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par

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Un Kiss pour le contrôle saisonnier de la reproduction

A Kiss for the seasonal control of reproduction

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" Ce n'est pas parce que les choses sont difficiles que nous n'osons pas,

c'est parce que nous n'osons pas qu'elles sont difficiles. "

Sénèque

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Abstract - English

To ensure the birth of the offspring at the most favourable time of year, photoperiod (or day-length) is "read" by most seasonal breeders to restrict their fertility to a particular season. Annual variations of the photoperiod are translated into the release of the pineal hormone melatonin, the nocturnal production of which relies on night-length. Previous experiments have demonstrated that the annual variations of melatonin secretion synchronise reproductive activity with seasons. For instance, exposure to a long nocturnal peak of melatonin for 8 to 10 weeks (like in short days conditions, SD) induces a marked gonadal atrophy in male Syrian hamsters (*Mesocricetus auratus*). Conversely, when transferred back to long days (LD), a condition in which the nocturnal peak of melatonin is short, hamsters become sexually active within 6 weeks. Despite its strong action on the reproductive axis, the precise sites and mechanisms of action of melatonin are still unclear. We recently reported that in the Syrian hamster, melatonin inhibits the expression of several hypothalamic genes, including *Kiss1*, which might mediate the photoperiodic message to the reproductive axis. *Kiss1* encodes a family of peptides, the kisspeptins (Kp), that all bind to the same receptor, Kiss1r. Kp play a crucial role in the activation of the gonadotropic since a loss-of-function mutation in *Kiss1r* prevents puberty humans and rodents.

My thesis work consisted in studying the implication of *Kiss1*-encoded peptides (kisspeptins, Kp) in the seasonal cycles of the reproductive activity. In a first part, the anatomy of the kisspeptinergic system was characterised in the Syrian hamster's brain in both LD sexually active and SD sexually inactive individuals. In a second part, I investigated how photoperiod affects *Kiss1* expression and finally, in a third part, I analysed the effects of exogenous administration of Kp and its site of action on the reproductive axis

First of all, the distribution of Kp fibres and cell bodies was characterised in the Syrian hamster's by immunohistochemistry and non-radioactive *in situ* hybridisation. Two populations of *Kiss1* neurones were identified in the Arcuate nucleus (ARC) and in the Anteroventral periventricular nucleus (AVPV). Kp immunoreactive fibres were observed in both nucleus and in other hypothalamic (preoptic median nucleus, preoptic median area, anterior hypothalamic area, paraventricular nucleus, dorsomedial hypothalamus) and extra hypothalamic structures (Bed nucleus of stria terminalis, paraventricular nucleus of the thalamus, central amygdala). Since *Kiss1* expression is highly dependent on photoperiod, the distribution of Kp immunoreactive fibres was also analysed in SD-adapted hamsters. The number of *Kiss1* and Kp neurones is strongly decreased in SD

sexually inactive hamsters. Fibres distribution is identical as in LD but fibres density is higher in SD, probably because of an accumulation of Kp into the fibres. To determine wether these fibres innervate fibres-containing structures or just go through them, we analysed the expression of cFos (a marker of cellular activity) after an intracerebroventricular (ICV) injection of Kp and we found that cFos expression is increased in the preoptic median nucleus, the preoptic medial area, the paraventricular nucleus, the dorsomedial hypothalamus, the bed nucleus of stria terminalis and the paraventricular nucleus of the thalamus. This indicates that these brain structures are innervated by Kp fibres and activated upon Kp release. The phenotype of the target neurones however remains to be determined.

In a second part, I analysed how photoperiod affects *Kiss1* expression in the Syrian hamster. First of all, we demonstrated that *Kiss1* mRNA is highly expressed in the ARC and AVPV in LD-adapted male or female hamsters, while exposition to SD condition drastically reduces the number of *Kiss1* neurones in both structure and sex. Daily melatonin injections mimicking SD-like melatonin levels reduces *Kiss1* expression in ARC and AVPV. By contrast pinealectomy in SD-adapted hamsters increases the number of *Kiss1* neurones in both nuclei, showing that melatonin mediates the effect of photoperiod on *Kiss1* neurones. The kinetic of melatonin effect on *Kiss1* neurones was also analysed and one or three weeks of daily melatonin injections do not affect *Kiss1* expression whereas after eight weeks of treatment, *Kiss1* expression reaches its minimal level. Noteworthy, SD exposure induces gonadal regression as well as a massive reduction in testosterone levels. In rats and mice, testosterone strongly regulates *Kiss1* expression in both ARC and AVPV and the SD- or melatonin-induced reduction in the number of *Kiss1* neurones could be secondary to variations in testosterone plasmatic concentration. As a consequence, I also analysed the effect of gonadal hormones on *Kiss1* expression in the Syrian hamster and I observed that testosterone up-regulates *Kiss1* expression in the AVPV and down-regulates the number of ARC *Kiss1* neurones. Thus, the SD- or melatonin-induced reduction in the number of ARC *Kiss1* neurones is not secondary to a reduction in testosterone level. By contrast, in the AVPV where testosterone is stimulatory, the SD- or melatonin-induced down-regulation of *Kiss1* expression was found to be mediated by the SD- or melatonin-induced decrease in gonadal hormones levels. All together these data indicate that the SD-induced reduction in the number of ARC *Kiss1* neurones is due to a direct effect of melatonin on the hypothalamus whereas the decrease in the number of AVPV *Kiss1* neurones is secondary to a reduction of

testosterone levels. The AVPV is a sexually dimorphic brain nucleus with *Kiss1* expression being higher in females. Thus, the effects of melatonin and oestradiol were analysed in females hamsters as well and similar results were observed.

Finally, in a third part, I analysed peripheral Kp sites of action. In the lab, we demonstrated that Kp chronic intracerebroventricular treatment for 4 weeks re-activates gonadal activity in SD-adapted male hamsters. This indicates that Kp overpass melatonin inhibitory effect on the reproductive axis. Previous studies showed that Kp trigger LH, FSH and sex steroids release when given centrally or peripherally. I thus studied the effect of several protocols of peripheral Kp long-term administration to SD-acclimated sexually inactive Syrian hamsters. We demonstrated that 2 daily IP injections of Kp during 5 weeks restore gonadal activity. To identify the structure(s) involved in the peripheral effect of Kp, we studied the expression of cFos after IP injection of Kp. The anterior pituitary was found to strongly express cFos and this Kp-induced activation is GnRH-dependent since a GnRH antagonist prevented it.

To conclude, this PhD work not only proved that the Kp system is strongly regulated by photoperiod and melatonin (via distinct mechanisms involving both direct effects and seasonal variations in gonadal hormones levels), but also that *Kiss1* neurones impacts the photoperiodic message on the reproductive axis, probably at the level of GnRH nerve terminals of the median eminence.

Résumé de thèse - Français

La plupart des espèces restreignent leur fertilité à une période limitée de l'année pour que la naissance des petits ait lieu au moment le plus favorable à leur survie. Par exemple, chez le hamster Syrien mâle (*Mesocricetus auratus*), l'exposition à une photopériode courte (PC; 10h lumière / 14h obscurité) mimant la saison hivernale cause une inhibition de l'axe reproducteur conduisant à une atrophie des testicules en 8 à 10 semaines. Les animaux ne sont alors plus en mesure de se reproduire. Chez les rongeurs saisonniers, la lecture du temps annuel se fait par l'intermédiaire de la mélatonine, une hormone produite par la glande pinéale et dont la libération est proportionnelle à la durée de la nuit. En hiver, lorsque les nuits sont longues (PC), le pic nocturne de mélatonine l'est aussi. Inversement, en été (photopériode longue, PL), lorsque les nuit sont courtes, la durée du pic nocturne de mélatonine est réduite. Le message photopériodique est ainsi converti en message hormonal capable de synchroniser la fonction de reproduction avec les saisons. Bien qu'exerçant un effet puissant sur l'axe gonadotrope, les sites d'actions de la mélatonine demeurent mal connus. Nous avons récemment démontré que la mélatonine contrôle l'expression de plusieurs gènes impliqués dans la fonction de reproduction. Parmi ces gènes, *Kiss1* code pour une famille de peptides, les kisspeptines (Kp), qui se lient tous au même récepteur, le Kiss1r. Les Kp jouent un rôle crucial dans l'activation de l'axe gonadotrope car il a été démontré qu'une mutation perte de fonction dans le gène *Kiss1r* empêche le déclenchement de la puberté aussi bien chez l'être humain que chez la souris.

Mon travail de thèse a consisté à étudier l'implication des Kp dans les cycles saisonniers d'activité de l'axe gonadotrope chez le hamster et s'est articulé autour de trois grands axes.

En premier lieu, la cartographie complète du système kisspeptinergique a été réalisée par immunohistochimie et hybridation *in situ* non radioactive chez le hamster Syrien mâle en PL (14h lumière / 10h obscurité) (**A1**). Deux populations de neurones exprimant l'ARNm du gène *Kiss1* et les Kp ont ainsi été identifiées dans le noyau arqué (ARC) et le noyau antéroventral péri-ventriculaire (AVPV). Des fibres à Kp ont été observées dans ces deux structures ainsi que dans d'autres régions hypothalamiques (noyau préoptique médian, aire pré-optique médiane, aire hypothalamique antérieure, noyau paraventriculaire, hypothalamus dorsomédian) et extra hypothalamiques (noyau du lit de la strie terminale, noyau paraventriculaire du thalamus, amygdale centrale). L'expression du système kisspeptinergique étant fortement dépendante de la photopériode, la cartographie de ce système a également été réalisée chez des hamsters

maintenus en PC (**A1**). Le nombre de neurones exprimant l'ARNm du gène *Kiss1* ou Kp est diminué en PC quand les hamsters sont sexuellement quiescents. Les fibres à Kp ont été identifiées dans les même régions qu'en PL mais avec une densité supérieure en PC, probablement en raison d'un processus d'accumulation lié à une diminution de la libération des peptides. Afin de déterminer si ces fibres sont des fibres de passage ou correspondent à une innervation spécifique de ces structures, l'expression de cFos (un marqueur d'activité cellulaire) a été analysée après l'injection intracérébroventriculaire de Kp (**A1**). C'est ainsi que l'expression de cFos est augmentée dans le noyau préoptique médian, l'aire préoptique médiane, le noyau paraventriculaire, l'hypothalamus dorsomédian, le noyau du lit de la strie terminale et le noyau paraventriculaire du thalamus, indiquant que ces régions sont activées par les Kp et que les fibres à Kp y libèrent probablement ces peptides. Le phénotypage des neurones cibles des Kp reste cependant à réaliser.

Dans un deuxième temps nous avons étudié comment la photopériode régule l'expression de *Kiss1* chez le hamster doré (**A3, A5**). Premièrement, nous avons démontré chez le hamster doré mâle et femelle que l'ARNm du gène *Kiss1* est exprimé dans l'ARC et l'AVPV et que l'exposition des hamsters à une PC inhibitrice durant 10 semaines diminue l'expression de *Kiss1* dans ces deux structures. L'injection quotidienne de mélatonine exogène à des hamsters maintenus en PL selon une cinétique mimant le rythme de mélatonine en PC, réduit l'expression de *Kiss1* à la fois dans l'AVPV et dans l'ARC; démontrant que la mélatonine est responsable des variations photopériodiques de l'expression de *Kiss1*. La cinétique de l'action de la mélatonine sur l'expression de *Kiss1* montre qu'une semaine d'injection quotidienne de mélatonine n'affecte pas l'expression de *Kiss1,* mais qu'à partir de 3 semaines d'injection, l'expression de *Kiss1* diminue jusqu'à atteindre un niveau minimal au bout de 8 semaines d'injection (**A3**). Il est à noter que l'atrophie testiculaire induite par la PC ou la mélatonine exogène s'accompagne d'une réduction massive du niveau de testostérone circulant. Or, chez le rat et la souris, les stéroïdes sexuels affectent l'expression de *Kiss1* dans l'ARC et l'AVPV. Par conséquent, l'inhibition de l'expression de *Kiss1* en PC ou induite par les injections de mélatonine peut être secondaire aux variations du taux de testostérone. Par conséquent, l'effet de la testostérone sur l'expression de *Kiss1* chez le hamster Syrien a également été analysé (**A3, A5**). Nous avons ainsi observé que la testostérone stimule l'expression de *Kiss1* dans l'AVPV mais l'inhibe dans l'ARC. Ainsi, dans l'ARC, la réduction de l'expression de *Kiss1* observée en PC ou suite aux injections de mélatonine ne peut être

imputée à la diminution du taux de testostérone. En revanche, dans l'AVPV où la testostérone exerce un rétrocontrôle positif, nous avons démontré que la diminution de l'expression de *Kiss1* en PC ou induite par la mélatonine est due à un effet indirect via la diminution des taux circulants de testostérone. L'ensemble de ces résultats indique que dans l'ARC, la PC réduit l'expression de *Kiss1* par un effet direct de la mélatonine sur l'hypothalamus alors que dans l'AVPV, la mélatonine diminue l'expression de *Kiss1* via une réduction du taux de testostérone.

L'AVPV est une des régions sexuellement dimorphiques du cerveau et l'expression de *Kiss1* est plus importante chez la femelle que chez le mâle. Afin d'approfondir nos données obtenues chez le mâle, l'effet de la mélatonine et de l'œstradiol sur l'expression de *Kiss1* dans l'AVPV a été étudié chez le hamster Syrien femelle et des résultats similaires ont étés obtenus (**A3**).

Dans une troisième partie, nous avons analysé les sites d'action périphériques des Kp (**A2**). Au laboratoire, nous avions démontré que l'administration chronique intracérébroventriculaire de Kp durant 4 semaines ré-active l'axe gonadotrope du hamster doré mâle maintenu en PC, indiquant que les Kp sont capables de surpasser l'effet inhibiteur de la mélatonine sur l'axe gonadotrope. Des études précédentes chez le rat et la souris ayant démontré que les Kp stimulent l'axe gonadotrope lorsqu'elles sont administrées par voie centrale (ICV) ou périphérique (intrapéritonéale), nous avons étudié l'effet de l'administration chronique périphérique de Kp sur l'axe gonadotrope du hamster mâle maintenu en PC (**A2**). Nous avons testé différents protocoles d'administration de Kp et démontré que deux injections quotidiennes de Kp durant 5 semaines ré-activent l'axe gonadotrope. Afin d'identifier les structures cibles des Kp périphériques, nous avons analysé l'expression de cFos après l'injection intrapéritonéale de Kp (**A2**). Nous avons ainsi mis en évidence une forte activation de l'hypophyse antérieure par les Kp, activation dépendante du GnRH car abolie en présence d'acyline, un antagoniste du GnRH.

En conclusion, ce travail de thèse a permit de démontrer non seulement que le système kisspeptinergique est fortement dépendant de la photopériode et de la mélatonine (via des mécanismes directs ou faisant intervenir les variations saisonnières du taux de stéroïdes sexuels circulants), mais aussi que les neurones à *Kiss1* répercutent l'information photopériodique sur l'axe reproducteur, en particulier au niveau des terminaisons des neurones à GnRH de l'éminence médiane.

Publications

- **A1: Ansel L**, Bartzen J, Streicher D, Klosen P, Mikkelsen JD, Simonneaux V. Neuroanatomy of the kisspeptinergic system and its seasonal variation in the Syrian hamster. *In preparation*
- **A2: Ansel L**, Bentsen AH, Bolborea M, Ancel C, Klosen P, Mikkelsen JD, Simonneaux V. Peripheral kisspeptin reverses short photoperiod-induced gonadal regression in Syrian hamsters by acting on GnRH release. *Journal of Neuroendocrinology, in revision.*
- **A3: Ansel L**, Bolborea M, Bentsen AH, Ancel C, Klosen P, Mikkelsen JD, Simonneaux V. Melatonin and testosterone-dependant variations in *Kiss1* expression drive seasonal reproduction in the Syrian hamster. *Journal of Biological Rhythms, in press.*
- **A4:** Revel FG, **Ansel L**, Klosen P, Saboureau M, Pevet P, Mikkelsen JD, Simonneaux V. Kisspeptin: a key link to seasonal breeding. *Rev Endocr Metab Disord* 2007; 8: 57-65.
- **A5:** Simonneaux V, **Ansel L**, Revel FG, Klosen P, Pevet P, Mikkelsen JD. Kisspeptin and the seasonal control of reproduction in hamsters. *Peptides* 2009; 30 (1):146-53
- **A6:** Mikkelsen JD, Bentsen AH, **Ansel L**, Simonneaux V, Juul A. Comparison of the effects of peripherally administered kisspeptins. *Regul Pept* 2009; 152 (1-3) : 95-100
- **A7:** Simonneaux V, Revel FG, **Ansel L**. La régulation de la reproduction saisonnière par la mélatonine nécessite un Kiss. *In press, "Métabolisme, Hormone, Diabète et Nutrition"*
- **A8:** Bentsen AH, **Ansel L**, Simonneaux V, Tena-Sempere M, Juul A, Mikkelsen JD. Maturation of kisspeptinergic neurons coincides with puberty onset in male rats. *Peptides, in press*
- **A9:** Bolborea M, **Ansel L**, Weinert D, Steinlechner S, Klosen P. The Bed Nucleus of the Stria Terminalis in the Syrian hamster (*Mesocricetus auratus*): Absence of vasopressin expression in standard and wild-derived hamsters and galanin regulation by seasonal changes in circulating sex steroids. *Neuroscience* 2010; 165 (3) : 819-830

Scientific communications

- **Ansel L**, Klosen P, Mikkelsen JD, Simonneaux V. Neuroanatomy of the kisspeptinergic system and its seasonal variations in the Syrian hamster. **Poster** presented at the annual meeting of the "Society for Neuroscience", Chicago, 17-21 october 2009.
- Bentsen AH, **Ansel L**, Simonneaux V, Tena-Sempere M, Juul A, Mikkelsen JD. Maturation of kisspeptin neurones coincide with puberty onset in male rats. **Poster** presented at the annual meeting of the "Society for Neuroscience", Chicago, 17-21 october 2009.
- Simonneaux V, Ancel C, **Ansel L**, Mikkelsen JD. The role of RFRP peptide in the seasonal regulation of reproduction. **Poster** presented at the annual meeting of the "Society for Neuroscience", Chicago, 17-21 october 2009.
- **Ansel L**, Bolborea M, Bentsen AH, Ancel C, Klosen P, Mikkelsen JD, Simonneaux V. Mechanisms of photoperiodic regulation of Kiss1 expression in the Syrian hamster. **Poster** presented at the XIth Congress of the "European Biological Rhythms Society", Strasbourg, August 2009.
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Abbreviations

5

5HT: 5-hydroxytryptamine (serotonin)

A

AAAD: Aromatic amino acid decarboxylase

AA-NAT: Arylalkylamine-N-acetyl transferase

ARC: Arcuate nucleus

AVP: Arginine vasopressine

AVPV: Anteroventral periventricular nucleus

B

bFGF: Basic fibroblast growth factor

bHLH: Basic helix-loop-helix

BMAL1: Brain and muscle ARNt protein 1

BNST: Bed nucleus of stria terminalis

C

cAMP: Cyclic adenosine monophosphate

cFOS: Finkel-Biskis-Jinkins murine osteosarcoma viral oncogene homolog

cGMP: Cyclic guanosine monophosphate

CHO: Chinese hamster ovary

CLOCK: Circadian locomotor output cycles kaput

CREB: cAMP response element binding protein

Crtc1: Creb1-regulated transcription co-activator1

Cry: Cryptochrome

D

DAG: Diacylglycerol

DARP-32: Dopamine and cAMP regulated phosphoprotein 32

DIO1, 2, 3: Deiodinase 1, 2, 3

DMH: Dorsomedial hypothalamus

Dyn: Dynorphin

E

ER: Oestrogen receptor

ERK: Extracellular-signal regulated kinases

F

FSH: Follicle stimulating hormone

G

GABA: Gamma amino butyric acid

GnIH: Gonadotropin inhibitory hormone

GnRH: Gonadotropin releasing hormone

H

HIOMT: Hydroxyindole-O-methyltransferase

HPG: Hypothalamic pituitary gonadal axis

I

ICV: Intracerebroventricular

IGF: Insulin-like growth factor

IGL: Intergeniculate leaflet

IML: Intermediolateral column

IP: Intraperitoneal

IP3: Inositol triphosphate

K

Kiss1R: Primate Kiss1 receptor

Kiss1r: Rodent and ovine Kiss1 receptor

KO: Knock-out

KOR: kappa opioid receptor

Kp: Kisspeptins

Kp-ir: Kisspeptins immunoreactive

L

LD: Long days

LH: Luteinising hormone

LS: Lateral septum

M

MAP: Mitogen activated protein

Mel_{1c}: Melatonin receptor type 1c

MPN: Medial preoptic nucleus

MPO: Medial preoptic area

$MT_{1, 2, 3}$: Melatonin receptor type 1, 2, 3

mTOR: Mammalian target of rapamycin

N

NKB: Neurokinin B

NFkB: Nuclear factor kappa B

NO: Nitric oxide

NPY: Neuropeptide Y

O

Ob-Rb: Leptin receptor

P

PACAP: Pituitary adenylate cyclase activating peptide

PAS: Per Arnt Sim

PCR: Polymerase chain reaction

Per: Period

PI: Phosphatidyl inositol

PINX: Pinealectomy

PT: Pars tuberalis

PVN: Paraventricular hypothalamic nucleus

PVT: Paraventricular thalamic nucleus

R

RFRP: RF amide-related peptide

ROR: Retinoic acid related orphan receptor

rT3: reverse T3

RT-PCR: Reverse transcriptase PCR

S

SCN: Suprachiasmatic nuclei

SCG: Superior cervical ganglia

SD: Short days

STAT3: Signal transducer and activator of transcription 3

T

T2: diiodotyronine

T3: triiodotyronine

T4: thyroxin

TGF: Transforming growth factor

TpOH: Tryptophan hydroxylase

TRH: Thyroid releasing hormone

TSH: Thyroid stimulating hormone

V

VIP: Vasoactive intestinal peptide

VMH: Ventromedial hypothalamus

Chapter 1 - General introduction

I. Genesis of seasonal rhythms

French poet and philosopher Jean-Jacques Rousseau wrote "Climate, seasons, sounds, colours, darkness, light, elements, noise, silence, movement, rest, everything acts on both our machinery and our soul." (Jean-Jacques Rousseau, *Les confessions*, 1769). These words written in the 18th century illustrate the sentiment that the light/dark cycle and seasons exert profound effects on physiological functions.

The annual revolution of our planet around the Sun and its daily rotation on a 23.5°-inclined axis cause important seasonal changes in various environmental parameters (temperature, hygrometry, day-length / photoperiod) in polar and temperate parts of the Earth. To survive, most species have developed adaptive mechanisms to anticipate these variations. During the harsh season, some species adapt their metabolic activity, fur colour and/or density, other hibernate and most of them restrict their reproductive function to a limited time of year to ensure the offspring's birth occurs at the most favourable season.

To anticipate climatic seasonal changes, species rely on a photo-neuro-endocrine system to measure the annual time and analyse environmental cues mirroring it. Photoperiod is the most reliable noise-free cue to establish the time of year and seasonal species read, integrate and convert it into a neuro-hormonal signal to synchronise biological functions with seasons. These processes are highly dependent on an endogenous circadian clock detecting the daily duration of the light phase and distributing the photoperiodic message to both brain structures and peripheral organs via nervous and hormonal outputs.

1. The clock in the brain

a. The suprachiasmatic nucleus is the master circadian clock

The first observation of an endogenous daily rhythm was made in 1735 by the French astronomer Jean-Jacques d'Ortous de Mairan who observed that mimosa plant continue to fold and unfold its leaflets each day, even in constant darkness. This daily rhythm is said "circadian" because first, its period is of approximatively 24 hours (from latin *circa* = near, *dies* = day) and second, this rhythm is endogenous, that is persisting even in the absence of external temporal cues (or zeitgeber). Since this first observation,

it was discovered that most organisms, from cyanobacteria to human, possess endogenous circadian clocks.

A typical example is the daily rhythm of locomotor activity in rodents. Nocturnal rodents are active during the dark phase and rest during the light phase. In laboratory conditions, the locomotor activity can be monitored and nocturnal rodents display a marked daily rhythm of locomotor activity synchronised to 24 hours exactly by the light/dark cycle (Figure 1). Like mimosa plants continuing to fold and unfold its leaflets when maintained in constant conditions, the locomotor activity rhythm persists when animals are kept in constant darkness with an endogenous period of approximately 23.5 hours (Figure 1), proving that this rhythm is controlled by an endogenous circadian clock. The exact location of this endogenous clock remained unknown until the 1970's when bilateral lesions of the suprachiasmatic nuclei of the hypothalamus (SCN) were shown to abolish the daily rhythm of locomotor activity, drinking behaviour (Stephan and Zucker, 1972) and corticosterone secretion (Moore and Eichler, 1972) in rats maintained in constant darkness.

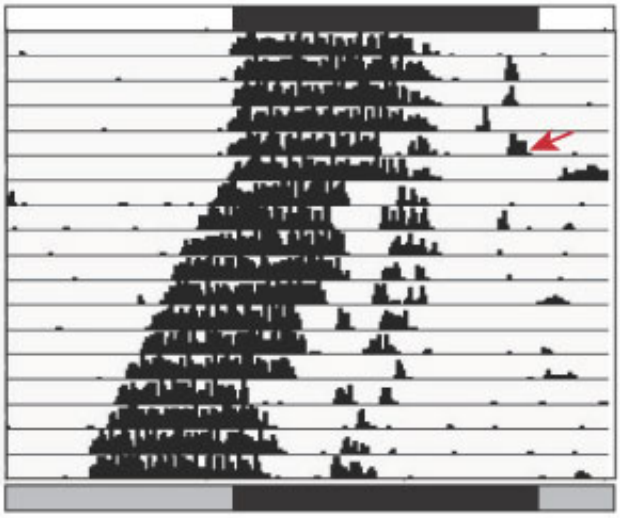

Figure 1: Locomotor activity rhythm in nocturnal rodents

Rodents display a marked rhythm of locomotor activity, nocturnal animals being active at night (black horizontal bars). Each line represent a day and each vertical black bar corresponds to a 10-minute period of locomotor activity monitored by a running wheel connected to an electronic recording system. The locomotor activity rhythm is synchronised by the alternation of a light/dark cycle (open and black horizontal bars). When animals are transferred to constant darkness (red arrow, subjective day: grey horizontal bar; subjective night: black horizontal bar), the endogenous period of the locomotor activity rhythm is expressed and is inferior to 24 hours.

In 1985, while they were working at the university of Oregon, Martin Ralph and Michael Menacker used to order Syrian hamsters (*Mesocricetus auratus*) from an american supplier. One day, Ralph noticed the unusual behaviour of an individual who

used to start its locomotor activity long before lights off. Intrigued by this behaviour, Ralph transferred this individual to constant darkness and observed that this hamster displayed a rhythmic activity but its endogenous period was of 20 hours instead of around 24 hours. He later crossed this individual with normal hamsters, and surprisingly, the offspring's endogenous period was also shorter than usual (22 hours). It appeared that the transmission of this phenotype follows Mendel's laws and is probably due to a mutation in a single gene, heterozygotes having a period of 22 hours and homozygotes having a period of 20 hours. This mutation was named after the Greek letter referring to a period: *Tau*. Later, it was discovered that the *Tau* mutation affects the casein kinase ∂ gene, which is involved in the molecular machinery of the clock (Ralph and Menaker, 1988). Interestingly, a SCN graft from a *Tau* mutant to a wild-type SCN-lesioned hamster restores the daily rhythm of locomotor activity with a period corresponding to the donor's period (Ralph et al., 1990). These observations prove that the endogenous circadian clock is located in the SCN and distributes its endogenous period to the rest of the organism.

The SCN are located in the anterior part of the hypothalamus, on each side of the third ventricle and just above the optic chiasm. Each nucleus contains from 8000 to 10000 neurones (Abrahamson and Moore, 2001; Moore et al., 2002) most of which are GABAergic (γ-aminobutyric acid; Moore and Speh, 1993). The SCN are constituted by a ventrolateral part and a dorsomedial part. Dorsomedial neurones express arginine vasopressin (AVP) in a circadian way with a peak during the light phase (Tominaga et al., 1992; Abrahamson and Moore, 2001; Moore et al., 2002). Ventrolateral neurones mainly express the vasoactive intestinal peptide (VIP) with a peak during the dark phase. However, this rhythm is not circadian because it does not persist in constant darkness (Abrahamson and Moore, 2001) (Figure 2). SCN express a variety of other neuropeptides such as somatostatin, cholecystokinin, substance P, galanin, neurotensin or calretinin (Abrahamson and Moore, 2001). Interestingly, when individual SCN neurones are cultured *in vitro*, each individual cells display a circadian rhythm of firing activity even after three weeks of culture (Welsh et al., 1995), indicating that each individual SCN cell contains the molecular machinery required to generate circadian oscillations.

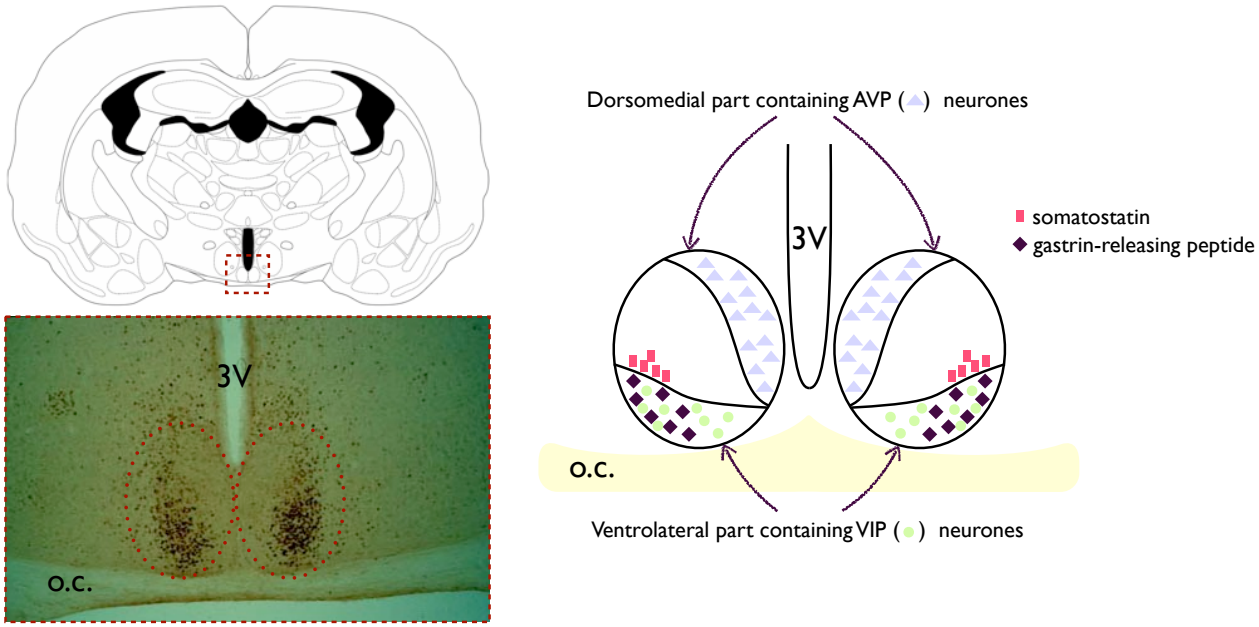

Figure 2: Localisation and neuropeptides content of the SCN

SCN are located just above the optic chiasm (o.c.) on each side of the third ventricle (3V). They are constituted of a ventrolateral part containing vasoactive intestinal peptide (VIP) neurones and of a dorsomedial part containing arginine vasopressin (AVP) neurones. The ventrolateral part also contains somatostatin and gastrin-releasing peptide.

b. The clock genes system

The clock molecular machinery in each individual SCN cell relies on a few genes called clock genes interacting together and dimerising cyclically in molecular positive and negative feedback loops with a period of approximatively 24 hours (Figure 3). It is in1971 that the first clock gene, *period* (*Per*) was identified in drosophila mutants (*Drosophila melanogaster*) (Konopka and Benzer, 1971). Twenty-three years later, the gene *Clock* (Circadian locomotor output cycles kaput) was identified in mammals (Vitaterna et al., 1994). Today, about ten genes are classified as clock genes in mammals: *Clock*, *Bmal1*, *Per1*, *Per2*, *Per3*, *Cry1*, *Cry2*, *Rev-erbα, Dec1* and *Dec2.* To be classified as a clock gene, four criteria have been defined: 1) the loss-of-function mutants are arrhythmic, 2) the expression of the mRNA / protein should display a circadian rhythmicity, 3) this rhythm of expression should be shifted by temporal cues, and 4) modifying the cellular amount of clock protein should modify the rhythm pattern. However, in reality, except *Bmal1* (Takahashi, 2004), none of the above-mentioned clock genes fully satisfies all four criteria.

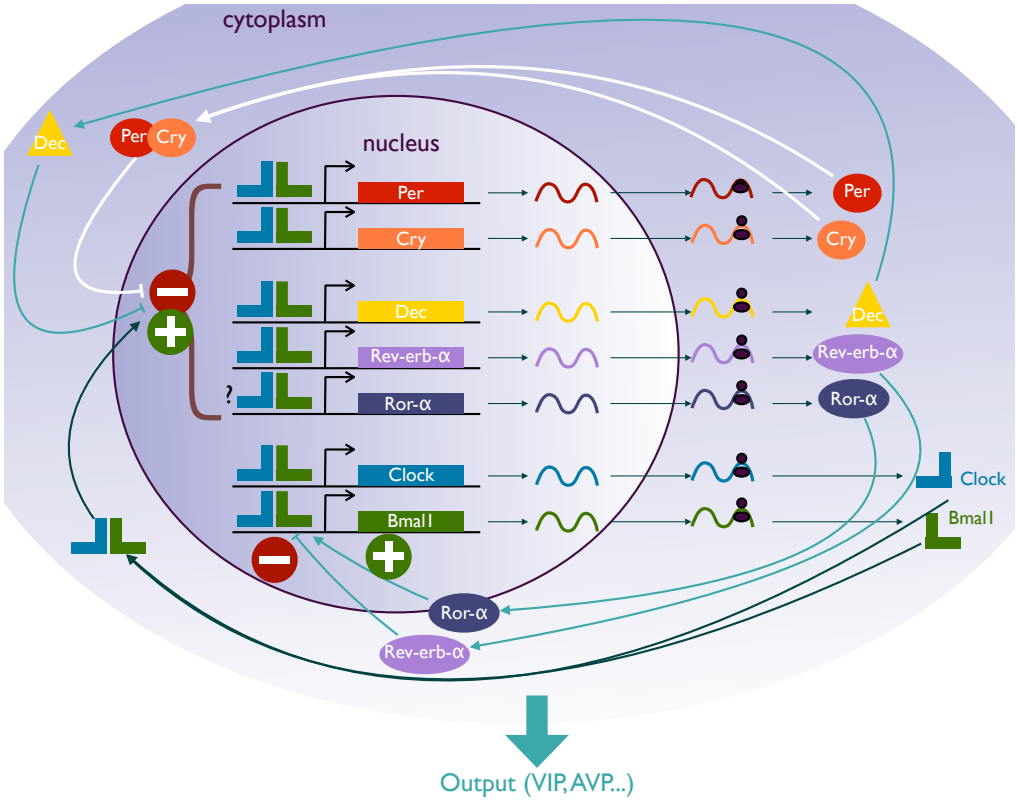

Figure 3: The clock genes machinery

CLOCK/BMAL1 dimers stimulate the transcription of *Per*, *Cry*, Dec, Rev-erb, and Ror genes via E-box elements in their promoter regions. Once translated, PER and CRY dimerise in the cytosol and enter into the nucleus to inhibit CLOCK/BMAL1 stimulatory effect and thus, repress their own synthesis. DEC proteins support this negative feedback loop. REV-ERB and ROR proteins inhibit and activate *Bmal1* transcription respectively. One auto-regulatory cycle takes about 24 hours and the temporal message is distributed to the whole organism via clock-controlled genes such as the genes encoding VIP and AVP..

i. Clock and Bmal1: the positive loop

Clock is constitutively expressed in the SCN in both light/dark cycle and constant darkness (Tei et al., 1997; Maywood et al., 2003) and its mutation leads to a large increase in the locomotor activity rhythm period, rhythm rapidly abolished in constant darkness (Vitaterna et al., 1994; Antoch et al., 1997; King et al., 1997). *Bmal1* ("Brain and muscle ARNt protein 1" also known as *Mop3*) expression in the SCN is highly circadian and peaks during the night (Abe et al., 1998; Honma et al., 1998). *Bmal1* invalidation causes a decrease in the locomotor activity in light/dark conditions and a total arrhythmia in constant darkness (Bunger et al., 2000).

CLOCK and BMAL1 proteins are PAS-bHLH transcription factors because they possess (Hirayama and Sassone-Corsi, 2005):

- a PAS (Period-Arnt-Sim) domain allowing the dimerisation with other PAS proteins
- a bHLH (basic Helix-Loop-Helix) domain, which is a DNA-binding domain.

Since CLOCK expression is constitutive and BMAL1 expression is highly rhythmic, the formation of CLOCK/BMAL1 dimers is also rhythmic. The CLOCK/BMAL1 dimers bind a consensus sequence of DNA called E-box (5'-CACGTG-3'). The temporal message is distributed via E-box containing genes which constitute the clock outputs. Many genes contain an E-box, including other clock genes, but the sole presence of an E-box does not guarantee a rhythmic expression. Among the genes whose expression is controlled by the CLOCK/BMAL1 dimer, are *Per* and *Cry* genes, the core of the molecular negative feedback loop.

ii. Per and Cry: the negative loop

Per gene mRNAs are rhythmically expressed in the SCN with successive peaks during the light phase (Shearman et al., 1997; Takumi et al., 1998a; Takumi et al., 1998b; Yan et al., 1999; Yamamoto et al., 2001). PER protein expression is maximal in late light / early dark phase (Field et al., 2000; Maywood et al., 2003). PER 1, 2, 3 proteins also possess a PAS but not a DNA binding bHLH domain (Hirayama and Sassone-Corsi, 2005) and thus, they are not considered as true transcription factors.

CRY (cryptochrome) proteins are members of the family of flavin-containing blue-light photoreceptors related to phospholyases (Cashmore, 2003). *Cry1* mRNA expression is circadian with a peak in late light phase (Kume et al., 1999; Okamura et al., 1999) whereas *Cry2* mRNA expression either peaks at the light/dark transition (Kume et al., 1999) or is not rhythmic in constant darkness (Okamura et al., 1999). CRY proteins are also expressed rhythmically with a peak at the light/dark transition (Kume et al., 1999).

CLOCK/BMAL1 dimers activate PER and CRY expression by binding to the E-box in the promoter regions of *Per* and *Cry* genes (Jin et al., 1999; Kume et al., 1999; Bunger et al., 2000). When the concentration of PER and CRY proteins is high enough, they dimerise and bind to the CLOCK/BMAL1 complex preventing its stimulatory action (Okamura et al., 2002). This results in an inhibition of *Per* and *Cry* transcription. When the level of PER and CRY proteins is low enough, CLOCK/BMAL1 exerts its stimulatory action again. In addition to the above-mentioned transcriptional regulations, post-translational regulations also exist. PER and CRY proteins in particular are rhythmically phosphorylated by kinases proteins, including the casein kinase ∂ (see Chapter 1, section

I 1 a). The alternation of the positive loop (CLOCK/BMAL1) and the negative loop (PER/CRY) creates 24-hour oscillations of the expression of clock-controlled genes (eg AVP). These genes contain an E-box in their promotor region and are rhythmically expressed under the control of the circadian clock. They distribute the circadian rhythmicity generated into the SCN to the whole organism.

In addition to the two main regulatory loops, secondary loops exist. In 2002, *Rev-erb*α was identified (Preitner et al., 2002) and it negatively regulates *Bmal1* expression. Two years later, *Ror*α was identified as an other component of the mammalian circadian clock machinery (Sato et al., 2004). Contrary to *Rev-erb*α it activates *Bmal1* transcription. By their opposite action on *Bmal1*, REV-ERB and ROR proteins maintain high amplitude of clock genes oscillations and are important stabilisers of these rhythms. However, *Rev-erb*α and *Ror*α do not appear necessary to the genesis of circadian rhythmicity in the SCN (Liu et al., 2008).

2. The photoperiod is integrated by the suprachiasmatic nuclei

a. The retino-hypothalamic tractus

In constant conditions, the endogenous period of the clock gene-generated oscillations is about 24 hours. These oscillations are synchronised to 24 hours exactly by temporal cues (or zeitgebers), the most powerful being the light/dark cycle. The rhythmic alternation of light and dark periods is perceived in the retina by a particular class of retinal ganglion cells that are directly photosensitive and which express the photopigment melanopsin (Provencio et al., 1998; Freedman et al., 1999; Lucas and Foster, 1999; Lucas et al., 1999; Mrosovsky et al., 2001; Hattar et al., 2002). The melanopsin retinal ganglion cells are known to detect changes in light intensity and adjust the pupilla diameter in consequence, phenomenon known as the pupillary light reflex (Hattar et al., 2003; Lucas et al., 2003). These cells also appear critical for photo-entrainment (Panda et al., 2002; Ruby et al., 2002; Gooley et al., 2003; Panda et al., 2003; Rollag et al., 2003). However, melanopsin knock-out (KO) mice are still entrained by light (Panda et al., 2002; Ruby et al., 2002) whereas rods/cones/melanopsin retinal ganglion cells-impaired mice no longer are (Hattar et al., 2003; Panda et al., 2003). This suggest that melanopsin retinal ganglion cells are not the only ones to convey the light information to the circadian clock and that classical photoreceptors play a yet unknown role in this process.

Interestingly, melanopsin retinal ganglion cells project directly on ventrolateral VIP-expressing neurones of the SCN. Following a light-induced activation, melanopsin retinal ganglion cells release glutamate and pituitary adenylyl cyclase activating peptide (PACAP) (Hannibal and Fahrenkrug, 2002, for review). The glutamate released by melanopsin retinal ganglion cells would mediate the effects of light on the circadian clock because application of NMDA glutamate receptor agonists mimics the effects of light on the clock (Ding et al., 1994; Mintz and Albers, 1997).

Beside direct projections to the SCN, the retinal ganglion cells also indirectly project to the SCN via the intergeniculate leaflets (IGL) (Swanson et al., 1974; Hickey and Spear, 1976; Pickard, 1985) or the olivary and posterior pretectal nuclei (Mikkelsen and Vrang, 1994).

b. The suprachiasmatic nuclei integrate day-length

The daily duration of the light phase (i.e. photoperiod) varies with seasons. The SCN integrate the photoperiodic message to distribute it to the whole organism.
The exposition of rodents to a light pulse during the dark phase but not during the day, induces the expression of cFos in the ventrolateral part of the SCN (Sumova et al., 1995; Vuillez et al., 1996). The time frame during which a light pulse induces cFos expression in the SCN is called the photosensitive phase. When the effect of a light pulse administered at different moments of the dark phase was investigated in long day (LD)- or short day (SD)-adapted animals, it was observed that the photosensitive phase of the SCN is longer in SD than in LD (Sumova et al., 1995; Vuillez et al., 1996), proving that the SCN integrates day-length (Figure 4). Similarly, cFos expression is constitutive in the dorsomedial part of the SCN with higher levels during the day and low levels during the dark phase. Interestingly, the profile of cFos expression is longer in LD-acclimated rats (Jac et al., 2000; Sumova et al., 2000). Electrophysiological recordings in isolated hypothalamic slices revealed that the firing activity in the SCN collected from LD-acclimated hamsters is of longer duration than that from SD (Jagota et al., 2000; Mrugala et al., 2000) (Figure 5), demonstrating the SCN's ability to integrate the photoperiod. Finally, this ability is also demonstrated by the observation that the clock genes expression profiles in the SCN considerably vary with photoperiod in several hamster species (Tournier et al., 2003; Johnston et al., 2005; Tournier et al., 2007; Tournier et al., 2009).

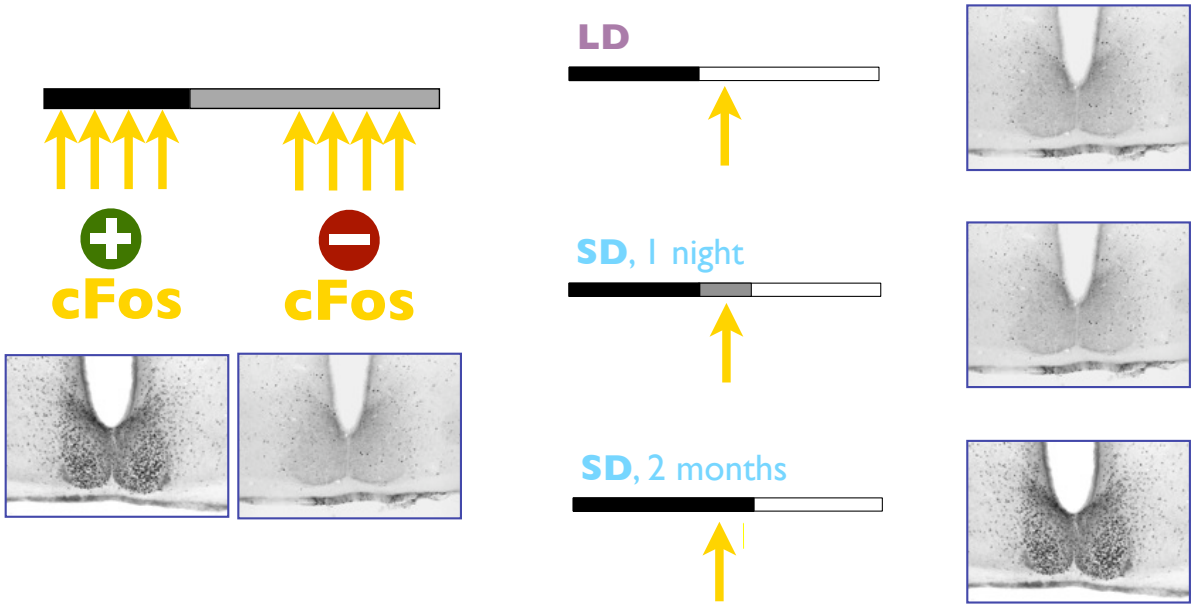

Figure 4: The SCN photosensitive phase depends on photoperiod

A light pulse administered during the subjective dark phase but not during the subjective light phase induces cFos expression in the SCN (left). The time lapse during which a light pulse activates the SCN is called the photosensitive phase. When animals are transferred from long days (LD) to short days (SD), the duration of the photosensitive phase is increased. An example of this is the administration of a light pulse 1 hour after lights on in LD which does not triggers cFos expression in the SCN (right). The same light pulse given at the same moment (now during the dark phase) to animals just transferred to SD also remains without effect. Conversely, this light pulse (now during the dark phase) given to SD-acclimated hamsters massively induces cFos expression, proving that the photosensitive phase of the SCN depends on photoperiod. Adapted from Vuillez et al., personal communication.

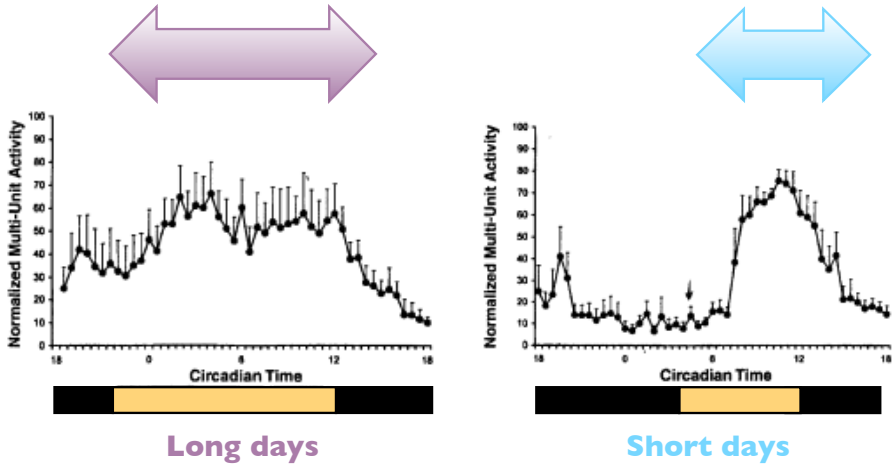

Figure 5: The duration of the firing activity of SCN slices depends on photoperiod

The peak of firing activity of SCN slices lasts longer in long day (LD) than in short day (SD)-acclimated Syrian hamsters. Adapted from Mrugala et al., 2000.

Different models have been proposed to explain how the SCN encodes day-length. One of them hypothesises that two oscillators exist in the SCN: one corresponding to the morning and the other to the evening, each one of them being constituted by different clock gene pairs whose expression changes with photoperiod (Daan et al., 2001). The photoperiodic message would be encoded by the time lapse between the peak of the morning or evening oscillator-controlled outputs. However, doubts remain on the exact distribution of the morning oscillator versus evening oscillator: the left and right SCN oscillators (de la Iglesia et al., 2000) versus rostral and caudal SCN oscillators (Jagota et al., 2000; Hazlerigg et al., 2005).

c. The suprachiasmatic nuclei distribute the seasonal message

The SCN distribute their temporal information by two means. First, the SCN release diffusible factors such as the prokineticin-2 or the TGFα (Transforming growth factor α), the latter being responsible for the circadian rhythm of locomotor activity as demonstrated by encapsulated SCN grafts experiments (Silver et al., 1996). Second, SCN send neuronal efferent projections to various brain areas.

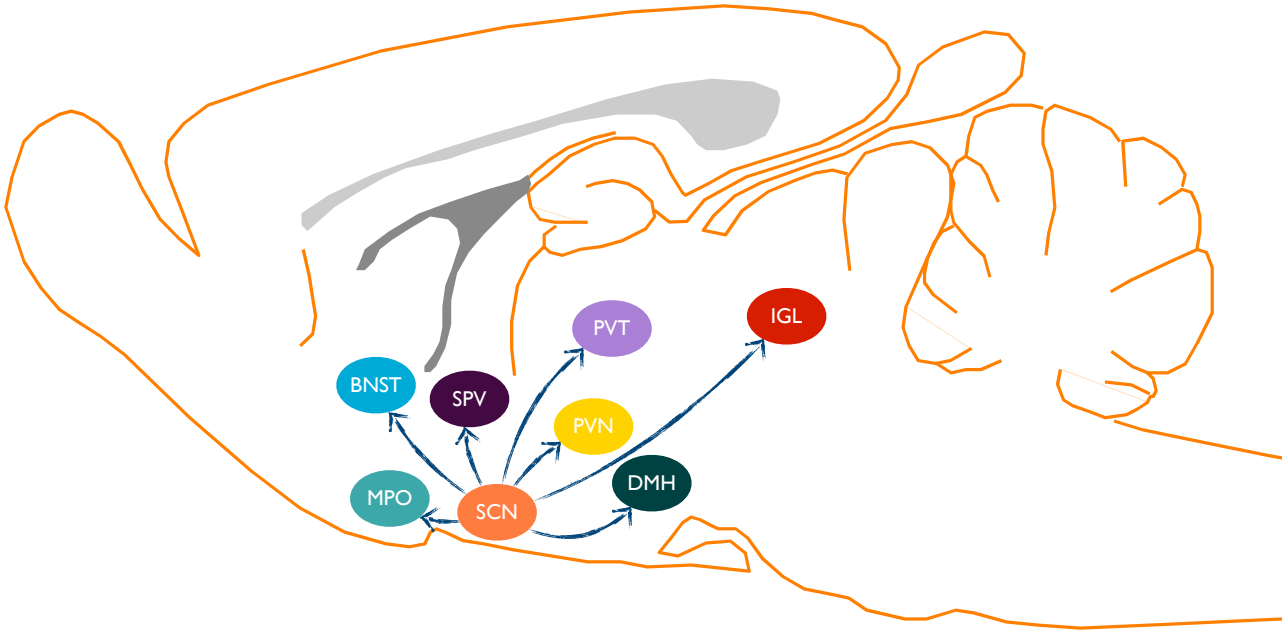

Figure 6: The efferent projections of the SCN

The projection areas of the SCN are the median preoptic area (MPO), the paraventricular hypothalamic nucleus (PVN), the sub-paraventricular hypothalamic nucleus (SPV), the dorsomedian hypothalamus (DMH), the paraventricular thalamic nucleus (PVT), the intergeniculate leaflets (IGL) and the bed nucleus of stria terminalis (BNST).

i. SCN efferent projections

The SCN send projections to many regions, but most concern hypothalamic nuclei (Figure 6) (Leak and Moore, 2001; Kalsbeek and Buijs, 2002). Three different types of neuronal targets have been distinguished (Kalsbeek and Buijs, 2002): endocrine neurones, autonomic neurones and intermediate neurones. The two first categories are thought to affect hormonal and autonomic functions. The third group probably integrates SCN circadian information with other hypothalamic inputs before this information is forwarded. The main projection areas of the SCN are the median preoptic area (MPO), the paraventricular hypothalamic nucleus (PVN), the sub-paraventricular hypothalamic nucleus (SPV), the dorsomedian hypothalamus (DMH) and the ventromedian hypothalamus (VMH). The SCN also innervates the paraventricular thalamic nucleus (PVT), the intergeniculate leaflets (IGL), the lateral septum (LS) and the bed nucleus of stria terminalis (BNST) (Abrahamson and Moore, 2001; Leak and Moore, 2001; Kalsbeek and Buijs, 2002). These neuronal efferences are essential for most circadian functions studied so far such as oestrous cyclicity and rhythmic corticosterone and melatonin production (Hakim et al., 1991; Meyer-Bernstein et al., 1999).

ii. Melatonin synthesis is controlled by the SCN

Melatonin was discovered in the late 1950s and is an amphiphilic molecule synthesised by the pineal gland from the amino acid tryptophan. There is a circadian rhythm in the synthesis and release of melatonin in nearly all vertebrates. The melatonin rhythm is driven by the SCN and is the same is diurnal and nocturnal species with higher levels at night and low levels during the day (Klein and Moore, 1979). Because of its amphiphilic nature, melatonin is not stored but immediately released by the pineal gland exclusively at night. Moreover, its short plasmatic half-life (20 min in rats) (Gibbs and Vriend, 1981; Skene et al., 2001) allows rapid dynamic changes in circulating melatonin levels which make of this hormone a reliable temporal messenger. Indeed, the duration of the nocturnal peak of melatonin is in direct proportion to night-length and this hormone thus provides an endocrine representation of seasons (Reiter, 1980; Goldman, 2001; Simonneaux and Ribelayga, 2003, for review). Although melatonin is also produced in other peripheral organs such as the retina, the pineal gland is the main source of

plasmatic melatonin because a pinealectomy (PINX) completely abolishes the daily rhythm of circulating melatonin (Lewy et al., 1980).

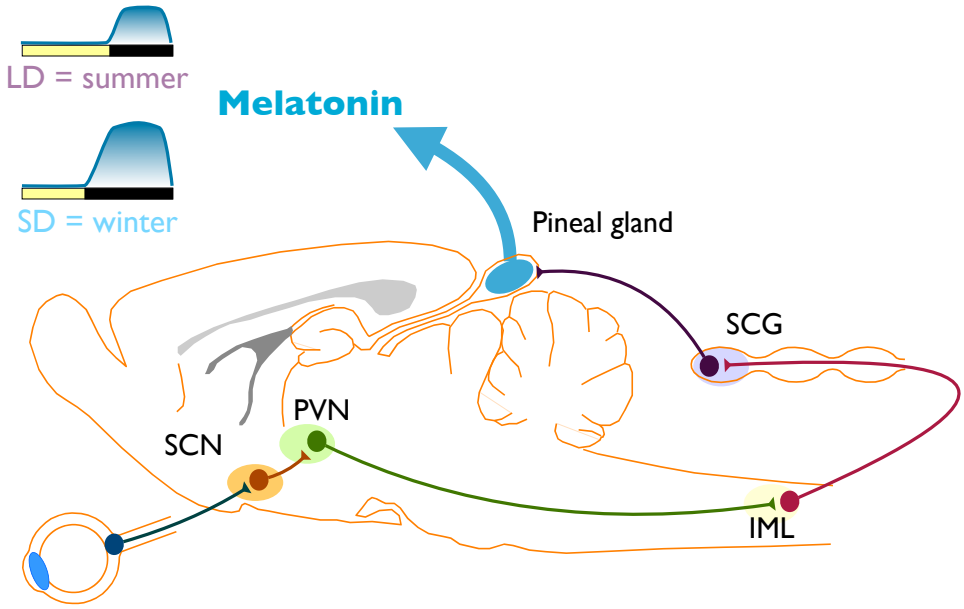

Figure 7: The photo-neuro-endocrine system

The melanopsin retinal ganglion cells relay the photoperiodic information to the SCN. In turn, the SCN controls the pineal gland activity via a multi-synaptic pathway involving the paraventricular hypothalamic nucleus (PVN), the intermediolateral column (IML) and the superior cervical ganglia (SCG). The SCG releases noradrenaline during the dark phase which activates melatonin synthesis and release by the pineal gland. Melatonin synthesis is proportional to night-length and melatonin thus provides an endocrine representation of seasons.

The SCN control the pineal gland activity via a multi-synaptic pathway involving the PVN, the intermediolateral column (IML) of the spinal chord and the superior cervical ganglia (SCG). This pathway, along with the retino-hypothalamic tractus, constitutes the photo-neuro-endocrine system (Larsen et al., 1998; Teclemariam-Mesbah et al., 1999) (Figure 7).

The increased firing rate in the SCN neurones during the day (Jagota et al., 2000; Mrugala et al., 2000) reflects an increased GABA release. Interestingly, GABA antagonist infusion in the PVN stimulates melatonin synthesis during the day and GABA agonist inhibits it at night (Kalsbeek et al., 1996; Kalsbeek et al., 1999; Kalsbeek et al., 2000). Beside GABA, glutamate also regulates melatonin synthesis because NMDA receptor antagonist infusion into the PVN decreases melatonin synthesis during the night (Perreau-Lenz et al., 2004). As the SCN neurones firing rate decreases in late afternoon, the PVN firing rate increases and stimulates the IML cell of the thoracic levels via AVP and oxytocin fibres (Teclemariam-Mesbah et al., 1997; Larsen et al., 1998; Larsen, 1999). In turn, IML innervates the rostral part of the SCG via cholinergic fibres (Strack et al., 1988;

Reuss et al., 1989; Kasa et al., 1991). Finally, SCG-originating noradrenergic fibres project to the pineal gland to activate melatonin synthesis and release (Larsen, 1999).

iii. Melatonin synthesis pathway

Noradrenaline released at night under the control of the SCN/PVN is the most powerful stimulator of melatonin synthesis. It is released into the pineal gland from the sympathetic nerve terminals and activates two types (α1 and ß1) adrenergic receptors. In all mammalian species, the activation of the ß1 receptor on pinealocytes membrane by noradrenaline activates the adenylate cyclase via a Gs protein, which in turn induces a massive rise in intracellular cyclic AMP (cAMP) levels. This is followed by the activation of the cAMP-dependent protein kinase A (PKA). Stimulation of the α1 receptor does not change cAMP levels on its own but potentiates the ß1 effect via a rise in intracellular Ca^{2+} concentration and activation of the protein kinase C (PKC) (Vanecek et al., 1985; Schomerus et al., 1995). This initiates melatonin production via an activation of the arylalkylamine-N-acetyltransferase (AA-NAT), the limiting enzyme of the hormone synthesis (Klein et al., 1997). In the first step of melatonin synthesis, pinealocytes capture tryptophan which is hydroxylated into 5-hydroxytryptophan by the tryptophan hydroxylase (TpOH) expressed in the pineal mitochondria (Sato et al., 1967). 5-hydroxytryptophan is converted into 5-hydroxytryptamine (or serotonin, 5HT) by the aromatic amino acid decarboxylase (AAAD) and acetylated by the AA-NAT into N-acetylserotonin. Finally N-acetylserotonin is O-methylated by the hydroxyindole-O-methyltransferase (HIOMT) to form melatonin (N-acetyl-methoxytryptamine) (Klein et al., 1981, for review) (Figure 8).

The AA-NAT is the rhythm-generating enzyme of melatonin synthesis and it is mainly expressed in pinealocytes (Borjigin et al., 1995; Klein et al., 1997; Liu et al., 2004). In all species studied, the rhythm of melatonin synthesis mirrors the rhythm of AA-NAT activity in the pineal gland. AA-NAT activity is very low during the day and is increased at night (Klein et al., 1997; Simonneaux and Ribelayga, 2003, for review).

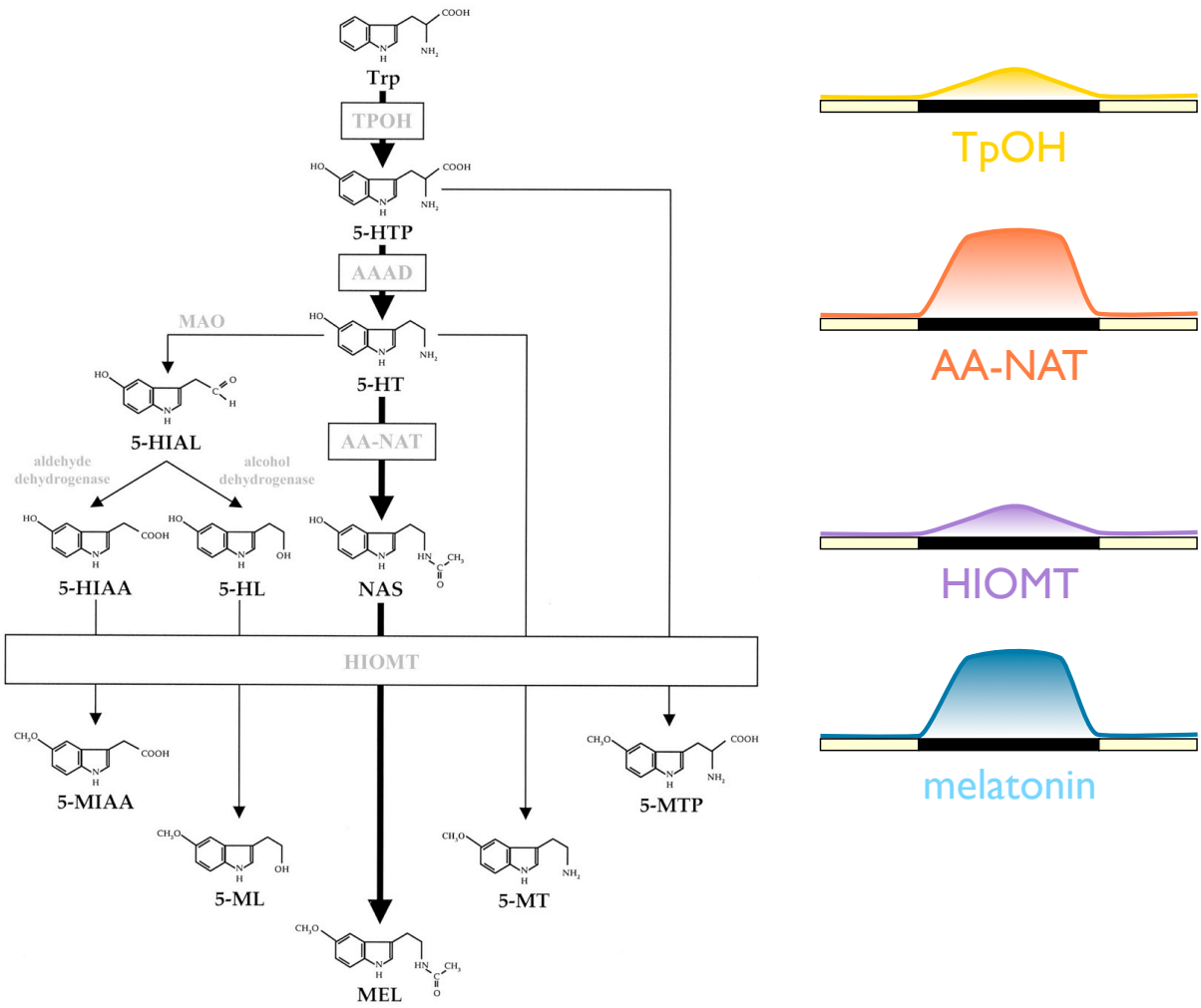

Figure 8: The melatonin biosynthesis pathway

Melatonin synthesis from tryptophan involves 4 enzymes: the tryptophan hydroxylase (TpOH), the aromatic amino acid decarboxylase (AAAD), the arylalkylamine-N-acetyltransferase (AA-NAT) and the hydroxyindole-O-methyltransferase (HIOMT). On the right panel, schematic daily variations of TpOH, AA-NAT and HIOMT activity levels are represented as well as the daily rhythm of melatonin synthesis. Modified from Balemans, 1979.

In ungulates, *aa-nat* mRNA levels are constitutively high and vary little over 24 hours (Coon et al., 1995; Craft et al., 1999). By contrast, AA-NAT activity differs considerably between day and night, suggesting post-translational regulatory mechanisms (Klein et al., 1997). AA-NAT protein is constitutively synthesised and also constitutively degraded by proteosomal proteolysis. At the beginning of the night, noradrenergic stimulation inhibits AA-NAT degradation by cAMP-PKA dependent mechanisms. The phosphorylation of AA-NAT by PKA protects the enzyme from degradation by promoting the binding of AA-NAT to 14-3-3 protein which acts as a shield (Ganguly et al., 2001; Obsil et al., 2001; Zheng et al., 2001). The 14-3-3 protein also increases the affinity of AA-NAT for its substrate (Ganguly et al., 2005). Consequently,

intracellular levels of AA-NAT protein increases, along with AA-NAT activity, and melatonin synthesis occurs. At the end of the night, noradrenaline release stops which induces a de-protection of AA-NAT by the 14-3-3 protein and a degradation of AA-NAT (Gastel et al., 1998; Stehle et al., 2001).

In rodents, the synthesis of melatonin is regulated by transcriptional mechanisms. The activation of adrenergic receptors induce the activation of the PKA which in turn phosphorylates the cAMP response element binding protein (CREB) (Roseboom and Klein, 1995). Phosphorylated CREB enhance the transcription of the *aa-nat* gene via a cAMP response element in its promoter region (Baler et al., 1997). Consequently, *aa-nat* mRNA levels are undetectable during the day but massively increase at night, which allows the synthesis of AA-NAT protein. The daily rhythms of *aa-nat* mRNA and AA-NAT activity are driven by the SCN since they persist in constant darkness (Klein et al., 1997; Simonneaux and Ribelayga, 2003, for review) and are abolished by SCN lesions (Moore and Klein, 1974; Klein and Moore, 1979).

In SD, the nocturnal peaks of *aa-nat* mRNA and AA-NAT activity lengthen which increases the duration of the peak of melatonin synthesis until a maximal duration dependent on species (Illnerova and Vanecek, 1980; Brainard et al., 1982; Illnerova et al., 1984; Vivien-Roels et al., 1992; Miguez et al., 1995; Ribelayga et al., 2000). In SD, not only is the duration of the peak increased, but also is its amplitude. The AA-NAT does not appear to be responsible for this increase because its levels of transcription and activity are globally decreased in SD (Illnerova et al., 1984; Ribelayga et al., 2000). By contrast HIOMT activity is enhanced in SD in several hamster species and this enzyme thus appears to be responsible for the increased amplitude of the peak of melatonin in SD (Ribelayga et al., 1998, 2000). To summarise, while the AA-NAT controls the initiation and the arrest of melatonin synthesis (ON/OFF switch), the HIOMT determines the amplitude of the nocturnal peak of melatonin.

iv. Melatonin receptors

The temporal message is distributed rapidly to all central and peripheral structures via melatonin release into the bloodstream and in the cerebro-spinal fluid (Tricoire et al., 2002; Tricoire et al., 2003). Radio-iodinated melatonin ligand (^{125}I-melatonin) binding studies revealed that about 110 cerebral structures express melatonin binding sites. Two different types of ^{125}I-melatonin binding sites have been identified:

- the high affinity sites (with a constant of dissociation comprised between 20 and 200 pM) identified as melatonin receptors type 1 and 2 (MT_1, MT_2). A third type of melatonin receptor known as Mel_{1c} also exists in non-mammalian vertebrates (Reppert et al., 1996, for review). The orphan receptor GPR50 identified in mammals and classified as melatonin-related receptor, appears to be the mammalian orthologue of Mel_{1C} (Dufourny et al., 2008) even if it does not bind melatonin (Drew et al., 1998).
- the low affinity sites (with a constant of dissociation in the nanomolar range), previously known as melatonin receptor type 3 (MT_3), which was since described as the quinone reductase 2 enzyme (Nosjean et al., 2000). It is a rationale explanation of the anti-oxidant properties of melatonin (Tan et al., 2007).

The MT_1 and MT_2 receptors are G-protein-coupled receptors with seven transmembrane domains and they are negatively coupled to the adenylate cyclase system (Reppert et al., 1994; Reppert et al., 1995). MT_1 activates parallel signalling pathways. First via a $G_{q/11}$ G-protein coupled to phospholipase Cß activity (Godson and Reppert, 1997) and second, via a pertussis toxin insensitive inhibition of cAMP mediated by a G_z protein, as described in sheep PT (Morgan et al., 1989). MT_2 activation also inhibits cGMP (cyclic guanosine monophosphate) via the guanylate cyclase pathway (von Gall et al., 2002).

v. Melatonin receptors distribution

Melatonin binding sites have been identified in the SCN of nearly all mammals but the highest density in melatonin binding sites has been observed in the pars tuberalis (PT) of the anterior pituitary with the notable exception of human. A great variability of melatonin binding sites exists among species in both the distribution and the intensity of labelling (Weaver et al., 1991; Masson-Pevet et al., 1994; Morgan et al., 1994; Vanecek, 1998, for reviews). In the Syrian hamster, specific labelling was observed in the SCN, the DMH, the PVN, the PVT and the medial part of the lateral habenular nucleus. The PT is also intensely labelled as in all mammals (Weaver et al., 1989; Williams et al., 1989). In the Djungarian hamster (*Phodopus sungorus*), specific binding was found in the SCN, the PVN, the nucleus of stria medullaris and the PT (Weaver et al., 1989). In the rat, a signal

was observed in the SCN, the PVN, the subiculum, the area postrema, and the PT (Weaver et al., 1989; Klosen et al., 2002).

Whereas MT_1 is mainly expressed in the SCN and the PT (Masson-Pevet and Gauer, 1994; Weaver and Reppert, 1996), MT_2 is mainly expressed in the retina (Reppert et al., 1995) and probably in the brain and SCN as well (Dubocovich et al., 1998; Isobe et al., 2001). Interestingly, a nonsense mutation occurs in the MT_2 coding gene in Djungarian and Syrian hamsters and the receptor is not functional in these species (Weaver et al., 1996). In mice, all ^{125}I-melatonin binding sites in the brain and pituitary disappear in MT_1-deficient animals (Liu et al., 1997), supporting a preponderant role of MT_1.

Finally, melatonin receptors are also expressed in peripheral organs such as the Harderian gland, spleen, testis, ovary, vascular system, intestines, smooth muscle, and some cells of the immune system (Vanecek, 1998, for review)

II. Reproduction as a seasonal function

1. The reproductive axis

The reproductive axis (Figure 9) is constituted of GnRH neurones dispersed in the POA, the diagonal band of Broca and the organum vasculosum of lamina terminalis (Witkin et al., 1982; Merchenthaler et al., 1984; Wray and Hoffman, 1986). These neurones release the decapeptide from nerve terminals located in the external part of the median eminence (Hahn and Coen, 2006), at the level of the hypothalamic-hypophyseal portal system. GnRH release is characterised by its pulsatility which is crucial for a proper activation of the reproductive axis. Indeed, fertility is impaired when GnRH pulsatility is altered by chronic malnutrition, excessive caloric expenditure or ageing (Tsutsumi and Webster, 2009, for review).

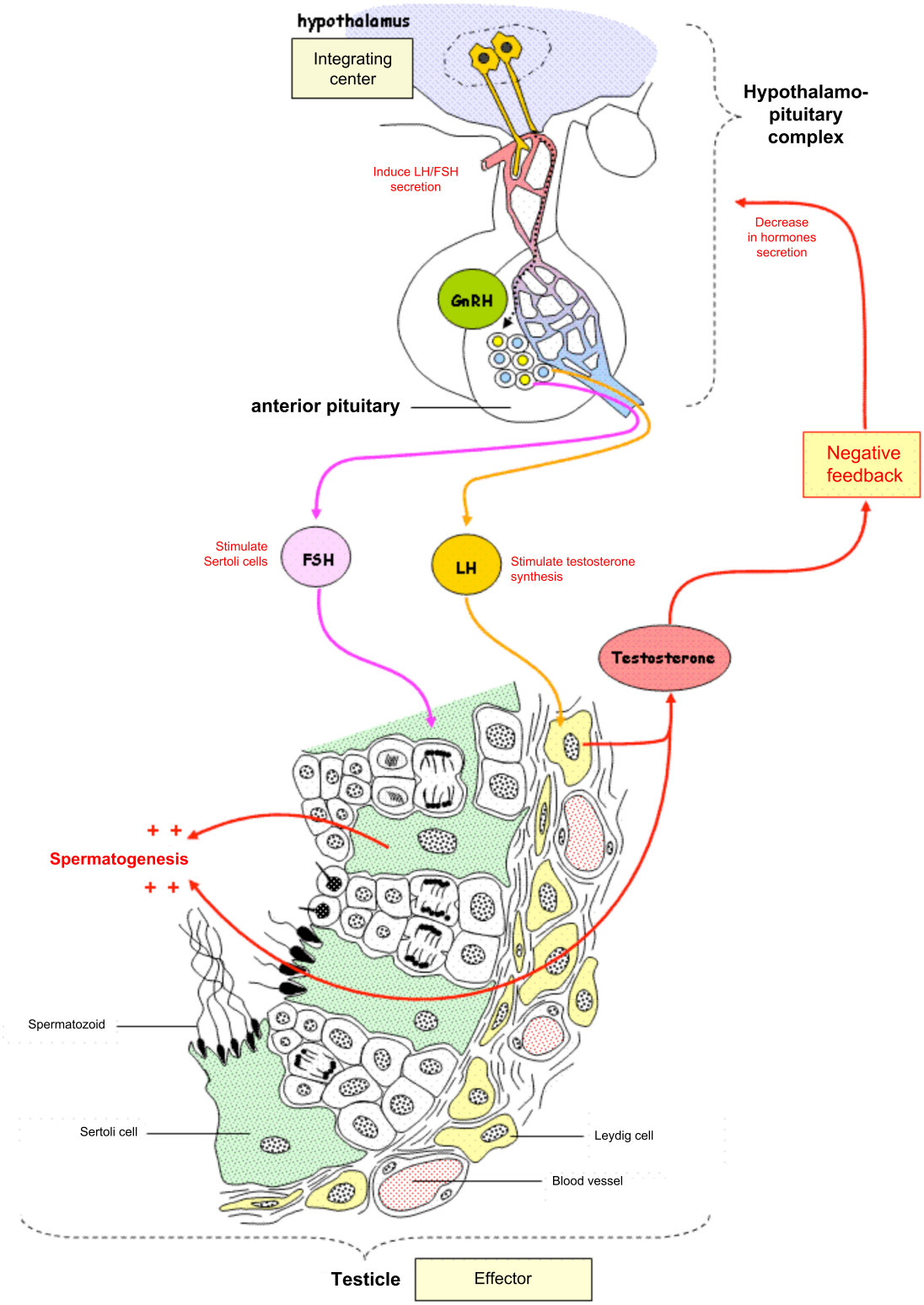

Figure 9: The hypothalamic-pituitary gonadotropic axis in males

Hypothalamic GnRH (gonadotropin-releasing hormone) neurones release GnRH into the portal hypophyseal system. The GnRH triggers the secretion of LH (luteinising hormone) and FSH (follicle-stimulating hormone) from the anterior pituitary. LH/FSH released in the bloodstream act on the testes. LH acts on Leydig cells and induces the secretion of testosterone which feedbacks on the hypothalamus. FSH acts on Sertoli cells to control spermatogenesis. Modified from http://svt.ac-dijon.fr/schemassvt.

The portal blood carries GnRH to the anterior pituitary where it binds to its membrane receptor expressed by gonadotrophs. GnRH receptor (GnRHR) is a seven-transmembrane G-protein-coupled receptor that stimulates the beta isoform of the phosphoinositide phospholipase C which mobilises Ca^{2+} and protein kinase C. Upon GnRH activation, gonadotrophs synthesise and release luteinising hormone (LH) and follicle stimulating hormone (FSH) into the bloodstream. LH and FSH are heterodimeric glycoproteins constituted of two glycopeptidic sub-units. The alpha sub-unit contains 92 amino acids in human and is common to other glycoproteic hormones such as thyroid-stimulating hormone (TSH). The beta sub-unit is specific to each glycoprotein hormone and confers its specific biologic action and is responsible for the specificity of the interaction with the hormone receptor. In males, LH interacts with its receptor expressed by Leydig cells to activate testosterone production by the testes. FSH acts on Sertoli cells to stimulate spermatogenesis. In females, FSH increases the expression and the activity of the aromatase in ovarian granulosa cells which catalyses the conversion of androgens into oestrogen. FSH is also responsible for follicle maturation preceding ovulation which is triggered by a massive secretion of LH. In addition, during the luteal phase, LH also controls the corpus luteum development and thus, the production of progesterone (Figure 10).

Both androgens and oestrogens exert feedback effects on their own production/release both at the level of the anterior pituitary and of the hypothalamus. In males, testosterone exerts a constant negative feedback and in females, during the most important part of the cycle (Figure 10), oestrogens exert a negative feedback as well. However, when oestrogen levels reach a minimum value, the feedback switches from negative to positive and this leads to the pre-ovulatory LH surge. It was tempting to speculate that GnRH are directly sensitive to sex steroids. However, no classical sex steroid receptors have been identified in GnRH neurones so far and sex steroid feedbacks must involve interneurones (Figure 11).

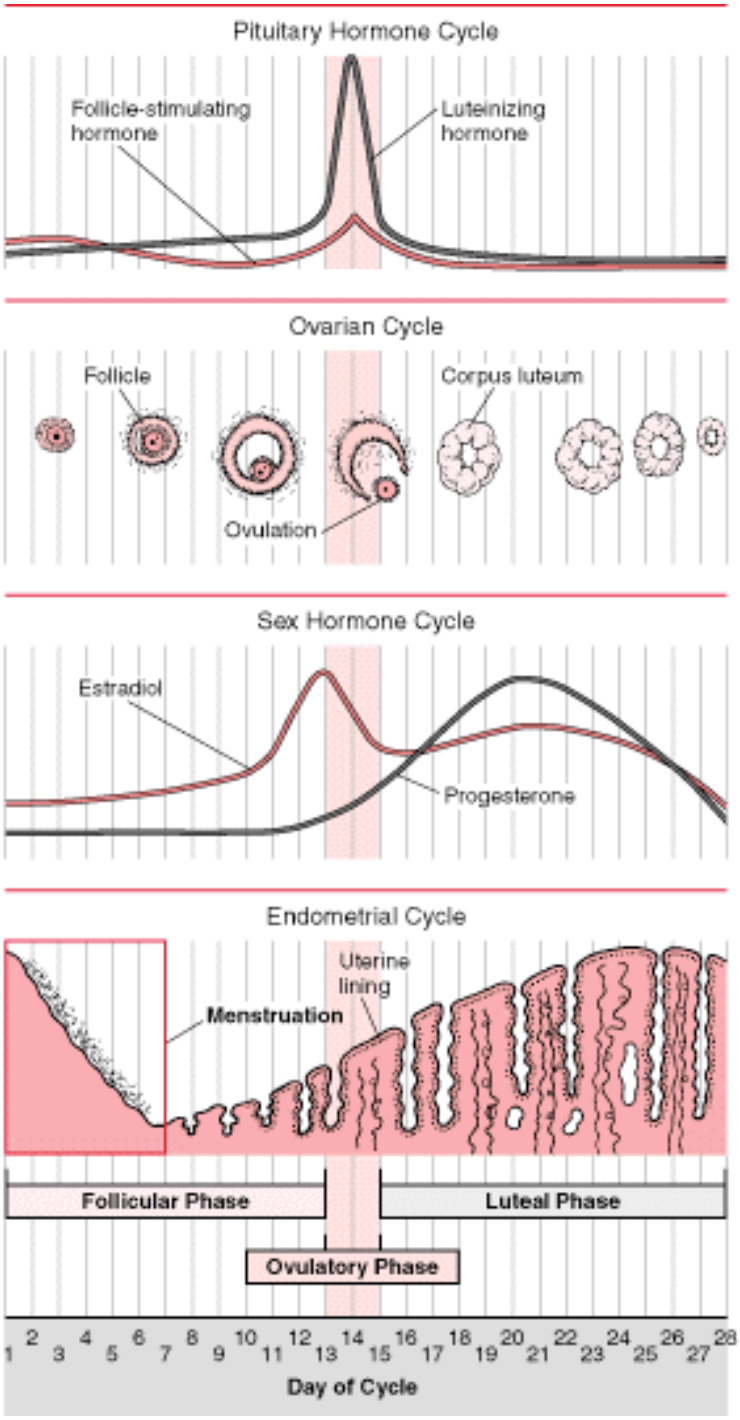

Figure 10: Reproductive cycles in female primates

Females display variations in gonadal hormones throughout the reproductive cycle. During the first part of the cycle (the follicular phase), follicle stimulating hormone (FSH) stimulates the maturation of the follicle which produces oestradiol. OEstradiol levels progressively raise to attain the threshold which activates the oestrogen positive feedback. In response, luteinising hormone (LH) levels dramatically increase in the middle of the cycle and this triggers ovulation. During the luteal phase, progesterone is produced by the *corpus luteum*. The cycle ends either with the nidification of the embryo or with the uterine lining desquamation (menstruation).

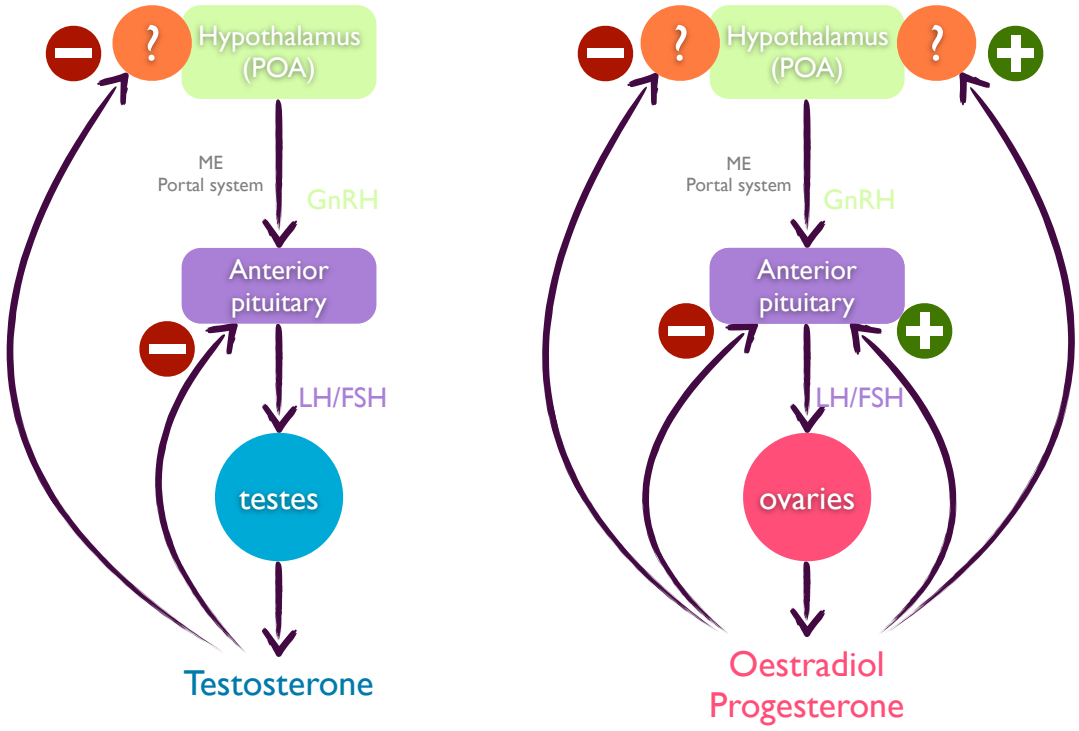

Figure 11: Sex steroids exert feedbacks on their own synthesis

GnRH (gonadotropin-releasing hormone) neurones located in the preoptic area (POA) of the hypothalamus release GnRH into the portal hypophyseal system at the level of the median eminence (ME). The GnRH triggers the secretion of LH (luteinising hormone) and FSH (follicle-stimulating hormone) from the anterior pituitary. LH/FSH released in the bloodstream act on the gonads which produce sex steroids. In males and females, gonadal hormones negatively feedbacks on the pituitary and the hypothalamus at the level of interneurones forwarding this inhibitory message to GnRH neurones. Additionally, shortly before ovulation, oestradiol exerts a positive feedback on the pituitary and on intermediate neurones of the hypothalamus which forward the stimulatory message to GnRH neurones.

2. Seasonality of reproduction

Seasonal variations of the reproductive activity allows species survival by restricting the offspring's birth at the most favourable time of year. To measure the annual time, most (but not all) seasonal species rely on photoperiod. Depending on the duration of embryonic development (and thus, gestation length), two categories of seasonal breeders can be distinguished: long-day breeders (short gestation time or gestation lasting around one year) that are sexually active in late winter/spring, and short-day breeders that are sexually active in fall/winter (gestation length of a few months). However, within different species of LD (or SD) breeders, mechanisms involved in the seasonal reproduction vary considerably. In this thesis manuscript, we will focus on a

model of SD-breeders (the sheep) and two examples of LD-breeders, the Syrian and the Djungarian hamsters.

a. In long-day breeders: hamsters

i. Effect of photoperiod on the reproductive axis

Syrian hamsters synchronise their reproductive activity to seasons via seasonal changes in photoperiod (Honrado et al., 1991). When photoperiod falls below the critical photoperiod (12.5 hours) the male Syrian hamster experiences a complete gonadal regression within eight to ten weeks. Gonadal regression is characterised by a ten-fold reduction of testes weight, reduced seminiferous tubules diameter and a dramatic drop in gonadotropin and sex steroid plasmatic levels. Inversely, when transferred back to LD condition reproductive activity is restored within six weeks (Goldman, 2001) (Figure 12). Similarly, in female Syrian hamster, exposure to short winter-like days (SD) induces a disruption of the oestrous cycle (Sorrentino and Reiter, 1970; Bridges and Goldman, 1975) a one-third reduction of uteri size (Hoffman and Reiter, 1966) and the ovaries stop functioning (Reiter and Johnson, 1974).

It appears that SD-induced changes in gonadotropin levels are secondary to changes in hypothalamic function because in vivo and in vitro studies revealed that the pituitary's response to GnRH is not an important factor causing the SD-induced reduction in LH and FSH levels (Bartke and Steger, 1992). Indeed, male hamsters maintained in SD for ten weeks respond to GnRH injections similarly to hamsters kept in LD (Pickard and Silverman, 1979). Finally, it was demonstrated that cultured anterior pituitaries from SD-acclimated hamsters can still release LH and FSH in response to GnRH (Bacon et al., 1981; Steger et al., 1983; Steger and Gay-Primel, 1990). It is thus hypothesised that SD inhibits GnRH release without affecting the sensitivity of the reproductive axis to exogenous GnRH.

The expression of the SD phenotype in Syrian hamsters is modulated by several other parameters such as ambient temperature, previous photoperiod history, strain and social environment of the animals (Li et al., 1987; Pevet et al., 1989; Urbanski et al., 1990; Vitaterna and Turek, 1993; Larkin et al., 2002).

Interestingly, if Syrian hamsters kept in LD remain sexually active, when they are kept in SD for a prolonged period (over 20 weeks), testicular functions are re-activated

despite the inhibitory photoperiod. This phenomenon is know as photo-refractoriness because animals become insensitive to the photoperiod. To become sensitive again to photoperiod, Syrian hamsters have to be kept in LD for a minimal time interval. The precise mechanisms involved in this process remain however unclear.

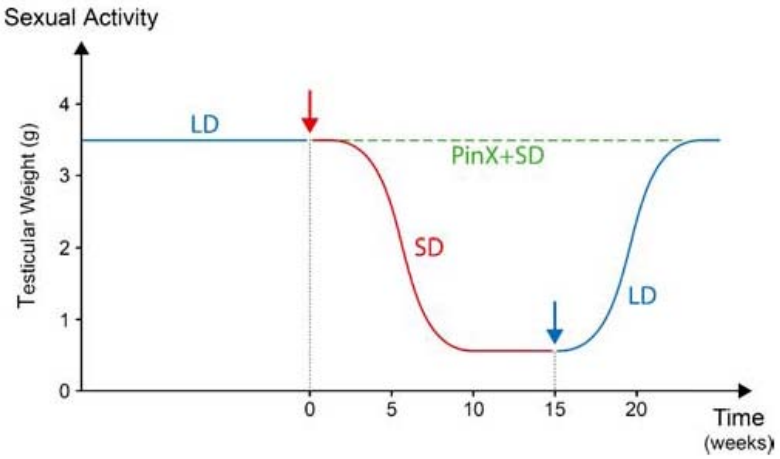

Figure 12: Photoperiodic control of testicular activity

When exposed to a short day photoperiod (SD) for 8 to 10 weeks, male Syrian hamsters undergo a complete inhibition of the reproductive activity assessed by very low testes weight and low gonadal hormones levels. Conversely, when transferred back to long days (LD), high testes weight are restored within 6 weeks. The pineal hormone melatonin controls seasonal cycle of the reproductive activity. Pinealectomy (PinX) prevents the SD-induced inhibition of reproductive activity.

Aside Syrian hamsters, several other hamster species constitute interesting models to study seasonal reproduction. For instance, in Djungarian hamsters, gonadal regression is also observed when photoperiod decreases. Noteworthy, in this hamster species, the absolute value of photoperiod is not the critical parameter. Rather, Djungarian hamsters compare the present photoperiod to the past one and it is the difference that is read by these animals (Duncan et al., 1985; Hoffmann and Illnerova, 1986; Stetson and Watson-Whitmyre, 1986; Prendergast et al., 2000; Prendergast et al., 2004). For instance, a 14 hours light / 10 hours dark cycle can be interpreted either as a LD or a SD photoperiod, depending on the previous day-length. In this hamster species, photoperiod also affects fur colour and body weight.

Finally, in both Syrian and Djungarian hamsters SD represent the active photoperiod (i.e that inhibits reproduction), and in natural conditions, the re-activation of the reproductive axis does not directly depends on photoperiod but rather on the establishment of photo-refractoriness.

ii. Melatonin drives seasonal variations of reproductive activity

Melatonin, whose production by the pineal gland is tightly controlled by the light/dark cycle, provides an endocrine representation of photoperiod and synchronises reproductive activity to seasons (Reiter, 1980). Indeed, pinealectomised Syrian hamsters fail to undergo gonadal regression when transferred to SD (Figure 12). Conversely, administration of exogenous melatonin to LD Syrian hamsters with a kinetic mimicking a SD-like peak of melatonin, induces a complete gonadal atrophy after 8 weeks of treatment (Reiter et al., 1976; Tamarkin et al., 1976; Sackman et al., 1977; Tamarkin et al., 1977; Stetson and Tay, 1983). However, the timing of injection is crucial since melatonin injections induce gonadal atrophy only when given during late afternoon (Stetson and Tay, 1983; Watson-Whitmyre and Stetson, 1983). Taken together, these data demonstrate that melatonin relay the photoperiodic information on the reproductive axis.

Interestingly, in photo-refractory hamsters, the melatoninergic signal remains that of SD. Exogenous melatonin injections to SD-acclimated Syrian hamsters for 7 weeks did not prevent the increase in gonadal activity, indicating that the reproductive axis becomes insensitive to melatonin. Similar insensitivity has also been observed in cerebral structures in the Djungarian hamster. Noteworthy, in *Tau* mutant Syrian hamsters (see Chapter 1, section I 1 a) testicular regression and photo-refractoriness occur earlier than in wild-type animals (Vitaterna and Turek, 1993; Loudon et al., 1998). If we consider that a day lasts 20 hours in homozygote mutants (20 hours corresponding to the animals' endogenous period) and 24 hours in wild-type hamsters, testicular regression and photo-refractoriness occurs after the same number of circadian days spent in SD (Loudon et al., 1998). This has led to the hypothesis that the SCN may control these processes.

b. In a model of short day-breeder: the sheep

i. Effect of photoperiod on the reproductive axis

The sheep (*Ovies aries*) is classified as a short-day breeder because the decreasing day-length in autumn is stimulatory for the reproductive axis. The arrest of the reproductive season occurs with photo-refractoriness to SD. When artificially transferred to LD, sheep experience a complete inhibition of their reproductive function, suggesting that LD could also be directly inhibitory in this species. These phenomena restrict the

offspring birth to spring while food resources are the most abundant. Interestingly, ewes maintained in constant photoperiod show cycles of reproductive activity with a period of approximately one year, revealing the presence of a circannual clock (Karsch et al., 1989; Wayne et al., 1990; Jansen and Jackson, 1993). Sheep rely on photoperiod to synchronise this annual rhythm of reproductive activity to one year exactly.

Under natural photoperiod, ewes begin the breeding season in the late summer / fall, after exposure to a sequence of photoperiodic events including increasing day-length before and decreasing day-length after the summer solstice (Malpaux et al., 1989). The breeding season is characterised by a succession of 16-18 day long oestrous cycles which are composed by a succession of four different phases (pro-oestrus, oestrus, metoestrus and dioestrus). This is then followed by the anoestrus season in late winter / spring. During the anoestrus season, no ovarian or behavioural cyclicity is observed (Thiery et al., 2002). The end of the breeding season occurs with the onset of the photo-refractoriness to SD, and this transition is known to involve thyroid hormones.

ii. Melatonin drives photoperiodic changes of reproductive activity

As in hamsters, melatonin controls seasonal changes in reproductive activity. Pinealectomy does not prevent the appearance of the reproductive cycles, but the injection of melatonin (or melatonin hypothalamic micro-implants) is sufficient to re-synchronise the cycle. Therefore, the pineal gland is required for the synchronisation of the rhythms of reproductive activity but not for the generation of the rhythm itself.

In the sheep, melatonin controls the change of the hypothalamic pituitary gonadal (HPG) axis activity by modifying the pulsatile LH secretion. LH pulse frequency is reduced during the anoestrus season, partly due to an increased estradiol negative feedback (Goodman et al., 1982). In ovariectomised ewes with a subcutaneous implant releasing constant amount of estradiol throughout the year, LH pulse frequency is reduced during the non-breeding season. This indicates that melatonin adjust seasonal reproduction by two mechanisms. First, via changes in the negative sex steroid feedback on GnRH secretion and second, via a direct sex steroid-independent modulation of GnRH release (Malpaux et al., 2001).

3. Melatonin sites of action on the reproductive axis

a. In long-day breeders: hamsters

In the Syrian hamster, it rapidly appeared that GnRH neurones could not be a direct target of melatonin for the seasonal control of reproduction. Indeed, GnRH neurone localisation does not overlap with melatonin binding sites distribution (see Chapter 1, section I 2 c v). Second, photoperiod does not affect GnRH mRNA level (Brown et al., 2001), nor the number or the morphology of GnRH neurones (Urbanski et al., 1991) nor pituitary responsiveness to exogenous GnRH (Pickard and Silverman, 1979). It appears that in SD conditions, GnRH neurones are still responsive to excitatory stimuli by excitatory amino acids (Meredith et al., 1991; Hui et al., 1992; Urbanski, 1992). This indicates that photoperiod/melatonin affects GnRH release rather than GnRH production and discards a direct effect of melatonin on these neurones.

Because the synthesis of specific rodent melatonin receptors antibodies has never been successful, the cellular localisation of melatonin receptors remains difficult to establish. Thus melatonin receptors have only been localised by autoradiography, or in some cases, using non radioactive *in situ* hybridisation in structures displaying high levels of melatonin receptor expression such as the PT (Klosen et al., 2002; Dardente et al., 2003a).

Indeed, melatonin acts on its receptors of the PT to control seasonal functions such as prolactin secretion. In addition, several studies indicate that the PT could be involved in the seasonal control of reproduction via thyroid stimulating hormone (TSH) release by PT cells. TSH would then act on the hypothalamus to locally regulate thyroid hormones levels which would in turn control GnRH release (a detailed explanation of the PT model for the seasonal control of reproduction can be found Chapter 6, section II 2 d)

In addition to the PT melatonin receptors, melatonin binding sites are found in the DMH of Syrian hamsters. Interestingly, DMH lesion prevents the SD-induced testicular atrophy (Maywood and Hastings, 1995) as well as the inhibitory effect of exogenous melatonin (Reiter and Sorrentino, 1972; Maywood et al., 1996). These data indicate that melatonin's target for the control of the HPG axis is seated in the DMH in the Syrian hamster (Figure 13).

However, important disparities are observed across species. For instance, DMH lesions do not prevent the SD-induced gonadal atrophy in the Djungarian hamster but

SCN bilateral lesion prevents the melatonin-induced gonadal atrophy as well as melatonin effect on body mass (Bartness et al., 1991; Bittman et al., 1991; Maharaj et al., 1992; Song and Bartness, 1996). This indicates that SCN mediates melatonin action on the HPG axis in the Djungarian hamster. Conversely, SCN lesions do not prevent gonadal regression induced by three daily melatonin injections in Syrian hamsters (Bittman et al., 1979; Bittman et al., 1989). Interestingly, lesions of other structure such as the olfactory bulbs also prevent SD-induced gonadal regression in Syrian hamsters (Pieper et al., 1984; Pieper et al., 1986a; Pieper et al., 1986b). Nonetheless, from all the above-mentioned structures, the DMH appears as the most interesting candidate in the Syrian hamster because 1) its lesion prevents the SD-induced gonadal regression, 2) it contains melatonin binding sites and 3) several photoperiod-controlled genes that could mediate melatonin action on the reproductive axis (Ross et al., 2004; Revel et al., 2006b; Revel et al., 2006a; Revel et al., 2008) are expressed in the DMH/VMH/Arcuate nucleus (ARC) complex. One of these genes is *Kiss1* which encodes several peptides called kisspeptins (Kp).

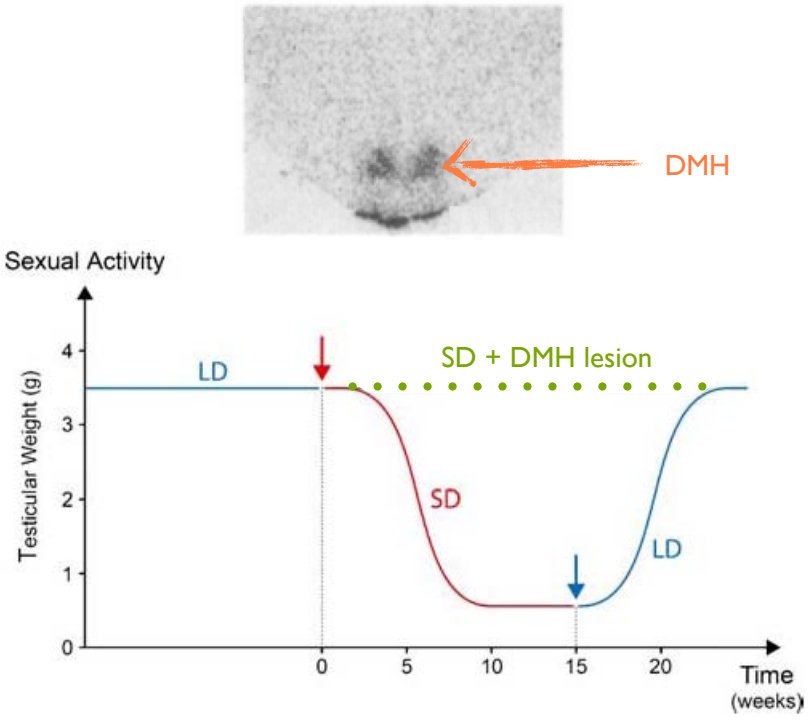

Figure 13: Melatonin target for the control of reproduction in the Syrian hamster

In the Syrian hamster, melatonin binding sites have been identified in the mediobasal hypothalamus (MBH), more specifically in the dorsomedial hypothalamus (DMH) and the *pars tuberalis* of the pituitary (PT). Lesion of the DMH prevents the short day (SD)-induced gonadal regression, indicating that these melatonin binding sites mediate melatonin's effect on the reproductive axis. Adapted from Maywood & Hastings, 1995 and 1996.

b. In a model of short day breeder: the sheep

The pineal gland is required for the synchronisation of reproductive cycles in sheep. Melatonin micro-implants placed in the MBH, but not other brain regions, induce SD-like changes in gonadotropin secretion (Lincoln, 1992; Lincoln and Maeda, 1992b; Lincoln and Maeda, 1992a; Malpaux et al., 1993; Lincoln, 1994; Malpaux et al., 1994; Malpaux et al., 1995). This suggests MBH mediates melatonin control of the reproductive hormone release. More detailed studies with melatonin micro-implants identified the target site of melatonin as the pre-mammillary hypothalamic area. Interestingly, micro-implants located one mm or more outside the pre-mammillary hypothalamic area are ineffective (Malpaux et al., 1998), indicating a very limited and precise region of action. This region is known to bind ^{125}I-melatonin (Stankov et al., 1991; Chabot et al., 1998), most presumably by MT_1 receptors (Migaud et al., 2005).

Although melatonin micro-implants placed in the PT have no effect on gonadotropin secretion, this structure is known to highly express MT_1 receptors in all species studied, including the sheep. Melatonin can thus act directly on the PT. Indeed, clock genes are expressed in the PT and their pattern of expression is dramatically influenced by melatonin (Morgan et al., 1998; Messager et al., 2000; Messager et al., 2001; Lincoln et al., 2002; Dardente et al., 2003b; Johnston et al., 2006). The onset of melatonin secretion at dusk induces the expression of *Cry1* (Dardente et al., 2003b; Hazlerigg et al., 2004) and the termination of the melatonin signal at dawn triggers the expression of *Per1* (Messager et al., 1999; Messager et al., 2000). As a consequence, photoperiodic changes in melatonin signal duration lead to a change in the relative timing of the peak expression of these two clock genes. This suggests that, depending on the interval between dusk and dawn which is relayed via the melatonin signal, the extent of the daily formation of PER / CRY dimers (see Chapter 1, section I 1 b ii) varies throughout the year and may mediate the photoperiodic message within the PT (Figure 14).

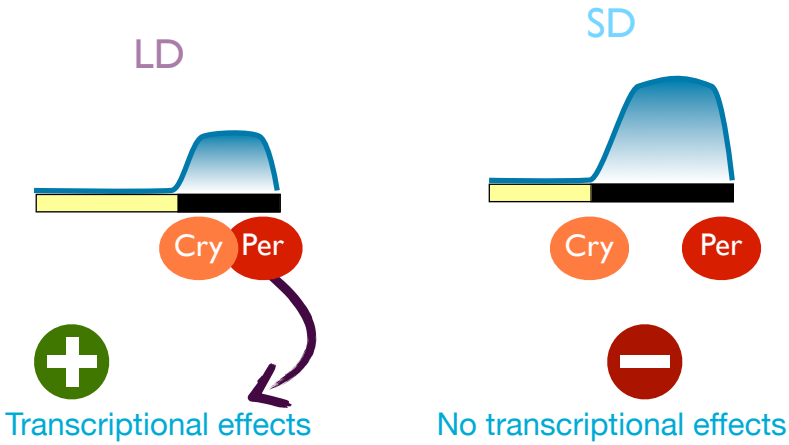

Figure 14: Model of melatonin effect on the pars tuberalis

Melatonin induces CRY expression at dusk whereas termination of the melatonin signal at dawn induces PER expression. In LD, the short duration of the nocturnal peak of melatonin allows an important formation of PER / CRY dimers which induces transcriptional effects mediating melatonin signalling within the pars tuberalis. By contrast, the long duration of the nocturnal peak of melatonin in SD does not facilitate the formation of PER / CRY dimers and no transcriptional effects are observed. Adapted from Hazlerigg & Wagner, 2006.

Finally, the *Kiss1* gene is also expressed in the MBH of the sheep and this important regulator of the reproductive activity is also involved in the seasonal reproduction in ovine (see Chapter 6, section I 3). It thus remains to be determined how the clock genes / PT and the MBH models interact together to mediate melatonin action on the reproductive axis (see Chapter 6, section III) both in ovine and rodents.

III. Biological functions of kisspeptins

1. Introduction to kisspeptins

a. Discovery

Kiss1 was discovered in 1996 (Lee et al., 1996) and named after a famous product of Hershey (Pennsylvania): chocolate Kisses. The *Kiss1* gene codes for a family of peptides called kisspeptins (Kp). Kisspeptins receptor was known as GPR54 and it was first described in 1999 (Lee et al., 1999). It is only 2 years later that GPR54 ligand was identified as Kp (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). GPR54 no longer being an orphan receptor, a new nomenclature was recently adopted and GPR54

is now designated as Kiss1r or Kiss1R for non-primate or primate forms respectively (Gottsch et al., 2009a).

It is in 2003 that two independent groups discovered that loss-of-function mutation of *Kiss1r* prevents the normal maturation of the HPG axis in humans (de Roux et al., 2003) and mice (Seminara et al., 2003). In both humans and mice, loss-of-function mutation into the *Kiss1r* gene induces a hypogonadic phenotype characterised by immature gonads (Figure 15), low gonadotropin and testosterone levels (Figure 16). This discovery unveiled the crucial role of the *Kiss1*/*Kiss1r* system in the maturation of the reproductive axis and was corroborated by the observations first, that mice with loss-of-function mutations in *Kiss1* itself fail to undergo normal puberty (d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007) and second, humans with an “activating” mutation of Kiss1R develop precocious puberty (Teles et al., 2008).

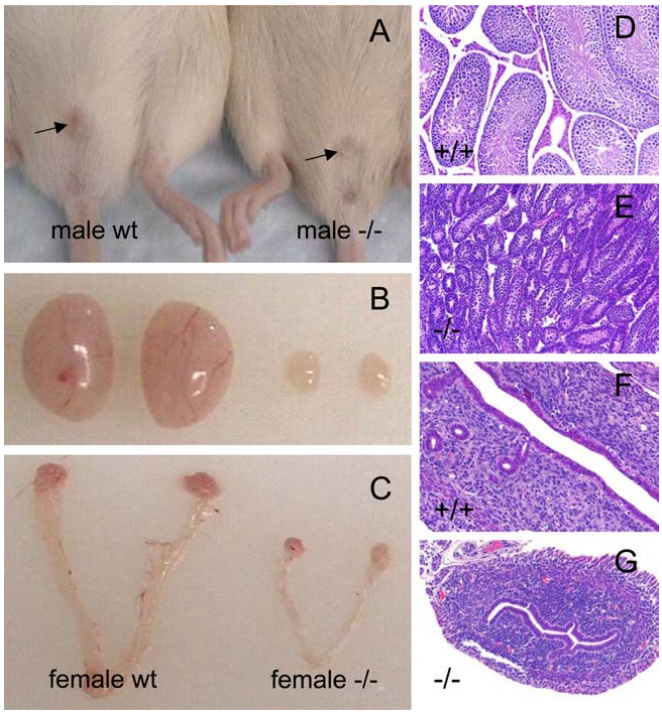

Figure 15: Analysis of the reproductive organs of *Kiss1r* KO mice

Gross and microscopic analysis of the reproductive organs of 30-day-old wild-type and *Kiss1r* -/- male and female mice. (A) External view of male wt and *Kiss1r* -/- mice, showing reduced penis size (arrows). (B) Testes from 30-day-old homozygous mice were reduced in size compared to the wild-type litter mates. (C) Uterine horns and ovaries from 30-day-old homozygous mice were reduced in size compared to the wild-type litter mates. (D–G) Histological analysis of the testes, uterus, and ovaries of male and female 30-day-old wild-type and Kiss1r -/- mutant mice. Hematoxylin and eosin stained sections from wild-type (D) and Kiss1r -/- testis (E), and wild-type (F), and Kiss1r -/-ovary (G). Funes et al., 2003.

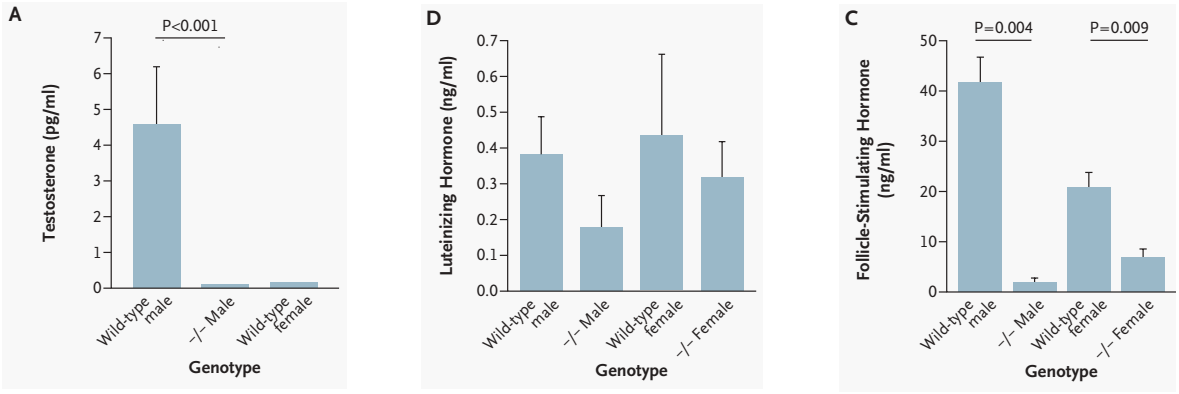

Figure 16: Gonadal hormones levels in *Kiss1r* KO mice

Testosterone (left), luteinising (LH; middle) and follicle stimulating (FSH; right) hormones levels in male and female wild type (WT) and Kiss1r -/- mice. Seminara et al., 2003.

b. Kisspeptins structure

The *Kiss1* gene encodes a 145-amino acid pro-peptide cleaved, in humans, in a 54-amino acid known as kisspeptin-54 (Kp54) as well as shorter peptides of 14, 13 or 10 amino acids. Kp belong to the RF-amide peptide super family because the C-terminal extremity is constituted by an arginine-phenylalanine amidated motif, hallmark of this family of peptides (Figure 17). Interestingly, the amid motive in the C-terminal position plays an important role in the receptor activation since the un-amidated Kp54 form shows only a very weak biological activity (Ohtaki et al., 2001). No endogenous cleavage sites have been identified in Kp54 and the shorter forms could be degradation products of the longest form or constitute purification artefacts (Kotani et al., 2001; Ohtaki et al., 2001; Bilban et al., 2004). Whatever the truth is, the endogenous form(s) present in both the central nervous system and in peripheral organs still remain(s) to be identified.

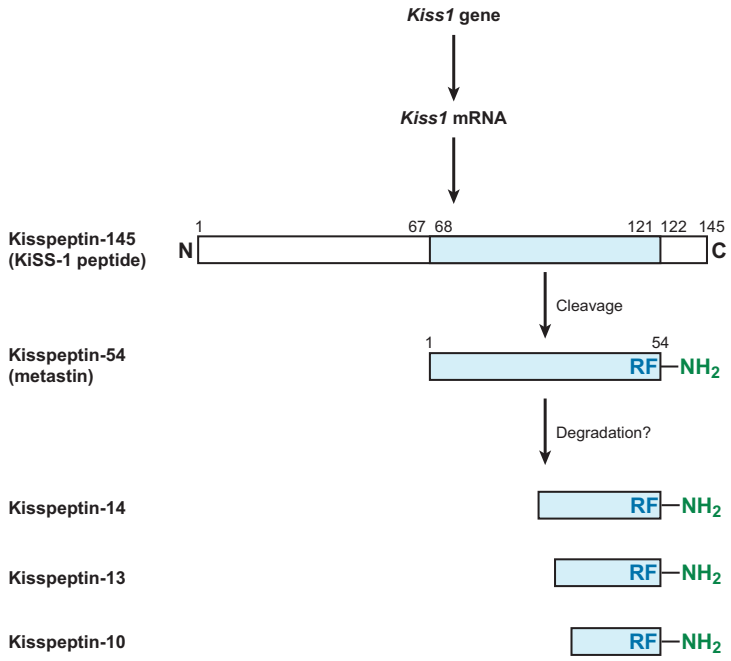

Figure 17: Products of the *Kiss1* gene in humans

Kiss1 mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid pro-peptide. Shown are cleavage sites on the pro-peptide 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 pro-peptide that would lead to synthesis of the shorter peptides, such peptides may be degradation products of kisspeptin-54. Popa et al., 2008

In rodents, the precursor is post-translationally spliced into a 52-amino acid form (Kp52) which includes a disulfid bridge between position 4 and 16 (Ohtaki et al., 2001). Additionally, the C-terminal amino acid differs between human and rodents. In rodents, the C-terminal residue is a tyrosine (Tyr) whereas it is a phenylalanine (Phe) in humans. Shorter fragments (i.e. 10, 13 and 14-amino acid peptides) have also been purified and shown to bind to Kiss1r with the same affinity as the longer form (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001), indicating that the ten final C-terminal residues determines the biological activity of the peptides. This ideas is supported by the observation that N-terminally truncated peptides (Kp14 and Kp10) are three to ten times more active than shorter fragments (i.e. Kp9) to elicit a Ca^{2+} response in Chinese hamster ovary (CHO)/h175 cells, proving the crucial role played by the C-terminal sequence from Tyr45 to Phe54 in the receptor interaction (Ohtaki et al., 2001). The N-terminal extremity does not appear crucial for receptor interaction but may play a role against proteolytic degradation of the longer forms. All Kp forms induce a LH and FSH release as well as testosterone in males when injected centrally or peripherally (Gottsch et al., 2004; Matsui

et al., 2004; Thompson et al., 2004; Navarro et al., 2005b; Mikkelsen et al., 2009) (Figure 18). However, when given peripherally, longer forms have a slower onset of action and a longer lasting effect on testosterone release (Mikkelsen et al., 2009), supporting the hypothesis that the N-terminal extremity protects the longer forms against proteolytic degradation.

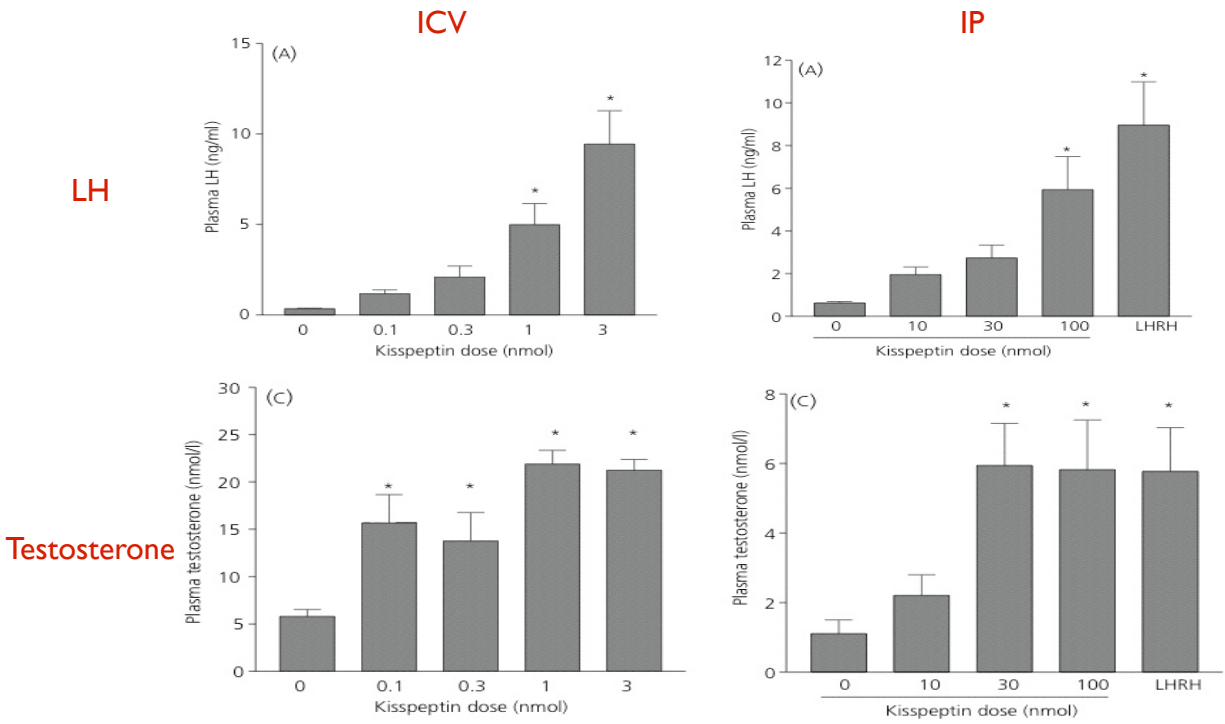

Figure 18: Effect of kisspeptins on LH and testosterone levels

Both central (ICV) and peripheral (IP) injections trigger luteinising hormone (LH), follicle stimulating hormones (FSH) and testosterone. Thompson et al., 2005

c. Kisspeptins expression

i. In peripheral organs

In periphery, *Kiss1* is highly expressed in the placenta, the thymus, testes and ovaries. *Kiss1* mRNA can also be detected at lower levels in the liver, the pancreas, the small intestine, the spleen and the colon (Ohtaki et al., 2001).

ii. In the brain

The *Kiss1* gene is expressed in a restricted number of areas of the central nervous system. In rodents, *Kiss1* mRNA can be found mainly in the ARC and the anteroventral periventricular nucleus (AVPV) (Gottsch et al., 2004; Smith et al., 2005a; Smith et al., 2005b; Revel et al., 2006b; Mason et al., 2007). *Kiss1*-positive neurones are also observed in the periventricular nucleus, the anterodorsal preoptic nucleus and the medial amygdala (Gottsch et al., 2004) (Figure 19).

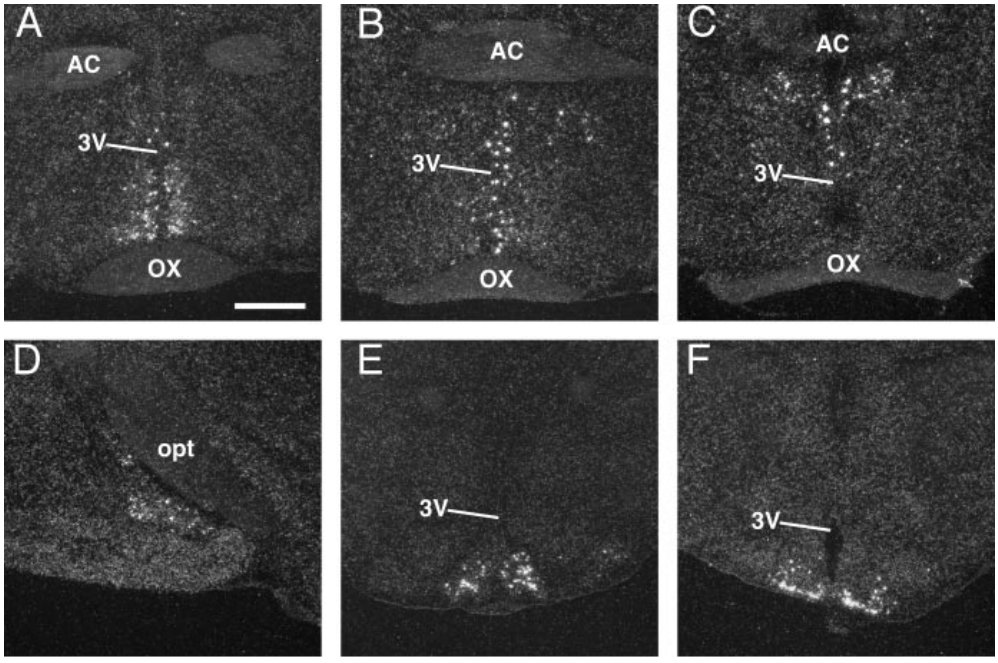

Figure 19: Distribution of *Kiss1* mRNA in the male mouse brain

Kiss1 expressing neurones are found in the anteroventral periventricular nucleus (AVPV; A), the periventricular nucleus (PeN, B), the anterodorsal preoptic nucleus (C), the medial amygdala (D) and the arcuate nucleus (ARC; E & F). Gottsch et al., 2004

In the sheep, Kp immunoreactive neurones are found predominantly in the ARC but a second population of Kp neurones exists in the preoptic area (Franceschini et al., 2006; Pompolo et al., 2006). Similarly, in the mare, Kp immunoreactive neurones are also present in the ARC with an extension toward the pre-mammillary region. A second smaller population of Kp immunoreactive neurones exists in the preoptic periventricular zone of the hypothalamus (Magee et al., 2009).

In primates, *Kiss1* is expressed in the infundibular nucleus (the primate homologue of the ARC) in the rhesus monkey (*Macaca mulatta*) (Plant, 2006; Shibata et al., 2007), the cynomologus monkey (*Macaca fascicularis*) (Rometo et al., 2007) and humans (Rometo et

al., 2007). Few scattered *Kiss1* positive neurones are also found in the medial preoptic area (Rometo et al., 2007).

d. Kisspeptins receptor: Kiss1r

i. Kiss1r signalling pathways

Kiss1r is a G protein-coupled receptor with seven transmembrane domains. Studies involving heterologous cell models expressing rat or human Kiss1r revealed that this receptor is coupled to a pertussis toxin insensitive $G_{q/11}$ protein and which typically activates the phospholipase C (PLC) leading to inositol triphosphate (IP3) accumulation, calcium (Ca^{2+}) mobilisation and subsequent protein kinase C (PKC) activation (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Castano et al., 2009) (Figure 20). A further study in COS7 cells transfected with the murine *Kiss1r* suggests that it is the PLC ß which is activated by the receptor (Stafford et al., 2002).

Interestingly, when a mutated form of Kiss1R causing isolated hypogonadic hypogonadism is transfected into a heterologous cell model (HEK293), it shows a severe reduction in binding affinity for Kp as well as a complete incapacity to normally increase phosphatidyl inositol (PI) turnover like wild-type Kiss1R does (Tenenbaum-Rakover et al., 2007). Conversely, an autosomal dominant activating mutation in Kiss1R was described to cause central precocious puberty in a little girl. When expressed in COS7 cells, the mutated receptor led to a prolonged activation of intracellular signalling pathways in response to Kp with a more sustained elevation of PI levels. Furthermore, the receptor was found to remain on the surface of the cell for a longer period than non-mutated Kiss1R, suggesting a resistance to receptor desensitisation (Teles et al., 2008). These observations emphasise the importance of the proper intracellular signalling associated with Kiss1r for an adequate functioning of the reproductive axis.

In the same study, Teles et al. also reported a prolonged activation of the mitogen activated protein (MAP) kinases pathway (Teles et al., 2008). This indicates the ability of Kiss1r to activate additional signalling pathways including the MAP kinase signalling cascade. Indeed, previous studies revealed a sustained stimulation of the MAP kinases ERK1 and ERK2 as well as a weak stimulation of p38 MAP kinase phosphorylation in CHO cells expressing Kiss1r or Kiss1R exposed to Kp (Kotani et al., 2001). Activation of the MAP kinase pathway by Kp was also described in thyroid cancer cells (Ringel et al.,

2002). In hypothalamic ex-vivo tissues, blockade of ERK1/2 and p38 fully prevents Kp-induced GnRH release indicating that this signalling pathway is active in GnRH neurones and is important for the decapeptide release (Castellano et al., 2006a).

Finally, Kiss1r activation was also shown to activate the phospholipase A2 (PLA2) which induce arachidonic acid production (Kotani et al., 2001; Muir et al., 2001).

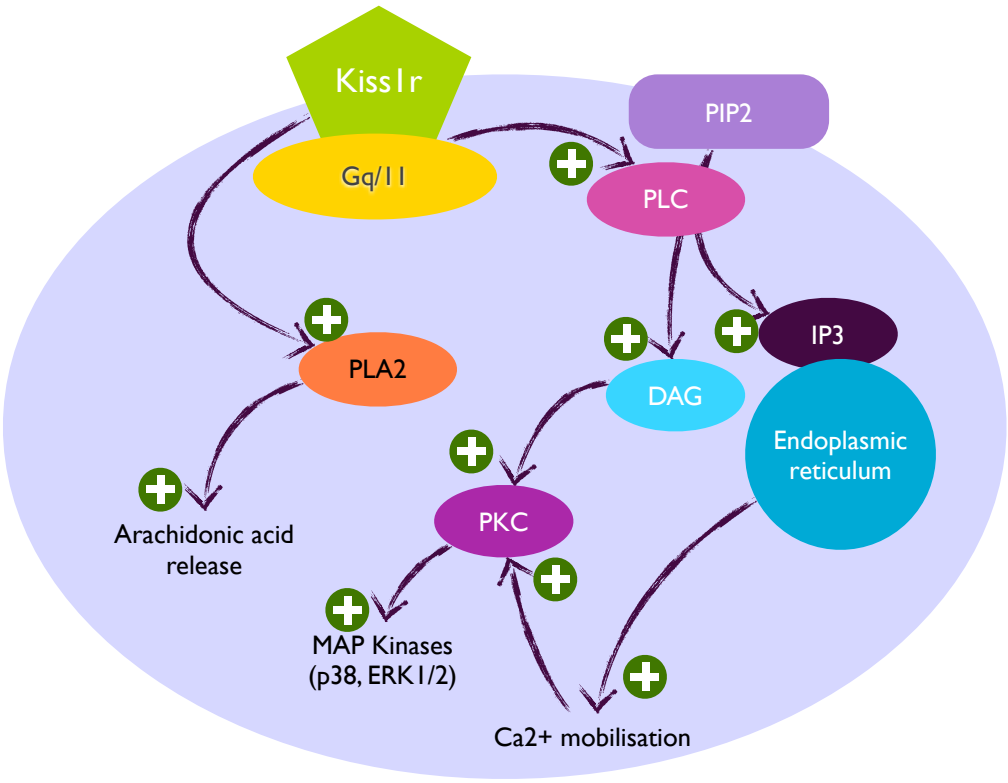

Figure 20: Kiss1r signalling pathways

Kiss1r is a G protein-coupled receptor with seven transmembrane domains. It is coupled to a $G_{q/11}$ protein. It activates the phospholipase C which catalyses the hydrolysis of phosphatidyl inositol diphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 induces Ca^{2+} mobilisation from intracellular stores and DAG activates the protein kinase C which activate the MAP kinases pathway. It also activates the phospholipase A which stimulates arachidonic acid release

ii. Kiss1r expression: localisation

Northern blot, *in situ* hybridisation and quantitative RT-PCR studies revealed that *Kiss1r* mRNA is widely expressed in the central nervous system. In the rat, the highest levels of expression were observed in hypothalamic and amygdaloid nuclei. *Kiss1r* mRNA is highly expressed in the zona incerta, ventral tegmental area, dentate gyrus, ARC, DMH, primary olfactory cortex, lateral habenular nucleus, lateral hypothalamic area, locus coeruleus, and the cortical and medial nuclei of the amygdala. *Kiss1r* mRNA is also expressed in the superior colliculus, medial preoptic area, anterior hypothalamic area,

posterior hypothalamic nucleus, periaqueductal gray, parafascicular thalamic nucleus, parabrachial nucleus, and ventral pre-mammillary nucleus (Lee et al., 1999; Muir et al., 2001) (Figure 21). In peripheral organs, *Kiss1r* is highly expressed in the pancreas and the placenta. *Kiss1r* expression is also found at lower levels in testes and ovaries, the spleen, the small intestine, adrenal glands, lymph nodes and peripheral blood leukocytes (Kotani et al., 2001; Ohtaki et al., 2001; Funes et al., 2003)

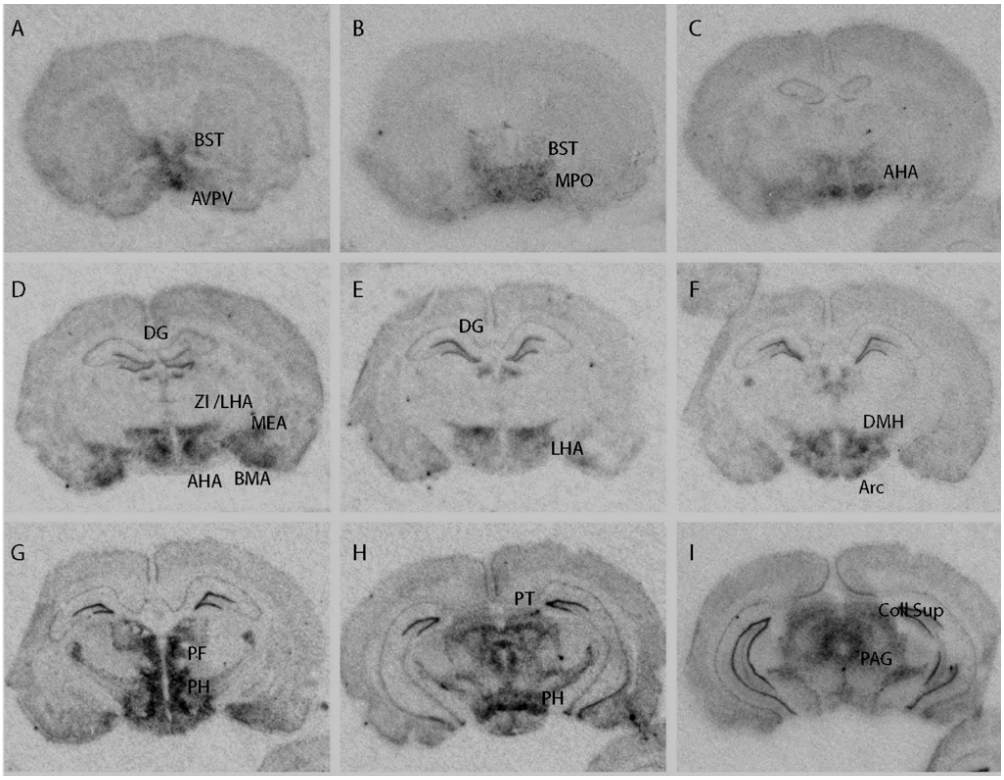

Figure 21: Kiss1r mRNA distribution in the male rat brain

Kiss1r mRNA distribution was assessed by radioactive *in situ* hybridisation. Labelled structures are the bed nucleus of stria terminalis (BST), the anteroventral periventricular nucleus (AVPV), the median preoptic nucleus (MPO), the anterior hypothalamic ares (AHA), the dentate gyrus (DG), the lateral hypothalamic area (LHA), the medial amygdala (MEA), the dorsomedian hypothalamus (DMH), the arcuate nucleus (ARC), the posterior hypothalamus (PH) and the periaqueducal grey (PAG). Mikkelsen et al., unpublished.

2. Kisspeptins' functions

a. Anti-metastatic actions

Historically, *Kiss1* gene discovery is tightly linked to cancer research, the double "s" in Kiss referring to its anti-metastatic properties. The largest Kp, Kp54 was first known as metastin for similar reasons. In 2001, the level of *Kiss1* expression was assessed in

human melanocytic tumours and *Kiss1* mRNA could be found only in 56% of the metastasis tumours and in 50% of the primary melanoma tissues, suggesting a down-regulation of *Kiss1* in these tumours (Shirasaki et al., 2001). Reduced *Kiss1* expression was also observed in many cancer types reviewed by Makri and colleagues (Makri et al., 2008). The reduction in *Kiss1* expression might account for metastases formation and this hypothesis is supported by the following case report. A patient with skin melanoma metastases was successfully treated using topical imiquimod. A biopsy from skin lesions before and after imiquimod treatment was analysed by RT-PCR and *Kiss1*, matrix metalloproteinase 1 and tissue inhibitor of metalloproteinase 1 were up-regulated. The authors suggest the healing action of imiquimod may be partially due *Kiss1* up-regulation (Hesling et al., 2004). Furthermore, matrix metalloproteinases are strongly involved in tissue remodelling such as trophoblast implantation, bone development, angiogenesis and metastases formation. Interestingly, *Kiss1* was shown to negatively regulate the expression of matrix metalloproteinase 9 via a reduced NFκB trans-activation (Yan et al., 2001), and this process probably accounts for Kp anti-metastatic properties.

Kiss1r activation has been shown to negatively regulate the chemotactic activity of the CXCR4 receptor. CXCR4 is a G-protein-coupled receptor which is known to direct organ-specific migration of tumour cells leading to metastasis formation (Navenot et al., 2005). Similarly Kp54 also inhibits chemotaxis and invasion of CHO cells as well as the development of pulmonary metastasis in Kiss1r-transfected B16-BL6 melanomas (Hori et al., 2001). However, the precise mechanisms involved in the anti-metastatic properties of Kp remain to be deciphered.

One model relies on the well known role of Akt in cancer. Akt, a family of protein kinases involved in various biological processes, is known to be involved in cellular survival pathway by inhibiting apoptotic processes (Song et al., 2005). The phosphorylation of Akt proteins was shown to overcome cell cycle arrest in G1 and G2 phases (Ramaswamy et al., 1999; Kandel et al., 2002). Thereby, Akt has been implicated as a major factor in many cancer types. Interestingly, Kiss1r activation is known to promote cell cycle arrest as well as apoptosis (Becker et al., 2005) and Akt phosphorylation is inhibited by Kiss1r activation in CHO cells (Navenot et al., 2005). The inhibition of Akt protein probably accounts for Kp anti-metastatic properties but further studies are required to identify all other actors of Kp anti-metastatic effect.

b. Trophoblast invasion

Kp levels are dramatically increased in pregnant women, especially during the first trimester (Horikoshi et al., 2003) (Figure 22). In light of Kp anti-metastasis properties, it was rapidly hypothesised that these peptides would play an important role in the regulation of trophoblast invasion of the uterine wall, an essential process for foetal development which closely mimics the invasion of cancer cells.

Indeed, both *Kiss1* and *Kiss1r* are highly expressed in the placenta and several studies revealed higher *Kiss1R* expression levels in early stage placentas (where the invasive capacity of early trophoblasts needs to be tightly controlled; Figure 23) as compared to term placentas (Janneau et al., 2002; Terao et al., 2004; Qiao et al., 2005). As a consequence, a role for *Kiss1*/*Kiss1R* has been proposed in the pathogenesis of pre-eclampsia, the most common pregnancy complication where the placenta is shallowly implanted. Pre-eclampsia is characterised by disturbed and inadequate remodelling of uterine spiral arteries by invading trophoblasts thus reducing blood flow which results in pregnancy-induced hypertension and foetal hypoxia. By contrast profuse invasion results in abnormally deep utero-placental adhesion, such as placenta *accreta*, *increta* and *percreta*. Thus, proper trophoblast invasion is crucial for maternal health and adequate development of the foetus. Many studies detected decreased *Kiss1* or Kp levels in placental tissues or blood of pre-eclampsia patients (Farina et al., 2006; Hiden et al., 2007; Armstrong et al., 2009; Reynolds et al., 2009), supporting the idea these peptides control trophoblast invasion. *Kiss1* is also expressed in a cycle-dependant and a region-specific manner in the rat oviduct (Gaytan et al., 2007) suggesting that *Kiss1* could be involved in the prevention of extra-uterine implantation.

How Kp precisely regulate the depth of placental implantation remains to be determined. It appears that Kp10 is the key kisspeptin. Only Kp10, but not Kp13, 14 nor Kp54 stimulated intracellular Ca^{2+} release in primary trophoblasts of the early placenta indicating Kp10 is the physiological activator of Kiss1R in these cells (Bilban et al., 2004). In the placenta, the invasive behaviour of trophoblasts is controlled by metalloproteinases among others (Bischof et al., 2000). As mentioned previously (see Chapter 1, section III 2 a) *Kiss1* negatively regulates matrix metalloproteinases (Yan et al., 2001), and this is a plausible pathway to explain Kp10 effects on trophoblast invasion.

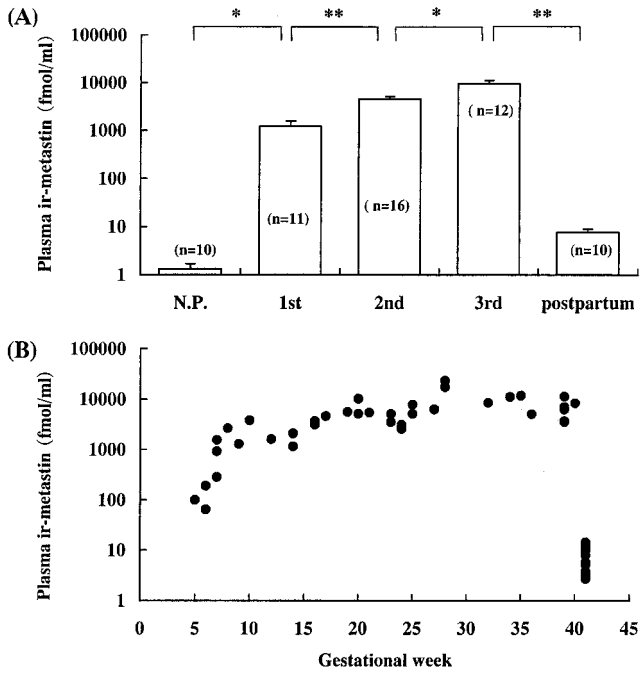

Figure 22: Plasmatic kisspeptins levels and immunoreactivity during pregnancy

Kisspeptins levels dramatically increase during the first semester of pregnancy (1st) when compared to non-pregnant women (N.P.). Kisspeptins levels are elevated throughout pregnancy (2nd, 3rd) and rapidly decrease five days after birth (postpartum). Horikoshi et al., 2002.

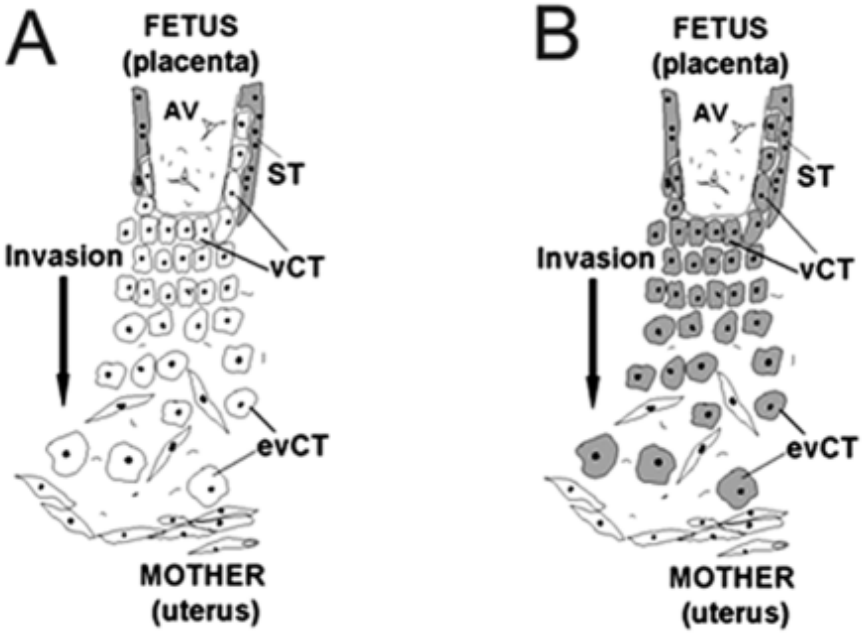

Figure 23: Trophoblast invasion of the uterine wall

At early stages of pregnancy (A), syncytiotrophoblasts (ST) as well as extravillous (evCT) and villous (vCT) trophoblasts, the placental epithelial cells, migrate into the uterine wall. As a result, the embryo is anchored in the maternal uterus and maternal oxygen and nutrients reach the embryo (B). Bilban et al., 2004

c. Puberty initiation

i. Puberty in primates

The onset of puberty is generally defined as the activation (or in some species, a re-activation) of the previously dormant neuroendocrine reproductive axis. *Kiss1* and *Kiss1R/r* proper functioning is a prerequisite for normal pubertal development. Indeed, loss-of-function mutations in *Kiss1R* prevent the normal maturation of the HPG axis in humans and mice (de Roux et al., 2003; Funes et al., 2003; Seminara et al., 2003). Since these initial observations, more cases of patients with *Kiss1R* invalidating mutations were also reported (Semple et al., 2005; Tenenbaum-Rakover et al., 2007). Conversely, several studies reported that activating mutations in *Kiss1R* are associated with precocious puberty (Luan et al., 2007; Teles et al., 2008) and exogenous Kp administered to juvenile monkeys induces precocious release of GnRH and LH (Shahab et al., 2005). This suggests Kp synthesis and secretion may be enhanced at puberty to increase GnRH release. The observation that *Kiss1* mRNA levels increase across pubertal development in both male and female monkeys (Shahab et al., 2005) supports this hypothesis. Finally neural Kp secretion, measured by radioimmunoassay, from hypothalamic explants from female monkeys is higher in pubertal compared to juvenile monkeys (Keen et al., 2008). Since Kp secretion was shown to be pulsatile and to pulse in synchrony with GnRH pulses (Keen et al., 2008), it was hypothesised that increased Kp pulsatility drives increased GnRH pulsatility which triggers puberty in the monkey.

In contrast to the pubertal changes in *Kiss1* expression, which were similar in male and female monkeys, a pubertal increase in hypothalamic expression of *Kiss1R* was only observed in females (Shahab et al., 2005). Interestingly, persistent pulsatile secretion of LH with normal frequency but low amplitude were observed in a female patient suffering from hypogonadism due to a homozygous *Kiss1R* mutation (Tenenbaum-Rakover et al., 2007). It was then suggested that Kiss1R activation by Kp only enhances the amplitude of GnRH pulses at puberty, rather than triggering the pulses.

Taking together, these findings suggest that increased Kp signalling at the Kiss1R receptor occurs in the primate hypothalamus at the time of puberty and that reduced Kp tone during childhood may contribute to the juvenile hypogonadic state.

ii. Puberty in rodents

Similar to primates, proper Kp signalling is a prerequisite for normal pubertal development. First, invalidation of Kiss1r gene prevents pubertal development in mice (Funes et al., 2003; Seminara et al., 2003). Of note, *Kiss1r* KO mice have normal distribution and content of GnRH (Messager et al., 2005), suggesting that deletion of *Kiss1r* does not significantly affect GnRH neurone migration or the decapeptide synthesis and indicates that the GnRH system is functional in these mice. Second, Kp induce LH and FSH release in prepubertal male and female rats and mice (Castellano et al., 2005; Han et al., 2005; Navarro et al., 2005a). Third, repeated Kp central administration to juvenile female rats leads to advanced vaginal opening (Navarro et al., 2004b). It is then tempting to speculate that Kp signalling triggers puberty onset.

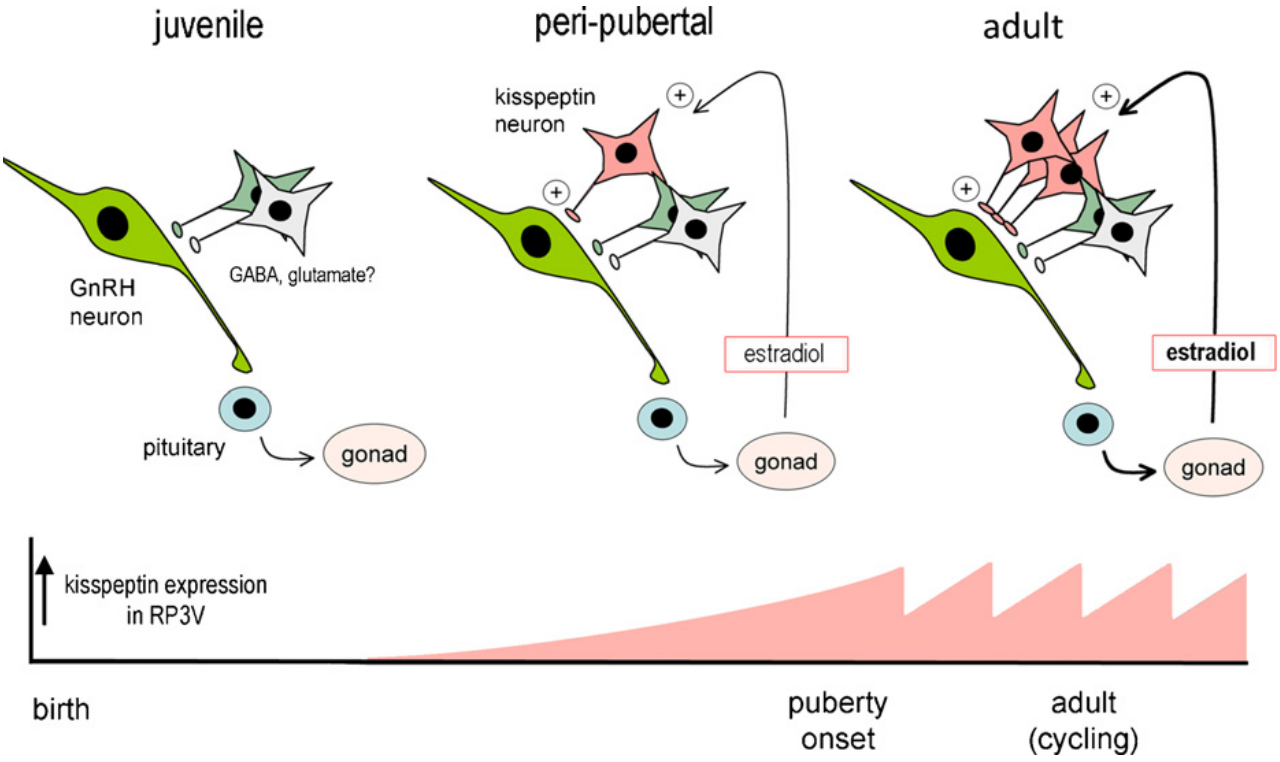

Figure 24: Activation of AVPV Kp neurones by oestrogen across development in female mice

From birth to the early peri-pubertal period there is no Kp expression in the AVPV (represented by the pink colour at the bottom of the figure). Neurotransmitters such as GABA and glutamate may provide the initial activation of the GnRH neurones, which results in an increase in circulating estradiol levels. The rising levels of estradiol act to increase Kp expression the AVPV that then amplifies GnRH neurone activity in a positive feedback manner leading to the completion of puberty onset. After puberty onset, Kp levels in the AVPV fluctuate with the cyclical levels of estradiol to drive the generation of the pre-ovulatory GnRH/LH surge. GABA, γ-Aminobutyric acid; glu, glutamate. Clarkson et al., 2010

This hypothesis is supported by developmental changes in both *Kiss1* and *Kiss1r* expression. Analyses of *Kiss1* mRNA levels were first performed by RT-PCR and these levels were found to be higher in total hypothalamus from adults rats compared to juveniles individuals (Navarro et al., 2004a; Navarro et al., 2004b), suggesting increased Kp synthesis during puberty.

In the AVPV/PeN nuclei, *Kiss1* mRNA levels measured by *in situ* hybridisation are higher in adult than juvenile males mice (Han et al., 2005). However, because AVPV/PeN *Kiss1* expression is stimulated by sex steroids, it is not clear if the higher *Kiss1* expression in adults reflects higher circulating sex steroid levels at this age. A precise time course of Kp expression was recently performed in female mice and revealed that the number of Kp immunoreactive neurones in the AVPV/PeN remains low until post-natal day 25 and reaches its maximal level at pot-natal day 30 (Clarkson et al., 2009a). Similar results were observed in the male rat using *in situ* hybridisation (Bentsen et al., 2010). Interestingly, ovariectomy at post-natal day 15 (while Kp neurones are few) prevents the increase in the number of Kp neurones at puberty and estradiol treatment in ovariectomised female mice restores high levels of Kp expression at puberty (Clarkson et al., 2009a). This suggest that the higher Kp expression in post-pubertal rodents reflects higher levels of circulating sex steroids (Figure 24).

Kiss1 mRNA levels were found to be higher in the ARC of adult male rat when compared to juvenile individuals (Bentsen et al., 2010) whereas no significant differences between adult and juvenile male mice were observed (Han et al., 2005). Interestingly, in male rats, a slight difference in the magnitude of the increase of *Kiss1* mRNA signal is observed between ARC and AVPV, as a moderate signal is detected in the ARC at post-natal day 15, when very little signal is seen in the AVPV (Bentsen et al., 2010). This different timing suggests that the maturation of Kp neurones in these two areas might occur via distinct mechanisms.

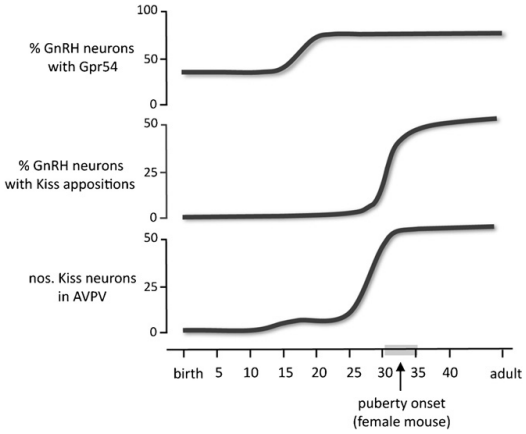

Figure 25: Temporal pattern of GnRH neurones sensitivity to kisspeptins

Schematic diagram showing the temporal pattern of *Kiss1r* expression in GnRH neurones (top panel), Kp inputs to GnRH neurones (middle panel) and the numbers of Kp neurones in the anteroventral periventricular nucleus (AVPV; bottom panel) during postnatal development in the mouse. The onset of puberty in the female mouse is typically around postnatal days 30–35 as indicated. GnRH, gonadotropin-releasing hormone. Clarkson et al., 2010

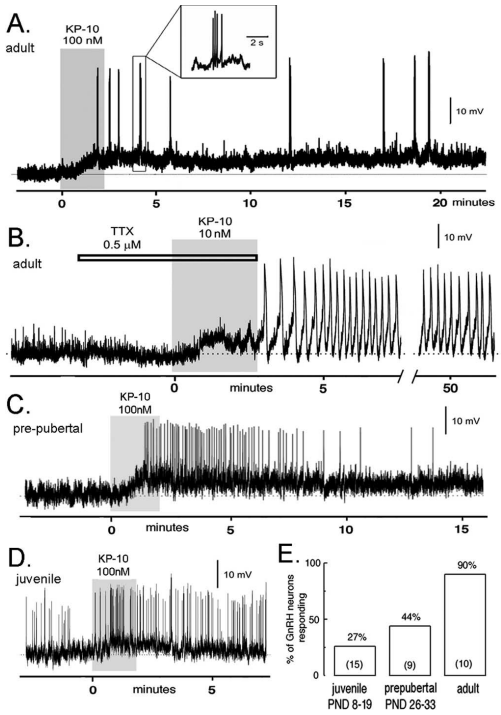

Figure 26: GnRH neurones are depolarised by kisspeptins

A, An adult male mouse GnRH neurone is depolarised by 100 nM kisspeptin-10 (KP-10) for over 20 min and exhibits short bursts of action potentials (inset). **B**, Adult male GnRH neurone exhibiting a depolarising response and pronounced membrane oscillations to 10 nM kisspeptin-10 (KP-10) in the presence of the Na^+ channel blocker tetrodotoxin (TTX). **C**, A prepubertal (post-natal day 30) GnRH neurone exhibiting a depolarising response associated with increased firing in response to 100 nM kisspeptin-10 (KP-10). **D**, Spontaneously active juvenile (Post-natal day 9) GnRH neurone responding to kisspeptin-10 (KP-10) with a transient depolarisation. **E**, Developmental recruitment of kisspeptin-sensitive GnRH neurones across puberty. The histogram shows the percentage of male juvenile, prepubertal, and adult GnRH neurones activated by KP-10. PND, Post-natal day. Han et al., 2005

As mentioned previously, it is hypothesised that increased Kp pulsatility drives increased GnRH pulsatility which triggers puberty. Indeed the number of Kp immunoreactive fibres that come in close apposition with GnRH neurones increases throughout postnatal development (Clarkson and Herbison, 2006) (Figure 25). In addition GnRH neurones may also become more sensitive to Kp stimulation since electrophysiological recordings from GnRH neurones in slice preparations show an increased responsiveness to Kp in older mice. While GnRH neurones from juvenile animals are transiently depolarised by Kp, GnRH neurones from adult mice display a long-lasting (over 30 min) depolarisation (Han et al., 2005) (Figure 26). This observation is consistent with the finding that Kp is less potent in stimulating LH in pre-pubertal rats or mice when compared with adults (Navarro et al., 2004a; Han et al., 2005; Navarro et al., 2005a). The increase in GnRH neurones sensitivity to Kp at puberty is probably not the result of an increase in *Kiss1r* mRNA in mice GnRH neurones. Indeed, around 40% of GnRH neurones express *Kiss1r* at birth and until post-natal day 5. Fifteen days later, the percentage of GnRH neurones that express *Kiss1r* is of 80 %, which is similar to adult levels as shown using the *Kiss1r*-LacZ knock-in mouse (Herbison et al.; Messager et al., 2005) or by dual *in situ* hybridisation (Han et al., 2005) (Figure 25). Then, the enhanced sensitivity to Kp around puberty may be due to post-transcriptional regulation of *Kiss1r* such as translation or insertion in the plasma membrane.

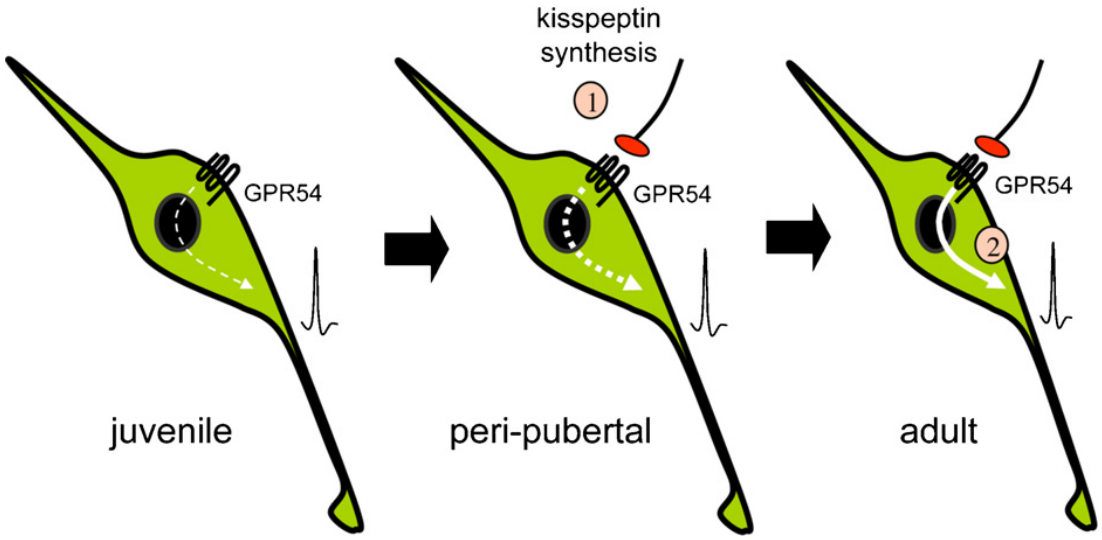

Figure 27: Two-step mechanism of Kp-controlled puberty onset

Schematic diagram illustrating the two-step mechanism of Kp activation of GnRH neurones leading to puberty onset. During the pubertal period, there is (1) the emergence of Kp signalling to GnRH neurones and (2) the maturation of the electrical response of the GnRH neurones to Kp. Clarkson et al., 2010

Taken together, these data indicates that puberty would be triggered in a two-step process. First, Kp input to GnRH neurones increases and second, GnRH neurones become more sensitive to Kp stimulation (Figure 27). However, it has to be remembered that puberty is a complex sequence of events affected by many factors. As a consequence, the *Kiss1/Kiss1r* system certainly does not act alone to initiate puberty but rather is a permissive element in a complex network of transmitters and hormones (Figure 28).

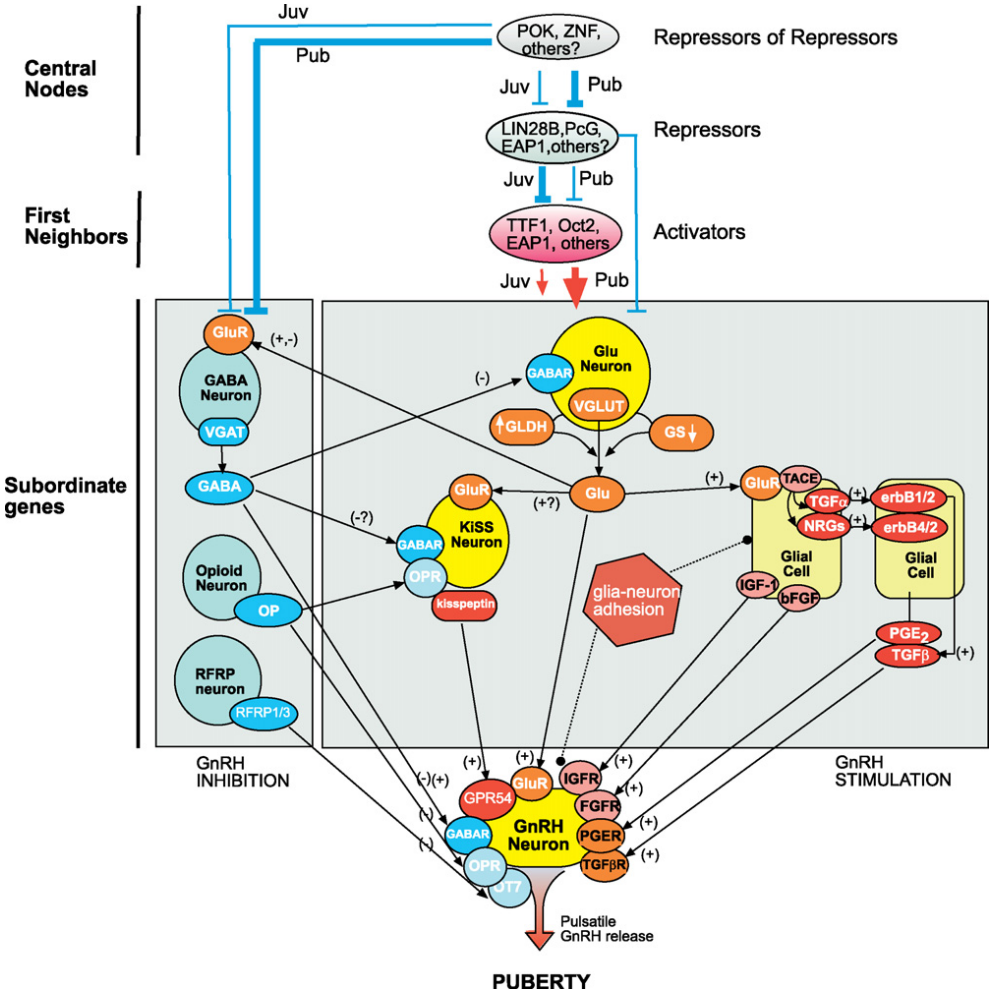

Figure 28: Genes network involved in the neuroendocrine control of puberty

A speculative view of the general structure of a gene network involved in the neuroendocrine control of mammalian puberty. It is envisioned that the highest level of control is exerted by repressors of repressors, and that the influence of these genes on the inhibitory neuronal component of puberty is low during juvenile development (Juv), increasing at puberty (Pub). A similar temporal change in repressive influence would occur in the control of a second, less "central" level of control formed by "repressors" that may act during juvenile development to prevent the premature activation of "puberty-inducing" genes. As a result of these changes in repressive tone, expression of subordinate genes sub-serving the inhibitory trans-synaptic control of GnRH secretion would decrease at puberty, whereas expression of key genes involved in the transsynaptic and glial stimulation of GnRH secretion would be enhanced. Ojeda et al., 2009.

c. Regulation of the reproductive axis activity

i. Kisspeptins directly act on GnRH neurones

Kp are powerful activators of the HPG axis activity since doses as low as 1 fmole markedly increase LH and testosterone levels when given directly in brain ventricle (Gottsch et al., 2004). Except GnRH itself, Kp are the only GnRH regulators to exert such a powerful action.

In rats and mice, around 80 % of GnRH neurones express cFos after central Kp administration and express *Kiss1r* mRNA (Herbison et al.; Irwig et al., 2004; Han et al., 2005; Messager et al., 2005). Similar results were found in the cichlid fish (Parhar et al., 2004). In rodents, over 90 % of GnRH neurones are also depolarised after Kp application (Han et al., 2005) (Figure 26) and Kp-immunoreactive fibres are found in close apposition to GnRH cell bodies (Kinoshita et al., 2005; Clarkson and Herbison, 2006). Altogether, these data suggest that Kp can directly act on GnRH cell bodies.

The presence of Kp-immunoreactive fibres in the median eminence in sheep (Franceschini et al., 2006), the expression of ß-Galactosidase in the median eminence of mice expressing this enzyme under the control of the *Kiss1r* promoter (d'Anglemont de Tassigny et al., 2008), and the ability of Kp to induce a GnRH release from mediobasal hypothalamus (d'Anglemont de Tassigny et al., 2008), indicate that Kp can also directly act on GnRH neurone nerve terminals. Furthermore, *Kiss1r* mRNA was also detected by RT-PCR in the ARC-median eminence complex in female rats (Kinoshita et al., 2005) and one can extrapolate that *Kiss1r* mRNA could be transported along GnRH axons and Kiss1r could be expressed in GnRH nerve terminals of the median eminence. The presence of Kiss1r in the median eminence remains however difficult to assess due to a lack of specific anti-serum for this membrane receptor.

ii. ARC Kiss1 neurones mediate sex steroid negative feedback on GnRH neurones

It is well established that sex steroids exert both positive and negative feedback effects on GnRH neurones. However, these neurones express neither oestrogen (ER) nor androgen receptors nor progesterone 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), and therefore, the feedbacks exerted by gonadal hormones must involve at least one intermediate.

In view of Kp powerful effects on the reproductive axis and more specifically on GnRH neurones, *Kiss1* neurones rapidly appeared as interesting candidates to mediate gonadal hormones feedbacks on GnRH neurones. First, it was shown by RT-PCR that *Kiss1* mRNA is inhibited by testosterone in hypothalamic tissue fragments from male rat and by estradiol in the female rat (Navarro et al., 2004a). In the same line, gonadectomy increases *Kiss1* mRNA levels, whereas replacement with gender-appropriate sex steroids reverses this effect (Navarro et al., 2004a). Second, *in situ* hybridisation studies revealed that sex steroids inhibit *Kiss1* expression in the ARC (Irwig et al., 2004; Smith et al., 2005a; Smith et al., 2005b; Revel et al., 2006b; Adachi et al., 2007; Rometo et al., 2007; Smith et al., 2007). In the male, testosterone effect involve both the AR and the ER (Smith et al., 2005b). Indeed, dihydrotestosterone, a non-aromatisable androgen that binds to AR, and estradiol, which binds to ERα, can both inhibit the castration-induced increase in *Kiss1* mRNA in the ARC. However, dihydrotestosterone only partially inhibits the castration-induced rise in *Kiss1* mRNA levels in the ARC, whereas estradiol fully reverses it. This suggest that the primary effect of testosterone on *Kiss1* expression is mediated by ERα, presumably reflecting the conversion of testosterone into estradiol and the subsequent binding of estradiol to ERα. Later, *Kiss1* neurones were found to co-express both ER and AR in the ARC (Smith et al., 2005b).

iii. AVPV Kiss1 neurones mediate sex steroid positive feedback on GnRH neurones

Gonadectomy reduces *Kiss1* expression in the AVPV while sex steroid treatment up-regulates it, demonstrating the positive feedback exerted by gonadal hormones on AVPV/PeN *Kiss1* neurones (Smith et al., 2005a; Smith et al., 2005b). The existence of a positive feedback of sex steroids on *Kiss1* neurones is of high importance in females. Interestingly *Kiss1* expression in the adult AVPV/PeN is sexually dimorphic (females displaying a higher number of *Kiss1* neurones than males) whereas that in the ARC is not (Clarkson and Herbison, 2006; Kauffman et al., 2007) (Figure 29). Across the oestrous cycle, oestrogens levels varies considerably. From low levels in the beginning, oestrogens concentrations progressively raises up to trigger the positive oestrogen feedback leading

to the pre-ovulatory LH surge on the evening of the pro-oestrous day. It is commonly admitted that GnRH neurones do not express classical sex steroid receptors and the positive feedback of oestrogens must then involve intermediate neurones. Since AVPV *Kiss1* neurones express oestrogen receptor α (ERα) (Smith et al., 2005a; Smith et al., 2005b; Smith et al., 2006b) they could mediate this positive feedback.

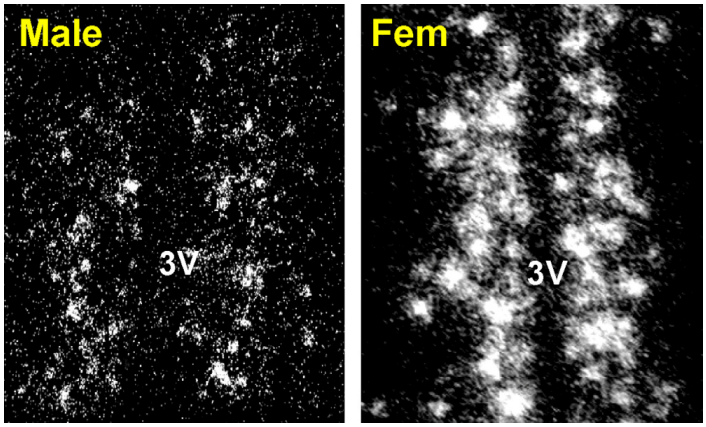

Figure 29: AVPV *Kiss1* expression is sexually dimorphic

Adult female ovariectomised and estradiol-treated mice have significantly more *Kiss1* neurones in the AVPV than adult castrated and testosterone-treated males. 3V: third ventricle. Kauffman et al., 2010

Indeed, central infusion of Kp antiserum or antagonist blocks the pre-ovulatory LH surge (Kinoshita et al., 2005; Pineda et al., 2010) and *Kiss1* expression in the AVPV is increased, along with cFos in *Kiss1* neurones concomitantly with the pre-ovulatory LH surge (Smith et al., 2006b; Robertson et al., 2009). The observation that *Kiss1* KO mice fail in generating an LH surge in response to estradiol treatment (Clarkson et al., 2008) emphasises the role of Kp signalling in the positive oestrogen feedback.

Finally, the pre-ovulatory LH surge is known to be gated by the circadian clock in the SCN (de la Iglesia and Schwartz, 2006) and it was recently shown that AVPV/PeN *Kiss1* neurones of ovariectomised estradiol-treated female mice display a significant circadian pattern of *Kiss1* expression and cFos expression in *Kiss1* neurones in direct synchrony with the circadian timing of LH secretion (Robertson et al., 2009). Interestingly, the circadian activation of AVPV/PeN *Kiss1* neurones is dependent on the presence of estradiol (Robertson et al., 2009), suggesting oestrogen constitute a permissive condition for a clock-controlled Kp production (Figure 30).

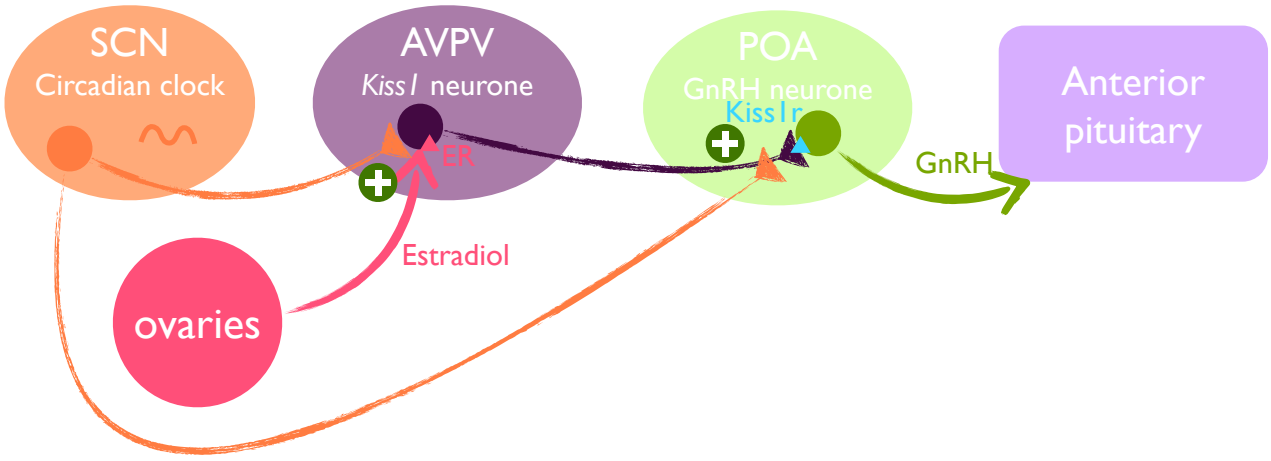

Figure 30: Model of the role of *Kiss1* in the generation of the GnRH/LH surge in female rodents

According to this model, AVPV *Kiss1* neurones receive daily late afternoon stimulatory circadian signal from the SCN. When estradiol levels reaches a minimal level, which occurs every four day, the joint effect of the circadian and estradiol stimulatory signals activates the oestrogen positive feedback leading to the pre-ovulatory GnRH/LH surge. From Smith et al., 2006

iv. ARC vs. AVPV Kiss1 neurones: different roles and targets for the regulation of the reproductive axis

It was suggested that the ability of estradiol to inhibit *Kiss1* expression in the ARC and stimulate *Kiss1* in the AVPV reflects different modes of intracellular estradiol signalling in each region, with a “classical” estradiol signalling in AVPV *Kiss1* neurones and a “non-classical” estradiol signalling in ARC *Kiss1* cells (Gottsch et al., 2009b). This suggests different roles of ARC and AVPV *Kiss1* neurones in the control of the reproductive axis activity. Indeed the two *Kiss1* populations appear to act on different parts of the GnRH neurone: AVPV *Kiss1* neurones would project to GnRH cell bodies, whereas ARC *Kiss1* neurones would rather project to GnRH nerve terminals in the median eminence (Figure 31) (Wintermantel et al., 2006; d'Anglemont de Tassigny et al., 2008; Ramaswamy et al., 2008). It then appears that AVPV *Kiss1* neurones are involved in the surge mode of the reproductive axis stimulation whereas ARC *Kiss1* neurones would rather drive pulsatile activation of GnRH neurones (see Chapter 1, section III 2 d v).

However, it has to be remembered this model only applies to rodents. In the sheep, the POA *Kiss1* neurones does not appear to be critical for the oestrogen positive feedback. Rather, the ovine caudal ARC has been implicated in mediating the pre-ovulatory LH surge (Smith et al., 2009). Interestingly, ARC *Kiss1* population seems to be sexually dimorphic because male and female sheep contain different numbers of *Kiss1*

neurones in the ARC (ewes having more than twice as many neurones than rams), raising the possibility of an important sex difference in the ovine ARC *Kiss1* system (Cheng et al., 2010).

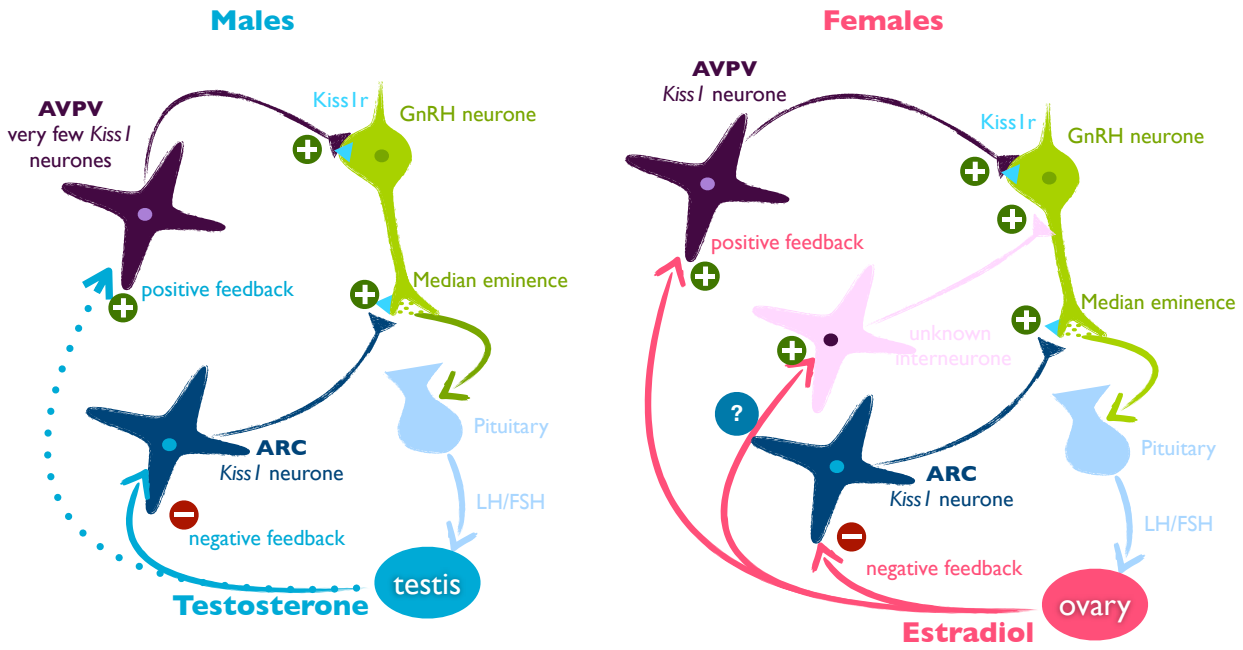

Figure 32: Model of *Kiss1/Kiss1r* signalling in male and female rodents' brain

In both (a) males and (b) females, *Kiss1* neurones in the ARC probably drive GnRH pulsatile release and are negatively regulated by gonadal sex steroids, thereby providing the cellular basis for the negative feedback effects of testosterone and oestrogen on GnRH secretion (and hence, LH and FSH secretion from the pituitary). In the AVPV, males possess very few *Kiss1* neurones, even when levels of sex steroids are high, whereas females possess numerous *Kiss1* cells in this region. In contrast to the ARC, *Kiss1* expression in the AVPV is stimulated by the presence of sex steroids, providing the cellular basis for the positive feedback induction by oestrogen of the pre-ovulatory GnRH surge in females. It should be noted that this rodent model of *Kiss1/Kiss1r* signalling might not apply to sheep and primates in which positive feedback signalling is probably derived from Kp neurones in the MBH rather than the more anterior AVPV/POA region. Kauffman et al., 2007

To conclude, it is possible to propose a model of Kiss1/Kiss1r signalling in the brain of male and female rodents (Figure 32). In both males and females, ARC *Kiss1* neurones would drive GnRH pulsatile release (see Chapter 1, section III 2 d v) and are negatively regulated by gonadal sex steroids, thereby providing the cellular basis for the negative feedback effects of testosterone and oestrogen on GnRH secretion (and hence, LH and FSH secretion from the pituitary) (see Chapter 1, section III 2 d ii). In the AVPV, males possess very few *Kiss1* neurones, even when levels of sex steroids are high, whereas females possess numerous *Kiss1* cells in this region. In contrast to the ARC,

Kiss1 expression in the AVPV is stimulated by sex steroids, providing the cellular basis for the oestrogen-induced positive feedback leading to the pre-ovulatory GnRH surge in females.

v. Kisspeptins drive GnRH pulsatility

GnRH pulsatile release is mandatory for the proper functioning of the reproductive axis but the precise mechanisms driving GnRH pulsatility remains unknown. However, it was shown that hypothalamic deafferentation isolating the anterior part of the ARC from the MBH abolishes LH pulses while foetal MBH transplants with no GnRH immunoreactivity restores pulsatile LH release in female rats (Ohkura et al., 1992). Thus it was speculated that a mechanism generating GnRH pulses may consist of non-GnRH neurones residing in the MBH. Since ERα KO mice are infertile (Lubahn et al., 1993) with elevated plasma LH levels (Rissman et al., 1997), whereas ERβ KO mice are fertile with a pulsatile LH/FSH release (Hewitt and Korach, 2003), the GnRH pulse generator appears to consist of non-GnRH neurones that express ERα.

The ARC *Kiss1* neurones are interesting candidates for being a GnRH pulse generator because they are negatively regulated by oestrogen (see Chapter 1, section III 2 d ii), they express ERα (Smith et al., 2005a; Smith et al., 2005b; Franceschini et al., 2006; Pompolo et al., 2006) and the dense distribution of Kp fibres in the ARC (Franceschini et al., 2006; Clarkson et al., 2009b) could synchronise *Kiss1* neurones activity which is necessary for a pulsatile Kp release from the median eminence.

Since intermittent Kp administration is more effective at maintaining GnRH secretion in juvenile monkeys than is continuous Kp infusion, which results in Kiss1R desensitisation, endogenous Kp release may be pulsatile (Plant et al., 2006; Seminara et al., 2006). A recent study in rodents revealed that under basal conditions, GnRH neurones exhibit spontaneous baseline oscillations in intracellular Ca^{2+} concentration. When exposed to Kp, GnRH neurones exhibit an increase in the frequency of Ca^{2+} spiking indicating that a plasma membrane Ca^{2+} oscillator is spontaneously operative in the majority of GnRH neurones and is facilitated by Kp (Constantin et al., 2009). Kp pulsatile secretion was assessed in the monkey and Kp pulses were found to be in synchrony with GnRH pulses (Keen et al., 2008). Finally rhythmic bursts of electrical activity coinciding with LH pulses were observed in the ARC (Ohkura et al., 2009). These observations suggest the ARC Kp neurones may be the intrinsic source of the GnRH pulse generator.

ARC *Kiss1* neurones co-express neurokinin B (NKB) and dynorphin A (Dyn) (Goodman et al., 2007; Rometo et al., 2007; Gottsch et al., 2009b; Navarro et al., 2009; Wakabayashi et al., 2010). These neuropeptides have been shown to differentially regulate GnRH secretion. While NKB promotes bursts of electrical activity of ARC neurones associated to GnRH/LH pulses (Ohkura et al., 2009), Dyn inhibits spontaneous bursts of electrical activity in the ARC (Wakabayashi et al., 2010). *In situ* hybridisation studies revealed that the expression of the genes coding for Dyn, NKB, Dyn receptor KOR (kappa opioid receptor) and NKB receptor (NK3) are inhibited by estradiol in the ARC (Navarro et al., 2009). Moreover, studies in Dyn and KOR KO mice revealed that long-term disruption of Dyn/KOR signalling prevents the ovariectomy-induced rise in LH levels (Navarro et al., 2009). Similarly, exogenous administration of a Dyn agonist inhibits Kp/NKB/Dyn neurones, thus reducing GnRH and LH secretion (Navarro et al., 2009). Interestingly, the Kp/NKB/Dyn neurones form a bilateral interconnected network of sex-steroid responsive neurones which project to GnRH terminals in the median eminence (Wakabayashi et al., 2010).

Taken together, these data allow to propose a model involving ARC Kp/NKB/Dyn neurones in the synchronisation of pulsatile release of GnRH (Figure 33). According to this model, Kp/NKB/Dyn neurones in the ARC send axons to GnRH terminals in the median eminence, whereas their collaterals and dendrites form a neural circuit connecting the ensemble to synchronise firing activity in the whole population of Kp/NKB/Dyn neurones. When sex steroids levels are low, the spontaneous activity of *Kiss1* neurones is amplified by positive auto-synaptic feedback via NKB/NK3 signalling, which serves as accelerator. This would be followed by a delayed inhibition of Kp/NKB/Dyn neurones, mediated by Dyn acting through KOR. Dyn/KOR signalling thus serves as brake. Dyn probably acts on the cell bodies, as well as on the presynaptic structure of axonal collaterals, which extinguishes the NKB-induced bursting. This would cause Dyn release to cease and lift its inhibitory effect, re-activating the *Kiss1*/NKB/Dyn neurones and initiating another cycle of activity, followed by inhibition. Each time *Kiss1*/NKB/Dyn neurones would undergo a burst of activity, a pulse of Kp, Dyn, and NKB would be released in the median eminence, where Kp and NKB would act directly on GnRH fibres. To limit the release of GnRH to a discrete pulse, the Kp-induced activity must be extinguished either via an unidentified inhibitory neurotransmitter, or via NKB/NK3 signalling which would activate a delayed inhibitory pathway, as indicated in figure 33. Dyn does not appear to act directly on GnRH neurones because they do not seem to express KOR. According to this model, any

dysfunction of the NKB/Dyn system would compromise pulsatile Kp and GnRH/LH release.

Taken together, these data point to Kp as crucial regulators of GnRH release and suggest that these peptides may act directly on GnRH cell bodies or on GnRH nerve terminals of the median eminence. As a consequence, Kp control GnRH production as well as its release and these peptides are involved in long-term GnRH regulation (i.e seasonal reproduction) as well as short-term control of GnRH release (i.e GnRH pulsatile release).

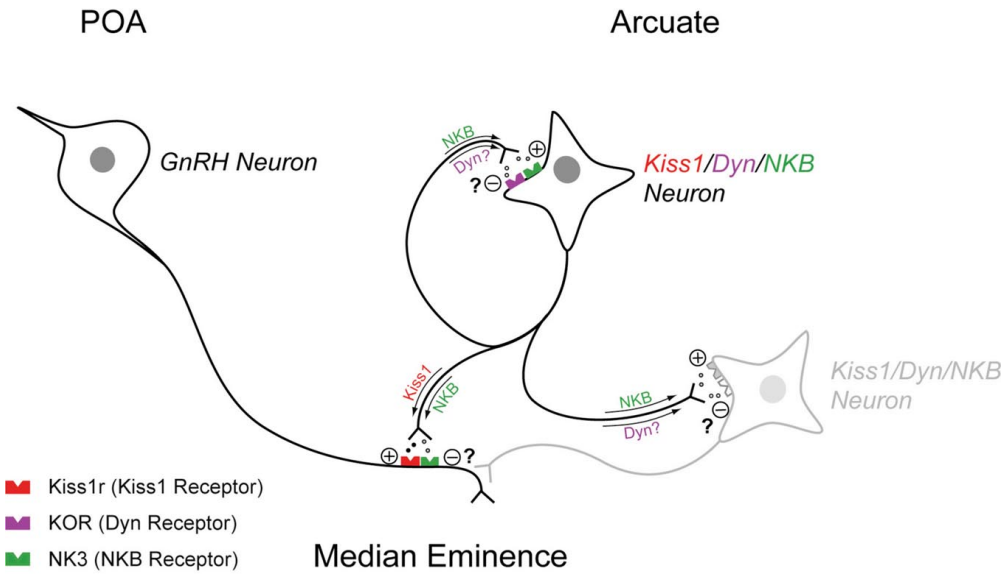

Figure 33: Role of ARC Kp/NKB/Dyn neurones in the pulsatile GnRH release

According to this model, Kp/NKB/Dyn neurones in the ARC form a neural circuit by their collaterals and dendrites. Within the neural circuit, NKB/NK3 signalling plays the role of accelerator, whereas Dyn/KOR signalling serves as a brake on activation of *Kiss1*/NKB/Dyn neurones. Through the reciprocal actions of NKB/NK3 and Dyn/KOR signalling, rhythmic oscillation of neural activity is generated in *Kiss1*/NKB/Dyn neurones, which in turn induces pulsatile Kp release in the median eminence and hence pulsatile GnRH release into the portal circulation. Thus, ARC *Kiss1*/NKB/Dyn neurones would act as the GnRH pulse generator through the coordinated interaction between three peptides. Navarro et al., 2009

vi. Peripheral kisspeptins sites of action on the reproductive axis

The observation that Kp activates the HPG axis when injected centrally or peripherally (Gottsch et al., 2004; Matsui et al., 2004; Thompson et al., 2004; Navarro et al., 2005b; Mikkelsen et al., 2009) raises the issue of peripheral Kp sites of action. To date it is commonly admitted that peripheral Kp act on GnRH nerve terminals of the median eminence since 1) this structure is accessible to circulating hormones / peptides and 2)

Kp-immunoreactive fibres are present in the median eminence in sheep (Franceschini et al., 2006) and Kiss1r seem to be expressed in this structure in the mouse (d'Anglemont de Tassigny et al., 2008).

However a direct effect of Kp on the pituitary cannot be excluded. This hypothesis has already been subject of investigation but with conflicting results. Initially, RT-CPR studies revealed that Kiss1R/r is expressed in adult human (Kotani et al., 2001; Muir et al., 2001) and rat (Kinoshita et al., 2005; Gutierrez-Pascual et al., 2007) pituitary gland. In cultured bovine and porcine pituitary glands, exogenous Kp10 triggered a LH release but only at the highest doses (> 1 μM). In cultured pituitary cells from male and female peri-pubertal rats application of 10 nM of Kp10 also elicited a LH release (Gutierrez-Pascual et al., 2007). Eventually, in static incubation of pituitaries from pubertal male rats, 10 nM of Kp10 increased LH release but did not affect FSH levels (Navarro et al., 2005b). However, two different groups investigated the effect of exogenous Kp10 on incubated anterior pituitaries (Thompson et al., 2004) or on primary culture of anterior pituitaries (Matsui et al., 2004) from adult male and female rats and both failed to show an increase in LH or FSH even at doses as high as 1 μM. Since *Kiss1r* expression is increased around puberty in rats hypothalamus (Navarro et al., 2004a), one can extrapolate developmental differences in *Kiss1r* expression in the anterior pituitary and a higher sensitivity of the gland to Kp around puberty, which could explain the aforementioned discrepancies.

Finally, both *Kiss1* and *Kiss1r* are expressed in the gonads and a direct effect of Kp on testes or ovaries is still possible. For instance, in the female rat ovaries, Kp could act as a local regulator of ovulation because its expression is increased in that organ in the afternoon of the pro-oestrous day (Castellano et al., 2006c). Similarly, chronic Kp54 peripheral treatment induces a testicular long-lasting degeneration in the rat as well as a significant decrease in the circulating levels of the testes-derived hormone inhibin B (Thompson et al., 2006). In this study, the unaffected LH and FSH levels in Kp54 treated rats tends to exclude a decreased GnRH release which could also explain the altered testicular histology. Thus, direct Kp effect on the gonads appears plausible in both male and female rats.

e. Metabolism and reproduction

In most species, a negative energy balance inhibits the reproductive axis by either a delay in pubertal onset or an interruption of normal reproductive function in adults

(Castellano et al., 2009). Information about the metabolic status is communicated to the reproductive axis via circulating factors produced by peripheral organs such as leptin or insulin. Because of their position in the reproductive axis, it has been hypothesised that *Kiss1* neurones could be sensitive to the metabolic status and transmit this information to downstream components of the HPG axis. If so, it is expected that *Kiss1* is regulated by fasting or metabolic hormones (Castellano et al., 2009).

It was demonstrated by RT-PCR that in pubertal male and female rats, a 72-hour fasting significantly decreases *Kiss1* mRNA and LH levels (Castellano et al., 2005). Similarly, in adult female rats, a 18-hour food-deprivation also decreases *Kiss1* mRNA levels (Brown et al., 2008) and in adult male mice, a 12-hour fasting already reduces *Kiss1* expression (Luque et al., 2007). By contrast, a 72-hour fasting increases *Kiss1r* expression in the pubertal rat (Castellano et al., 2005) which is consistent with the observation that Kp administration exerts a more potent effect in fasting than in *ad libitum*-fed animals (Castellano et al., 2005). However, it was also shown that 12 to 48 hours of fasting decreases *Kiss1r* expression in the adult mouse (Luque et al., 2007). It is possible that a reduction in *Kiss1r* expression constitute an immediate effect of fasting, which would be followed by a secondary increase in *Kiss1r* mRNA levels. Furthermore, streptozotocin (STZ)-injected rats, a model of severe diabetes, develop a state of hypogonadism associated with a significant decrease in *Kiss1* mRNA levels (Castellano et al., 2006b). Conversely, exogenous Kp treatment in these rats could reverse the hypogonadal state as attested by increased testicular and prostate weights and normal LH and testosterone levels (Castellano et al., 2006b). Finally, during lactation, a state of negative energy balance, *Kiss1* mRNA and Kp protein levels are decreased in the ARC (Yamada et al., 2007). The decreased Kp signalling in lactating rats prevents the proper functioning of the HPG axis and thus prevents a new pregnancy until the litter is weaned. Interestingly, Kp stimulates LH release in lactating rats. However Kp effect is less potent in lactating rats when compared to non-lactating controls (Roa et al., 2006; Yamada et al., 2007), *Kiss1r* mRNA levels in the AVPV of lactating rats being reduced (Yamada et al., 2007). Taken together, these observations indicate that *Kiss1* neurones are highly sensitive to the metabolic status and relay the subsequent stimulation or inhibition to downstream structures constituting the HPG axis.

This hypothesis is supported by the finding that *Kiss1* expression is regulated by metabolic hormones such as leptin. Leptin is secreted by the white adipose tissue in proportion to the amount of body energy stores and functions as satiety factor

(Casanueva and Dieguez, 1999). Indeed, leptin-deficient (ob/ob) mice show decreased *Kiss1* mRNA levels in the ARC which could be partially reversed by leptin treatment (Smith et al., 2006a). It was also shown that leptin intracerebroventricular (ICV) infusion to STZ-injected rats normalises *Kiss1* expression and increases gonadal hormones levels (Castellano et al., 2006b), the stimulatory effect of leptin on *Kiss1* neurones being confirmed by in vitro experiments since leptin can increase *Kiss1* mRNA expression in the murine hypothalamic cell line N6, and human GnRH-secreting neuroblasts (Luque et al., 2007; Morelli et al., 2008). Leptin probably directly acts on ARC *Kiss1* neurones since 40% of them express the leptin receptor (Ob-Rb) (Smith et al., 2006a; Luque et al., 2007). The exact mechanism of leptin action on *Kiss1* neurones remains to be deciphered. However, it was demonstrated that intracerebral administration of leptin to adult female rats induces phosphorylation of transcription factor STAT3 in the AVPV (Quennell et al., 2009). Similarly, Crtc1 is the Creb1-regulated transcription co-activator-1 and it probably also mediates leptin effects on *Kiss1* neurones. Crtc1 KO mice are known to be obese and hyperphagic as well as infertile. Interestingly, leptin dephosphorylates Crtc1, which in turn stimulates the recruitment of Crct1 and leads to *Kiss1* gene transcription (Altarejos et al., 2008).

Beside leptin, other metabolic factors affect the activity of the reproductive axis. For instance, the neuropeptide Y (NPY) is an orexigenic neuropeptide involved in the regulation of the reproductive axis activity (Bauer-Dantoin et al., 1992b; Bauer-Dantoin et al., 1992a; Catzeflis et al., 1993). Interestingly, *Kiss1* expression levels are decreased in NPY KO mice (Luque et al., 2007) and stimulation of the hypothalamic N6 cells with NPY increases *Kiss1* mRNA levels (Luque et al., 2007). Ghrelin is a circulating orexigenic factor that signals negative energy balance, which has emerged as putative gatekeeper of the timing of puberty (Tena-Sempere, 2008a, b). Ghrelin mediates an inhibitory signal to the HPG axis (Tena-Sempere, 2008a, b) and inhibits hypothalamic GnRH secretion (Fernandez-Fernandez et al., 2005). This effect could involve *Kiss1* neurones because it was shown that ghrelin can suppress *Kiss1* gene expression (Forbes et al., 2009). The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that operates as sensor of the cellular energy status coupled to cell growth and proliferation (Schmelzle and Hall, 2000; Martin and Hall, 2005). mTOR has been involved in the control of puberty onset via modulation of *Kiss1* expression (Roa et al., 2009). Indeed blockade of mTOR disrupts the normal timing of puberty in female rats and inhibition of mTOR signalling prevents the permissive effects of leptin on puberty onset, and suppresses *Kiss1* mRNA

levels mainly in the ARC (Roa et al., 2009). Interestingly mTOR has been proposed as transducer for leptin effects on energy homeostasis and food intake in the ARC (Cota et al., 2006). Hence, leptin-mTOR-Kp pathway appears to play an important role in the metabolic control of puberty (Figure 34).

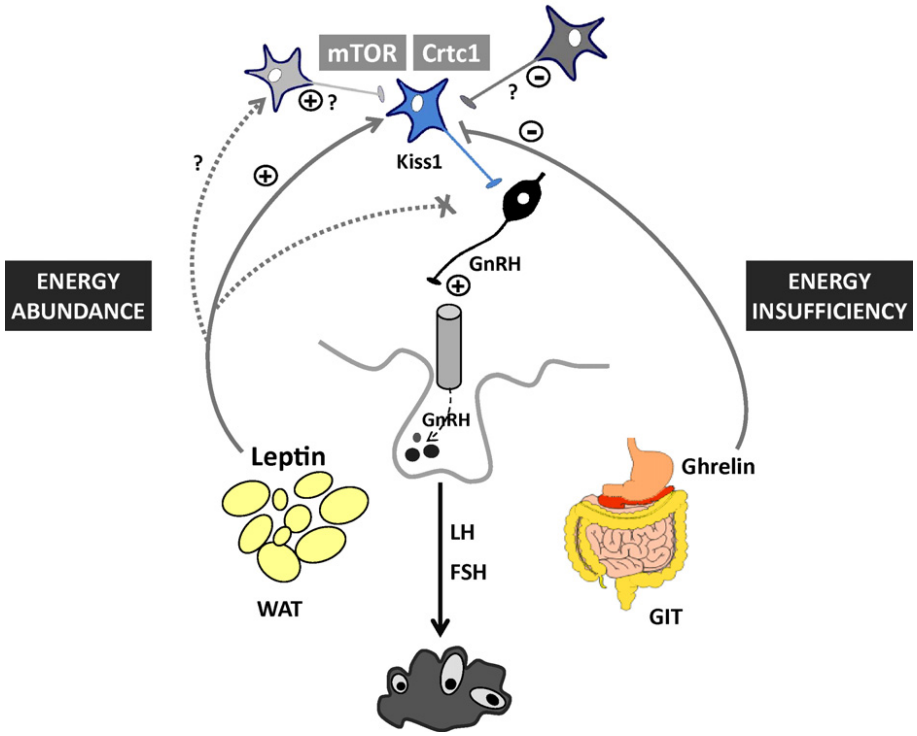

Figure 34: Model for the metabolic control of the reproductive axis

Kiss1 neurones have been proposed as targets for the regulatory actions of leptin (as prominent signal for energy abundance; permissive/stimulatory factor) and ghrelin (as putative signal of energy insufficiency; inhibitory factor) on the reproductive axis. The role of *Kiss1* neurones as mediators for leptin effects is reinforced by the fact that leptin receptors are not expressed in GnRH neurones, which excludes the possibility of direct effects of leptin at this site. Notwithstanding, it remains plausible that part of leptin actions on Kiss1 neurones may be indirectly conducted via intermediary neuronal pathways, whose nature and physiological relevance is yet to be elucidated. In addition, the potential involvement of mTOR and Crtc1 in transmitting metabolic (leptin) information onto *Kiss1* neurones is also indicated. For sake of simplicity, discrimination between different populations of *Kiss1* neurones (i.e., ARC vs. AVPV) is not provided in the scheme; yet, it remains possible that not all *Kiss1* neurones are similarly regulated by different metabolic cues. Roa et al., 2009

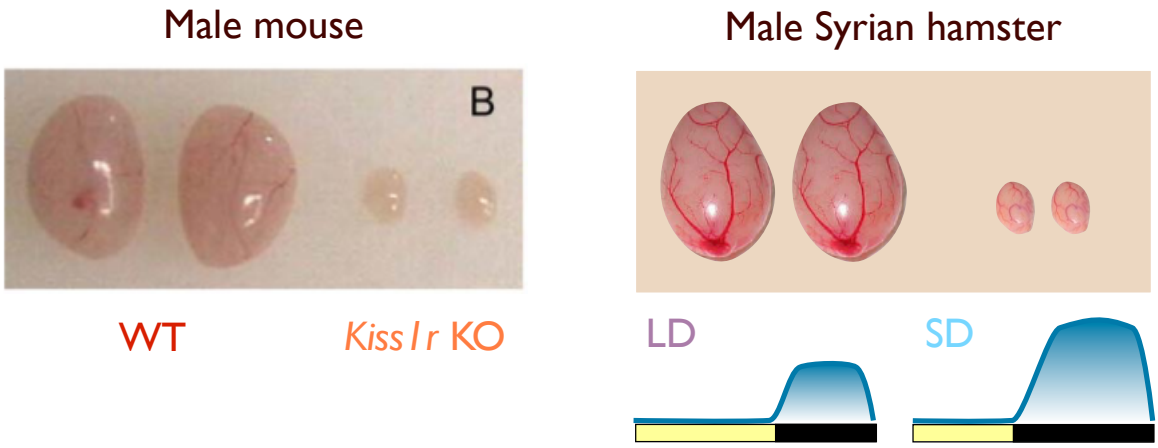

Figure 35: Kiss1r KO mice and photo-inhibited hamsters

Loss-of-function mutations in the Kiss1r gene in mice induce a hypogonadic phenotype characterised by undeveloped gonads and very low gonadal hormones levels, a phenotype extremely similar to SD-acclimated hamsters. This observation led us to investigate the role of Kp in seasonal reproduction.

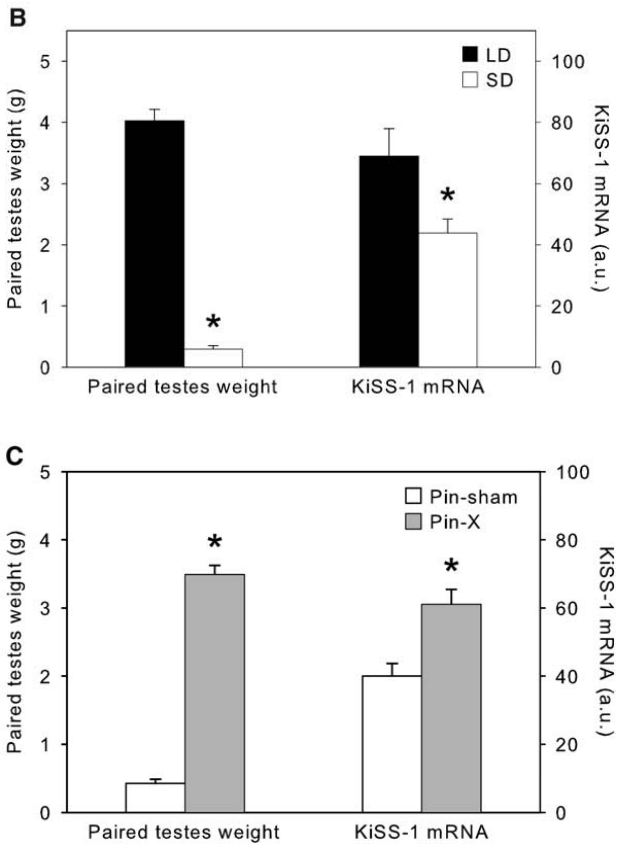

Figure 36: Effect of photoperiod and melatonin on ARC Kiss1 expression in the Syrian hamster

The effects of photoperiod and melatonin on ARC *Kiss1* neurones were investigated by radioactive *in situ* hybridisation. *Kiss1* mRNA levels are reduced after 8 weeks of short days (SD) exposure as compared to long day (LD) controls (upper panel). Interestingly, the SD-induced reduction Kiss1 mRNA levels appears mediated by melatonin since pinealectomy (Pin-X) prevents it (lower panel). Revel et al., 2006.

f. Seasonal reproduction

The observation that Kiss1r loss-of-function mutations in mice induce a hypogonadic phenotype characterised by undeveloped gonads and very low gonadal hormones levels (Funes et al., 2003; Seminara et al., 2003), a phenotype extremely similar to SD-acclimated hamsters (Figure 35) led us to investigate the role of Kp in seasonal reproduction. First, ARC *Kiss1* expression was analysed in both LD and SD-adapted male Syrian hamsters. *Kiss1* mRNA levels were found to be reduced after 8 weeks of SD exposure. Interestingly, this SD-induced reduction appears mediated by melatonin since pinealectomy prevents it (Revel et al., 2006b) (Figure 36). One could hypothesised that melatonin-induced reduction in circulating sex steroid could mediate melatonin effect on ARC *Kiss1* neurones. However, in the Syrian hamster, as observed in all rodent species, castration up-regulates *Kiss1* expression in the ARC (Revel et al., 2006b) (Figure 37). Consequently, the melatonin or SD-induced decrease in ARC *Kiss1* expression seems due to a steroid-independent action of melatonin. Second, exogenous chronic ICV administration of Kp for 4 weeks to SD-acclimated sexually-inactive hamsters restored gonadal activity despite the inhibitory photoperiod (Revel et al., 2006b) (Figure 38). Taken together, these data emphasise the role of Kp in the seasonal control of reproduction (an extensive review and discussion of the comparative role of Kp in other seasonal species can be found in the Chapter 6, section I 1, 2 and 3). However, at this point, little was known about the cartography of the kisspeptinergic system in rodents, the effect of photoperiod on AVPV *Kiss1* neurones and peripheral Kp effects in the Syrian hamster. During my PhD work, all three points were assessed and the results are described in the next section.

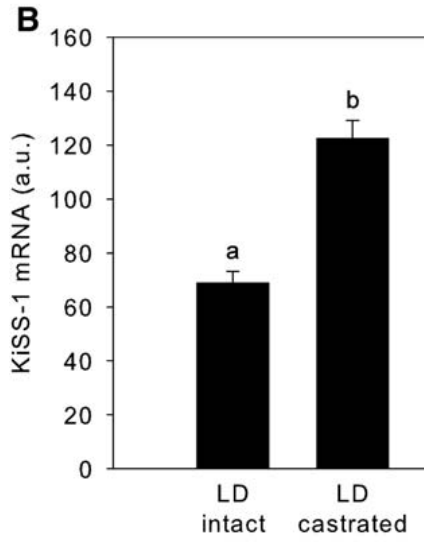

Figure 37: Effect of castration on ARC *Kiss1* expression in the Syrian hamster

The effect of testosterone on ARC *Kiss1* neurones was investigated by *in situ* hybridisation. Since castration up-regulates *Kiss1* mRNA level, it demonstrates testosterone negatively regulates *Kiss1* expression in the ARC. Revel et al., 2006

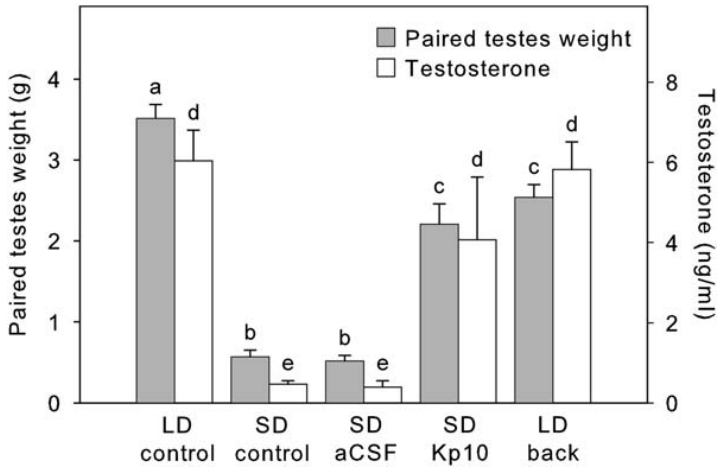

Figure 38: Chronic Kp treatment restores gonadal activity

Exogenous chronic intracerebroventricular delivery of Kp to short-day (SD)-acclimated sexually inactive hamsters (SD Kp10) restores high paired-testes weight when compared to SD or SD-vehicle (SD aCSF) groups. Kp treated-hamsters have testosterone levels and testes weight similar to those of SD-acclimated hamsters that were transferred back to long days (LD; LD back) during the treatment duration. Revel et al., 2006

Chapter 2 - Objectives

Syrian hamsters synchronise their reproductive activity with season via the photoperiodic time measurement mechanisms described in the introduction. These mechanisms involve changes in the secretion of the pineal hormone melatonin. However, little is known about the cellular site(s) of action of melatonin on the reproductive axis. My thesis work consisted in studying the implication of *Kiss1*-encoded peptides in mediating melatonin effects on the reproductive axis in this species.

First, the neuroanatomy of the kisspeptinergic system had to be characterised in that species. Both mRNA and protein distribution was studied and the exact knowledge of Kp neurones and fibres distribution enabled us to investigate the effect of photoperiodic variations of *Kiss1* and Kp expression.

We next analysed the mechanisms involved in the photoperiodic variations in *Kiss1* expression. Indeed, changes in day-length induce variations of circulating levels of both sex steroids and melatonin. In rats and mice, it is well established that gonadal hormones strongly regulate *Kiss1* expression. As a consequence, the effects of sex steroids on *Kiss1* neurones needed to be determined in the Syrian hamster. Then, the effects of melatonin on *Kiss1* neurones was assessed. Because *Kiss1* expression is sexually dimorphic in the AVPV of rodents, we conducted these studies in both male and female hamsters.

We demonstrated that melatonin regulates *Kiss1* expression in the Syrian hamster, suggesting that Kp convey the inhibitory melatoninergic signal to the gonadotropic axis. Florent Revel, Jens Mikkelsen and Valérie Simonneaux previously demonstrated that chronic Kp ICV administration restores testicular activity in SD photo-inhibited male hamsters demonstrating that seasonal variations in Kp production and release mediate melatonin action on the reproductive axis. However, little was known about Kp sites of action in the Syrian hamster. Particularly, Kp central and peripheral exogenous administration strongly induces LH/FSH and testosterone/oestrogen release in rats and mice. This raises the issue of peripheral Kp sites of action. Hence, we checked wether chronic Kp peripheral treatment could also restore gonadal activity in SD male hamsters and analysed the brain region expressing cFos after acute Kp peripheral injection.

Chapter 3 - Mapping of the kisspeptinergic system in the Syrian hamster

I. Introduction

1. Introduction to kisspeptins

Kiss1 codes for a family of peptides of variable length (from 10 to 54 amino acids) called Kp. Disruption of either the Kiss1 gene or the Kiss1r gene generates a hypogonadic phenotype in mice (Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007) and humans (de Roux et al., 2003) while gain of function mutations causes precocious puberty (Teles et al., 2008). Kp also strongly activate the HPG axis in adults since a dose as low as 1 fmol triggers a LH release in rodents (Gottsch et al., 2004). Furthermore, Kp integrate the effect of many factors affecting reproduction such as sex steroid levels (Smith, 2008), nutritional status (Castellano et al., 2009), stress (Kinsey-Jones et al., 2009) or photoperiod (Revel et al., 2006b; Revel et al., 2007; Simonneaux et al., 2009; Ansel et al., 2010). As a consequence, Kp play a pivotal role in the maturation as well as in the regulation of the HPG axis.

2. Introduction to seasonal reproduction

Syrian hamsters synchronise their reproductive activity to seasons via predictable changes in photoperiod. Annual variations of the photoperiod are translated into the release of the pineal hormone melatonin, the nocturnal production of which relies on night-length. When exposed to SD for eight to ten weeks, the male Syrian hamster experiences a complete gonadal regression (Goldman, 2001) attested by low paired-testes weight and low testosterone levels. Inversely, when transferred back to LD condition reproductive activity is restored within 6 weeks (Goldman, 2001). We have reported that *Kiss1* expression varies according to photoperiod with higher level of expression in LD, when hamsters are sexually active (Revel et al., 2006b; Revel et al., 2007; Simonneaux et al., 2009; Ansel et al., 2010). Moreover, central (ICV) exogenous administration of Kp to sexually inactive SD-adapted male Syrian hamsters reverses the inhibitory effect of SD exposure by re-activating the HPG axis (Revel et al., 2006b), indicating that photoperiodic variations of *Kiss1* expression drive seasonal changes in the HPG axis activity.

3. Aims of the study

To identify Kp central sites of action for the seasonal control of reproduction, a complete mapping of the kisspeptinergic system in the hamster is required. Our study is thus aimed at establishing the complete neuroanatomy of the kisspeptinergic system using two different highly specific antibodies targeted against the central part of mouse Kp52 or against the C-terminal extremity of Kp10. The use of the two different antiserum allow to discriminate between the different Kp forms putatively expressed in the central nervous system. This approach was completed by the analysis of cFos expression in the brain following an acute ICV injection of Kp. Since Syrian hamsters display strong photoperiodic differences in *Kiss1* expression, all experiments were conducted in LD and SD-adapted animals.

II. Material and methods

1. Animals and tissue collection

Male Syrian hamsters weighing 100 - 140 g were raised in our animal facilities with food (Safe 105, rodent pellets, Safe, Augy, France) and water *ad libitum*, under controlled temperature (22 ± 1°C) and long photoperiod (LD; 14 hours light (200 lux) / 10 hours dark (2 lux, dim red light) cycle). When required, hamsters were exposed to a short photoperiod (SD; 10 hours light / 14 hours dark cycle) for ten weeks.

All experiments were conducted in accordance with the French National Law (licence n° 67-32) implementing the European Communities Council Directives of November, 24 1986 (86/609/EEC).

2. Experimental designs

To analyse the distribution of Kp-immunoreactive (Kp-ir) neurones and fibres, groups (n = 3) of male Syrian hamsters were either kept in LD condition or exposed to SD for ten weeks before sacrifice. Hamsters were killed as described below and testes weight was measured to check for photoperiod integration.

To identify Kp central sites of action, male Syrian hamsters were either kept in LD (n = 7) or exposed to SD for ten weeks (n = 7) and implanted with a cannula (outer diameter: 0.7 mm; inner diameter: 0.4 mm; length: 1.7 mm) in the lateral ventricle under a Zoletil® (100 mg/kg, Virbac, Carros, France) and Rompun® (6 mg/kg, Bayer Pharma, Puteaux, France) anaesthesia. The cannula was implanted at the following coordinates: 0.8 mm anterior to the bregma, 2 mm lateral to bregma and 3 mm under pial surface. After a one-week recovery, hamsters were anaesthetised with 3 % isoflurane (AErrane, Baxter, France) and N2O, and 2 µl of Ringer's solution or a 500 µM Kp54 solution were injected intracerebroventrically at the rate of 1 µl/min using a microinjection pump (CMA 400, CMA, Acton, MA, USA). After the injection, the syringe was left in place for 3 min before removal. Hamster were killed 2 hours after the ICV injection as described below.

3. Immunohistochemistry

Hamsters were killed by pentobarbital overdose and perfused transcardially with 4 % formaldehyde in 0.1 M phosphate-buffer pH 7.4. The brains were post-fixed for 24 hours at 4°C in 4 % formaldehyde in 0.1 M phosphate buffer pH 7.4, rinsed overnight in phosphate buffer, dehydrated and embedded in polyethylene glycol as described by Klosen et al., 1993 (Klosen et al., 1993). Microtome sections (12 µm) were cut and processed (one every 120 µm) for immunohistochemistry. When, required (with #566 antiserum) antigenic reactivation was first performed in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) for 2 hours at 95 °C. After cooling for 1 hour at room temperature, non specific binding sites were saturated for 1-hour with 3 % dry skimmed milk. Primary anti cFos (1:2000, Santa Cruz biotechnologies, Santa Cruz, CA, USA) or anti-Kp #566 (1:50 000, gift of Dr Caraty, Nouzilly, France; (Franceschini et al., 2006)) or JLV-1 (1:600; (Mikkelsen and Simonneaux, 2009)) rabbit polyclonal antisera was applied overnight. The sections were then incubated for 1 hour with a secondary biotinylated donkey anti-rabbit antibody (1:2000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) followed by a 1 hour-incubation with streptavidin-peroxydase (1:2000, Roche, Basel, Switzerland). Peroxydase activity was detected by using 3,3-diaminobenzidine (Sigma, Saint Louis, MO, USA) as a chromogen. The slides were then dehydrated and mounted with Eukitt® and the number of Kp-ir neurones was hand-counted and the density of Kp-ir fibres was evaluated on a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) by a person naive to the experiment. For cFos immunohistochemistry, photomicrographs

were taken with a 5x objective and quantified with ImageJ software (Rasband, W.S., US National Institutes of Health, Bethesda MD, USA) as described by Salingre et al., 2009 (Salingre et al., 2009). Briefly, pictures were thresholded according to the intensity of the background and the same threshold was applied for each slide. A particle analysis was performed and particles whose diameter was inferior to 30 pixels were excluded. The sum of the integrated density of each immunoreactive particle (i.e. cFos positive cell) was calculated and divided by the total area quantified to obtain a mean integrated density per surface unit.

4. Statistical analysis

Results are shown as mean ± SEM. Data were analysed by ANOVA followed by Tukey's multi-comparisons or Student's t test.

III. Results

1. Distribution of Kp-ir neurones and fibres

In LD-acclimated sexually active male hamsters, Kp-ir neurones were identified in the AVPV, the PeN and the ARC of the hypothalamus (Figure 39). Strikingly, more Kp neurones were revealed with the JLV-1 than with the #566 antiserum in the ARC. Extremely few (<1 neurone/section) immunoreactive neurones were present in the AVPV/PeN with the #566 antibody while a few more neurones could be counted in the AVPV/PeN with the JLV-1 antiserum (1-3 neurones/section). Very few neurones were observed in the subfornical organ with JLV-1 antiserum (1-3 neurones/section).

A more dense network of Kp-ir fibre was observed with the #566 antiserum than with the JLV-1. With the former, Kp-ir fibres were observed in the accumbens nucleus, the diagonal band of Broca (DBB), the BNST, the AVPV, the medial preoptic nucleus (MPN), the MPO, the paraventricular nucleus of the thalamus (PVT), the PeN, the paraventricular nucleus of the hypothalamus (PVN), the anterior hypothalamic area (AH), the basomedial (BM), the basolateral (BL), the central (Ce) and the lateral (LA) amygdaloid nuclei, the DMH, the ARC, the median eminence and the medial periaqueducal grey (MPAG) (Figure

39). Rare fibres were labelled with JLV-1 in the AVPV, the MPN, the MPO, the DMH, the ARC and the median eminence.

2. cFos expression in the brain following acute ICV Kp injection

To identify the Kp sites of action suggested by the distribution of Kp-ir fibres, cFos expression was analysed 2 hours following an acute ICV injection of Kp54 (Figure 40). The cFos protein expression was higher after Kp-treated animals as compared to vehicle injection in the BNST ($p = 0.003$), the PVT ($p = 0.030$) and the DMH ($p = 0.034$) of LD hamsters.The cFos protein expression was higher in SD-adapted Kp-treated animals in the MPN/MPO ($p = 0.030$) and the DMH ($p = 0.023$). No effect of Kp injection has been observed on cFos expression in the AVPV or the ARC.

3. Photoperiodic variations in Kp-ir neurones and fibres

As expected, in SD-adapted sexually inactive hamsters, the number of Kp-ir neurones in the ARC was decreased by about 50% (JLV-1 and #566 antisera, $p = 0.030$ and $p = 0.010$ respectively) when compared to LD sexually active animals (Figure 41 and 42). Fibres density was higher in SD-adapted hamsters in the AVPV, the BNST, the PVT, the PVN, the DMH the ARC and the median eminence (Table 1).

IV. Discussion

This study is the first one reporting the neuroanatomical and functional sites of action of Kp in a male rodent, the Syrian hamster. It reveals Kp projections and action in various brain regions, in particular the BNST, the MPO/MPN, the PVT and the DMH, and demonstrates that the kisspeptinergic system is altered by photoperiod in this seasonal species.

We report a dense Kp innervation in the BNST and the MPN/MPO as well as the presence of Kp-ir fibres in the ARC and AVPV region, the PVT and the DMH. Recently, a complete mapping of the kisspeptinergic system reported a quite similar Kp distribution with the additional presence of Kp-ir fibres in the ventral lateral septum, the supraoptic

nucleus and the subfornical organ (Clarkson et al., 2009b) that were not observed in the Syrian hamster. These differences can either be gender-specific or species-specific.

As expected from previous *in situ* hybridisation studies (Simonneaux et al., 2009; Ansel et al., 2010), Kp-ir neurones were found in the AVPV and the ARC of Syrian hamsters as well as mice (Clarkson et al., 2009b). Noteworthy, Kp-ir cells were observed in the DMH of the female mouse with the #566 antiserum (Clarkson et al., 2009b) while we did not observe such Kp-ir cell bodies neither with #566 nor with JLV-1 antiserum. Since no *Kiss1* mRNA has been detected in the DMH of any rodent (Gottsch et al., 2004; Smith et al., 2005b; Ansel et al., 2010), one can speculate that these mouse DMH immunoreactive cells may result from a cross-reaction with other RFamide peptides (Revel et al., 2008; Mikkelsen and Simonneaux, 2009). Indeed, dot blot tests showed that #566 antiserum recognises the RFamide related peptide-3 (RFRP-3) which is specifically expressed in the DMH of various species (Hinuma et al., 2000; Yano et al., 2003; Revel et al., 2008; Smith et al., 2008b). Furthermore, DMH Kp-ir cells disappear when the antiserum is used at high dilution (1:50,000) and on thin sections (< 20 µm). Finally, these cells are not observed with the JLV-1 antiserum which is targeted against the central part of the mouse Kp52 peptide preventing cross-reactions with the other RFamides sharing a common C-terminal motif with Kp. Altogether, these observations support the hypothesis of a cross-reaction of the #566 antiserum in the mouse DMH (Mikkelsen et al., unpublished). Surprisingly, Kp-ir cells were observed in the subfornical organ in the male Syrian hamster using JLV-1 antiserum, not the #566.

Interestingly, more fibres are observed with the #566 than with the JLV-1 antiserum. As mentioned, the JLV-1 antibody is targeted against the central part of the Kp52 peptide. Thus, this antiserum is rather specific of long Kp forms (Kp52 and Kp54). By contrast, #566 antiserum is targeted against the final ten amino acids of Kp and thus recognises both long and short Kp forms. The difference observed in Kp-ir fibres density may result from a different cellular distribution of the various Kp forms. Since JLV-1 recognises long Kp forms and reveals mainly Kp-ir cell bodies, it suggests that longer Kp forms are present in Kp cell bodies and less in Kp fibres. On the other hand, the #566 antiserum recognises both forms and detects both cell bodies and fibres, suggesting that longer Kp forms are processed in Kp cell bodies into shorter forms that are transported along the axon of Kp neurones to be released at the nerve terminals. However, the exact nature of the Kp form(s) present in the hypothalamus remains to be established and purification experiments should assess that issue.

As expected, a dense network of Kp-ir fibres was found in the MPO/MPN and the median eminence were GnRH cell bodies and nerve terminals respectively are present. However, the wide distribution of Kp fibres does not support the idea of a restricted action of Kp on GnRH release exclusively. Rather, Kp may also control reproduction-associated behaviours such as parental care or mating. The dense Kp innervation of the BNST support this hypothesis. Indeed, the BNST is involved in the control of several social- and reproduction-related behaviours: scent marking, vocalisation, parental behaviours, sexual behaviours, pair-bonding and mate choice, offensive aggression, social recognition and divergent social strategies (Goodson and Bass, 2001; Simerly, 2002). Additionally, BNST cells project to GnRH neurones in rats (Simerly and Swanson, 1986; Polston et al., 2004) and sheep (Pompolo et al., 2005). Thus BNST might be part of the Kp/GnRH/gonadotropin pathway (Beltramino and Taleisnik, 1980; Raitiere et al., 1997).

The Kp-ir fibres mapping is consistent with Kiss1r distribution. Indeed, *Kiss1r* mRNA has been found in the rat POA, DMH and amygdala (Lee et al., 1999). Unfortunately, this mapping is not extensive and a more precise knowledge of *Kiss1r* distribution is required. Similarly, Kiss1r protein distribution remains unknown since no specific Kiss1r antiserum has been produced yet. However, the use of a transgenic mouse line expressing the ß-galactosidase under the control of *Kiss1r* promotor region revealed that Kiss1r is present in the mouse median eminence and pituitary pars tuberalis (d'Anglemont de Tassigny et al., 2008). A recent study using the same mouse model established Kiss1r expression (Herbison et al.), but important discrepancies exist between this study and the rat Kiss1r mapping (Lee et al., 1999). Indeed, no Kiss1r has been detected in any of Kp fibres-containing regions with the above-mentioned transgenic mouse model. Whether these discrepancies are due to species-difference or to a different sensitivity of the technique remains to be determined.

To determine whether Kp are active into Kp fibres-containing structures, we analysed cFos expression in these brain regions after an acute central Kp54 injection. This experiment revealed that most structure containing Kp fibres respond to the peptide administration, suggesting that the observed fibres release Kp in these areas. However, the possibility of an indirect action of Kp via interneurones cannot be excluded. Interestingly, the sensitivity of these brain structures varies with photoperiod. The BNST and the PVT are more sensitive to Kp in LD, and the POA (MPN/MPO) is more sensitive to Kp in SD sexually inactive hamsters. Finally, the DMH equally responds to Kp in LD or SD.

These results are quite surprising since Kp fibres density is increased in SD in all structures analysed. Indeed, the number of Kp-ir neurones is decreased in SD which is consistent with what we previously reported (Revel et al., 2006b; Simonneaux et al., 2009; Ansel et al., 2010). Hence, the increased density in Kp-ir fibres in SD probably results from an accumulation of the peptides. Alternatively, seasonal variations in *Kiss1r* expression may account for the photoperiodic differences in the response of Kp target structures and it would be of high interest to analyse the expression of *Kiss1r* in both photoperiod to assess that issue. The biological signification of such difference is not known yet but, the identification of the phenotype of Kp-innervated cells should provide a new insight.

In conclusion our data demonstrated the wide distribution of Kp-ir fibres in the hamster's brain which support the hypothesis of extra GnRH Kp targets. In addition, cFos expression study following Kp ICV injection established that Kp fibres are not mere passage fibres, but they release Kp in the analysed structures. Our data also evidence subtle photoperiodic variations in the sensitivity of Kp target structures to the peptides. Finally, the use of two different antisera targeted agains different parts of Kp suggests that Kp longer forms (Kp54 and Kp52) are processed into shorter forms which are released at Kp neurones nerve terminals.

V. Acknowledgements

The authors wish to thank Caroline Ancel for English correction. This work was supported by the "Region Alsace", the French "Agence Nationale de la Recherche" (grant n° BLAN-07-0056), the NOVO Nordisk Foundation, the Sawmill owner Jeppe Juhl and wife Ovita Juhls' Foundation and the European Doctoral College of Strasbourg. This study was conducted at the Animal Resource and Experimentation Plateform / Chronobiotron of Strasbourg Institute of Neuroscience (IFR37).

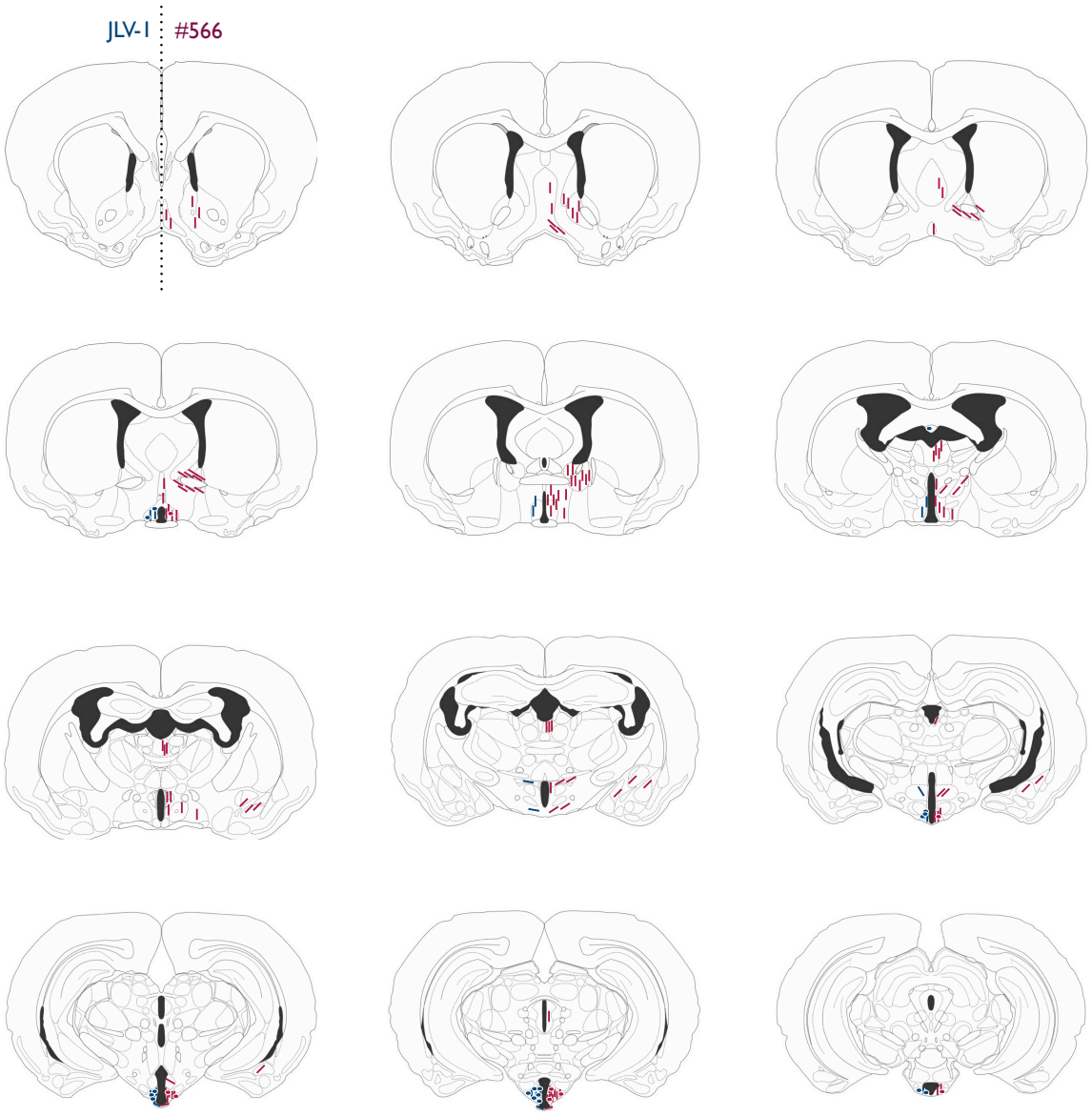

Figure 39: Distribution of Kp immunoreactive cells and fibres in Syrian hamsters

Kp immunoreactive neurones are represented as dots and Kp fibres as bars. Blue dots and bars on the left part of each brain coronal section represent Kp immunoreactivity detected with the JLV-1 antiserum. Purple dots and bars on the right part of each brain coronal section represent Kp immunoreactivity observed with #566 antiserum.

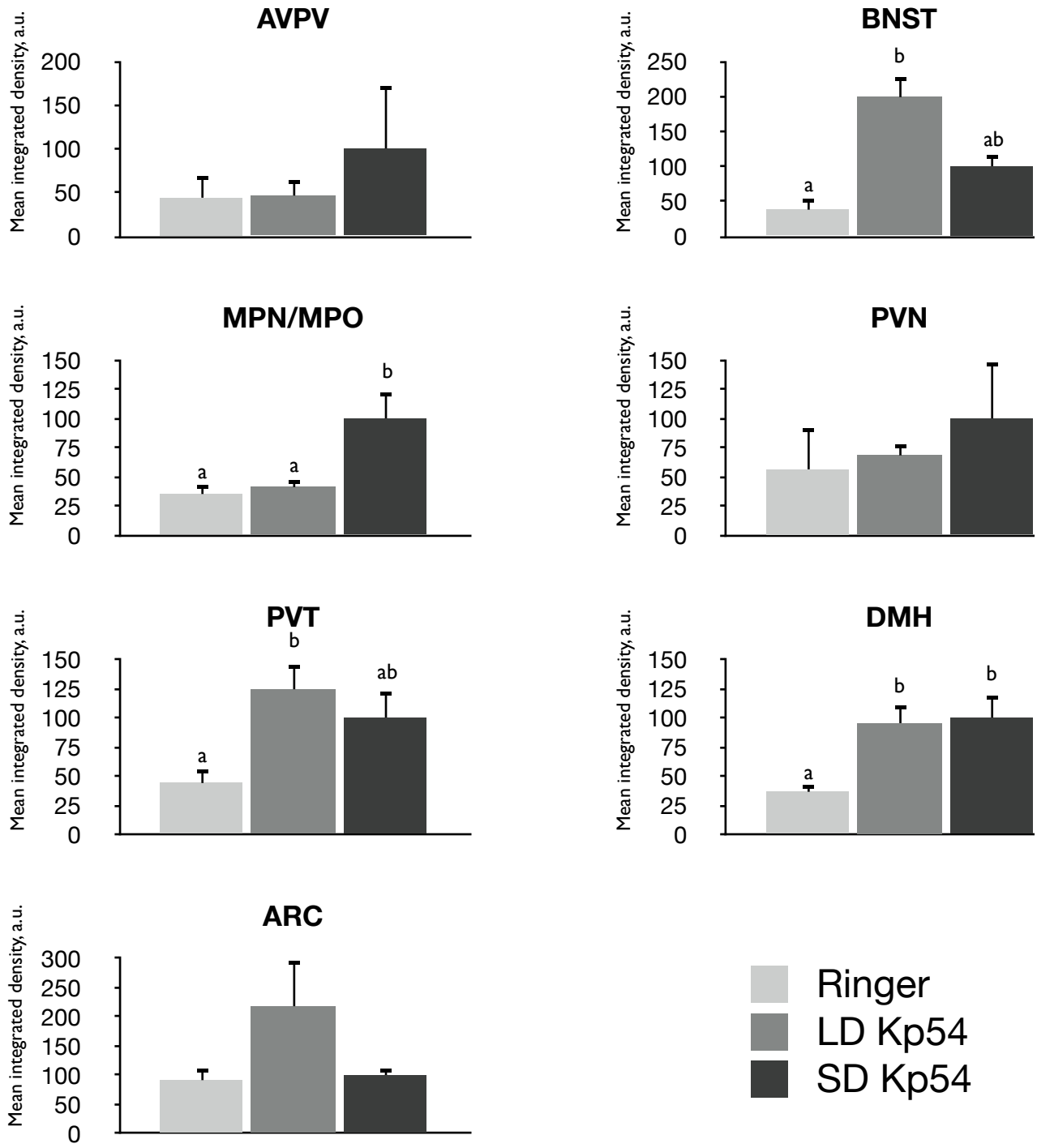

Figure 40: Effect of acute ICV injection of Kp54 or vehicle (Ringer) on cFos expression

The mean integrated density was measured with the ImageJ software on pictures of each brain region containing Kp fibres, both in LD or SD-acclimated male Syrian hamsters. Values are mean ± SEM (n = 4-6). Different letters indicate a statistically significant difference. a.u., arbitrary units.

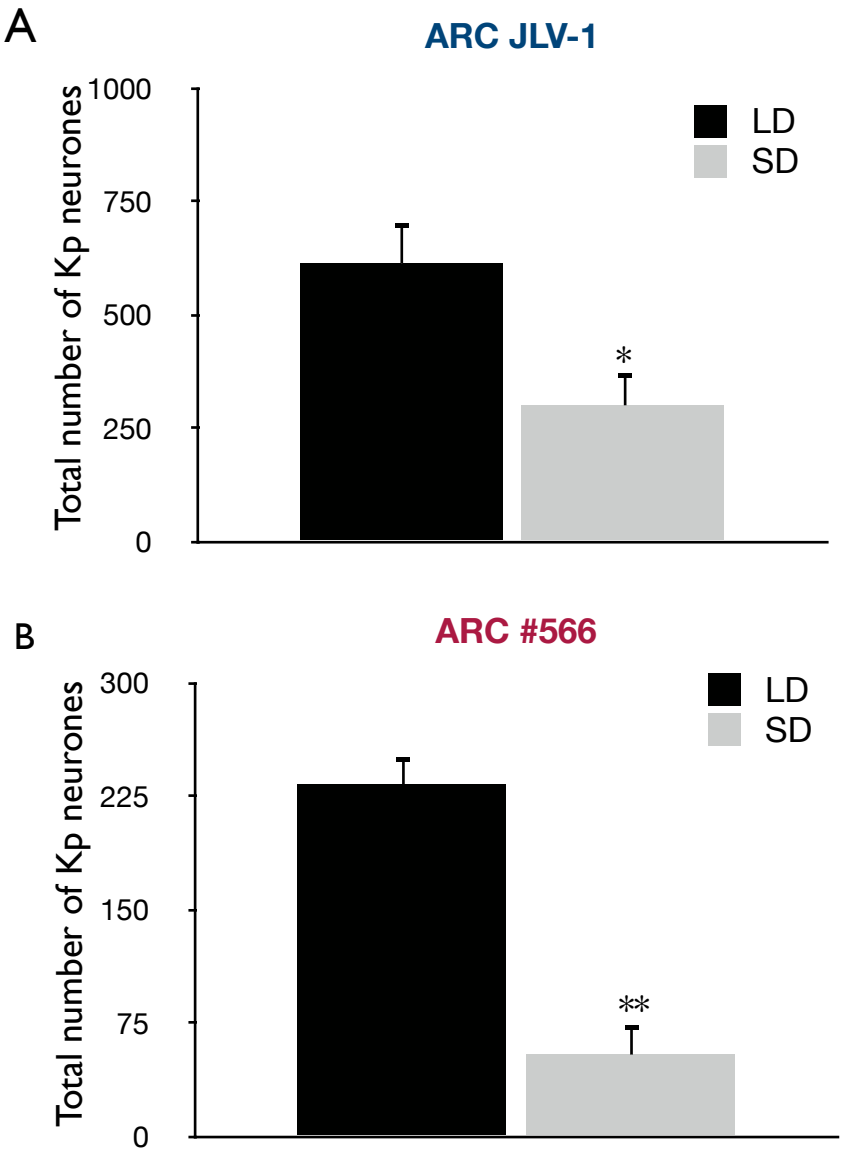

Figure 41: Effect of photoperiod on the number of Kp neurones

The total number of Kp neurones labelled with the JLV-1 (upper graph) or the #566 (low graph) antibody was quantified in LD and SD-acclimated male Syrian hamsters. Values are mean ± SEM (n = 4-6), * < 0.05 and ** p < 0.01 when compared to LD hamsters.

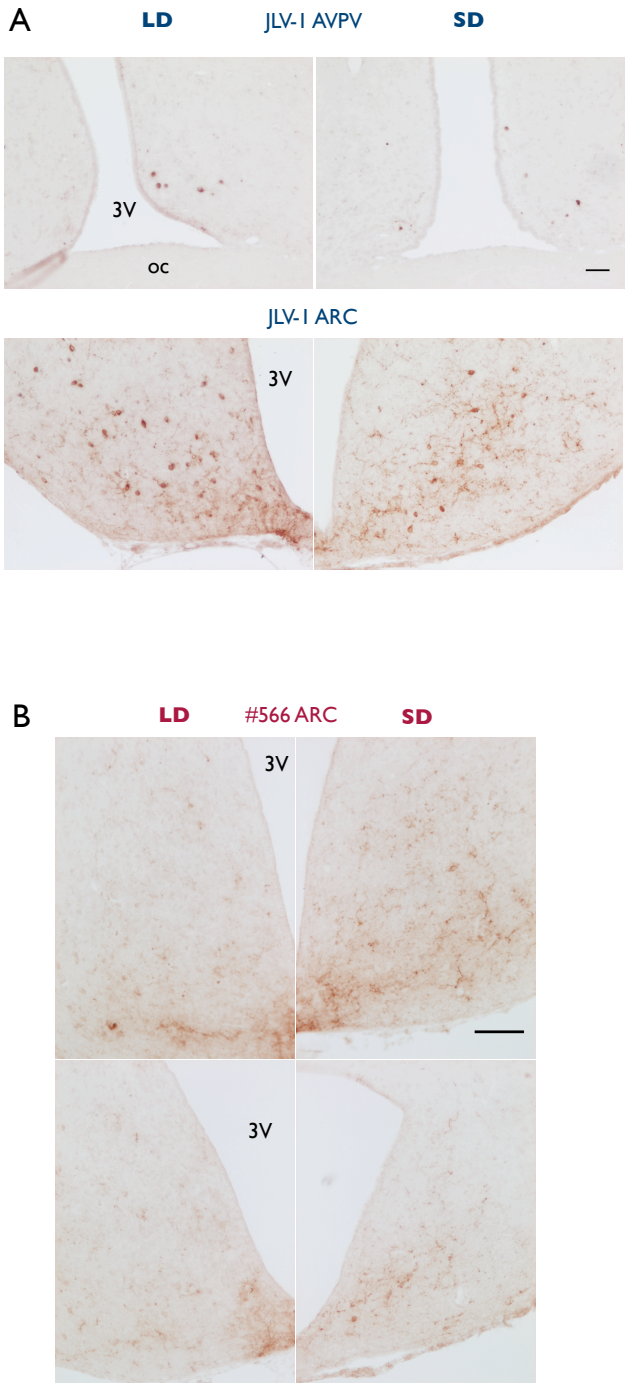

Figure 42: Effect of photoperiod on Kp expression in the male Syrian hamster

A: Picture showing Kp immunoreactive cells labelled with JLV-1 antiserum in the AVPV and the ARC of LD or SD acclimated hamsters. Scale bar: 100 µm. B: Pictures showing Kp immunoreactive cells labelled with #566 antiserum in the ARC of LD or SD-acclimated animals. Scale bar: 100 µm. 3V, third ventricle; oc, optic chiasm.

	AVPV	BNST	MPN/ MPO	PVT	PVN	DMH	ARC	ME
LD	+	+	++	+	+	-	+	+
SD	++	++	+++	++	++	+	++	++

Table 1: Effect of photoperiod on Kp fibres density

Kp fibres density was evaluated by a person blind to the experiment in the AVPV, the BNST, the MPN/MPO, the PVT, the PVN, the DMH, the ARC and the median eminence.

Chapter 4 - Regulation of *Kiss1* expression by melatonin and gonadal hormones

I. Introduction

1. Introduction to kisspeptins

As mentioned in the general introduction chapter, *Kiss1* codes for a family of peptides of variable length (from 10 to 54 amino acids) called kisspeptins. Disruption of either the *Kiss1* gene or the *Kiss1r* gene (also referred to as *Gpr54*) generates a hypogonadic phenotype in mice (Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007) and humans (de Roux et al., 2003), indicating that kisspeptins play a pivotal role in the maturation and the regulation of the HPG axis. In rodents, the *Kiss1* gene is expressed in two hypothalamic nuclei: the ARC and the AVPV nuclei (Gottsch et al., 2004; Smith et al., 2005a; Smith et al., 2005b) where *Kiss1* expression is sexually dimorphic with higher levels in females (Clarkson and Herbison, 2006; Adachi et al., 2007; Kauffman et al., 2007).

2. Kisspeptins: a key link between melatonin and seasonal reproduction ?

Syrian hamsters synchronise their reproductive activity to seasons via seasonal changes in photoperiod. When exposed to short winter-like days (SD) for eight to ten weeks, the male Syrian hamster experiences a complete gonadal regression (Goldman, 2001). Similarly, in female Syrian hamsters, SD exposure induces a disruption of the oestrous cycle (Sorrentino and Reiter, 1970; Bridges and Goldman, 1975). We have reported that *Kiss1* expression varies according to photoperiod with higher levels of expression in LD, when hamsters are sexually active (Revel et al., 2007; Simonneaux et al., 2009). Moreover, exogenous administration of kisspeptins to sexually inactive SD-adapted male Syrian hamsters reverses the inhibitory effect of SD exposure by re-activating the HPG axis (Revel et al., 2006b), indicating that photoperiodic variations of *Kiss1* expression drive seasonal changes in the HPG axis activity.

3. Aim of the study

In addition to photoperiod, *Kiss1* expression is regulated by several factors including nutritional status (Castellano et al., 2005; Forbes et al., 2009), stress (Kinsey-Jones et al., 2009) and sex steroid levels (Smith, 2008). Indeed, *Kiss1* expressing neurones mediate testosterone and oestrogen feedbacks on gonadotropin-releasing hormone (GnRH) neurones and differentially respond to sex steroid, *Kiss1* expression being stimulated in the AVPV and inhibited in ARC (Smith et al., 2005a; Smith et al., 2005b). Our study is aimed at deciphering how photoperiod drives seasonal changes in *Kiss1* expression. We focused on the role of melatonin whose production by the pineal gland is tightly controlled by the light/dark cycle, providing an endocrine representation of photoperiod and synchronising reproductive activity to seasons (Reiter, 1980). Since changes in day-length affect both the duration of the nocturnal peak of melatonin and the level of circulating sex steroid, we also analysed the effect of gonadal hormones on *Kiss1* expression in male Syrian hamsters. Since *Kiss1* expression is sexually dimorphic in the AVPV, this study was conducted in female Syrian hamsters as well.

II. Material and methods

1. Animals and tissue collection

Male and female Syrian hamsters weighing 100 - 180 g were raised in our animal facilities with food (Safe 105, rodent pellets, Safe, Augy, France) and water *ad libitum*, under controlled temperature (22 ± 1°C) and long photoperiod (LD; 14 hours light (200 lux) / 10 hours dark (2 lux, dim red light) cycle). When required, hamsters were exposed to a short photoperiod (SD; 10 hours light / 14 hours dark cycle) for eight or ten weeks.

Male hamsters were killed by decapitation under deep isoflurane anaesthesia. Brains were immediately removed, frozen on dry ice and non radioactive Kiss1 in situ hybridisation was performed. Trunk blood was collected, plasmas extracted, and the testes were weighed to check for photoperiod integration. To improve the morphology in histological sections, female hamsters were perfused transcardially with 4 % formaldehyde in 0.1 M phosphate-buffer pH 7.4, their brains post-fixed in the same fixative and polyethylene glycol-embedded as described by Klosen et al. (Klosen et al.,

1993). Brains were then processed for Kiss1 non radioactive in situ hybridisation as described below.

All experiments were conducted in accordance with the French National Law (licence n° 67-32) implementing the European Communities Council Directives of November, 24 1986 (86/609/EEC).

2. Experimental designs

a. Effect of photoperiod on *Kiss1* expression

Groups (n = 7-9) of male or female Syrian hamsters were either kept in LD conditions or exposed to SD for ten weeks before sacrifice. In their paper, Smith et al. (Smith et al., 2006b) reported that the number of *Kiss1* neurones in the female mouse AVPV is quite stable throughout the oestrous cycle but increased significantly in the evening of the pro-oestrous day. To avoid this transient increase in *Kiss1* expression in the AVPV, all female hamsters were sacrificed in the morning (between zeitgeber time (ZT) 2 and ZT 5).

b. Effect of sex steroid on *Kiss1* expression

Male or female hamsters (n = 4-9 / group) either remained in LD or were transferred to SD for eight weeks. Then, LD male hamsters were castrated (OCHX) or sham-operated and SD male hamsters received either an empty silastic capsule or a silastic capsule filled with testosterone. Testosterone levels were measured to check that castration and implants were done correctly. Testosterone levels were as follows: LD = 5.99 ± 1.01 pg/ml, LD OCHX = 0.11 ± 0.005 pg/ml, SD = 0.24 ± 0.08 pg/ml, SD + T= 5.96 ± 1.28 pg/ml. LD female hamsters were either ovariectomised (OVX) or left intact and half of the SD females received a silastic capsule filled with estradiol. Hamsters were killed four weeks after surgery.

c. Effect of melatonin on *Kiss1* expression

One group of male Syrian hamsters (n = 5-8) adapted to SD were OCHX and given a testosterone implant to avoid an indirect effect of melatonin via circulating testosterone

and to maintain a basal level of Kiss1 expression in the AVPV. A week later, half of these hamsters were pinealectomised (PINX) and half were sham-operated. Both groups were killed four weeks after the second surgery.

A second group of male hamsters (n = 4-6 / group) was either OCHX or left intact. Each hamster of both subgroups received daily intraperitoneal (IP) injections of either vehicle (Ringer - 5 % ethanol) or melatonin (50 µg in Ringer - 5 % ethanol, Sigma, Saint-Louis, MO, USA) one hour and a half before lights off during one, three or eight weeks. Daily vehicle or melatonin injections started two weeks after surgery.

A third group of female Syrian hamsters (n = 5-8) were either pinealectomised or sham-operated and killed four weeks later.

3. Surgical procedures

Hamsters were anaesthetised with 3 % isoflurane (AErrane, Baxter, France) and N2O to perform OCHX, OVX or sex steroid replacement. For sex steroid replacement, testosterone (4-androsten-17β-ol-3one, Sigma, Saint-Louis, MO, USA) or estradiol (1,3,5 [10]-estratriene-3,17ß-estradiol, Sigma, Saint-Louis, MO, USA)-filled silastic capsules (i.d. 1.47 mm; o.d. 1.95 mm; length: 13 mm) were subcutaneously implanted while control hamsters received an empty implant. For PINX, hamsters were anaesthetised with a mixture of Zoletil® (100 mg/kg, Virbac, Carros, France) and Rompun® (6 mg/kg, Bayer Pharma, Puteaux, France) injected IP. A circular hole was drilled in the skull and the pineal gland was removed with microdissection tweezers (PINX) or left in place (sham-PINX). After removal, the pineal gland was carefully examined to check for complete removal.

4. *Kiss1 in situ* hybridisation

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-labelled nucleotides (Roche, Meylan, France) according to the manufacturer's protocol. For male hamsters, four series of coronal brain sections (16 micrometers) were cut with a cryostat (Leica Microsystem, Rueil-Malmaison, France) through the ARC and AVPV. Sections were thaw-mounted on SuperFrost® ultraplus slides (Menzel-Gläser, Braunschweig, Germany) and stored at -80°C until *in situ* hybridisation. One series of sections out of four was post-fixed for twenty minutes at room temperature in 4 %

phosphate-buffered formaldehyde, rinsed in phosphate-buffered saline (PBS), dehydrated and rehydrated in ethanol five-minute baths (70°, 95°, 100°, 95°, and 70° ethanol). After three rinses in PBS, sections were acetylated twice for ten minutes with 0.25 % acetic anhydride in 100 mM triethanolamine. After two rinses in PBS, the sections were equilibrated in 5X saline sodium citrate buffer (SSC), 0.05 % Tween-20 before hybridisation. Hybridisation was performed for forty hours at 60 °C with 200 ng/ml of sense or antisense probe in 50 % formamide, 5X SSC, 5X Denhardts' solution and 1 mg/ml salmon sperm DNA. Six stringency rinses were performed for ten minutes in 0.1X SSC 0.05 % Tween-20 at 72 °C. In some experiments (effect of melatonin injections in castrated hamsters) the sections were also treated with RNAse A (20 µg/ml in 400 mM NaCl, 10 mM Tris-HCl, 2.5 mM ethylenediaminetetraacetic acid for thirty minutes at 37 °C) before stringency rinses to improve tha background-to-signal ratio. Digoxigenin-labelled bound probes were detected with an alkaline phosphatase-labelled antidigoxigenin antibody (Roche, Meylan, France). Alkaline phosphatase activity was detected with bromo-chloro-indolyl phosphate and nitroblue tetrazolium in the presence of 5 % polyvinyl alcohol (70.000-100.000 MW, Sigma, St Louis, MO, USA).

For female hamsters, 16 micrometer-thick polyethylene glycol sections were cut through the ARC and AVPV. Serial one in ten (ARC) or one in five (AVPV) sections were slide-mounted and stored at 4°C in PBS containing 0.02% dimethyl pyrocarbonate (DMPC, Sigma, Saint-Louis, MO, USA). Sections were then post-fixed for ten minutes at room temperature in 4 % phosphate-buffered formaldehyde, rinsed in PBS and digested for thirty minutes at 37 °C with 1 µg/ml proteinase K (Roche, Meylan, France). Proteinase K digestion was stopped with 2 % phosphate-buffered formaldehyde. Sections were then acetylated and processed as described above.

After detection of alkaline phosphatase activity, slides were mounted and the number of *Kiss1* neurones was hand-counted on a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France). In each experimental group, every AVPV or ARC section containing *Kiss1* neurones was quantified, and the total number of *Kiss1* neurones was calculated for each structure. To allow a direct comparison between the experiments, for each experiment data are given as the total number of *Kiss1* neurones in each structure. Variations in the number of *Kiss1* neurones in the control group (LD intact) may however occur because *in situ* hybridisation of all experiments were performed over two years and sensitivity of the technique varies across the different assays.

5. Testosterone assay

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

6. Statistical analysis

Results are shown as mean ± SEM. Data were analysed by ANOVA followed by Tukey's *post-hoc* analysis. Two-tailed p-values are expressed as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

III. Results

1. Effect of photoperiod on *Kiss1* expression

To analyse the effect of photoperiod on *Kiss1* expression, male and female Syrian hamsters were kept in LD or transferred to SD for ten weeks prior to sacrifice. The number of *Kiss1* positive neurones in the AVPV is larger in females than in males (Figure 43 A and Figure 44 A) when compared to the number of ARC *Kiss1* population. In both male and female hamsters, SD exposure significantly decreased *Kiss1* expression in both the AVPV and ARC (Males: Figure 43 A, B, $p < 0.001$ and $p = 0.005$ respectively. Females: Figure 44 A, B, $p = 0.020$ and $p = 0.024$ respectively). No labelling was observed with the sense probe (data not shown).

2. Effect of sex steroid on *Kiss1* expression

To analyse the regulation of *Kiss1* expression by sex steroid, male and female hamsters were either kept in LD and gonadectomised (or sham-gonadectomised), or kept in SD and sex steroid-supplemented (or sham-supplemented). In male ARC, OCHX of LD hamsters increased the number of *Kiss1* neurones (Figure 45 A, $p = 0.014$) but testosterone replacement in SD hamsters did not diminish *Kiss1* expression (Figure 45 B, $p = 0.564$) when compared to control animals. In the male AVPV, OCHX of LD hamsters

reduced *Kiss1* expression (Figure 45 A, $p < 0.001$) whereas testosterone replacement in SD animals increased the number of *Kiss1* neurones when compared to control counterpart (Figure 45 B, $p = 0.001$). In females, OVX increased the number of *Kiss1* neurones in the ARC (Figure 46 A, $p = 0.023$) but did not alter *Kiss1* expression in the AVPV (Figure 46 A). Conversely, oestrogen replacement in SD female hamsters increased the number of *Kiss1* neurones in the AVPV (Figure 46 B, $p = 0.007$) and strongly decreased it in the ARC (Figure 46 B, $p = 0.003$) as compared to control animals.

3. Effect of melatonin on *Kiss1* expression

To analyse the effect of melatonin on *Kiss1* expression, PINX was performed in SD-adapted male or female hamsters. In both sexes, pineal gland removal markedly increased *Kiss1* expression in the ARC (Figure 47 A, $p = 0.006$ and Figure 47 B, $p = 0.038$, respectively) but did not change the number of AVPV Kiss1 neurones (Figure 47 A, B).
Eight weeks of daily melatonin injections to LD-adapted male hamsters significantly decreased testes weight (Figure 48 A, $p < 0.001$) and circulating testosterone (Figure 48 B, $p = 0.034$) together with a reduction in the number of *Kiss1* neurones in the ARC (Figure 48 C, $p = 0.025$) and the AVPV (Figure 48 D, $p = 0.041$). Shorter (one or three weeks) duration of melatonin administration had no effect on any of these parameters. Strikingly, when hamsters were castrated, melatonin administration had a more potent and a more rapid inhibitory effect on *Kiss1* expression in the ARC (Figure 48 E, $p = 0.007$) but had no effect in the AVPV (Figure 48 F) even after eight weeks of treatment.

IV. Discussion

We have previously reported that *Kiss1* expression in the ARC of male Syrian hamsters is inhibited in SD (Revel et al., 2006b) and we demonstrate here that it is true in the AVPV as well. Furthermore, we report here that *Kiss1* expression is likewise down-regulated in the ARC and AVPV of female Syrian hamsters. Since changes in day-length affect both the duration of the nocturnal peak of melatonin and the level of circulating sex steroids, we analysed how melatonin and testosterone / oestrogen regulate *Kiss1* expression in the ARC and AVPV in male and female Syrian hamsters.

In the ARC of sexually active LD-adapted male hamsters, OCHX further increased the number of *Kiss1* neurones, indicating that testosterone inhibits *Kiss1* expression in

that structure as already reported in other species (Irwig et al., 2004; Smith et al., 2005b; Rometo et al., 2007; Smith et al., 2007). However, testosterone treatment of sexually inactive SD-adapted male hamsters did not significantly diminish *Kiss1* expression probably because the number of ARC *Kiss1* neurones was already minimal due to SD exposure. Our data are consistent with the absence of effect of castration in SD in another hamster species (Greives et al., 2008b). Photoperiodic changes in ARC *Kiss1* expression thus do not appear to be mediated by changes in testosterone production. Indeed, low circulating testosterone levels in SD would result in an increased *Kiss1* expression, which is opposite to the observed reduction in the number of ARC *Kiss1* neurones. By contrast, PINX markedly increased *Kiss1* expression in the ARC of castrated SD-adapted male hamsters and PINX before SD exposure prevented *Kiss1* down-regulation (Revel et al., 2006b). Furthermore, long-term melatonin injections in LD-adapted hamsters inhibited *Kiss1* expression. These observations indicate that melatonin drives photoperiodic changes in *Kiss1* expression in the ARC. Strikingly, in castrated hamsters, the inhibitory effect of melatonin on *Kiss1* expression is much faster and more potent, with a maximal decrease reached after one week of treatment. This observation could indicate that low testosterone levels may potentiate melatonin effect. Indeed, melatonin receptor density and/or affinity may be altered by testosterone in our model since it has been reported in the Syrian hamster that castration in LD increases and testosterone-supplementation in SD reduces the density of melatonin binding sites in the *pars tuberalis* (Recio et al., 1998). Furthermore, in the European hamster (*Cricetus cricetus*), entry into hibernation, which depends on melatonin/short photoperiod, is not observed until gonadal regression has occurred, and high testosterone levels prevent hibernation (Darrow et al., 1988). Further studies including melatonin injections in LD castrated and testosterone-supplemented Syrian hamsters are required to determine to which extent gonadal hormones impact on melatonin effect on *Kiss1* expression in the ARC.

In the ARC of female Syrian hamsters, OVX in LD animals significantly increased *Kiss1* expression whereas oestrogen treatment in SD hamsters reduced the number of *Kiss1* neurones. Furthermore, PINX in SD female hamsters led to a large increase in ARC *Kiss1* expression. As a consequence, the decrease in the number of ARC *Kiss1* neurones observed in sexually inactive SD-adapted female hamsters is not caused by the decrease in circulating oestrogen but rather by the increase in melatonin production.

Kiss1 expression is sexually dimorphic in the hamster's AVPV, females having a higher number of *Kiss1* neurones when compared to males, as reported in other rodent species (Clarkson and Herbison, 2006; Gottsch et al., 2006; Adachi et al., 2007; Kauffman et al., 2007). Similar to the ARC, *Kiss1* expression in the AVPV of male and female Syrian hamsters is also significantly reduced in SD conditions. The stimulatory effect of testosterone replacement and the inhibitory effect of castration on the number of *Kiss1* neurones in respectively SD- and LD-adapted male hamsters demonstrate the positive feedback effect exerted by testosterone on the AVPV in the Syrian hamster as already reported in mice (Smith et al., 2005b). Oestrogen replacement in SD-adapted females strongly up-regulated *Kiss1* expression, indicating that sex steroids exert a positive feedback on *Kiss1* neurones in females hamsters' AVPV as previously described in rats and mice (Kinoshita et al., 2005; Smith et al., 2005a). However OVX in LD-adapted female hamsters did not decrease the number of AVPV *Kiss1* neurones, maybe because intact hamsters were not in the evening of the pro-oestrus day, a period when *Kiss1* expression is enhanced (Smith et al., 2006b). Therefore the LD-induced up-regulation of AVPV *Kiss1* expression could be explained by the sole stimulatory effect of sex steroids. This observation raises the issue of the mode of action of melatonin on these neurones. Eight weeks of daily melatonin injections inhibited AVPV *Kiss1* expression in intact LD animals but had no effect in OCHX LD animals. Furthermore, PINX in sexually inactive hamsters did not alter AVPV *Kiss1* expression. These findings suggest that *Kiss1* expression in the AVPV is down-regulated upon SD exposure following a reduction in circulating sex steroids. However, it cannot be excluded that a direct effect of melatonin in the AVPV could not be detected because of the experimental protocol. Indeed since castration strongly down-regulates *Kiss1* expression in the AVPV, melatonin inhibitory effect may not be detected in the AVPV of OCHX LD hamsters. Similarly, because testosterone supplementation in SD hamsters up-regulates the number of AVPV *Kiss1* neurones, further stimulation by PINX may not be detectable.

Taken together, our data suggest a differential photoperiodic regulation of *Kiss1* expression in the ARC and AVPV of the Syrian hamster. In SD conditions, the increased production of melatonin would inhibit *Kiss1* expression in the ARC and according to our previous study (Revel et al., 2006b), this is sufficient to inhibit gonadal activity. Consequently, the decrease in circulating sex steroid would lift their stimulatory feedback effects on the AVPV neurones, hence decreasing *Kiss1* expression.

It is well established in the Syrian hamster that melatonin does not affect GnRH mRNA levels (Brown et al., 2001), the number and morphology of GnRH neurones (Urbanski et al., 1991) nor the pituitary responsiveness to GnRH (Pickard and Silverman, 1979). Thus, melatonin inhibits reproductive activity probably via a regulation of GnRH release. In rodents, AVPV *Kiss1* neurones are thought to project directly to GnRH cell bodies (Clarkson and Herbison, 2006; Wintermantel et al., 2006) whereas ARC *Kiss1* neurones probably project toward GnRH nerve terminals of the median eminence to locally control GnRH release (d'Anglemont de Tassigny et al., 2008). It is therefore tempting to speculate that melatonin inhibits ARC *Kiss1* expression, which downstream reduces GnRH release.

To date, no melatonin receptors have been identified in the ARC of the Syrian hamster, thus a direct effect of melatonin on *Kiss1* neurones appears unlikely. Melatonin sites of action for the seasonal control of reproduction in the Syrian hamster have been found in the mediobasal hypothalamus, an area comprising the dorsomedial hypothalamus. This region contains melatonin binding sites and its lesion prevents the SD-induced gonadal regression (Maywood and Hastings, 1995) and abolishes hamsters' ability to respond to exogenous melatonin (Reiter and Sorrentino, 1972; Maywood et al., 1996). We recently reported that neurones in hamsters' dorsomedial hypothalamus express the gene encoding RFamide-Related Peptides (RFRP) with a melatonin-driven inhibition in SD conditions (Revel et al., 2008). In rats, RFRP-3 inhibits LH secretion (Johnson et al., 2007; Anderson et al., 2009). Therefore, RFRP neurones may be part of the pathway between melatonin and the seasonal control of reproduction in Syrian hamsters.

Besides the Syrian hamster, photoperiodic variations in *Kiss1* expression (Simonneaux et al., 2009) and Kp immunoreactivity (Greives et al., 2007; Mason et al., 2007) have been reported in another seasonal rodent, the Djungarian hamster. However, *Kiss1* expression in the ARC is decreased in LD, when animals are sexually active, whereas it is increased in the AVPV (Greives et al., 2007; Simonneaux et al., 2009). Such discrepancies may reflect species differences in melatonin sites of action for the control of seasonal reproduction. Indeed, in Djungarian hamsters, suprachiasmatic nucleus lesion prevents the inhibitory effect of exogenous melatonin infusions (Bartness et al., 1991), whereas its lesion does not compromise responsiveness to exogenous melatonin in Syrian hamsters (Bittman et al., 1989), suggesting that in the former species, the suprachiasmatic nucleus may mediate melatonin effects on the reproductive axis. In

rodents, the suprachiasmatic nucleus sends vasopressin projections to AVPV neurones (Kalsbeek and Buijs, 2002) and regulates *Kiss1* neurones' activity in mice AVPV (Robertson et al., 2009). Therefore, AVPV *Kiss1* neurones might mediate the effect of melatonin on the reproductive axis in the Djungarian hamster. This hypothesis is supported by the important number of Kp-expressing neurones in the Djungarian hamster's AVPV when compared to the ARC (Greives et al., 2007; Simonneaux et al., 2009) and the observation that melatonin could act on GnRH production in that species (Porkka-Heiskanen et al., 1997; Bernard et al., 1999).

In conclusion, our study demonstrates that the regulation of *Kiss1* expression by sex steroids in the Syrian hamster is similar to other rodents, with a positive feedback in the AVPV and a negative one in the ARC. However, sex differences between males and females were observed in the combined effects of photoperiodic and sex steroid manipulations. Importantly, our data also demonstrate that AVPV and ARC *Kiss1* neurones differentially respond to melatonin and point to ARC *Kiss1* neurones as key gatekeepers of the seasonal control of the reproductive function in the Syrian hamster.

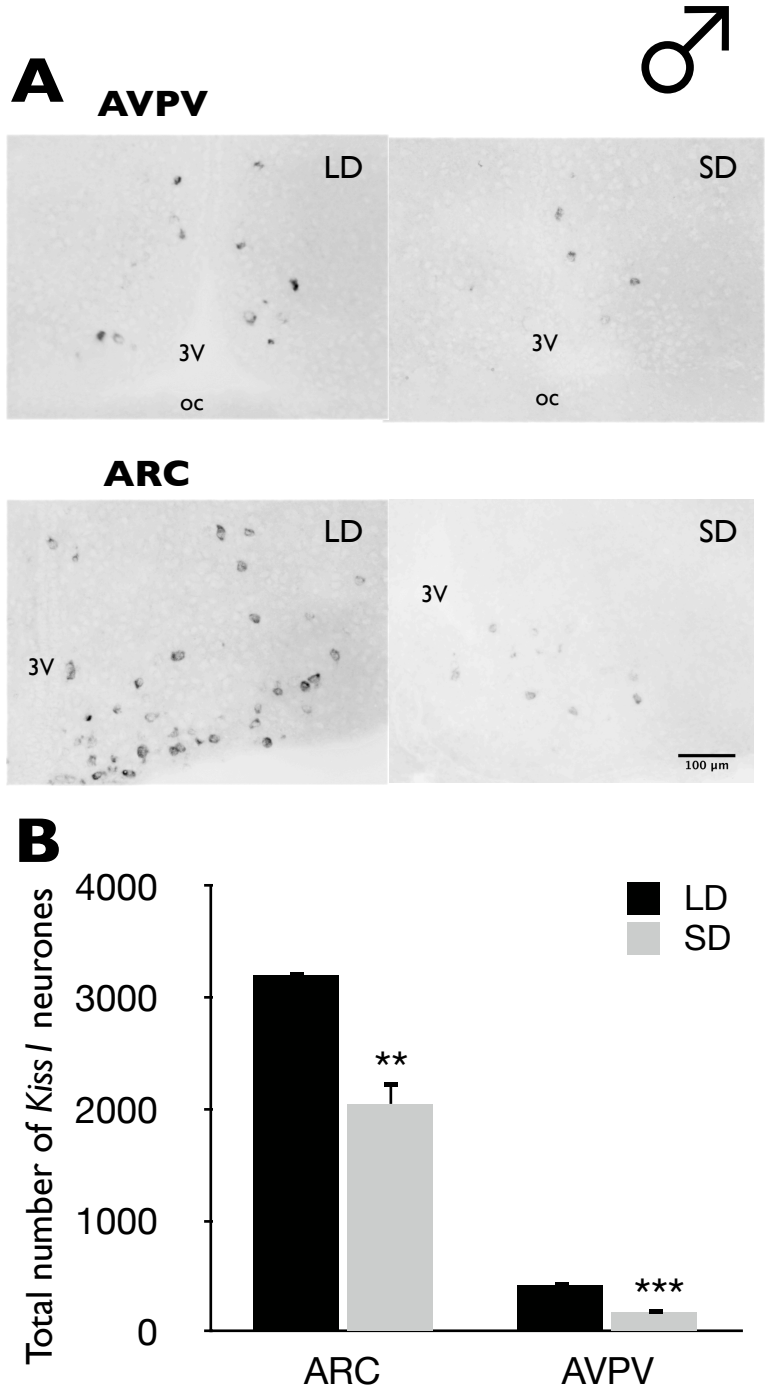

Figure 43: Effect of photoperiod on *Kiss1* expression in the ARC and AVPV of male Syrian hamsters.

A: Pictures showing *Kiss1* in situ hybridisation in the ARC and AVPV of LD or SD-adapted male Syrian hamsters (3V: third ventricle; oc: optic chiasm). B: Total number of *Kiss1* neurones in the ARC and AVPV of LD-adapted hamsters. Values are mean ± SEM (n = 7-9) for each structure, ** $p < 0.01$, *** $p < 0.001$ when compared to LD-adapted hamsters.

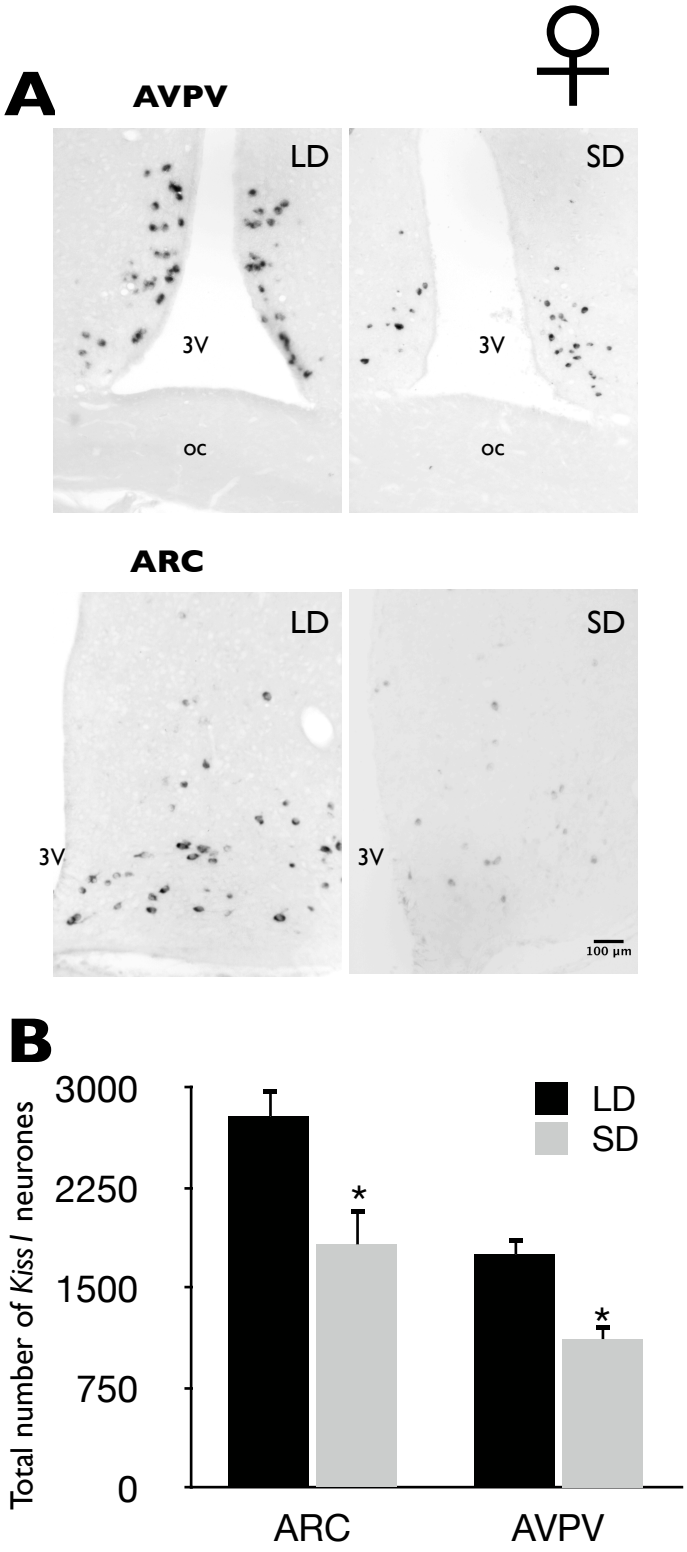

Figure 44: Effect of photoperiod on *Kiss1* expression in the ARC and AVPV of female Syrian hamsters.

A: Pictures showing *Kiss1* in situ hybridisation in the ARC and AVPV of LD or SD-adapted female Syrian hamsters (3V: third ventricle; oc: optic chiasm). B: Total number of *Kiss1* neurones in the ARC and AVPV of LD-adapted hamsters. Values are mean ± SEM (n = 7-9) for each structure, * $p < 0.05$ when compared to LD-adapted hamsters.

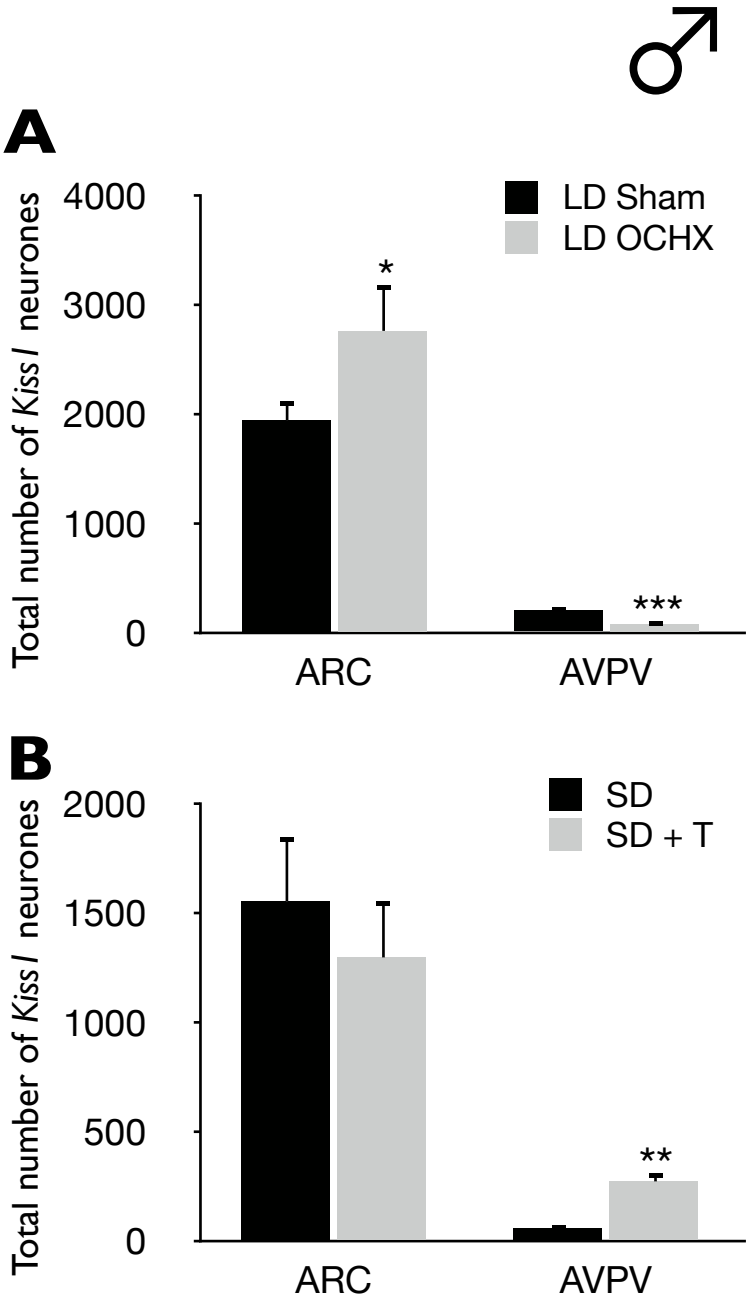

Figure 45: Effect of testosterone and castration on Kiss1 expression in the ARC and AVPV of male Syrian hamsters

A: Number of *Kiss1* neurones in the ARC and AVPV of intact (Sham) or castrated (OCHX) LD male hamsters.. Values are mean ± SEM (n = 5-7) for each structure, * $p < 0.05$ and *** $p < 0.001$ when compared to LD sham hamsters. B: Total number of *Kiss1* neurones in the ARC and AVPV of SD-adapted male hamsters treated or not with testosterone implants (T). Values are mean ± SEM (n = 4-7) for each structure, ** $p < 0.01$ when compared to untreated hamsters.

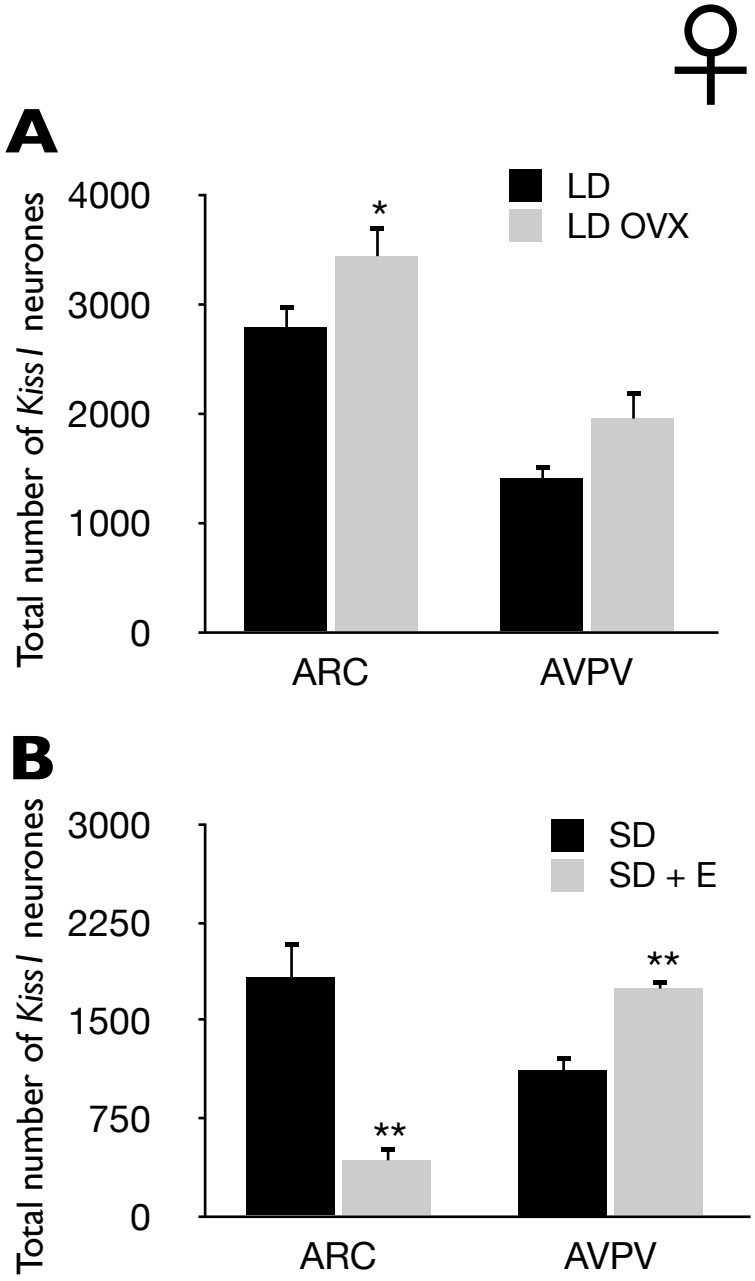

Figure 46: Effect of oestrogen and ovariectomy on *Kiss1* expression in the ARC and AVPV of female Syrian hamsters

A: Total number of *Kiss1* neurones in the ARC and AVPV of intact or ovariectomised (OVX) long day (LD) female hamsters. Values are mean ± SEM (n = 4-9) for each structure,* $p < 0.05$. B: Total number of *Kiss1* neurones in the ARC and AVPV of short day (SD)-adapted female hamsters treated or not with oestrogen implants (E). Values are mean ± SEM (n = 4-9) for each structure, ** $p < 0.01$ when compared to untreated hamsters.

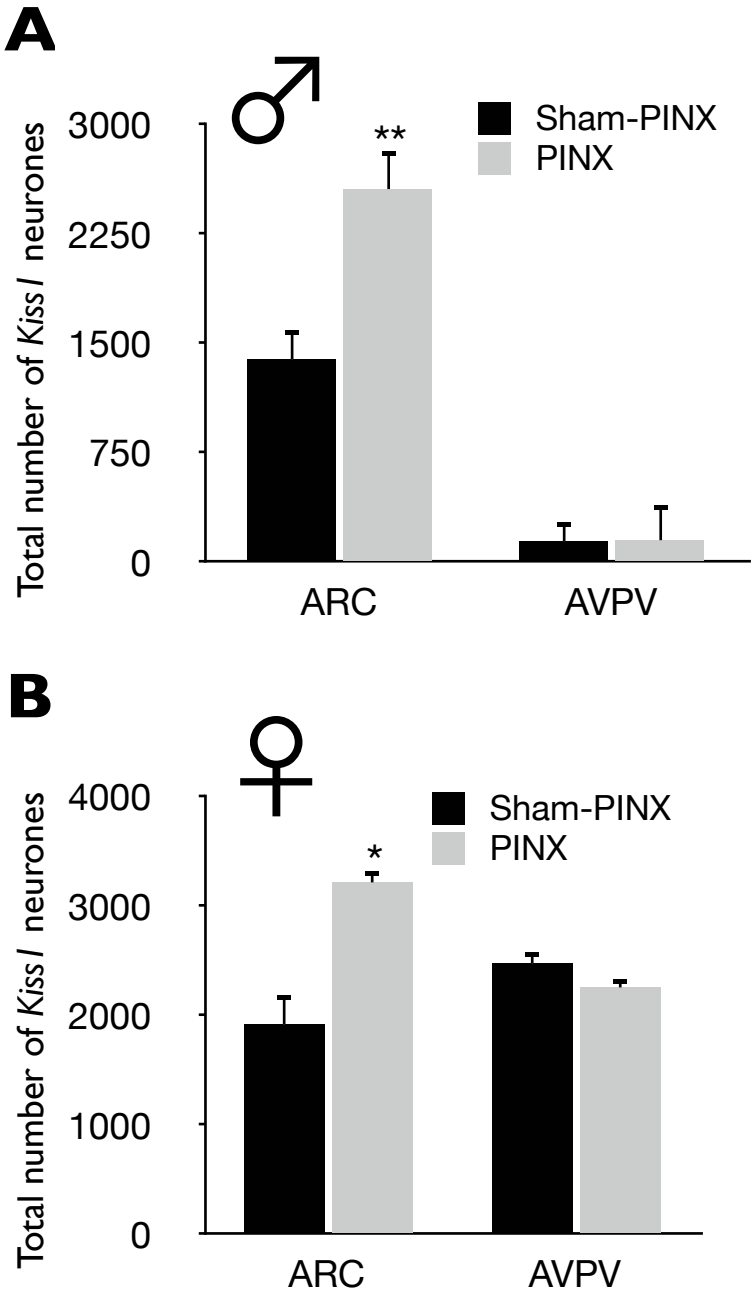

Figure 47: Effect of pinealectomy on *Kiss1* expression in the ARC and AVPV of male and female Syrian hamsters.

SD-adapted hamsters were either PINX or sham-PINX four weeks before sacrifice. The total number of *Kiss1* neurones was quantified in the ARC and AVPV of castrated SD-adapted male (A; n = 5-7) or intact female (B; n = 7-8) Syrian hamsters. Values are mean ± SEM, * $p < 0.05$, ** $p < 0.01$ when compared to sham-operated hamsters.

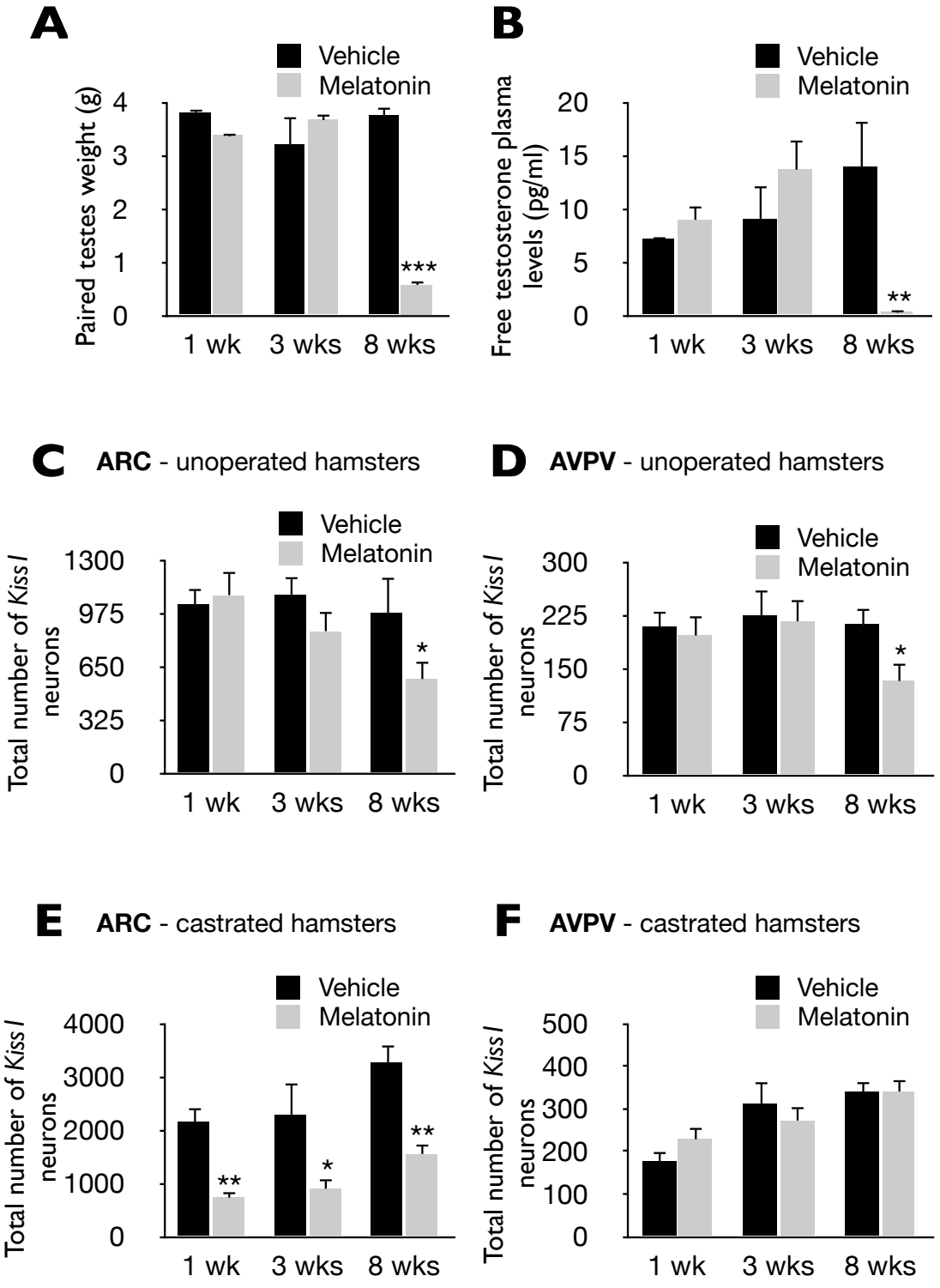

Figure 48: Effect of daily melatonin injections on the reproductive axis of long day-adapted male Syrian hamsters.

The effect of IP injection of melatonin (50 µg) or vehicle (Ringer 5% ethanol) given every late afternoon for 1, 3 or 8 weeks (wks) was analysed on paired-testes weight (A), plasma free-testosterone (B), *Kiss1* expression in the ARC (C) or AVPV (D) of intact male hamsters and on *Kiss1* expression in the ARC (E) or AVPV (F) of castrated male hamsters. Values are mean ± SEM (n = 4-6), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to corresponding vehicle-treated animals.

Chapter 5 - Effects and sites of action of peripheral kisspeptins

I. Introduction

1. Photoperiodic regulation of the reproductive function

Important seasonal variations in various environmental parameters such as photoperiod, temperature or hygrometry occur throughout the year. Annual changes in photoperiod is used by most seasonal breeders to restrict their fertility to a particular season, so as to ensure the birth of the offspring at the most favourable time of year. Thus, transfer of male Syrian hamsters from long days (LD) to short days (SD) inhibits their reproductive activity within 8 to 10 weeks with a 10-fold reduction of testes weight and a dramatic drop in gonadotropins and sex steroid plasmatic levels (Goldman, 2001). Inversely, when transferred back to LD condition reproductive activity is restored within 6 weeks (Goldman, 2001). Therefore, SD-adapted Syrian hamsters constitute an adult model of reversible hypogonadism. Annual variations of the photoperiod are translated into a release of the pineal hormone melatonin, the nocturnal production of which relies on night-length. Previous experiments have demonstrated that the annual variations of melatonin secretion synchronise reproductive activity to season (Reiter, 1980). However, the sites and mechanisms of action of melatonin on the hypothalamo-pituitary gonadotropic (HPG) axis are still unclear. We recently reported that in the Syrian hamster, melatonin inhibits the expression of several hypothalamic genes (Revel et al., 2006b; Revel et al., 2006a; Revel et al., 2008; Simonneaux et al., 2009), including Kiss1, which might be potential intermediates between photoperiod and the reproductive function.

2. *Kiss1* and the reproductive function

Disruption of either the Kiss1 gene or the Kiss1receptor (r) gene (also referred to as Gpr54) generates a hypogonadic phenotype in mice (Funes et al., 2003; Seminara et al., 2003; d'Anglemont de Tassigny et al., 2007; Lapatto et al., 2007) and humans (de Roux et al., 2003), demonstrating the key role of the Kiss1/Kiss1r system in the regulation of the HPG axis. The Kiss1 gene is expressed in two hypothalamic nuclei: the anteroventral periventricular (AVPV) and arcuate (ARC) nuclei (Gottsch et al., 2004; Smith et al., 2005a; Smith et al., 2005b). It is translated into a 145 amino-acid protein processed to generate one or several biologically active peptides of various lengths named Kisspeptins (Kp).

Both Kp10 and Kp54 shares a 10 amino-acid common C-terminus and bind to the Kiss1r with a similar very high affinity (Kotani et al., 2001). Kp are the most potent activators of the HPG axis since central administration of doses as low as 1 fmol triggers the release of LH (Gottsch et al., 2004).

3. Aim of the study

In the Syrian hamster, we have shown that the Kiss1 mRNA level in AVPV and ARC is significantly reduced in SD by melatonin (Ansel et al. 2010) and that central chronic administration of Kp to SD hamsters reverses the inhibitory effect of melatonin by re-activating the HPG axis, pointing to Kp as pivotal gate-keepers of seasonal reproduction (Revel et al. 2006a, Simonneaux et al. 2009). Since peripheral administration of Kp has been reported to activate the HPG axis as does central infusion (Thompson et al. 2004, Navarro et al. 2005, Mikkelsen et al. 2009), we have investigated whether long term intraperitoneal administration of Kp can restore gonadal activity in SD photo-inhibited male hamsters and further analysed the sites of action of peripheral Kp.

II. Material and methods

1. Animals

Male Syrian hamsters weighing 100 - 180 g were raised in our animal facilities (Strasbourg, France) with food and water *ad libitum*, under controlled temperature (22 ± 1°C) and long photoperiod (LD; 14h light (200 lux) / 10h dark (2 lux, dim red light) cycle with lights on at 06.00). When required, hamsters were exposed to short photoperiod (SD; 10h light / 14h dark cycle with lights on at 10.00) for 10 weeks. All experiments were conducted in accordance with the French National Law (licence n° 67-32) implementing the European Communities Council Directives of November, 24 1986 (86/609/EEC).

2. Peptide and reagents

Human Kp54 was used to investigate the effect of peripheral Kp on the gonadotropic axis because it exerts a long lasting effect on the gonadotropic axis

(Mikkelsen et al., 2009). It was synthetised by GenScript (Piscataway, NJ, USA).) GnRH was purchased from Sigma (Saint Louis, MO, USA) and acyline, a selective GnRH antagonist (Herbst, 2003), was kindly provided by Drs John Amory and William J Bremner (University of Washington, Seattle, WA, USA).

3. Experimental designs

a. Effect of long term peripheral administration of Kp54

To examine whether peripheral administration of Kp54 could restore reproductive activity of photo-inhibited hamsters, two types of long term administration were tested. Hamsters (n = 6-7 / group) were transferred to SD for 10 weeks, after which testicular regression was verified by scrotal palpation. In the first protocol, subcutaneous chronic administration was tested with osmotic minipumps (Alzet, Charles River France, model 2004, 4-week treatment) filled with either vehicle (Ringer) or Kp54 (140 µM, i.e 10 nmoles / hamster / day) delivered at a rate of 2,5 µl/h over a period of 28 days. The dose of Kp54 was chosen according to previous studies in rats where 50 nmoles per day induced testicular degeneration (Thompson et al., 2006). The hamsters were anaesthetised with 3 % isoflurane and a small incision of the skin was made in the interscapular region. A haemostat was used to create a pocket into which the pump was inserted with the flow moderator facing away from the incision. Xylocain was administered in the wound before the skin was sutured with wound clips. In the second protocol, hamsters were IP injected (10 nmoles / hamster) twice a day (1h after lights on and at lights off) with either vehicle (Ringer) or Kp54 (100 µM). The hamsters were kept in SD for the four or five weeks (first and second protocol respectively) of Ringer or Kp54 administration and testes size was checked every week by scrotal palpation. In daily IP-injected hamsters, four weeks of treatment increased testes size as checked by scrotal palpation. Injections were performed for one more week to maximise Kp54 effect. Hamsters' body weight was measured at the beginning and at the end of the period of Kp54 chronic administration. At the end of the experiments, hamsters were killed by decapitation after deep isoflurane anaesthesia (AErrane, Baxter, France). Trunk blood was taken in heparinised tubes. The plasma was separated and stored at –80°C until testosterone assay. The testes were immediately dissected and weighed.

b. Effect of an acute peripheral administration of Kp54

To identify peripheral Kp sites of action, we examined the effect of peripheral administration of Kp54 on cFos expression in the pituitary. Hamsters (n = 5 / group) were intraperitoneally (100 µl / hamster) injected with a vehicle solution (Ringer) or 150 µM Kp54 solution (15 nmoles / hamster). Two hours after injection, hamsters were deeply anaesthetised with isoflurane vapours and perfused transcardially with 4 % formaldehyde in 0.1 M phosphate buffer pH 7.4. The two hour delay between injection and sacrifice is required for the expression of cFos protein. Entire pituitary tissue (both anterior and posterior lobes) was examined for cFos expression by immunohistochemistry.

To determine whether the effect of Kp54 on the pituitary is direct or mediated via GnRH, five groups of hamsters (n = 4-6 / group) were intraperitoneally injected (100 µl / hamster) first with either vehicle (Ringer) or 50 µg of the GnRH receptor antagonist acyline, then, one hour later, with vehicle or 300 µM GnRH or 150 µM Kp54. Two hours after the last injection, animals were killed as described previously and pituitary tissues were examined for cFos expression. Moreover, to find out if pituitary cell activation is correlated to downstream activation of the HPG axis, blood was taken by cardiac puncture. The plasma was extracted, stored at -80°C and processed for testosterone assay. Testosterone was preferred to LH to assess the downstream activation of the HPG axis because peripheral Kp effect on LH release is no longer detectable 60 minutes after the injection (Navarro et al. 2005) whereas Kp54 exerts a long-lasting effect on testosterone secretion in the rat (Mikkelsen et al. 2009).

To phenotype the cFos responsive cells, the pituitaries of three GnRH-injected and three Kp54-injected hamsters were further processed for dual cFos and ßLH/ßFSH immunohistochemistry. Three sections per pituitary were processed and for each section, three different areas were analysed. The total number of cFos positive cells and ßLH/ßFSH positive cells was hand-counted. The proportion of gonadotrophs expressing cFos and the proportion of cFos positive cells expressing ßLH/ßFSH were calculated.

c. Effect of central or peripheral Kp on cFos expression in the preoptic area

To determine wether peripheral Kp54 acts on GnRH neurones cell bodies and/or GnRH nerve terminals, we compared the ability of centrally or peripherally-given Kp54 to

elicit a cFos response in the preoptic area which contains many GnRH neurone cell bodies in the Syrian hamster.

Two groups of hamsters (n = 6 / group) were maintained in LD and were implanted with a cannula (outer diameter: 0.7 mm; inner diameter: 0.4 mm; length: 1.7 mm) in the lateral ventricle under a Zoletil® (100 mg/kg, Virbac, Carros, France) and Rompun® (6 mg/kg, Bayer Pharma, Puteaux, France) anaesthesia. The cannula was implanted at the following coordinates: 0.8 mm anterior to the bregma, 2 mm lateral to bregma and 3 mm under pial surface. After a one-week recovery, hamsters were anaesthetised with 3 % isoflurane (AErrane, Baxter, France) and N2O, and 2 µl of Ringer's solution or a 500 µM Kp54 solution were injected intracerebroventrically at the rate of 1 µl/min using a microinjection pump (CMA 400, CMA, Acton, MA, USA). After the injection, the syringe was left in place for 3 min before removal. Hamsters were deeply anaesthetised and killed 2 hours after the intracerebroventricular injection as described previously.

Two other groups of hamsters (n = 6 / group) kept in LD received a single intraperitoneal 100 µl-injection of either vehicle (Ringer) or 150 µM Kp54. Two hours after the injection, the hamsters were deeply anaesthetised with pentobarbital (5 ml/kg) and perfused transcardially with 4 % formaldehyde as described previously.

d. Effect of photoperiod on Kp-induced cFos expression in the pituitary

Hamsters were kept in LD or SD for 10 weeks (3 groups per photoperiod, n = 6 / group). For each photoperiod, groups of hamsters received a single intraperitoneal injection of either vehicle (Ringer), 300 µM GnRH or 150 µM Kp54. Two hours after the injection, the hamsters were deeply anaesthetised with pentobarbital (5 ml/kg) and perfused transcardially with 4 % formaldehyde in 0.1 M phosphate buffer pH 7.4. The fixed testes were dissected and weighed to check for photoperiodic regression. The pituitaries were removed and processed for cFos immunohistochemistry.

4. Immunohistochemistry

a. Single immunohistochemistry

After transcardiac perfusion of 4 % formaldehyde in 0.1 M phosphate buffer pH 7.4, brains were immediately removed from the skull, post-fixed for 24 hours at 4°C in 4

% formaldehyde in 0.1 M phosphate buffer pH 7.4, rinsed overnight in phosphate buffer, dehydrated and embedded in polyethylene glycol as described by Klosen et al., 1993 (Klosen et al., 1993). Microtome sections (12 µm) were cut and processed (one every 120 µm) for immunohistochemistry. For cFos detection, non specific binding sites were saturated for 1-hour with 3 % dry skimmed milk. Primary anti cFos rabbit polyclonal antibody (1:2000, Santa Cruz biotechnologies, Santa Cruz, CA, USA) was applied overnight. The sections were then incubated for 1 hour with a secondary biotinylated donkey anti-rabbit antibody (1:2000, Jackson Immunoresearch Laboratories, West Grove, PA, USA**)** followed by a 1 hour-incubation with streptavidin-peroxydase (1:2000, Roche, Basel, Switzerland**)**. Peroxydase activity was detected by using 3,3-diaminobenzidine (Sigma, Saint Louis, MO, USA) as a chromogen. The slides were then dehydrated and mounted with Eukitt®.

b. Dual immunohistochemistry

For dual cFos and ßLH/ßFSH immunohistochemistry, endogenous alkaline phosphatase was inhibited with 15 % acetic acid for 5 minutes. The sections were first processed for cFos immunohistochemistry as described previously except for the enzyme label (streptavidin-alkaline phosphatase, 1:2000, Roche, Basel, Switzerland**)**. The enzymatic activity was detected for 40 minutes using Naphtol AS MX phosphate as substrate and Fast Blue BB as a chromogen. The cFos and the biotinylated donkey anti-rabbit antibodies were then eluted with 100 mM glycine and 0.3 % Triton X-100 for twice 15 minutes. Gonadotrophs were labelled with a mix of anti-ßLH and anti-ßFSH antibodies (ßLH: 1:60000 rabbit polyclonal antibody; ßFSH: 1:40000 rabbit polyclonal antibody, both from National Hormone and Peptide Program). ßLH and ßFSH presence was detected with peroxydase and DAB as described above.

c. Quantification

Photomicrographs were taken with a Leica DMRB microscope (Leica microsystems, Rueil-Malmaison, France) equiped with an Olympus DP50 digital camera (Olympus France, Rungis, France). For quantification, all parameters of the microscope and the camera software (Viewfinder Lite, Olympus) were standardised. For cFos immunohistochemistry, photomicrographs were taken with a 5x objective and quantified

with ImageJ software (Rasband, W.S., US National Institutes of Health, Bethesda MD, USA) as described by Salingre et al., 2009 (Salingre et al., 2009). Briefly, pictures were thresholded according to the intensity of the background and the same threshold was applied for each individual experiment. A particle analysis was performed on the pituitary and particles whose diameter was inferior to 30 pixels were excluded. The sum of the integrated density of each immunoreactive particle (i.e. cFos positive cell) was calculated and divided by the total area quantified to obtain a mean integrated density per surface unit. The same procedure was applied on preoptic area sections extending from 0.8 to 0.2 mm anterior to Bregma.
For cFos and ßLH/ßFSH dual immunohistochemistry, the total number of cFos positive cells and ßLH/ßFSH positive cells were hand-counted.

5. Testosterone assay

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

6. Statistical analysis

Student's t test or one-way ANOVA analysis was carried out followed by Tukey's multicomparison. Two-tailed p-values are expressed as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

III. Results

1. Effect of long term peripheral administration of Kp54

To examine whether peripheral administration of Kp54 could restore reproductive activity of photo-inhibited hamsters, two types of long term administration were tested. Chronic delivery (4 weeks) of Kp54 via subcutaneous mini-pumps failed to increase testes weight and plasma testosterone of male hamsters kept in SD. Kp54 treatment even tended to decrease testes weight when compared to vehicle treated animals although without reaching statistical significance ($p = 0.07$, Figure 49 A). By contrast, daily Kp54

injections for five weeks significantly increased testes weight ($p = 0.001$; Figure 49 B) and increased plasma levels of testosterone ($p = 0.07$; Figure 49 B) when compared to vehicle injected hamsters. This protocol had no effect on body weight (Figure 50).

2. Effect of an acute peripheral administration of Kp54

Induction of cFos expression in the pituitary and the production of testosterone were analysed after a single Kp54 intraperitoneal injection. Kp54 strongly increased the number of cFos immunoreactive cells in hamsters' anterior pituitary when compared to vehicle-treated hamsters ($p = 0.005$; Figure 51 A, B). Dual cFos and ßLH/ßFSH labelling of pituitaries from Kp54 injected animals revealed that over 90 % of cFos-positive cells were gonadotrophs (Figure 51 C) and gonadotrophs activation was correlated to a large downstream release of testosterone ($p < 0.001$, Figure 51 D). Strikingly, the effect of acute peripheral injection of GnRH on pituitary cFos expression and testosterone production was qualitatively and quantitatively very similar to those of Kp54 (Figure 51 B,C,D). This finding suggests that peripheral Kp may act via a release of GnRH. To test this hypothesis, the effect of Kp54 and GnRH was tested in the presence of a GnRH receptor antagonist, acyline.

Similarly to Kp54, GnRH acute injection induced cFos expression in the anterior pituitary when compared to vehicle-treated hamsters ($p = 0.005$; Figure 52). Pretreatment with acyline not only completely blocked GnRH stimulatory effect, but also Kp54 effect ($p = 0.017$ and $p = 0.028$ respectively; Figure 52).

3. Effect of central or peripheral Kp on cFos expression in the preoptic area

To determine wether peripheral Kp54 acts on GnRH cell bodies, we analysed the effect of peripherally and centrally-administered Kp on cFos expression in the preoptic area. Kp54 intraperitoneal injection failed to induce cFos expression in the preoptic area when compared to vehicle treated counterparts ($p = 0.948$, Figure 53 A). By contrast, intracerebroventricular injection of Kp54 induced a significant increased in cFos expression in the preoptic area as compared to vehicle treated hamsters ($p = 0.043$, Figure 53 B).

4. Effect of photoperiod on Kp54-induced cFos expression

To analyse the effect of photoperiod on GnRH- or Kp54-induced cFos expression in the pituitary, an intraperitoneal injection of vehicle (Ringer), GnRH or Kp54 was given to LD or SD-adapted hamsters. GnRH intraperitoneal injection induced a similar level of cFos expression in the anterior pituitary of LD and SD hamsters ($p = 0.999$; Figure 54). By contrast, Kp54 induced a significantly larger expression of cFos in SD hamsters as compared to LD hamsters ($p = 0.033$; Figure 54).

IV. Discussion

Our study reports that peripheral long term administration of Kp54 re-activates the HPG axis of photo-inhibited SD hamsters, providing the protocol is appropriately defined. In a first experiment we observed that continuous subcutaneous administration of Kp54 to SD male hamsters using minipumps failed to re-activate the HPG axis as attested by low testes weight and low plasmatic testosterone levels. These data are consistent with the finding that in SD Siberian hamsters, continuous peripheral administration of Kp10 failed to increase testes weight (Greives et al. 2008). Since the stability of Kp54 in the Alzet osmotic minipumps for several weeks was previously established (Revel et al. 2006a, Thompson et al. 2006), it appears unlikely that the lack of Kp54 effect in this protocol is due to the degradation of the peptide. Hence, one might speculate that the dose used was not appropriate. However, a previous study reported that continuous administration of a similar dose of Kp54 induced testicular degeneration in rats (Thompson et al. 2006), which is consistent with the observed tendency for lower paired-testes weight in Kp54-treated hamsters. Therefore, failure of continuous administration of Kp54 to activate testicular activity may result from a down-regulation / desensitisation of the Kiss1r. Indeed, it has been previously reported that a preliminary continuous intravenous administration of Kp10 in male rhesus monkey prevented the LH rise induced by a further bolus of Kp10 (Seminara et al. 2006, Ramaswamy et al. 2007). Moreover, continuous Kp exposure of mice MBH explants induced a sustained GnRH release for 4 hours followed by a decrease, suggesting a desensitisation of the Kiss1r in the median eminence area (d'Anglemont de Tassigny et al. 2008). Alternatively, one might speculate that constant exposure to Kp induces a constant release of GnRH, which is known to desensitise pituitary GnRH receptor (Belchetz et al. 1978) and this could also explain the

absence of effect of chronic Kp54 administration on gonadal activity in photo-inhibited hamsters. In the second experiment, we observed that two daily injections of Kp54 for five weeks to SD-acclimated, sexually inactive, Syrian hamsters significantly increased testes weight, indicating a re-activation of the HPG axis despite the inhibitory photoperiod. The stimulatory effect of repeated versus continuous peripheral administration of Kp54 indicates an absence of desensitisation / down-regulation of the Kiss1r or GnRH receptor with this protocol. This hypothesis is supported by the finding that intermittent infusions of Kp10 to juvenile agonadal male monkey induce sustained GnRH-dependant LH pulsatile release (Plant et al. 2006) and repeated intravenous administration of low doses of Kp10 to adult male rats increase LH levels without evidence of desensitisation (Tovar et al. 2006).

A previous study reported that one daily intraperitoneal injection of Kp10 failed to re-activate the HPG axis in photo-inhibited Siberian hamsters (Greives et al. 2008). Discrepancies between this study and ours can be explained by the form of the peptide used and the frequency of peripheral administration. Firstly, longer forms of the human peptide tend to act in a prolonged way when compared to shorter forms (Mikkelsen et al. 2009). Secondly, one daily injection might not be enough to induce a sustained activation of the HPG axis. Indeed, so far only a frequency of two daily peripheral bolus of Kp10 over 60 hours caused ovulation in seasonally acyclic ewes (Caraty et al. 2007), underlining the importance of the frequency of administration.

To elucidate the target site(s) of peripheral Kp, we chose cFos as a marker of cellular activation which further allows an anatomical analysis. We examined cFos expression in the brain and pituitary and observed that peripheral Kp strongly induced cFos expression in the anterior pituitary, an effect followed by the downstream increase in testosterone levels reflecting increased LH levels. Dual labelling experiment showed that Kp-induced cFos expression in the anterior pituitary is quite restricted to gonadotrophs. This observation together with the finding that long term administration of Kp54 had no effect on hamster body weight excludes an effect of the peptide on the hypothalamo-pituitary somatotrope axis in contrast with what is described in peripubertal rats (Gutierrez-Pascual et al. 2007). To activate gonadotroph cells, peripheral Kp may act either on GnRH neurones and/or directly on the pituitary, in a GnRH-independant manner. A direct effect of Kp on the pituitary is still a matter of debate. In cultured pituitary cells from peripubertal rats (Gutierrez-Pascual et al. 2007) or glands from pubertal rats (Navarro et al. 2005) application of 10 nM Kp10 increased LH release but did not affect FSH levels.

However, on cultured anterior pituitaries (Thompson et al. 2004) or dissociated cells of anterior pituitaries (Matsui et al. 2004) from adult rats exogenous Kp10 failed to increase LH or FSH even at doses as high as 1 μM. Since Kiss1r expression is increased around puberty in rat hypothalamus (Navarro et al. 2004), one might expect a similar regulation in the anterior pituitary with a higher sensitivity of the gland around puberty, which would explain the aforementioned discrepancies. In the adult Syrian hamster, the effect of Kp54 on cFos immunoreactivity in the anterior pituitary was completely blocked by acyline, a GnRH antagonist. Thus, peripheral Kp54 activates the pituitary in a GnRH-dependent manner. Our results are in line with previous studies in sheep showing that peripheral Kp induced GnRH release in cerebrospinal fluid (Caraty et al. 2007) but failed to induce LH release in hypothalamo-pituitary disconnected animals (Smith et al. 2008), and that in rodents, acyline prevented Kp-induced LH release (Gottsch et al. 2004, Irwig et al. 2004, Mikkelsen et al. 2009). These observations indicate that peripheral Kp54 activates the pituitary gonadotrophs via the release of GnRH and exclude a direct effect of the peptide on the pituitary. However, the precise site of action of peripheral Kp on GnRH neurones remains unknown. To determine wether peripheral Kp acts on GnRH cell bodies or on GnRH nerve terminals, we analysed cFos expression in the preoptic area, where most GnRH cell bodies are located, after peripheral Kp injection. We found that peripheral Kp did not increase cFos in the preoptic area, in line with a previous study in mice revealing that peripheral Kp failed to induce cFos expression in GnRH neurone cell bodies (Mikkelsen et al. 2009). By contrast, central Kp significantly increased cFos expression in the preoptic area which suggests that central Kp induces cFos in GnRH cell bodies as previously demonstrated in rats (Irwig et al. 2004). Taken together, these observation suggests that peripheral and central Kp acts on different parts of the GnRH neurone. While central Kp would act on GnRH neurone cell bodies (and putatively on GnRH nerve terminals as well), peripheral Kp would act on GnRH nerve terminals of the median eminence to induce GnRH release. Due to the lack of specific antibodies for Kiss1r at this point, the localisation of the receptor remains difficult. However, the observation that Kp can induce GnRH release from mice mediobasal hypothalamus explants despite tetrodotoxin pre-treatment (d'Anglemont de Tassigny et al. 2008) strengthens this hypothesis.

Because Syrian hamsters display marked photoperiodic variations in Kiss1 expression with lower levels in SD (Revel et al. 2006a, Simonneaux et al. 2009, Ansel et al. 2010), we investigated whether gonadotrophs responsiveness to Kp may depend on

photoperiod. An acute injection of Kp54 induced more cFos positive cells in the pituitary of SD than of LD-adapted animals. The increased cFos immunoreactivity in SD animals is not due to a photoperiodic change in the anterior pituitary's responsiveness to GnRH since there was no effect of photoperiod on GnRH-induced cFos expression. Insufficient dose of GnRH cannot be accounted for the lack of photoperiodic difference in GnRH effect on cFos expression in our study since a dose as low as 0.1 nmole of GnRH given subcutaneously induces a massive LH release in Syrian hamsters (Pickard & Silverman 1979). Moreover, previous studies reported no photoperiodic difference of pituitary response to exogenous GnRH (Pickard & Silverman 1979). Therefore, Kp54 sensitivity to photoperiod probably occurs at the level of the GnRH nerve terminals of the median eminence. One might speculate that low levels of Kp in SD hamsters (Revel et al. 2006a) could up-regulate / re-sensitise Kiss1r at the GnRH terminals of the median eminence. Indeed in fasting prepubertal rats, while *Kiss1* mRNA is decreased, *Kiss1r* mRNA is increased (Castellano et al. 2005) and Kp10 is more potent to induce LH release (Castellano et al. 2005), indicating a higher sensitivity of the HPG axis in undernutrition. However, a feedback effect of testosterone as well as a direct effect of photoperiod / melatonin on GnRH neurones sensitivity to Kp cannot be excluded.

In conclusion, our data demonstrate that central and peripheral exogenous Kp have different sites of action. While centrally given Kp would target GnRH neurone cell bodies and/or nerve terminals, exogenous peripheral Kp re-activate a photo-inhibited HPG axis by stimulating GnRH release probably from median eminence nerve terminals (Figure 55), with a photoperiod-dependent sensitivity. Taken together, our data confirm the key role played by Kp in the seasonal control of reproduction in the Syrian hamster and point to the necessity of clearly defining protocols (form of the peptide, dose, frequency) to study the effect of long term administration of Kp in a clinical perspective.

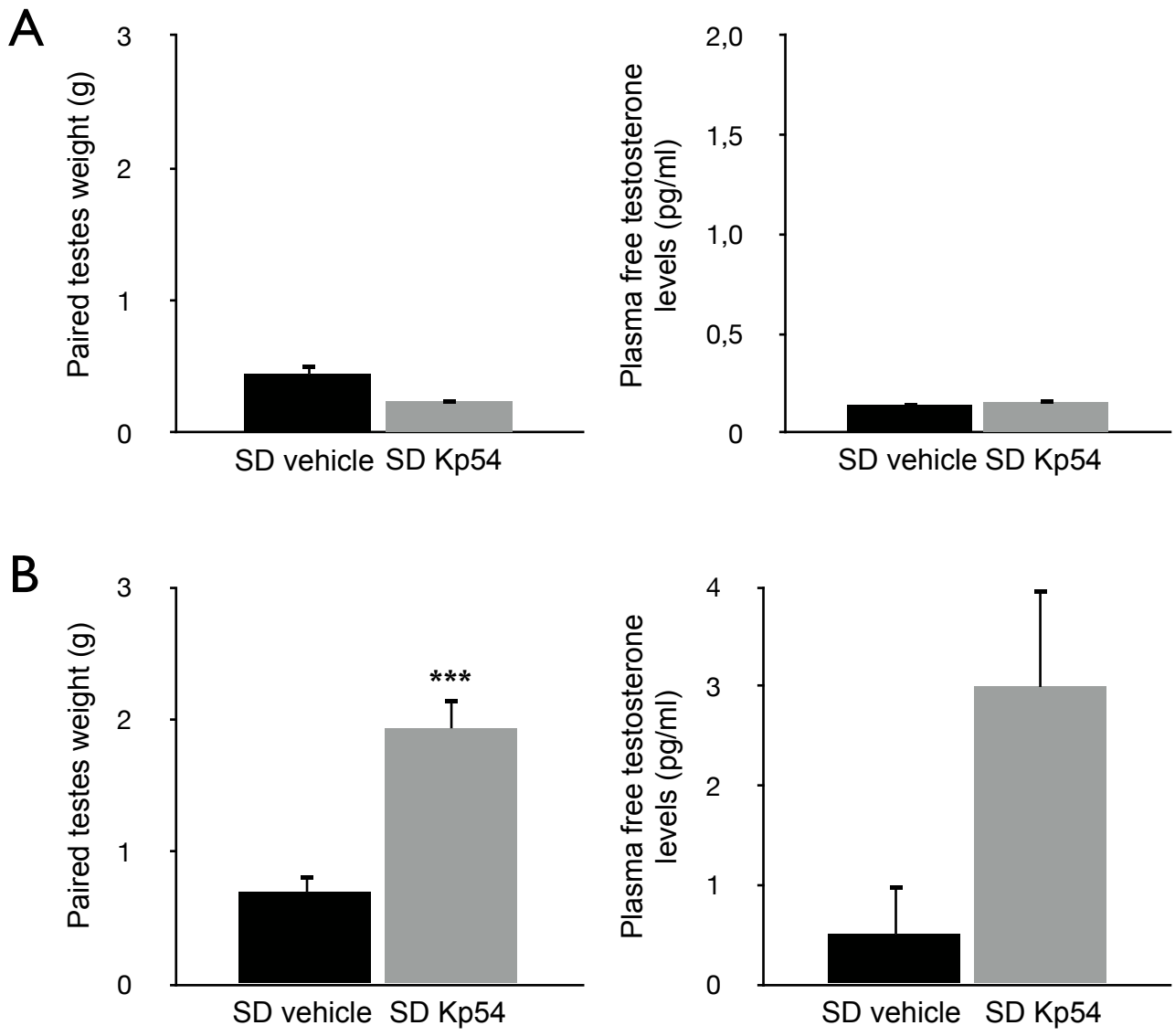

Figure 49: Effect of continuous or repeated long term peripheral administration of Kp54 to SD-adapted hamsters

A: Mean value of paired-testes weight (left pannel) and concentration of free plasma testosterone (right pannel) were measured in short days (SD) Syrian hamsters after four weeks of chronic subcutaneous administration of vehicle (Ringer) or Kp54 (10 nmoles / hamster / day) via osmotic minipumps. Values are mean ± SEM (n = 5-6). B: Mean value of paired-testes weight (left panel) and concentration of plasma free testosterone (right panel) were measured in SD Syrian hamsters after five weeks of daily IP injections of vehicle (Ringer) or Kp54 (twice 10 nmoles / hamster). Values are mean ± SEM (n = 5-7), *** $p < 0.001$ when compared to vehicle-injected hamsters (Student t test).

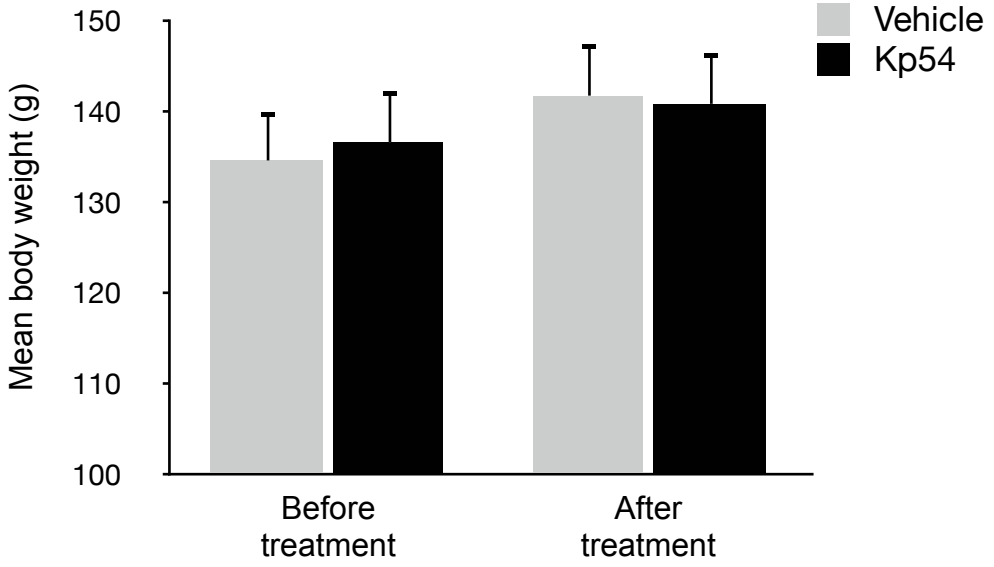

Figure 50: Effect of five weeks of Kp54 daily injections on the body weight of short day-adapted hamsters

Animals were kept in SD and weighed before and after five weeks of two daily injections of vehicle (Ringer) or Kp54 (10 nmoles / hamster). Body weight is expressed as mean ± SEM (n = 5-7)

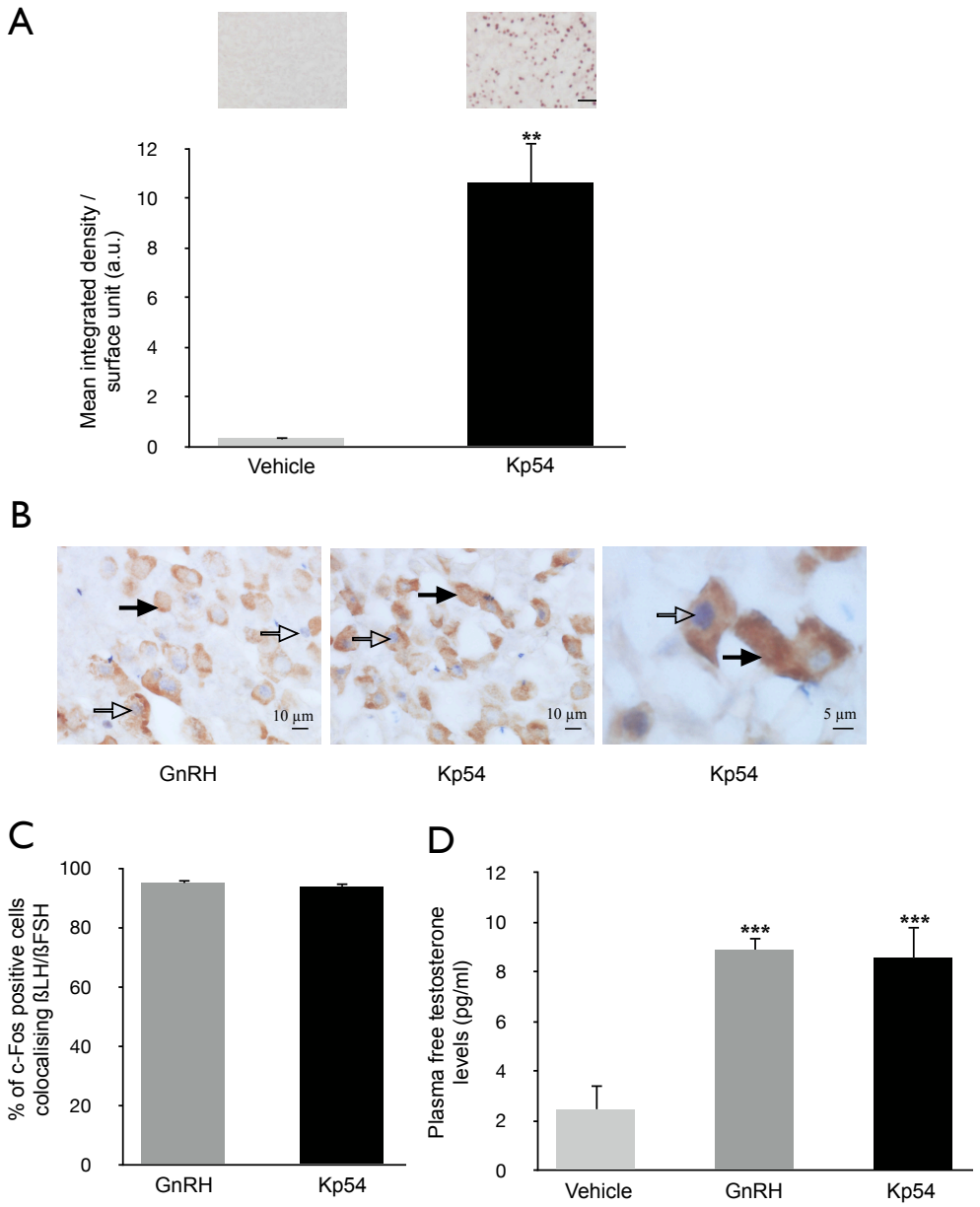

Figure 51: Effect of acute peripheral injection of Kp54 or GnRH on pituitary cFos expression and testosterone levels

A: Mean value of cFos immunoreactivity (arbitrary units, a.u.) in the pituitary of long day-adapted Syrian hamsters was analysed two hours after an intraperitoneal injection of vehicle (Ringer) or Kp54 (15 nmoles / hamster). Values are mean ± SEM (n = 5), ** $p < 0.01$ when compared to vehicle-injected hamsters (Student t test). *B*: Phenotype of cFos positive cells after GnRH or Kp54 intraperitoneal injection in the pituitary of Syrian hamsters. Representative images of dual immunohistochemistry for cFos (blue, open arrows) and ßLH/ßFSH (brown, black arrows) in the anterior pituitary of long day-adapted Syrian hamsters two hours after intraperitoneal injection of GnRH (30 nmoles / hamster) or Kp54 (15 nmoles / hamster). *C*: Proportion of cFos positive cells colocalising ßLH/ßFSH in the anterior pituitary of Syrian hamsters two hours after an intraperitoneal injection of GnRH (30 nmoles / hamster) or Kp54 (15 nmoles / hamster). Values are mean ± SEM (n = 3). *D*: Concentration of free plasma testosterone (pg/ml) two hours after vehicle (Ringer), GnRH (30 nmoles / hamster) or Kp54 (15 nmoles / hamster) intraperitoneal injection. Values are mean ± SEM (n = 5-6), *** $p < 0.001$ when compared to vehicle-injected hamsters (ANOVA followed by Tuckey post hoc analysis)

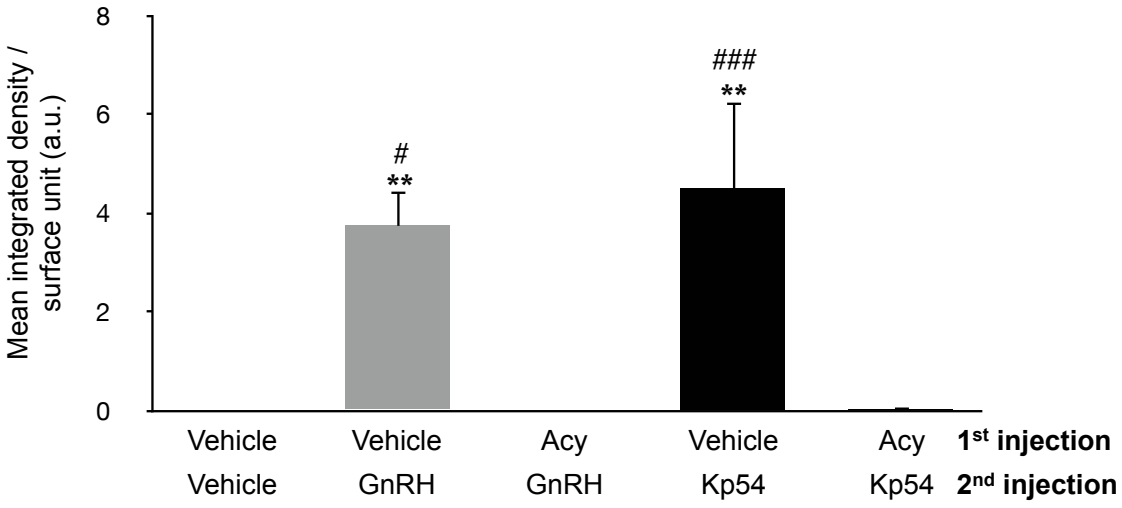

Figure 52: Effect of a GnRH antagonist on Kp-induced cFos expression in the pituitary

Mean value of cFos immunoreactivity (a.u.) in the pituitary of long day-adapted Syrian hamsters pretreated or not with acyline, a GnRH receptor antagonist (50 µg / hamster), then injected intraperitoneally with GnRH (30 nmoles / hamster) or Kp54 (15 nmoles / hamster) two hours before sacrifice. Values are mean ± SEM (n = 3-6), ** $p < 0.01$ when compared to vehicle-injected hamsters (ANOVA followed by Tuckey post hoc analysis); # $p < 0.05$, ### $p < 0.001$ when compared to acylin-pretreated hamsters (ANOVA followed by Tuckey post hoc analysis).

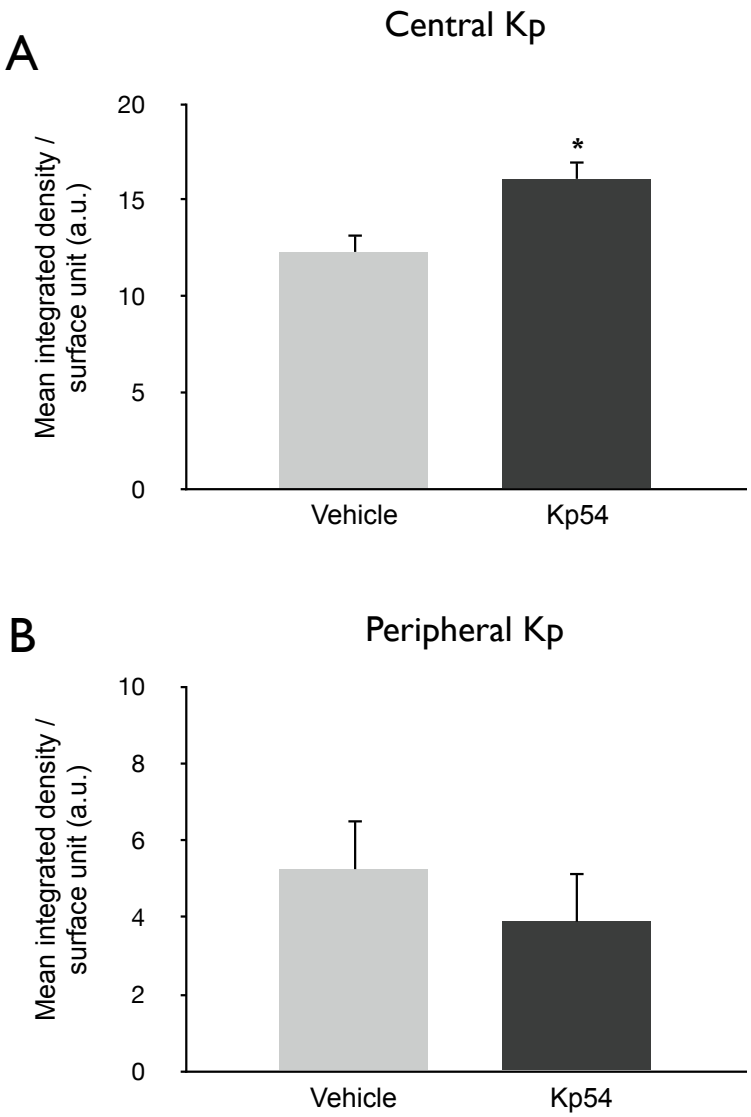

Figure 53: Effect of central of peripheral Kp on cFos expression in the preoptic area

A: Mean value of cFos immunoreactivity (arbitrary unit, a.u.) in the preoptic area of Syrian hamsters kept in long days injected centrally with vehicle or Kp54 (1 nmole / hamster) two hours before sacrifice. Values are mean ± SEM (n = 6), * $p < 0.05$, when compared to Kp54-injected LD hamsters (Student t test). *B*: Mean value of cFos immunoreactivity (arbitrary unit, a.u.) in the preoptic area of Syrian hamsters kept in long days injected intraperitoneally with vehicle or Kp54 (15 nmoles / hamster) two hours before sacrifice. Values are mean ± SEM (n = 6).

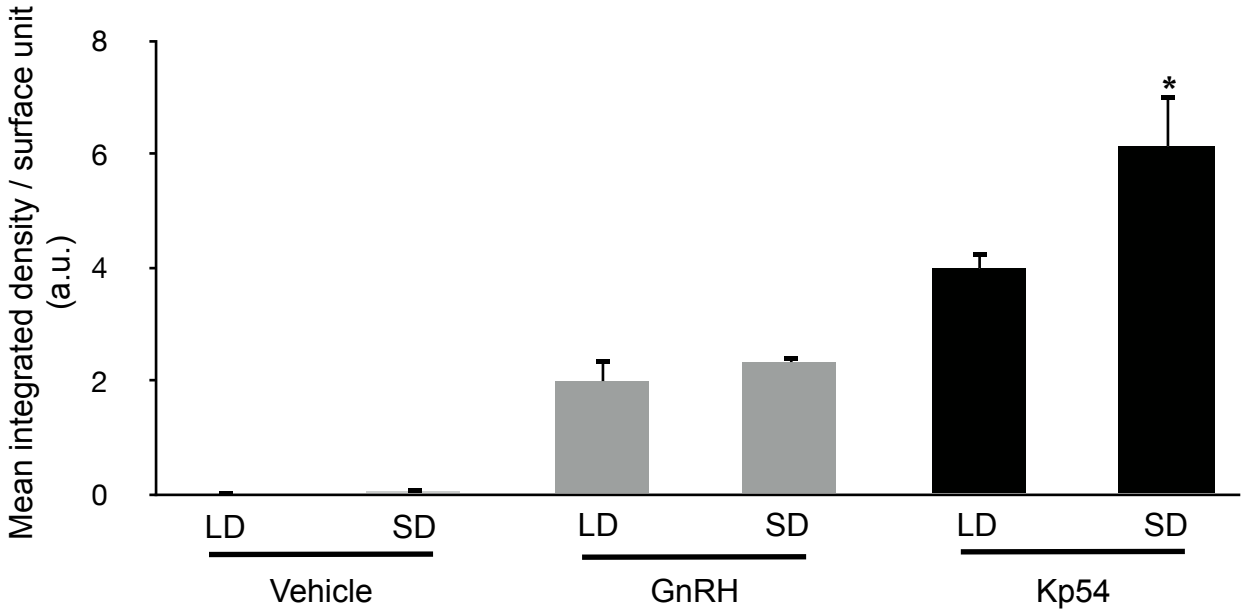

Figure 54: Effect of photoperiod on GnRH or Kp54-induced pituitary cFos

Mean value of cFos immunoreactivity (arbitrary unit, a.u.) in the anterior pituitary of Syrian hamsters kept in long (LD) or short days (SD) injected intraperitoneally with GnRH (30 nmoles / hamster) or Kp54 (15 nmoles / hamster) two hours before sacrifice. Values are mean ± SEM (n = 5-6), * $p < 0.05$, when compared to Kp54-injected LD hamsters (Student t test).

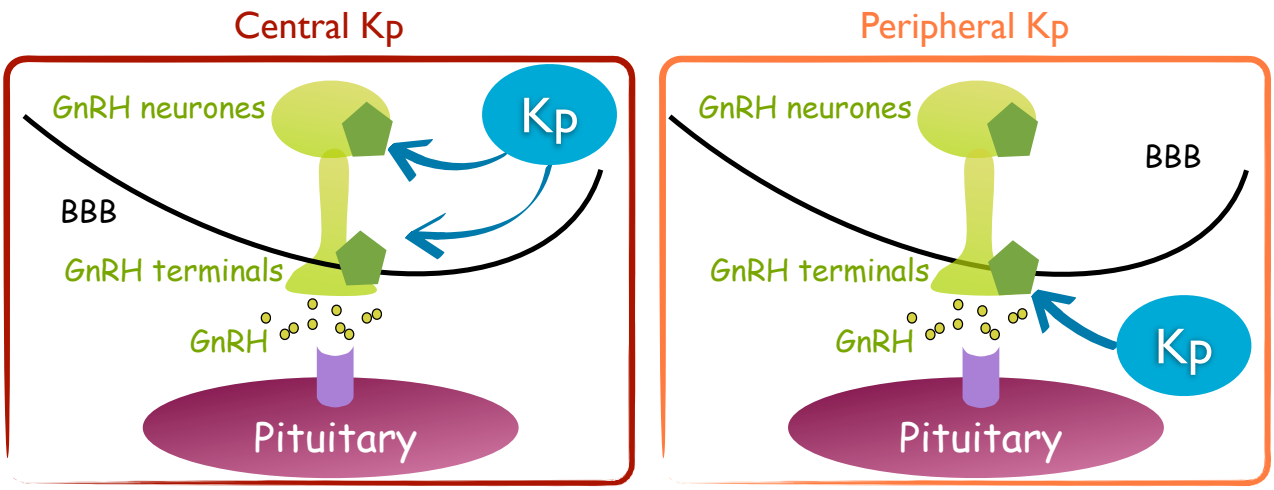

Figure 55: Central and peripheral exogenous Kp target different parts of the GnRH neurone

While central Kp would act directly on GnRH neurone cell bodies and/or nerve terminals, peripheral Kp effect on GnRH neurones appear restricted to nerve terminals located in the median eminence.

Chapter 6 - General discussion

I. Roles of kisspeptins in the seasonal reproduction

1. In Syrian hamsters

It is well established in the Syrian hamster that melatonin does not affect GnRH mRNA levels (Brown et al., 2001), the number and morphology of GnRH neurones (Urbanski et al., 1991) nor the pituitary responsiveness to GnRH (Pickard and Silverman, 1979). Thus, melatonin inhibits reproductive activity probably via a regulation of GnRH release. However, melatonin binding sites do not overlap GnRH neurones distribution and the pineal hormone acts via other neurones, *Kiss1* neurones, to control GnRH release.

a. Roles of ARC *Kiss1* neurones

i. ARC Kiss1 neurones integrate the melatonin signal

In Syrian hamsters, the number of neurones expressing *Kiss1* mRNA and Kp protein is markedly down-regulated by SD exposure in the ARC of both sexes (Figures 43 and 44). Several hypothesis can explain a reduction in Kp production in SD. First, this could be a consequence of the decreased levels of circulating sex steroids. However, Revel et al. demonstrated that photoperiodic changes in ARC *Kiss1* expression do not appear to be mediated by testosterone because low circulating levels of this inhibitory hormone in SD would result in an increased *Kiss1* expression, which is opposite to the observed reduction in the number of ARC *Kiss1* neurones (Revel et al., 2006b). According to the second hypothesis, melatonin would inhibit ARC *Kiss1* expression. Indeed, pinealectomy prior to SD transfer prevents the decrease in *Kiss1* expression (Revel et al., 2006b). Similarly pinealectomy in castrated SD-acclimated hamsters markedly increases *Kiss1* expression in the ARC (Figure 47) and long-term melatonin injections in LD-adapted hamsters inhibit *Kiss1* expression (Figure 48). These observations indicate that melatonin drives photoperiodic changes in *Kiss1* expression in the ARC via steroid-independent mechanisms. However, since the inhibitory effect of melatonin on *Kiss1* expression is much faster and more potent in absence of testosterone, it could indicate that low gonadal hormones levels may potentiate melatonin effect. Indeed, melatonin receptor density and/or affinity is probably altered by testosterone since this hormone affects the density of melatonin binding sites in the PT (Recio et al., 1998).

ii. ARC Kiss1 neurones mediate the melatonin signal to the reproductive axis

Kp reactivate the reproductive axis in SD sexually inactive hamsters. It indicates that Kp overpass melatonin inhibitory effect. Since *Kiss1* neurones are highly sensitive to melatonin, they appear to convey the inhibitory SD signal to GnRH neurones. However, how Kp controls GnRH seasonal release is not precisely known. Several evidence indicate that Kp probably act on the median eminence to do so. First, peripheral Kp long-term administration re-activates the HPG axis (Figure 49, a discussion regarding the mechanisms of Kp re-activating effects on the HPG axis can be found Chapter 5, section IV). Second, Kp acute injection strongly induces cFos expression in the pituitary (Figure 51) and increases LH and FSH plasmatic levels (Ansel et al., unpublished). This GnRH-dependent effect indicates that peripheral Kp trigger GnRH-release from a structure accessible to circulating factors, that is, located outside the blood brain barrier. Since the median eminence contains GnRH nerve terminals and is accessible to peripheral peptides, Kp probably act on the median eminence. Third, Kiss1r are expressed in the median eminence and exogenous Kp releases endogenous GnRH from MBH explants (d'Anglemont de Tassigny et al., 2008). Finally, peripherally given Kp do not induce cFos in GnRH neurones (Mikkelsen et al., 2009) whereas ICV Kp induce cFos expression in GnRH neurones cell bodies . This suggest that exogenous peripheral Kp act on GnRH nerve terminals.

As mentioned previously (Chapter 1, section III 2 d iv), while AVPV *Kiss1* neurones seem to directly contact GnRH cell bodies, ARC *Kiss1* neurones would rather project on GnRH nerve terminals of the median eminence (Figure 31). Hence, ARC *Kiss1* neurones appear to mediate melatonin effect on GnRH release from nerve terminals via Kp fibres present in the median eminence, and melatonin drives seasonal changes in the reproductive function via a marked effect on ARC *Kiss1* neurones.

c. Roles of AVPV *Kiss1* neurones

Melatonin does not alter AVPV *Kiss1* mRNA expression in gonadectomised animals (Figures 47 and 48), indicating that melatonin does not directly regulate *Kiss1* expression in the AVPV. Sex steroids being stimulatory in that structure, reduced *Kiss1* expression in

the AVPV upon SD exposure can be explained by the sole reduction in circulating sex steroids.

i. Sexual dimorphism of Kiss1 expression

Kiss1 expression is sexually dimorphic in the hamster's AVPV, females having a higher number of *Kiss1* neurones when compared to males (Figures 43 and 44) , as reported in other rodent species (Clarkson and Herbison, 2006; Gottsch et al., 2006; Adachi et al., 2007; Kauffman et al., 2007) (Figure 29). The stimulatory effect of sex steroid replacement and the inhibitory effect of gonadectomy on the number of *Kiss1* neurones in respectively SD- and LD-adapted male and female hamsters demonstrate the positive feedback effect exerted by gonadal hormones in the AVPV of the Syrian hamster as already reported in mice (Kinoshita et al., 2005; Smith et al., 2005a; Smith et al., 2005b).

The existence of a positive feedback of sex steroids on *Kiss1* neurones is of high importance in females. Across the oestrous cycle, oestrogens level varies considerably. From low levels in the beginning, oestrogens concentration progressively raises up to trigger the positive oestrogen feedback leading to the pre-ovulatory LH surge occurring in the evening of the pro-oestrous day. AVPV *Kiss1* neurones are thought to mediate the estradiol positive feedback on GnRH neurones because 1) they express ERα (Smith et al., 2005a; Smith et al., 2005b; Smith et al., 2006b), 2) *Kiss1* expression in the AVPV is increased, along with cFos, concomitantly with the pre-ovulatory LH surge (Smith et al., 2006b; Robertson et al., 2009), 3) central infusion of Kp antiserum or antagonist blocks the pre-ovulatory LH surge (Kinoshita et al., 2005; Pineda et al., 2010) and 4) *Kiss1* KO mice fail in generating an LH surge in response to estradiol treatment (Clarkson et al., 2008).

ii. Kisspeptins and the circadian timing of ovulation

In rodents, the pre-ovulatory LH surge is known to be gated by the circadian clock in the SCN. Indeed SCN lesion abolishes oestrous cyclicity as well as the pre-ovulatory LH surge in females, and the daily LH surge in ovariectomised estradiol-treated females (Stetson and Watson-Whitmyre, 1976; Brown-Grant and Raisman, 1977; Wiegand et al., 1980; Ronnekleiv and Kelly, 1988). Since discrete knife cuts dorsocaudal to the SCN disrupt circadian LH secretion and reproductive cycles in rats (Watts et al., 1989), it is

believed that specific efferent neuronal fibres from the SCN convey the output signal for this function. This hypothesis is confirmed in hamster since SCN grafts (Meyer-Bernstein et al., 1999) fail to restore circadian LH secretion. AVP signalling seems to be involved in the circadian regulation of reproductive cycles because AVP receptor V1a mRNA is expressed in the female rat POA (Kalamatianos et al., 2004), AVP induces an LH surge when it is administered ICV to ovariectomised estradiol-treated rats (Palm et al., 1999; Palm et al., 2001), and AVP rhythmic release may drive GnRH secretion in SCN/ POA co-cultures in vitro (Funabashi et al., 2000). Beside AVP, VIP also seems to mediate circadian rhythmicity to GnRH neurones because VIP-containing SCN neurones directly innervate GnRH neurones (van der Beek et al., 1993; van der Beek et al., 1994), GnRH neurones express the VIP receptor VPAC2 (Smith et al., 2000), and central administration of antisense mRNA (Harney et al., 1996) or antiserum (van der Beek et al., 1999) to VIP delays and diminishes the LH surge in ovariectomised estradiol-treated rats. Interestingly, the two peptides are synthesised in separate dorsal and ventral subdivisions of the SCN, respectively (see Chapter 1, section I 1 a) and circadian oscillations between subdivisions can be functionally dissociated (Shinohara et al., 1995; Nagano et al., 2003; de la Iglesia et al., 2004; Albus et al., 2005; Nakamura et al., 2005), suggesting that AVP and VIP might represent differentially timed neural signals to the HPG axis. One can speculate that AVP SCN neurones would innervate AVPV *Kiss1* cells whereas VIP signalling would target GnRH neurones.

Although direct connexions exist between SCN and GnRH neurones, the AVPV, which is interposed between the SCN and MPO (Watson et al., 1995), has been proposed as an integrative centre for oestrogen and circadian signals (Petersen et al., 2003). Indeed, ERα are highly expressed in this nucleus and appear to be the ER subtype critical for the positive feedback effects of oestrogens and the occurrence of the LH surge. As mentioned in the previous section, AVPV contains *Kiss1* neurones and they are thought to convey circadian rhythmicity to GnRH neurones. Indeed it was recently shown that AVPV *Kiss1* neurones of estradiol-treated female mice display a significant circadian pattern of *Kiss1* expression and cFos expression in *Kiss1* neurones in direct synchrony with the circadian timing of LH secretion. Interestingly, the circadian activation of AVPV/PeN *Kiss1* neurones is dependent on the presence of estradiol (Robertson et al., 2009). It is therefore tempting to speculate that the AVPV neurones contacted by SCN neurones are, at least partially, *Kiss1* neurones. However, it is possible that ERα expressing neurones in the AVPV do not all co-localise *Kiss1* mRNA.

Additionally, GnRH neurones show immunoreactivity for PER1 (Olcese et al., 2003) and the GT1–7 immortalised GnRH cell line exhibits circadian rhythms of clock gene expression after the cells are synchronised (Chappell et al., 2003; Gillespie et al., 2003; Olcese et al., 2003). Importantly, these cells also have a circadian rhythm of GnRH mRNA expression (Gillespie et al., 2003), and GnRH pulsatility is disrupted after transient transfection of the cells with the dominant-negative mutant CLOCK protein from homozygous clock mutant mice (Chappell et al., 2003). The precise role of clock gene expression in GnRH neurones in regard to the pre-ovulatory LH surge remains to be determined as well as how this observation fits in with the crucial role played by Kp.

c. Roles of kisspeptins' target structures in seasonal reproduction

In addition to the ARC and the AVPV, Kp-immunoreactive fibres have been identified in several brain regions including the BNST and the PVT (Figure 39). Kp ICV acute injection also induces cFos expression in these nuclei (Figure 40) suggesting that Kp fibres release the peptides in these structures. Although the identity of Kp cellular targets in the BNST and PVT remains to be determined, both structures seem involved in seasonal reproduction.

i. Role of the BNST

BNST bilateral lesions in the Syrian hamster inhibit SD-induced testicular regression (Raitiere et al., 1997). If the lesion is performed when the animals are already sexually inactive, this does not affect subsequent photoperiodic responses, suggesting that BNST lesions interrupts the transmission of the inhibitory signal to the reproductive axis (Raitiere et al., 1997). The important density of Kp fibres observed in that brain region supports this hypothesis.

The BNST is part of the limbic system and is thus involved in the response to stress and anxiety. The BNST is also involved in the control of several social- and reproduction-related behaviours: scent marking, vocalisation, parental behaviours, sexual behaviours, pair-bonding and mate choice, offensive aggression, social recognition (Goodson and Bass, 2001; Simerly, 2002). One can thus hypothesise that these behaviours may be regulated via a dense BNST Kp innervation.

Because BNST is not a target structure of melatonin in hamsters, the inhibitory SD signal on reproductive-related behaviours could be mediated by Kp. In addition, BNST expresses sex steroid receptors that were directly incriminated in BNST physiological roles. ERα seems to be important in regulating reproductive behaviours of the BNST such as mating and parental care (Ogawa et al., 1997; Champagne et al., 2006; Trainor et al., 2007). Melatonin could control BNST-related reproductive behaviours via seasonal changes in circulating gonadal hormones levels and Kp innervation (see Chapter 3, section III 3).

Additionally, the BNST projects to GnRH neurones in rats (Simerly and Swanson, 1986; Polston et al., 2004) and sheep (Pompolo et al., 2005). Thus BNST might take part in the regulation of GnRH and gonadotropin secretion (Beltramino and Taleisnik, 1980; Raitiere et al., 1997). Kp innervation in the BNST can thus constitute an alternative pathway enhancing Kp effects on GnRH neurones (Figure 54).

ii. Role of the PVT

In addition to BNST, PVT also displays a dense Kp innervation. PVT is a multi-sensorial structure receiving inputs from different nuclei from the brain stem, as well as the prefrontal cortex, the amygdala and many hypothalamic cell groups including the SCN (Bentivoglio et al., 1991; Groenewegen and Berendse, 1994). Efferents from the SCN project to the posterior PVT (Watts and Swanson, 1987; Watts et al., 1987). The reciprocal connexions of PVT with the SCN, along with the inputs to the PVT from other components of the circadian system, suggest that PVT might play a role in the regulation of circadian rhythms. However, to date, such a role has not been elucidated.

Interestingly, this structure contains melatonin binding sites (Weaver et al., 1989; Bittman and Weaver, 1990; Recio et al., 1996) and is involved in the response to photoperiod changes (Teubner and Freeman, 2007). Although the phenotype of Kp-innervated and melatonin receptor-expressing cells remains unknown, one can hypothesise that the kisspeptinergic and melatoninergic signals converge to that particular structure to reinforce the inhibitory effect of SD on the reproductive axis. Interestingly, the PVT are known to send neuronal efferences to the ARC.

iii. Roles of kisspeptins target structure: conclusion

The Kp fibres wide distribution does not support the idea of an exclusive effect of Kp on GnRH neurones. Noteworthy, Kp long-term administration is more potent to reactivate the photo-inhibited HPG axis when given centrally (ICV) than when given peripherally (IP) (Compare Revel et al., 2006 and Figure 49). This can be explained by peripheral degradation of the peptides,. Alternatively, central but not peripheral Kp reach other targets than GnRH neurones and this would potentialise the peptides effects on the reproductive axis. The cellular phenotype of Kp targets remains to be determined as well as the origin of Kp fibres (ARC versus AVPV). Castration and sex steroid supplementation having opposite effects on ARC and AVPV *Kiss1* neurones, such experiments could clarify the origin of Kp fibres in each target structures.

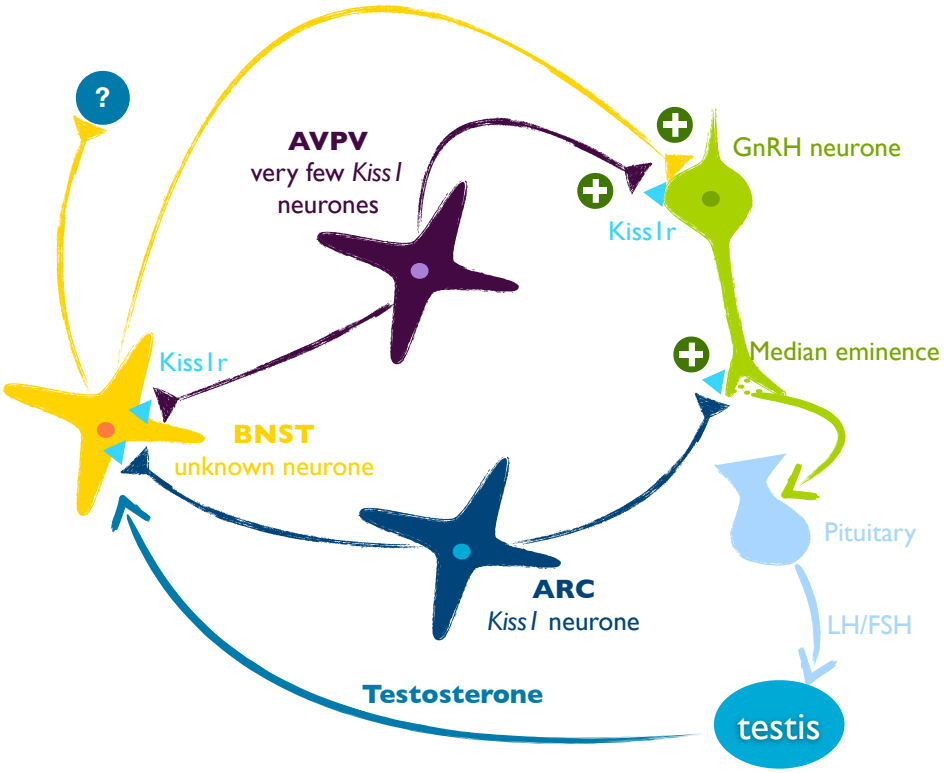

Figure 56: Role of BNST as mediator of Kp effect on GnRH neurones

ARC and/or AVPV Kiss1 neurones project to the BNST on yet unknown neurones. BNST sends efferent projections to GnRH neurones. As a consequence, BNST neurones could constitute an alternative pathway potentialising Kp effects on GnRH secretion.

d. Roles *Kiss1* neurones in the Syrian hamster: conclusion

Taken together, our data suggest a differential photoperiodic regulation of *Kiss1* expression in the ARC and AVPV of the Syrian hamster (Figure 57). In SD conditions, the increased production of melatonin would inhibit *Kiss1* expression in the ARC and this is sufficient to inhibit gonadal activity. Consequently, the decrease in circulating sex steroid would lift their stimulatory feedback effects on the AVPV neurones, hence decreasing *Kiss1* expression.

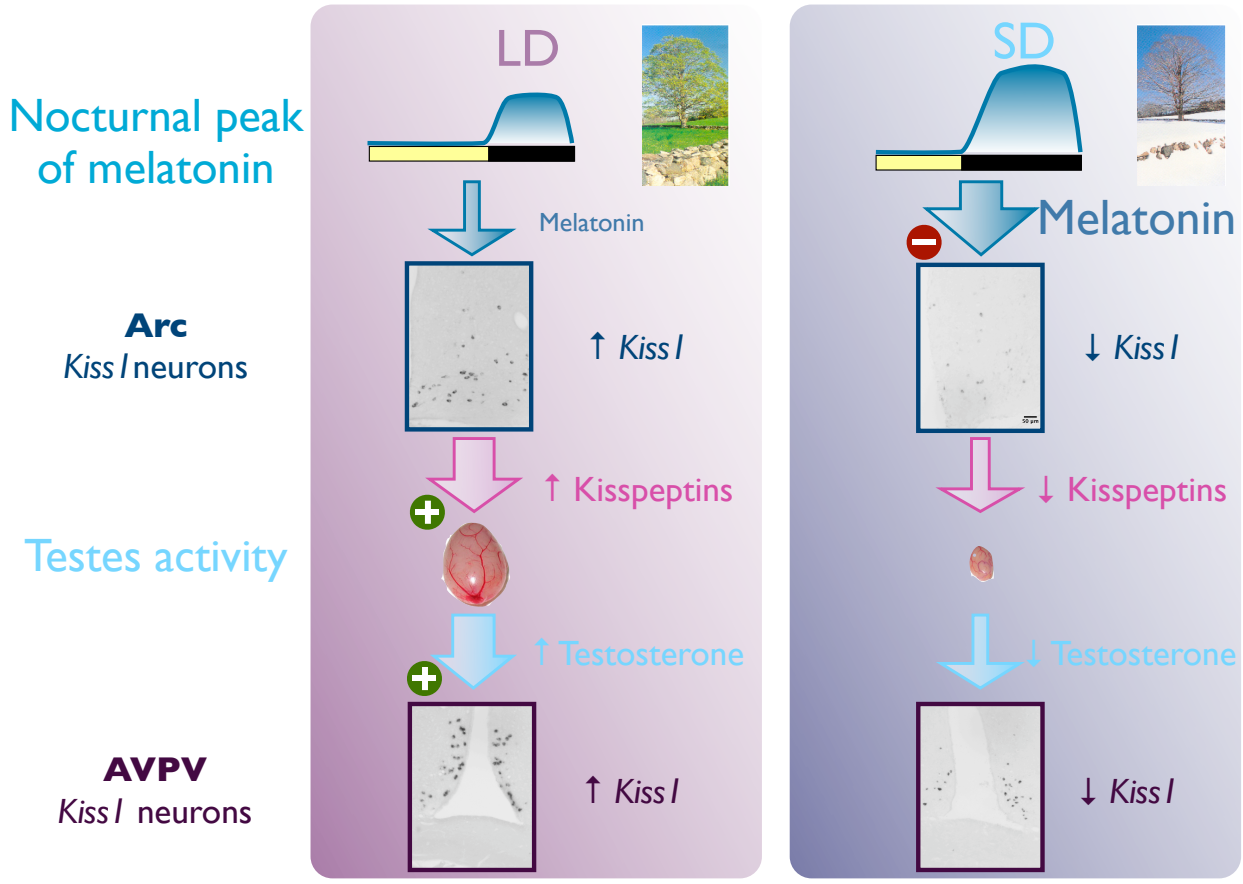

Figure 57: Model of the regulation of *Kiss1* expression by melatonin

In SD conditions, the increased production of melatonin inhibits *Kiss1* expression in the ARC and this is sufficient to inhibit gonadal activity. Consequently, the decrease in circulating sex steroids levels lift their stimulatory feedback effects on the AVPV neurones, hence decreasing *Kiss1* expression in that structure.

2. In Djungarian hamsters

Besides the Syrian hamster, photoperiodic variations in *Kiss1* expression (Simonneaux et al., 2009) and Kp immunoreactivity (Greives et al., 2007; Mason et al.,

2007) have been reported in another seasonal rodent, the Djungarian hamster. However, in contrast to the Syrian hamster, *Kiss1* expression in the ARC is decreased in LD, when animals are sexually active (Greives et al., 2007; Simonneaux et al., 2009) (Figure 58). This suggests that the inhibitory effect of melatonin on the reproductive axis does not involve ARC *Kiss1* neurones. Rather, these neurones seem exclusively regulated by sex steroids in Djungarian hamsters.

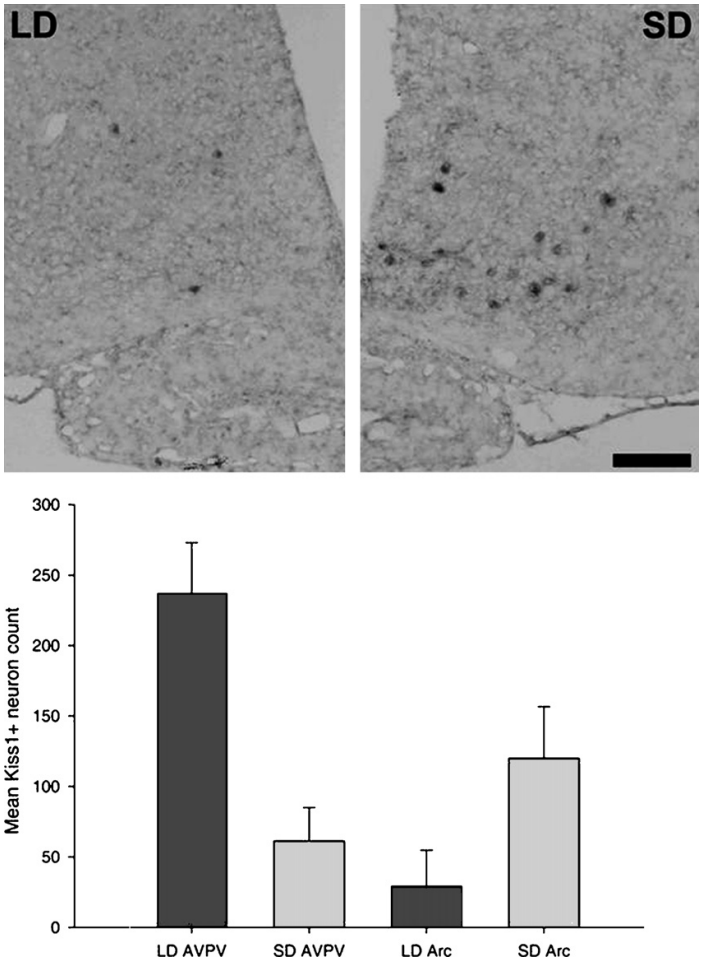

Figure 58: Photoperiodic variations of *Kiss1* expression in the Djungarian hamster

Top: non-radioactive in situ hybridisation of *Kiss1* mRNA in the ARC of adult male Djungarian hamster raised under long (LD) or short (SD) days (scale bar = 100 µm). Bottom: Number of *Kiss1* neurones in the AVPV or ARC in male Djungarian hamsters raised under LD or SD. Simonneaux et al., 2008

Such discrepancies may reflect species differences in melatonin sites of action for the control of seasonal reproduction. Indeed, in Djungarian hamsters, SCN lesion prevents the inhibitory effect of exogenous melatonin infusions (Bartness et al., 1991), whereas its lesion does not compromise responsiveness to exogenous melatonin in Syrian hamsters (Bittman et al., 1989), suggesting that in the former species, the SCN

may mediate melatonin effects on the reproductive axis. In rodents, the SCN sends AVP projections to AVPV neurones (Kalsbeek and Buijs, 2002) and regulates *Kiss1* neurones' activity in mice AVPV (Robertson et al., 2009). Therefore, AVPV *Kiss1* neurones might mediate the effect of melatonin on the reproductive axis in the Djungarian hamster. This hypothesis is supported by the important number of Kp expressing neurones in the Djungarian hamster's AVPV when compared to the ARC, and the inhibitory effect of SD on AVPV *Kiss1* neurones (Greives et al., 2007; Simonneaux et al., 2009). The observation that melatonin could act on GnRH production in that species (Porkka-Heiskanen et al., 1997; Bernard et al., 1999) reinforces the idea that it is AVPV *Kiss1* neurones that mediate melatonin effect, since AVPV *Kiss1* neurones are thought to act directly on GnRH cell bodies.

Peripheral Kp injection increases gonadotropin release in both LD and SD-acclimated male Djungarian hamsters, demonstrating that the GnRH system remains equally sensitive to Kp regardless of reproductive condition (Greives et al., 2007). By contrast, similar Kp injections result in higher cFos expression in the pituitary (and putative gonadotropin levels) in SD than in LD-acclimated Syrian hamsters, indicating that the GnRH system seems more sensitive to Kp in SD in that species. Such discrepancies cannot be explained by the fact that the dose of Kp used in the Djungarian hamster may have been too high and mask potential subtle differences in hypothalamic sensitivity to the peptide. Indeed a dose of 2 nmoles/100g was used in Djungarian hamsters which is much lower to the 15 nmoles/100g used in Syrian hamsters. Hence, Djungarian hamsters, contrary to Syrian hamsters, do not seem to exhibit seasonal variations in GnRH neurones sensitivity to Kp in males. In female Djungarian hamsters, not only is Kp expression altered in the AVPV and the ARC in response to changes in day-length, but the sensitivity of the GnRH system to this peptide is also changed since females with a regressed reproductive axis do not respond to peripheral Kp injections with an elevation in LH concentrations. Females appear to have evolved a mechanism to ensure that pregnancy will not occur during inappropriate times of year.

Another considerable difference between Syrian and Djungarian hamsters, is that daily IP injection of Kp10 fails to re-activate the HPG axis in photo-inhibited Djungarian hamsters (Greives et al., 2008a) contrary to the Syrian hamster. Several hypothesis can be elaborated. First, doses used in Djungarian hamsters were much lower than that used in the Syrian hamster. The absence of Kp effect in Djungarian hamsters may be due to the fact that only one injection per day was performed and this may not allow sufficient

activation of the reproductive axis. Second, two daily injections were necessary to re-activate the reproductive axis in Syrian hamsters. Finally, Kp may not play the same role as in the Syrian hamsters. In the latter species, Kp are crucial for the seasonal cycles of reproduction. In Djungarian hamsters, melatonin may involve other pathways such as thyroid hormones (see Chapter 6, section II 2), Kp playing a necessary but not sufficient role.

Finally, Djungarian, but not Syrian hamsters display marked seasonal variations in body weight. Interestingly the ARC monitors current energy state and relays this information to the reproductive axis (Smith and Grove, 2002). In times of reduced energy availability, reproduction is inhibited (Bartness, 1996; Schneider et al., 2000). ARC *Kiss1* neurones respond to various metabolic signals such as leptin (Smith et al., 2006a). It is possible that they are altered in response to energy status in addition to modulation through negative feedback in response to sex steroids.

3. In a short-day breeder: the sheep

Kp also drive seasonal cycles of the reproductive function in sheep. In the ewe, there is a marked seasonal change in the negative feedback effect of oestrogen with season, and this is fundamental for the transition from breeding (SD) to non-breeding (LD) season and vice versa (Legan et al., 1977). Since Kp neurones possess ERα, they constitute excellent candidates for the transmission of the negative feedback effect of oestrogen, and thus the transmission of the melatoninergic signal, to GnRH cells.

Interestingly, like in the Syrian hamster, *Kiss1* mRNA levels are strongly increased in the ARC of ewes during the breeding season (Wagner et al., 2008), and this effect does not only involve sex steroids because *Kiss1* expression is also reduced during the non-breeding season in ovariectomised estradiol-treated ewes (Smith et al., 2008b). Similarly, Kp immunoreactivity in the POA is increased in SD (Chalivoix et al., 2009). The increase in *Kiss1* expression in the POA and ARC is due to increased peptide synthesis, as attested by colchicine experiments (Chalivoix et al., 2009).

In sheep, two sub-populations of *Kiss1* neurones can be distinguished in the ARC. First, the rostral and mid-ARC contain *Kiss1* neurones that are negatively regulated by sex steroids (Smith et al., 2008b). Second, caudal ARC contains *Kiss1* neurones involved in the oestrogen positive feedback (Estrada et al., 2006). Interestingly, oestrogen treatment reduces *Kiss1* expression in both LD and SD in the rostral ARC. However, in the caudal

ARC, estradiol inhibits *Kiss1* expression only during the non-breeding season (Smith et al., 2008b). This suggests that caudal ARC *Kiss1* neurones can mediate both the estradiol positive feedback effect during the breeding season and the negative feedback effect during the non-breeding season. In sheep, melatonin acts on the pre-mammillary hypothalamic area to control the seasonal reproduction (see Chapter 1, section II 3 b) (Malpaux et al., 1998; Sliwowska et al., 2004). It is not yet clearly established how this inhibits GnRH release during the non-breeding season but it is interesting to note that in ovine's brain, the pre-mammillary area contains the caudal part of the ARC.

Kp cells project directly to GnRH neurones. Dual immunocytochemistry studies determined that the number of Kp-immunoreactive terminals that appear to directly contact GnRH neurones is higher in the breeding season than in the non-breeding season (Smith et al., 2008b). The data obtained to date indicate that *Kiss1* neurones are likely mediators of the seasonal change in oestrogen feedback, but whether these cells are direct targets for melatonin is not clear yet.

Acyclicity in ewes during the non-breeding season is associated with reduced Kp function in the ARC. Interestingly, intravenous infusion of Kp elevates gonadotropin secretion and causes ovulation during the non-breeding season (Caraty et al., 2007). It indicates that Kp stimulate GnRH/LH secretion during the non-breeding season which then activates the ovaries, leading to initiation of positive feedback circuits in the brain and subsequent induction of pre-ovulatory LH surges.

II. Melatonin and seasonal reproduction: complementary hypotheses

To date, no melatonin receptors have been identified in the ARC or the AVPV of the Syrian hamster, thus a direct effect of melatonin on *Kiss1* neurones appears unlikely and it is currently hypothesised that melatonin is acting on intermediate neurones to transmit the photoperiodic message to the *Kiss1* neurones and downstream structures of the reproductive axis.

1. The RF-amide related peptides hypothesis

Melatonin sites of action for the seasonal control of reproduction in the Syrian hamster have been found in the MBH, an area comprising the DMH. This region contains

melatonin binding sites and its lesion prevents the SD-induced gonadal regression (Maywood and Hastings, 1995) and abolishes hamsters' ability to respond to exogenous melatonin (Reiter and Sorrentino, 1972; Maywood et al., 1996) (see Chapter 1, section II 3 a). It was recently reported that neurones in hamsters' DMH/VMH express the gene encoding RFamide-Related Peptides (RFRP) with a melatonin-driven inhibition in SD conditions (Revel et al., 2008). The *rfrp* gene codes for a preproprotein which is able to generate three mature peptides: RFRP-1, RFRP-2 and RFRP-3, although RFRP-2 is not expressed in rodents (Hinuma et al., 2000; Fukusumi et al., 2006). These peptides belong the large RF-amide family. In this family, there is a group of peptides whose members share a LPXRF-amide (X = L or P) C-terminal. In 2000, the first LPXRF-amide peptide was identified in the Japanese quail (*Coturnix japonica*) and termed Gonadotropin Inhibitory Hormone (GnIH). GnIH was shown to inhibit, in a dose-dependent manner, gonadotropin release from cultured quail anterior pituitaries (Tsutsui et al., 2000). The *rfrp* gene is the mammalian ortholog of the avian *gnih* gene. The peptides RFRP-1 and RFRP-3 were later isolated and sequenced in the rat and were found to be highly expressed in the hypothalamus, notably in the DMH (Hinuma et al., 2000; Yano et al., 2003). In rats, RFRP-1 stimulates prolactin release and plays a role in nociception (Liu et al., 2001) and RFRP-3 reduces plasma levels of LH and increases growth hormone (Johnson et al., 2007). Recently, a central inhibitory effect of RFRP-3 on the HPG axis specifically during the estradiol-induced GnRH/LH surge was demonstrated in rats (Anderson et al., 2009). Similar inhibitory effect of RFRP-3 on LH secretion were observed in ovine and bovine (Clarke et al., 2008; Kadokawa et al., 2009).

Whether RFRP peptides are involved in the direct control of gonadotropin secretion from the pituitary or via a control on GnRH neurones in mammals is not yet clear. RFRP-3 immunoreactive fibres exist in the external median eminence of the sheep (Clarke et al., 2008) and RFRP-3 immunoreactive fibres are found in close proximity to most GnRH neurone cell bodies in rats (Johnson et al., 2007) and sheep (Smith et al., 2008b). A recent study in mice supports a role for RFRP-3 in the regulation of mammalian gonadotropin secretion via the modulation of GnRH neurone activity (Ducret et al., 2009). Hence, it is possible that substantial species differences exist in regard to the role of LPXRF-amides at the pituitary. The receptor for RFRP peptides is NPFF1. There are two subtypes of NPFF receptors: NPFF1 and NPFF2. NPFF2 has been associated with pain modulation and opiate tolerance (Simonin et al., 2006). In rats, NPFF1 is expressed in the

periacqueductal grey matter, DMH and VMH, supraoptic nucleus, ARC, PVT and PeN (Gouarderes et al., 2002).

Interestingly, the *rfrp* mRNA is also expressed in the DMH/VMH region in the Syrian hamster. In this species, DMH contains melatonin receptors that are important to relay the melatonin signal to the reproductive axis (see Chapter 1, section II 3 a). In Syrian hamsters, the *rfrp* gene is modulated by photoperiod, with very low mRNA levels and fewer immunoreactive cell bodies in SD compared to LD (Revel et al., 2008) (Figure 59). Pinealectomised hamsters fail to show a SD-induced decline in *rfrp* mRNA level and injections of LD hamsters with melatonin provoke inhibition of *rfrp* expression down to SD levels, indicating that melatonin mediates the photoperiodic regulation of *rfrp* expression (Revel et al., 2008) (Figure 60). Considering that the DMH/VMH is the key region mediating the photoperiodic effects of melatonin on reproduction, it is hypothesised that melatonin act on *rfrp* neurones in the DMH/VMH to regulate the reproductive axis maybe via an action on ARC *Kiss1* neurones.

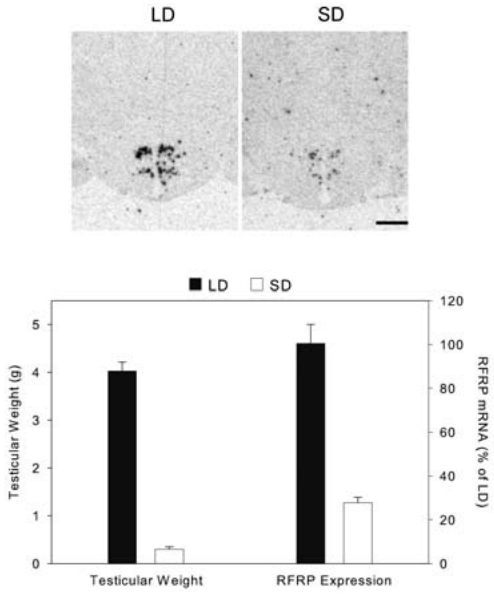

Figure 59: Photoperiodic variations of *rfrp* expression in the Syrian hamster

Top: in situ hybridisation of *rfrp* mRNA in the DMH of adult male Syrian hamsters raised under long (LD) or short (SD) days . Bottom: Testicular weight and *rfrp* mRNA expression levels in the DMH of male Syrian hamsters raised under LD or SD. Revel et al., 2008

Recent unpublished data indicate that RFRP-3 exerts a stimulatory effect on gonadotropin release in Syrian hamsters (Ancel et al., unpublished) which is surprising in light of the above-mentioned studies carried out in other species. Hence, it is possible that substantial species differences exist in regard to the role of LPXRF-amides.

Stimulatory effect of RFRP on the HPG axis is further demonstrated by the observation that acute ICV injections of the peptide to SD hamsters induces cFos expression in the ARC where most *Kiss1* neurones are found (Ancel et al., unpublished). In addition, RFRP-3 induces cFos expression in the PVT which has been shown to express NPFF1 (Gouarderes et al., 2002) and which sends neuronal projections to the ARC (Van der Werf et al., 2002). Eventually, RFRP-3 chronic administration to SD photo-inhibited male Syrian hamsters significantly increase testes weight and the number of ARC *Kiss1* neurones. The effect of RFRP-3 on *Kiss1* neurones does not involve circulating sex steroids because if so, the increased levels of testosterone would inhibit *Kiss1* expression in the ARC. Whether the effect of RFRP-3 is direct or involves other interneurone(s) remains to be determined. However, strong neuronal connexions exist between the DMH and the ARC and, most importantly, NPFF1 (RFRP-3 receptor) is expressed in this brain region. Dual labelling for *Kiss1* and NPFF1 should assess that issue. Interestingly, the presence of Kp immunoreactive fibres in the DMH (Figure 39) and the ability of central Kp to induce cFos expression in that structure (Figure 40) suggest the existence of a feedback exerted by (ARC ?) *Kiss1* neurones on *rfrp* cells.

These data enabled us to elaborate a working model (Figure 61) according to which, melatonin would repress *rfrp* expression. Reduced RFRP signalling to ARC *Kiss1* neurones would induce a dramatic reduction in Kp production causing the gonadal atrophy observed in SD. Studies are currently in progress to test this hypothesis.

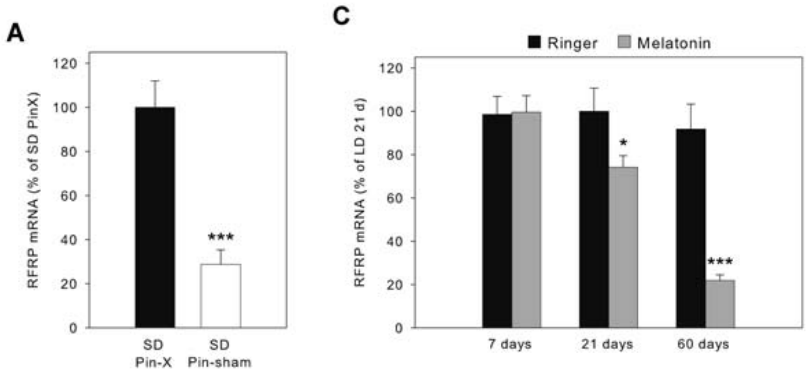

Figure 60: Regulation of *rfrp* expression by melatonin in the Syrian hamster

A, SD-induced reduction of *rfrp* expression was prevented by ablating the pineal gland of Syrian hamsters (Pin-X), relative to sham-operated animals (Pin-sham). Animals underwent surgery before being transferred from LD to SD, and were killed 10 weeks later. C, Groups of Syrian hamsters held in LD were injected daily with ringer (Ringer-5% ethanol) or melatonin (50 µg in ringer-5% ethanol) 1.5 h before lights off. Animals were treated for 7, 21 or 60 days. Quantification of *rfrp* mRNA level showing that hamsters injected with melatonin for 21 and 60 days had *rfrp* expression significantly lower than vehicle-treated animals. Data are expressed as percentage of the maximum value observed for the group ringer 21 days, and represent the mean +/- SEM. *, $P < 0.05$; ***, $P < 0.001$. Revel et al., 2008

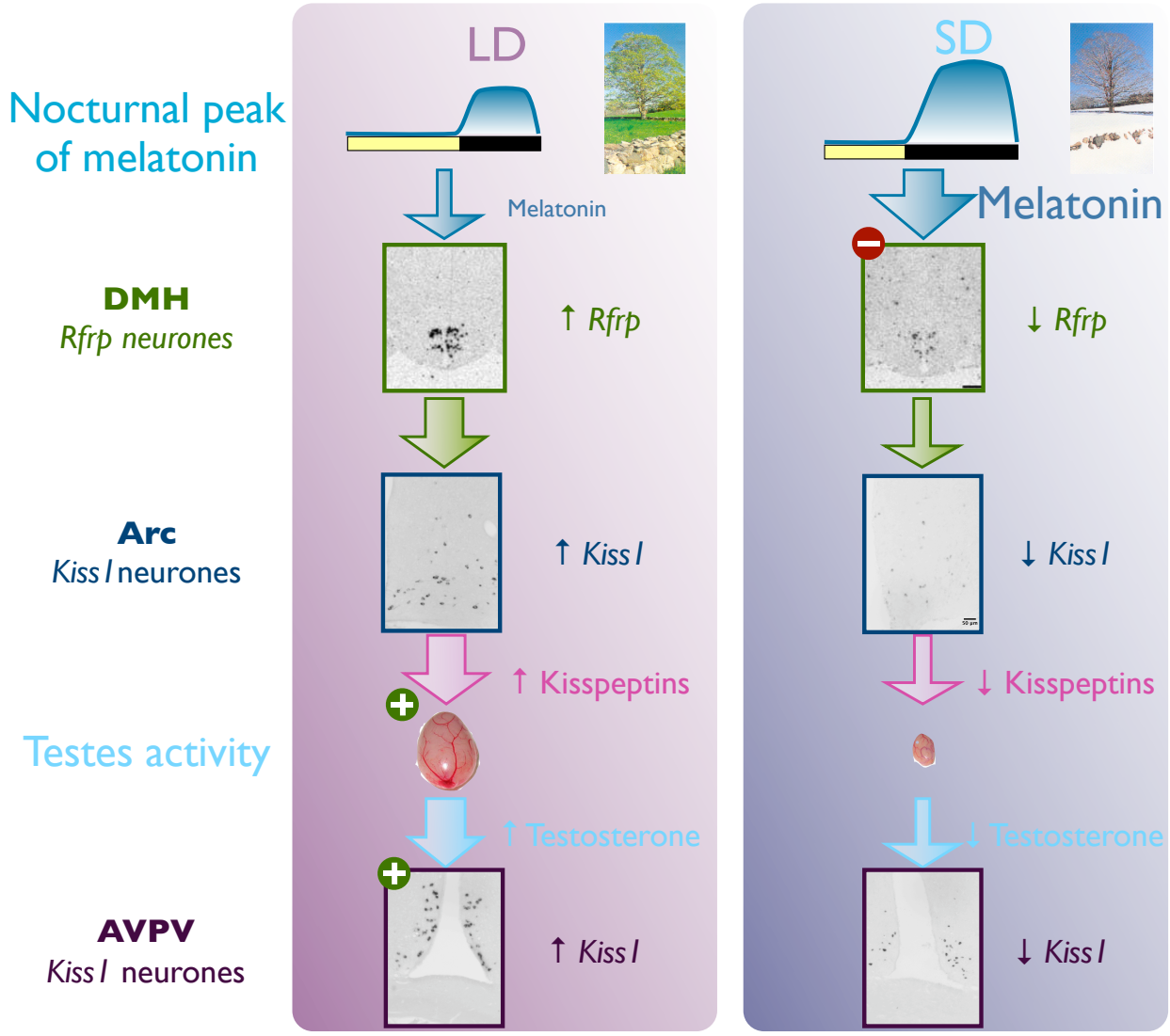

Figure 61: A model of melatonin action on the reproductive axis of the Syrian hamster

According to this working model, in SD, melatonin would inhibit *rfrp* expression in the DMH, resulting in a decreased expression of *Kiss1* mRNA in the ARC. The consecutive inhibition of the reproductive axis leading to a dramatic drop in testosterone levels, AVPV *Kiss1* mRNA levels are reduced as well in inhibitory SD.

2. The thyroid hormones hypothesis

Another target of melatonin for the seasonal control of reproduction involves thyroid hormones. Indeed, thyroid hormones regulate the reproductive function in several species of LD or SD-breeders (Freeman et al., 2007).

a. Introduction to thyroid hormones

Thyroid hormones control various physiological functions from growth to metabolism. Thyroid hormone synthesis and release is controlled by the hypothalamic-pituitary thyroid axis (Figure 61). Hypothalamic neurones located in the PVN synthesise thyroid-releasing hormone (TRH) which is secreted into the hypophyseal portal system. In response, thyroid stimulating hormone (TSH) is secreted from the thyrotrophs of the anterior pituitary into the blood stream. TSH reaches the thyroid gland where it stimulates the synthesis and the release of thyroid hormones (Yen, 2001).

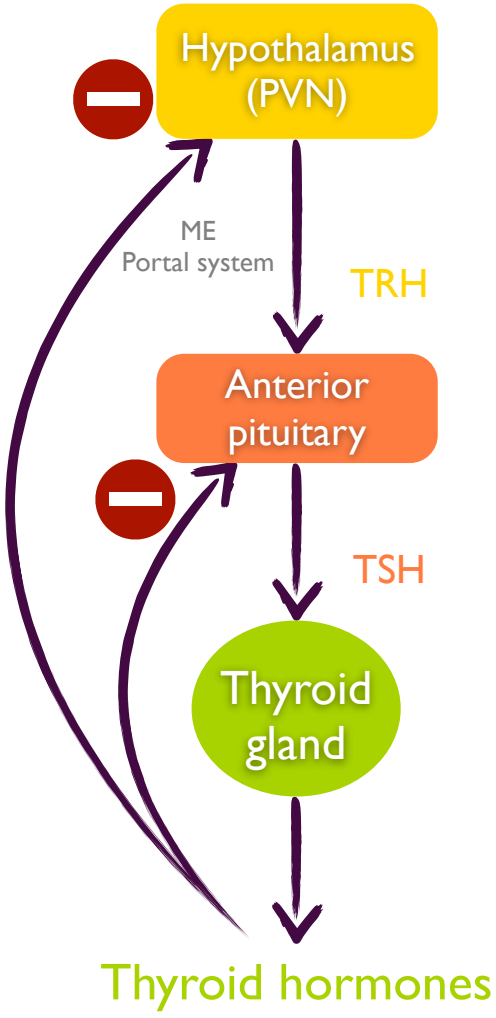

Figure 61: The hypothalamic pituitary thyroid axis

Hypothalamic neurones located in the PVN synthesise thyroid-releasing hormone (TRH) which is secreted into the hypophyseal portal system. In response, thyroid stimulating hormone (TSH) is released into the blood stream where it reaches the thyroid gland and stimulated the secretion of thyroid hormones.

Thyroid hormones are synthesised in the thyroid gland from the amino acid tyrosine and the thyroid hormones family comprises thyroxin (T4), triiodotyronine (T3), reverse T3 (r-T3) and diiodotyronine (T2). The thyroid gland mainly releases T4 but in target organs, T4 is de-iodinated into T3 which is the most biologically active thyroid hormone. They are generated by a sub-set of seleno-enzymes called deiodinases 1, 2 or 3 (dio1, 2 or dio3) (Bianco et al., 2002; Kohrle, 2002) (Figure 60).

- Dio1 catalyses the de-iodination of T4 into active form T3 or inactive form r-T3. However, it may also play a role in the degradation of iodityronines.
- Dio2 converts T4 into biologically active T3 in various tissues including the central nervous system.
- Dio3 catalyses the conversion of T4 into inactive rT3 and T3 into inactive T2.

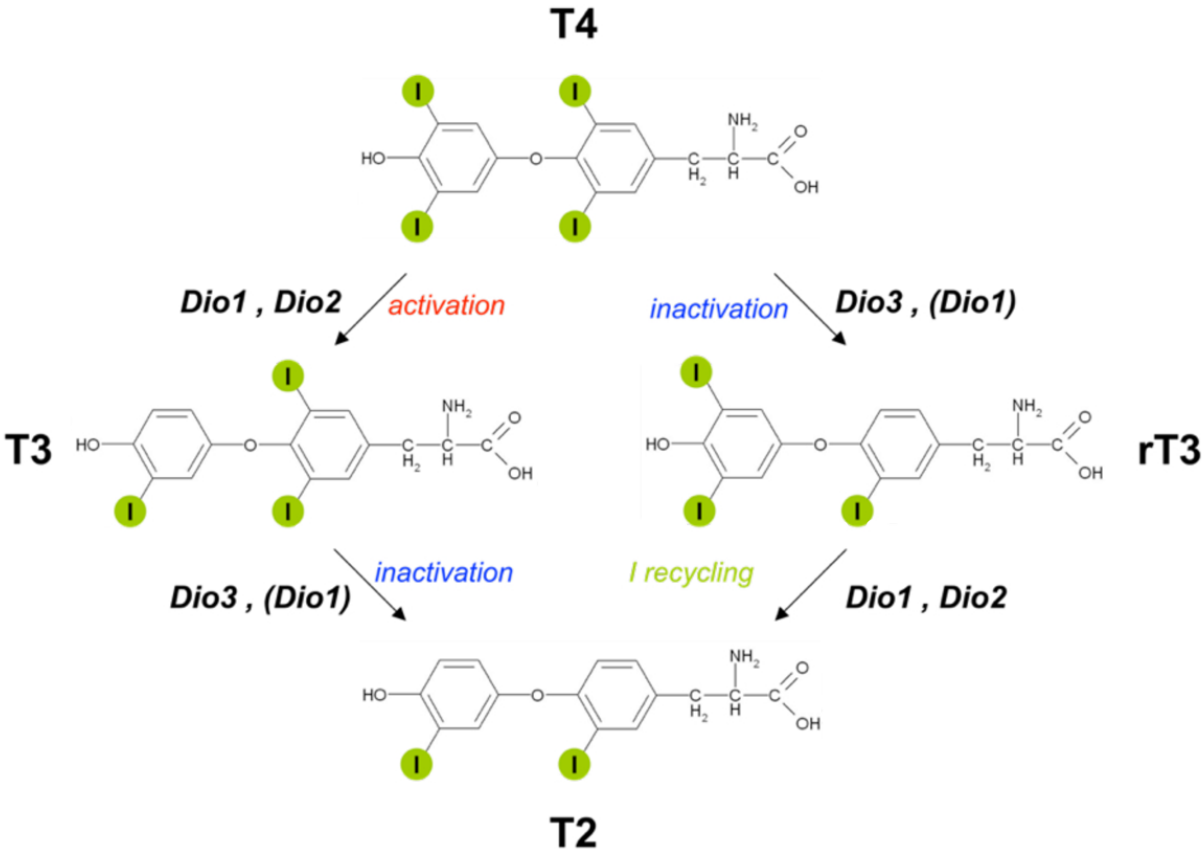

Figure 60: Thyroid hormone metabolism

Iodothyronine deiodinases catalyse the conversion from one form to another as indicated. Dio1: iodothyronine deiodinase type 1; Dio2: iodothyronine deiodinase type 2; Dio3: iodothyronine deiodinase type 3; T4: 3,3',5,5'-tetraiodo-L-thyronine (thyroxine); T3: 3,5,3'-triiodo-L-thyronine (triiodothyronine); rT3: 3,5',3'-triiodo-L-thyronine (reverse T3); T2: 3,3'-diiodo-L-thyronine. Modified from Bianco et al., 2002.

b. Role of thyroid hormones in the seasonal reproduction

In many species including birds and mammals, thyroid hormones affect the seasonal cycles of the reproductive activity (Falconer, 1963; Tavernor et al., 1968; Fraser and McNeilly, 1982; Nicholls et al., 1988; Karsch et al., 1995; Dawson, 2001). In various avian species (*Coturnix japonica*, *Sturnus vulgaris*, *Spizella arborea*), treatment with thyroid hormones of SD-acclimated individuals mimics the effects of LD on the reproductive function (Follett and Nicholls, 1984; Goldsmith and Nicholls, 1984; Wilson and Reinert, 1995; Yoshimura et al., 2003). Furthermore, the expression of *Dio2* and *3* is dependent on photoperiod in the MBH, *Dio2* levels of expression being reduced in SD and *Dio3* being up-regulated in the quail in SD (Yoshimura et al., 2003). Similarly, in sheep, the entry into the an-oestrous season requires the presence of thyroid hormones (Karsch et al., 1995; Lehman et al., 1997). In this species, thyroid hormones site of action for the inhibition of the reproductive function is located in the pre-mammillary hypothalamic region (Malpaux et al., 1998) and in the ventromedial preoptic area (Anderson et al., 2003).

In Syrian hamsters, *Dio2* is expressed in the ependymal cell layer lining the third ventricle wall with lower levels in SD-acclimated sexually inactive individuals (Figure 61). Interestingly, the SD-induced reduction in *Dio2* expression is mediated by melatonin since daily melatonin injections to LD hamsters for 1 week also down-regulate *Dio2* expression (Revel et al., 2006a) (Figure 62). In Djungarian hamsters, Dio3 levels of expression increases in SD while Dio2 levels remain constant (Barrett et al., 2007). This also results in decreased local T3 levels in SD. The photoperiodic regulation of this enzyme expression also probably reflects photoperiodic variations in the conversion of T4 into T3 and reinforces the idea that T3 plays a crucial role in the seasonal cycles of the reproductive axis activity in mammals. Indeed T3 implants in Djungarian hamsters' hypothalamus prevent the SD-induced inactivation if reproductive activity (Barrett et al., 2007; Freeman et al., 2007).

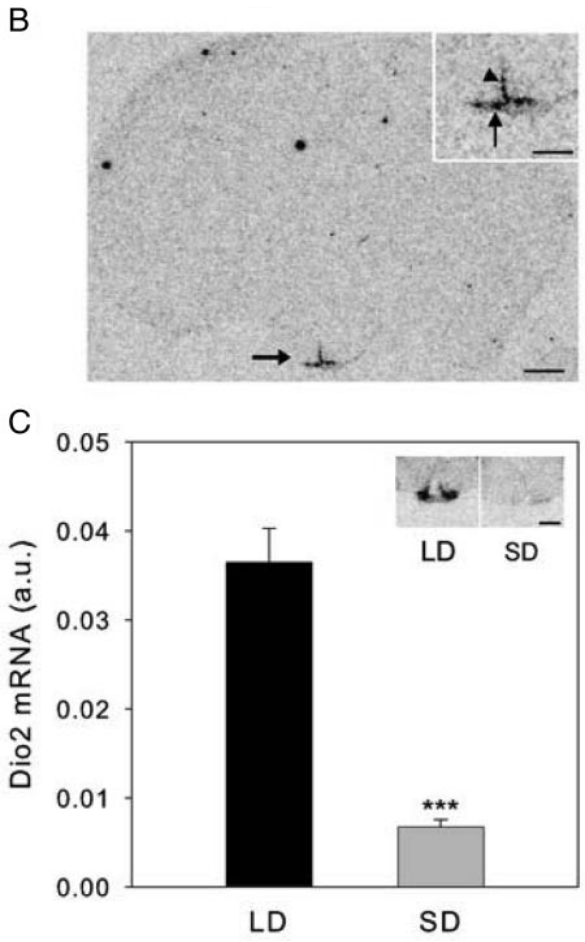

Figure 61: Photoperiodic expression of *Dio2* in the Syrian hamster's MBH

Dio2 expression was quantified in the region of the median eminence/ARC of LD or SD-acclimated male Syrian hamsters. Values show mean +/- SEM. Revel et al., 2006b

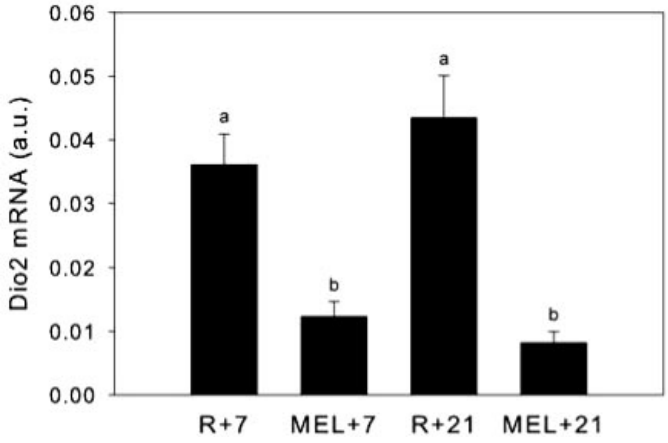

Figure 62: Effect of melatonin injections on *Dio2* expression

Syrian hamsters held in LD were injected daily with vehicle (R, Ringer-5% ethanol) or melatonin (MEL, 50 µg in Ringer-5% ethanol) 1.5 hours before lights off. Animals were treated for 7 days (R+7, MEL +7) or 21 days (R+21, MEL+21), and *Dio2* expression was quantified in the region of the median eminence/ARC. Melatonin-injected hamsters have low *Dio2* mRNA levels, compared with vehicle-treated animals. Values show mean +/- SEM. Revel et al., 2006b

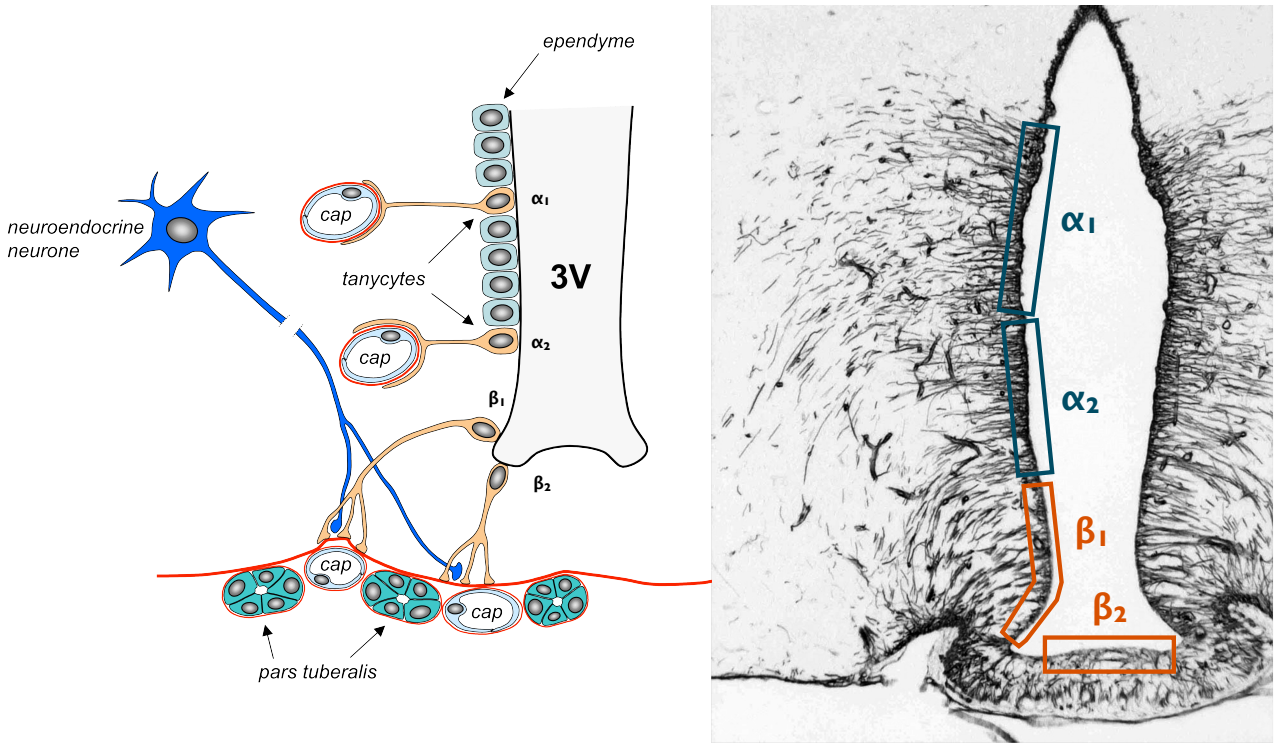

Figure 63: Tanycytes subtypes

Left:Localisation and distribution of α and β tanycytes in the MBH. Right: in vivo tanycytes subtypes. Provided by M. Bolborea.

c. Tanycytes, central Dio2/Dio3 expressing cells types

In the hypothalamus, dual labelling studies combining specific markers of tanycytes (DARP-32, dopamine and cyclic adenosine monophosphate regulated phosphoprotein; and vimentin) revealed that *Dio2* is expressed in this specialised type of glial cells (Guadano-Ferraz et al., 1997; Tu et al., 1997). Tanycytes are bipolar elongated ependymal cells having a proximal pole in the ependymal wall of the third ventricle and a distal pole contacting portal vessels (Horstmann, 1954). Four types of tanycytes exist, all of them having their cell bodies embedded in the ependymal wall of the third ventricle (Figure 63). This classification is based on localisation, spatial relationships, morphology, cytochemistry and functions (Akmayev and Fidelina, 1976; Rodriguez et al., 1979; Rodriguez et al., 2005):

- Alpha 1 tanycytes processes contact capillaries and neurones within the DMH and VMH.
- Alpha 2 tanycytes send their processes to the ARC and a few of them terminate on the lateral side of the tubero-infundibular sulcus.
- Beta 1 tanycytes line the lateral corner of the infundibular recess and their processes are in close contact with the capillaries of the portal blood system of the lateral parts of the median eminence (Amat et al., 1999). However, some processes cross the basal lamina of meninges to establish cell-to-cell contacts with specific secretory cells located in the PT (Guerra et al., 2010).
- Beta 2 tanycytes line the floor of the infundibular recess and their processes contact the capillaries of the hypophyseal portal system of the medial part of the median eminence.

Recently, tanycytes have been shown to extract substances from the cerebrospinal fluid by absorptive processes. Conversely, tanycytes can also extract substances from the bloodstream and concentrate them into the cerebrospinal fluid (Mitro and Palkovits, 1981; Fukagawa et al., 1995; Wittkowski, 1998; Rodriguez et al., 2005). These exchange processes concern thyroid hormones. Thus tanycytes are considered as local «gatekeeper» of thyroid hormone transfer into the brain (Yamamura et al., 2004).

d. Tanycytes as local managers of thyroid hormone concentration

In seasonal mammals, a model of melatonin action on the reproductive axis involving tanycytes and thyroid hormones has been elaborated (Hanon et al., 2008; Ono et al., 2008; Yasuo et al., 2009; Hanon et al., 2010) (Figure 64). According to this model, melatonin from the pineal gland act on its receptors in the PT to induce a release of TSH from the PT cells. The TSH is then captured by capillaries of the pituitary portal system. These capillaries form loops into the median eminence up to the sub-ependymal layer before joining the portal veins leading to the secondary capillary plexus in the pituitary (Murakami et al., 1987). Thus TSH acts on the tanycytes since these cells express TSH receptor (Ono et al., 2008; Yasuo et al., 2009; Hanon et al., 2010). TSH receptor in turn activates the transcription of the *Dio2* gene (Figure 64). This results in the conversion of inactive T4 into active T3 (Nakao et al., 2008). T3 can act on GnRH neurones or on other nearby hypothalamic neurones (*Kiss1* or RFRP neurones). T3 may also induce seasonal morphological remodelling of tanycytes.

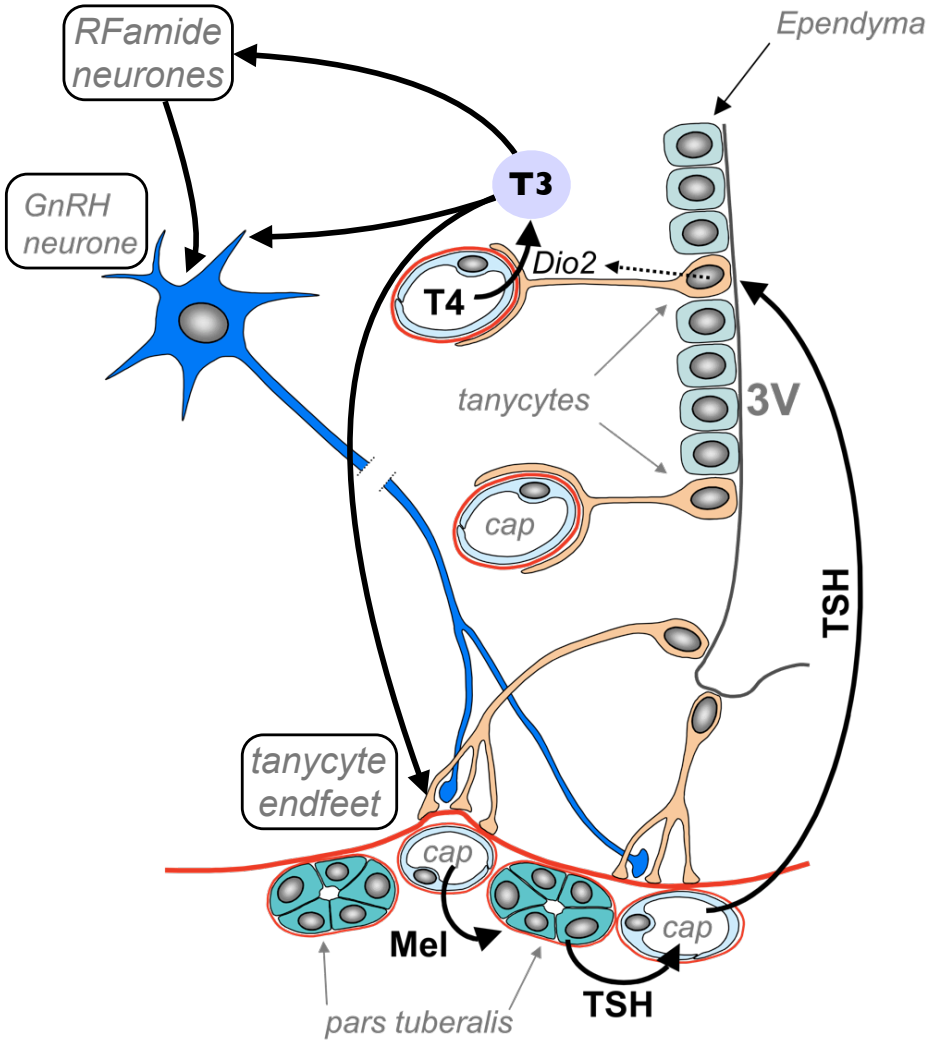

Figure 64: Pars tuberalis model of melatonin action on the reproductive axis

Low melatonin (Mel) levels in LD lift the inhibitory effect of the pineal hormone on the pars tuberalis cells. In response these cells release thyroid-stimulating hormone (TSH) which bind to its receptor (TSH-R) expressed by tanycytes. This results in the activation of *Dio2* (deiodinase 2) transcription, and the conversion of thyroid hormone T4 in the active form T3. Increased local T3 levels would then act on nearby RFamide neurones. Alternatively, T3 could induce remodelling of tanycyte morphology regulating GnRH release. Adapted from Bolborea.et al., personal communication.

e. Tanycytes: the remodelling theory

A series of studies mention that plastic changes of tanycytes morphology are involved in the release of hypothalamic hormones from their terminals contacting the capillaries of the hypophyseal portal system (Flament-Durand and Brion, 1985; McQueen and Wilson, 1994; Wittkowski, 1998; Garcia-Segura et al., 1999; Rodriguez et al., 2005) Indeed, in the quail, electron microscopy studies revealed important cellular remodelling in the tanycytes of SD-acclimated sexually quiescent animals (Kameda et al., 2003;

Yamamura et al., 2004). In this species, tanycyte glial processes of SD photo-inhibited animals detach GnRH nerve terminals from the capillaries of the pituitary portal system in the median eminence region. It thus creates a physical barrier for GnRH release under SD conditions. In LD, glial terminals retract which allows the access of GnRH nerve terminals to the capillaries and GnRH may be released again (Yoshimura, 2006) (Figure 65). Interestingly, it was demonstrated in the Japanese quail that T3 implants placed in the MBH directly promote the access of GnRH terminals to the pituitary portal system via morphological changes of tanycyte processes (Yamamura et al., 2006). This suggest that thyroid hormones control seasonal changes of tanycytes morphology. However, a direct effect of melatonin on tanycytes cannot be excluded provided that tanycytes express MT1 receptor which is present in the MBH (Kameda et al., 2003).

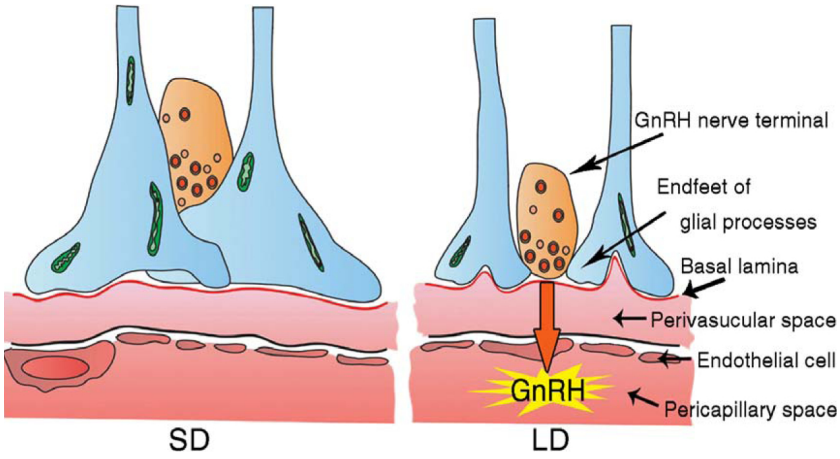

Figure 65: Photoperiodic tanycyte remodelling in the Japanese quail

Tanycyte glial processes of SD photo-inhibited animals detach GnRH nerve terminals from the capillaries of the pituitary portal system in the median eminence region. It thus creates a physical barrier for GnRH release under SD conditions. In LD, glial terminals retract which allows the access of GnRH nerve terminals to the capillaries and GnRH is released again. Yoshimura, 2006

Strikingly, similar observations were made across the oestrous cycle (Prevot, 2002). When gonadotropin levels are low, most GnRH nerve terminals do not access capillaries of the portal system because tanycytes processes create a physical barrier. At the time of the pre-ovulatory LH surge, GnRH nerve terminals sprout toward the capillaries endothelial wall and tanycytes retract their processes. The combination of both phenomena allow enhanced GnRH release at the time of the pre-ovulatory surge (Prevot, 2002) (Figure 66).

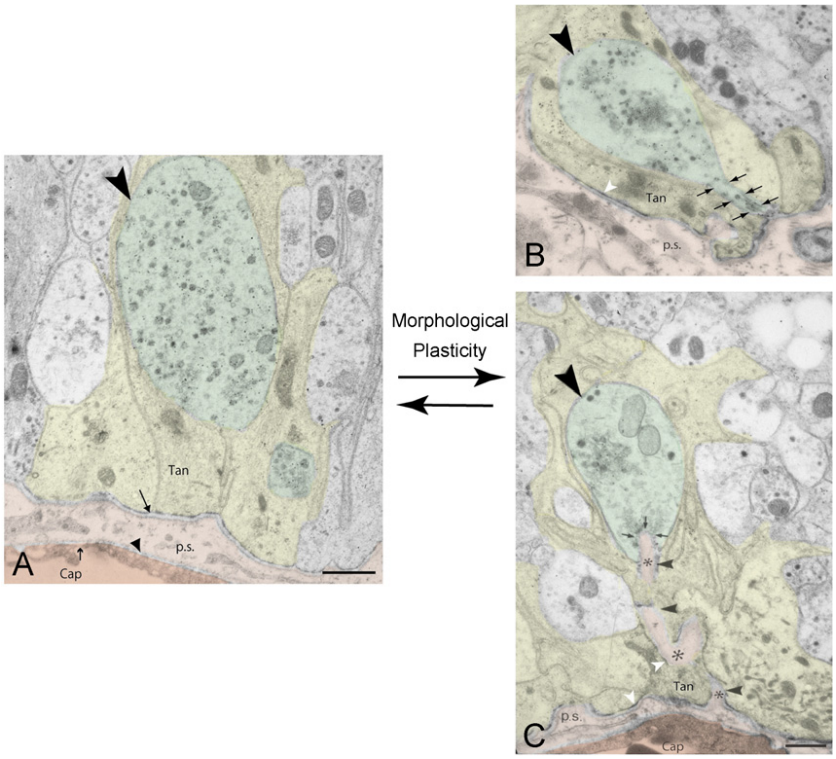

Figure 66: Changes of tanycytes morphology throughout the rat oestrous cycle

Electron micrograph of GnRH-immunoreactive terminals (large arrowhead, green) in the external zone of the median eminence in close proximity of the fenestrated capillaries (Cap, red) of the portal vasculature. At most stages of the reproductive cycle, GnRH nerve terminals are entirely embedded in tanycytic endfeets (Tan, yellow), which prevent them from contacting the peri-capillary space (p.s., pink). Arrowhead, endothelial basal lamina; short arrows, fenestration of the endothelium; scale bar: 0.5 mm. (B, C) On pro-estrus, a significant fraction of GnRH nerve endings (large arrowhead, green) directly contact the peri-capillary space (p.s., pink) either through filopodial extension of the nerve terminal (arrows) (B) or (C) by evaginations of the parenchymatous basal lamina (small black arrowheads) that allows the peri-capillary space (p.s., asterisk) to penetrate into the nervous parenchyma; scale bar: 0.5 mm. Prevot et al., 2007

Tanycyte morphological plasticity involve various signalling pathways. They include transforming growth factor (TGF) α, TGFß1, TGFß2, basic fibroblast growth factor (bFGF) or insulin-like growth factor 1 (IGF1). All these compounds are known to modulate GnRH release and to be expressed by tanycytes (Marchetti, 1997; Melcangi et al., 2001; Melcangi et al., 2002; Galbiati et al., 2003; Rodriguez et al., 2005).

For instance, TGFα and TGFß1 have opposite effects on tanycyte plasticity in vitro (Prevot et al., 2003). While TGFß1 induces retraction of tanycytic processes, TGFα promotes tanycytic growth. Interestingly, longer exposure of tanycytes to TGFα causes tanycytic retraction. Since this retraction is abolished by application of TGFß1 antiserum, the retraction is attributable to TGFα-induced TGFß1 formation (Prevot et al., 2003).

Interestingly, photoperiodic variations of TGFα expression in the tanycytes of the Japanese quail have been observed and TGFα promotes gonadal activity independently from thyroid hormones (Takagi et al., 2007).

In addition to tanycytes, capillaries endothelial cells also release diffusible factors (NO) inducing plasticity in the external part of the median eminence (De Seranno et al., 2004). The involvement of seasonal variations of NO release by endothelial cells in the seasonal variations of GnRH secretion remains to be established.

III. Conclusion: the *Rfrp*/*Dio2*/*Kiss1* network

In conclusion, the MBH of the Syrian hamster expresses several genes under photoperiodic control: *Kiss1*, *Dio2* and *Rfrp*, all of them impacting on GnRH release. The MBH thus appear to contain a gene network crucial for the photoperiodic control of reproduction in seasonal breeders. Although the importance of each gene has not been investigated in a same species, we propose a general model to explain the mechanisms involved in the seasonal control of reproduction in LD-breeder rodents (Figure 67). According to this working model, melatonin would control the activity of the reproductive axis via different complementary pathways:

- In SD, melatonin inhibits pars tuberalis cells. In LD, the inhibitory signal is lifted which allows the production of TSH by pars tuberalis cells. THS acts on tanycytes to activate *Dio2* expression. This leads to increased local concentrations in T3. In turn, T3 would act directly on GnRH neurones or on hypothalamic neurones impacting on GnRH synthesis/release. TSH or T3 activation of tanycytes could also involve important morphological remodelling regulating the access of GnRH nerve terminals to the capillaries according to the season. However, a direct effect of melatonin on tanycyte biochemistry and/or plasticity cannot be excluded.
- Melatonin could act directly on *Rfrp* neurones of the DMH. In LD, when the inhibitory melatoninergic signal is lifted, increased RFRP-3 and Kp levels induce GnRH release. RFRP-3 could impact on GnRH release via an effect on *Kiss1* neurones or a direct effect on GnRH cells.
- *Kiss1* neurones (which could also be sensitive to local T3 levels) send projections to various brain areas such as the BNST, which is known to project toward GnRH neurones. Kp efferent projections can either constitute alternative pathways for the regulation of GnRH release and/or mediate melatonin effect on reproduction-

associated functions (reproductive behaviours). Alternatively, a feedback effect of Kp on their own synthesis remains possible and it would involve these structures.

This hypothetic model is designed from our studies in the Syrian hamster and it may present considerable species differences. In particular, the relative importance of each gene in the seasonal control of reproduction may be species-dependent. For instance, as the central sites of action for seasonal reproduction is different between Syrian and Djungarian hamsters, on might expect a different role of Kp, RFRP-3 and T3 in those species.

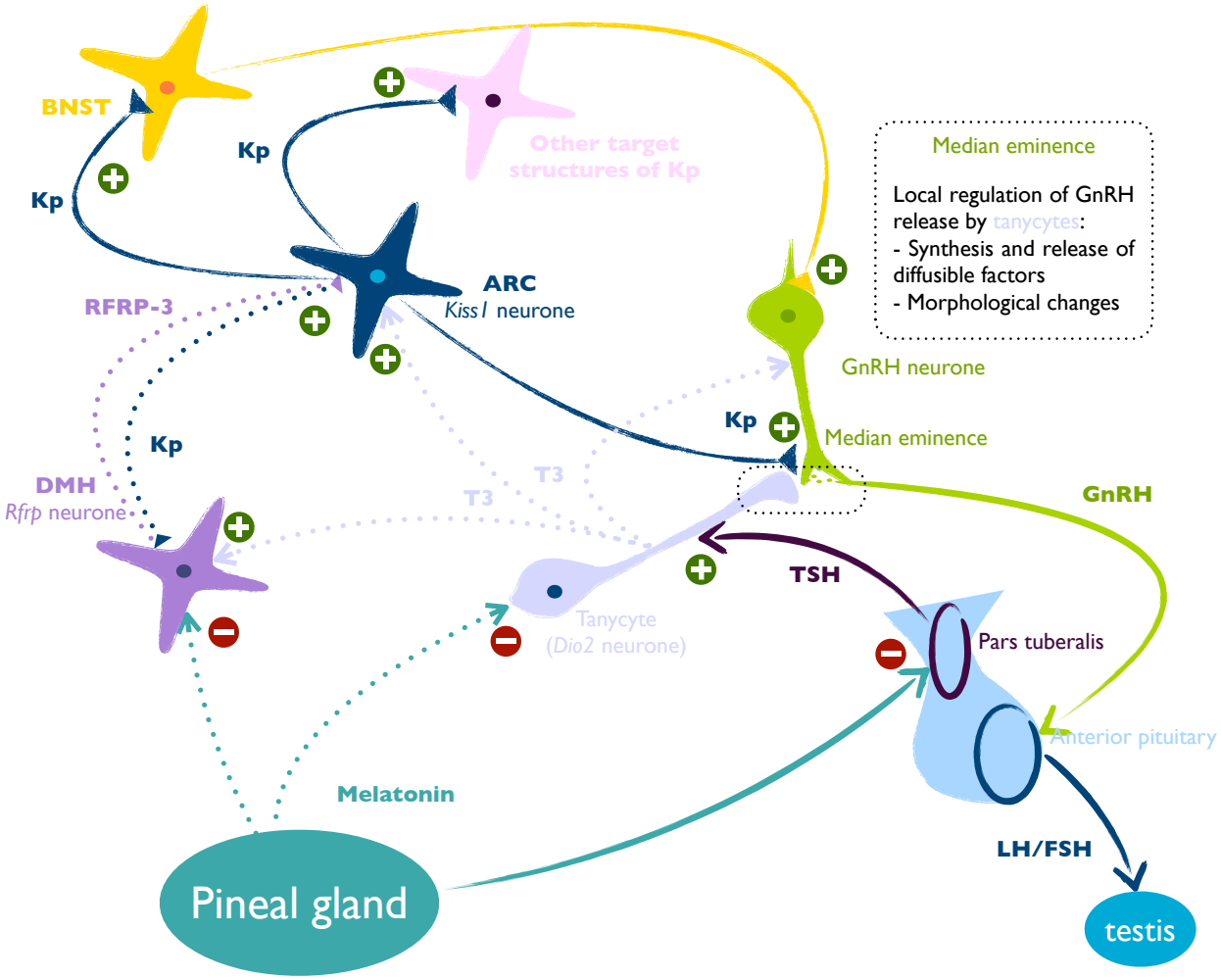

Figure 67: General model of melatonin action on the reproductive axis

In SD, melatonin inhibits pars tuberalis cells. In LD, the inhibitory signal is lifted which allows the production of TSH by pars tuberalis cells. THS act on tanycytes to activate *Dio2* expression. This increases local concentrations in T3. In turn, T3 would act directly on GnRH neurones or on hypothalamic neurones impacting on GnRH synthesis/release. TSH or T3 activation of tanycytes could also involve important morphological remodelling regulating the access of GnRH nerve terminals to the capillaries according to the season. However, a direct effect of melatonin on tanycyte biochemistry and/or plasticity cannot be excluded. Melatonin could act directly on *Rfrp* neurones of the DMH. In LD, when the inhibitory melatoninergic signal is lifted, increased RFRP-3 and Kp levels induce GnRH release. RFRP-3 could impact on GnRH release via an effect on *Kiss1* neurones or a direct effect on GnRH cells. *Kiss1* neurones (which could also be sensitive to local T3 levels) send projections to various brain areas such as the BNST, which is known to project toward GnRH neurones. Kp efferent projections can either constitute alternative pathways for the regulation of GnRH release and/or mediate melatonin effect on reproduction-associated functions (reproductive behaviours). Alternatively, a feedback effect of Kp on their own synthesis remains possible and it would involve these structures.

Chapter 7 - Perspectives

This PhD work has clarified and emphasised the role of Kp in the seasonal control of reproduction.

Melatonin controls seasonal variations of reproductive activity by impacting on ARC *Kiss1* neurones which relay the photoperiodic information to GnRH neurones. The observation that *Kiss1* mRNA does not overlap melatonin binding sites indicates that melatonin does not act directly on Kp production. As mentioned above, *Rfrp* DMH neurones or tanycyte-born T3 might constitute a relay between melatonin and *Kiss1* neurones. Hence, the presence of melatonin receptors in *Rfrp* neurones, the presence of RFRP receptors in *Kiss1* cells as well as direct connexions between RFRP fibres and *Kiss1* neurones have to be investigated.

Especially, the distribution of Kp neurones and fibres was analysed and revealed an important number of Kp fibre-containing structures. It suggests that Kp function may not be exclusively restricted to the control of GnRH release. Rather, the presence of Kp fibres in brain regions such as the BNST indicates that Kp could also affect reproductive behaviours and opens the door for a role of Kp in pheromone-induced sexual behaviours. This is why it is crucial to determine the phenotype of Kp target neurones. In regard of photoperiodic control of reproduction, it is of high importance to focus on the role of the PVT in the Syrian hamster. Indeed, this structure contain melatonin binding sites, Kp and RFRP immunoreactive fibres, suggesting that PVT may constitute a secondary integrating centre for seasonal control of reproduction.

Thyroid hormones exert a profound effect on the reproductive axis. According to the model proposed in figure 67, tanycytes would control the local production of T3 which could act on nearby neurones. However, the presence of thyroid hormone receptors on *Kiss1* neurones or the effect of thyroid hormones on *Kiss1* expression have never been investigated. The evidence of a direct link between thyroid hormones and Kp production would strengthen the model proposed above.

In addition, many evidence point to AVPV Kp as regulators of the pre-ovulatory LH surge. Since the pre-ovulatory LH surge is gated by the circadian clock, further studies investigating the link between SCN and Kp would be of high interest in regard of the lab's thematic. First, the presence of reproductive abnormalities could be assessed in clock gene KO mice. Second, the expression of clock genes in AVPV *Kiss1* neurones can be characterised. Third, the SCN output(s) targeting AVPV *Kiss1* neurones has to be identified. The clarification of the effect of the SCN on AVPV *Kiss1* neurones is clinically

interesting since many women whose working schedule is erratic present ovulation troubles. The re-synchrony of AVPV *Kiss1* neurones may constitute a therapeutic target.

To conclude, *Kiss1* neurones act as integrative centres for the control of the reproductive function. Because of this strategic position in the reproductive axis, *Kiss1* neurones constitute interesting object of studies in the context of seasonality which involves photoperiodic changes in many biological parameters putatively impacting on the reproductive function.

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