTION

Animals living in temperate and boreal latitudes experience marked seasonal changes in their environment. To overcome these environmental changes, thus increasing their chances of survival, they show seasonal changes in several aspects of their physiology i.e. reproduction, metabolism, and behavior. Annual variations in day length are translated into an endocrine message, namely the nocturnal secretion of the pineal hormone melatonin, which acts as a potent seasonal synchronizer of biological functions, especially reproductive activity (Carter and Goldman, 1983b)(Hoffman and Reiter, 1965)(Malpaux et al., 2001)(Pevet, 1988).

The effects of photoperiod on reproductive function have long been established and recent studies have made much progress in describing key components and pathways involved in this adaptive process. The RFamide peptide kisspeptin (kp), a very potent stimulator of gonadotropin releasing hormone (GnRH) release, shows photoperiodic variations and was therefore thought to be a likely candidate for the photoperiodic control of reproduction in seasonal breeders, but increasing evidence now points towards another RFamide peptide, the RFamide-related peptide (RFRP) as the critical intermediate between the melatonin-dependent photoperiodic signal and central control of the reproductive axis (Klosen et al., 2013)(Ancel et al., 2012)(Ubuka et al., 2012)(Hazlerigg and Simonneaux, 2015).

Herein we review our current understanding of the RFRP system in seasonal breeders, reporting the mechanisms through which melatonin impacts on RFRP synthesis and the effects of RFRP peptides in the seasonal control of reproduction including species-dependent and sex-specific variations in the RFRP system.

THE PINEAL HORMONE MELATONIN SYNCHRONIZES REPRODUCTION WITH SEASONS

In seasonal species reproductive activity is restricted to a particular time of the year so that birth occurs when warmer temperatures and better accessibility to food increases the chances of survival of the offspring. Early studies have demonstrated that synchronization of reproductive activity with season is driven by the pineal hormone melatonin (Hoffman and Reiter, 1965). Photic information reaches the pineal gland via the retino-hypothalamo-pineal pathway that during nighttime generates a release of norepinephrine, which acts as a potent and reliable regulator of the rhythmic release of melatonin from the pineal gland. As a consequence, melatonin is synthesized and secreted in a diurnal fashion with a dramatic increase during nighttime that returns to nearly undetectable levels at daytime with the duration of elevated melatonin depending on night length (Karsch et al., 1984)(Carter and Goldman, 1983b)(Hazlerigg and Simonneaux, 2015)(Hoffman and Reiter, 1965)(Malpaux et al., 2001)(Pevet, 1988). In long photoperiod (LP)-breeders, i.e. the Syrian hamster (*Mesocricetus auratus*) that is widely used as a rodent model to study seasonal reproduction, short day

lengths represented by a long nocturnal duration of melatonin secretion inhibits the reproductive axis and removal of the melatonin signal by pinealectomy prevents this short day inhibition of reproductive activity (Hoffman and Reiter, 1965). In contrast to small rodents, larger mammals with a longer gestation time, like the sheep, are sexually active in short photoperiod (SP) and becomes sexually quiescent after transfer to LP conditions (Bittman and Karsch, 1984). Although the reproductive timing is opposite in hamsters and sheep, in both cases the photoperiodic changes in circulating levels of melatonin synchronize reproduction with seasons.

MELATONIN MODES AND SITES OF ACTION ON THE REPRODUCTIVE AXIS

Three melatonin receptor subtypes have been characterized so far; MT1 (Mel1a), MT2 (Mel1b) and Mel1c with its mammalian ortholog GPR50 (Dufourny et al., 2008)(Reppert et al., 1996). Using the highly specific 2-[¹²⁵I]-iodomelatonin, high affinity melatonin binding sites have been found in the hypothalamus and the *pars tuberalis* (PT) of mammals and amongst species the highest concentration of melatonin receptors is found in the PT (Hazlerigg and Simonneaux, 2015)(Weaver et al., 1989)(Vaněcek et al., 1987)(Vaněcek, 1988)(Williams et al., 1989). The MT1 subtype seems to be dominantly expressed throughout species and is known to be responsible for the neuroendocrine integration of season (Poirel et al., 2003)(Cogé et al., 2009). Indeed, Siberian hamsters (also known as the Djungarian hamster or *Phodopus sungorus*) show seasonal reproductive responses to melatonin despite lacking a functional MT2 receptor (Weaver et al., 1996). Maywood and Hastings found that site specific lesions of iodomelatonin-binding sites in the mediobasal hypothalamus (MBH) prevent testicular regression in Syrian hamsters exposed to SP (Maywood et al., 1996) and in Siberian hamsters, melatonin infusion into or lesions of the suprachiasmatic nucleus (SCN), alter the reproductive response to seasonal changes (Bartness et al., 1991)(Badura and Goldman, 1992). Finally in sheep, the premammillary region of the hypothalamus contains melatonin binding sites (de Reviers et al., 1989)(Malpaux et al., 1998) and melatonin implantations in the area of this structure, but not the PT, were shown to prevent synchronization of reproduction with photoperiod (Lincoln and Maeda, 1992)(Malpaux et al., 1998)(Malpaux et al., 1993)(Malpaux et al., 1995). Altogether these data have pointed towards the potential importance of these hypothalamic regions for proper integration of the melatonin-dependent photoperiodic signal onto the reproductive axis. However, it has not been possible to determine whether and how melatonin would act directly on these hypothalamic sites. Although a direct hypothalamic effect of melatonin cannot be excluded, accumulating evidence now point towards the PT as the major site for the hypothalamic integration of the melatonin signal in seasonal breeders (Johnston et al., 2005)(Hanon et al., 2008a)(Dardente et al., 2014)(Hazlerigg and Simonneaux, 2015).

TSH, THYROID HORMONES AND THE MELATONIN-DRIVEN REPRODUCTIVE ACTIVITY

In 2003, Yoshimura and colleagues made a remarkable finding that unveiled a link between the thyroid stimulating hormone (TSH) pathway and seasonal reproduction. They showed that light-induced type 2 thyroid hormone deiodinase (Dio2) expression in the MBH and subsequent hormone conversion of thyroxine (T4) into the bioactive triiodothyronine (T3) regulates the photoperiodic response of gonads in birds (Yoshimura et al., 2003). Since this discovery it has been shown that TSH expression in the PT is regulated by photoperiod in a melatonin-dependent and TSH stimulates Dio2 expression in seasonal mammals (Nakao et al., 2008a)(Revel et al., 2006b)(Hanon et al., 2008a). MT1-expressing cells in the PT synthesize TSH and its production in the PT is strongly inhibited by the SP pattern of melatonin (Dardente et al., 2003)(Wittkowski et al., 1988)(Böckers et al., 1995). Recent work moreover disclosed that melatonin regulates the photoperiodic changes in TSH expression in the PT via differential effects on clock gene expression and on the transcription of the co-activator EYA3 (Hazlerigg et al., 2004)(Dardente et al., 2010)(Masumoto et al., 2010). Another primary response to photoperiodic changes in melatonin is the opposite regulation of Dio2 and type 3 thyroid hormone deiodinase (Dio3) expression in the MBH (Revel et al., 2006b)(Yasuo et al., 2007)(Yoshimura et al., 2003)(Köhrle, 1999)(Watanabe et al., 2004)(Hanon et al., 2010)(Hanon et al., 2008a). While Dio2 catalyzes the conversion of T4 into T3, Dio3 catalyses the conversion of T3 to the biological inactive T2. Thus, in concert Dio2 and Dio3 regulate the hypothalamic T4/T3 balance according to photoperiod, with a higher production of T3 in LP as compared to SP (Barrett et al., 2007)(Hanon et al., 2010)(Saenz de Miera et al., 2013)(Klosen et al., 2013). Thyroid hormones have long been known to be important for the transitions between the breeding and non-breeding state i.e. in 1940 thyroidectomy in starlings was reported to result in persistent breeding (Woitkewitsch, 1940). Similarly, thyroidectomized sheep remain in the breeding state when changing from spring to anestrus and T4 replacement can reverse these effects, but interestingly, thyroidectomy displays no effect in the transition from the anestrus state to the breeding state (Moenter et al., 1991)(Dahl et al., 1994b)(Webster et al., 1991)(Billings et al., 2002). Moreover, T3 administration in the LP-breeding Siberian hamster blocks the SP-induced gonadal regression (Barrett et al., 2007)(Freeman et al., 2007)(Murphy et al., 2012).

Both deiodinases are highly expressed in a population of specialized glial cells in the ependymal cell layer lining the third ventricle named tanycytes (Rodríguez et al., 2005). Interestingly, tanycytes co-express the TSH receptor (TSHR) and recent data clearly shows that activation of these receptors increases *Dio2* expression, thereby increasing levels of T3 in the MBH, in a number of seasonal mammals (Hanon et al., 2008a)(Klosen et al., 2013)(Bolborea et al., 2015)(Ono et al., 2008a)(Hanon et al., 2010)(Nakao et al., 2008c)(Revel et al., 2006b)(Dardente, 2012). In line with these observations a recent study shows that PT-derived TSH, in contrast to *pars distalis*-derived TSH, does not stimulate the thyroid gland, but rather acts via TSHR on the tanycytes (Ikegami et al., 2014). Altogether these

studies have unveiled a conserved photoperiodic transduction pathway explaining how the melatonin signal is integrated in the PT and transduced into a local thyroid message in the MBH (Hazlerigg and Simonneaux, 2015), (figure 1).

Thyroid hormones mediate their neuroendocrine effects through still undefined hypothalamic sites since no cellular phenotyping of their receptors have been reported in the hypothalamus. Interestingly however, a recent study found that chronic TSH administration in SP-adapted Siberian and Syrian male hamsters reactivates the reproductive axis while at the same time increasing the expression of two known hypothalamic regulators of reproductive output, RFRP and Kp, suggesting that the melatonin signal reaches the reproductive axis via the TSH/thyroid hormone pathway acting on these neurons (Klosen et al., 2013) rather than via direct hypothalamic effect as previously suggested (Maywood et al., 1996).

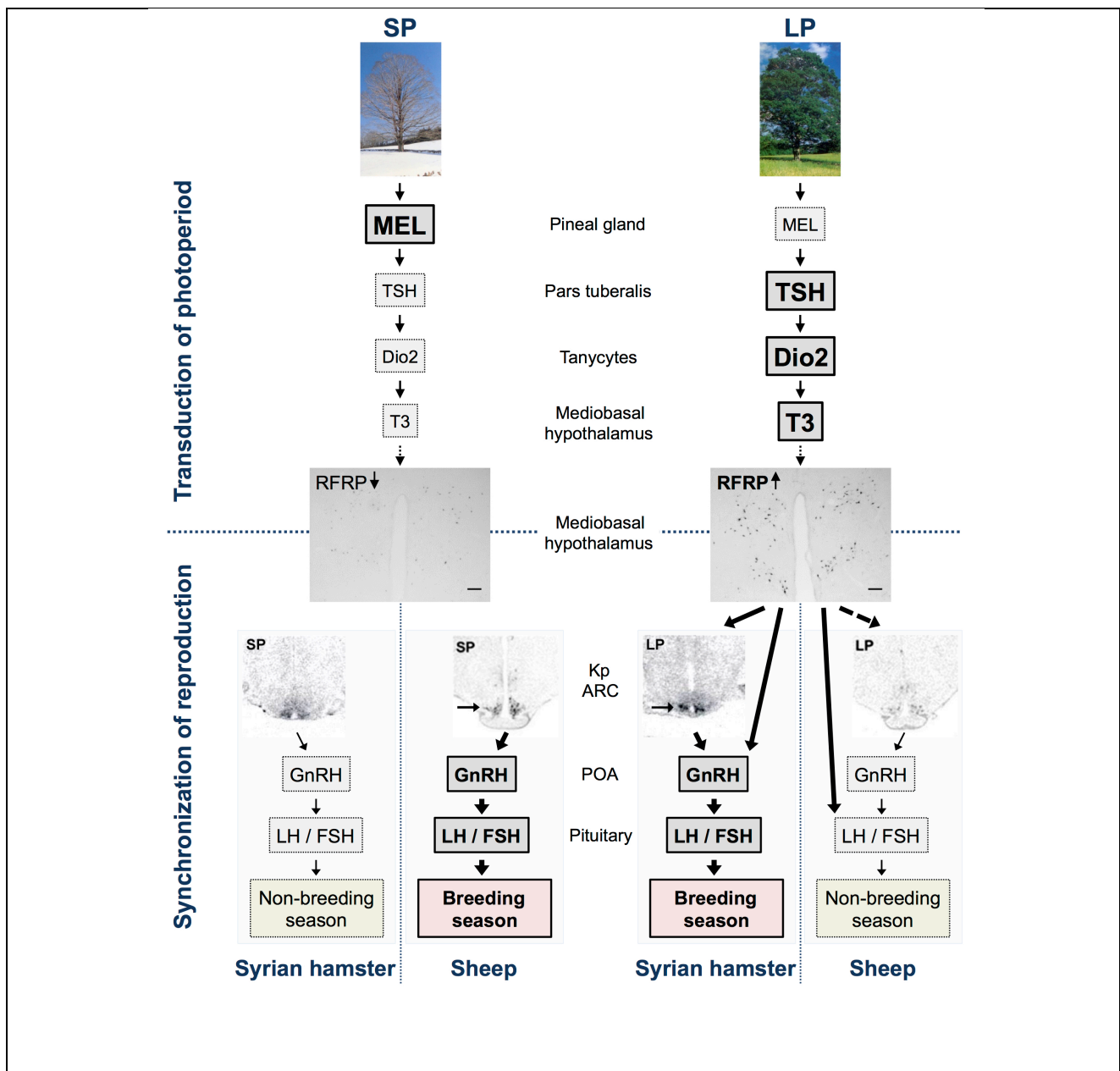


Figure 17. Model of the transduction of photoperiod and seasonal regulation of the reproductive axis in long (Syrian hamsters) and short day (sheep) breeders. In short photoperiod (SP), the large production of melatonin from the pineal gland inhibits TSH synthesis in the *pars tuberalis*, whereas the lower production of melatonin in long photoperiod (LP) allows the synthesis and release of TSH. TSH is transmitted via TSH receptors expressed in tanycytes surrounding the 3rd ventricle and activates the enzyme deiodinase 2 (Dio2). Dio2 ultimately controls and increases the local availability of the active form of the thyroid hormone, T3, in the mediobasal hypothalamus. T3 subsequently regulates the expression of RFRP also in the mediobasal hypothalamus so that there is a high expression in LP and a low expression in SP in both LP and SP breeders, as demonstrated with pictures of RFRP-ir neurons in brains from Syrian hamster kept in LP and SP (scale bar 100µm, taken from Henningsen *et al.*, 2015). In Syrian hamsters, RFRP subsequently acts either directly on GnRH neurons or indirectly *via* kisspeptin (kp) neurons (indicated by arrows) or other interneurons in the arcuate nucleus (ARC) to synchronize reproduction with season. In sheep, RFRP regulates the reproductive axis directly at the level of the pituitary (indicated by arrow) and possibly also directly or indirectly via kp neurons (indicated by dotted arrow) and/or GnRH neurons. Expression of the gene encoding kp in the ARC displays an opposite photoperiodic regulation in the two species being elevated in both LP-adapted sexually active Syrian hamsters (see arrow in picture taken from Revel *et al.*, 2006) and SP-adapted sexually active sheep (see arrow in picture taken from Wagner *et al.*, 2008). High levels of expression are highlighted using bold letters and arrows, as opposed to low levels of expression that are indicated by narrow letter and arrows. This model does not describe specific effects of RFRP-3 reported in each species, sex and photoperiod, and readers are referred to table 3 for a detailed summary.

HYPOTHALAMIC REGULATION OF SEASONAL REPRODUCTION

Hypothalamic control of the reproductive axis is commonly regulated amongst species through the release of gonadotropin releasing hormone (GnRH) from GnRH fiber terminals projecting to the median eminence. GnRH is released into the portal blood system from which it regulates the synthesis and release of pituitary gonadotropins. Despite the marked decrease of GnRH release during sexual quiescence most seasonal species display an unchanged number and level of GnRH neurons and GnRH-immunoreactivity (ir) in the different photoperiods (Urbanski et al., 1991)(Barrell et al., 1992). GnRH synthesis and release is regulated upstream by various signals, especially two RF-amide peptides released from respectively RFRP neurons in and around the dorso/ventromedial hypothalamus (DMH/VMH) and kp neurons in the anteroventral periventricular nucleus (AVPV) and medial preoptic nucleus (MPN), and in the arcuate nucleus (ARC). Kp peptides are potent stimulators of the reproductive axis and acts directly on GnRH neurons through their cognate receptor GPR54 (Liu et al., 2008)(Piet et al., 2015a). Interestingly, kp expression both in the MPN/AVPV and ARC was found to be significantly down-regulated by melatonin in SP-adapted sexually inactive Syrian hamsters (Ansel et al., 2010b)(Revel et al., 2006a). In the SP-breeding sheep, kp expression is oppositely up-regulated in SP (Wagner et al., 2008)(Smith et al., 2008)(Chalivoix et al., 2010), suggesting that ARC kp expression reflects the breeding state rather than the seasonal state of the animal (figure 1). In both hamsters and sheep continuous infusion of kp during sexual quiescence fully restores reproductive activity (Revel et al., 2006a)(Ansel et al., 2011a)(Caraty et al., 2007)(Sébert et al., 2010) and kp neurons are thus a pivotal component between the photoperiodic signal and seasonal activation of GnRH neurons. This statement is however complicated by results showing a lowered ARC kp expression in LP-breeding Siberian (Mason et al., 2007)(Greives et al., 2008) and European (Saenz et al., 2014) hamsters as compared to SP. While these differences in the photoperiodic regulation of ARC kp might be explained by different feedback mechanisms of sex steroids, it also suggests that these neurons are differently implicated in the photoperiodic control of reproduction from one species to another. Therefore it seems unlikely that kp neurons are solely responsible for mediating the melatonin-dependent seasonal signal onto the reproductive axis. By contrast, increasing results demonstrate that the photoperiodic regulation of RFRP expression within the DMH/VMH is conserved among seasonal species (Simonneaux and Ancel, 2012) suggesting that RFRP neurons may be potential candidates for integration of the photoperiodic signal.

THE RFRP SYSTEM

RF (Arg-Phe) amide-related peptides (RFRPs) were discovered in birds and mammals in 2000 and found to be primarily expressed in neurons located in the paraventricular nucleus (PVN) and in between the DMH and VMH in birds and rats, respectively (Tsutsui et al., 2000)(Hinuma et al., 2000). In birds, the peptide was shown to inhibit gonadotropin secretion from cultured quail pituitaries and

thus termed gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000). The avian GnIH precursor encodes one GnIH and two GnIH related peptides (GnIH-RP-1 and GnIH-RP-2). GnIH and GnIH-RP-1 contain a LPLRFa motif in the C-terminal whereas GnIH-RP-2 contains a LPQRFa motif. The mammalian gene named *RFamide-related peptide (Rfrp)* encodes RFRP-1, -2 and -3 peptides, of which RFRP-1 (containing a LPLRFa motif) and RFRP-3 (containing a C-terminal LPQRFa motif) are functional peptides (Fukusumi et al., 2001a)(Hinuma et al., 2000)(Ukena et al., 2002). RFRP-3 and GnIH have been shown to inhibit GnRH neuron activity and gonadotropin release in several seasonal (sheep, hamster, quail) and non-seasonal (rat, mouse) species (Anderson et al., 2009)(Clarke et al., 2008)(Ducret et al., 2009)(Johnson et al., 2007)(Kriegsfeld et al., 2006) (Tsutsui et al., 2000)(Pineda et al., 2010a)(Leon et al., 2014)(Tsutsui et al., 2015). There is moreover evidence of a hypophysiotropic effect of GnIH and RFRP-3 in birds and ewes, respectively, although the effect in ewe is still of controversy since in one study intravenous (iv) infusion of RFRP-3 inhibits pulsatile LH secretion in ovariectomized ewes (Clarke et al., 2008), whereas two other studies find no variation in LH plasma concentrations in neither ovariectomized nor intact ewes injected either intracerebroventricular (icv) or iv with RFRP-3 (Caraty et al 2012)(Decourt et al., 2016a). Two recent studies reported a stimulatory effect of central administration of RFRP-3 in male Syrian and Siberian hamster, indicating that the effects of the peptide are species-dependent (Ancel et al., 2012)(Ubuka et al., 2012). Strikingly, while RFRP-3 activates the reproductive axis in male Syrian hamsters (Ancel et al., 2012), the avian GnIH inhibits LH secretion in ovariectomized females (Kriegsfeld et al., 2006), adding a supplementary sex-difference in the effects of the peptides.

Recently, a study of Tena-Sempere and colleagues (Leon et al., 2014) reported that mouse KO for GPR147, the cognate receptor for RFRP peptides, do not display strong reproductive phenotypic alterations as compared to wild type mice. Moderate changes are however observed in GPR147 deficient mice as during pubertal transition male KO mice exhibit increased LH levels, and in adulthood FSH levels are higher in both female and male KO mice as compared to wild type mice. Moreover, litter sizes from KO mice are slightly increased as compared to wild type litter sizes. Interestingly, the male KO mice moreover show an increased level of *kp* expression in the ARC, but not in the MPN/AVPV, suggesting that in male mice RFRP neurons provide a tonic inhibition on ARC *kp* neurons.

RFRP MODES AND SITES OF ACTION

RFRP neurons are mainly found in and around the DMH/VMH from where they project to multiple regions of the rodent brain. RFRP-ir fibers are found especially in the preoptic area/organum vasculosum of the lamina terminalis (POA/OVLT), MPN/AVPV, the anterior part of the SCN, PVN, anterior hypothalamus, VMH and ARC as well as in the bed nucleus of the stria terminalis, habenular nuclei, and paraventricular nucleus of the thalamus (Henningsen et al., 2015)(Kriegsfeld et al.,

2006)(Ubuka et al., 2012). RFRP terminals make apparent contact to 20-40% of GnRH somas in rodents and sheep (Kriegsfeld et al., 2006)(Ubuka et al., 2012)(Rizwan et al., 2012)(Smith et al., 2008) and in female mice, around 20% of MPN/AVPV kp neurons and 35% of ARC kp neurons receive RFRP-fiber contacts (Poling et al., 2013)(Rizwan et al., 2012). In mice, RFRP-3 application to brain slices inhibits the firing rate of 41% GnRH neurons and stimulates the firing rate of 12% of the GnRH neurons (Ducret et al., 2009), whereas in male Syrian hamster icv infusion of RFRP-3 induces c-FOS expression in 20-30% GnRH neurons but also in non-kisspeptinergic neurons of the ARC (Ancel et al., 2012).

RFRP peptides bind preferentially to the G-protein coupled receptor, GPR147 (also known as NPFF1). GPR147 has been found to couple to both stimulatory and inhibitory G protein subunits *in vitro* (Gouarderes et al., 2007)(Bonini et al., 2000) and in GPR147 transfected CHO cells, hRFRP-1 induces a maximal inhibition of a forskolin-induced cAMP accumulation, indicating that RFRP-1 might inhibit adenylate cyclase through a G α i-bound receptor complex (Mollereau et al., 2002). However, the exact signaling events occurring downstream of GPR147 in its natural cellular environment still remains unknown. GPR147-encoding mRNA is widely distributed in the brain, however particular strong expression is observed in hypothalamic regions as the POA/OVLT, MPN/AVPV, SCN, PVN, anterior hypothalamus, VMH and ARC, and outside the hypothalamus in the posterior part of the bed nucleus of the stria terminalis, habenular nuclei, and the pyramidal cell layer of the hippocampus (Henningsen et al., 2015)(Gouarderes et al., 2004)(Dardente et al., 2008). Interestingly, GPR147 has been shown to be expressed in 15-33% of mice GnRH neurons and a subpopulation of kp neurons in the AVPV (5-16%) and the ARC (25%) (Poling et al., 2012)(Poling et al., 2013)(Rizwan et al., 2012). Altogether these studies suggest that RFRP peptides can act directly on these central neuroendocrine regulators of reproduction.

RFRP: A CRITICAL SWITCH BETWEEN MELATONIN AND THE REPRODUCTIVE AXIS

GnIH AND AVIAN SEASONAL REPRODUCTION

GnIH inhibits LH release from cultured quail pituitaries (Tsutsui et al., 2000) and to further support a direct pituitary effect of GnIH in quail, GnIH-ir fibers have been found to project to the median eminence in this species (Ukena et al., 2003). Also in quail, studies have revealed that GnIH expression and release is directly regulated by melatonin acting on Mel1c receptors specifically expressed in GnIH neurons (Ubuka et al., 2005). In contrast to mammalian seasonal species, GnIH expression is increased by melatonin and consequently GnIH-ir expression is increased in SP as compared to LP (Ubuka et al., 2005). *In vitro* studies furthermore show that GnIH release has a diurnal rhythm and is increased during nighttime in quail hypothalamic explants (Chowdhury et al., 2010). In house and song sparrows GnIH-ir neurons are reported bigger towards the end of the breeding season (Bentley et al., 2003). By

contrast in wild Australia zebra finches there is no variation in neither GnIH-ir nor *GnIH* expression between the breeding and non-breeding state (Perfito et al., 2011).

RFRP IN SEASONAL MAMMALIAN SPECIES

Photoperiodic variations in the RFRP system

There are no circadian or day-to-night variations in RFRP mRNA expression in male Syrian (Revel et al., 2008) and European (Saenz de Miera et al., 2014) hamsters, respectively. RFRP expression is however strongly regulated by photoperiod and is down-regulated in SP in several seasonal breeders (Janati et al., 2013)(Ancel et al., 2012)(Revel et al., 2008)(Saenz de Miera et al., 2014)(Simonneaux and Ancel, 2012)(Smith et al., 2008). Studies in male Syrian and Siberian hamsters show the SP down-regulation to be melatonin-dependent (Ubuka et al., 2012)(Revel et al., 2008). Recent findings have revealed that in female Syrian hamsters as well, RFRP expression is down-regulated in SP, probably driven by the same mechanisms (Henningsen et al., 2015). Interestingly, RFRP expression is similarly down-regulated in SP in short day breeders like the sheep (Dardente et al., 2008)(Smith et al., 2008). This suggests that the SP pattern of circulating melatonin displays a conserved inhibition on RFRP expression independently of whether mammals are long or short day breeders (figure 1). Importantly, the photoperiodic/melatonin regulation of RFRP expression, in contrast to kp, may not be modulated by the gonadal hormone feed back because, although RFRP neurons express sex steroid receptors (Kriegsfeld et al., 2006), neither gonadectomy nor sex-hormone implants alter RFRP expression in male (Revel et al., 2008) and female (Henningsen et al., unpublished) Syrian hamsters.

We have recently shown that GPR147 mRNA levels in the Syrian hamster's brain also depends on photoperiod being down-regulated in SP condition, and interestingly, this down-regulation is much stronger and consistent in females as compared to males (Henningsen et al., 2015). In Siberian hamsters and sheep the amount of GnRH neurons receiving RFRP fiber contacts is decreased in SP conditions (Clarke et al., 2008)(Ubuka et al., 2012), however in the Syrian hamster we did not find any photoperiodic variations in numbers of RFRP-ir fibers projecting specifically to the OVLT or the ARC (Henningsen et al., 2015).

Species-specific differences in the effects of RFRP on seasonal reproduction

We were the first to show that in male Syrian hamster, RFRP-3 is capable of stimulating the reproductive axis (Ancel et al., 2012). In more details, RFRP-3 was found to stimulate GnRH neuronal activity, LH and FSH release and testosterone production independently of the photoperiodic condition, although to a lesser extent in SP animals. Moreover, chronic central administration of RFRP-3 in SP-adapted male Syrian hamsters reactivated the reproductive axis via an increase in ARC kp expression, despite the animals being kept in SP-inhibitory conditions. The stimulatory effect of RFRP-3 observed in male Syrian hamsters fits well with the high RFRP expression in sexually active LP

animals and our data furthermore indicate that the stimulation of reproductive activity could be mediated via the ARC kp neurons. Thus in the male Syrian hamster, RFRP neurons appear to integrate and transfer the seasonal input towards kp neurons. In another hamster species, the male Siberian hamster, RFRP-3 displays reverse effects depending on the photoperiodic condition, stimulating LH release in SP but decreasing LH levels in LP conditions (Ubuka et al., 2012). Although the mechanism underlying such photoperiod-dependent effect of RFRP-3 in this species are unknown it might help to explain the up regulation of ARC kp in the sexually inactive SP-adapted Siberian hamsters (Simonneaux et al., 2009)(Greives et al., 2007)(Mason et al., 2007). These observations indicate that in the two hamster species RFRP-3 either has opposite sites of action in LP-adapted animals or is integrated differently along the hypothalamo-pituitary-gonadal axis.

Despite the controversies of RFRP's effect in sheep, one study reported that iv administration of ovine RFRP-3 peptide (also referred to as GnIH3) inhibits LH release in ovariectomized ewe (Clarke et al., 2012), which indicates that RFRP peptides might have a hypophysiotropic effect in ovine species, similarly to what is observed in avian species. Indeed, RFRP-fibers have been shown to project to the median eminence and RFRP peptides are detected in the portal blood of sheep (Sari et al 2009)(Smith et al 2012). As previous studies have reported there are no evidence of peripheral effects of RFRP peptides on hamster's reproduction (Ancel et al., 2012), thus describing another fundamental difference in how the RFRP signal is integrated in the hypothalamo-pituitary axis among mammalian seasonal species. Interestingly, the opposite effects and photoperiodic regulation of RFRP between sheep and hamsters support our hypothesis that a similar neuroendocrine pathway is conserved between LP- and SP-breeders with the RFRP peptides playing a pivotal role in adapting reproductive activity to the environment (figure 1). Further analyses are required to test this hypothesis, in particular whether the inhibitory effect of RFRP-3 account for the lower expression of kp in LP-adapted sexually inactive sheep. The reported effects of RFRP-3 in seasonal mammals are summarized in table 3.

Species	Sex and Status	Effects of RFRP-3	Reference
Siberian hamster	Male	Central administration inhibits LH release in LP Central administration stimulates LH release in SP	Ubuka et al., 2012
Syrian hamster	Male	Central acute and continuous administration stimulates LH release in LP and SP. No peripheral effect.	Ancel et al., 2012
	Female OVX	Central and peripheral administration (GnIH) inhibits LH release in LP.	Kriegsfeld et al., 2006
Sheep	Female	Peripheral injection or a 4hour perfusion has no effect on kisspeptin-mediated increase in LH in LP.	Decourt et al., 2016
		Peripheral administration inhibits LH release in SP.	Clarke et al., 2012
	Female OVX	Repeated peripheral injection has no effect on pulsatile LH release in LP. 24-hour perfusion has no effect on E2-induced LH surge in SP.	Decourt et al., 2016
		Central and peripheral administration has no effect on LH release in SP and LP.	Caraty et al., 2012
		Peripheral administration inhibits E2-induced LH surge in SP.	Clarke et al., 2008
		Peripheral administration inhibits pulsatile LH release in SP.	Clarke et al., 2012
		Peripheral administration inhibits GnRH-induced LH release. RFRP-3 is detected in the portal blood in SP and LP, with higher conc. detected in LP.	Smith et al., 2012
Table 3. Overview of the <i>in vivo</i> effects of RFRP-3 on reproduction in seasonal mammals.			

A fascinating issue is to disclose how RFRP peptides can have opposite seasonal and species-dependent effects. As previously mentioned, GPR147 has been found to couple to both stimulatory and inhibitory G proteins *in vitro* (Gouarderes et al., 2007)(Bonini et al., 2000), but it remains to be established if there are fundamental species-specific differences in the downstream signaling cascades after activation of the GPR147. Alternatively it is likely that the cellular response to RFRP is conserved among species, but mediated via different targets. In male Syrian hamsters, acute injections of RFRP-3 induce c-Fos expression in a subset of GnRH neurons but also in an unidentified population of neurons in the ARC (Ancel et al., 2012) and one can speculate whether the observed stimulatory effect of RFRP-3 in the male Syrian, but also Siberian hamsters, arise through inhibition of inhibitory interneurons, thus resulting in the stimulatory outcome. Future studies should aim at phenotyping downstream targets of RFRP in order to understand better its various effects on the reproductive axis of seasonal breeders.

Sex differences in seasonal rodents

In contrast to the stimulatory effect of RFRP-3 we have observed in the male Syrian hamsters (Ancel et al., 2012), central injections of the avian RFRP peptide, GnIH, inhibits LH release in ovariectomized females (Kriegsfeld et al., 2006). It should be stressed that GnIH contains a -LPLRFa motif similar to that of the mammalian RFRP-1 and not RFRP-3. However, we recently found that central RFRP-3 administration in the intact female Syrian hamster inhibits LH release similarly to the effects observed with the GnIH ortholog (Henningsen *et al.*, unpublished). These observations add supplementary sex-specific differences in the acute effects of RFRP peptides, at least in Syrian hamsters. In order to understand such opposite effect of RFRP-3, we explored other potential sex-differences in the Syrian hamster RFRP system. We found that the number of RFRP neurons and the intensity of the immunoreactive labeling were markedly higher in females than in males adapted to LP conditions. In SP conditions however, RFRP expression is down-regulated to a similar low level in both sexes (Henningsen et al., 2015). The number of RFRP-ir fibers projecting specifically to the MPN/AVPV is increased in SP as compared to LP in female, but not in male. Moreover, we found that the overall levels of GPR147 mRNA were higher in females than in males, regardless of photoperiod, and that the SP-induced down-regulation of GPR147 mRNA levels were stronger in females than in males (Henningsen et al., 2015). A similar sex-specific difference in RFRP expression is also reported in the non-seasonal rat, where RFRP-1 expression is found to be higher in females as compared to males (Jorgensen et al., 2014). Altogether these findings point towards a particular importance of the RFRP system in seasonal as well as non-seasonal females.

RFRP and circadian changes in female reproductive activity

RFRP neurons project to the MPN/AVPV (Henningsen et al., 2015)(Kriegsfeld et al., 2006)(Rizwan et al., 2012) where kp neurons provide the stimulatory signal onto the GnRH neurons causing the surge of LH and thereby ovulation (Smith et al., 2006c). The surge of LH requests high circulating E2 levels, as an indicator of ovarian maturation, but its timing is also gated by a circadian signal to occur at the end of the resting period (Simonneaux and Bahougne, 2015). It has been suggested that in female mammals, RFRP neurons mediate a SCN-generated circadian output onto the MPN/AVPV kp neurons, thereby modulating the timing and generation of the LH surge (Gibson et al., 2008). Indeed, in the female Syrian hamster a decrease in RFRP expression occurs around the time of the LH-surge (Gibson et al., 2008) and in rat and ewes, RFRP expression is similarly reduced during the pre-ovulatory period (Clarke et al., 2012)(Jorgensen et al., 2014). Interestingly, a recent study showed that SCN-derived vasoactive intestinal peptide (VIP)-ergic terminal fibers projections are found in the area of where RFRP neurons are expressed in the female Syrian hamster and more importantly that central VIP administration markedly suppress RFRP cellular activity in the evening, but not in the morning (Russo et al., 2015). Altogether these data point towards a specific circadian rhythm in RFRP expression and

release in females, adding a supplementary role of RFRP in regulating reproductive activity in seasonal female species.

CONCLUSION

Over the past nearly two decades RF-amide related peptides have been extensively studied for their putative involvement in the regulation of the reproductive axis (Tsutsui et al., 2015). Initially, RFRPs were thought to act with a similar inhibitory effect as the avian ortholog GnIH in all species, and is still widely referred to as GnIH, despite its well-documented stimulatory effect in Siberian and Syrian hamster species (Ancel et al., 2012)(Ubuka et al., 2012). These recent findings have challenged the conception of a conserved role of RFRPs throughout species and underline the necessity to delineate its effects in one species, sex and physiological condition, such as season.

The marked down-regulation of RFRP expression observed in seasonal mammals, independently of sex, species and breeding behavior, provides evidence for a distinct and conserved role of this peptide in the integration of the photoperiodic melatonin signal via a TSH-dependent regulation of thyroid hormone locally in the MBH (Simonneaux and Ancel, 2012)(Klosen et al., 2013)(Hazlerigg and Simonneaux, 2015), (figure 1).

In the Syrian hamster, RFRP has opposite effects in females and males and there are strong sex-specific differences in the RFRP system manifested by a higher expression and stronger photoperiodic variations in the RFRP system in females as compared to male. Few studies have investigated the regulation and role of the RFRP system in female reproduction, however, findings so far strongly suggest that RFRP neurons play a critical role in female reproduction in regards to the timing of both daily and seasonal synchronization of their reproductive activity.

PhD Thesis

Jo Beldring Henningsen

The roles of RFRP in the central control of reproduction:
photoperiodic and sex-specific differences.

Rôle du RFRP dans le contrôle central de la reproduction saisonnière en fonction du sexe
et de la photopériode.

ABSTRACT

RFRP neurons regulate the reproductive axis, however, their effects depend on species and sex. Here, we aimed at providing a neuroanatomical description of the RFRP system in the Syrian hamster and at investigating the role of RFRP in the daily and seasonal control of female reproduction.

We show that besides being regulated by annual changes in photoperiod, the RFRP system is more strongly expressed in females than in males. In line with this, we unveil that RFRP has multiple roles in regulating female reproduction. RFRP neuronal activity is specifically reduced at the time of the pre-ovulatory LH surge and central RFRP-3 administration prior to the surge decreases LH peak levels, altogether pointing towards a daily down-regulation of the inhibitory RFRP signal necessary for proper generation of the LH surge. Moreover, chronic RFRP-3 infusion in sexually inactive females, with endogenous low RFRP expression, completely reactivates the reproductive axis.

Taken together, we demonstrate that RFRP is a key component in the seasonal control of reproduction while at the same time specifically regulating cyclic events controlling reproductive activity in females.

RESUMÉ

Le RFRP est un neuropeptide impliqué dans la régulation de l'axe reproducteur, mais ses effets varient en fonction du sexe et des espèces. Le but de cette étude était de décrire en détails l'organisation du système RFRP et de caractériser son rôle dans le contrôle circadien et saisonnier de l'axe reproducteur de hamsters femelles.

Les résultats montrent que le système RFRP est régulé par la photopériode et que son niveau d'expression est plus élevé chez les femelles que chez les mâles. Cela se traduit par des actions spécifiques sur l'axe gonadotrope femelle. En effet, l'activité des neurones à RFRP est diminuée au moment du pic pré-ovulatoire de LH et des injections centrales de RFRP-3 dans l'heure qui précède le pic de LH induisent une diminution de l'amplitude de la sécrétion de LH, démontrant une implication du RFRP dans la régulation circadienne du pic pré-ovulatoire de LH. Par ailleurs, des infusions chroniques de RFRP-3 chez des hamsters femelles sexuellement inactifs sont capables de réactiver le fonctionnement de l'axe reproducteur, ce qui montre que le RFRP a également un rôle régulateur essentiel dans le contrôle saisonnier de la reproduction.