The effect of RFRP-3 on the gonadotrophic axis of the Syrian hamster : sex-dependent differences and modes of action

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Le RFRP-3 et l'axe gonadotrope du hamster Syrien : effets genre-dépendants et modes d'action

The effect of RFRP-3 on the gonadotrophic axis of the Syrian hamster: sex-dependent differences and modes of action

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"Happiness does not come from doing easy work but from the afterglow of satisfaction that comes after the achievement of a difficult task that demanded our best."

Theodore Isaac Rubin

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ABBREVIATIONS

5-HT: 5-hydroxytryptamine, or serotonin
5-HTP: 5-hydroxytryptophan
AA-NAT: aralkylamine N-acetyltransferase
Ach: acetylcholine
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR: androgen receptor
ARC: arcuate nucleus of the hypothalamus
AVP: arginine vasopressin
AVPV: anteroventral periventricular nucleus of the hypothalamus
Bmal1: brain and muscle ARNT-like protein 1
CSF: cerebrospinal fluid
Cry: Cryptochrome
Dio2: deiodinase 2
DMH: dorsomedial nucleus of the hypothalamus
ER: estrogen receptor
FSH: follicle-stimulating hormone
GABA: γ-aminobutyric acid
GHT: geniculohypothalamic tract
GnRH: gonadotrophin-releasing hormone
HIOMT: hydroxyindole O-methyltransferase
HPG: hypothalamic-pituitary-gonadal
IGL: intergeniculate leaflet
IML: intermediolateral nucleus
KO: knock-out
Kp: kisspeptins
LD: long-day photoperiod
LH: luteinizing hormone
MEL: melatonin
MBH: mediobasal hypothalamus
NA: noradrenaline
NAS: N-acetylserotonin
NKB: neurokinin B
NMDA: N-methyl-D-aspartate
NPY: neuropeptide Y
OVX: ovariectomised
PACAP: pituitary adenylate cyclase-activating polypeptide
Per: Period
PG: pineal gland
POA: preoptic area of the hypothalamus
PT: pars tuberalis of the adenohypophysis
PVN: paraventricular nucleus of the hypothalamus
PVT: paraventricular nucleus of the thalamus
RFRP: RFamide-related peptide
RGCs: retinal ganglion cells
RHT: retino-hypothalamic tract
SCG: superior cervical ganglia
SCN: suprachiasmatic nuclei of the hypothalamus
SD: short-day photoperiod
T_3: triiodothyronine
T_4: thyroxine
Trp: tryptophan
TSH: thyroid stimulating hormone
VP: vasopressin
VIP: vasoactive intestinal peptide

INTRODUCTION

THE CONCEPT OF SEASONAL RHYTHMS

Every organism living on the planet Earth is submitted to daily and seasonal variations in environmental conditions. The daily variations result from the 24-hour rotation of the Earth around its own axis, leading to the alternation between day and night. The seasonal changes result from the yearly revolution of the Earth around the Sun and the 23.5° tilt of the Earth's axis relative to the plane of revolution (Figure 1). The combination of these two factors affects the intensity and duration of sunlight that reaches the Earth's surface and therefore results in seasonal variations in temperature, humidity and food availability. The seasonal variations in environmental conditions are more extreme with increasing latitude.

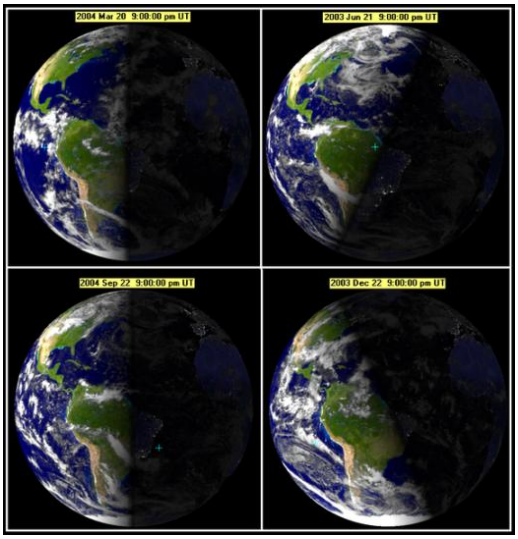

FIGURE 1 - GEOPHYSICAL PHENOMENA RESULT IN SEASONAL VARIATIONS OF THE ENVIRONMENT

Because the Earth's axis is tilted at 23.5° relative to the plane of revolution and because the Earth rotates around the Sun, the solar radiation varies along the year. In addition, solar radiation is maximal when the sun beams are perpendicular to the surface of the Earth, and decreases with increasing latitude. The combination of these factors results in the seasonal variations of the environment, which are increasingly marked with increasing latitude. *Picture by Tom Ruen, Full Sky Observatory.*

In order to survive, most species have developed a mechanism to anticipate the seasonal variations of the environment so as to adapt their physiology and behaviour accordingly. Examples of seasonal adaptations include hibernation, migration, moult and the restriction of reproduction to a certain time of the year. Indeed, the availability of food around the time of birth is a critical factor for offspring survival, hence producing offspring at the wrong time of year would compromise species survival. Wild organisms restrict their fertility to a certain time of the year to ensure that their progeny are born during the most favourable season (i.e. spring), and the period of sexual activity therefore depends on the duration of gestation. Animals can be classified into two categories:

- Long day breeders: stimulation of reproductive activity occurs when day length increases, in early spring and summer. This category includes horses, hamsters and most species of birds living in temperate regions.
- Short day breeders: stimulation of reproductive activity occurs when day length decreases, in autumn and winter. This category includes sheep, goats, deer, foxes and badgers.

Because maintaining a fully working set of reproductive organs is highly energy-consuming, seasonally-breeding animals undergo gonadal regression during the non-breeding season. This is manifested by a reduction of the size of the gonads and of circulating levels of sex steroids.

In order to anticipate the seasonal variations in the environment, animals rely on the most stable long-term indicator of the seasons: photoperiod (i.e. day length). Unlike temperature, rainfall or food availability, photoperiod is highly reproducible from one year to another and mammals use a photoneuroendocrine system to translate the light information into an endocrine message.

In addition to the adaptative response to photoperiodic variations, a number of species possess an endogenous mechanism of seasonal time measurement. Two types of seasonal strategies have been described:

- In some species, and particularly in long-lived ones, a circannual clock is responsible for synchronising seasonal rhythms. In this case, the rhythms are

fully endogenous, and persist in constant conditions with a period of approximately 12 months.

- In seasonal species which do not have a circannual clock, a mechanism termed photorefractoriness is responsible for the reactivation of the reproductive function after prolonged exposure to photoinhibitory conditions. This phenomenon is particularly important in animals which spend winter hibernating, and therefore do not see the increase in day length as spring arrives.

In both cases, the annual variations in photoperiod synchronise these endogenous events to one year exactly. It is important to note that the notions of "long" and "short" photoperiod are subjective, and depend on the species. Indeed, every species has its own critical photoperiod, which corresponds to the amount of daylight per 24-hour period above which the signal will be translated to long day information.

The Syrian hamster (*Mesocricetus auratus*) is a classic model for the study of seasonal reproduction. Because gestation lasts approximately 3 weeks, this species will be sexually active during springtime and summer, which corresponds to a long-day photoperiod (LD) (Figure 2). In the Syrian hamster, the critical photoperiod corresponds to 12.5h of light per day. On the other hand, exposure to a short-day photoperiod (SD) results in an inhibition of the reproductive function within 8-10 weeks, as manifested by low circulating levels of gonadal hormones and a massive decrease in testes size (Figure 2). Prolonged exposure to SD results in a spontaneous reactivation of the reproductive function, a phenomenon known as photorefractoriness (Figure 2) (Turek et al., 1975, Stetson et al., 1976, Prendergast et al., 2000). The photoperiodic regulation of reproduction is controlled by the nocturnal secretion of melatonin (MEL) from the pineal gland, because removal of the pineal gland prior to exposure to SD conditions prevents the SD-induced gonadal regression (Czyba et al., 1964, Hoffman and Reiter, 1965).

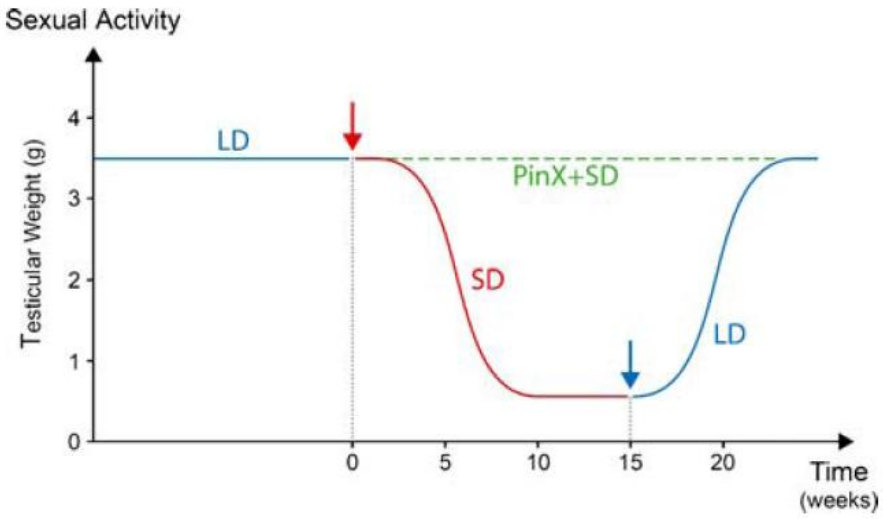

FIGURE 2 - PHOTOPERIODIC REGULATION OF REPRODUCTION IN THE SYRIAN HAMSTER

In long-day breeders, including the Syrian hamster, sexual activity is promoted by exposure to a long-day photoperiod (LD), whereas exposure to a short-day photoperiod (SD) inhibits reproductive activity within 8-10 weeks. This phenomenon is controlled by the nocturnal release of melatonin, because pinealectomy prior to transfer to SD abolishes the SD-induced gonadal regression (PinX+SD). Exposure to SD for over 15 weeks results in photorefractoriness, and a reactivation of the reproductive axis. *Revel et al., 2007.*

DECODING PHOTOPERIOD

To decode photoperiod, mammals rely on a photoneuroendocrine system in which neural pathways originating in retinal ganglion cells (RGCs) relay photoperiodic information to the pineal gland, where the neural message is transduced to a humoral message through the nocturnal release of MEL (Figure 3).

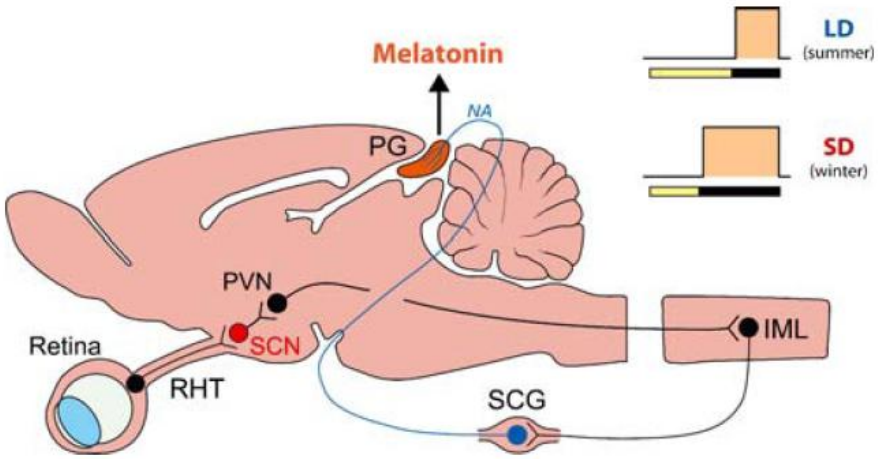

FIGURE 3 - MAMMALS USE A PHOTONEUROENDOCRINE SYSTEM IN ORDER TO DECODE PHOTOPERIOD

Cells originating in the retina project, via a multisynaptic pathway including the master circadian clock, to the pineal gland where melatonin is produced exclusively at night. Because the duration of the night varies according to the seasons, the duration of the nocturnal peak of melatonin provides a stable indication of the seasons. Thus, in summer (long-day conditions, LD) there is a short peak of melatonin and in winter (short-day conditions, SD) there is a long peak of melatonin.

IML: intermediolateral nucleus of the upper thoracic spinal cord; NA: noradrenaline; PG: pineal gland; PVN: paraventricular nucleus of the hypothalamus; RHT: retino-hypothalamic tract; SCG : superior cervical ganglia; SCN: suprachiasmatic nuclei. *Revel et al., 2007, adapted from Hoffman & Reiter, 1965.*

The light/dark information is perceived by the retina and transmitted to the master circadian clock located in the hypothalamus, specifically within the suprachiasmatic nuclei (SCN). The photoperiodic information is conveyed to the SCN via the retino-hypothalamic tract (RHT) which represents a fraction of the optic nerve and is mostly constituted by the projections from RGCs. These cells are intrinsically photoreceptive and express the photopigment melanopsin which was originally discovered in the specialized light sensitive cells of frog skin (Provencio et al., 1998, Freedman et al., 1999, Lucas et al., 1999, Mrosovsky et al., 2001, Hattar et al., 2002). The discovery of this new class of photosensitive RGCs led to the concept that the visual system is composed of two photoreceptive systems: an image-forming system and a non-image-forming system. The image-forming system relies on rods and cones for the detection of colours, shapes, and movements of objects in the environment. In contrast, the non-image-forming system relies mostly on intrinsically photoreceptive RGCs to detect the gross changes in luminance in the environment to adjust the biological clock, as well as the pupillary light reflex and other behavioural and physiological responses (Panda et al., 2002, Ruby et al., 2002, Berson, 2003, Gooley et al., 2003, Hattar et al., 2003, Panda et al., 2003). However, melanopsin knock-out (KO) mice remain entrained by light (Panda et al., 2002, Ruby et al., 2002) whereas rods/cones/melanopsin KO mice do not (Hattar et al., 2003, Panda et al., 2003), suggesting that the image-forming system is also involved somehow in conveying the light information to the SCN.

Melanopsin-containing RGCs send direct projections to the SCN via the RHT (Moore and Lenn, 1972) which releases glutamate (Ebling, 1996) and pituitary adenylate cyclase-activating polypeptide (PACAP) (Hannibal et al., 1997) as neurotransmitters (Figure 4). Studies using glutamate and glutamate receptor agonists and antagonists have shown that glutamate is the main neurotransmitter conveying photic information from the RHT to the SCN (Ding et al., 1994, Abe and Rusak, 1994, Mintz et al., 1999). Depolarization of melanopsin-containing RGCs by light induces glutamate release from RHT axon terminals, which then binds to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. This induces a depolarization in SCN neurons, causing a Ca^{2+} influx that activates intracellular signalling pathways.

The RGCs also project to the SCN via the geniculohypothalamic tract (GHT), a distinct indirect pathway originating in the intergeniculate leaflet (IGL) (Card and Moore, 1989), which releases neuropeptide Y (NPY) and GABA (Figure 4)(Moore and Speh, 1993).

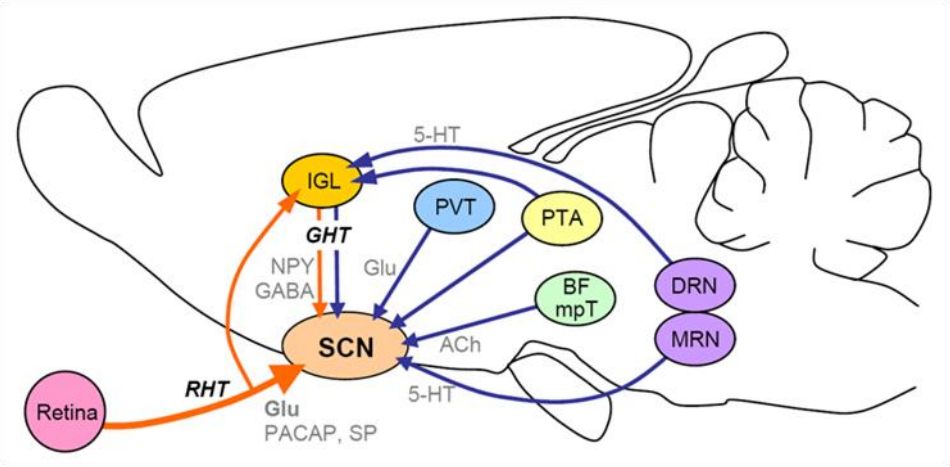

FIGURE 4 - MAJOR SCN AFFERENT PROJECTIONS

The orange and blue arrows represent photic and non-photic inputs respectively. Melanopsin-containing RGCs send direct projections to the SCN via the RHT. They also project indirectly to the SCN via the GHT which originates in the IGL. The MRN sends direct projections to the SCN and the DRN projects indirectly to the SCN via the IGL.

5-HT: 5-hydroxytryptamine, or serotonin; Ach: acetylcholine; BF: basal forebrain; DRN: dorsal raphe nucleus; GABA: γ-aminobutyric acid; GHT: geniculo-hypothalamic tract; Glu: glutamate; IGL: intergeniculate leaflets; mpT: mesopontine tectum; MRN: medial raphe nucleus; NPY: neuropeptide Y; PACAP: pituitary adenylate cyclase-activating polypeptide; PTA: pretectal area; PVT: paraventricular nucleus of the thalamus; RHT: retino-hypothalamic tract; SCN: suprachiasmatic nucleus; SP: substance P.

The SCN are the seat of the master circadian clock in most vertebrates, including mammals. The SCN are two small nuclei located in the anterior part of the hypothalamus, just above the optic chiasm and bilateral to the third ventricle. They are composed of a ventrolateral and a dorsomedial part which are coupled by a GABAergic (γ-aminobutyric acid) mechanism (Moore and Speh, 1993). The dorsomedial neurons express arginine vasopressin (AVP) in a circadian manner with a peak during the light phase (Tominaga et al., 1992, Abrahamson and Moore, 2001, Moore et al., 2002), whereas ventrolateral neurons mainly express vasoactive intestinal peptide (VIP) with a peak during the dark phase (Abrahamson and Moore, 2001). The ventrolateral SCN is involved in relaying the light information throughout the SCN, whereas the dorsomedial SCN have an endogenous 24-hour rhythm that can persist under constant darkness.

Initial studies, showing that electrolytic lesion of the SCN produced arrhythmicity, suggested that the SCN were a necessary component of the central circadian oscillator (Moore and Eichler, 1972, Stephan and Zucker, 1972). It was later shown, both *in vivo* and *in vitro*, that the SCN contain an autonomous circadian pacemaker (Schwartz and Gainer, 1977, Inouye and Kawamura, 1979, Green and Gillette, 1982, Groos and Hendriks, 1982, Shibata et al., 1982, Prosser et al., 1989) and subsequent studies went on to demonstrate that transplanted SCN can restore circadian function in SCN-lesioned animals (Lehman et al., 1987, Ralph et al., 1990). Moreover, single SCN cells exhibit independent rhythms in their firing rate when cultured *in vitro*, indicating that each individual cell contains the molecular machinery required to generate circadian oscillations (Welsh et al., 1995).

The circadian rhythm in the SCN is controlled by clock proteins, which dimerise cyclically in molecular feedback loops with a period of approximately 24 hours (Figure 5). In the first loop, two transcriptional activators Bmal1 (brain and muscle ARNT-like protein 1) and Clock form heterodimers in the cytoplasm and enter the nucleus where they bind to E-box sequences in the promoters of *Period* (*Per1*,*2*) and *Cryptochrome* (*Cry1*,*2*) genes, contributing to the activation of their expression. In the cytoplasm various combinations of Per and Cry proteins interact with each other, enter the nucleus

and inhibit the activity of Bmal1/Clock complex, hence Per and Cry proteins shut off their own transcription. A second loop regulates the expression of the *Bmal1* gene. Bmal1/Clock heterodimers bind to E-boxes present in the promoters of genes that encode the retinoic acid-related orphan nuclear receptors Rev-erbα and Rorα, which compete for the ROR element (RORE) in the *Bmal1* promoter. Rorα activates *Bmal1* expression, while Rev-erbα represses it.

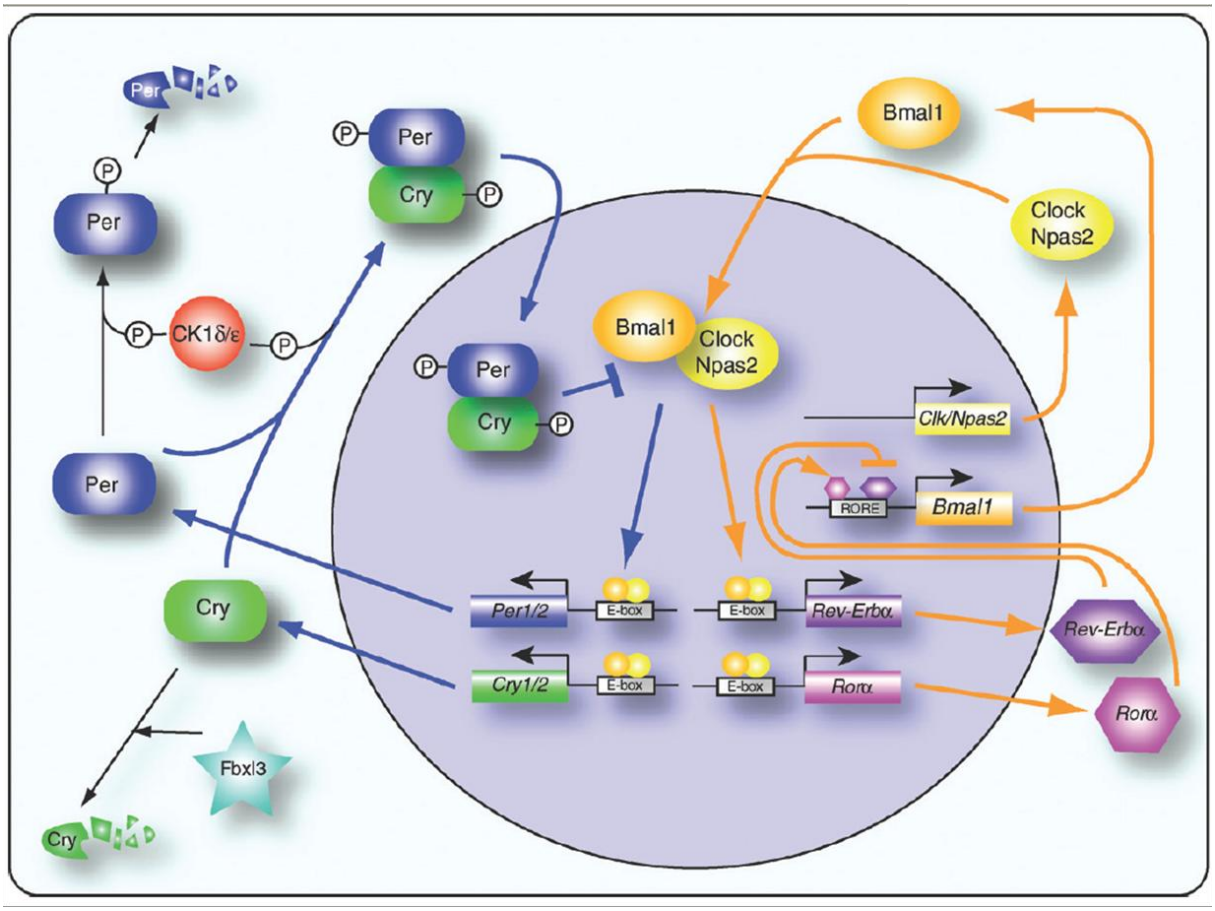

FIGURE 5 - MOLECULAR MACHINERY OF THE MAMMALIAN CIRCADIAN CLOCK LOCATED IN THE SCN

Clock and Bmal1 dimerise and stimulate the transcription of *Per*, *Cry*, *Rev-erbα* and *Rorα* genes. In turn, Per and Cry form dimers and inhibit the activity of the Bmal1/Clock complex, and therefore repress their own synthesis. Rev-erbα and Rorα activate and repress *Bmal1* expression, respectively. *Albrecht & Ripperger.*

The expression of most clock genes and clock-controlled genes is dependent on the time of day, and the circadian clock is entrained to a 24 hour cycle by the light-induced release of glutamate and PACAP (although metabolic factors are also able to synchronise the clock). However, the light/dark cycle undergoes seasonal changes and this photoperiodic variation induces a differential 24 hour expression of clock and clock-controlled genes in the SCN of mammals (Figure 6)(Messager et al., 2000, Steinlechner et al., 2002, Lincoln et al., 2002, Sumova et al., 2003, Tournier et al., 2003, Tournier et al., 2007). These studies have described differential patterns of expression of clock and clock-controlled genes in animals acclimated to LD or SD conditions, indicating that the SCN are able to integrate photoperiodic information. Moreover, it has been shown that the photosensitive phase of the SCN, which is the time frame during which a light pulse induces c-Fos expression in the SCN, is modulated by photoperiod. Indeed, exposure of rodents to a light pulse during the dark phase induces c-Fos expression in the SCN, whereas the same protocol carried out during the subjective day has no effect on c-Fos in the SCN (Rusak et al., 1990). Interestingly, in animals maintained in a SD photoperiod, the photosensitive phase is longer than in animals maintained in a LD photoperiod (Sumova et al., 1995, Vuillez et al., 1996), further supporting the concept that the SCN integrate day-length.

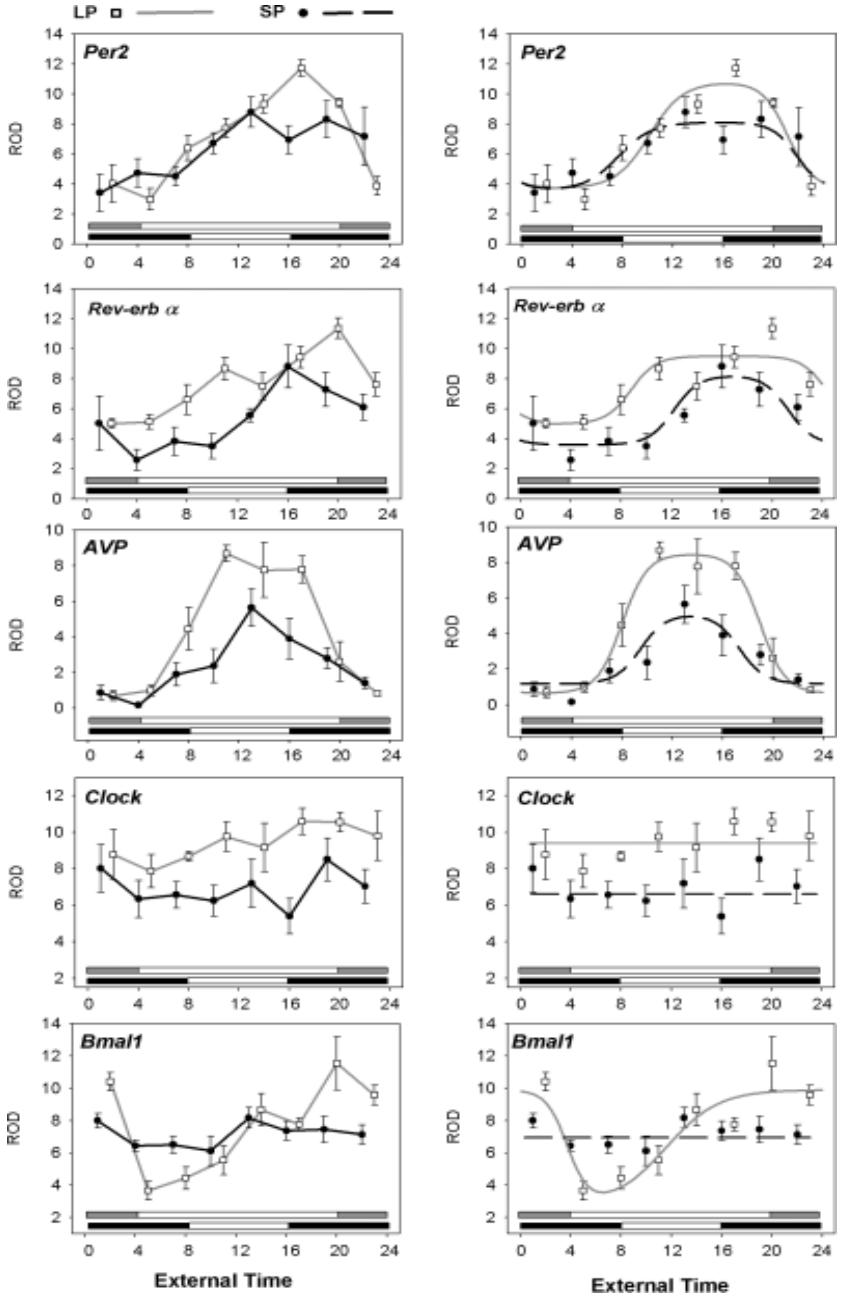

FIGURE 6 - PHOTOPERIOD AFFECTS DIURNAL EXPRESSION OF CLOCK AND CLOCK-CONTROLLED GENES IN THE SUPRACHIASMATIC NUCLEI OF EUROPEAN HAMSTERS

Horizontal solid and open bars represent dark and light phases of the light/dark cycle, respectively. ROD, relative optical density. Left: mRNA levels determined by *in-situ* hybridization in the SCN in either long (LP) (gray line and □) or short (SP) (black line and •) natural photoperiod. Each time point represents the mean ± SEM of four to six European hamsters. Right: Non-linear regression of mRNA levels in either LP (gray line and □) or SP (black line and •). *Tournier et al., 2007*.

FROM THE SCN TO THE PINEAL GLAND

The SCN relay the photoperiodic information via two pathways:

- Through the release of diffusible factors, such as TGFα or prokineticin-2, molecules which are thought to be involved in the regulation of locomotor activity (Kramer et al., 2001, Snodgrass-Belt et al., 2005, Cheng et al., 2002, Zhou and Cheng, 2005).
- Via neural connections to multiple hypothalamic brain areas, to control endocrine and autonomic functions (Buijs and Kalsbeek, 2001, Kalsbeek and Buijs, 2002). The SCN direct the 24-hour secretion rhythm of various hormones, including MEL, which will be the focus in this part.

The paraventricular nucleus of the hypothalamus (PVN) is the main relay between the SCN and the pineal gland. Indeed, lesions of the PVN abolish the rhythm of MEL synthesis in the pineal gland (Klein et al., 1983), an electrical stimulation of SCN cells activates PVN neurons (Hermes et al., 1996) and VIP or VP administration in the PVN modulates MEL release (Kalsbeek et al., 1993). GABA appears to be involved in transmitting signals from the SCN to the PVN since infusion of a GABA antagonist during the subjective day in the PVN area stimulates MEL synthesis, whereas infusion of GABA during the night inhibits night-time MEL secretion (Kalsbeek et al., 1996, Kalsbeek et al., 1999, Kalsbeek et al., 2000). More recently, glutamatergic signalling within the PVN was shown to play a role in MEL synthesis (Perreau-Lenz et al., 2004).

Retrograde tracing studies have shown that the PVN controls MEL synthesis from the pineal gland through a multisynaptic pathway (Larsen et al., 1998, Larsen, 1999). PVN neurons form AVPergic and oxcytocinergic synaptic contacts with cells in the intermediolateral nucleus (IML) of the upper thoracic spinal cord (Teclemariam-Mesbah et al., 1997). The IML neurons connect via cholinergic fibres to postsynaptic sympathetic neurons in the superior cervical ganglia (SCG) (Strack et al., 1988) which constitute the final input to the pineal gland via noradrenergic fibres (Larsen, 1999). Noradrenalin is a very potent stimulator of MEL synthesis.

MELATONIN SYNTHESIS

In mammals, MEL is mainly synthesised by the pineal gland although other peripheral organs, notably the retina and the Harderian gland, also produce low levels of the hormone which are not released into the blood. In rodents, the pineal gland is located at the intersection of the cerebral hemispheres and the cerebellum and is innervated with nervous fibres of various origins which contain a variety of neurotransmitters, but the main neurotransmitter is noradrenalin (Simonneaux and Ribelayga, 2003). Sympathetic noradrenergic fibres originating in the SCG stimulate the synthesis of MEL via two types of adrenergic receptors (α1 and β1). MEL is an amphiphilic molecule and is therefore immediately released by the pineal gland, and because its plasmatic half-life is very short (approximately 20 minutes), the changes in circulating MEL levels are rapid and dynamic. MEL is secreted exclusively at night and for a duration proportional to night length, and is responsible for spreading the photoperiodic message to various organs.

MEL is synthesised from the amino acid tryptophan. Tryptophan is converted into serotonin and serotonin is converted into MEL, via the successive actions of the enzymes AA-NAT (aralkylamine N-acetyltransferase) and HIOMT (hydroxyindole O-methyltransferase) (Figure 7). The activity of AA-NAT displays circadian variations of high amplitude (Klein and Weller, 1970, Gastel et al., 1998), whereas HIOMT activity is very stable over a 24-hour period and varies according to photoperiod (Ribelayga et al., 1999a, Ribelayga et al., 1999b). Therefore, AA-NAT drives the daily rhythm in MEL secretion, and is considered as the MEL "rhythm-generating enzyme", whereas HIOMT is involved in the photoperiodic modulation of the amplitude of the nocturnal MEL peak (Figure 7).

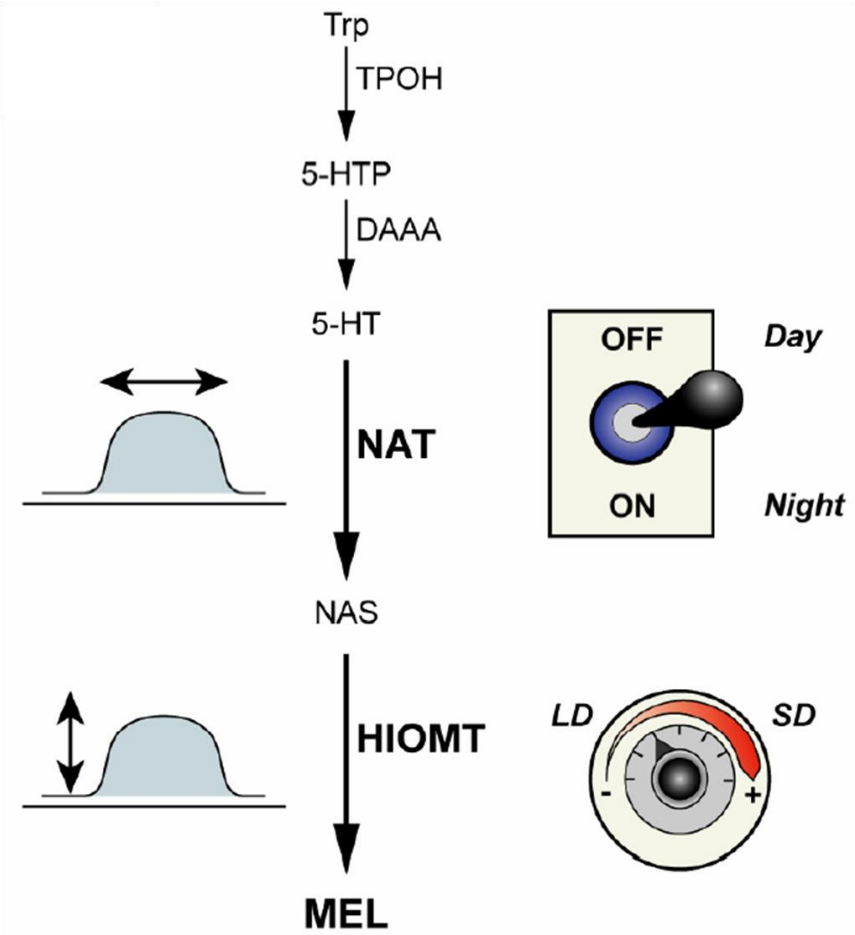

FIGURE 7 - AA-NAT AND HIOMT REGULATE THE DAILY AND PHOTOPERIODIC PATTERNS OF MELATONIN SYNTHESIS

Model for the respective roles of AA-NAT and HIOMT in the daily and photoperiodic regulation of melatonin synthesis. The marked onset of AA-NAT activity at the beginning of the night and its offset at the end of the night drive the duration of the nocturnal melatonin peak, whereas photoperiodic variations of HIOMT activity, with lower values in LD, drive the amplitude of the nocturnal peak of melatonin.

5-HT: 5-hydroxytryptamine, or serotonin; 5-HTP: 5-hydroxytryptophan; LD: long photoperiod; MEL: melatonin; NAS: N-acetylserotonin; SD: short photoperiod; Trp: tryptophan.

MEL is released into the bloodstream and into the cerebrospinal fluid (CSF), and can therefore act on peripheral organs as well as central targets. The precise localisation of MEL receptors has proven complicated, because no antibody is available and because the level of expression of the receptor in the brain is too low for in situ hybridisation detection. In the brain, two types of MEL binding sites have been identified using a radioiodinated MEL ligand: low-affinity and high-affinity sites. The low-affinity binding sites are no longer considered to be MEL-binding receptors and have since been described as the quinone reductase 2 enzyme (Nosjean et al., 2000).

Following the binding studies, cloning of the genes coding for MEL receptors made it possible to identify three types of G-protein-coupled high-affinity receptors, which are coupled negatively to the adenylate cyclase system ($G_{i/o}$ subunit):

- MT1 (Reppert et al., 1994): widely expressed throughout the brain, with significant species-dependent differences in the distribution.
- MT2 (Reppert et al., 1995): localised mainly in the retina, but also in the SCN. Interestingly, this subtype is not functional in Syrian and Siberian hamsters (Weaver et al., 1996), suggesting that this receptor is not critical for the regulation of seasonal functions.
- Mel_{1c} (Ebisawa et al., 1994): only found in non-mammalian vertebrates (Reppert et al., 1996).

As mentioned above, the distribution of MEL receptors varies greatly depending on species. A large number of species have been investigated, and MEL binding sites have been identified in over 100 central and 30 peripheral structures (Masson-Pevet and Gauer, 1994, Morgan et al., 1994, Morgan and Mercer, 1994, Vanecek, 1998). Only two structures have been found to consistently contain MEL receptors in mammals: the SCN and the pars tuberalis of the adenohypophysis (PT) (Morgan and Williams, 1989, Bartness et al., 1993, Masson-Pevet and Gauer, 1994, Morgan et al., 1994, Morgan and Mercer, 1994, Masson-Pevet et al., 1996). In the Syrian hamster, in addition to the SCN and the PT, MEL binding sites have been identified in the dorsomedial nucleus of the hypothalamus (DMH), PVN, paraventricular nucleus of the thalamus (PVT) and the

medial part of the lateral habenular nucleus (Figure 8) (Weaver et al., 1989, Williams et al., 1989).

As previously mentioned, MEL plays a crucial role in the seasonal regulation of reproduction. Indeed, removal of the pineal gland prior to exposure to SD conditions prevents the SD-induced gonadal regression in the Syrian hamster (Czyba et al., 1964, Hoffman and Reiter, 1965), indicating that MEL is responsible for transmitting the photoperiodic information to the reproductive axis. However, the site(s) of action of MEL for the seasonal regulation of reproduction are still a matter of debate, and this issue will be addressed in further detail later in this manuscript.

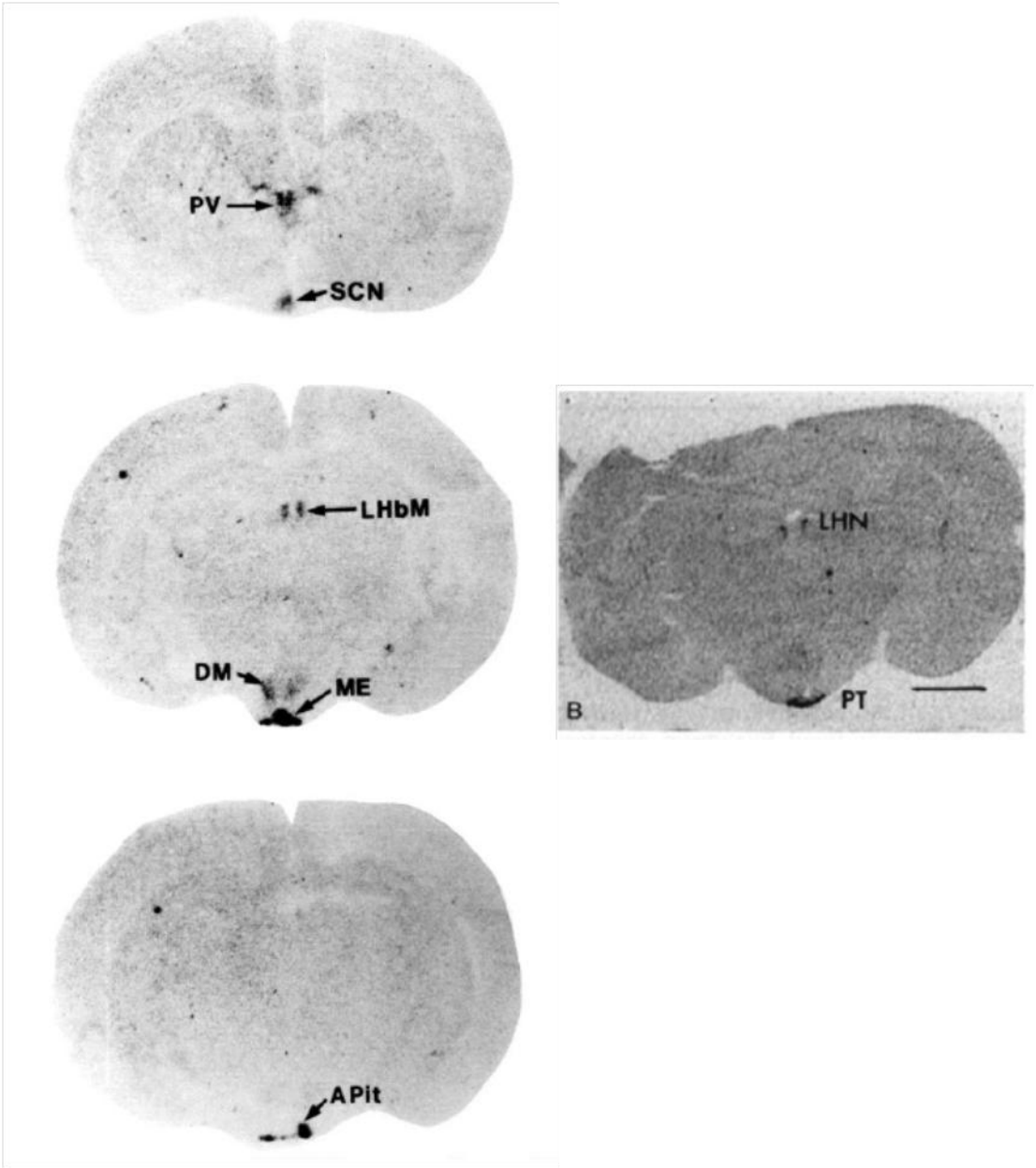

FIGURE 8 - DISTRIBUTION OF I-MEL BINDING SITES IN THE SYRIAN HAMSTER BRAIN

Left panel. Autoradiograms illustrate the areas of specific I-MEL binding. Nonspecific binding was homogeneous and equalled section background. APit: anterior pituitary gland; DM: dorsomedial nucleus of the hypothalamus; LHbM: medial part of the lateral habenular nucleus; ME: median eminence; PV: paraventricular nucleus of the thalamus; SCN: suprachiasmatic nucleus. *Weaver at al., 1989*. The labelling of the *pars tuberalis* of the adenohypophysis was mistaken for labelling in the median eminence in this study.

Right panel. The pars tuberalis (PT) of the pituitary which adheres to the median eminence at the base of the hypothalamus is darkly labelled compared to the medial region of the lateral habenular nuclei (LHN) which are present in the same section. Bar: *2* mm. *Williams et al., 1989*.

THE MAMMALIAN HYPOTHALAMIC-PITUITARY-GONADAL AXIS

Reproductive activity is controlled by the hypothalamic-pituitary-gonadal (HPG) axis in which gonadotrophin-releasing hormone (GnRH) neurons located in the hypothalamus control the production and release of gonadotrophins from the anterior pituitary, which subsequently regulate gonadal function (Figure 9).

GNRH NEURONS

The GnRH neurons (~1000 in higher mammals) are scattered throughout the preoptic area (POA), the diagonal band of Broca and the organum vasculosum of the lamina terminalis (Witkin et al., 1982, Merchenthaler et al., 1984, Wray and Hoffman, 1986). GnRH is a decapeptide which is released in pulses from the nerve terminals located in the external part of the median eminence (Hahn and Coen, 2006) into the hypophyseal portal system. Adequate pulsatile secretion of GnRH is mandatory for proper attainment and maintenance of reproductive function (Knobil et al., 1980, Kelch et al., 1985, Santoro et al., 1986, Bakker et al., 2010).

The synchronized release of GnRH bursts is due to the GnRH pulse generator, a hypothalamic network that includes GnRH neurons as well as other afferents, and which enables the pulsatile secretion of GnRH (Knobil, 1980). The anatomy of the pulse generator has been the subject of active investigation, and evidence suggests that GnRH secretory patterns are not solely dictated by the intrinsic activity of GnRH neurons, but also require the contribution of additional hypothalamic afferents (Maeda et al., 2010, Terasawa et al., 2010). The emerging concept is that the pulsatile secretion of GnRH results from the dynamic balance between excitatory and inhibitory signals (Ojeda et al., 2006, Ojeda et al., 2010, Christian and Moenter, 2010).

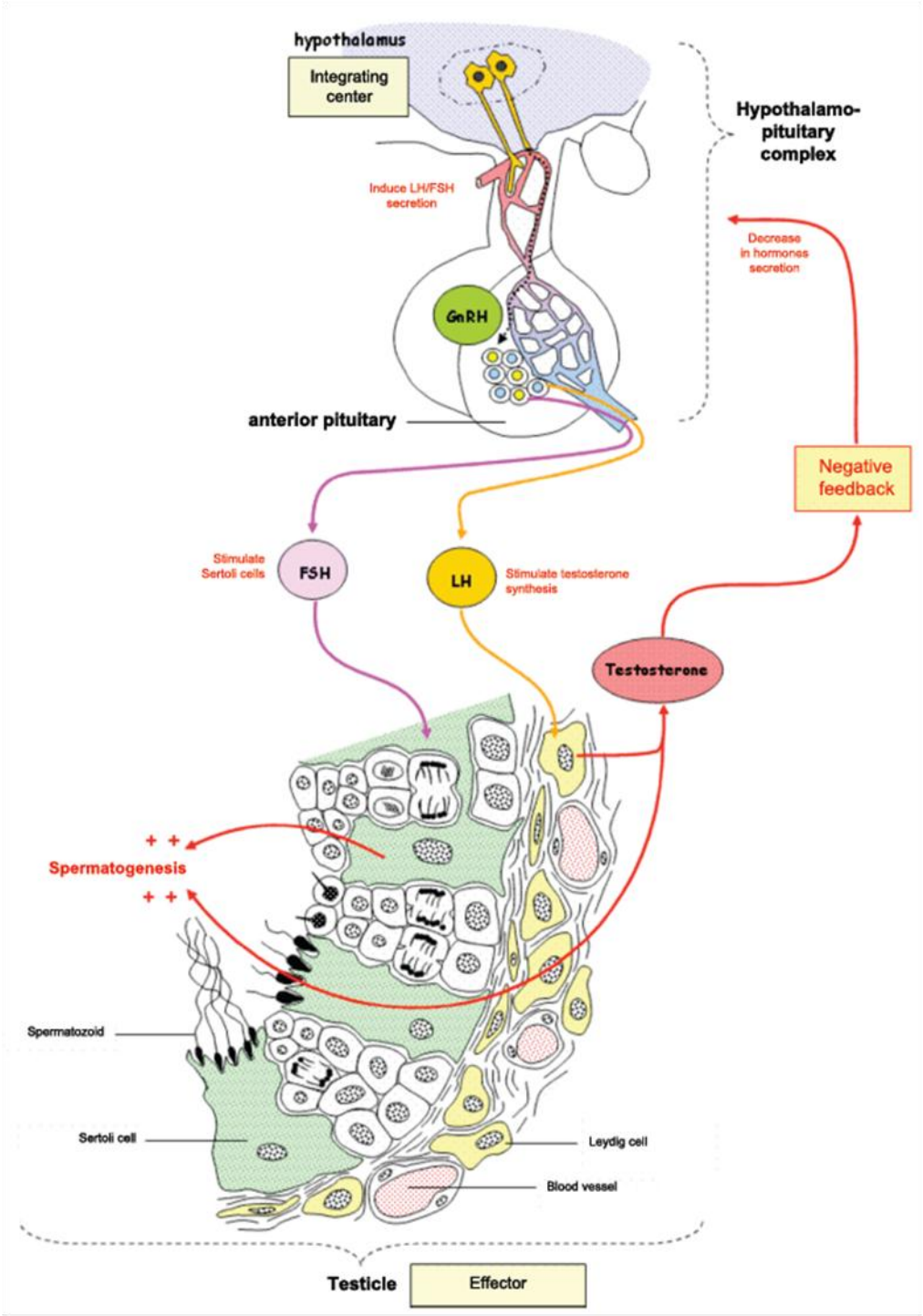

FIGURE 9 - HYPOTHALAMIC-PITUITARY-GONADAL AXIS OF MALES

GnRH neurons release GnRH into the portal blood, where GnRH acts at the level of the anterior pituitary to stimulate the release of LH and FSH into the bloodstream. In turn, LH and FSH act on the testes where testosterone will be produced by the Leydig cells in response to LH and spermatogenesis will be induced in the Sertoli cells in response to FSH. *Adapted from http://svt.ac-dijon.fr/schemassvt.*

GnRH secretion is controlled by a variety of interacting trans-synaptic and glial inputs, such as glial-derived growth factors and glutamate (Ojeda et al., 2006, Ojeda et al., 2010). Moreover, neuronal transmitters also play a central role in synchronizing the pulsatile release of GnRH. Glutamate, norepinephrine, GABA, endogenous opioids, NPY, nesfatin-1, neurokinin B (NKB), kisspeptins (Kp) and RFamide-related peptides (RFRP) have all been shown to regulate GnRH secretion in mammals (Herbison and Moenter, 2011, Clarke et al., 2009, Navarro et al., 2006, Smith and Clarke, 2010, Garcia-Galiano et al., 2010, Pralong, 2010, Lehman et al., 2010).

GNRH SITES OF ACTION AND EFFECTS

The portal blood vessels carry GnRH to the anterior pituitary where it binds to its G-protein-coupled receptor located on the gonadotrophs. GnRH binding sites have also been described in the gonads, placenta, breast and brain, but their precise physiological relevance remains to be elucidated. In the pituitary, the GnRH receptor is coupled to a $G_{q/11}$ protein which activates the phospholipase C pathway, which stimulates the release of intracellular calcium via the protein kinase C and IP3 system. GnRH induces the synthesis and release of gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary into the bloodstream. In males, LH stimulates the production of testosterone by the Leydig cells in the testes and FSH induces spermatogenesis in the Sertoli cells (Figure 9). In females, LH stimulates the production of androgens and estrogens from the theca cells of the ovaries and FSH initiates follicular growth and participates in stimulating ovulation.

SEX STEROIDS FEED-BACK TO THE HPG AXIS

The female reproductive cycle is characterised by variations in pituitary and ovarian hormone levels (Figure 10). The variations in gonadotrophin and sex steroid concentrations are due to feed-back effects of estrogen and progesterone. Indeed, during the first part of the cycle, FSH stimulates the maturation of the follicle, which in turn

produces estrogen. Throughout most of the cycle, estrogen exerts a negative feed-back effect on the brain, therefore maintaining low levels of LH and FSH. However, when estrogen levels rise to a given threshold, the feed-back effect switches from negative to positive and stimulates LH secretion, therefore triggering the preovulatory LH surge leading to subsequent ovulation. If fertilisation does not take place, the corpus luteum begins to degenerate and produces estrogen and progesterone, which in turn apply negative feed-back effects to LH and FSH secretion.

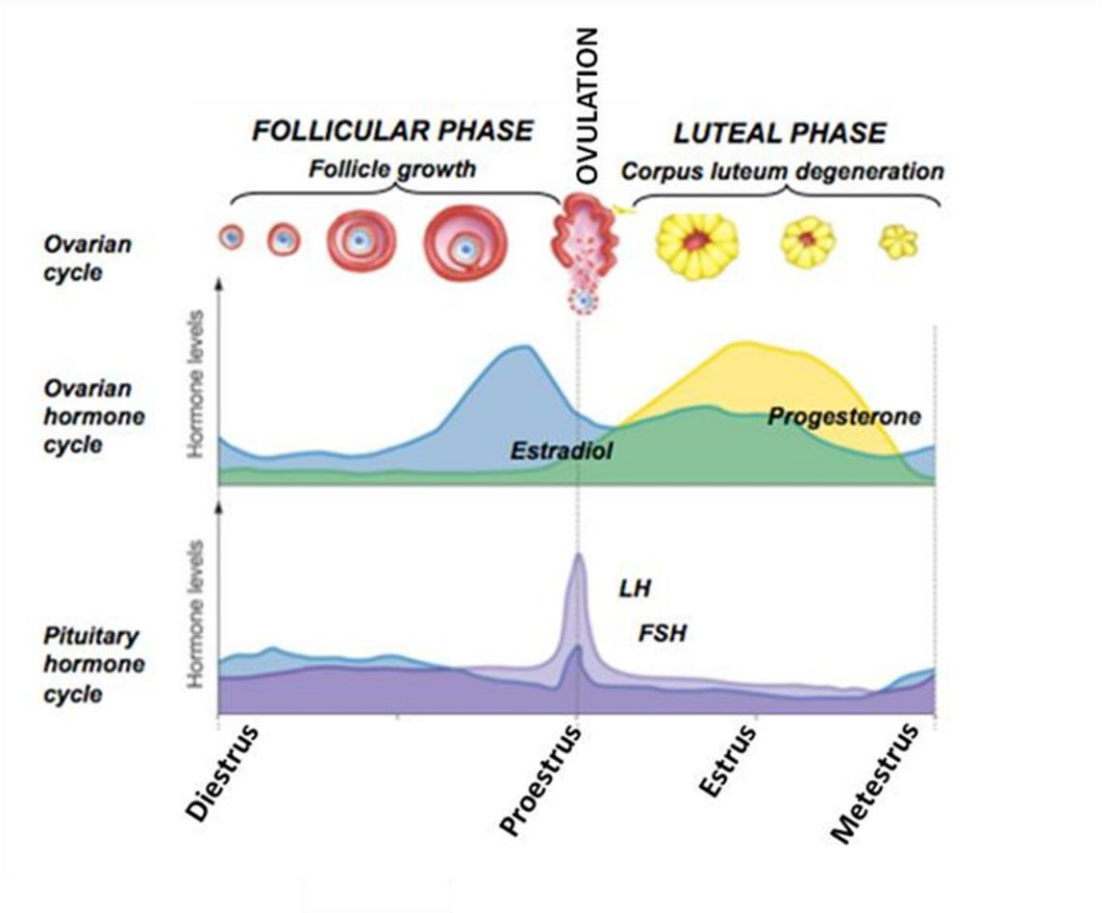

FIGURE 10 - THE FEMALE REPRODUCTIVE CYCLE, OR ESTROUS CYCLE

In female rodents, the estrous cycle lasts approximately 4 days and is composed of 4 stages. During diestrus, FSH stimulates the maturation of the follicle, which in turn produces estrogen. When estrogen levels rise to a given threshold, their feed-back effect switches from negative to positive, and this leads to the preovulatory LH surge which occurs on the day of proestrus, leading to subsequent ovulation. Subsequently, on the day of estrus, females will be sexually receptive. If fertilization does not occur, the corpus luteum degenerates and produces estrogen and progesterone, which exert inhibitory feed-back effects on LH and FSH secretion. *Figure 48-13 from Silverthorn Human Physiology.*

In rodents, the estrous cycle lasts four days and is composed of four stages: diestrus, proestrus, estrus and metestrus. On the day of proestrus, the LH surge which occurs in the late afternoon triggers ovulation. On the following day, females will be sexually receptive and this stage is termed estrus.

In males, testosterone inhibits its own production through inhibitory feed-back effects.

Because GnRH neurons do not contain sex steroid receptors, hypothalamic interneurons must be responsible for transmitting feed-back information to GnRH neurons.

THE SEASONAL REGULATION OF REPRODUCTION

As previously mentioned, wild species have developed a mechanism to restrict their fertility to a certain time of the year, to ensure that the offspring are born during the most favourable season. The seasonal regulation of reproduction therefore ensures species survival.

The Syrian hamster is a long day breeder, and sexual activity is therefore stimulated by exposure to a LD photoperiod. The MEL-induced changes in gonadotrophin levels have been shown to result from changes in hypothalamic function. Indeed, GnRH injections to male Syrian hamsters in LD and SD conditions produce the same effects (Pickard and Silverman, 1979) and cultured anterior pituitaries from SD hamsters can still release LH and FSH in response to GnRH (Bacon et al., 1981, Steger et al., 1983, Steger and Gay-Primel, 1990). This suggests that the photoperiodic regulation of the HPG axis activity is mediated by GnRH neurons. However, MEL binding sites have not been identified in the POA of Syrian hamsters (Weaver et al., 1989, Williams et al., 1989), indicating that GnRH neurons are probably not a direct target of MEL. This is supported by results in the Syrian hamster indicating that photoperiod does not affect *gnrh* mRNA levels (Brown et al., 2001) or the number and morphology of GnRH neurons (Urbanski et al., 1991). Photoperiod, via MEL, would therefore indirectly affect the release of GnRH rather than its production. The mediobasal hypothalamus (MBH) seems to be involved in the photoperiodic regulation of reproduction in the Syrian hamster, as MEL receptors have

been identified in this area and a lesion of this region abolishes the SD-induced gonadal regression (Maywood and Hastings, 1995, Maywood et al., 1996). The precise cellular targets of MEL in the MBH of the Syrian hamster remain unknown, but possible candidates have been identified in recent years and this will be addressed in further detail later in this manuscript. It is worth noting that in Siberian hamsters, lesions of the MBH do not prevent the SD-induced gonadal atrophy, indicating that species-differences might exist in the sites of action of MEL for the control of seasonal reproduction. Interestingly, SCN lesions prevent the MEL-induced gonadal regression, whereas they do not in the Syrian hamster (Bittman et al., 1979, Bittman et al., 1989, Bartness et al., 1991, Bittman et al., 1991, Maharaj et al., 1992, Song and Bartness, 1996).

The sheep is a short day breeder, and decreasing day-lengths in autumn stimulate reproductive activity, whereas exposure to a LD photoperiod inhibits the reproductive function. Unlike the Syrian hamster, sheep possess a circannual clock which is synchronised by photoperiod to adjust the annual rhythms in activity to exactly one year. Indeed, when maintained in constant photoperiodic conditions for a prolonged period, ewes show a cycle of reproductive activity with a period of approximately one year (Karsch et al., 1989, Wayne et al., 1990, Jansen and Jackson, 1993). The mechanisms underlying seasonal reproduction have only been studied in ewes, in which the reproductive cycle lasts 16-18 days during the breeding season, and is composed of four different phases like in hamsters (proestrus, estrus, metestrus and diestrus). During the non-breeding season, or anestrus, no ovarian cyclicity is observed (Thiery et al., 2002). In sheep, MEL is responsible for synchronising the rhythm in reproductive activity, but not for generating it. Indeed, pinealectomy does not prevent the seasonal cycle of reproduction (due to the involvement of a circannual clock), but MEL injections re-synchronise it. The pre-mammillary hypothalamic area could be involved in the MEL-mediated photoperiodic regulation of reproduction, as this region contains MEL receptors (Stankov et al., 1991, Chabot et al., 1998, Migaud et al., 2005) and MEL micro-implants placed in this area induce SD-like changes in gonadotrophin secretion (Malpaux et al., 1998). However, the cells expressing MEL receptors in the pre-mammillary hypothalamic area of the sheep have not been phenotyped, and so the exact site of action of MEL remains unknown.

Because it appears that MEL controls GnRH release upstream from these neurons, work carried out in recent years has focused on identifying potential upstream regulators of GnRH neuronal activity, which could be involved in the photoperiodic regulation of the reproductive function. Two hypothalamic neuropeptides, members of the RFamide family of peptides, have been proposed to mediate MEL's effect on GnRH neurons: kisspeptins and RFamide-related peptide.

DISCOVERY

In 2003, the *GPR54* gene (now named *Kiss1r*), which codes for the receptor for peptides encoded by the *Kiss1* gene, was identified as a central player in the development of puberty. Indeed, a loss-of-function mutation of the *Kiss1r* gene was shown to induce a hypogonadic phenotype in mice and humans (Figure 11) (Seminara et al., 2003, de Roux et al., 2003, Funes et al., 2003).

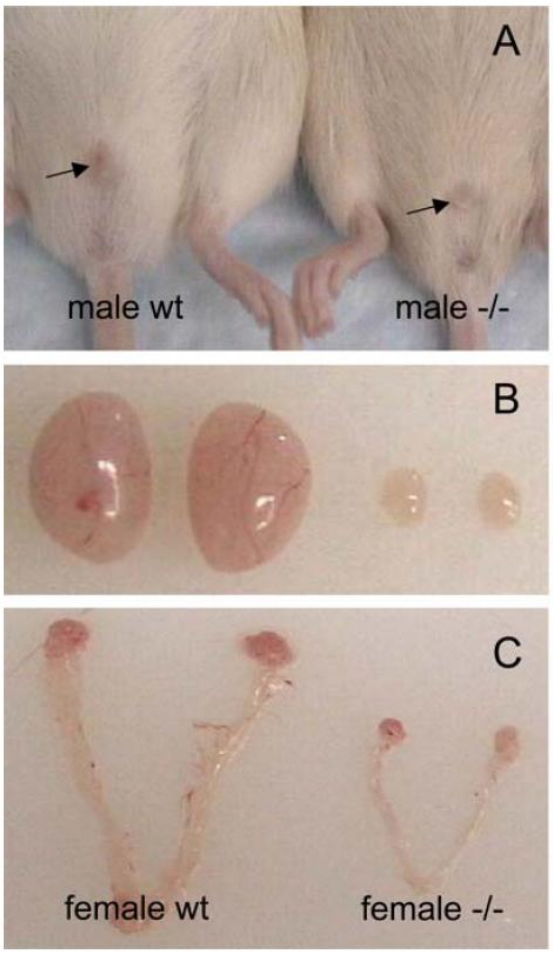

FIGURE 11 - GROSS ANALYSIS OF THE REPRODUCTIVE ORGANS OF 30-DAY-OLD WILD-TYPE AND GPR54 KNOCK-OUT MALE AND FEMALE MICE

(A) External view of male wild-type (wt) and GPR54 (or Kiss1r) knock-out (-/-) mice, showing reduced penis size (arrows). (B) Testes from 30-day-old homozygous mice were reduced in size compared to the wild-type littermates. (C) Uterine horns and ovaries from 30-day-old homozygous mice were reduced in size compared to the wild-type littermates. *Funes et al., 2003.*

It was later shown that *Kiss1r* gene inactivation did not induce defects in GnRH neuronal migration, GnRH synthesis, or pituitary responsiveness to GnRH (Seminara et al., 2003, d'Anglemont de Tassigny et al., 2007). This indicates that the *Kiss1* system is an

essential, excitatory upstream regulator of GnRH neurons and that absence of *Kiss1*/*Kiss1r* signalling results in suppressed GnRH secretion.

KISS1 EXPRESSION AND KISSPEPTIN STRUCTURE

Kiss1 neurons are mainly localised in the arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) of the hypothalamus in rodents (Gottsch et al., 2004, Smith et al., 2005a, Smith et al., 2005b, Revel et al., 2006b, Mason et al., 2007), although smaller populations have been detected in the periventricular nucleus, anterodorsal preoptic nucleus and medial amygdala (Gottsch et al., 2004).

The *Kiss1* gene produces a family of peptides called kisspeptins (Kp), which vary in size from 54 (52 in rodents) to 10 amino acids (Figure 12) (Kotani et al., 2001, Ohtaki et al., 2001, Muir et al., 2001). These peptides belong to the large family of RFamide peptides, which share a common Arg-Phe-NH_2 motif. The Kiss1r (or GPR54) is coupled with a $G_{q/11}$ protein which activates the phospholipase C cascade and ultimately activates protein kinase K (Kotani et al., 2001, Muir et al., 2001, Ohtaki et al., 2001, Castano et al., 2009). The different forms of Kp bind Kiss1r with the same affinity, and induce potent increases in LH and testosterone secretion when administered centrally or peripherally (Gottsch et al., 2004, Matsui et al., 2004, Thompson et al., 2004, Navarro et al., 2005b, Mikkelsen et al., 2009).

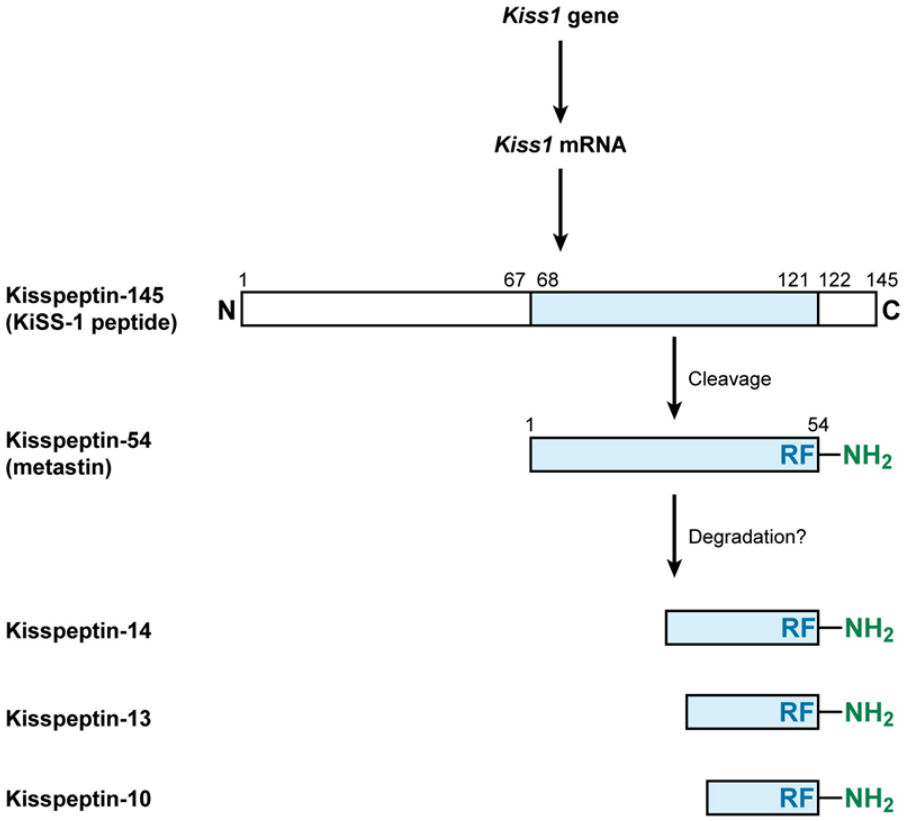

FIGURE 12 - PRODUCTS OF THE *KISS1* GENE

Kiss1 mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid propeptide called kisspeptin-145. Shown are cleavage sites on the propeptide that lead to the production of the RF-amidated kisspeptin-54, also known as metastin. Shorter peptides (such as kisspeptin-10, -13, and -14) were identified by mass spectrometry. These peptides share a common C terminus and RF-amidated motif with kisspeptin-54. Because no putative cleavage sites have been identified on the propeptide that would lead to synthesis of the shorter peptides, such peptides may be degradation products of kisspeptin-54. *Popa et al., 2008.*

KP AND THE REPRODUCTIVE AXIS

The discovery of the effects of *Kiss1r* mutations on puberty onset led to intensive study of the involvement of Kp in the regulation of adult reproductive function. A large number of studies indicate that Kp administration, both centrally and systemically, potently stimulates gonadotrophin secretion in all the mammalian species studied to date, including mice, rats, sheep, goats, pigs, cows, monkeys and humans (Gottsch et al., 2004, Matsui et al., 2004, Navarro et al., 2004, Thompson et al., 2004, Dhillo et al., 2005, Messager, 2005, Messager et al., 2005, Shahab et al., 2005, Ezzat Ahmed et al., 2009, Hashizume et al., 2010, Lents et al., 2008).

Kp stimulate gonadotrophin and gonadal steroid secretion via a direct effect on GnRH neurons. Indeed, Kp-immunoreactive (-ir) fibres are found in close apposition to GnRH cell bodies (Kinoshita et al., 2005, Clarkson and Herbison, 2006) and over 90% of GnRH neurons are depolarised following Kp application in rodents (Han et al., 2005). Moreover, approximately 80% of GnRH neurons express c-Fos after Kp administration and also express *Kiss1r* mRNA (Herbison et al., 2010, Irwig et al., 2004, Han et al., 2005, Messager et al., 2005). In addition, the effects of Kp on gonadotrophin secretion are completely abolished by pretreatment with GnRH receptor antagonists (Gottsch et al., 2004, Matsui et al., 2004, Navarro et al., 2005a, Navarro et al., 2005b, Shahab et al., 2005). The fact that Kp is able to induce GnRH release from MBH explants which contain GnRH nerve terminals (but not cell bodies) (d'Anglemont de Tassigny et al., 2008) has led to the speculation that Kp could act both at the level of GnRH cell bodies in the hypothalamus and nerve terminals in the median eminence. However, because there is no selective antibody for the Kiss1r, the presence (or not) of this receptor in the median eminence is difficult to assess.

Recent evidence from mouse, rat, goat and monkey studies indicates that a majority of *Kiss1* neurons co-express NKB and Dynorphin. They are thus named KNDy neurons. These KNDy neurons could be a component of the GnRH pulse generator (Ramaswamy et al., 2008, Roseweir et al., 2009, Martinez-Chavez et al., 2008, Navarro et al., 2009, Wakabayashi et al., 2010, Choe et al., 2013).

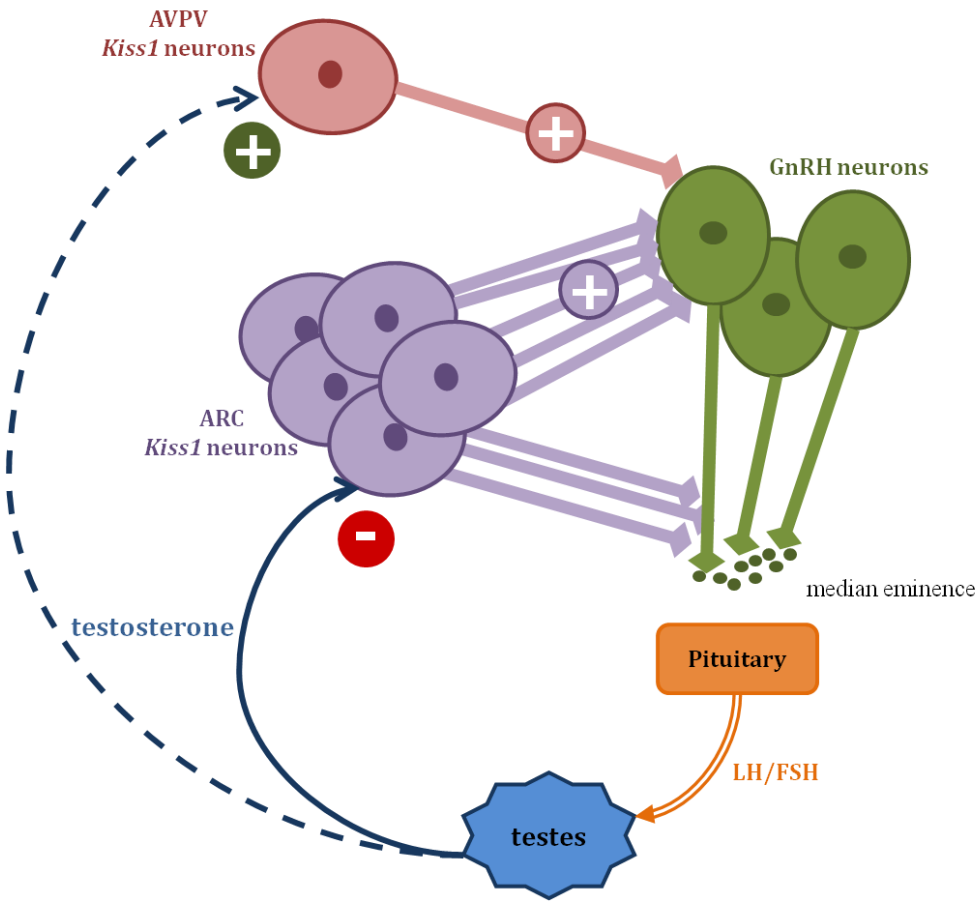

FIGURE 13 - *KISS1* SIGNALLING IN THE MALE RODENT BRAIN

Kiss1 neurons in the ARC drive GnRH pulsatile release and subsequent LH and FSH secretion from the anterior pituitary. They are the site of action of testosterone for its inhibitory feed-back effects on GnRH secretion. In the male rodent, the AVPV *Kiss1* neuronal population is very sparse, and therefore although their expression is increased by testosterone, this positive feed-back effect presumably has little functional significance in males.

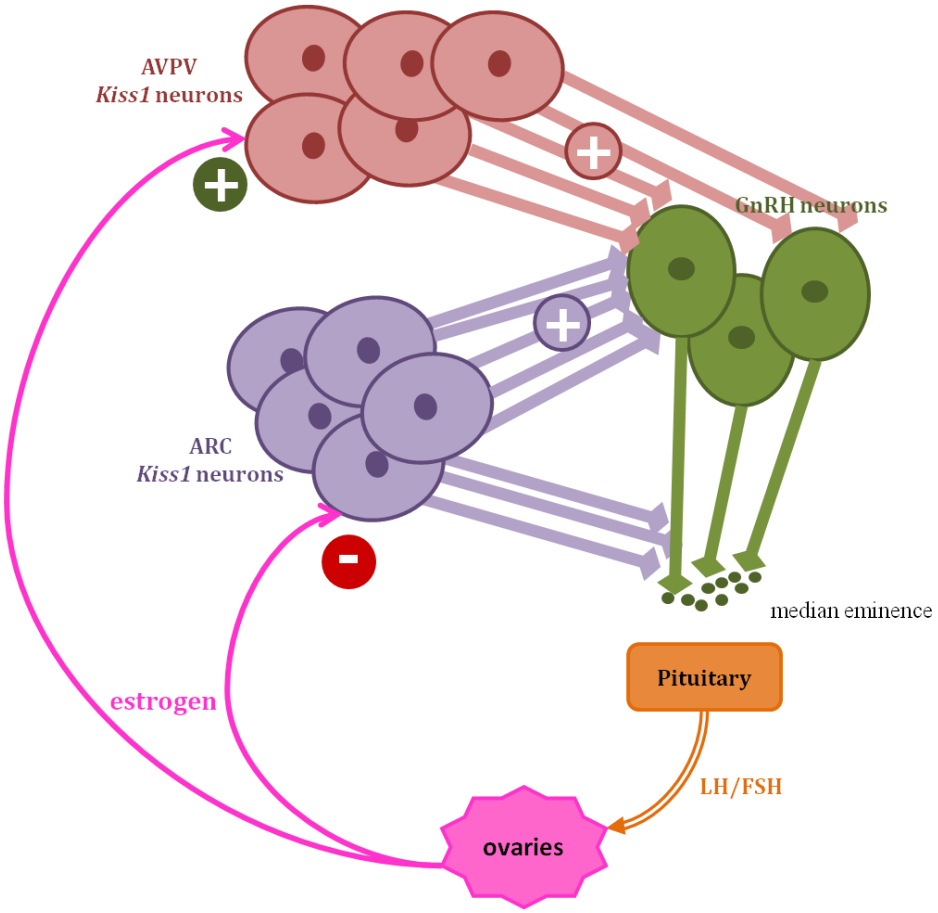

FIGURE 14- *KISS1* SIGNALLING IN THE FEMALE RODENT BRAIN

In female rodents, ARC and AVPV *Kiss1* neuronal populations play different roles in the estrogen-mediated feed-back effects regulating GnRH secretion. The ARC neurons are the site of action of the negative feed-back effect of estrogen and progesterone during the luteal phase. During the follicular phase, the negative feed-back effect of estrogen switches to a positive feed-back effect; the AVPV *Kiss1* neurons are therefore responsible for generating the preovulatory GnRH and subsequent LH surges.

KP NEURONS AND SEX STEROID FEED-BACK EFFECTS

It is well established that the positive and negative feed-back effects of sex steroids are mediated via the GnRH neurons, however these neurons do not express sex steroid receptors (Shivers et al., 1983, Fox et al., 1990, Leranth et al., 1992, Huang and Harlan, 1993, Herbison et al., 1996, Skinner et al., 2001), indicating that there must be at least one intermediate in transmitting the feed-back effects of gonadal hormones.

Accumulating evidence indicates that the *Kiss1* neurons in the hypothalamus are the site of action of sex steroids for the central feed-back effects. Interestingly, the ARC and AVPV neuronal populations are involved in different aspects of the feed-back effects of gonadal hormones. In the ARC, *Kiss1* neurons co-express both estrogen receptors (ER) and androgen receptors (AR) (Smith et al., 2005b), and this neuronal population has been shown to mediate the negative feed-back effects of gonadal hormones (Figures 13 & 14). Indeed, in both male and female rodents, sex steroids inhibit arcuate *Kiss1* expression whereas gonadectomy increases *Kiss1* mRNA levels (Navarro et al., 2004, Irwig et al., 2004, Smith et al., 2005a, Smith et al., 2005b, Revel et al., 2006b, Ansel et al., 2010). The AVPV neuronal population is sexually dimorphic, with a high number of neurons in females versus a low number in males (Figure 15) (Clarkson and Herbison, 2006, Kauffman et al., 2007). Moreover, the number of *Kiss1* neurons and their activation are increased in the AVPV at the time of the preovulatory LH surge (Smith et al., 2006b, Robertson et al., 2009). In addition, administration of a Kp antagonist blocks the preovulatory LH surge in rats (Pineda et al., 2010a). It has been suggested that the *Kiss1* neurons of the AVPV are involved in the positive feed-back effect of estrogen, because gonadectomy reduces *Kiss1* expression whereas sex steroid treatment up-regulates it in mice and hamsters (Smith et al., 2005a, Smith et al., 2005b, Ansel et al., 2010). This is supported by the observation that AVPV *Kiss1* neurons express ERα (Smith et al., 2005a, Smith et al., 2005b). Taken together, these data indicate that the ARC *Kiss1* neurons mediate negative feed-back effects of sex steroids in male and female rodents, whereas the AVPV *Kiss1* neurons are responsible for mediating the positive feed-back effects of sex steroids in females, and therefore play a central role in generating the preovulatory LH surge (Figure 14).

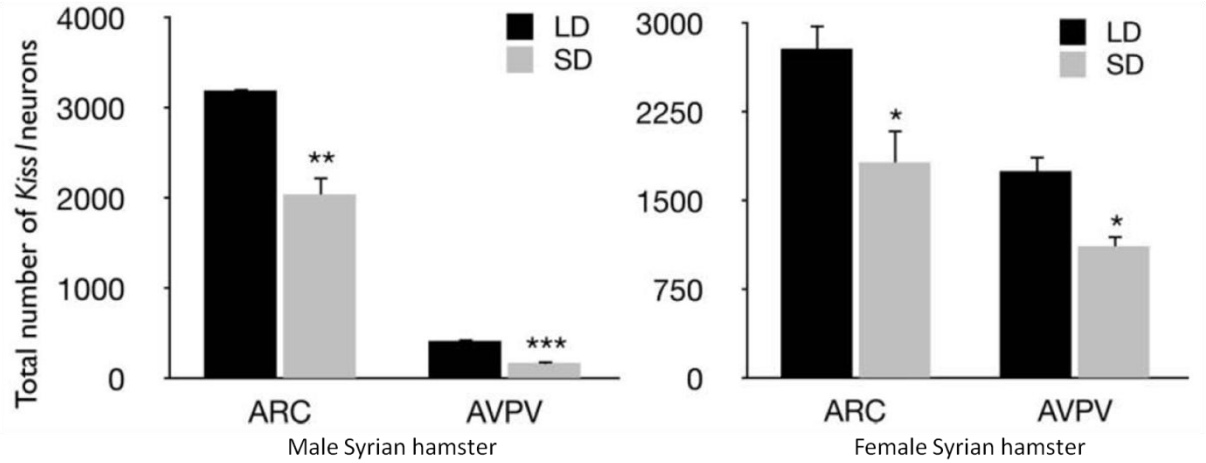

FIGURE 15 - EFFECT OF PHOTPERIOD AND GENDER ON *KISS1* EXPRESSION IN THE ARC AND AVPV

In both male and female Syrian hamsters, *Kiss1* expression is down-regulated in SD conditions. The AVPV neuronal population is sexually-dimorphic, with higher cell numbers in females than in males in both LD and SD conditions. *Ansel et al., 2010.*

KISS1 AND SEASONAL REPRODUCTION

In the Syrian hamster, Kp have been shown to play a central role in the seasonal regulation of reproduction (Revel et al., 2006a). In this species, *Kiss1* expression is down-regulated in SD through two different mechanisms in the ARC and the AVPV (Figure 15). In the ARC, MEL is responsible for the decrease in *Kiss1* expression in SD, whereas the decrease observed in *Kiss1* expression in the AVPV results from secondary changes in sex steroid feed-back effects (Revel et al., 2006b, Ansel et al., 2010). Indeed, because *Kiss1* expression is down-regulated in the ARC in SD, this results in a decrease in circulating levels of gonadal hormones. In turn, this decrease in circulating sex steroid levels is responsible for the reduction in AVPV *Kiss1* expression, because the positive feed-back effect is suppressed (Ansel et al., 2010). The reduction of *Kiss1* expression in SD conditions is involved in the subsequent inhibition of reproductive activity, because continuous administration of Kp to sexually inactive male Syrian hamsters maintained in SD reactivates the reproductive axis (Revel et al., 2006b). However, the fact that the ARC

and AVPV do not contain MEL receptors in the Syrian hamster suggests that there must be another intermediate in the seasonal control of reproduction.

In other seasonal mammals, the *Kiss1*/Kiss1r system has also been shown to be regulated by photoperiod. This is notably the case in the Siberian hamster (Simonneaux et al., 2009, Greives et al., 2007, Mason et al., 2007) and the sheep (Wagner et al., 2008, Smith, 2008, Chalivoix et al., 2010). The involvement of the Kp system in the seasonal regulation of reproduction will be further addressed in the general discussion of this manuscript.

OTHER FUNCTIONS OF *KISS1* NEURONS

Kiss1 neurons have been shown to play a role in modulating reproductive activity in response to metabolic and/or environmental cues. Notably, *Kiss1* neurons might be involved in integrating information regarding metabolic disturbances and adapting reproductive function accordingly (Pinilla et al., 2012). It has also been suggested that *Kiss1* neurons might be involved in the functional changes of the HPG axis observed in conditions of acute stress and immune/inflammatory challenge (Pinilla et al., 2012).

RFAMIDE-RELATED PEPTIDE-3

This chapter has been submitted as a review in Current Trends in Endocrinology: Ancel C & Simonneaux V, *The role of RFamide-related peptide-3 (RFRP-3) in the regulation of the reproductive function: versatile effects and new perspectives*.

The gonadotrophin-releasing hormone (GnRH) neurons in the rostral hypothalamus (preoptic area and organum vasculosum of lamina terminalis) represent the final common pathway in the neural regulation of the hypothalamo-pituitary-gonadal (HPG) axis. These neurons release GnRH into the portal blood system, inducing the downstream secretion of gonadotrophins luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. Finally, LH and FSH regulate the production of sex steroids by the gonads, which will in turn feedback at various levels of the gonadotrophic axis. GnRH neurons are the target of various neurotransmitters, neuropeptides, and peripheral hormones known to modulate their function in order to fine-tune the activity of the HPG axis in accordance with environmental, metabolic and endocrine signals.

In recent years, the characterisation of GnRH neuron activity regulators has significantly progressed, notably with the discovery of kisspeptins (Kp). In 2003, two studies concurrently indicating that the Kp receptor (Kiss1R), and therefore its ligands, were essential for normal reproduction (de Roux et al., 2003, Seminara et al., 2003) prompted intensive research on the involvement of Kp in the regulation of the HPG axis. The presence of Kiss1R in GnRH neurons (Irwig et al., 2004, Han et al., 2005) and the fact that Kp fibres come into close apposition to GnRH cell bodies in the preoptic area and fibres in the median eminence (Ramaswamy et al., 2008) suggest that Kp could be acting directly at the level of GnRH neuron cell bodies and via GnRH nerve terminals in the median eminence (d'Anglemont de Tassigny et al., 2008). It has now been thoroughly demonstrated that Kp are potent stimulators of HPG axis activity and that they are central gatekeepers of key aspects of reproductive function (Pinilla et al., 2012).

Although it is now apparent that Kp are central players in the regulation of GnRH neuron activity, other modulators of HPG axis activity have been identified. Notably, novel

peptides of the RFamide family of peptides of which Kp is also a member, which share a common C-terminal LPXRFamide (X=L or Q) motif, have been identified in mammals. RFamide-related peptide-1 and -3 (RFRP-1 and RFRP-3) were isolated in mammals in 2000 (Hinuma et al., 2000) and since then a large number of studies have sought to identify the role of these peptides in the regulation of endocrine functions.

DISCOVERY, LOCALISATION AND SITES OF ACTION OF RFRP-3

DISCOVERY AND EVOLUTIONARY HISTORY

RFRP-3 is part of the large family of RFamide peptides, which share a common Arg-Phe-NH_2 motif at their C-terminus. The first RFamide peptide was discovered in the clam *Macrocallista nimbosa* and reported to exert cardioexcitatory effects (Price and Greenberg, 1977a). Since then, other RFamide peptides have been isolated from invertebrates (Walker, 1992) and the first report of an RFamide peptide in a vertebrate was made some 30 years ago: LPLRFamide was isolated from chicken brain (Dockray et al., 1983) and shown to have vasopressor and stimulatory effects on neurons in mammals (Price and Greenberg, 1977b, Barnard and Dockray, 1984). Since then at least five different genes encoding RFamide peptides have been identified in mammals: PrRP, NPFF, QRFP/26RFa, Kp and RFRP (Yang et al., 1985, Perry et al., 1997, Hinuma et al., 1998, Panula et al., 1999, Hinuma et al., 2000, Liu et al., 2001, Kotani et al., 2001, Ohtaki et al., 2001, Chartrel et al., 2003, Fukusumi et al., 2003, Jiang et al., 2003). One of these genes, *RFamide-related peptide* (*rfrp*), was identified in mammals in 2000 (Hinuma et al., 2000) concurrently with the discovery of its avian ortholog, *gonadotrophin-inhibitory hormone* (*gnih*) (Tsutsui et al., 2000). The *rfrp* gene encodes a precursor which produces two peptides of various sizes in mammals: RFRP-1 and RFRP-3 (Table 1)(Hinuma et al., 2000). Because GnIH was shown to inhibit gonadotrophin release from cultured quail pituitaries (Tsutsui et al., 2000), the involvement of RFRP-1 and RFRP-3 in the regulation of neuroendocrine functions in mammals was examined. Since an initial study in rats showed that RFRP-1 had no effect on gonadotrophin secretion (Hinuma et al., 2000), studies have aimed at investigating the involvement of RFRP-3 in the regulation of mammalian reproduction. However, recent evidence indicates that the effect of RFRP-1 on the gonadotrophic axis could be species-dependent (Ancel et al., 2012, Ubuka et al.,

2012), and therefore the relative role of this peptide in the regulation of the reproductive function deserves further investigation. Nevertheless, accumulating evidence now indicates that RFRP-3 is involved in the regulation of the hypothalamo-pituitary-gonadal axis in mammals, and this will be addressed in detail in this review.

MPHSFAN**LPLRFa**	Human RFRP-1	Ubuka *et al*., 2009
VPN**LPQRFa**	Human RFRP-3	Ubuka *et al*., 2009
SGRNMEVSLVRQVLN**LPQRFa**	Monkey RFRP-3	Ubuka *et al*., 2009
SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN**LPLRFa**	Bovine RFRP-1	Fukusumi *et al*., 2001
AMAHLPLRLGKNREDSLSRWVPN**LPQRFa**	Bovine RFRP-3	Yoshida *et al*., 2003
SVTFQELKDWGAKKDIKMSPAPANKVPHSAAN**LPLRFa**	Rat RFRP-1*	Hinuma *et al*., 2000
ANMEAGTMSHFPS**LPQRFa**	Rat RFRP-3	Ukena *et al*., 2002
SPAPANKVPHSAAN**LPLRFa**	Siberian hamster RFRP-1	Ubuka *et al*., 2012
TLSRVPS**LPQRFa**	Siberian hamster RFRP-3	Ubuka *et al*., 2012
SPAPANKVPHSAAN**LPLRFa**	Syrian hamster RFRP-1*	Kriegsfeld *et al*., 2006
ILSRVPS**LPQRFa**	Syrian hamster RFRP-3*	Kriegsfeld *et al*., 2006
SIKPSAY**LPLRFa**	Quail GnIH	Tsutsui *et al*., 2000
SLNFEEMKDWGSKNFMKVNTPTVNKVPNSVAN**LPLRFa**	Quail GnIH-RP-1*	Satake *et al*., 2001
SSIQSLLN**LPQRFa**	Quail GnIH-RP-2	Satake *et al*., 2001

Table 1 - Alignment of amino acid sequences of LPXRFa (X = L or Q) peptides in mammals and the quail.

*, Putative LPXRFa peptides hypothesized from their precursor mRNA sequences.

LOCALISATION OF *RFRP* NEURONS IN THE MAMMALIAN BRAIN

Both *in situ* hybridization studies and immunohistochemical mapping experiments have been carried out to localise RFRP-expressing cells, however it is important to bear in mind that the immunohistochemical findings could be affected by a possible variation in specificity of the antibodies used. Indeed, a variety of antibodies has been characterized for the study of RFRP-immunoreactive (-ir), including a polyclonal antibody raised against avian GnIH (Tsutsui et al., 2000), an antiserum against the rat RFRP precursor peptide (Rizwan et al., 2009), a white crowned sparrow GnIH antiserum (Smith et al., 2008, Kriegsfeld et al., 2006) and an antibody raised in guinea pigs against human RFRP-3 (Qi et al., 2009). Moreover, because of the differences in RFRP-like sequences among

mammalian species (Table 1), a given antibody could result in variable labeling from one species to another.

In the mouse brain, RFRP-ir cells have been localised in the diencephalon, pons, medulla and dorsomedial nucleus of the hypothalamus (DMH) (Ukena and Tsutsui, 2001). In rats, RFRP-ir cells are located mainly in the DMH and in regions surrounding the ventromedial nucleus and tuberomammillary nucleus (Johnson et al., 2007, Rizwan et al., 2009). In rats, *rfrp* mRNA has been detected in cells located in the DMH and dorsomedial parts of the ventromedial nucleus with cells extending rostral to the anterior hypothalamus and the ventral perifornical area (Legagneux et al., 2009). In another study, RFRP-ir and mRNA were detected in the DMH in Syrian hamsters, mice and rats (Kriegsfeld et al., 2006, Revel et al., 2008). In Siberian hamsters, RFRP-ir cell bodies are distributed in the medial region of the hypothalamus spanning from the anterior hypothalamic area to the DMH and premammillary nucleus (Ubuka et al., 2012). In sheep, *in situ* hybridisation has shown RFRP-expressing cells in the ventral region of the paraventricular nucleus and DMH (Clarke et al., 2008, Dardente et al., 2008, Smith et al., 2008). A similar distribution was described using immunohistochemistry (Smith et al., 2008, Qi et al., 2009).

Although inter-species differences appear in the distribution of RFRP neurons, possibly due to antibody specificity issues, the MBH, and particularly the DMH, appears to be a key region containing these neurons. This is of interest as the DMH has been implicated in a variety of behavioural and physiological responses, including those associated with feeding, reproduction, stress, circadian rhythms, and thermogenesis. Moreover, the DMH receives inputs from a large number of hypothalamic regions, suggesting that neurons in this area could integrate environmental and physiological signals to regulate endocrine responses.

SITES OF ACTION OF RFRP-3 IN MAMMALS

In various mammalian species including humans, RFRP fibre networks are found in multiple brain regions including the preoptic area, the arcuate nucleus, the lateral septum, the anterior hypothalamus and the bed nucleus of the stria terminalis (Ukena and Tsutsui, 2001, Kriegsfeld et al., 2006, Johnson et al., 2007, Mason et al., 2010).

Notably, RFRP-ir fibres make apparent contact with a subpopulation of GnRH neurons in rodents and sheep (Kriegsfeld et al., 2006, Smith et al., 2008, Ubuka et al., 2012, Poling et al., 2012, Rizwan et al., 2012) suggesting that RFRP-3 acts centrally to control the reproductive axis (Table 2).

There is still a debate on a possible hypophysiotrophic effect of RFRP-3 in mammals as reported in birds. A large body of evidence now reports the absence of fibres in the median eminence of mice, rats and Siberian hamsters (Ukena and Tsutsui, 2001, Yano et al., 2003, Rizwan et al., 2009, Ubuka et al., 2012). In another study, only sparse RFRP fibre innervation was observed in the median eminence of mice, rats and Syrian hamsters (Kriegsfeld et al., 2006) (Table 2). On the other hand, in the sheep RFRP fibres terminating in the median eminence have been identified and RFRP has been detected in the portal blood (Clarke et al., 2008, Sari et al., 2009, Smith et al., 2012) (Table 2). These data suggest the likelihood of species-dependent differences in the modes of action of RFRP-3, and it is possible that sheep and rodents evolved this system differently.

The RFRP peptides bind with high affinity to GPR147 (also known as NPFF1R) and with a lower affinity to GPR74 (also known as NPFF2R), which were first identified as neuropeptide FF receptors (Hinuma et al., 2000, Liu et al., 2001, Engstrom et al., 2003). The GPR147 receptor couples with $G\alpha_{i3}$ or $G_{\alpha s}$ proteins (Gouarderes et al., 2007) suggesting that GPR147 can have both inhibitory and stimulatory downstream effects on cellular activity. However, in CHO cells, activation of the receptor inhibits forskolin-stimulated cAMP accumulation (Mollereau et al., 2002).

NPFF receptors have been detected in rodent, lagomorph, and monkey brains suggesting that they are conserved (Gouarderes et al., 2004b). Importantly, however, remarkable variations in GPR147 and GPR74 receptor contents and distribution exist from one species to another and from one strain to another among the same species (Gouarderes et al., 2004b, Gouarderes et al., 2004a). Early studies describing the autoradiographic distribution of GPR147 in mice and rats indicated that the receptor was present throughout the hypothalamus (Gouarderes et al., 2002, Gouarderes et al., 2004b, Gouarderes et al., 2004a). Recent studies have made it possible to localise RFRP sites of action in more detail in various rodent species. Indeed, RFRP-3 fibres are in contact with

20-40% of GnRH neurons in rats and hamsters (Ubuka et al., 2012, Rizwan et al., 2012) and about 25% of GnRH neurons express *Gpr147* but not *Gpr74* in mice, rats and hamsters (Rizwan et al., 2012, Poling et al., 2012, Ubuka et al., 2012). In another study in mice expressing GnRH-green fluorescent protein-tagged neurons, RFRP-3 was found to exert a direct inhibitory effect on the firing rate of 41% of GnRH neurons, while 12% increased their firing rate, and the remainder were unaffected (Ducret et al., 2009). Furthermore, we demonstrated that central injection of RFRP-3 to Syrian hamsters induces c-Fos expression in 30% of the GnRH neurons (Ancel et al., 2012). Whether this effect is due to a direct action of RFRP-3 on GnRH neurons or whether it is linked to an effect on upstream regulators of the reproductive axis remains to be determined. Indeed, in the same study, although c-Fos expression was not observed in Kp neurons following acute RFRP-3 administration, the continuous central administration of RFRP-3 led to an increase in *Kiss1* expression in the arcuate nucleus (Ancel et al., 2012). Moreover, in rats RFRP-3 fibres are in contact with Kp neurons, a subpopulation (20%) of which expresses the *Gpr147* gene (Rizwan et al., 2012). It is of note to say that in our analysis of c-Fos expression in the Syrian hamster brain following icv RFRP-3 administration we found an increase in non-Kp neurons in the arcuate nucleus (Ancel et al., 2012).

Only a few studies have addressed the question of the distribution of GPR147 in peripheral tissues. The receptor has been localised in the Syrian hamster pituitary (Gibson et al., 2008) although only a very low level of pituitary expression has been reported in rats (Hinuma et al., 2000, Quennell et al., 2010). More recently, ovine pituitary cells have been shown to express *Gpr147* (Smith et al., 2012). These data further support the hypothesis that RFRP-3 could have a direct hypophysiotrophic effect in sheep and not in rodents, although additional studies will be required in order to provide an answer to this controversial question.

RFRP-3 AND THE REPRODUCTIVE AXIS

LESSONS FROM NON-MAMMALIAN VERTEBRATES

As previously mentioned, GnIH was discovered in birds in 2000 and termed accordingly because of its inhibitory effect on gonadotrophin secretion (Tsutsui et al., 2000). Indeed, GnIH administration reduces plasma LH concentrations *in vivo* in quails and sparrows (Tsutsui et al., 2000, Osugi et al., 2004, Ubuka et al., 2006) and inhibits gonadotrophin synthesis and release *in vitro* from cultured quail and chicken pituitaries (Ciccone et al., 2004, Ubuka et al., 2006). Taken together, these data indicate that GnIH inhibits gonadotrophin synthesis and release in birds, probably via a direct inhibitory effect at the level of the pituitary (Tsutsui et al., 2009, Tsutsui et al., 2010a, Tsutsui et al., 2012).

In 2002, goldfish GnIH was discovered in teleosts (Sawada et al., 2002) leading to investigation of the involvement of this peptide in gonadotrophin secretion in fish. In goldfish, intraperitoneal administration of GnIH peptide induced a decrease in serum LH levels (Zhang et al., 2010), however goldfish GnIH and its related peptides stimulated the release of LH and FSH from cultured pituitary cells of sockeye salmon (Amano et al., 2006). These results raise interesting questions of possible species-dependent differences in the effects of GnIH on gonadotrophin secretion in vertebrates. Additional studies should aim at determining whether these conflicting data are due to the different methods of investigation used, or whether they reflect a physiological reality in the effect of GnIH on the reproductive axis.

ACTIONS OF RFRP-3 ON GONADOTROPHIN SYNTHESIS AND RELEASE IN MAMMALS

The discovery that GnIH was a potent regulator of gonadotrophin synthesis and release in non-mammalian vertebrates led to intensive research on the possible roles of RFRP-1 and RFRP-3 in the regulation of the mammalian reproductive axis. Because RFRP-3 is closest to avian GnIH as regards its sequence, focus was initially directed towards the role of RFRP-3 in the regulation of mammalian reproduction, to the detriment of RFRP-1. Moreover, an initial study in rats, indicating that icv RFRP-1 stimulated prolactin secretion but not other pituitary hormones (Hinuma et al., 2000), suggested that this peptide might be involved in the regulation of other endocrine functions rather than reproduction. In recent years, a large number of studies have demonstrated in a range of

mammalian species that RFRP-3 plays a role in the regulation of the hypothalamo-pituitary-gonadal axis (Table 2) (Tsutsui et al., 2010a, Bentley et al., 2010)(for reviews). In mice, RFRP-3 was found to exhibit rapid and repeatable inhibitory effects on the firing rate of a subpopulation of GnRH neurons in hypothalamic slices (Ducret et al., 2009). In male rats, icv RFRP-3 significantly suppresses all facets of sex behaviour and also significantly reduces plasma levels of LH (Johnson et al., 2007, Pineda et al., 2010b). In female rats, chronic icv infusion of RFRP-3 causes a dose-dependent inhibition of GnRH neuronal activation at the LH surge peak and also suppresses neuronal activation in the anteroventral periventricular region, which provides stimulatory input to GnRH neurons (Anderson et al., 2009). Taken together, these results point to a central inhibitory effect of RFRP-3 on the HPG axis, via the GnRH neurons in the POA/OVLT brain region.

However, there are contradictory data about a possible hypophysiotrophic effect of the peptide in mammals. In ovariectomised (OVX) rats, intravenous administration of RFRP-3 significantly reduces plasma LH concentrations (Murakami et al., 2008), while in another study the same protocol had no effect on basal LH secretion and minimal effects on GnRH-stimulated secretion (Rizwan et al., 2009). *In vitro*, RFRP-3 was shown to inhibit LH secretion from cultured pituitary cells when GnRH is present, but did not have a significant effect on basal LH levels in the same study (Murakami et al., 2008). In another study, RFRP-3 did not have a direct suppressive effect on LH secretion in rat cultured anterior pituitary cells (Anderson et al., 2009). In OVX female Syrian hamsters, a study has shown that peripheral injections of GnIH significantly inhibit LH secretion (Kriegsfeld et al., 2006), but in the male hamster we reported no effect of RFRP-3 on LH secretion when injected peripherally, nor on the basal or GnRH-stimulated production of LH from isolated pituitary glands (Ancel et al., 2012). In sheep and cattle, intravenous RFRP-3 administration inhibits gonadotrophin release (Clarke et al., 2008, Kadokawa et al., 2009) although another study failed to replicate these results in sheep (Caraty et al., 2012). Interestingly, RFRP-3 is released into the portal blood in sheep and appears to induce a marked inhibition of gonadotrophin secretion *in vitro* (Clarke et al., 2008, Sari et al., 2009, Smith et al., 2012). To date, no consensus has been reached on the subject of RFRP-3 sites of action for the control of mammalian reproduction and it is possible that species-dependent differences exist with regard to the hypophysiotrophic effect of

RFRP-3 in mammals. Additional studies, using similar experimental protocols in rodents and sheep could help to answer some of the pending questions.

Until recently, and based on the plethora of publications supporting this hypothesis, it was assumed that RFRP-3 functioned in mammals as GnIH functioned in birds and served as an inhibitory component regulating the hypothalamo-pituitary-gonadal axis. However, we have recently reported novel findings in the male Syrian hamster (Ancel et al., 2012) which have led to question this assumption, concurrently with another group working on the male Siberian hamster (Ubuka et al., 2012). In the male Syrian hamster kept in long-day photoperiodic (LD) conditions, we reported that acute icv administration of RFRP-3 stimulates GnRH cell activity, gonadotrophin release and testosterone production (Ancel et al., 2012). Similarly, in short-day photoperiodic (SD) conditions, a single central injection of RFRP-3 increases gonadotrophin release (unpublished data). In the Siberian hamster, while administration of RFRP-3 in LD conditions inhibits gonadotrophin release, the same protocol stimulates gonadotrophin secretion in SD conditions (Ubuka et al., 2012). Remarkably, these findings of a stimulatory action of RFRP-3 on the male hamster reproductive axis are in sharp contrast with a previous study reporting an inhibitory effect of icv GnIH on LH secretion in OVX female Syrian hamsters (Kriegsfeld et al., 2006), raising the question of a possible sex-dependent difference in the effect of RFRP-3 on the reproductive axis.

Reproductive activity of female rodents displays a well-described oestrous cycle, characterised by varying levels of circulating gonadotrophins and sex steroids. It has been hypothesised that the RFRP neuronal system might be involved in the estrogen-mediated positive feedback which regulates the oestrous cycle. Indeed, the number of RFRP neurons and their level of activity are decreased at the time of the LH surge in the Syrian hamster (Gibson et al., 2008). Furthermore, *rfrp* mRNA expression is reduced in OVX mice treated with estrogen (Molnar et al., 2011). However, a study in rats showed no difference in *rfrp* mRNA levels of females that were OVX versus OVX and treated with estrogen or diestrus (Quennell et al., 2010). In addition, in OVX ewes, estrogen treatment does not significantly alter *rfrp* mRNA expression levels (Smith et al., 2008). These observations suggest that there could be another level of complexity in the involvement of the RFRP neuronal system in the regulation of the reproductive system, according to

the gender of the animal analyzed. In this context it would be interesting to determine whether the effect of RFRP-3 on the female reproductive axis depends on the stage of the oestrous cycle at which it is administered.

Next page: **Table 2 - Summary of the effects of RFRP-3 on LH secretion and of the sites of action of the peptide in mammals.**

In seasonal breeders, reproduction is restricted to a specific time of the year to ensure that the birth of the offspring occurs during the most favourable season. In order to synchronise their reproductive activity with the seasons, mammals use the annual variations in photoperiod. To decode photoperiod, mammals rely on a photoneuroendocrine system in which cells originating in the retina project, via a multisynaptic pathway, to the pineal gland where MEL is released exclusively at night. As a result, the duration of the nocturnal release of MEL is proportional to night duration, therefore giving a stable indication of the seasons (Simonneaux and Ribelayga, 2003).

Syrian and Siberian hamsters are classic models for the study of seasonal rhythms. In these species, sexual activity is promoted by exposure to a LD and exposure to a SD induces an inhibition of the reproductive function within 8-10 weeks. Although it is now well established that the seasonal regulation of reproduction is mediated via MEL, its precise sites of action remain unknown.

In the Syrian hamster, the MBH appears to be an important brain region in the photoperiodic control of reproduction. Indeed, MEL receptors are localised in this hypothalamic area and an electrolytic lesion of the MBH prevents the SD-induced gonadal regression (Maywood and Hastings, 1995, Maywood et al., 1996). Interestingly, *rfrp* neurons are localised in this same brain region and we have shown that both *rfrp* mRNA and RFRP protein levels are down-regulated by MEL in a SD photoperiod in the Syrian hamster (Figure 16) (Revel et al., 2008). Recently, a similar MEL-driven down-regulation of *rfrp* mRNA levels and RFRP-ir content has been reported in the male Siberian hamster (Ubuka et al., 2012). It is worth noting that in both hamster species these photoperiodic variations of *rfrp* expression are independent of the photoperiodic variation in circulating levels of testosterone. Taken together, these data suggest that *rfrp* and its product, RFRP-3, might be involved in the MEL-driven seasonal regulation of reproduction in hamsters.

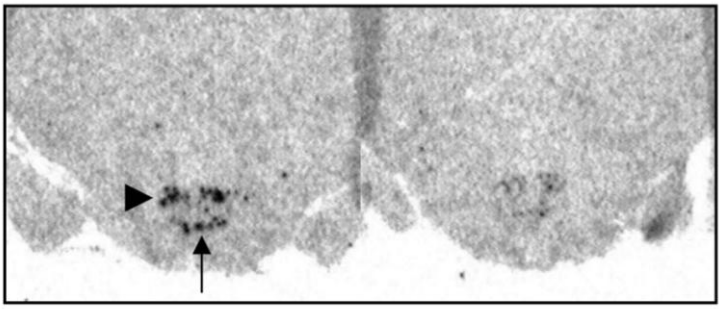

FIGURE 16 - *RFRP* EXPRESSION IS DOWN-REGULATED IN THE SYRIAN HAMSTER BRAIN IN SD

The expression of *rfrp* is down-regulated by melatonin in SD conditions compared to LD conditions. This down-regulation is mediated by melatonin, as demonstrated with pinealectomy and melatonin injection experiments. Gonadal steroids do not affect *rfrp* expression, as demonstrated using orchidectomy and testosterone implant experiments. The *arrow* shows expression in the dorsomedial division of the ventromedial hypothalamic nucleus and the *arrowhead* shows expression in the dorsomedial nucleus of the hypothalamus. *Revel et al., 2008.*

We have recently investigated the role of RFRP-3 in the seasonal control of reproduction. Male Syrian hamsters were placed in photoinhibitory conditions and implanted with osmotic minipumps releasing a constant flow of RFRP-3 in the lateral ventricle. Within 5 weeks, RFRP-3 administration had fully reactivated the reproductive function compared to the administration of vehicle, manifested by an increase in *Kiss1* expression in the arcuate nucleus, paired testes weight and plasma testosterone concentrations (Ancel et al., 2012). These results indicate that *rfrp* neurons are likely candidates in mediating the MELergic information to the reproductive axis. However, additional experiments are required in order to determine whether MEL is acting directly upon *rfrp* neurons or whether there are other intermediates involved in the MEL-driven regulation of the reproductive function. In particular, it has been proposed that the pars tuberalis plays a central role in the photoperiodic control of seasonal functions. Indeed, in seasonal species abundant MEL receptors are present in the pars tuberalis of the anterior pituitary, and MEL-responsive cells in the pars tuberalis control the production of thyrotrophin which acts locally on cells in the adjacent MBH, leading

to increased expression of type II thyroid hormone deiodinase (Dio2) in LD conditions in Syrian and Siberian hamsters (Revel et al., 2006c, Watanabe et al., 2004). Because Dio2 catalyzes the conversion of thyroxine (T_4) to the bioactive form triiodothyronine (T_3), this photoperiodic regulation results in elevated levels of T_3 during the breeding season, compared to the non-breeding season. In photoinhibited Siberian hamsters T_3 administration reactivates the reproductive function (Freeman et al., 2007), indicating that this pathway could be involved in the regulation of seasonal reproduction. Additional studies investigating the effect of T_3 administration on RFRP expression and the presence or not of T_3 receptors on RFRP neurons could help clarify the hierarchical organization of the T_3/RFRP systems.

Contrary to hamsters, sheep are short day breeders; that is to say that sexual activity is promoted by exposure to a SD photoperiod and inhibited upon exposure to a LD photoperiod. In this species, like hamsters, *rfrp* expression is down-regulated in SD conditions, when sheep are sexually active, and elevated in LD conditions, when they are sexually inactive (Dardente et al., 2008, Smith et al., 2008). These observations are in line with the findings indicating that acute administration of RFRP-3 has an inhibitory effect on the reproductive function in sheep (Clarke et al., 2008, Sari et al., 2009, Kadokawa et al., 2009). However, a possible seasonal role has to date not been addressed using continuous infusions of the peptide. In the future, it will be interesting to determine whether RFRP-3 is a regulator of seasonal reproduction in sheep as it appears to be the case in hamsters. Moreover, as previously mentioned, *rfrp* neurons are likely candidates in mediating the melatonergic information to the reproductive axis in hamsters, and it will be fascinating to find out whether they play a central role in transmitting seasonal information to the gonadotrophic axis in sheep. Indeed, although it is well established that MEL controls the seasonal regulation of the hypothalamo-pituitary-gonadal axis in seasonally-breeding species, the precise mechanisms through which the same MELergic signal produces opposite behavioural responses remain unclear. It is reasonable to hypothesise that RFRP neurons are the switch point in converting the same MELergic signal into a stimulatory or an inhibitory output to the reproductive axis in seasonally-breeding mammals.

RFRPS AND OTHER FUNCTIONS

The DMH is involved in a variety of behavioural and physiological responses, thus the involvement of RFRPs in the regulation of other endocrine functions has been investigated. Notably, the peptides have been found to be implicated in functions which indirectly affect or are affected by the reproductive status of the animal, including feeding, stress and nociception.

RFRPS AND FEEDING

Because the DMH plays an important role in the control of energy metabolism and RFRP neurons are located in the DMH in mammalian species, it seems likely that RFRP-1 and/or RFRP-3 may play a role in the regulation of feeding behaviour. In the sheep brain, RFRP fibres are found to have close appositions with neuropeptide Y, proopiomelanocortin, orexin, and melanin-concentrating hormone neurons (Qi et al., 2009), all of which are known to play important roles in the control of food intake. Moreover, the administration of RFRPs induces c-Fos expression in the arcuate nucleus in rats and hamsters (Yano et al., 2003, Ancel et al., 2012), a brain region well-known for its key role in the regulation of feeding behaviour.

Only a few studies have investigated the behavioural effect of RFRP peptide injections in mammals. In rats, icv RFRP-3 administration induces an increase in food intake (Johnson et al., 2007, Murakami et al., 2008) and in body weight (Johnson and Fraley, 2008). However, in another study central RFRP-1 injection resulted in food intake decrease in rats (Kovacs et al., 2012). Given that RFRP-1 applied icv to chicks significantly reduced both food intake and water intake (Newmyer and Cline, 2009), it is reasonable to speculate that RFRP-1 and RFRP-3 might have variable effects on food intake in mammals. Indeed, in the Syrian hamster we have shown that continuous icv administration of RFRP-3 for 5 weeks did not affect food intake or body weight (Ancel et al., 2012).

RFRPS AND STRESS

The DMH is also involved in the control of stress responses (DiMicco et al., 2002) and the involvement of RFRP in the control of stress has been reported. Exposure to stressful

stimuli induces an increase in *rfrp* expression and an activation of RFRP neurons in the hypothalamus (Kaewwongse et al., 2010, Kirby et al., 2009). Moreover, RFRP fibres appear to project directly to cells containing corticotrophin-releasing hormone or oxytocin in the hypothalamus, hormones which are known for their role in stress responses (Qi et al., 2009). Administration of RFRP-1 and RFRP-3 induces c-Fos expression in the hypothalamic paraventricular nucleus and in oxytocin neurons, and induces the secretion of adrenocorticotropic hormone and oxytocin into the peripheral circulation (Kaewwongse et al., 2010). Interestingly, similar patterns of c-Fos expression and hormone release are observed after stressful stimuli (Onaka, 2000). In addition, central administration of RFRP induces anxiety-related behaviours (Kaewwongse et al., 2010). On the other hand, initial work indicated that central RFRP-1 application increased prolactin secretion in rats (Hinuma et al., 2000). Given the anti-stress and anxiolytic properties of prolactin, this aspect of RFRP peptide function deserves further investigation. Taken together, these data are consistent with the view that RFRPs are involved in neuroendocrine and behavioural responses to stressful stimuli.

RFRPS AND NOCICEPTION

Two peptides of the RFamide family of peptides have been shown to play important roles in the control of pain and analgesia, namely NPFF and NPAF (Panula et al., 1996, Panula et al., 1999, Roumy and Zajac, 1998). These peptides were initially identified as the endogenous ligands for GPR74 and GPR147 (Elshourbagy et al., 2000, Bonini et al., 2000, Hinuma et al., 2000), but it was later shown that NPFF and NPAF had a lower affinity for GPR147 than RFRP peptides (Hinuma et al., 2000, Liu et al., 2001, Engstrom et al., 2003). In 2001, a study showing that RFRP-1 is more potent in attenuating morphine-induced analgesia than NPFF when injected icv (Liu et al., 2001) suggested that RFRP peptides could play a role in nociception. More recently, using neuroblastoma cells transfected with GPR147 a similar opioid-attenuating activity was observed for RFRP-3 (Kersante et al., 2006), further supporting the possibility that RFRP peptides are involved in the control of pain and analgesia. Unfortunately, only a few studies have aimed at clarifying this aspect of RFRP peptide function, and it is therefore difficult to conclude on the implication of these peptides in nociception. However, a recent report indicating that RF9, a dipeptide with subnanomolar affinities towards GPR147, exhibited a potent in vivo preventive effect on opioid-induced hyperalgesia at low dose (Gealageas

et al., 2012) indicates that GPR147 may be a key partner of an anti-opioid system that modulates the antinociceptive properties of the opioid system. Since the endogenous ligands for GPR147 are RFRP peptides, this branch of research deserves further investigation and might lead to the discovery of an additional function for RFRP peptides, besides the regulation of the gonadotrophic axis.

CONCLUSION AND PERSPECTIVES

The discovery of *rfrp* and its product RFRP-3 in mammals led to a new direction in investigating the regulation of GnRH neuron activity and therefore of the HPG axis. Although it was initially hypothesised that RFRP-3 might act as an inhibitory component regulating the reproductive axis in mammals, it now appears that this is not always the case. Indeed, recent evidence indicates that there are probable species-dependent differences in the effect of the peptide on the gonadotrophic axis. Further investigations will be required in order to answer the questions raised by the contradictory results observed in mammals, notably: 1) what is the functional significance of these opposing effects? 2) through which mechanisms does RFRP-3 induce either a stimulatory or an inhibitory effect on the gonadotrophic axis? 3) apart from GnRH neurons, what are the central sites of action of the peptide? and 4) to what extent is RFRP-3 involved in the regulation of non-reproductive functions?

The future development of highly selective pharmacological and molecular tools should help answer the question as to whether the species- and gender-dependent differences in the physiological effects of RFRP-3 might be mediated by differences in the modes and sites of action of the peptide on the HPG axis.

AIM OF THE STUDY

The observation that *rfrp* expression is down-regulated by MEL in SD in male Syrian hamsters (Revel et al., 2008), when animals are sexually inactive, is in contradiction with studies in rats and sheep reporting that RFRP-3 has an inhibitory effect on the gonadotrophic axis (Johnson et al., 2007, Murakami et al., 2008, Clarke et al., 2008, Sari et al., 2009, Pineda et al., 2010b). Therefore, the initial aim of my work was to determine the acute effects of RFRP-3 administration on the male Syrian hamster gonadotrophic axis. In parallel, we sought to characterise possible sites of action of RFRP-3 in the Syrian hamster brain and pituitary.

In line with this reasoning, and because *rfrp* expression is elevated in LD when hamsters are sexually active, my work also focused on the seasonal aspect of reproduction. Indeed, the sites of action of MEL for the photoperiodic control of reproduction are not known. However, MEL receptors have been detected in the MBH of Syrian hamsters and a lesion of this area abolishes the SD-induced gonadal regression (Maywood and Hastings, 1995, Maywood et al., 1996), suggesting that this brain region could be involved in mediating MEL's effect on the gonadotrophic axis. Interestingly, *rfrp* neurons are located in this area, and because MEL regulates *rfrp* expression in the Syrian hamster, we hypothesised that RFRP-3 could play a role in the seasonal regulation of reproduction. Therefore, my work also addressed the chronic effect of RFRP-3 administration on reproductive function.

In the female Syrian hamster, two recent studies suggested that the involvement of RFRP-3 in the regulation of the HPG axis might be more complex than initially expected. Firstly, in OVX female Syrian hamsters, central administration of GnIH inhibits LH secretion (Kriegsfeld et al., 2006). Secondly, RFRP-ir levels vary throughout the estrous cycle, with reduced levels at the time of the preovulatory LH surge (Gibson et al., 2008). These observations led us to speculate that in female Syrian hamsters the effect of RFRP-3 on the gonadotrophic axis might vary in the course of the estrous cycle. In order to address these points, we first analysed *rfrp* expression in LD and SD, in order to compare the effect of photoperiod in males and females. We then went on to characterise *rfrp* expression throughout the estrous cycle. Finally, we examined the effect of RFRP-3 administration on LH secretion in intact female Syrian hamsters, at different stages of the estrous cycle. Indeed, to date all the studies on the effect of RFRP-3 on the gonadotrophic axis carried out in females have been performed in OVX animals, in order to

bypass the feed-back effects of sex steroids. However, the results obtained might not be functionally significant.

The last part of my work aimed at identifying RFRP-3 modes and sites of action in the Syrian hamster hypothalamus. Indeed, little is known about the central targets mediating the effects of RFRP-3 on the gonadotrophic axis in hamsters and other rodents. Specifically, there is a debate on possible interactions between RFRP-3 and other RFamide receptors. In order to determine whether the effects of the peptide on the Syrian hamster reproductive axis are mediated via GPR147, which is thought to be the receptor for RFRPs, we carried out experiments using an antagonist for this receptor. At the same time, we sought to analyse GPR147 distribution in the Syrian hamster hypothalamus.

The results obtained in the course of my PhD will be presented as scientific articles, which have been or are in the process of being published:

Chapter 1

- Effects of acute and chronic RFRP-3 administration on the male Syrian hamster gonadotrophic axis
- Sites of action of RFRP-3 in the male Syrian hamster: central and peripheral targets

Chapter 2

- Photoperiodic regulation of *rfrp* expression in the female Syrian hamster and effect of gonadal steroids
- *rfrp* expression at the time of the LH surge on the day of proestrus compared to diestrus
- Effects of acute RFRP-3 administration on the female Syrian hamster
- Characterisation of a possible hypophysiotrophic effect of RFRP-3 in the female Syrian hamster

Chapter 3

- Does RFRP-3 exert its effects on the reproductive axis via GPR147 in the Syrian hamster? Antagonist studies
- What are the sites of action for RFRP-3 in the Syrian hamster hypothalamus? GPR147 distribution

CHAPTER 1 – STIMULATORY EFFECT OF RFRP-3 ON THE GONADOTROPHIC AXIS IN THE MALE SYRIAN HAMSTER: THE EXCEPTION PROVES THE RULE

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ABSTRACT

In seasonal mammals, a distinct photoneuroendocrine circuit that involves the pineal hormone MEL tightly synchronises reproduction with seasons. In the Syrian hamster, a seasonal model in which sexual activity is inhibited by short days, we have previously shown that the potent GnRH stimulator, kisspeptin, is crucial to convey MEL's message; however the precise mechanisms through which MEL affects kisspeptin remain unclear. Interestingly, *rfrp* gene expression in neurons of the dorsomedial hypothalamic nucleus, a brain region in which MEL receptors are present in the Syrian hamster, is strongly down-regulated by MEL in short days. Because a large body of evidence now indicates that RFRP-3, the product of the *rfrp* gene, is an inhibitor of gonadotrophin secretion in various mammalian species, we sought to investigate its effect on the gonadotrophic axis in the Syrian hamster. We show that acute central injection of RFRP-3 induces c-Fos expression in GnRH neurons and increases LH, FSH and testosterone secretion. Moreover, chronic central administration of RFRP-3 restores testicular activity and *Kiss1* levels in the arcuate nucleus of hamsters despite persisting photoinhibitory conditions. By contrast RFRP-3 does not have a hypophysiotrophic effect. Overall, these findings demonstrate that, in the male Syrian hamster, RFRP-3 exerts a stimulatory effect on the reproductive axis, most likely via hypothalamic targets. This places RFRP-3 in a decisive position between the melatonergic message and *Kiss1* seasonal regulation. Additionally, our data suggest for the first time that the function of this peptide depends on the species and the physiological status of the animal model.

In vertebrates, the reproductive system is controlled by the gonadotrophic axis, in which gonadotrophin-releasing hormone (GnRH) secretion from the hypothalamus stimulates the production of LH and FSH from the pituitary gland. Recently, two peptides of the RFamide family have been identified as important regulators of the gonadotrophic axis. The first one, kisspeptin (Kp), is a potent stimulator of GnRH secretion and the principal conduit for mediating sex steroid feedback. The second one, gonadotrophin-inhibitory hormone (GnIH), was identified as an inhibitor of gonadotrophin release in the quail (Tsutsui et al., 2000). The mammalian ortholog of avian *gnih*, termed *RFamide-related peptide* (*rfrp*), encodes a precursor that produces two peptides, RFRP-1 and RFRP-3 (Clarke et al., 2008, Hinuma et al., 2000, Kriegsfeld et al., 2006, Ukena et al., 2002, Yoshida et al., 2003, Ukena and Tsutsui, 2005, Fukusumi et al., 2001). Initial work in rats indicated that intracerebroventricular (icv) RFRP-1 increased prolactin release (Hinuma et al., 2000) and a large body of evidence now indicates that RFRP-3 inhibits LH secretion in various mammalian species (Ducret et al., 2009, Wu et al., 2009, Johnson et al., 2007, Johnson and Fraley, 2008, Kriegsfeld et al., 2006, Anderson et al., 2009, Murakami et al., 2008, Clarke et al., 2008, Kadokawa et al., 2009, Sari et al., 2009, Pineda et al., 2010b). The emerging concept is that Kp and GnIH/RFRP would have antagonistic roles in regulating the gonadotrophic axis (Clarke, Bentley et al., 2012, Kriegsfeld et al., 2010, Tsutsui et al., 2010b, Tsutsui et al., 2010a, Smith and Clarke, 2010).

In seasonal breeders, photoperiod (i.e. day-length) tightly regulates reproduction to ensure that birth occurs at the most favourable time of the year (Goldman, 2001). In photosensitive rodents like the Syrian hamster (*Mesocricetus auratus*) reproductive activity is promoted by exposure to long summer days (LD) and inhibited by short winter days (SD) (Goldman, 2001, Gaston and Menaker, 1967, Pevet, 1988). These variations in photoperiod modify the circulating levels of the pineal hormone MEL to synchronise reproductive activity with the seasons (Bartness et al., 1993, Goldman, 2001). We have shown that *Kiss1* expression in the arcuate nucleus (ARC) is strongly down-regulated by MEL and that this is responsible for the photo-inhibition of reproductive activity in SD conditions (Revel et al., 2006b, Ansel et al., 2010). However, MEL receptors are distributed through the dorso/ventromedial hypothalamus in this species and thus do not overlap with *Kiss1*-expressing neurons in the ARC (Maywood

and Hastings, 1995, Hanon et al., 2008). Interestingly, we have recently demonstrated that in hamsters *rfrp* is expressed in the dorso/ventromedial hypothalamus, where its expression is downregulated by MEL in SD (Revel et al., 2008). This observation that sexually inactive animals have low *rfrp* levels challenges the current hypothesis that RFRP-3 is a negative regulator of the gonadotrophic axis, at least in this species. To document this prospect, we thus investigated how RFRP-3 regulates the reproductive axis of the Syrian hamster.

MATERIALS AND METHODS

ANIMALS

The animals were adult male and female Syrian hamsters (*Mesocricetus auratus*) bred in-house. From birth, they were maintained in a LD photoperiod consisting of 14h light and 10h dark, with lights on at 0500h, at 22±2°C with *ad libitum* access to water and food. The SD photoperiod to which some groups were transferred consisted of 10h light and 14h dark. 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 with the rules of the European Committee Council Directive of November 24, 1986 (86/609/EEC).

ICV INJECTIONS

Syrian hamsters were anaesthetised using a mixture of Zoletil 20 (Virbac, Carros, France) and Rompun (Bayer Pharma, Puteaux, France) and positioned in the stereotaxic apparatus. The head of the animal was shaved and prepared for aseptic surgery. A single incision was made on the midline of the scalp. Once the area had been prepped, a stainless steel 30-gauge cannula was placed in the lateral ventricle at 2 mm lateral to the midline, 0.8 mm posterior 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. The cannula was blocked with a metallic wire and protected with a plastic cap. The animals were allowed a week to recover from the surgery. The injections (2µL/animal; flow rate 1µL/min) were given in the morning using a 30-gauge stainless steel cannula attached to polyethylene tubing and a 50 µl Hamilton syringe (Hamilton Inc., Reno, NV, USA) under light anaesthesia with isoflurane vapour for the duration of the injections. The effect of anaesthesia alone on LH secretion was analysed in animals which were submitted or not to the same anaesthetic protocol as the one used for the icv injections. The results indicate that our anaesthesia using isoflurane vapour has no significant effect on LH secretion [LH in ng/ml for non-anaesthetised animals 1.14 ± 0.16 (n = 5) vs. anaesthetized animals 0.92 ± 0.11 (n = 4)].

OVARIECTOMIES

Female Syrian hamsters were anaesthetised using a mixture of Zoletil 20 (Virbac, Carros, France) and Rompun (Bayer Pharma, Puteaux, France) and bilateral ovariectomy was carried out under sterile conditions. The animals were then placed in a stereotaxic apparatus and implanted with a cannula in the lateral ventricle as described above. Animals were left to recover for 7 days before receiving icv injections as described above.

ICV INFUSION

Infusions were performed as previously reported (Revel et al., 2006b). Three days prior to implantation, osmotic minipumps (model 2006; flow rate: 0.15 µL/hr.; duration: 6 weeks; Durect, Cupertino, CA) were filled with aCSF with or without RFRP-3 and stored at 37°C in Ringer Lactate (B. Braun Medical, Boulogne, France) until the surgery.

HORMONE MEASUREMENTS

Free testosterone was measured in plasma using a direct RIA kit (DPC coat-a-count RIA method; Siemens Medical Solutions, Mölndal, Sweden) as previously described (Mikkelsen et al., 2009).

Serum LH and FSH levels were determined in a volume of 25–50µL using a double-antibody method and RIA kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labelled with ^{125}I using Iodo-gen® tubes, following the instructions of the manufacturer (Pierce, Rockford, IL, USA). Hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and inter-assay coefficients of variation were, respectively, < 8 and 10% for LH and <6 and 9% for FSH. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. Accuracy of determinations

was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

LH secretion in pituitary cell cultures was determined using an ENZYME-linked immunosorbent assay (ELISA). After culturing for 6 h, media were collected and subjected to ELISA for LH. In brief, microtiter plates were filled with 100µL of rat LH High Purity in coating buffer, at a concentration of 10ng/100µL, and incubated overnight at 4°C. Excess LH was removed, and the plates were washed using 200µL/well of 10 mM phosphate buffer saline (PBS) with 0.1% Tween-20. The plates were blocked with 200µL/well of 10 mM PBS containing 1% BSA and 0.1% Tween-20 for 1 h at room temperature. 200µL of sample or various concentrations of standard rLH-RP-3, diluted in assay buffer, were preincubated with 200µL of LH antiserum (rLH-S-11 - 1:3000 in assay buffer) for 18 h at 4°C. 100µL of preincubated samples, standards and controls were added per well in triplicate and incubated overnight at 4°C. After washing, 100µL of donkey anti-rabbit IgG conjugated to horseradish peroxidase was added at 1:1000 dilution and incubated for 1 h at 37°C. The plates were again washed, and 100µL of 3,3',5,5'–tetramethylbenzidine substrate was added to all the wells. The colour reaction was allowed to develop for 30 min in the dark. The enzyme was stopped by adding 50µL of 0.5% sulphuric acid per well and the optical density of each well was immediately read at 492nm. Intra- and inter-assay coefficients of variation were, respectively, < 8 and 11%.

IMMUNOHISTOCHEMISTRY

The animals were deeply anesthetised with CO_2 vapour and their thorax opened. Heparin (250 IU per animal; Liquemine®, Roche, Meylan, France) was injected directly into the left ventricle, and a cannula was placed in the aorta. Blood was washed out with 100ml of PBS 1X (pH 7.4) and the tissues were fixed by perfusing 250ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed from the skull, post-fixed for 24 h at 4°C in 4 % formaldehyde in 0.1 M phosphate buffer (pH 7.4), then transferred to 0.05 M phosphate buffered saline (PBS). Brains and pituitaries were cryoprotected in 30% sucrose–PBS solution. Brains were frozen on the stage of a sliding

microtome, and 4 sets of 40-µm-thick coronal sections were cut containing the ARC and the preoptic area (POA), and kept as free-floating sections in antifreeze solution at -20°C. Series of brain sections were processed for by means of the avidin–biotin immunohistochemical procedure. Prior to the immunohistochemical steps, the sections were rinsed for 3 X 10 min in 0.01 M PBS and incubated in 1% H_2O_2/PBS for 10 min. Before incubation in the primary antiserum, the sections were treated in a blocking solution containing PBS with 0.3% Triton X-100, 5% swine serum, and 1% bovine serum albumin (BSA) for 20 min. Then the sections were incubated at 4°C for 24 h with a primary rabbit polyclonal antisera directed against c-Fos at 1:2000 previously characterised (Mikkelsen et al., 1998). After incubation in primary antiserum the sections were washed in PBS with 0.1% TX, and incubated for 60 min in biotinylated donkey anti-rabbit (Jackson Labs, 711-066-152) diluted 1:1000 in the same buffer with 0.3% BSA. After a wash, the sections were incubated for 60 min in streptavidin–horseradish peroxidase complexes, and washed again. Finally, after a careful wash they were incubated in nickel-enhanced 0.05% diaminobenzidine (DAB) (Sigma–Aldrich, St. Louis) with 0.05% H_2O_2 in 0.05 M Tris–HCl buffer (pH 7.6) for 10 min and then washed twice in PBS. This generates a black precipitate within the nucleus. For the second immunolabelling, sections were washed in 1% hydrogen peroxide to quench any remaining peroxidase and incubated with a polyclonal rabbit anti-GnRH antibody (1/2000; AB1567, Chemicon, Temecula, CA) or an antiserum raised against full-length rat kisspeptin-52 (1/200; JLV-1)(Mikkelsen and Simonneaux, 2009, Desroziers et al., 2010). The sections were then washed in PBS with 0.1% TX, and incubated for 60 min in biotinylated donkey anti-rabbit (Jackson Labs, 711-066-152) diluted 1:1000. After a wash, the sections were incubated for 60 min in streptavidin–horseradish peroxidase complexes, and washed again. Finally, immunoreactivity was revealed using DAB alone that resulted in a brown precipitate within the cytoplasm of the labelled cell. The free-floating brain sections were mounted on gelatinised glass slides, dried, and coverslipped in Pertex.

The relative number of GnRH and kisspeptin neurons containing c-Fos-immunoreactivity was counted manually in two sections/animal at the level of the POA and the ARC, respectively, by an observer blind to the treatment of the individual animals. Furthermore, the total number of c-Fos positive cells was counted in a defined area containing the Kp-immunoreactive neurons (see Fig. 5A).

IN SITU HYBRIDISATION (ISH)

Animals were deeply anesthetised with CO_2 vapour and killed by decapitation. Brains were removed from the skull, snap-frozen on dry ice, and stored at -80°C until sectioning. Brains were sectioned using a cryostat (Leica, Leica microsystems, Rueil-Malmaison, France) at -20°C. Four sets of ARC serial sections (16µm) were cut and thaw-mounted on SuperFrost®Plus (Menzel-Gläser, Braunschweig, Germany) slides and stored at -80°C until ISH.

Sense and antisense riboprobes were transcribed from linearised plasmids containing a 270-bp rat *Kiss1* cDNA (90-359 of Genbank NM_181692) in the presence of digoxigenin-labeled nucleotides (Roche, Meylan, France) according to the manufacturer's protocol. In brief, the sections were fixed in 4% paraformaldehyde, acetylated in triethanolamine buffer, and dehydrated in graded ethanols. After the riboprobe was denatured and mixed with hybridization medium (200 ng/mL) it was applied to slides and incubated for 40 h at 60°C. Six stringency rinses were performed at 72°C. Digoxigenin-labelled bound probes were detected with an alkaline phosphatase-labelled antidigoxigenin antibody (Roche). Alkaline phosphatase activity was detected with bromochloroindolyl phosphate and nitroblue tetrazolium in the presence of 5% polyvinyl alcohol (70.000-100.000 MW; Sigma).

The slides were then mounted and the total number of *Kiss1* cells in the ARC was manually counted on a Leica DMRB microscope (Leica microsystems, Rueil-Malmaison, France).

PITUITARY CELL CULTURE

The pituitary glands were sampled and immediately dissociated as previously described by Simonneaux et al. (Simonneaux et al., 1999). Briefly, cells were dissociated by enzyme dispersion and pipette trituration in a saline solution containing collagenase (0.8 mg/mL), trypsin (0.2 mg/mL) and DNAse (10 mg/mL) at 37°C. After the glands were entirely dissociated, cells were resuspended in Dulbecco's modifed Eagle's medium (DMEM) containing 8% horse serum, 2% fetal calf serum and 40 mg/mL gentamicin, plated at a density of 700 000 viable cells/well and maintained at 37°C under a mixture

of 95% air and 5% CO2 at 100% humidity. After preincubation for 48 h, the medium was changed, followed by incubation for 6 h in culture medium alone as control or three different concentrations of RFRP-3 (10 pM, 1 nM, 100 nM; Abgent, San Diego, USA). In another experiment, cells were incubated in culture medium with three concentrations of RFRP-3 (10 pM, 1 nM, 100 nM) with 1 nM of GnRH (Sigma). After culturing for 6 h, media were collected and subjected to ELISA for LH. The pituitaries were incubated for 6 h after we performed a time-course of the effect of GnRH incubation on LH release in which we found that an incubation time ranging from 3 to 9h was optimal to detect an effect on LH secretion.

STATISTICAL ANALYSES

Results are shown as mean ± SEM. All statistical analyses were performed using Statistica (StatSoft Inc., USA). Data were analyzed by *t* test or one-way ANOVA, followed by post-hoc analysis: Tukey's Honestly Significant Difference test, as appropriate. Statistical significance was set at $P_{value} < \alpha = 0.05$.

Syrian Hamster RFRP-3*	NMEAMILSRVPSLPQRF-NH2	Abgent, San Diego, USA	Kriegsfeld et al., 2006
Syrian Hamster RFRP-1*	SPAPANKVPHSAANLPLRF-NH2	Abgent, San Diego, USA	Kriegsfeld et al., 2006
Rat RFRP-3	ANMEAGTMSHFPSLPQRF-NH2	CASLO Laboratory ApS, Lyngby, Denmark	Ukena et al., 2002
GnIH	SIKPSAYLPLRF-NH2	Provided by Dr. Tsutsui	Tsutsui et al., 2000
Rat Kp10	YNWNSFGLRY-NH2	CASLO Laboratory ApS, Lyngby, Denmark	Terao et al., 2004
Human Kp54	GTSLSPPPESSGSRQQPGLS APHSRQIPAPQGAVLVQRE KDLPNYNWNSFGLRF-NH2	GenScript Corporation, Piscataway, USA	Terao et al., 2004

TABLE 3 – AMINO ACID SEQUENCES OF THE RFAMIDE PEPTIDES UDED IN THE STUDY.

*The sequences of the mature peptides are not known for Syrian hamster RFRP-1 and RFRP-3. The amino acid sequence of the precursor is predicted from its mRNA sequence.

CENTRAL, BUT NOT PERIPHERAL, RFRP-3 STIMULATES THE GONADOTROPHIC AXIS OF MALE SYRIAN HAMSTERS.

Based on studies in mice, rats and sheep, RFRP-3 is considered as an inhibitor of the gonadotrophic axis, through blockade of GnRH release (Ducret et al., 2009, Wu et al., 2009, Johnson et al., 2007, Johnson and Fraley, 2008, Kriegsfeld et al., 2006, Anderson et al., 2009, Murakami et al., 2008, Clarke et al., 2008, Kadokawa et al., 2009, Sari et al., 2009, Pineda et al., 2010b). As similar effects have been reported in the female Syrian hamster (Kriegsfeld et al., 2006), we first verified that RFRP-3 also inhibits gonadotrophin release in males. To do so, we examined the effects of Syrian hamster RFRP-3 (Table 1) injected icv on LH, FSH and testosterone secretion in adult, sexually active male hamsters. Surprisingly, RFRP-3 dose-dependently increased plasma LH and FSH levels after 30 min, with the 1500 ng dose giving the maximal response (Figures 17A and 17B).

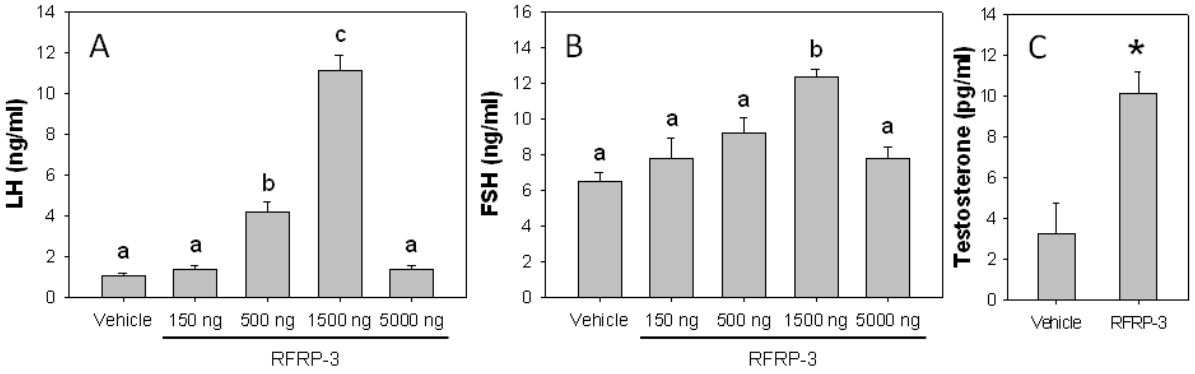


FIGURE 17 - SYRIAN HAMSTER RFRP-3 STIMULATES LH, FSH AND TESTOSTERONE SECRETION IN THE MALE SYRIAN HAMSTER

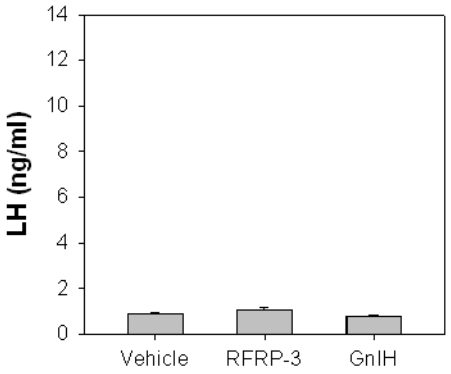
(A,B) Centrally-administered hamster RFRP-3 (150-5000 ng, icv) dose-dependently increased LH (A) and FSH (B) secretion after 30 min in male Syrian hamsters. Data represent the mean ± SEM (n = 6/group). Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis). (C) Similarly, icv RFRP-3 (1500 ng) significantly elevated plasma testosterone after 2 h. Data represent the mean ± SEM (n = 6/group). *, $p < 0.05$ compared with the vehicle-injected group by Student's t-test.

Similarly, RFRP-3 (1500 ng) significantly increased plasma testosterone levels after 30 minutes (vehicle-injected group 2.83pg/ml ± 0.95 vs. RFRP-3-injected group 4.21pg/ml ± 0.65, n = 6/group, $p < 0.05$ compared with the vehicle-injected group by Student's t-test) and 2 h (Figure 17C). These data suggest that central RFRP-3 does not inhibit, but rather activates the gonadotrophic axis of male Syrian hamsters, contrasting with females and other species studied so far.

Because this result is in contradiction with the available literature, in particular in female Syrian hamsters, we analysed the effect of acute RFRP-3 (1500 ng) and GnIH (2000 ng) administered centrally in ovariectomised female Syrian hamsters. Interestingly, neither RFRP-3 nor GnIH affected plasma LH levels after 30 min (Figure 18). This result indicates that the effects of RFRP-3 administration on the gonadotrophic axis may be sex-dependent, at least in the Syrian hamster.

FIGURE 18 - SYRIAN HAMSTER RFRP-3 AND GNIH DO NOT AFFECT LH SECRETION IN THE FEMALE SYRIAN HAMSTER

Centrally-administered hamster RFRP-3 (1500 ng, icv) and avian GnIH (2000 ng, icv) do not affect LH secretion in ovariectomised female Syrian hamsters after 30 min. Data represent the mean ± SEM (n = 6/group).



Conflicting data exist on whether or not peripherally-administered RFRP-3 affects the gonadotrophic axis (Rizwan et al., 2009, Pineda et al., 2010b, Murakami et al., 2008, Anderson et al., 2009). Thus, we investigated how intraperitoneal (ip) injections of RFRP-3 (5-500 µg) affect LH secretion in sexually active adult male hamsters, in comparison to Kp54 (300 µg) that was used as a positive control. Whereas Kp54 increased plasma LH significantly after 30 min, RFRP-3 did not alter LH levels significantly at any of the concentrations tested, although it appeared to slightly inhibit basal LH levels (Figure 19A). This suggests that in this species RFRP-3 does not influence the pituitary gland directly. To confirm this observation *in vitro*, we analysed the effects of RFRP-3 on LH secretion directly from cultured pituitary cells. Incubation with RFRP-3 for 6 h (10 pM -100 nM) had no effect on LH concentrations (Figure 19B), in contrast to GnRH (1 nM) that stimulated LH secretion significantly (Figure 19C).

Similarly, RFRP-3 did not prevent GnRH from stimulating LH secretion (Figure 19C). Taken together, these observations show that in male Syrian hamsters, RFRP-3 exerts its effect on the gonadotrophic axis through central targets.

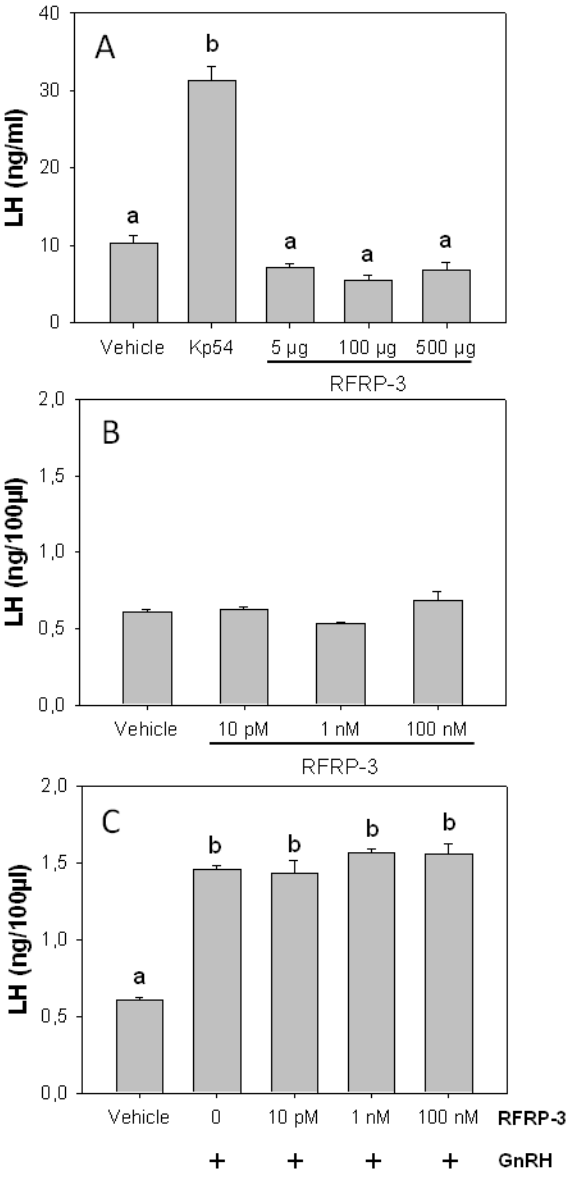


FIGURE 19 - RFRP-3 DOES NOT HAVE A HYPOPHYSIOTROPHIC EFFECT IN THE MALE SYRIAN HAMSTER

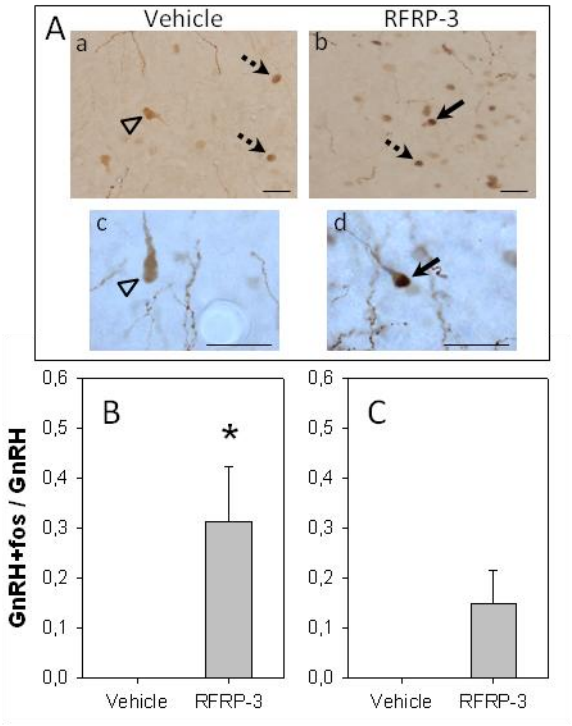
(A) Peripheral Kisspeptin 54 (Kp54; 300 µg in 100µl, ip) increased LH secretion significantly, whereas 3 concentrations of RFRP-3 (5-500 µg, ip) had no significant effect. Data represent the mean ± SEM (n = 5/group). Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis). (B) RFRP-3 (10 pM – 100 nM) did not significantly alter the basal secretion of LH by pituitary cell in culture. Hamster pituitary cells were incubated for 6 h with RFRP-3 and LH secretion was assayed in the supernatant. Data represent the mean ± SEM (n = 4/group). (C) Similarly, RFRP-3 did not significantly affect GnRH-induced LH secretion from cultured hamster pituitary cells. The cells were incubated for 6 h with GnRH (1 nM) alone or with 3 concentrations of RFRP-3 (10 pM – 100 nM) and LH secretion was assayed in the supernatant. Data represent the mean ± SEM (n = 4/group). Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis).

CENTRAL RFRP-3 ADMINISTRATION ACTIVATES GNRH NEURONS.

To determine whether GnRH and Kp neurons are targets for the central action of RFRP-3 in the male Syrian hamster, we examined the effects of RFRP-3 on c-FOS expression, a widely-used marker of neuronal activation (Kovacs, 2008), in the hypothalamus of sexually active adult hamsters. Two hours after giving RFRP-3 (1500 ng) icv, c-Fos was

determined in two neurochemical populations of hypothalamic neurons, the GnRH neurons in the POA and the Kp neurons in the ARC. In the POA, RFRP-3 increased c-Fos immunoreactivity in GnRH neurons, as shown by dual labelling immunohistochemistry (Figures 20A, B and C). While virtually no GnRH neurons were co-labelled with c-Fos in animals injected with vehicle in LD, about 20-30% of the GnRH neurons detected in the POA co-stored c-Fos (Figure 20B).

FIGURE 20 - GNRH CELLS ARE ACTIVATED BY RFRP-3 ADMINISTRATION



(A) *a* In vehicle-injected hamsters, GnRH-ir neurons (arrowhead) do not co-express c-Fos (dotted arrow). *b* In RFRP-3-treated hamsters, about 20-30% of the GnRH neurons detected in the POA co-stored c-Fos (dotted arrow showing a c-Fos-positive cell nucleus, black arrow showing a GnRH neuron co-expressing c-Fos). *c*, *d* Enlargements showing a GnRH cell (arrowhead) in a vehicle-injected hamster (c) and a GnRH cell co-expressing c-Fos in an RFRP-3-injected hamster (d). *Scale bar*, 30 µm. An acute central injection (2µl) of RFRP-3 (1500ng) to male Syrian hamsters maintained in long day (B) produced a significant increase in the number of GnRH neurons co-expressing c-Fos 2 h after injections compared to vehicle-injected hamsters, whereas the same treatment in short day (C) produced a slight but non-significant increase in the number of GnRH neurons co-expressing c-Fos. Mean ± SEM of ratio (n = 6/group). *, $p < 0.05$ compared with the vehicle-injected group by Student's t-test.

In contrast, although c-Fos immunoreactivity was slightly but non-significantly increased following RFRP-3 administration in the area of the ARC containing the Kp-immunoreactive neurons in LD and SD (Figures 21A, B and C), c-Fos immunoreactivity did not co-localise with Kp-positive neurons (Figure 21A). Accordingly, these data suggest that RFRP-3 delivered icv stimulates gonadotrophin release via GnRH neuron activation, while it does not appear to activate *Kiss1*-expressing neurons in the ARC, at least directly.

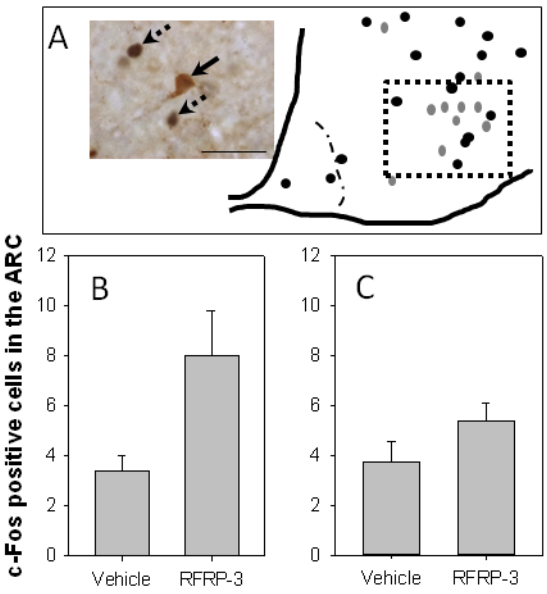


FIGURE 21 - RFRP-3 ADMINISTRATION INDUCES C-FOS IN THE ARC WHICH DOES NOT CO-LOCALISE WITH KISSPEPTIN NEURONS

(A) c-Fos activity was analysed in the area of the ARC containing the Kp-immunoreactive neurons. The c-Fos-positive neurons (dotted arrows) do not co-localise with kisspeptin-positive neurons (black arrow). *Scale bar*, 30 µm. An acute central injection (2µl) of RFRP-3 (1500ng) to male Syrian hamsters maintained in long day (B) and short day (C) produced a slight but non-significant increase in the number c-Fos-positive neurons in the subregion of the ARC shown in A 2 h after injections. Mean ± SEM of cell number (n = 6/group).

RAT KP-10, RAT RFRP-3 AND AVIAN GNIH STIMULATE LH AND TESTOSTERONE SECRETION.

RFRP-3 is a member of the RFamide family of peptides which contains 5 members that all share a common RFamide N-terminal motif (Greives et al., 2008, Dockray, 2004, Fukusumi et al., 2006). To partially exclude non-specific effects of RFRP-3, we examined if other members of this family (Table 1) equally stimulate the gonadotrophic axis of hamsters. Whereas rat Kp-10 (rKp10; 800 ng) and rat RFRP-3 (500 ng) given icv significantly increased plasma LH (Figures 22A and B) after 30 min, hamster RFRP-1 (500 ng, icv) had no significant effect on LH secretion (Figure 22C). Interestingly, the avian ortholog of RFRP, GnIH (2 µg, icv) also increased plasma LH (Figure 22D) and testosterone (vehicle-injected group 2.83pg/ml ± 0.95 vs. GnIH-injected group 5.63pg/ml ± 0.49, n = 6/group, $p < 0.05$ compared with the vehicle-injected group by Student's t-test) after 30 min. These data show that although it is derived from the same propeptide, RFRP-1 does not affect LH secretion, in contrast to the orthologous peptide GnIH, supporting the specificity of RFRP-3 effects on the gonadotrophic axis.

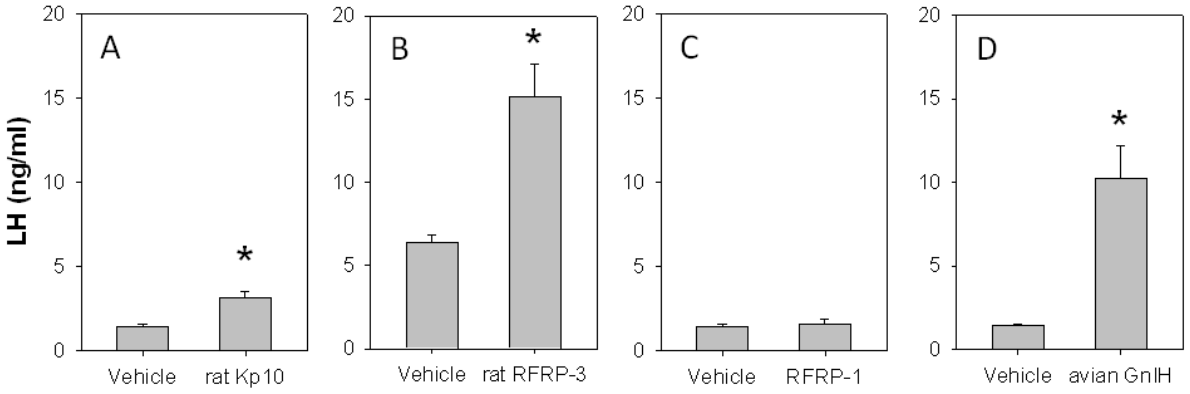


FIGURE 22 - THE STIMULATORY EFFECT ON LH SECRETION IN THE MALE SYRIAN HAMSTER IS NOT COMMON TO ALL RFAMIDE PEPTIDES

(A,B) Centrally-administered rat Kisspeptin10 (Kp10; 800 ng in 2 µL icv) (A) and rat RFRP-3 (500 ng in 2 µL, icv) (B) increased LH plasma levels significantly. Mean ± SEM (n = 6/group). *, $p < 0.05$ compared with the vehicle-injected group by Student's t-test. (C) In contrast, centrally-administered hamster RFRP-1 (500 ng in 2 µL, icv) did not affect LH secretion after 30 minutes. Mean ± SEM (n = 6/group). (D) Similar to RFRP-3, avian GnIH (2000 ng in 2 µL, icv) significantly increased LH plasma levels. Data represent the mean ± SEM (n = 6/group). *, $p < 0.05$ compared with the vehicle-injected group by Student's t-test.

RFRP-3 REACTIVATES THE REPRODUCTIVE AXIS OF PHOTOINHIBITED SYRIAN HAMSTERS.

Given that *rfrp* expression is strongly downregulated in sexually quiescent hamsters (Revel et al., 2008) and that acute RFRP-3 stimulates LH, FSH and testosterone secretion, we questioned whether a chronic administration of RFRP-3 to photoinhibited SD Syrian hamsters restores reproductive activity. For this, male Syrian hamsters were placed in SD conditions for 8 weeks to inhibit the gonadotrophic axis, as verified by scrotal palpation of testicular size. The animals were then divided into three groups: SD-aCSF, SD-RFRP-3 and LD-back. The animals of the SD-aCSF and SD-RFRP-3 groups were implanted subcutaneously with an osmotic minipump connected to a cannula implanted into the right lateral ventricle and filled with artificial cerebrospinal fluid (aCSF) alone or with hamster RFRP-3 (12µg/day), respectively. The SD-aCSF and SD-RFRP-3 groups were kept in SD conditions, whereas the LD-back group was transferred back to LD conditions. After 5 weeks of treatment, testes weight and plasma testosterone levels

were both reduced in SD-aCSF animals (Figures 23A and B). In contrast, hamsters receiving a chronic administration of RFRP-3 (SD-RFRP-3) underwent significant testicular reactivation, both in terms of testicular size (Figure 23A) and circulating levels of testosterone (Figure 23B). This effect was fully comparable to transferring photoinhibited SD animals back to stimulatory LD conditions for the same duration, and contrasted with hamsters receiving vehicle only and kept in SD. This experiment was repeated twice with lower doses and the results indicated a dose-dependent effect of the administration.

These effects of chronic RFRP-3 are identical to those obtained with rKp10 administered under similar conditions (Revel et al., 2006b). This raises the question of whether RFRP-3 exerts its effects via an increase in *Kiss1* expression. Interestingly, *Kiss1* expression was significantly increased in the ARC of the hamsters with chronic RFRP-3 as compared to vehicle-treated animals, and attained levels identical to those of the LD control animals (Figures 23C and D). This suggests that in male Syrian hamsters chronic RFRP-3 is able to reactivate the gonadotrophic axis upstream from the *Kiss1* neurons by blocking the inhibitory effect of MEL.

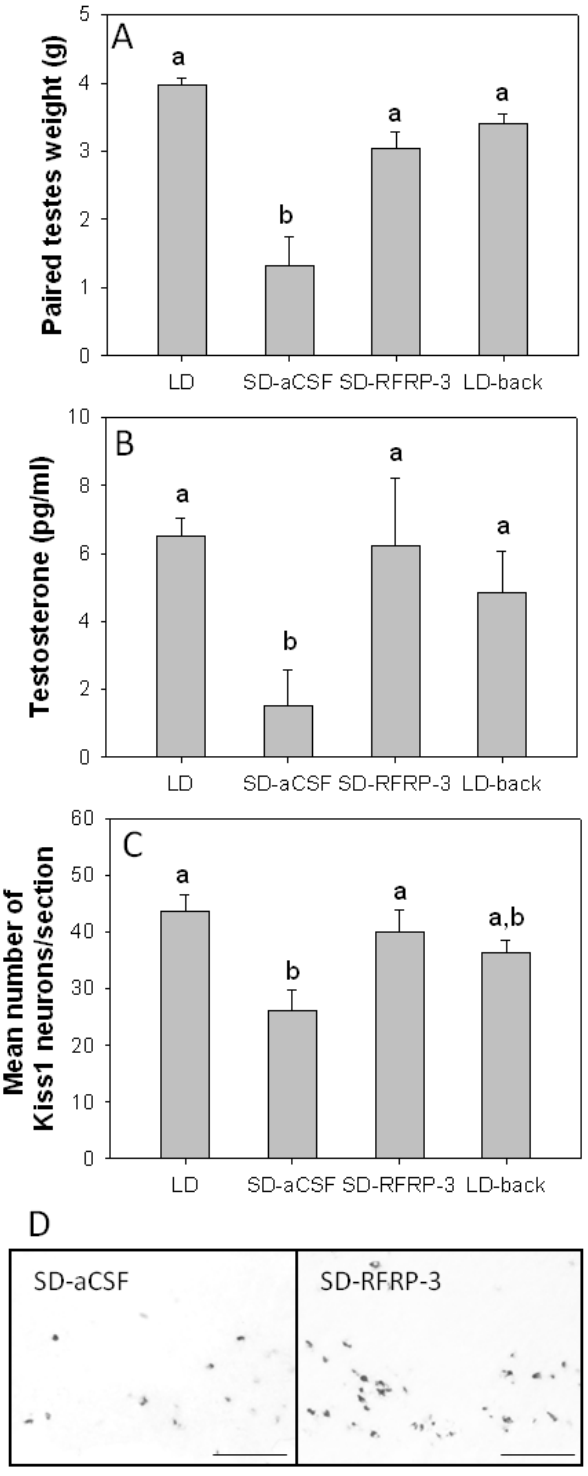


FIGURE 23 - RFRP-3 RESTORES TESTICULAR ACTIVITY AND INCREASES *KISS1* EXPRESSION IN THE ARCUATE NUCLEUS OF PHOTOINHIBITED SYRIAN HAMSTERS

Three groups of animals (n = 6/group) were placed in short day (SD) conditions for 8 weeks after which gonadal atrophy was verified by scrotal palpation. Animals were implanted with an intracerebroventricular cannula linked to an osmotic minipump (flow rate: 0.15 µl/hr) filled with either aCSF (SD-aCSF) or aCSF + hamster RFRP-3 (SD-RFRP-3; concentration: 1 mM) and were returned to SD conditions. A control group was transferred back to long day (LD) conditions on the day of surgery (LD-back). After 5 weeks of treatment, weighing the testes and dosing plasma testosterone allowed the level of reproductive activity to be monitored. A fifth group of hamsters was left in LD (LD; n = 6) for the whole experiment (13 weeks) for comparison. Administering RFRP-3 for 5 weeks to SD hamsters was sufficient to restore testicular activity [testes weight (A) and circulating testosterone (B)] despite persisting photoinhibitory conditions. This treatment was equivalent to transferring animals back into photostimulatory conditions. Data represent the mean ± SEM. Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis). (C) The chronic administration of RFRP-3 upregulated *Kiss1* expression in the ARC to levels comparable to those observed in the LD control and LD-back groups, as determined by *in situ* hybridisation. The administration of vehicle (SD-aCSF) did not produce any effect on *Kiss1* expression. Data represent the mean ± SEM. Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis). (D) Representative brain sections of SD-aCSF and SD-RFRP-3 animals processed for *in situ* hybridization with antisense riboprobe for *Kiss1* mRNA. *Scale bar*, 30 µm.

Whereas RFRP-3 has been shown to display inhibitory effects on the reproductive axis of all mammalian species investigated (Kriegsfeld et al., 2006, Clarke et al., 2008, Ducret et al., 2009, Wu et al., 2009, Johnson et al., 2007, Johnson and Fraley, 2008, Anderson et al., 2009, Murakami et al., 2008, Kadokawa et al., 2009, Sari et al., 2009), apart from a small percentage of GnRH neurons that were stimulated by RFRP-3 in mice (Ducret et al., 2009), we show that RFRP-3 stimulates the gonadotrophic axis in the male Syrian hamster. Acute central injection of hamster and rat RFRP-3 and avian GnIH stimulates LH/FSH and testosterone secretion in the adult male Syrian hamster. Central injection of hamster RFRP-3 produces a dose-dependent increase in LH and FSH plasma concentrations, but the highest dose had no effect. This could indicate a changing effect with increasing dose, however this seems unlikely as the inhibitory effects of the peptide observed in previous studies remain in the same dose-range as the one used in the present study (Kriegsfeld et al., 2006, Anderson et al., 2009, Pineda et al., 2010b, Johnson et al., 2007). Interestingly, hamster RFRP-1 had no significant effect on LH/FSH secretion. Even though this is in accordance with the literature (Samson et al., 2003, Hinuma et al., 2000, Kaewwongse et al., 2010) it is unclear why divergent effects between peptides binding to the same receptor are obtained. Whereas the stimulatory effect of Kp10 on the gonadotrophic axis has been reported in all the mammalian species in which it has been studied, including the Syrian hamster (Caraty and Franceschini, 2008, Smith et al., 2006a), our findings indicate that the effect of RFRP-3 varies across species and/or the biological status (i.e. gender) of a species. To further support this idea, we carried out icv injections of RFRP-3 and GnIH in ovariectomised female Syrian hamsters, because it has previously been shown that GnIH reduced LH plasma concentrations in ovariectomised female Syrian hamsters (Kriegsfeld et al., 2006). We were unable to demonstrate any stimulatory or inhibitory effect of the two peptides on LH secretion, suggesting that the effects observed may indeed depend on the sex and/or physiological status of the species. This idea is substantiated by the observation that in female mice and hamsters, RFRP-3 neuronal activity is modulated by estrogen levels over the course of the oestrous cycle (Kriegsfeld et al., 2006, Gibson et al., 2008, Molnar et al., 2011), indicating that the administration of RFRP-3 could have differing effects according to the phase of the cycle. It is of interest to remark that in both male and

female rats, RFRP-3 has been shown to modestly inhibit gonadotrophin secretion (Pineda et al., 2010b), unlike the sex-specific effect observed in the Syrian hamster. This could be explained by the fact that there is no effect of estrogen concentrations on RFRP mRNA levels in the female rat (Quennell et al., 2010), contrary to the female Syrian hamster. Overall, these observations suggest that the effect of RFRP-3/GnIH on the reproductive axis might not only vary across species, but might also include sex-specific effects in the same species. Interestingly, it has been shown that GnIH stimulates gonadotrophin release in salmons (Amano et al., 2006), indicating that the stimulatory effect of the peptide on the reproductive axis is not observed exclusively in the male Syrian hamster.

Moreover, our data provide evidence for a previously uncharacterized role of RFRP-3 in the regulation of seasonal reproduction. Indeed, chronic infusion of RFRP-3 reactivated the reproductive axis of male Syrian hamsters kept under photoinhibitory SD conditions, which is consistent with the observation that *rfrp* expression is increased in LD when these animals are sexually active (Revel et al., 2008). The magnitude of the effect obtained with a chronic administration of RFRP-3 is similar to that obtained with rKp10 administered under similar chronic conditions (Revel et al., 2006b). As continuous central administration of RFRP-3 also increases *Kiss1* expression in the ARC, it is likely that the stimulatory effect on gonadal activity results from increased Kp neurotransmission. We can exclude the possibility that the effect on *Kiss1* expression in the ARC results from the increase in sex steroid feedback as a result of testicular reactivation, since testosterone inhibits *Kiss1* expression in the male and female Syrian hamster (Ansel et al., 2010). Therefore, our hypothesis is that, at least in this species, RFRP-3 relays the photoperiod-driven MEL signal towards arcuate *Kiss1* neurons for the seasonal control of reproduction.

In mice, RFRP immunoreactive cells have been localised in the diencephalon, pons, medulla and dorsomedial hypothalamic nucleus (DMH) (Ukena and Tsutsui, 2001). In rats and hamsters, RFRP cell bodies are located in the DMH (Kriegsfeld et al., 2006, Revel et al., 2008, Rizwan et al., 2009). In the sheep brain, RFRP-expressing cells have been found in the paraventricular nucleus and DMH (Clarke et al., 2008, Dardente et al., 2008, Smith et al., 2008). In rodents, RFRP fibre networks are found in multiple brain regions (Johnson et al., 2007, Ukena and Tsutsui, 2001, Mason et al., 2010) and RFRP-ir fibres make apparent contact with GnRH neurons (Kriegsfeld et al., 2006). This suggests that

RFRP-3 acts centrally to control the hypothalamo-pituitary-gonadal axis. There is still uncertainty as to whether RFRP-3 also exerts a hypophysiotrophic effect in mammals, although a large body of evidence now reports the absence of fibres in the median eminence (Kriegsfeld et al., 2006, Yano et al., 2003, Ukena and Tsutsui, 2001, Smith et al., 2010). However, a couple of studies have identified RFRP fibres terminating in the median eminence in the Syrian hamster and sheep (Gibson et al., 2008, Clarke et al., 2008), suggesting a release into the portal circulation and involvement in the regulation of pituitary function. In the rat, RFRP-ir nerve fibres have not been reported in the median eminence (Johnson et al., 2007, Rizwan et al., 2009) and RFRP has not yet been demonstrated in the portal blood. Conflicting results on the effect of peripheral administration of RFRP-3, and *in vitro* studies of the effect of RFRP-3 on rat pituitary cells, make it difficult to conclude on a possible hypophysiotrophic role of RFRP-3 in rats. While intravenous RFRP-3 to ovariectomised rats had no effect on basal LH secretion and minimal effects on GnRH-stimulated secretion in one study (Rizwan et al., 2009), it significantly reduced plasma LH in another (Murakami et al., 2008). Along the same line, RFRP-3 modestly decreased serum LH levels in orchidectomised male rats after peripheral administration (Pineda et al., 2010b). Moreover, although RFRP-3 was shown to inhibit LH secretion from cultured pituitary cells when GnRH is present (Murakami et al., 2008), the peptide did not have a significant effect on basal LH levels in the same study. In another study, RFRP-3 did not have a direct suppressive effect on LH secretion in rat cultured anterior pituitary cells (Anderson et al., 2009). In our study, ip injections of RFRP-3 at three doses did not induce any effect on LH secretion. We started the lowest dose at the highest dose used icv (5 µg), to account for the dilution in the body and also tested two higher doses (100 and 500 µg), to make sure not to miss a possible effect if the dose tested was too low. We consider that the lack of effect does not result from a too high or too low dose, because with a similar amount of Kp54 (300 µg) we obtain a strong stimulatory effect in the experiment. Moreover, incubation of pituitary cells with RFRP-3 did not affect LH levels in the culture medium. Overall, these results indicate that RFRP-3 does not have a direct hypophysiotrophic effect in the Syrian hamster, rather suggesting a centrally-mediated effect.

The c-Fos data indicate that the peptide targets GnRH neurons in the POA, since the icv administration of RFRP-3 increased the number of c-Fos-positive GnRH neurons in LD Syrian hamsters. This is in line with a recent study showing that the density of RFRP-ir

fibre staining in the lateral septum, POA, anterior hypothalamus and paraventricular nucleus of the thalamus significantly increases in LD Syrian hamsters (Mason et al., 2010), however the effect observed on GnRH neurons could be direct or indirect. Our results also indicate that RFRP-3 could be acting via *Kiss1* or non-*Kiss1* neuron targets in the ARC. Indeed, the c-Fos data indicate that the peptide activates cells in the ARC that are not *Kiss1* neurons, which is in line with the literature in which it has been demonstrated that icv administration of RFRP-3 for two weeks to male rats had no effect on *Kiss1* mRNA expression (Johnson and Fraley, 2008). However, because we show that continuous central administration of RFRP-3 for 5 weeks in the male Syrian hamster increases *Kiss1* expression in the ARC, we cannot exclude a possible effect of RFRP-3 on Kp neurons which might reflect a more profound mechanism requiring a longer time to be detectable. It is also possible that RFRP-3 might affect *Kiss1* neurons via a signalling pathway that does not involve c-Fos expression, accounting for the lack of co-localisation in our c-Fos experiment. The receptor for RFRP peptides, NPFF1R, is coupled to an inhibitory G-protein (Bonini et al., 2000), and therefore direct activation of the receptor may not lead to activation of Ca^{2+}-dependent signalling and increase c-Fos. This does not cast doubt on our observations in which RFRP-3 induces an increase in GnRH neuron activation; however we are unable to conclude whether this effect of the peptide is direct or not. The fact that RFRP-3 does not affect c-Fos expression in *Kiss1* neurons but that the chronic administration of the peptide enhances *Kiss1* mRNA levels indicates that the effect of the peptide on these neurons is most likely indirect. To date, studies have identified various brain regions in which NPFF1R is expressed in different species (Gouarderes et al., 2002, Gouarderes et al., 2004b, Gouarderes et al., 2004a); however in the Syrian hamster the brain regions expressing NPFF1R have not yet been investigated. In order to understand the mechanisms of action of RFRP-3 on the reproductive axis in the Syrian hamster, it will be crucial to identify the brain regions expressing NPFF1R and to phenotype the cells expressing the receptor.

Our findings comprehensively demonstrate a stimulatory action of RFRP-3 on the reproductive axis, both acutely and under long-term changes in photoperiod. This suggests that the effect of this peptide on the reproductive function may depend on the physiological status of the animal model and the species tested. Furthermore, there is still controversy over a possible direct effect of RFRP-3 at the level of the pituitary, but our results clearly indicate that this is not the case in the Syrian hamster. Rather, a

central effect at the level of GnRH neurons or other sites in the hypothalamus is most likely. Finally, our data suggest that RFRP-3 neurons are part of the missing link between MEL and *Kiss1* in the seasonal control of reproduction, at least in the Syrian hamster. From a more general perspective, our work challenges the current theory according to which RFRP-3 and Kp exert opposing effects regarding the control of mammalian reproduction. This may not be as universal as previously assumed, and RFRP-3 appears not to be the opposite counterpart of Kp in every given physiological situation. In line with our conclusions, a paper has just been published reporting the effects if GnIH in the Siberian hamster (Ubuka et al., 2012). Overall, these findings raise questions on possible species- and sex-dependent differences in the role of RFRP-3 in the regulation of the reproductive function, especially at the seasonal level.

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CHAPTER 2 – RFRP-3 EXPRESSION AND EFFECTS IN FEMALE SYRIAN HAMSTERS ARE DEPENDENT ON THE REPRODUCTIVE STATUS

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INTRODUCTION

The activity of the hypothalamo-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 many environmental and metabolic factors, and the GnRH neurons are the final common pathway integrating these cues to regulate sexual behaviour accordingly. The identification of GnRH neuron activity regulators has therefore been the focus of many studies, and various neurotransmitters, neuromodulators and hormones have been shown to modulate the activity of GnRH neurons.

A novel peptide involved in the regulation of the hypothalamo-pituitary-gonadal axis was discovered in birds 2000 (Tsutsui et al., 2000). This peptide was termed gonadotrophin-inhibitory hormone (GnIH) because of its ability to inhibit gonadotrophin secretion from cultured quail pituitaries (Tsutsui et al., 2000). The mammalian ortholog of the avian *gnih* gene, named *RFamide-related peptide* (*rfrp*), was discovered concurrently and shown to produce two peptides in rodents, RFRP-1 and RFRP-3 (Hinuma et al., 2000). A large body of data now indicates that RFRP-3 is involved in the regulation of gonadotrophic activity in various mammalian species (Bentley et al., 2010, Kriegsfeld et al., 2010, Tsutsui et al., 2010b, Simonneaux and Ancel, 2012). In intact and gonadectomised (GNX) males and in ovariectomised (OVX) female rats, intracerebroventricular (icv) administration of RFRP-3 inhibits LH secretion (Pineda et al., 2010b). This effect is probably mediated via central targets, as RFRP-immunoreactive (-ir) fibres contact GnRH neurons in rats (Kriegsfeld et al., 2006, Rizwan et al., 2012) and RFRP-3 inhibits GnRH neuronal activation at the LH surge peak (Anderson et al., 2009). In contrast, in male Syrian and Siberian hamsters icv administration of RFRP-3 activates GnRH neurons and stimulates LH secretion (Ancel et al., 2012, Ubuka et al., 2012), indicating that the peptide has species-dependent differences in its effects on the gonadotrophic axis. Interestingly, in OVX female Syrian hamsters icv injection of GnIH inhibits LH secretion (Kriegsfeld et al., 2006), suggesting that the effect of RFRP-3 on the Syrian hamster gonadotrophic axis could be sex-dependent.

Hamsters are a classic model for the study of seasonal reproduction, an evolutionary mechanism which ensures that the birth of offspring occurs at the most favorable time of year. Hamsters are long-day breeders, thus reproductive activity is stimulated by exposure to a long day (LD) photoperiod whereas exposure to a short day (SD) photoperiod induces an inhibition of reproductive activity. In the male Syrian hamster, an electrolytic lesion of the area where RFRP neurons are localized renders the animals blind to the inhibitory short photoperiod (Maywood and Hastings, 1995, Maywood et al., 1996) and *rfrp* levels of expression are markedly down-regulated by MEL in SD-adapted male hamsters (Revel et al., 2008, Ubuka et al., 2012). Therefore, the first aim of this study is to determine whether the photoperiodic MEL-dependent regulation of *rfrp* expression which is observed in male Syrian hamsters is also seen in female Syrian hamsters.

In rodents, female reproduction is characterized by a well-defined estrous cycle which is composed of 4 distinct stages: metestrus, diestrus, proestrus and finally estrus, which corresponds to the time during which the female will be sexually receptive. On the day of proestrus, the LH surge which occurs in the late afternoon is responsible for the subsequent ovulation. In the female Syrian hamster, it has recently been hypothesized that the RFRP system might play a role in modulating the LH surge and ovulation (Khan and Kauffman, 2012). Because it has been shown that GnIH inhibits LH secretion in ovariectomised (OVX) female Syrian hamsters (Kriegsfeld et al., 2006), we hypothesized that an injection of RFRP-3 during different stages of the estrous cycle might have variable effects on gonadotrophin secretion. To test this hypothesis, we compared the effects of icv injections of the peptide carried out on the day of diestrus or during the LH surge on the day of proestrus.

We have demonstrated that chronic central infusion of RFRP-3 to SD-adapted male hamsters rescues their reproductive activity (Ancel et al., 2012), suggesting that RFRP neurons are critical for seasonal synchronization of reproduction. We therefore decided to investigate the effect of acute icv injections of RFRP-3 in both male and female Syrian hamsters kept in LD or SD conditions, in order to shed light on possible sex-dependent differences in the role of RFRP-3 in the seasonal regulation of reproduction.

To date, the precise sites of action of RFRP peptides for the control of the reproductive function remain unknown. Although it is now widely accepted that the peptide acts via GnRH neurons in the hypothalamus in rodents (Kriegsfeld et al., 2006, Smith et al., 2008,

Ubuka et al., 2012, Poling et al., 2012, Rizwan et al., 2012, Ancel et al., 2012), the question of whether RFRP-3 has a hypophysiotrophic effect is still open. In the male Syrian hamster, we have shown that peripheral injections of RFRP-3 have no effect LH secretion and that the peptide does not affect LH release from cultured pituitary cells (Ancel et al., 2012), therefore ruling out the possibility of a hypophysiotrophic effect of RFRP-3. However, a study indicating that peripheral injections of GnIH to OVX female Syrian hamsters inhibit LH secretion (Kriegsfeld et al., 2006) raises the question of possible sex-dependent differences also in the modes of action of RFRP peptides. We therefore decided to analyze the effect of RFRP-3 on LH release from cultured female Syrian hamster pituitary cells.

MATERIALS AND METHODS

ANIMALS

The animals were adult Syrian hamsters (*Mesocricetus auratus*) bred in-house. From birth, they were maintained in a LD photoperiod consisting of 14h light and 10h dark, with lights on at 0500h, at 22±2°C with *ad libitum* access to water and food. The SD photoperiod to which some groups were transferred consisted of 10h light and 14h dark for 10 weeks. 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 with the rules of the European Committee Council Directive of November 24, 1986 (86/609/EEC).

SURGICAL PROCEDURES

Female Syrian hamsters were anesthetized with 3% isoflurane (AErrane; Baxter, Maurepas, France) and N_2O to carry out OVX or sex steroid replacement. For OVX, the animals were left a week to recover from surgery prior to the beginning of the experiment (i.e. maintained in LD conditions or transferred to SD conditions). For sex steroid replacement, estradiol (1,3,5[10]-estratriene-3,17β-estradiol; Sigma)–filled silastic capsules (i.d. 1.47 mm; o.d. 1.95 mm; length: 13 mm) were subcutaneously implanted to animals prior to transfer to SD conditions.

In some experiments, the oestrous stage was determined by carrying out daily vaginal smears over two weeks prior to sacrifices.

ICV INJECTIONS

Syrian hamsters were anaesthetised using a mixture of Zoletil 20 (Virbac, Carros, France) and Rompun (Bayer Pharma, Puteaux, France) and positioned in the stereotaxic apparatus. The head of the animal was shaved and prepared for aseptic surgery. A single

incision was made on the midline of the scalp. Once the area had been prepped, a stainless steel 30-gauge cannula was placed in the lateral ventricle at 2 mm lateral to the midline, 0.8 mm posterior to the Bregma and 3 mm inferior to the dura mater. The cannula was kept in place on the skull by dental cement and bone screws. The cannula was blocked with a metallic wire and protected with a plastic cap. The animals were allowed a week to recover from the surgery. The injections (2µL/animal; flow rate 1µL/min) were given in the afternoon between 15:00 and 16:00 (which corresponds to the time frame of the LH surge on the day of proestrus) using a 30-gauge stainless steel cannula attached to polyethylene tubing and a 50 µl Hamilton syringe (Hamilton Inc., Reno, NV, USA) under light anaesthesia with isoflurane vapour for the duration of the injections.

IMMUNOHISTOCHEMISTRY (IHC) AND *IN SITU* HYBRIDISATION (ISH)

RFRP-ir and *rfrp* mRNA levels were analysed in female Syrian hamsters during diestrus or at the time of the LH surge on the day of proestrus. On the day of the sacrifice the animals were deeply anesthetised with CO_2 vapour and their thorax opened. Blood was taken by intracardial puncture for subsequent LH assay. Heparin (250 IU per animal; Liquemine®, Roche, Meylan, France) was injected directly into the left ventricle, and a cannula was placed in the aorta. Blood was washed out with 100ml of 1X phosphate buffered saline (PBS; pH 7.4) and the tissues were fixed by perfusing 250ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was removed from the skull, post-fixed for 24 h at 4°C in 4 % formaldehyde in 0.1 M phosphate buffer (pH 7.4), transferred to 0.05 M PBS and rinsed overnight. The brains were then dehydrated and embedded in polyethylene glycol as previously described (Klosen et al., 1993). Serial 16-µm-thick coronal sections were cut throughout the DMH and 1 in 6 was mounted on SuperFrost®Plus (Menzel-Gläser, Braunschweig, Germany) slides. Two series of sections were then processed for IHC or ISH.

IHC The sections were processed using the peroxidase-antiperoxidase (PAP) method. Before incubation in the primary antiserum, non-specific binding sites were saturated for 1h with 3% dry skimmed milk in TBS containing 0.05% Tween 20 (TBS-Tween, Sigma, St. Quentin Fallavier, France). The slides were then incubated overnight

with a primary rabbit polyclonal antibody raised against the rat RFRP precursor peptide at 1:15000 (Rizwan et al., 2009) diluted in TBS-Tween with 1% fetal calf serum. After incubation with the primary antiserum the sections were washed in TBS-Tween and incubated for 60min in a donkey-anti-rabbit IgG secondary antibody diluted at 1:200 in the same buffer, and washed again in TBS-Tween. Finally, the PAP complex diluted 1:800 in TBS-Tween with 0.2% cold water fish skin gelatin was used to detect the secondary antibody for 1 h. After a final rinse in TBS-Tween, peroxidase activity was detected using 3,3'-diaminobenzidine (Sigma, St. Quentin Fallavier, France) as a chromogen and 0.01% urea hydrogen peroxide (Acros Organics, Geel, Belgium) as a substrate. The slides were then mounted and RFRP-ir was quantified.

ISH Sense and antisense riboprobes were transcribed from linearised plasmids containing a *Phodopus sungorus rfrp* cDNA (87-529 of GenBank JF727837) in the presence of digoxigenin-labelled nucleotides (Roche, Meylan, France) according to the manufacturer's protocol. In brief, the sections were fixed in 4% paraformaldehyde, digested for 30 min at 37 °C with 1 µg/ml proteinase K (Roche, Meylan, France) in PBS, postfixed in cold 2% paraformaldehyde and acetylated in triethanolamine buffer. After the riboprobe had been denatured and mixed with hybridization medium (200 ng/mL) it was applied to slides and incubated for 40 h at 60°C. Six stringency rinses were performed at 72°C. Digoxigenin-labelled bound probes were detected with an alkaline phosphatase-labelled antidigoxigenin antibody (Roche). Alkaline phosphatase activity was detected with bromochloroindolyl phosphate and nitroblue tetrazolium in the presence of 5% polyvinyl alcohol (70.000-100.000 MW; Sigma). The slides were then mounted and *rfrp* mRNA levels were quantified.

QUANTIFICATION The total number of RFRP-ir and *rfrp* labelled cells was counted manually throughout the DMH by an observer blind to the estrous status of the individual animals. For the quantification of the integrated density of individual cells, photomicrographs were taken on a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) with an Olympus DP50 digital camera (Olympus France, Rungis, France). All lighting parameters on the microscope and the camera software (Viewfinder Lite, Olympus) were standardized to ensure consistent stable lighting throughout the image capture procedure. A background image of the slide without a section was taken for each slide and the background image was subtracted from the corresponding sample image to compensate for heterogeneous illumination of the image field. The images were

then analyzed using ImageJ software (Rasband, W. S., National Institutes of Health, Bethesda, MD). In brief, a fixed-size circle was laid over labelled cells, and the mean labelling intensity was determined by measuring all of the labelled cells which had been hand-counted. Once the number of *rfrp*/RFRP cells had been counted and the intensity of the labelling measured, the total amount of RFRP-ir of *rfrp* mRNA was obtained by multiplying the number of cells by the mean integrated density for each animal.

PITUITARY CELL CULTURE

The pituitary glands of female Syrian hamsters were sampled and immediately dissociated as previously described by Ancel et al. (Ancel et al., 2012). In short, cells were dissociated by enzyme dispersion and pipette trituration in a saline solution containing collagenase (0.8 mg/mL), trypsin (0.2 mg/mL) and DNAse (10 mg/mL) at 37°C. After the glands were entirely dissociated, cells were resuspended in Dulbecco's modifed Eagle's medium (DMEM) containing 8% horse serum, 2% fetal calf serum and 40 mg/mL gentamicin, plated at a density of 700 000 viable cells/well and maintained at 37°C under a mixture of 95% air and 5% CO2 at 100% humidity. After preincubation for 48 h, the medium was changed, followed by incubation for 6 h in culture medium alone as a control, RFRP-3 (1 nM; Abgent, San Diego, USA), GnRH (1nM; Sigma) or RFRP-3 and GnRH (1nM of each).. After culturing for 6 h, media were collected and subjected to ELISA for LH.

HORMONE MEASUREMENTS

Serum LH levels were determined in a volume of 25–50µL using a double-antibody method and RIA kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 was labelled with ^{125}I using Iodo-gen® tubes, following the instructions of the manufacturer (Pierce, Rockford, IL, USA). Hormone concentrations were expressed using the reference preparation LH-RP-3 as standard. Intra- and inter-assay coefficients of variation were, respectively, < 8 and

10% and the sensitivity of the assay was 5 pg/tube. Accuracy of determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

LH secretion in pituitary cell cultures was determined using an enzyme-linked immunosorbent assay (ELISA). After culturing for 6 h, media were collected and subjected to ELISA for LH. In brief, microtiter plates were filled with 100µL of rat LH High Purity in coating buffer, at a concentration of 10ng/100µL, and incubated overnight at 4°C. Excess LH was removed, and the plates were washed using 200µL/well of 10 mM phosphate buffer saline (PBS) with 0.1% Tween-20. The plates were blocked with 200µL/well of 10 mM PBS containing 1% BSA and 0.1% Tween-20 for 1 h at room temperature. 200µL of sample or various concentrations of standard rLH-RP-3, diluted in assay buffer, were preincubated with 200µL of LH antiserum (rLH-S-11 - 1:3000 in assay buffer) for 18 h at 4°C. 100µL of preincubated samples, standards and controls were added per well in triplicate and incubated overnight at 4°C. After washing, 100µL of donkey anti-rabbit IgG conjugated to horseradish peroxidase was added at 1:1000 dilution and incubated for 1 h at 37°C. The plates were again washed, and 100µL of 3,3',5,5'–tetramethylbenzidine substrate was added to all the wells. The colour reaction was allowed to develop for 30 min in the dark. The enzyme was stopped by adding 50µL of 0.5% sulphuric acid per well and the optical density of each well was immediately read at 492nm. Intra- and inter-assay coefficients of variation were, respectively, < 8 and 11%.

STATISTICAL ANALYSES

Results are shown as mean ± SEM. All statistical analyses were performed using Statistica (StatSoft Inc., USA). Data were analyzed by *t* test or one-way ANOVA, followed by post-hoc analysis: Tukey's Honestly Significant Difference test, as appropriate. Statistical significance was set at $P_{value} < \alpha = 0.05$.

RFRP EXPRESSION IS REGULATED BY PHOTOPERIOD INDEPENDENTLY OF CIRCULATING SEX STEROID LEVELS

In this study, we sought to determine whether the photoperiodic regulation of *rfrp* expression follows the same profile in female Syrian hamsters as in male hamsters. To do so, female Syrian hamsters were placed in LD or SD conditions for 10 weeks prior to sacrifice, in order to analyse *rfrp* mRNA levels. In parallel, the involvement of gonadal steroids in the photoperiodic modulation of *rfrp* expression was analysed in female hamsters which were OVX in LD or SD conditions or implanted with E_2 capsules in SD conditions. Our results indicate that *rfrp* expression is down-regulated in SD conditions compared to LD conditions in the female Syrian hamster (Figure 24). As in males, this down-regulation is not due to secondary changes in sex steroid concentrations, as OVX in LD and SD and E_2 implants in SD do not alter *rfrp* levels of expression compared to control animals (Figure 24).

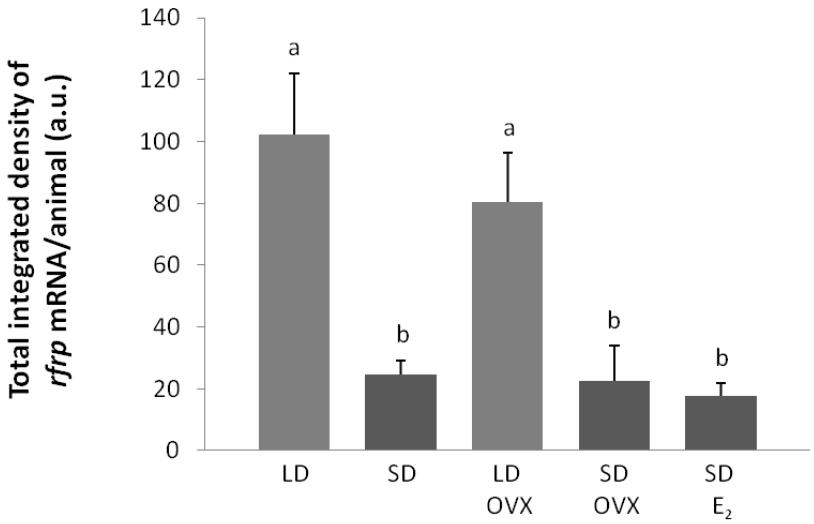

FIGURE 24 - *RFRP* EXPRESSION IS DOWN-REGULATED IN SD COMPARED TO LD IN THE FEMALE SYRIAN HAMSTER

Quantification of *rfrp* mRNA levels showing that *rfrp* expression is reduced in SD compared with LD animals. Moreover, OVX carried out in LD and SD conditions and estradiol implants in SD conditions indicate that the reduction in *rfrp* expression in SD conditions is not due to secondary changes in gonadal steroid concentrations. Data represent mean ± SEM (n = 6/group). *Bars with differing letters* differ significantly ($P < 0.05$ by one-way ANOVA followed by Tukey's analysis).

Because in our previous experiment the estrous cycle of females in LD conditions was not followed, we decided to investigate whether *rfrp* mRNA and peptide content varied according to the estrous stage, as suggested in a previous study (Gibson et al., 2008). On the day of diestrus or proestrus, animals were sacrificed between 15:00 and 16:00, which corresponds to the time frame at which the preovulatory LH surge occurs in this species. Plasma LH levels were subsequently assayed and indicate that the animals were indeed sacrificed at the time of the LH surge on the day of proestrus (Figure 25A). In situ hybridization for *rfrp* mRNA analysis indicates that the total amount of *rfrp* mRNA is identical between proestrus and diestrus females (Figure 25B), suggesting that there is no difference in RFRP synthesis. Immunohistochemical analysis of RFRP-ir revealed that there were no differences between proestrus and diestrus animals (Figure 25C), indicating that there is no difference in RFRP storage.

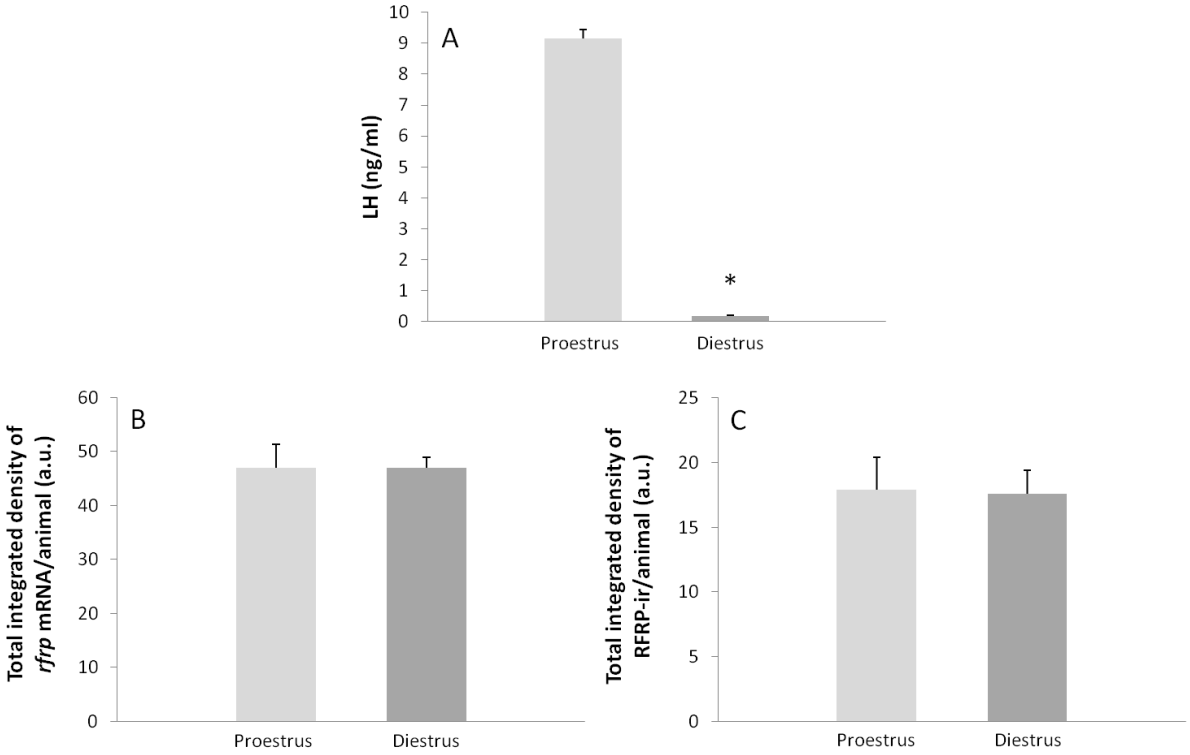

FIGURE 25 - *RFRP* MRNA AND RFRP-IR LEVELS IN THE DORSOMEDIAL NUCLEUS OF THE HYPOTHALAMUS OF THE FEMALE SYRIAN HAMSTER ARE SIMILAR IN PROESTRUS AND DIESTRUS

At the time of the LH surge on the day of proestrus (A), the total amount of *rfrp* mRNA (B) and RFRP-ir (C) do not significantly differ from levels at the same time point on the day of diestrus in the female Syrian hamster. Data represent mean ± sem (n = 7/group). *, $P < 0.05$ compared with the proestrus group by Student's *t* test.

THE EFFECT OF CENTRAL RFRP-3 ADMINISTRATION ON LH SECRETION DEPENDS ON THE ESTROUS STAGE

We carried out a study in a physiological model in which the effect of RFRP-3 on the gonadotrophic axis would be evaluated in intact female hamsters according to the stage of the estrous cycle. The estrous cycle of adult female Syrian hamsters was followed and an icv injection of RFRP-3 (1500 ng) was carried out between 15:00 and 16:00 on the day of diestrus or proestrus. When carried out on the day of diestrus, central administration of RFRP-3 does not have any effect on LH secretion (Figure 26A). However, when the injection is performed during the preovulatory LH surge on the day of proestrus, it induces a highly significant decrease in plasma LH concentrations within 30 minutes of administration (Figure 26A). This observation indicates that the effect of RFRP-3 on the female Syrian hamster hypothalamo-pituitary-gonadal axis depends on the estrous stage.

THE EFFECT OF RFRP-3 ADMINISTRATION ON LH SECRETION DOES NOT DEPEND ON THE PHOTOPERIOD

Since RFRP-3 administration induces variable effects in LD conditions on LH secretion in the female Syrian hamster, we decided to investigate the effect of the peptide under SD conditions. Icv administration of RFRP-3 under SD conditions to female Syrian hamsters had no effect on plasma LH concentrations (Figure 26A), similar to the effect observed in diestrus. In contrast, an icv injection of RFRP-3 in both LD and SD conditions results in a significant increase in plasma LH concentrations in the male Syrian hamster (Figure 26B). These results suggest that the photoperiodic effect of RFRP-3 on the reproductive axis is sex-dependent.

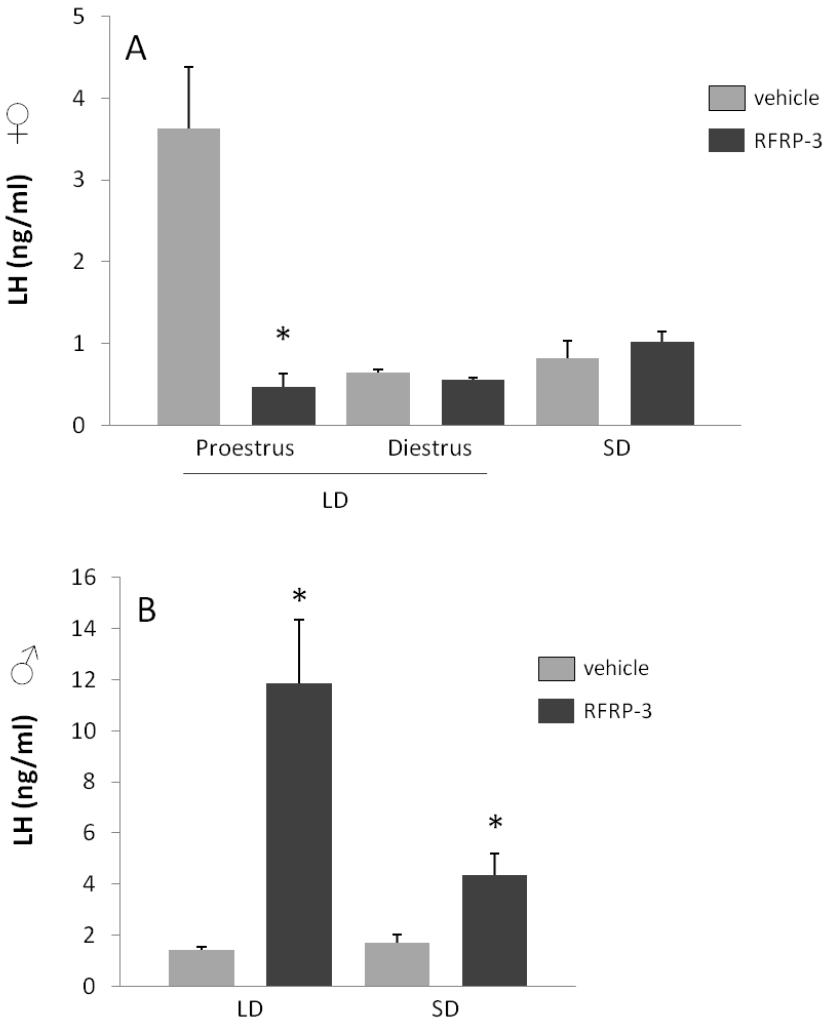

FIGURE 26 - (A) RFRP-3 ADMINISTRATION TO FEMALE SYRIAN HAMSTERS HAS VARIABLE EFFECTS ON GONADOTROPHIN SECRETION ACCORDING TO THE REPRODUCTIVE STATE AND (B) THE EFFECT OF RFRP-3 ON GONADOTROPHIN SECRETION IS STIMULATORY IN BOTH LD AND SD CONDITIONS IN THE MALE SYRIAN HAMSTER

(A) A central injection of RFRP-3 (1500 ng, icv) to female Syrian hamsters in LD has no effect in diestrus or in SD conditions but inhibits LH secretion at the time of the LH surge on the day of proestrus. Data represent mean ± sem (n = 7/group). *, $P < 0.05$ compared with the vehicle-injected group by Student's *t* test.

(B) A central injection of RFRP-3 (1500 ng, icv) to male Syrian hamsters stimulates LH secretion after 30 min in LD and SD conditions. Mean ± sem (n = 6/group). *, $P < 0.05$ compared with the vehicle-injected group by Student's *t* test.

We have previously demonstrated that RFRP-3 did not have a hypophysiotrophic effect in the male Syrian hamster (Ancel et al., 2012), and this is in contrast with a study indicating that peripheral administration of GnIH inhibits LH secretion in OVX hamsters (Kriegsfeld et al., 2006). We therefore decided to analyze the effects of RFRP-3 on LH secretion directly from cultured pituitary cells from intact female Syrian hamsters (Figure 27). Incubation with RFRP-3 for 6 h (1nM) had no effect on LH concentrations, in contrast to GnRH (1 nM) which stimulated LH secretion significantly. Similarly, RFRP-3 did not prevent GnRH from stimulating LH secretion. Taken together, these observations show that in female Syrian hamsters, RFRP-3 exerts its effect on the gonadotrophic axis through central targets.

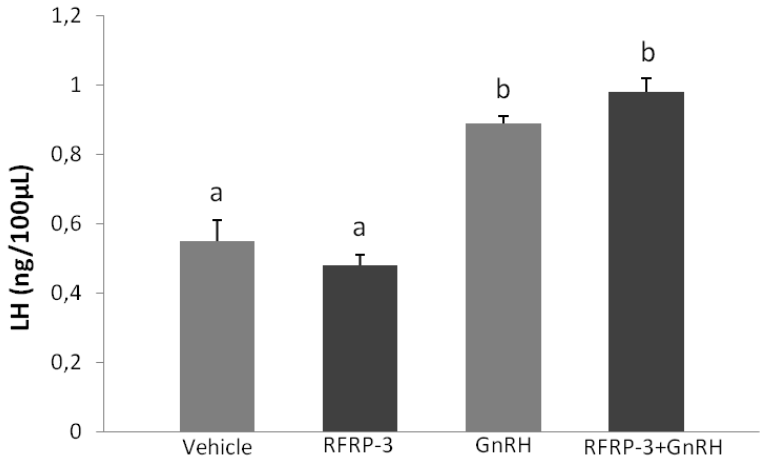

FIGURE 27 - RFRP-3 DOES NOT HAVE A HYPOPHYSIOTROPHIC EFFECT IN THE FEMALE SYRIAN HAMSTER

Hamster pituitary cells were incubated for 6 h with RFRP-3 alone (1 nM), GnRH alone (1 nM) or RFRP-3 in the presence of GnRH. LH secretion was assayed in the supernatant. RFRP-3 did not significantly alter the basal secretion of LH by female Syrian hamster pituitary cells in culture. Similarly, RFRP-3 did not significantly affect GnRH-induced LH secretion from cultured hamster pituitary cells. Data represent mean ± sem (n = 3/group). *Bars with differing letters* differ significantly ($P < 0.05$ by one-way ANOVA followed by Tukey's analysis).

DISCUSSION

The recent discovery of a novel player in the regulation of the gonadotrophic axis in mammals, namely RFRP-3, has led to intensive research on the precise function of this peptide in the regulation of the reproductive function. In females, reproductive activity is submitted to regular phases of activation and inactivation, therefore inducing a cycle in ovulation which is the basis of the estrous cycle in rodents. In addition, in wild animals reproductive activity is submitted to a seasonal regulation to ensure species survival. Consequently, in seasonal female rodents like the Syrian hamster there is a dual regulation of the reproductive function: estrous and seasonal.

It has been proposed that the RFRP neuronal network could play a role in the regulation of seasonal reproduction. In male Syrian and Siberian hamsters, *rfrp* expression is reduced by MEL in SD (Revel et al., 2008, Ubuka et al., 2012), and in Syrian hamsters the SD reduction in *rfrp* expression is independent of circulating levels of gonadal steroids (Revel et al., 2008). In the present work, we show that the photoperiodic regulation of *rfrp* expression is the same in the female Syrian hamster as it is in the male. Indeed, *rfrp* expression is reduced in SD conditions compared to LD conditions, independently of the circulating levels of estrogen. Therefore, the photoperiodic regulation of *rfrp* expression in the female Syrian hamster is likely to be controlled by MEL.

Because in our LD group we did not follow the estrous cycle, we went on to analyse *rfrp* expression and RFRP-ir at the time of the LH surge on the day of proestrus and in diestrus. In female Syrian hamsters, a previous study indicated that RFRP-ir cell numbers and their activational state are decreased at the time of the LH surge on the day of proestrus, compared to other time points on the day of proestrus and diestrus (Gibson et al., 2008). We were unable to replicate these results and in our hands both *rfrp* mRNA and RFRP protein levels were identical at the time of the LH surge on the day of proestrus and in diestrus. These diverging observations could be explained by the use of different antibodies, given that in our study we use a rabbit polyclonal antibody raised against the rat RFRP precursor peptide whereas Gibson et al. used a rabbit anti-white-crowned sparrow GnIH antibody (Gibson et al., 2008). However, the fact that we confirmed our immunohistochemical results with mRNA level analysis supports our observation in female hamsters. Moreover, our study of the photoperiodic regulation of *rfrp* expression confirms that gonadal steroids do not affect *rfrp* mRNA levels in both

male (Revel et al., 2008) and female Syrian hamsters. Similarly, a study in rats showed no difference in *rfrp* mRNA levels of females that were OVX versus OVX and treated with estrogen or progesterone (Quennell et al., 2010).

The present study is the first to investigate the effect of RFRP-3 in a female rodent in physiological conditions. Indeed, previous studies analysing the effect of the peptide in OVX females raise a number of questions regarding the physiological significance of the results. To date, the effect of RFRP-3 administration on the female reproductive axis has only been investigated in OVX animals, in order to bypass the potential interference due to varying levels of sex steroids (Pineda et al., 2010b, Murakami et al., 2008, Kriegsfeld et al., 2006). Nonetheless in both female OVX rats (Murakami et al., 2008, Pineda et al., 2010b) and Syrian hamsters (Kriegsfeld et al., 2006), central administration of RFRP-3 inhibits artificially-elevated LH plasma concentrations. However, female reproduction presents regular cycles of activation and inactivation. The female rodent estrous cycle is characterised by the preovulatory LH surge which occurs at a specific time window on the day of proestrus, under the influence of combined circadian signals arising in the SCN and estrogen-mediated feedback loops acting on multiple brain circuitries. During the other stages of the estrous cycle, circulating LH levels are constantly low. We hypothesised that the effect of RFRP-3 on the female reproductive axis might not be the same during different stages of the estrous cycle. The present results indicate that icv RFRP-3 inhibits LH secretion at the time of the LH surge on the day of proestrus, when LH levels are naturally elevated, but that the peptide has no effect on gonadotrophin secretion when LH levels are naturally low. It therefore appears that the RFRP neuronal network serves as an inhibitory component regulating the female Syrian hamster reproductive axis. There are two possible interpretations of these results: 1)the inhibitory effect of RFRP-3 is at its maximum during diestrus, and we are therefore unable to artificially induce a stronger inhibition; or 2)LH levels are already at their lowest level during diestrus and can therefore not be artificially inhibited. In proestrus however, comparable to OVX conditions, LH levels are endogenously elevated and exogenous RFRP-3 administration inhibits gonadotrophin secretion. Our results suggest that the RFRP neuronal system could be involved in the control of the LH surge in the female Syrian hamster. It is already established that the kisspeptin neurons in the arcuate (ARC) and anteroventral periventricular (AVPV) hypothalamic nuclei are key components regulating the estrous cycle and are involved in the positive-feedback

effects of estrogen leading to the preovulatory LH surge (Khan and Kauffman, 2012). It therefore appears that these two distinct neuronal populations, which produce two peptides of the RFamide family of peptides, could be central players in the mechanisms involved in generating the preovulatory LH surge. The combined effects of RFRPs and Kps might shape the LH secretion profile throughout the estrous cycle. This hypothesis is supported by the observation that in rats RFRP-3 fibres are in contact with kisspeptin neurons, a subpopulation (20%) of which expresses the *Gpr147* gene (Rizwan et al., 2012).

The present results raise interesting questions regarding possible sex-dependent differences in the effect of RFRP-3 on the reproductive axis. Indeed, in a previous study we have demonstrated that icv administration of the peptide induces a potent stimulatory effect on the male Syrian hamster reproductive axis (Ancel et al., 2012), and this stimulatory effect is confirmed in the present study in both LD and SD conditions. Here we show that icv RFRP-3 inhibits LH secretion in the female Syrian hamster at the time of the preovulatory LH surge and has no effect on gonadotrophin secretion during diestrus, a result which is similar to previous data obtained in OVX Syrian hamsters (Kriegsfeld et al., 2006). Possible explanations for these sex-related discrepancies in the effect of RFRP-3 on the gonadotrophic axis of Syrian hamsters could result from different GPR147 signalling, although to date there is no available data to support or contradict this hypothesis. The target cells of RFRP-3 could also be different in male and female hamsters, therefore resulting in different downstream effects. Finally, the sexual dimorphim in the Kp AVPV neuronal population could explain the discrepancies in the results obtained in male and female Syrian hamsters. Indeed, in rodents Kp AVPV cell numbers are higher in females than in males (Adachi et al., 2007, Kauffman et al., 2007, Clarkson and Herbison, 2006, Ansel et al., 2010). AVPV Kp neurons play a central role in the regulation of female reproduction. It is therefore possible that the Kp neurons in the ARC and AVPV are the targets mediating the effects of RFRP-3 on the reproductive axis in the Syrian hamster, inducing different effects in males and females.

Although it has now been demonstrated in a number of mammalian species that the effect of RFRP-3 on the gonadotrophic axis is mediated via central targets, in particular GnRH neurons in the rostral periventricular area (Ducret et al., 2009, Kriegsfeld et al., 2006, Rizwan et al., 2012, Poling et al., 2012, Anderson et al., 2009, Ubuka et al., 2012, Ancel et al., 2012), the question of a possible hypophysiotrophic effect of the peptide is

as yet unanswered. We have previously shown that RFRP-3 does not have a hypophysiotrophic effect in the male Syrian hamster (Ancel et al., 2012), but peripheral injections of GnIH inhibit LH secretion in OVX Syrian hamsters (Kriegsfeld et al., 2006) and GPR147 (also known as NPFF1; the receptor for RFRP peptides) has been detected in the pituitary of this species (Gibson et al., 2008). In this study, we show that RFRP-3 application to intact female Syrian hamster pituitary cells does not affect the basal or GnRH-stimulated LH release, indicating that the peptide does not have a hypophysiotrophic effect in the female Syrian hamster. This result is in accordance with our previous study in the male Syrian hamster (Ancel et al., 2012), and suggests that although the effects of RFRP-3 on the reproductive axis are sex-dependent, the modes of action of the peptide could be conserved. The effect of peripheral injections of GnIH to OVX female hamsters on LH secretion could be due to an action of the peptide at the level of GnRH nerve terminals in the median eminence, as is the case for kisspeptins in this species (Ansel et al., 2011). This hypothesis deserves further investigation, but it is supported by the observation that RFRP-ir fibres, although sparse, are present in the median eminence of female Syrian hamsters (Kriegsfeld et al., 2006).

The RFRP neuronal network has recently been shown to play a role in the regulation of seasonal reproduction. Indeed, continuous central infusion of RFRP-3 to male Syrian hamsters maintained in SD conditions reactivates the reproductive axis in spite of photoinhibitory conditions (Ancel et al., 2012). In this study, we show that RFRP-3 administration in SD conditions stimulates LH secretion in the same way as under LD conditions in the male Syrian hamster. On the other hand, icv administration of RFRP-3 to female Syrian hamsters kept under SD conditions has no effect on gonadotrophin secretion, in the same manner as in diestrus. Taken together, these observations raise a number of interesting questions regarding the sex-dependent differences in the role of the RFRP system in the regulation of reproduction.

The present results indicate that the RFRP system is involved in the regulation of ovulation in the female Syrian hamster. More specifically, it appears that RFRP neurons could act in concert with Kp neurons to generate the preovulatory LH surge. At the same time, the present studies are the first to identify a sex-dependent difference in the effect of RFRP-3 on the gonadotrophic axis and therefore suggest that the involvement of the RFRP neuronal network in the regulation of male and female reproduction is different. Moreover, from the viewpoint of seasonal reproduction, the RFRP system appears to

display striking species-dependent differences in its role. Indeed, even in rodents (Syrian and Siberian hamsters) belonging to the same subfamily (*Cricetinae*), although the photoperiodic regulation of *rfrp* expression is identical, the effect of central injections of RFRP-3 in LD and SD conditions is different (Ubuka et al., 2012). Overall, the present data show that the involvement of the RFRP network in the regulation of reproductive functions is far more complicated than initially expected and that many more investigations will be required to answer the numerous pending questions. In future experiments, it will be essential to bear in mind that there might be sex-dependent differences in the effect of RFRP-3 in other species than the Syrian hamsters.

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CHAPTER 3 – MODES AND SITES OF ACTION OF RFRP-3 IN THE SYRIAN HAMSTER BRAIN

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In preparation

Gonadotrophin-releasing hormone (GnRH) released into the portal blood system is responsible for the production and release of gonadotrophins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). GnRH neurons are therefore the final hypothalamic target for hypothalamic-pituitary-gonadal (HPG) axis activity regulators, such as neurotransmitters, neuropeptides, and peripheral hormones. It is well established that various regulators of GnRH release act on upstream targets in the brain. In recent years, it has emerged that kisspeptins (Kp) are key elements of the networks controlling GnRH secretion and mediating sex steroid feed-back effects in the brain. However, our understanding of the interactions of Kp with other regulatory signals of GnRH neurons remains incomplete.

In 2000, the *RFamide-related peptide* (*rfrp*) gene was identified in humans and shown to encode a precursor that produces two peptides, RFRP-1 and RFRP-3 (Hinuma et al., 2000). *Rfrp* is the mammalian ortholog of avian *gonadotrophin-inhibitory hormone* (*gnih*), which was identified in quails concurrently, the product of which was shown to inhibit gonadotrophin release from quail pituitaries (Tsutsui et al., 2000). The demonstration that GnIH is a potent inhibitor of gonadotrophin release in quails spurred great interest in the roles of RFRP-1 and RFRP-3 in the regulation of endocrine functions in mammals. A large number of studies now indicate that RFRP-3 inhibits the release of LH in mammals (Ducret et al., 2009, Johnson et al., 2007, Pineda et al., 2010b, Anderson et al., 2009, Clarke et al., 2008, Kadokawa et al., 2009). However, recent studies carried out in hamsters imply that there are species-dependent differences in the effect of the peptide on the gonadotrophic axis. Indeed, in Syrian and Siberian hamsters RFRP-3 appears to stimulate or inhibit gonadotrophin secretion depending on photoperiod-dependent factors (Ancel et al., 2012, Ubuka et al., 2012). These differences could be due to the seasonal component of reproduction; indeed, in hamsters reproduction is stimulated by exposure to a long day (LD) photoperiod whereas exposure to a short day (SD) photoperiod induces an inhibition of the reproductive function. Interestingly, in hamsters *rfrp* expression is down-regulated by MEL in a short day photoperiod (Ubuka et al., 2012, Revel et al., 2008), suggesting that this peptide could be involved in the seasonal regulation of reproduction. In any case, the involvement of RFRP peptides in the regulation of the hypothalamo-pituitary-gonadal axis of mammals is now thoroughly

documented but different effects are observed across sex, species and reproductive status; this raises a number of questions regarding the sites of action of the peptides.

In rodents, RFRP fibre networks are found in multiple brain regions (Ukena and Tsutsui, 2001, Kriegsfeld et al., 2006, Johnson et al., 2007, Mason et al., 2010) and RFRP-immunoreactive fibres make apparent contact with GnRH neurons (Ubuka et al., 2012, Kriegsfeld et al., 2006, Poling et al., 2012, Rizwan et al., 2012). In the Syrian hamster, we have shown that RFRP-3 administration induces c-Fos expression in GnRH neurons, suggesting that RFRP-3 acts centrally to control the hypothalamo-pituitary-gonadal axis (Ancel et al., 2012). In addition, RFRP-3 fibres are in contact with kisspeptin neurons in rats (Rizwan et al., 2012), and we have shown that continuous infusions of RFRP-3 to male Syrian hamsters increase arcuate *Kiss1* expression, concurrently with an increase of testicular activity (Ancel et al., 2012). This suggests that the RFRP neuronal system could be acting at different levels of the gonadotrophic axis to exert its effects on the reproductive function.

The RFRP peptides bind preferentially to GPR147 (also known as NPFF1) and with a lower affinity to GPR74 (also known as NPFF2). These receptors were initially identified as neuropeptide FF (NPFF) receptors but it was later shown that GPR147 has a higher affinity for RFRPs than for NPFF whereas GPR74 has a higher affinity for NPFF than for RFRPs. The GPR147 receptor couples with $G\alpha_{i3}$ and $G_{\alpha s}$ proteins (Gouarderes et al., 2007) suggesting that GPR147 can have both inhibitory and stimulatory downstream effects on cellular activity, although in CHO cells activation of the receptor inhibits forskolin-stimulated cAMP accumulation (Mollereau et al., 2002). Early studies describing the autoradiographic distribution of GPR147 in mice and rats indicated that the receptor was present throughout the hypothalamus (Gouarderes et al., 2002, Gouarderes et al., 2004b, Gouarderes et al., 2004a), but remarkable variations in GPR147 and GPR74 receptor contents and distribution exist from one species to another and from one strain to another among the same species (Gouarderes et al., 2004b, Gouarderes et al., 2004a). Recently, a few studies have provided a more detailed distribution of the receptor. In mice, rats, and Siberian hamsters, 25% of GnRH neurons express *Gpr147*, but not *Gpr74* (Rizwan et al., 2012, Poling et al., 2012, Ubuka et al., 2012) and in rats a subpopulation of Kp neurons expresses the *Gpr147* gene (Rizwan et al., 2012). However, a detailed distribution of GPR147 in the Syrian hamster

hypothalamus has not yet been carried out, and therefore a number of questions remain unanswered concerning potential RFRP-3 sites of action.

In recent years, novel tools have made it possible to investigate RFRP-3 modes of action in more detail, notably with the development of GPR147/74 antagonists. In 2006, a new compound with a potent antagonistic activity and similar binding affinity for GPR147 and GPR74 was discovered and termed RF9 (Simonin et al., 2006). This dipeptide was initially used to block the effects of NPFF on heart rate and blood pressure and to prevent opioid-induced hyperalgesia and tolerance in rats, phenomena that are mediated via GPR74 (Simonin et al., 2006). However, given that RF9 has similar binding affinity for GPR147 and GPR74, this compound could serve for the study of RFRP modes of action in the control of the reproductive function. In rats and mice, intracerebroventricular (icv) administration of RF9 increases circulating levels of gonadotrophins (Pineda et al., 2010c); however because the antagonist was not administered concurrently with RFRP-3 no conclusion can be drawn as to whether this effect is due to antagonisation of endogenous inhibitory RFRP-3 effects or whether it is a stimulatory effect of the dipeptide itself. Recently, a novel antagonist termed RF313 with a strong selectivity towards GPR147 has been discovered (Simonin et al., unpublished data). RF313 therefore appears as a valuable tool in the study of RFRP-3 modes of action for the control of the gonadotrophic axis.

In order to provide a better understanding of RFRP-3 sites and modes of action in the Syrian hamster, we carried out a detailed mapping of GPR147 in the Syrian hamster hypothalamus. Because *rfrp* expression is down-regulated by MEL in a short day photoperiod in the Syrian hamster (Revel et al., 2008), the mapping was quantified separately under LD and SD conditions, in order to detect a potential photoperiodic regulation in receptor content. Moreover, to determine whether the effects of RFRP-3 on the Syrian hamster gonadotrophic axis are mediated via GPR147, we analysed the effect of icv injections of RFRP-3 on LH secretion, in the presence or not of RF9 or RF313.

MATERIALS AND METHODS

ANIMALS

The animals were adult male Syrian hamsters (*Mesocricetus auratus*) bred in-house. From birth, they were maintained in a LD photoperiod consisting of 14h light and 10h dark, with lights on at 0500h, at 22±2°C with *ad libitum* access to water and food. The SD photoperiod to which some groups were transferred consisted of 10h light and 14h dark. 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 with the rules of the European Committee Council Directive of November 24, 1986 (86/609/EEC).

ICV INJECTIONS

Syrian hamsters were anaesthetised using a mixture of Zoletil 20 (Virbac, Carros, France) and Rompun (Bayer Pharma, Puteaux, France) and positioned in the stereotaxic apparatus. The head of the animal was shaved and prepared for aseptic surgery. A single incision was made on the midline of the scalp. Once the area had been prepped, a stainless steel 30-gauge cannula was placed in the lateral ventricle at 2 mm lateral to the midline, 0.8 mm posterior 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. The cannula was blocked with a metallic wire and protected with a plastic cap. The animals were allowed a week to recover from the surgery. The injections (2µL/animal; flow rate 1µL/min) were given in the morning using a 30-gauge stainless steel cannula attached to polyethylene tubing and a 50 µl Hamilton syringe (Hamilton Inc., Reno, NV, USA) under light anaesthesia with isoflurane vapour for the duration of the injections. Animals received two boluses which were administered 15 min apart. For each group, the treatment was as follows: vehicle group: vehicle-vehicle; RFRP-3 group: vehicle-RFRP-3; antagonist group: antagonist-antagonist; antagonist in the presence of RFRP-3 group: antagonist-antagonist with RFRP-3. The animals were sacrificed 30 min after the

injection of the second bolus and blood was taken by intracardiac puncture for subsequent LH assay.

HORMONE MEASUREMENTS

Serum LH levels were determined in a volume of 25–50µL using a double-antibody method and RIA kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 was labelled with ^{125}I using Iodo-gen® tubes, following the instructions of the manufacturer (Pierce, Rockford, IL, USA). Hormone concentrations were expressed using reference preparations LH-RP-3 as standard. Intra- and inter-assay coefficients of variation were, respectively, < 8 and 10%. The sensitivity of the assay was 5 pg/tube. Accuracy of determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

PREPARATION OF THE RIBOPROBES AND *IN SITU* HYBRIDIZATION

In order to quantify *Gpr147* mRNA expression by *in situ* hybridization, a GPR147 antisense riboprobe was transcribed with T7 RNA polymerase, according to the protocol provided with the MAXIscript® kit (Ambion, USA).

Animals were deeply anesthetised with CO_2 vapour and killed by decapitation. Brains were removed from the skull, snap-frozen on dry ice, and stored at -80°C until sectioning. Brains were sectioned using a cryostat (Leica, Leica microsystems, Rueil-Malmaison, France) at -20°C. Four sets of serial sections (16µm) were cut and thaw-mounted on SuperFrost®Plus (Menzel-Gläser, Braunschweig, Germany) slides and stored at -80°C until ISH.

Brain sections were treated with 4% paraformaldehyde in PBS for 15 min at room temperature, and then rinsed in PBS for 2 min. The slides were acetylated in 0.75% acetic anhydride in 0.1 M triethanolamine (pH 8.0) for 10 min and rinsed 2 min in PBS. Afterwards, slides were dehydrated in increasing concentrations of ethanol (70, 90, 95

and 100%) for 1 min in each. The slides were then dried at room temperature before hybridization. Sections were hybridized at 54°C for 16 h in a humid chamber with 400pM of the antisense *Gpr147* cRNA riboprobe labelled with [^{35}S]UTP (1250 Ci/mmol, NEN, France) in a solution containing 50% deionized formamide, 10% dextran sulfate, 50 mM dithiothreitol, Denhardt's solution 1X, SSC 2X, 0.5 mg/ml salmon sperm DNA, and 0.25 mg/ml yeast RNA. After incubation, the sections were rinsed for 5 min at room temperature in SSC 2X before being treated with ribonuclease A (0.15 Kunitz unit/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 in SSC 1X for 5 min followed by SSC 0.05X at 52°C for 30 min, to eliminate most of the non-specific labelling. Finally, sections were dehydrated in graded ethanol baths (70, 90, 95 and 100%, 1 min each), dried at room temperature and subsequently exposed to X-ray films (HyperfilmMP, Amersham) for 15 days concomitantly with microscale standards. Quantitative analysis of the autoradiogram was performed with a picture analysis system using ImageJ, as previously described (Gauer et al., 1998, Schuster et al., 2000, Poirel et al., 2002).

STATISTICAL ANALYSES

Results are shown as mean ± SEM. All statistical analyses were performed using Statistica (StatSoft Inc., USA). Data were analyzed by *t* test or one-way ANOVA, followed by post-hoc analysis: Tukey's Honestly Significant Difference test, as appropriate. Statistical significance was set at $P_{value} < \alpha = 0.05$.

RF313 ADMINISTRATION ABOLISHES THE STIMULATORY EFFECT OF RFRP-3 ON GONADOTROPHIN SECRETION IN THE MALE SYRIAN HAMSTER, WHEREAS RF9 DOES NOT.

We have previously demonstrated that RFRP-3 potently stimulates gonadotrophin secretion in male Syrian hamsters when administered icv. In order to determine whether this effect is mediated by GPR147, we carried out icv injections of RFRP-3 in the presence or not of RF9, a GPR147/GPR74 antagonist (Figure 28). As expected, icv administration of RFRP-3 (0.75 nM) induced a significant increase in LH secretion compared to the administration of vehicle. Surprisingly, when RF9 (8 nM) was injected alone it potently stimulated LH secretion to levels significantly higher than those observed following RFRP-3 administration. When injected simultaneously, RFRP-3 (0.75 nM) and RF9 (2 nM) induced an increase in LH secretion comparable to the levels observed with RF9 administration alone, and significantly higher than the levels observed with RFRP-3 alone. The same effect was obtained when injecting a lower dose of RF9 (2 nM) alone or concurrently with RFRP-3 (data not shown).

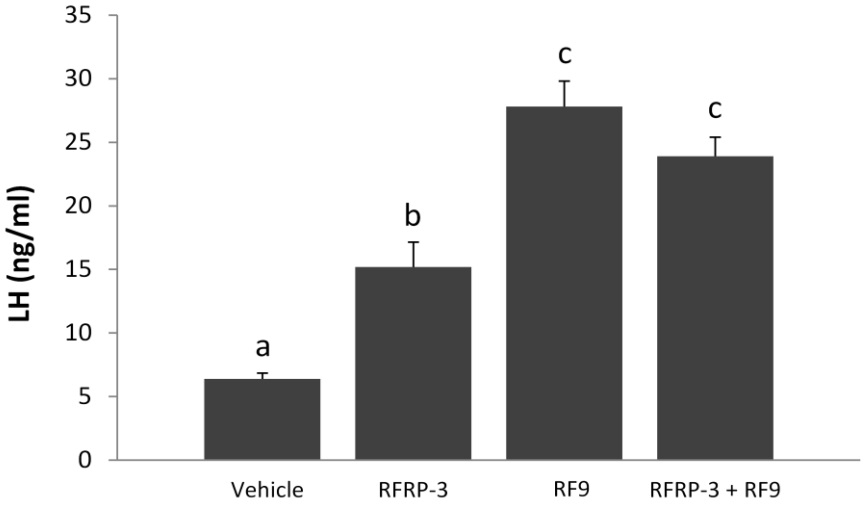

FIGURE 28 - EFFECT OF CENTRAL ADMINISTRATION OF RF9, IN THE PRESENCE OR NOT OF RFRP-3, ON LH SECRETION IN THE MALE SYRIAN HAMSTER

A central injection of RFRP-3 (0.75 nM, icv) to male Syrian hamsters in LD increases LH secretion significantly. A central injection of RF9 (8 nM, icv) induces a potent stimulatory effect on LH secretion, significantly higher than the effect obtained with RFRP-3. When administered concurrently, RFRP-3 and

RF9 powerfully stimulate LH secretion, to levels comparable to those obtained with RF9 alone. Data represent mean ± SEM (n = 6/group). Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis).

Because it appears that RF9 is not a suitable tool for the study of the modes of action of RFRP-3 in the Syrian hamster, we decided to test the effect of a recently discovered novel GPR147 antagonist, RF313. As previously described, icv administration of RFRP-3 (0.75 nM) induced a significant increase in LH secretion compared to the administration of vehicle (Figure 29). When injected alone, icv RF313 (10 nM) had no effect on LH secretion in male Syrian hamsters. However, when injected concurrently with RFRP-3 (0,75 nM), icv RF313 (10 nM) potently blocks the stimulatory effect of RFRP-3 on LH secretion.

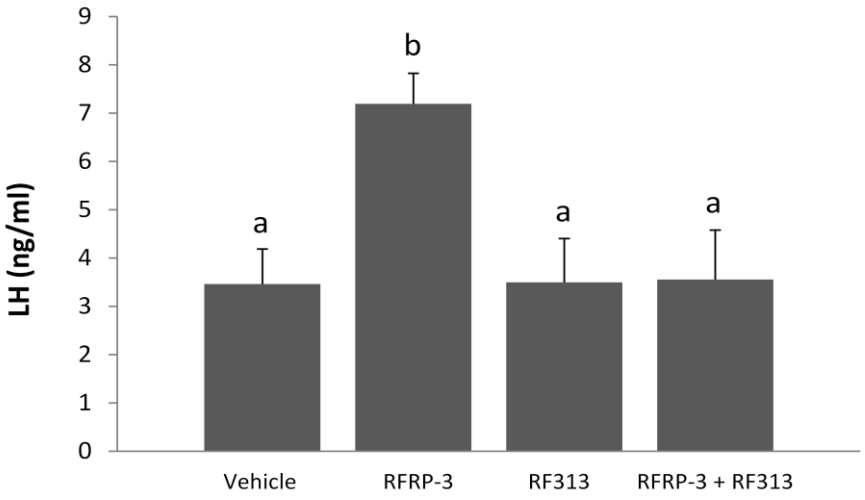

FIGURE 29 - EFFECT OF CENTRAL ADMINISTRATION OF RF313, IN THE PRESENCE OR NOT OF RFRP-3, ON LH SECRETION IN THE SYRIAN HAMSTER

A central injection of RFRP-3 (0.75 nM, icv) to male Syrian hamsters in LD increases LH secretion significantly. Administration of RF313 (10 nM, icv) alone has no effect on basal LH secretion, and simultaneous injection of RFRP-3 and RF313 abolishes the stimulatory effect on LH secretion obtained with RFRP-3 alone. Data represent mean ± SEM (n = 6/group). Bars with differing letters differ significantly ($p < 0.05$ by one-way ANOVA followed by Tukey's analysis).

Taken together, the results obtained using NPFF receptor antagonists indicate that: 1) RF9 is not a selective GPR147 antagonist and, 2) the effects of RFRP-3 on gonadotrophin secretion in the Syrian hamster are indeed mediated via GPR147.

The antagonistic properties of RF9 for GPR147 have already been questioned in a study carried out in GPR54 KO mice (Garcia-Galiano et al., 2012). Indeed, the stimulation of LH

release following RF9 administration is reduced in GPR54 KO mice compared to control wild-type mice, indicating that part of the effect observed is due to agonistic properties of RF9 on GPR54. This aspect will be discussed in further detail in the general discussion of this manuscript.

GPR147 MRNA EXPRESSION LEVELS SHOW PHOTOPERIODIC VARIATIONS IN THE MALE SYRIAN HAMSTER

In an attempt to investigate RFRP-3 sites of action, a [^{35}S]UTP-labelled riboprobe was used to map the localization of the receptor by *in situ* hybridization in the male Syrian hamster brain. Clear labelling was observed in various hypothalamic areas, particularly in the POA, SCN, PVN, anterior hypothalamus, VMH, DMH and ARC, but also in the lateral habenular nucleus and the PVT (Figure 30).

As previously mentioned, *rfrp* expression in the Syrian hamster is strongly down-regulated by MEL in SD and quantification of *Gpr147* mRNA expression in LD and SD was therefore carried out separately. Surprisingly, *Gpr147* mRNA levels appear to be higher in several hypothalamic structures in SD compared to LD, including the SCN, the PVN, the anterior hypothalamus, the VMH and the ARC (Figure 31).

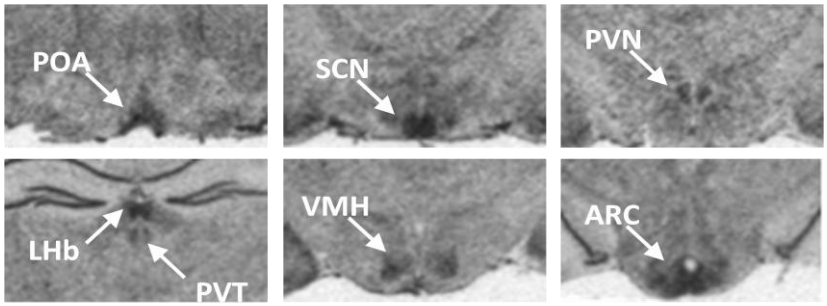

FIGURE 30 - REPRESENTATIVE PICTURES OF *GPR147* MRNA EXPRESSION IN THE SYRIAN HAMSTER HYPOTHALAMUS

Labelling for *Gpr147* mRNA was observed in the preoptic area (POA), suprachiasmatic nuclei (SCN), paraventricular nucleus of the hypothalamus (PVN), lateral habenula (LHb), paraventricular nucleus of the thalamus (PVT), ventromedial hypothalamic nuclei (VMH) and dorsomedial hypothalamic nuclei (DMH).

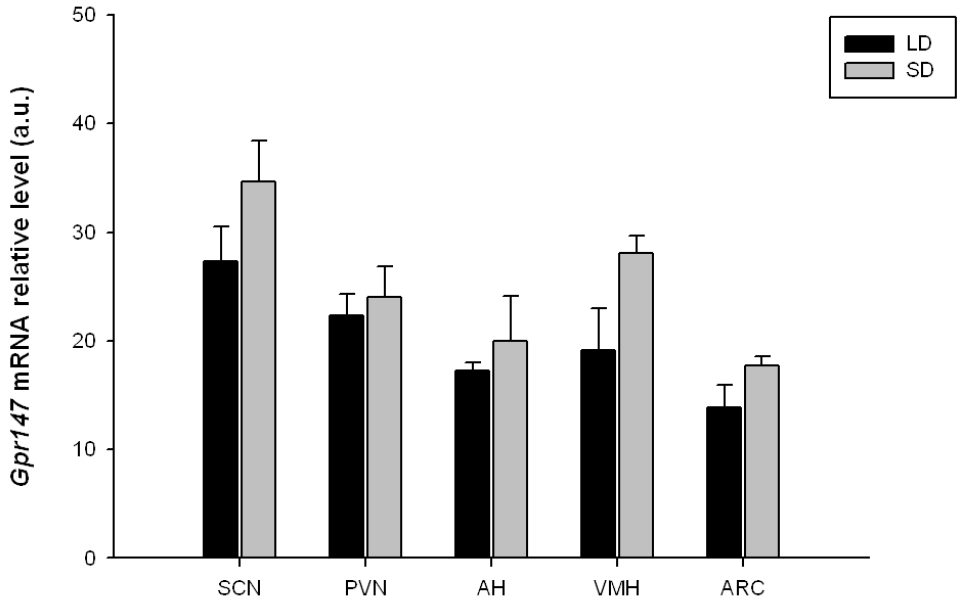

FIGURE 31 - EFFECT OF PHOTOPERIOD ON *GPR147* MRNA LEVELS IN THE SYRIAN HAMSTER HYPOTHALAMUS

The expression of *Gpr147* tends to be increased in SD conditions in the hypothalamus of the male Syrian hamster, compared to LD. Because there are only a small number of animals in each group, statistical analysis did not reveal a significant effect of photoperiod. Data represent mean ± SEM (n = 2/group in LD and 3/group in SD).

The preliminary mapping of GPR147 distribution in the Syrian hamster brain is concordant with the binding studies carried out in other rodents (Gouarderes et al., 2002, Gouarderes et al., 2004b, Gouarderes et al., 2004a). It appears that the receptor is widely expressed throughout the hypothalamus, including in the POA and ARC. This is of interest because GnRH neurons express *Gpr147* in mice, rats and Siberian hamsters (Rizwan et al., 2012, Poling et al., 2012, Ubuka et al., 2012) and a subpopulation of Kp neurons expresses the *Gpr147* gene in rats (Rizwan et al., 2012). Additional studies will be required in order to phenotype the cells expressing GPR147 in Syrian hamsters.

These results also indicate that GPR147 receptor content is regulated by photoperiod. In this preliminary experiment, no statistical significance was reached because the groups of animals were too small and a larger-scale experiment will be carried out in order to obtain a sufficient number of animals in each group. Nevertheless, the observation that GPR147 content in the Syrian hamster hypothalamus is photoperiodically-regulated

supports the hypothesis that the RFRP-3 system could be involved in the seasonal regulation of reproduction.

GENERAL DISCUSSION

SPECIES-DEPENDENT DIFFERENCES IN THE INVOLVEMENT OF RFRP-3 IN THE REGULATION OF THE HPG AXIS ACTIVITY

This work comprehensively demonstrates that RFRP-3 stimulates the male Syrian hamster gonadotrophic axis. Indeed, when administered acutely, central RFRP-3 injections stimulate LH, FSH and testosterone secretion both in LD and SD conditions. This effect on the HPG axis is likely to be mediated via the GnRH neurons in the POA, as the same type of administration induces c-Fos expression in GnRH neurons. These results, published concurrently with work carried out in the male Siberian hamster (Ubuka et al., 2012), are the first to indicate that the effect of RFRP-3 on the gonadotrophic axis could be species-dependent. Indeed, until recently only an inhibitory effect of the peptide had been reported on the reproductive axis of mammals, including rats (Johnson et al., 2007, Pineda et al., 2010b, Murakami et al., 2008), sheep (Clarke et al., 2008, Sari et al., 2009), and cattle (Kadokawa et al., 2009). The reasons for these species-related differences in the effect of RFRP-3 on the reproductive axis are not yet understood. Anyhow, it appears that different species have evolved the RFRP system differently. One hypothesis is that the RFRP neuronal network has taken on different functions in seasonal and non-seasonal breeders. The involvement of the RFRP system in the regulation of seasonal reproduction will be discussed later in the manuscript.

IN THE SYRIAN HAMSTER

The Syrian hamster is a seasonal breeder, in which sexual activity is stimulated by exposure to LD conditions. In this species, *rfrp* expression is down-regulated in SD by MEL, independently of secondary changes in circulating levels of gonadal steroids in both males (Revel et al., 2008) and females (present data). In male Syrian hamsters, continuous infusions of RFRP-3 in SD conditions induce a reactivation of the reproductive axis, manifested by increases in ARC *Kiss1* expression, paired testes weight, and circulating testosterone levels (Ancel et al., 2012). In the same line, acute injections of RFRP-3 stimulate LH secretion in both LD and SD conditions. Interestingly, the MBH (where RFRP neurons are located) is a key area in mediating the inhibitory effect of MEL on reproduction, because a lesion of this area abolishes the SD-induced gonadal regression (Maywood and Hastings, 1995, Maywood et al., 1996). Taken together, these elements point to *rfrp* neurons as central players in the seasonal regulation of reproduction in the Syrian hamster. The increase in *Kiss1* expression following continuous RFRP-3 infusion suggests that the effect on the reproductive axis is mediated via this hypothalamic target; however, further studies are required in order to determine 1)how RFRP-3 regulates *Kiss1* expression in the ARC; 2)whether MEL acts directly on RFRP neurons in the DMH or on other targets in the MBH. RFRP-3 is most likely not acting directly on Kp neurons in the ARC because GPR147 is coupled to an inhibitory G-protein. However, RFRP-ir fibres have been localised in the ARC (Kriegsfeld et al., 2006), GPR147 appears to be expressed in this area (present data), and RFRP-3 administration induces c-Fos expression in non-Kp neurons in the ARC (Ancel et al., 2012). Taken together, these data suggest that RFRP-3 is indeed acting on non-Kp neurons in the ARC, which could in turn modulate *Kiss1* expression.

IN OTHER SEASONAL SPECIES

In the Siberian hamster, a recent study has shown that *rfrp* expression is also down-regulated by MEL in SD conditions, compared to LD conditions (Figure 32) (Ubuka et al., 2012). However, in this species RFRP-3 administration inhibits LH secretion in LD whereas it stimulates LH secretion in SD (Ubuka et al., 2012). Although we are unable to provide an explanation for the diverging effects of RFRP-3 administration in Syrian and Siberian hamsters, they might reflect a different organization of the photoperiodic system. Indeed, whereas in Syrian hamsters *Kiss1* expression in increased in the ARC and AVPV in LD conditions (Revel et al., 2006b), in the Siberian hamster *Kiss1* expression and Kp-ir are reduced in the ARC in LD compared to SD and increased in the AVPV (Simonneaux et al., 2009, Greives et al., 2007, Mason et al., 2007). This suggests that in the Siberian hamster, *Kiss1* neurons are differently involved in the effect of MEL on the HPG axis. The discrepancies in *Kiss1* expression, as well as in the effect of RFRP-3 on LH secretion (Ancel et al., 2012, Ubuka et al., 2012), might reflect differences in the system relaying photoperiodic information onto the gonadotrophic axis. This hypothesis is supported by the observation that SCN lesions prevent the inhibitory effect of exogenous MEL infusions in the Siberian hamster (Bartness et al., 1991), but not in the Syrian hamster (Bittman et al., 1989). Conversely, a lesion of the MBH prevents the SD-induced gonadal regression in Syrian hamsters (Maywood and Hastings, 1995), but not in the Siberian hamster (Song and Bartness, 1996). Additional experiments will be necessary in order to shed light on the different pathways involved in transmitting photoperiodic information, via MEL, to the reproductive axis of both hamster species. It is of note to remark that the SD down-regulation of *rfrp* expression is also observed in other seasonal rodents, notably the European hamster (Figure 32) (Simonneaux and Ancel, 2012) and the jerboa (Janati et al., 2013).

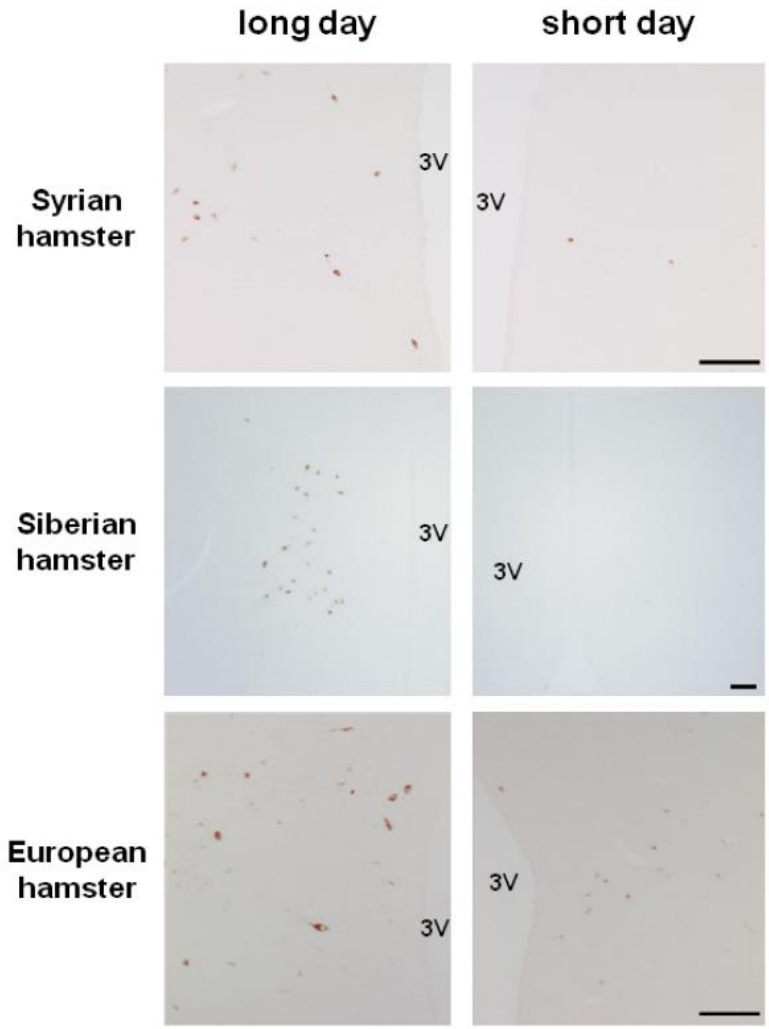

FIGURE 32 - PHOTOPERIODIC VARIATIONS IN RFRP IMMUNOREACTIVITY IN THE DORSOMEDIAL HYPOTHALAMUS OF MALE SYRIAN, SIBERIAN AND EUROPEAN HAMSTERS RAISED IN LONG DAY OR SHORT DAY CONDITIONS

Scale bar = 100 µM, 3V: third ventricle. *Simonneaux & Ancel, 2012*.

In sheep, gestation lasts approximately 5 months, and they will therefore be sexually active in SD conditions to ensure that the offspring is born during the most favourable season. Like in the Syrian hamster, *Kiss1* expression and Kp-ir are increased in the ARC and POA during the breeding season (Wagner et al., 2008, Smith, 2008, Chalivoix et al., 2010), indicating that Kp presumably play a similar role in sheep as in rodents. Interestingly, *rfrp* expression is decreased during the breeding season in the sheep compared with the non-breeding season (Dardente et al., 2008), supporting the hypothesis for an inhibitory effect of RFRP-3 on the reproductive function in this species.

It is well established that in both LD and SD breeders, MEL is responsible for transmitting the photoperiodic information to the gonadotrophic axis. However, so far the mechanisms which lead from an identical signal to a different behavioural response remain unclear. Indeed, why does a long peak of MEL inhibit reproductive activity in LD breeders and stimulate it in SD breeders? One hypothesis is that RFRP neurons in the hypothalamus might be the switchpoint, via one or more interneurons, in transmitting the MEL/seasonal information to the HPG axis (Figure 33). Indeed, in SD breeders such as the sheep, increased *rfrp* expression in LD conditions would result in a direct inhibitory effect on Kp neurons and/or GnRH neurons, therefore shutting down the reproductive function. On the other hand, in LD breeders such as the Syrian hamster, increased *rfrp* expression in LD conditions would result in the inhibition of an inhibitory interneuron, therefore stimulating the reproductive function. However, a number of questions remain unanswered: what, if any, interneurons are involved? is there a direct link between RFRP neurons and GnRH neurons? Finally, although MEL appears to be acting via RFRP neurons, it is not yet known whether the effect is direct or if it is mediated through other targets. It appears unlikely that MEL would act directly upon RFRP neurons, as MEL receptors have only been reported in the Syrian hamster in this brain region (Weaver et al., 1989, Williams et al., 1989).

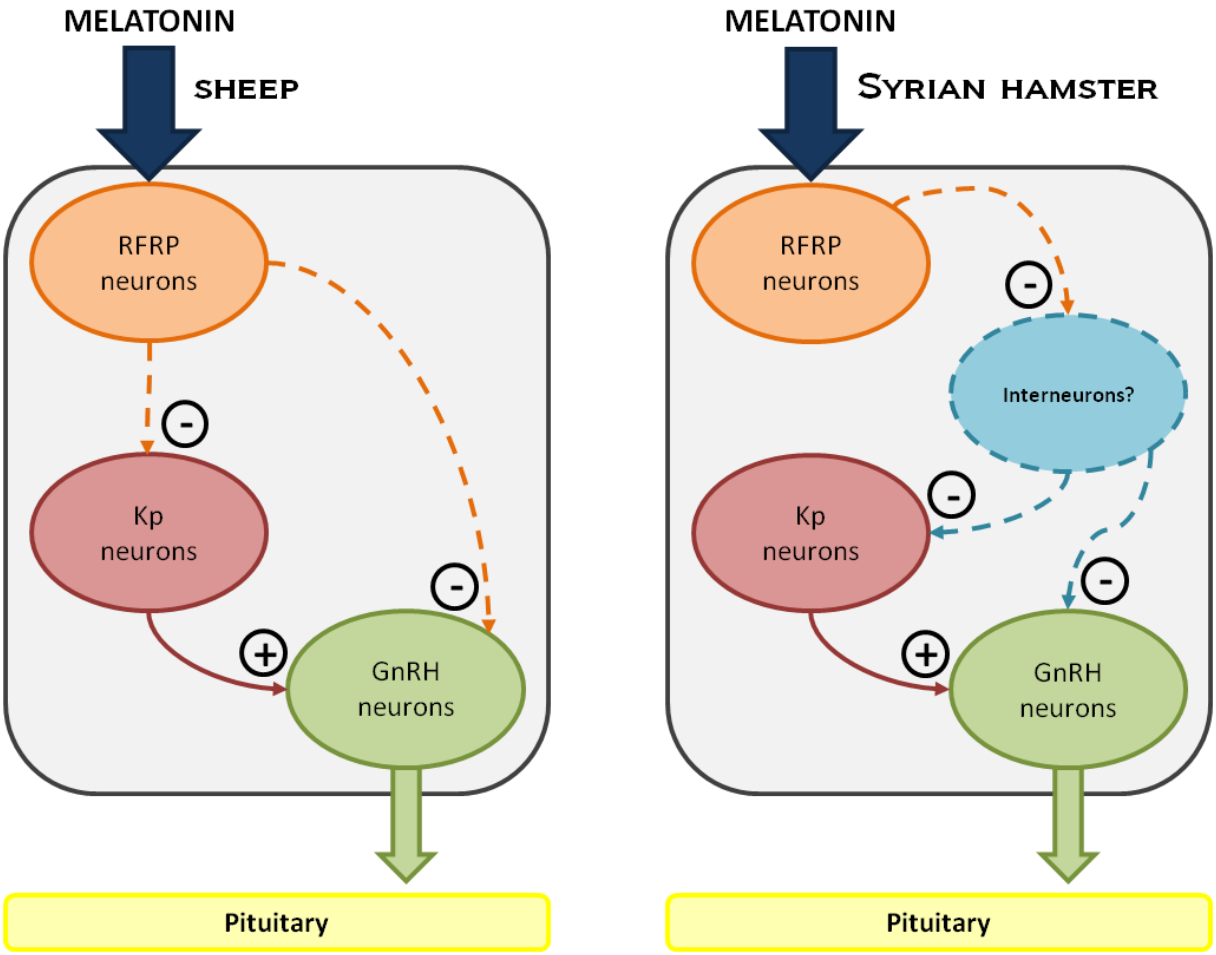

FIGURE 33 - SCHEMATIC MODEL OF THE ROLE OF RFRP-3 IN GENERATING A LONG OR SHORT DAY GONADAL RESPONSE TO MELATONIN

The central sites of action of melatonin for the seasonal control of reproduction are not known. However, because *rfrp* expression is down-regulated in a long day (LD) photoperiod in both long day and short day (SD) breeders, these neurons could be the site responsible for inducing the appropriate gonadal response to photoperiod. This is supported by the fact that RFRP-3 administration stimulates the gonadotrophic axis in hamsters whereas it inhibits the reproductive axis in sheep. Therefore, in sheep (left panel) RFRP-3 would inhibit the reproductive function in LD conditions via a direct or indirect action on Kisspeptin (Kp) and/or GnRH neurons. In hamsters (right panel), RFRP-3 stimulates the reproductive axis in LD conditions via unknown interneurons, which inhibit Kp and/or GnRH neurons directly or indirectly.

Regarding the exact site of action of MEL for the control of seasonal reproduction, the PT appears as a serious candidate, because MEL receptors have been identified in this area in all mammalian species investigated (Figure 34) (Morgan and Williams, 1989, Bartness et al., 1993, Masson-Pevet and Gauer, 1994, Morgan et al., 1994, Morgan and Mercer, 1994, Masson-Pevet et al., 1996).

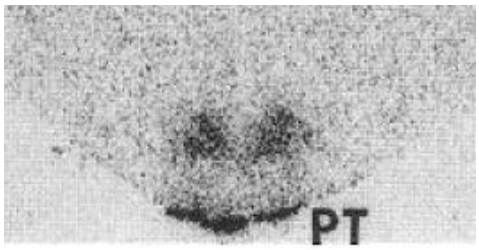

FIGURE 34 - AUTORADIOGRAPH OF SPECIFIC BILATERAL [^{125}I]IODOMELATONIN-BINDING WITHIN THE MEDIOBASAL HYPOTHALAMUS IN THE SYRIAN HAMSTER

In the Syrian hamster, melatonin binding sites have been localised in the mediobasal hypothalamus and pars tuberalis of the adenohypophysis (PT). *Maywood & Hastings, 1995.* The PT appears as a serious candidate in mediating the photoperiodic information to the reproductive axis, as melatonin receptors have been consistently identified in this region in all the mammalian species investigated.

Accumulating evidence now suggests that the PT could be involved in transmitting seasonal information via a pathway involving the thyroid stimulating hormone (TSH), the enzyme deiodinase 2 (Dio2) and the thyroid hormone triiodothyronine (T_3) (Figure 35). MEL receptors have been shown to co-localise with TSH-ir cells (Klosen et al., 2002) and TSH expression is modulated by photoperiod through MEL (Dardente et al., 2003, Dardente et al., 2010). An increase in TSH expression (or an exogenous administration of the hormone) induces an increase in Dio2 concentrations in the tanycytes (Hanon et al., 2008, Nakao et al., 2008, Klosen et al., 2013), a specialised type of glial cells which bridge the cerebrospinal fluid of the third ventricle. The enzyme Dio2 is responsible for the conversion of the inactive thyroid hormone thyroxine (T_4) to the bioactive form T_3, the increase in Dio2 concentrations therefore leading to a local increase in T_3 concentrations. A study carried out in Siberian hamsters indicates that the local increase

in T_3 concentrations is responsible for the photoperiodic reactivation of the reproductive axis, as implantation of a capsule releasing T_3 in the dorsomedial hypothalamus of short day-adapted male Djungarian hamsters induces a reactivation of the reproductive function (Barrett et al., 2007). In a recent study, TSH infusions were shown to reactivate the reproductive axis of male Syrian and Siberian hamsters kept in SD conditions, along with a restoration of LD-like levels in *rfrp* and *Kiss1* expression (Klosen et al., 2013). Thus, in Syrian hamsters *rfrp* expression in the DMH and *Kiss1* expression in the ARC are both increased. In the Siberian hamster, *rfrp* expression in the DMH is increased, whereas *Kiss1* expression in the ARC is decreased. These results indicate that RFRP neurons in the DMH are the primary target of MEL for the seasonal regulation of reproduction, and that Kp neurons are downstream modulators of reproductive activity. Taken together, these data indicate that the MEL receptors located in the TSH-expressing cells of the PT could be the conserved site of action of MEL for the seasonal control of reproduction.

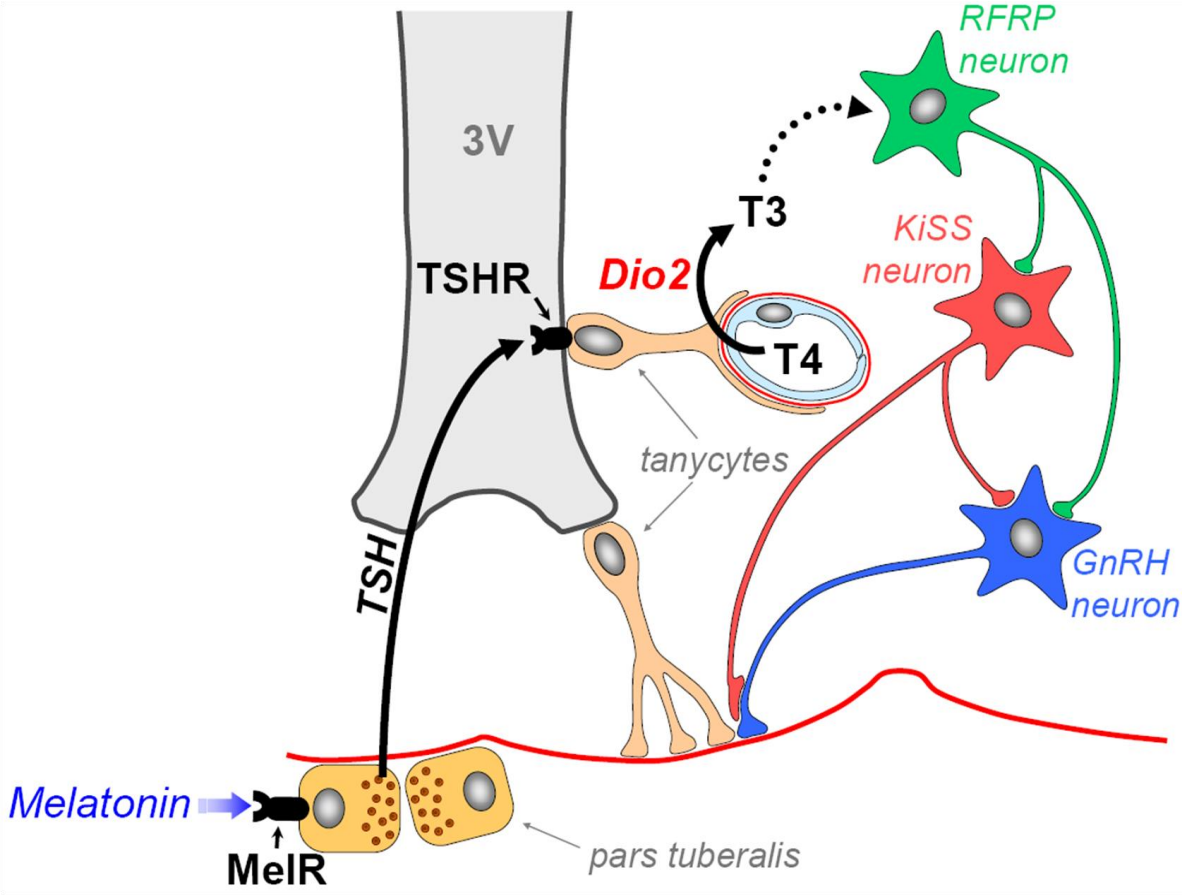

FIGURE 35 - MELATONIN-TSH-THYROID HORMONE SIGNALLING IN THE SEASONAL CONTROL OF REPRODUCTION OF MALE HAMSTERS

The photoperiodic signal is detected at the *pars tuberalis* of the adenohypophysis and converted into a TSH message transmitted to the tanycytes. This TSH message controls the expression of *Dio2*, which ultimately controls the local availability of the active form the thyroid hormones, T3. This local availability of T3 controls the gonadotrophic axis, hypothetically through the modulation of RFRP neurons, which appear to be the key switch for transitions from a non reproductive to a reproductive phenotype or reversely. Furthermore, RFRP neurons may act either directly on GnRH neurons and/or indirectly via kisspeptin neurons. *Klosen et al., 2013*.

INVOLVEMENT OF RFRP-3 IN THE REGULATION OF FEMALE REPRODUCTION

Alongside the species-dependent effects of RFRP-3 on the HPG axis, this work is the first to demonstrate a sex-dependent difference in the involvement of RFRP-3 in the regulation of reproduction. Indeed, whereas the peptide has a potent stimulatory effect on the male Syrian hamster gonadotrophic axis, the effect of RFRP-3 in the female Syrian hamster is variable. To date, the effect of RFRP-3 on the female gonadotrophic axis has only been investigated in OVX animals, including rats (Pineda et al., 2010b, Murakami et al., 2008), Syrian hamsters (Kriegsfeld et al., 2006) and sheep (Clarke et al., 2008, Sari et al., 2009). The reason underlying this protocol is that the levels of pituitary and gonadal hormones vary considerably throughout the reproductive cycle, and the feed-back effects of gonadal hormones are therefore also variable depending on the estrous stage. In order to rule out any side-effects of these mechanisms on the outcome of RFRP-3 administration, OVX is carried out prior to the injections. However, this experimental approach might not be adapted for the study of the effects of RFRP-3 on the mammalian reproductive axis because recent data suggest that *rfrp* neurons could be involved the estrogen-mediated positive feed-back loop.

THE ROLE OF RFRP NEURONS IN MEDIATING SEX STEROID FEED-BACK EFFECTS

The involvement of the RFRP neuronal system in the sex steroid feed-back effects on the brain has recently been investigated. A certain amount of data indicates that *rfrp* expression is not strongly regulated by circulating estrogen levels. Indeed, in estrogen- or progesterone-treated OVX versus untreated OVX rats, *rfrp* expression does not differ (Quennell et al., 2010), and in OVX ewes estrogen treatment does not affect *rfrp* expression either (Smith et al., 2008). In our work we did not find a difference in *rfrp* mRNA and RFRP-ir levels in the Syrian hamster after OVX or E_2 treatment or between diestrus or the time of the LH surge on the day of proestrus. However, a previous report indicates that RFRP-ir cell numbers are reduced in the female Syrian hamster at the time of the LH surge on the day of proestrus, compared to the day of diestrus (Gibson et al., 2008). In addition, in mice *rfrp* expression is down-regulated in estrogen-treated OVX

mice compared to OVX mice (Molnar et al., 2011). Whether the discrepancies in the effects of circulating levels of estrogen on *rfrp* expression are a result of species-dependent differences or other unaccounted factors remains to be determined. Indeed, a variety of antibodies has been characterized for the study of RFRP-ir in rodents, including a polyclonal antibody raised against avian GnIH (Tsutsui et al., 2000), an antiserum against the rat RFRP precursor peptide (Rizwan et al., 2009), a white crowned sparrow GnIH antiserum (Smith et al., 2008, Kriegsfeld et al., 2006) and an antibody raised in guinea pigs against human RFRP-3 (Qi et al., 2009); one can reasonably wonder whether the results obtained by immunohistochemistry can actually be compared. Nevertheless, the fact that 40% of RFRP neurons express ERα in Syrian hamsters (Kriegsfeld et al., 2006) suggests that these neurons only play a minor role in mediating sex steroid feed-back effects to the HPG axis.

In Syrian hamsters the level of activation of RFRP cells is reduced on the day of proestrus compared to diestrus (Gibson et al., 2008). Interestingly, in our study, although *rfrp* mRNA and RFRP-ir levels were similar at the time of the LH surge on the day of proestrus and in diestrus, preliminary data indicates that c-Fos expression is decreased in RFRP neurons at the time of the LH surge on the day of proestrus compared to diestrus. Therefore, although RFRP synthesis is unchanged, RFRP release might be decreased on the day of proestrus, leading to a reduced inhibition of the reproductive axis and therefore generating the preovulatory LH surge. Conversely, RFRP release might be increased during diestrus to inhibit GnRH neuron activity. These results led us to the hypothesis that RFRP-3 injections carried out at various stages of the estrous cycle might have variable effects on the gonadotrophic axis. The present work is the first to provide information regarding the administration of RFRP-3 to female rodents under physiological conditions. We show that RFRP-3 has no effect on LH secretion when administered during diestrus, but that it has a potent inhibitory effect on LH secretion at the time of the LH surge on the day of proestrus, when LH levels are endogenously high. As previously mentioned, the Kp neurons in the AVPV are central players in the positive feed-back effects of estrogen. Therefore, RFRP neurons and AVPV Kp neurons would act in concert to mediate the effects of estrogen onto GnRH neurons, leading to the preovulatory LH surge (Figure 36). In this model, the Kp neurons of the AVPV receive positive feed-back signals from rising levels of estrogen, and the

increase in *Kiss1* expression would lead to a subsequent stimulation of GnRH neurons in the POA. In parallel, the reduced inhibitory activity of RFRP neurons at the time of the preovulatory LH surge would potentiate the effects of Kp. Because 1)RFRP appears to have an inhibitory effect on the gonadotrophic axis in female rodents; 2)RFRP fibres contact GnRH neurons, a subpopulation of which expresses *Gpr147* (Kriegsfeld et al., 2006, Rizwan et al., 2012, Poling et al., 2012, Ubuka et al., 2012); and 3) RFRP fibres are in contact with Kp neurons, a subpopulation of which expresses the *Gpr147* gene (Rizwan et al., 2012); RFRP-3 would play a role in mediating the positive feed-back effects of estrogen because the increase in the inhibitory effect of estrogen on *rfrp* expression would alleviate its inhibitory effects on *Kiss1* expression in the AVPV and *gnrh* expression in the POA.

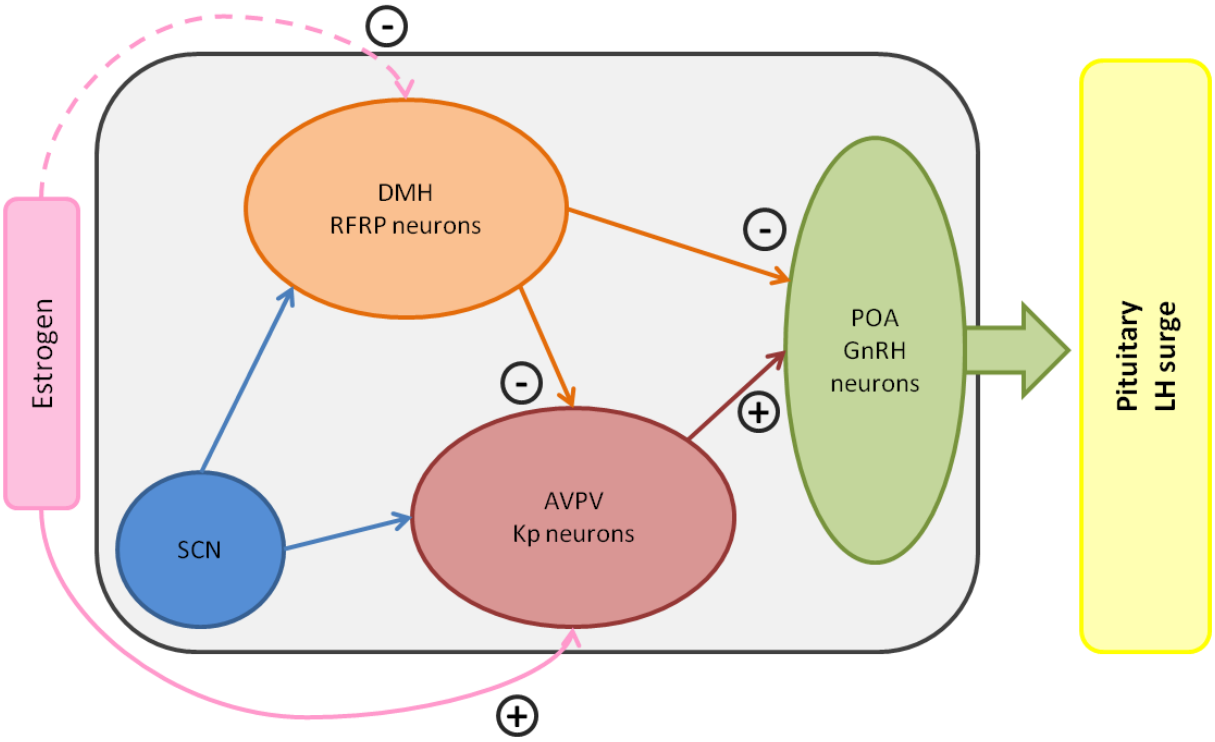

FIGURE 36 - RFRP AND KP NEURONS IN THE HYPOTHALAMUS ACT IN CONCERT TO MEDIATE THE POSITIVE FEED-BACK EFFECTS OF ESTROGEN TO DRIVE THE LH SURGE IN FEMALE RODENTS

AVPV: anteroventral periventricular nucleus of the hypothalamus; DMH: dorsomedial nucleus of the hypothalamus; LH: luteinizing hormone; POA: preoptic area; SCN: suprachiasmatic nuclei.

Although accumulating data substantiate this working hypothesis for the estrogen-mediated regulation of the LH surge, a number of questions remain unanswered.

Notably, because only 20% of Kp neurons express *Gpr147*, the effect of RFRP-3 on Kp secretion is most likely modulatory. The same comment can be made regarding GPR147 content of GnRH neurons. Finally, some Kp fibres have been found in the DMH, indicating a possible regulation of *rfrp* expression by Kp, and this aspect still needs to be examined in further detail.

THE ROLE OF RFRP NEURONS IN THE CIRCADIAN GATING OF THE PREOVULATORY LH SURGE

Approximately 60% of RFRP neurons in the DMH receive projections from the SCN in female Syrian hamsters (Gibson et al., 2008), suggesting that they might be involved in the circadian regulation of the preovulatory LH surge. In nocturnal rodents, the LH surge reliably occurs in the late afternoon or early evening of proestrus, just prior to female sexual behaviour which occurs when E_2 levels are elevated (i.e. late proestrus or early estrus). The SCN governs the timing of the preovulatory LH surge in rodents, as SCN lesions in Syrian hamsters and rats, and clock gene knock-out in mice, abolish the LH surge and subsequent ovulation (Brown-Grant and Raisman, 1977, Stetson and Watson-Whitmyre, 1976, Wiegand et al., 1980, Miller et al., 2004). The AVPV *Kiss1* neurons are involved in conveying circadian information to GnRH neurons, through an increase in their expression in the late afternoon/early evening linked with AVP signalling from the SCN (Robertson et al., 2009, Williams et al., 2011, Vida et al., 2010). Interestingly, the circadian activation of *Kiss1* neurons is dependent on the presence of elevated E_2 (Robertson et al., 2009, Williams et al., 2011), indicating that Kp neurons are integrators of both circadian and E_2 signals, two essential components of the LH surge.

Although the neurotransmitters used by the SCN to communicate with RFRP neurons are not known, the presence of SCN-derived fibres contacting RFRP cells indicates that they might be involved in the circadian component of the positive feed-back. A previous study carried out in female Syrian hamsters indicated that RFRP cell numbers and their level of activation are reduced at the time of the preovulatory LH surge (Gibson et al., 2008). As for Kiss1 expression, E_2 is required for this temporal regulation (Gibson et al., 2008). Although we unable to detect a difference in *rfrp* mRNA and RFRP preprotein

levels between diestrus and the time of the LH surge on the day of proestrus, we have preliminary data indicating that the activity of RFRP cells is increased during diestrus compared to proestrus. This issue needs to be addressed in further detail, and we are currently in the process of carrying out a more detailed analysis of c-Fos co-expression in RFRP neurons in the female hamsters used in our experiment. Furthermore, the link between the SCN and RFRP neurons should be characterised, and notably the type of neurotransmitter and the presence (or not) of the appropriate receptor on RFRP cells.

MODES OF ACTION OF RFRP-3

As previously mentioned, RFRP-3 is part of the large family of RFamide peptides, which share a common C-terminal Arg-Phe-NH_2 motif. In this family, there are five subgroups of peptides: PrRP, NPFF, QRFP/26RFa, Kp and RFRP (Yang et al., 1985, Perry et al., 1997, Hinuma et al., 1998, Panula et al., 1999, Hinuma et al., 2000, Liu et al., 2001, Kotani et al., 2001, Ohtaki et al., 2001, Chartrel et al., 2003, Fukusumi et al., 2003, Jiang et al., 2003). The receptors for these peptides are GPR10, GPR74, GPR103, GPR54 and GPR147, respectively. Because of the common C-terminal motif which characterises RFamide peptides, it has been hypothesised that the receptors might not be specific to a single subgroup of RFamide peptides. Up to now, only a very limited number of tools have been available to study the specificity of RFamide receptors for their peptides. In 2006, a selective NPFF receptor (GPR74 and GPR147) antagonist was described and shown to block the effects of NPFF on heart rate and blood pressure and to prevent opioid-induced hyperalgesia and tolerance in rats, phenomena that are mediated via GPR74 (Simonin et al., 2006). The discovery of this selective NPFF receptor antagonist received great attention from people working on the involvement of RFRP-3 in the regulation of the reproductive function. To date, only two studies have used this dipeptide *in vivo* and in both rats and sheep, icv administration of RF9 increases LH secretion significantly (Pineda et al., 2010c, Caraty et al., 2012). However, because RF9 was not administered simultaneously with RFRP-3, one cannot conclude that this effect on LH secretion is the consequence of an antagonistic effect of inhibitory actions of RFRP-3. In the Syrian hamster, RF9 potently stimulates LH secretion when administered alone, and does not antagonise the stimulatory effect of RFRP-3 on the gonadotrophic axis. This suggests that RF9 is not a selective antagonist for GPR147, on the contrary it could have agonistic effects on GPR147 or other RFamide receptors. This hypothesis is supported by two independent studies showing that 1)RF9 behaves as a full agonist of GPR74 receptors, and a partial agonist of GPR147 toward inositol triphosphate accumulation in African Green Monkey fibroblasts (Findeisen et al., 2012); and 2)RF9 did not antagonize the action of NPFF on the phosphorylation of MAPK/ERK1/2 and also did not itself affect this signalling pathway (Maletinska et al., 2013). Moreover, *in vivo*, RF9 produces a dose-

dependent anorectic effect in mice (Maletinska et al., 2013), further indicating an agonist-like property of this molecule on GPR147.

A novel selective antagonist for GPR147 has recently been engineered in Dr Férédric Simonin's laboratory and termed RF313. Although this molecule has not been published yet, it was readily made available to us, and we therefore tested its antagonistic properties concerning the effects of RFRP-3 on the Syrian hamster reproductive axis. The present results indicate that RF313 has no effect in itself on LH secretion and that it potently antagonizes the stimulatory effect of RFRP-3 on LH secretion. Although this antagonist requires further characterization, these encouraging results indicate that RF313 could be a valuable tool for the study of the effects of RFamide peptides *in vivo*. Moreover, our findings indicate that the effects of RFRP-3 on the Syrian hamster HPG axis are mediated by GPR147, and not via another RFamide receptor.

CENTRAL SITES OF ACTION OF RFRP-3

The effects of RFRP-3 on the reproductive axis appear to be mediated by central targets located in the hypothalamus. A number of studies indicate that RFRP cells project monosynaptically to GnRH neurons in mammals, including mice, rats, Syrian hamsters, and sheep (Kriegsfeld et al., 2006, Johnson et al., 2007, Smith et al., 2008). Our preliminary results show that *Gpr147* is present in the Syrian hamster POA, however ongoing studies will have to aim at determining whether the receptor co-localises with GnRH neurons. Indeed, GnRH neurons have recently been found to co-express *Gpr147* in mice, rats, and Siberian hamsters (Poling et al., 2012, Rizwan et al., 2012, Ubuka et al., 2012), indicating that RFRP-3 could have a direct effect on GnRH neurons. This hypothesis is supported by evidence in mice where direct application of RFRP-3 inhibits neuronal firing in a subset of GnRH cells even when amino acid transmission is blocked (Ducret et al., 2009, Wu et al., 2009). It is of interest to remark that in the same study, a subset of GnRH cells increased its firing rate in response to RFRP-3 (Ducret et al., 2009), indicating that the peptide is able to activate and/or inhibit GnRH neurons in mice. This observation might have a functional significance in explaining the species-dependent differences in the effect of RFRP-3 on the reproductive axis.

Hypothalamic *Kiss1* neurons are also emerging as possible RFRP-3 targets in the mammalian brain. In mice, rats, and Syrian hamsters RFRP fibres project to the ARC (Kriegsfeld et al., 2006). In the Syrian hamster, our preliminary data indicate that *Gpr147* is present in the ARC, however acute injections of RFRP-3 do not induce c-Fos in Kp neurons, whereas continuous administration of the peptide increases *Kiss1* expression in the ARC (Ancel et al., 2012). Evidence is still lacking in order to conclude on direct or indirect effects of RFRP-3 on *Kiss1* neurons, and the present data does not support one possibility over another. Indeed, the c-Fos data could indicate that RFRP-3 does not act on the HPG axis via Kp neurons, however it is also possible that a direct effect of RFRP-3 might not induce c-Fos expression. On the other hand, the increase in *Kiss1* expression following RFRP-3 infusions indicates that the reactivation of the reproductive axis is mediated (at least in part) by Kp neurons. However, additional studies will be required in order to precisely determine whether RFRP-3 acts on the reproductive axis via GnRH and/or Kp neurons. The development of new tools, notably selective antagonists and antibodies, will enable a more detailed study of the neuronal pathways involved in transmitting RFRP-3 effects onto the HPG axis.

It is worth remarking that RFRP-ir fibres have been identified in a number of hypothalamic areas (Kriegsfeld et al., 2006), and our preliminary data indicates that GPR147 is also widely distributed throughout the hypothalamus in the Syrian hamster. This suggests putative RFRP sites of action, but because of the involvement of RFRP-3 in the regulation of reproduction, work has focused up until now on Kp and GnRH neurons. Therefore, RFRPs could have other central sites of action, involved or not in the regulation of reproductive activity.

PERIPHERAL SITES OF ACTION OF RFRP-3

Sparse RFRP fibres have been localised in the median eminence of mice, rats and Syrian hamsters (Kriegsfeld et al., 2006), although a number of studies report the absence of fibres in the median eminence of rodents (Ukena and Tsutsui, 2001, Yano et al., 2003, Rizwan et al., 2009, Ubuka et al., 2012). These inconsistencies might result from the use

of different antibodies or different immunohistochemical procedures, but they raise the question of a possible hypophysiotrophic effect of RFRP-3 in rodents. GPR147 has been localised in the Syrian hamster pituitary (Gibson et al., 2008), however the relative level of expression is not known as it was not compared with hypothalamic expression. In rats, only a very low level of GPR147 pituitary expression has been reported compared to hypothalamic expression (Hinuma et al., 2000, Quennell et al., 2010), suggesting a minor role of the receptor in the pituitary. As previously mentioned, inconsistent data from *in vitro* pituitary culture experiments and *in vivo* peripheral administration procedures (Table 2) make it difficult to conclude on the question of the hypophysiotrophic effect of RFRP-3 in rats, although a larger amount of data does not support this hypothesis. In male Syrian hamsters, peripheral injections of RFRP-3 have no effect on LH secretion, and RFRP-3 does not affect LH secretion from cultured pituitary cells (Ancel et al., 2012), indicating that the peptide does not act directly at the level of the pituitary. In OVX female Syrian hamsters, peripheral administration of GnIH inhibits LH secretion (Kriegsfeld et al., 2006) but RFRP-3 does not affect LH secretion from pituitary cells *in vitro* (Ancel et al., submitted). A possible explanation for these surprising results could be that RFRP-3 stimulates GnRH secretion from the nerve terminals present in the median eminence, in a similar manner as Kp. This hypothesis would deserve further attention, and could be tested on MBH explants which contain GnRH nerve terminals (but not cell bodies) (d'Anglemont de Tassigny et al., 2008).

Here again, species-differences exist in regard to the hypophysiotrophic effect of RFRP-3. Indeed, in the sheep RFRP fibres have been identified in the median eminence and RFRP has been detected in the portal blood (Clarke et al., 2008, Sari et al., 2009, Smith et al., 2012). In addition, *Gpr147* is present in the pituitary (Smith et al., 2012) and peripheral administration of RFRP-3 potently inhibits LH secretion (Clarke et al., 2008, Sari et al., 2009). The fact that the modes of action of RFRP-3 differ among species could provide a physical basis explaining the species-dependent differences in the effects of the peptide on the HPG axis.

Although the peripheral expression of *rfrp* and *Gpr147* has not been extensively studied, a study in Syrian hamsters has shown that they are present in the seminiferous tubules of the testes (Zhao et al., 2010), suggesting a role in spermatogenesis. RFRP is also found

in the granulosa cells of mouse ovarian follicles during proestrus and estrus and in the luteal cells during diestrus (Singh et al., 2011), suggesting a role in follicular development.

CONCLUSIONS AND PERSPECTIVES

Overall, the present work indicates that species-dependent differences exist in both the effects and the modes of action of RFRP-3 on the HPG axis. Whereas RFRP-3 appears to inhibit the reproductive axis in rats and sheep, it has opposite effects in Syrian hamsters and variable effects in Siberian hamsters. In the future, studies should aim at determining the reason for these differences: do they result from the use of different peptides (i.e. GnIH vs. RFRP-3)? or do they reflect a functional reality, in which the role of RFRP-3 is not conserved among species?

Moreover, the studies carried out in female Syrian hamsters indicate that the effect of RFRP-3 on the gonadotrophic axis depends on the physiological status of the animal. Future work carried out in other rodents and non-rodents under different physiological conditions will be required in order to find out whether the sex-dependent differences are specific to the Syrian hamster, or whether they are a widespread characteristic of RFRP-3 effects on the HPG axis. In addition, the functional reason underlying the gender-related difference in the effect of the peptide on the reproductive axis deserves further attention. Specifically, the exact role of RFRP-3 in the regulation of the estrous cycle should be studied.

Finally, with the development of new tools we should be able to gain insight in the mechanisms and pathways involved in the regulation of the HPG axis by RFRP-3. Shedding light on the central actors targeting and targeted by RFRP neurons will certainly help understand the species-dependent differences in the role of RFRP-3 in the regulation of the reproductive function.

RFRP-3 AND OTHER FUNCTIONS

RFRP fibres are found in a large number of hypothalamic and extra-hypothalamic brain regions (Ukena and Tsutsui, 2001, Kriegsfeld et al., 2006, Johnson et al., 2007, Mason et al., 2010). Similarly, GPR147 is widely distributed throughout the hypothalamus in rodents (Gouarderes et al., 2002, Gouarderes et al., 2004b, Gouarderes et al., 2004a). It has recently been suggested that RFRP neurons could play a role in monitoring internal and external status and integrating this information to control reproductive functioning

precisely. In sheep, RFRP neurons project to neuropeptide- Y, pro-opiomelanocortin, orexin, and melanin concentrating cells (Clarke et al., 2009, Qi et al., 2009), which are important players in the regulation of metabolism. In addition, administration of RFRP-3 increases feeding in rats (Johnson et al., 2007). A recent study has shown that GABAergic neurons originating in the DMH are a key component of the food anticipatory behaviour, and some evidence suggests that these could be RFRP cells (Acosta-Galvan et al., 2011), although it remains to be determined whether these are RFRP neurons. On the other hand, the DMH has been shown to play a key role in coordinating responses to emotional stress (DiMicco et al., 2002). Interestingly, acute and chronic immobilization stress leads to an increase in *rfrp* mRNA and ir cell numbers in the DMH of rats, coincident with an increase in plasma corticosterone levels (Kirby et al., 2009). These stressors also cause activation of RFRP-3 neurons in rats (Kaewwongse et al., 2010). Finally, central administration of RFRP-3 increases ACTH (Kaewwongse et al., 2010) and corticosterone (Samson et al., 2003) secretion in rodents. Taken together, these data indicate that RFRP neurons in the DMH could play a role in transmitting metabolic and endocrine information to the reproductive axis, in order to adjust reproduction accordingly. The role of the RFRP system in regulating the reproductive axis in response to stress may not be common across species; indeed stress does not affect RFRP peptide or mRNA expression in sheep (Papargiris et al., 2010). Therefore, RFRP functions and effects on the reproductive axis appear to not be conserved among species.

WHAT ABOUT RFRP-1?

The *rfrp* gene encodes a precursor which produces two peptides, RFRP-1 and RFRP-3. The *rfrp* gene is the mammalian ortholog of avian *gnih*, and because GnIH is involved in the regulation of the reproductive axis in birds, work in mammals primarily aimed at determining the involvement of mammalian RFRPs in the regulation of reproduction. Because an initial study carried out in rats showed that central administration of RFRP-1 raised circulating levels of prolactin but did not affect the secretion of other pituitary peptides (Hinuma et al., 2000), subsequent studies focused mainly on the effect of RFRP-3 administration on endocrine functions. In the Syrian hamster, RFRP-1 administration has no effect on LH secretion (Ancel et al., 2012), but in Siberian hamsters RFRP-1 has

the same effect (although less potent) on LH secretion as RFRP-3 (Ubuka et al., 2012). This species-difference is interesting because contrary to Syrian hamsters, Siberian hamsters undergo marked photoperiodic changes in body weight and fur colour. Because RFRP-1 has been shown to regulate food intake in chicks (Newmyer and Cline, 2009) and rats (Kovacs et al., 2012), it is reasonable to suppose that RFRP-1 could be involved in the seasonal regulation of body weight in Siberian hamsters, in combination with the seasonal regulation of reproduction. Other lines of evidence suggest that RFRP-1 could be involved in neuroendocrine and behavioural responses to stressful stimuli. Ongoing work should aim at clarifying whether RFRP neurons are involved in transmitting metabolic and/or stressful information to the gonadotrophic axis.

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LIST OF FIGURES AND TABLES

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SCIENTIFIC PUBLICATIONS

Peripheral kisspeptin reverses short photoperiod-induced gonadal regression in Syrian hamsters by promoting GNRH release.
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Ancel C, Simonneaux V.
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Caroline ANCEL

Le RFRP-3 et l'axe gonadotrope du hamster Syrien : effets genre-dépendants et modes d'action

The effect of RFRP-3 on the gonadotrophic axis of the Syrian hamster: sex-dependent differences and modes of action

Résumé

Le peptide RFRP-3 joue un rôle dans la régulation de l'axe hypothalamo-hypophyso-gonadotrope des mammifères. Le but de cette étude était de déterminer l'implication du RFRP-3 dans la régulation de l'axe reproducteur du hamster Syrien. Nos résultats montrent que le RFRP-3 stimule l'axe gonadotrope chez le hamster Syrien mâle, tandis qu'il a des effets variables chez la femelle. En effet, chez la femelle le peptide inhibe l'axe reproducteur lorsqu'il est administré au moment du pic pré-ovulatoire de LH le jour du proestrus, et n'a pas d'effet pendant le diestrus. Nous avons poursuivi notre étude par la caractérisation des sites d'action du RFRP-3 chez le hamster Syrien, en démontrant que l'effet du peptide sur l'axe gonadotrope est médié directement ou indirectement par les neurones à GnRH. De plus, nous avons écarté l'hypothèse d'un effet hypophysiotrope du peptide chez cette espèce. Pour conclure, les résultats présentés soulèvent de nombreuses questions quant aux effets espèce- et genre-dépendants du RFRP-3 sur l'axe gonadotrope du mammifère.

Mots-clés : RFamide-related peptide, reproduction, hamster Syrien, axe gonadotrope, saisonnalité.

Summary

RFRP-3 has been shown to play a role in the regulation of the mammalian hypothalamic-pituitary-gonadal axis. The aim of this work was to determine the involvement of RFRP-3 in the regulation of the Syrian hamster reproductive axis. We report unprecedented results indicating that RFRP-3 stimulates the male Syrian hamster gonadotrophic axis, whereas it has variable effects in female Syrian hamsters. Indeed, in females the peptide inhibits the reproductive axis at the time of the LH surge on the day of proestrus, and has no effect during diestrus. We went on to characterize RFRP-3 sites of action in the Syrian hamster brain, and show that the effect of the peptide on the gonadotrophic axis is mediated directly or indirectly via GnRH neurons. Moreover, we clearly rule out the possibility of a hypophysiotrophic effect of RFRP-3 in this species. Taken together, the present data raise interesting questions regarding species- and sex-dependent effects of RFRP-3 on the mammalian gonadotrophic axis.

Key words: RFamide-related peptide, reproduction, Syrian hamster, gonadotrophic axis, seasonality.