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Ovulation in mammals

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■ **Controlling the timing of ovulation in farm mammals can improve fertility and the management of parturition, but current methods are based on the use of high doses of hormones. This review presents new paradigms for controlling the time of ovulation, respectful of the animal's physiology, without hormone supplementation based on the knowledge of physiological mechanisms.**

Introduction

Ovulation is the release of the female gamete. This phenomenon is preceded a few days before by a specific behaviour: the estrus behaviour often summarized by the term "estrus". Depending on the species, estrus behaviour may last a few hours after ovulation. During the estrus phase, the female displays behaviours to attract males (proceptivity) and accepts mating (receptivity). The term estrus derives from the Greek name *"οιστροξ" for* estrus, a dipteran of the family Oestridae, which inflicts bites on cattle in the summer. Females in estrus show a behaviour that is similar to that of animals annoyed by a large number of estrus or horseflies: irritation, tail scraping, etc. The term estrus was first used to describe the period when ovulation occurs by Walter Heape in his book on the different types of reproductive cycles in mammals (Heape, 1900). Heape defined estrus, mirroring rutting in the male, as *"the special period of sexual desire of the female"*. The other phases of the cycle are defined in relation to estrus: the proestrus and the post estrus which is now called metestrus.

Proestrus is the follicular phase during which one or more, waves of follicles grow and subsequently degenerate (atresia) or when the follicle(s) intended to continue growing (dominant follicle(s)) are selected. During proestrus, the follicles secrete estrogen but in insufficient quantity to induce sexual behaviour. Several follicular waves may follow one another during this phase. Metestrus corresponds to the luteal phase during which the follicular cells are transformed into luteal cells (luteinization), and form the corpus luteum that secrete progesterone. The estrus phase corresponds to the phase of expression of sexual behaviour when circulating estrogen levels are elevated and the dominant follicle(s) is (are) terminally growing. At the time of ovulation, the granulosa cells turn into luteal cells and lose their ability to secrete estrogen. The drop-off in plasma estrogen is accompanied by the cessation of sexual behaviour in some species and thus the refusal of mating. Knowing the precise time of ovulation increases the chances of fertilization when using artificial insemination (AI), especially when using cryopreserved semen because cryopreserved sperm have a shorter fertilization period.

In this review, we will describe the mechanisms leading to ovulation based on the most recent studies that address the intimate mechanisms of physiological regulation. A good knowledge of these biological bases makes it possible to consider new paradigms for the induction of ovulation in mammals.

1. Two types of ovulation in mammals

There are two types of ovulation in domestic mammals: spontaneous ovulation and induced ovulation. Spontaneous ovulation is endogenously triggered during the estrus

Box 1. ***Two modes of ovulation in mammals.***

Spontaneous ovulation species

Sow (*Sus scrofa*), Cow (*Bob taurus*),
Ewe (*Ovis aries*), Mouse (*Mus musculus*),
Macaque (*Macaca mulata*),
Dog (*Canis vulgaris*)

Induced ovulation species

Dromadery (*Camelus dormaderius*),
Rabbit (*Oryctolagus cuniculus*),
Alpaga (*Vicugna pacos*), Shrew (*Suncus murinus*),
Rhinoceros (*Diceros bicornis*)

In species with induced ovulation, the first male to mate with the female will fertilise the oocyte (mono-ovulating species) or the majority of oocytes (poly-ovulating species). In species with spontaneous ovulation, the male who mates with the female at the time t closest to ovulation will fertilise the oocyte or the majority of oocytes. However, males cannot predict the time of ovulation accurately, so it is likely that their sperm will compete with other males' sperm that precede or succeed them (Soulsbury, 2010). Thus, a higher level of male competition (e.g. sperm competition) is expected in spontaneously ovulating species than in induced ovulating species. The ability of males to monopolize paternity depends on several factors that have been well demonstrated such as the existence of estrus synchronization phenomena in females, family structure and the existence of male-female association. In males of spontaneously ovulating species, the intermittent male-female association is correlated with a loss of monopolization of paternity, which is not the case in males of induced ovulation species. Thus, the ovulation mode appears to be an evolutionary strategy of the species allowing a better parental monopolization of males (Soulsbury, 2010). However, the reasons why some species have evolved towards one or the other ovulation mode are not very clear. A number of species with induced ovulation live in groups and with male-female associations. The mode of induced ovulation makes it possible to reduce the level of sperm competition and therefore competition between males, but the factors responsible for the evolutionary strategy are not known. Induced ovulation also provides a benefit for the female who can choose the fertilizing male based on the level of stimulation during mating (Soulsbury, 2010).

period whether the female is in the presence of males or not. This is the case in most domestic mammals. In the case of induced ovulation, it occurs only when the female mates with a male. Thus, the duration of the estrus phase can be very variable, and only stops when the female mates. This is the case in rabbits, cats, and females of certain camelid species such as dromedaries, llamas and alpacas.

The existence of two modalities of ovulation induction in mammals can be discussed from the perspective of behavioural ecology and evolution. The different evolutionary strategies adopted by species are essentially based on optimising the production of viable offspring with good survival. Within this framework, various elements such as climate, availability of high quality feed resources and social behaviour of species, influence many parameters relating to the reproductive function (box 1).

■ 1.1 Spontaneous ovulation

It is classically accepted that ovulation is the consequence of a discharge of luteinizing hormone (LH) secreted by the pituitary gland. At the time of ovulation, the plasma concentration of LH reaches very high values, known as the pre-ovulatory LH peak. For example, in ewes, avererage LH concentration varies from 2 to 8 ng/mL, whereas during the pre-ovulatory peak it can reach 30 to 60 ng/mL. In ewes, the duration of the pre-ovulatory peak is approximately 12 hours (Caraty *et al.*, 1998). Ovulation occurs 24-36 hours after the maximum LH peak (Driancourt *et al.*, 2014). In the sow, LH peak lasts approximately 24 hours and ovulation occurs 24-64 hours after the onset of LH peak (Martinat-Botté *et al.*, 1997). In cows, LH peak lasts 12 to 45 hours depending on breed and physiological status (Wiltbank and Pursley, 2014), ovulation occurs within 24 to 30 hours after LH peak (Saumande and Humblot, 2005). An exception is the mare, where LH peak lasts several days and the maximum LH concentration value is reached 24 hours after ovulation (Noden *et al.*, 1975).

■ 1.2 Induced Ovulation

Several domestic mammals species display induced ovulation: cat, rabbit and some camelids. The hypothesis of a nervous origin of the ovulation reflex had been reinforced by the observation of numerous nerve endings around ovarian follicles and by the presence of muscle fibres in the follicle's fibrous layer in rabbit ovary. However, LH induces ovulation in rabbits, and it was concluded that the reflex leading to ovulation involved a hypothalamic factor that induced pituitary LH secretion. In alpaca, llama and dromedary, ovulation is also induced by coitus, but it involves a factor present in the seminal fluid of the male, the "ovulation *inducing factor*" (OIF). OIF injected intramuscularly or administrated in the uterus to female alpacas, induced a LH peak that started two hours after injection followed by ovulation 24 to 30 hours later (Tanco *et al.*, 2011). OIF has been identified as a major protein in alpaca seminal plasma: the β-nerve growth factor (β-NGF) (Kershaw-Young *et al.*, 2012).

Thus, in both spontaneously ovulating and mating induced ovulation mammals, the pituitary LH peak plays a major role in triggering ovulation.

2. Physiological regulation of ovulation

■ 2.1. Ovarian mechanisms

Ovulation is a complex process during which the resumption of oocyte meiosis, the expansion of the cumulus, the rupture of the apical pole of the follicle, and the differentiation of the granulosa and theca cells leading to the formation of the corpus luteum are induced. In a normal cycle, all these events must be coordinated to produce a mature, fertilizable oocyte and a corpus luteum capable of supporting early pregnancy.

A cascade of events leads to ovulation, but the initiator is the LH peak. At the level of the follicle(s) intended to ovulate, LH induces an increase in follicular diameter, which is due to the accumulation of follicular fluid, associated with the thinning of the follicular wall composed of granulosa and theca cells.

a. Functional changes in follicular cells

Only follicles that express a large number of LH receptors on granulosa cells' surface are able to ovulate in response to the pre-ovulatory LH peak (table 1). LH binds to its receptors and follicular cells respond to this signal by regulating the expression of several genes (figure 1). The binding of LH to its receptor increases intracellular levels of cyclic adenosine monophosphate (cAMP) and inositol tri-phosphate (Piquette *et al.*, 1991), but this latter pathway is only a potentiating pathway (figure 1) and activated only in the presence of high hormone concentrations. Thus, protein kinase A (PKA) activated by cAMP, induces a cascade of phosphorylation and activation

Figure 1. ***Ovarian mechanisms leading to ovulation.***

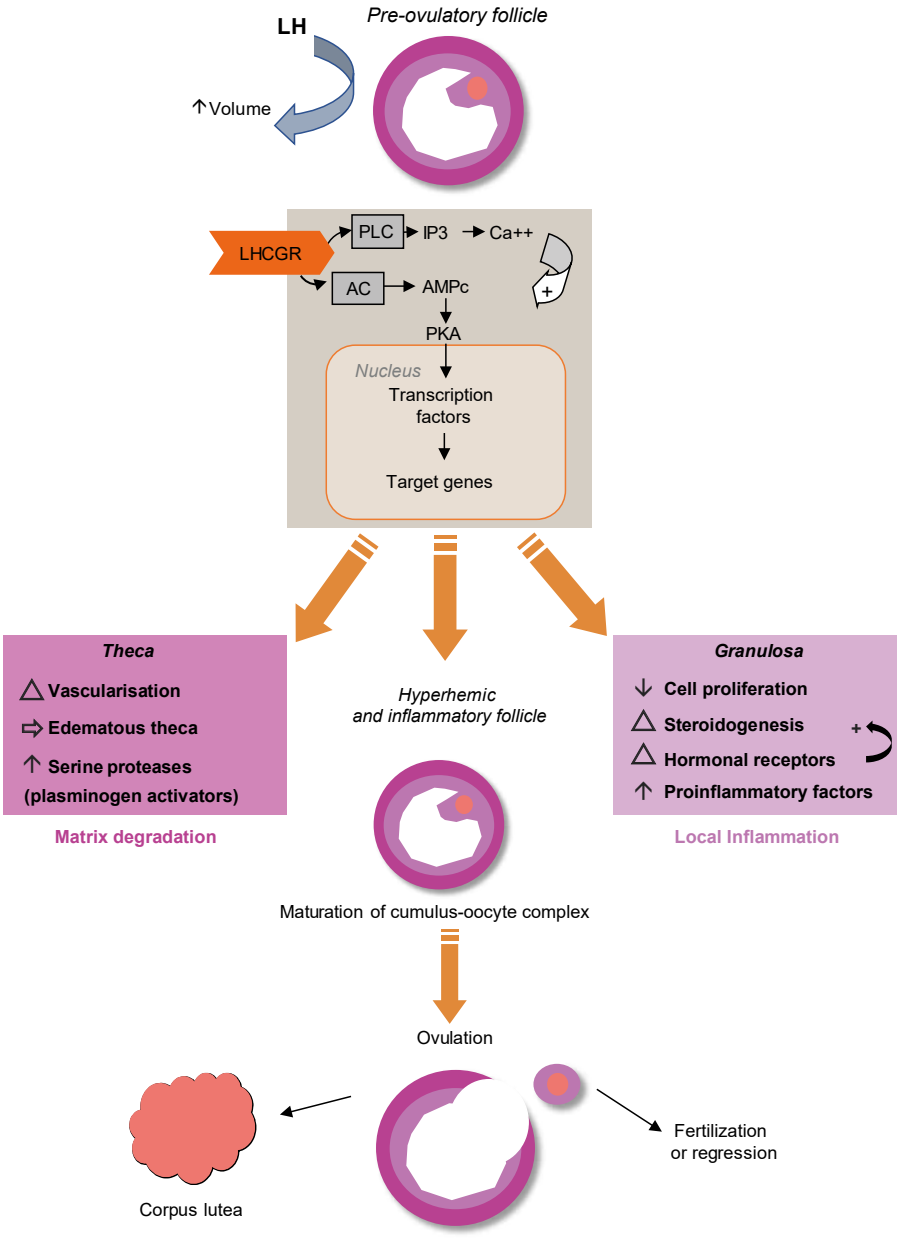


Table 1. ***Follicular diameters and acquisition of LH sensitivity.***

Species	Acquisition of LH receptors on the granulosa (diameter in mm)	Diameter at ovulation (diameter in mm)
Rate	0.5	0.6 à 0.8
Ewes	3 à 3.5	6 à 7
Sow	5	7 à 11
Cow	9	10 à 20
Mare	15	45
Woman	10 à 12	20

of transcription factors (CREB, SP1) that will inhibit or activate the expression of target genes coding for steroidogenesis enzymes. Thus, in the hours following circulating LH increase, serum estrogen and androgen levels collapse, while progesterone levels rise dramatically. In response to increased nitric oxide (NO; vasodilator activity) production, follicle theca becomes edematous and hyperhematous, increasing pre-ovulatory follicle blood flow and vascular permeability. Simultaneously, granulosa cells lose their FSH receptors, while LH receptor expression decreases transiently. In the rat, this "reprogramming" lasts approximately 7 hours (Richards and Hedin, 1988).

b. Follicular wall rupture

The ovulation process is associated with an inflammatory response type. Ovarian synthesis of inflammatory cytokines (IL, TNFα), prostaglandins and cortisol (with anti-inflammatory action) is enhanced in the pre-ovulatory follicle at the time of ovulation in mice, rabbits, sows, cows, mares and woman. Interleukin-1 (IL-1) is capable of inducing follicle rupture *ex vivo*, on infused ovaries of rat and rabbit (Brännström *et al.*, 1993; Takehara *et al.*, 1994) and *in vivo in* the mare after intrafollicular injection of dominant follicles (Martoriati *et al.*, 2003).

The mechanism of action of cytokines in pre-ovulatory maturation is probably similar to that observed during inflammation. In particular, they stimulate the production of prostaglandins, NO, and the activity of proteolytic enzymes. In addition, they are able to modulate steroidogenesis and are also involved in the maturation of the oocyte-cumulus complex in rabbits and mares (Gérard *et al.*, 2004).

Rupture of the follicular wall and expulsion of the oocyte into the oviduct requires the action of proteolytic enzymes that degrade the extracellular matrix. The local production of plasminogen activator, plasmin and collagenases (metalloproteinases MMP1 and MMP2) increases considerably. The plasminogen activator converts plasminogen into plasmin, which in turn activates collagenases (Beers *et al.*, 1975). The pre-ovulatory increase in the intrafollicular concentration of progesterone stimulates the synthesis and activity of two other proteases ADAMTS1 and CTSL1, which are involved in proteolysis. In parallel, the activity of serine protease inhibitors (SERPIN) and matrix metalloprotease inhibitors (TIMP1 and TIMP2) increases. The expression of these metalloproteases and TIMPs is regulated by steroids and prostaglandins. Thus, the expression of proteases and anti-proteases in the pre-ovulatory follicle allows temporal and spatial regulation of proteolytic activity prior to rupture (Curry and Smith, 2006).

c. Maturation of the oocyte-cumulus complex

In response to LH pre-ovulatory peak, the oocyte, blocked at the prophase stage of the first meiotic division, enters the maturation phase by resuming its meiosis. This stage called GVBD (germinal vesicle break-down) is visualized by the rupture of the nuclear membrane. In most mammalian species, the oocyte evolves to the metaphase stage of the second meiotic division and remains blocked at this stage until fertilization. The molecular mechanisms involved in nuclear and cytoplasmic oocyte maturation are complex and will not be discussed in this review. LH induces cumulus expansion, a phenomenon necessary for meiotic maturation and the acquisition of competence in oocyte development. When the pre-ovulatory follicle ruptures, the follicular fluid is expelled taking down with it the oocyte-cumulus complex that will be captured by the oviduct.

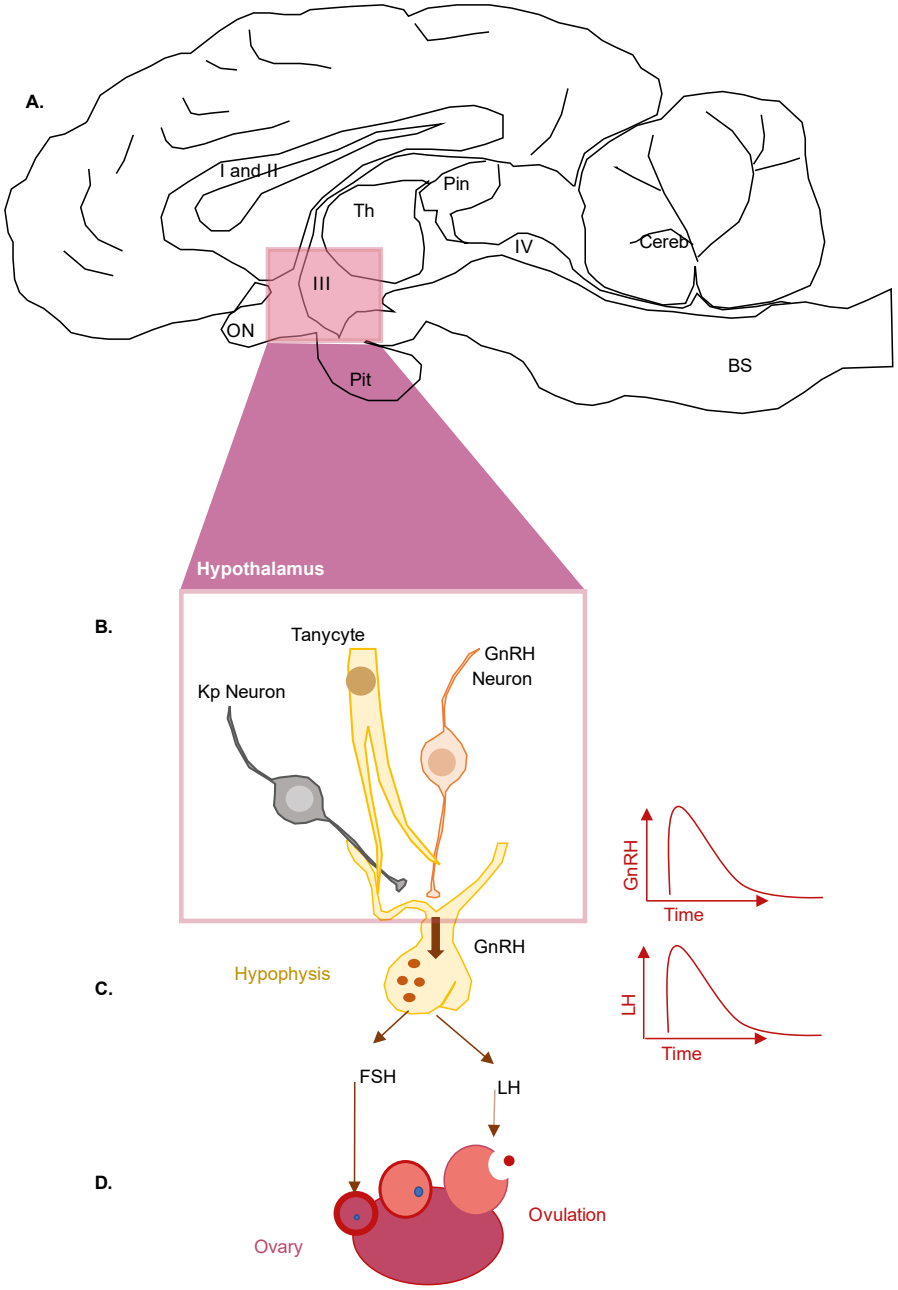
■ 2.2 Pituitary Mechanisms

Follicular growth and ovulation depend on gonadotropic hormones (gonadotropins), FSH and LH, secreted by the gonadotropic cells located in the pituitary gland. The synthesis and release of LH and FSH are dependent on a complex interaction of multiple endocrine and paracrine signals.

a. Role of GnRH (Gonadotropin Releasing Hormone)

The synthesis and release of the two gonadotropic hormones LH and FSH are dependent on the hypothalamic neurohormone, GnRH (see chapter 2.3.a). Its

Figure 2. ***Hypothalamo-hypophysio-ovarian axis.***



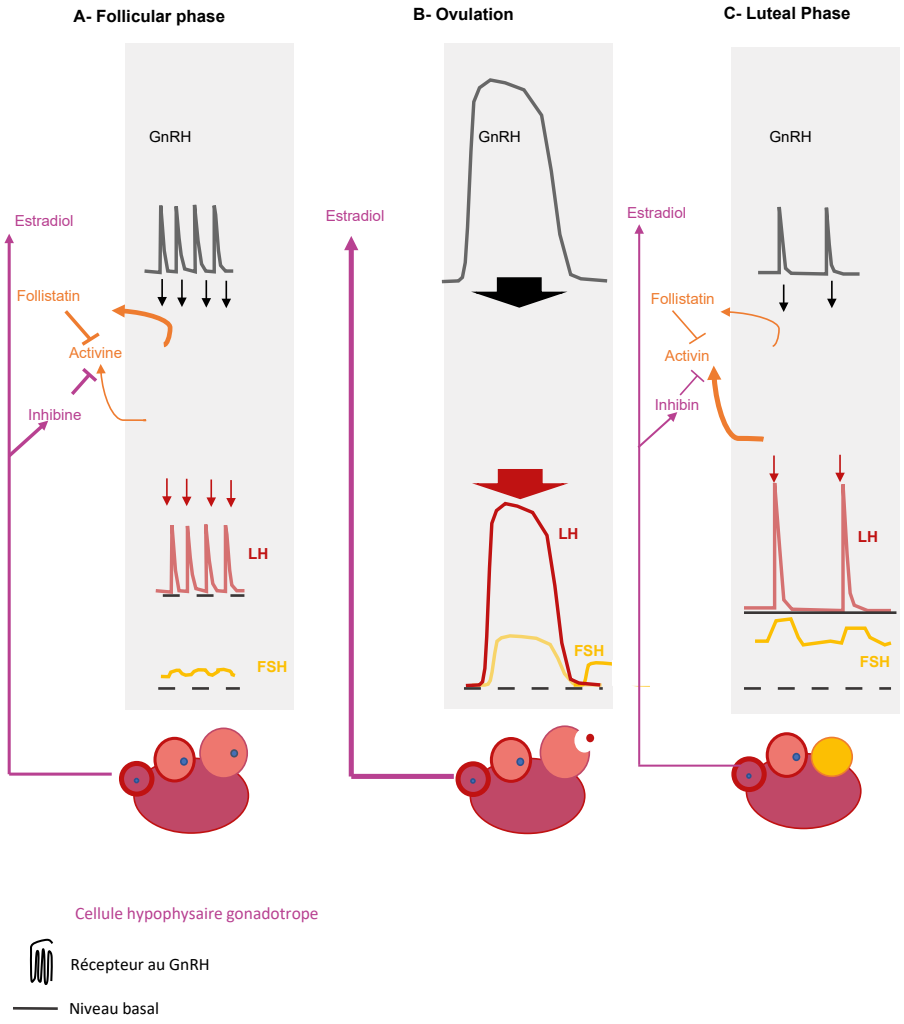
A. Schematic representation of a sagittal section of a sheep's brain, the red frame delineates the hypothalamus. I and II: lateral ventricles, III: 3rd ventricle, IV: 4th ventricle, ON: optic nerves, Pit: pituitary gland, Th: thalamus, Pin: pineal gland, Cereb: cerebellum, BS: brain stem.
B. Schematic representation of the hypothalamic actors regulating reproduction: GnRH neurons, Kp neurons and tanycytes. Tanycytes are glial cells lining the 3rd ventricle, they also possess properties of neural stem cells.
C. Schematic representation of the pituitary gland and LH and FSH release under the action of GnRH.
D. Schematic representation of the ovary with a pre-ovulatory follicle which ovulates under the action of the LH peak.

role has been well established through the use of GnRH antagonists (Brooks *et al.*, 1993) or anti-GnRH antibodies (Caraty *et al.*, 1984; Molter-Gérard *et al.*, 1999). These treatments induce immediate suppression of LH release, disappearance of the pre-ovulatory peak and blockade of ovulation. GnRH is released in a pulsatile manner by GnRH neurons and reaches the pituitary via the hypothalamic-pituitary portal blood system (figure 2). It in turn induces a pulsatile release of gonadotropic hormones. The frequency of GnRH pulses varies during the estrous cycle (figure 3): in ewes, the average frequency is of one pulse/hour during the follicular phase, and then becomes continuous in the period prior to ovulation. After ovulation, this frequency decreases and reach an average of one pulse every six hours during the luteal phase in the ewe (figure 3). The pulse frequency determines the pattern of gonadotropic hormones release. A high frequency favours the release of LH, whereas a low frequency favours the expression and release of FSH (figure 3) (Dalkin *et al.*, 1989). During the pre-ovulatory peak of LH, there is a small amplitude FSH peak (figure 3) which allows the recruitment of follicles for the next cycle. While the synthesis and release of LH is intimately dependent on GnRH, FSH synthesis and relase depend also on other regulations (see §2.2.4).

b. GnRH acts via a specific receptor

GnRH acts in the pituitary via a specific receptor (GnRH-R) located on the surface of gonadotropic cells. GnRH-R belongs to the family of G protein-coupled receptors. The binding of GnRH to its receptor initiates an intracellular signalling cascade involving several proteins whose activation induces the transcription of genes coding for LH and FSH and/or the release of these hormones. The importance of GnRH-R is highlighted in cases of hypogonadism induced by mutations present on the GnRH-R gene inactivating its functionality (Chevrier *et al.*, 2011). The number of GnRH-R receptors on the surface of gonadotropic cells varies with the frequency of GnRH pulses. High frequencies increase the number of receptors while low frequencies decrease the number of receptors (Katt *et al.*, 1985). Different intracellular signalling pathways are thought to be activated as a function of the number of receptors,

Figure 3. ***Schematic representation of the regulation of the synthesis and secretion of the gonadotropic hormones LH and FSH during the follicular phase (A.), ovulation (B.) and the luteal phase (C.).***



and thus the pulse frequency, leading to differential expression of the LHß and FSHß subunits and the release of LH and FSH (Stamatiades and Kaiser, 2018).

c. The essential role of estradiol

At the beginning of the follicular phase, the circulating concentrations of steroid hormones, mainly 17-β-estradiol (estradiol or E2), produced by the follicles remain low. E2 then exerts negative feedback control on the hypothalamic-pituitary complex by inhibiting the secretion of GnRH. At the end of the follicular phase, the concentration of E2 rises until it reaches a critical value at which the feedback becomes positive, generating the pre-ovulatory peak of LH, which causes ovulation.

d. Other factors regulating the secretion of gonadotropic hormones

In addition to the major role played by GnRH and estradiol, other factors are likely to modulate the synthesis and secretion of gonadotropic hormones. Growth factors of the *Transforming Growth Factor ß* (TGF-ß) family, in particular inhibin and activin, modulate the synthesis and release of FSH (figure 3). Inhibin B, produced by granulosa cells, inhibits the secretion of FSH at the end of the follicular phase. This action is essential for the selection of the dominant follicle(s). During the luteal phase, the corpus luteum secretes inhibin A, which reduces the release of FSH, thus preventing early maturation of the follicles. In addition, FSH synthesis is closely dependent on activin, produced in the pituitary gland. The absence of this factor leads to a drastic decrease in FSH concentrations whereas the massive injection of activin induces a shortening of the estrous cycle and an increase in the number of antrum follicles with premature superovulation (Erickson *et al.*, 1995). The action of activin is modulated by a binding protein, follistatin, but also by inhibin, which prevents it from binding to its receptors. A slow frequency of GnRH pulses promotes the synthesis and release of activin (Dalkin *et al.*, 1999).

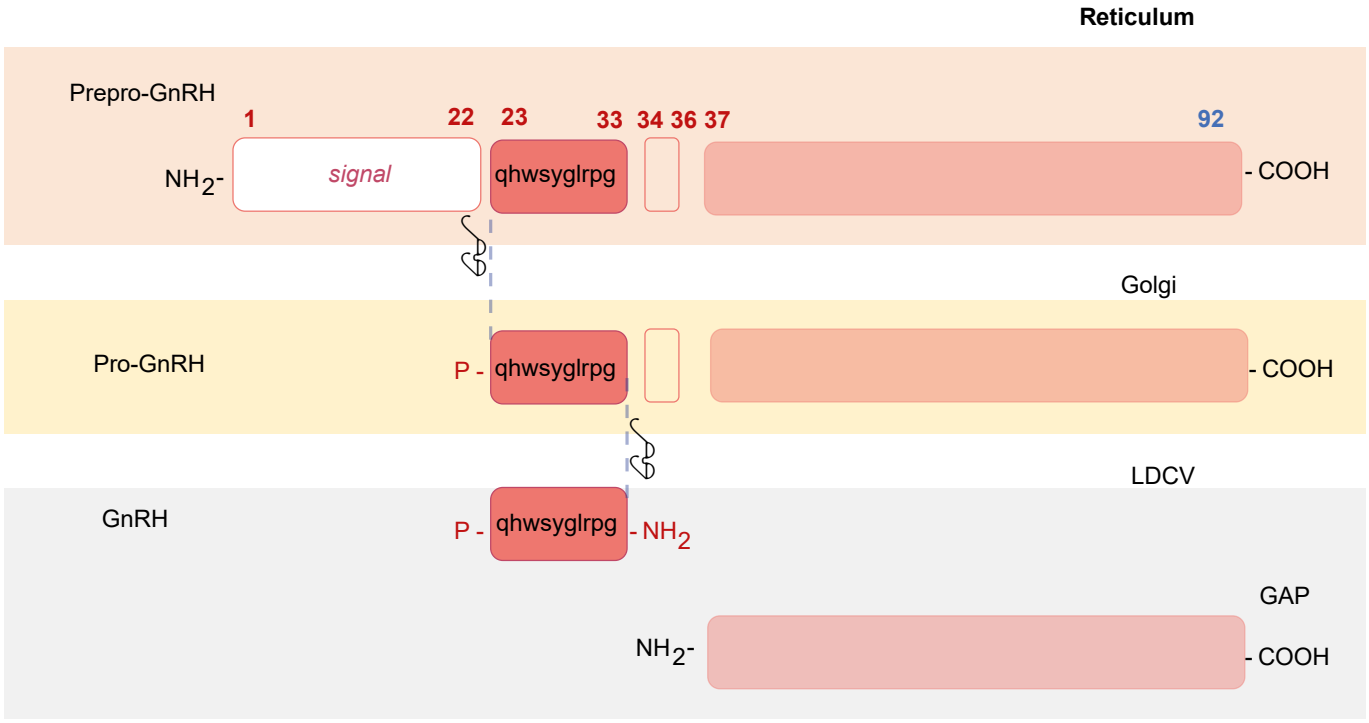
■ 2.3 Hypothalamic mechanisms

Whether ovulation is induced or spontaneous, it is triggered by the LH peak initiated by a GnRH secretion peak.

a. The GnRH conductor of ovulation induction

GnRH is a ten-amino acid peptide neuro-hormone whose sequence is highly conserved in mammals (for a review seeDuittoz and Prévot, 2014). This hormone has a particular horse-shoe-shaped conformation that is important for binding to its receptor and for carrying out its biological activity. GnRH undergoes two post-translational modifications: cyclisation of N-terminal glutamine to pyro-glutamine and amidation of C-terminal glutamic acid (figure 4). These two post-translational modifications prevent the action of exopeptidases, but the peptide remains sensitive to endopeptidases capable of rapidly degrading it. As a consequence, GnRH half-life is less than five minutes and its secretion in small quantities makes its detection in peripheral blood impossible. GnRH neurons are distributed in a particular way within brain structures, they form a hypothalamic continuum with a diffuse distribution from the pre-optic area (PAO) to

Figure 4. ***GnRH synthesis diagram.***



P- = pyro-glutamine; GAP = "GnRH Associated peptide"; LDCV = "Large Dense Core Vesicles".

the Ventromedial Hypothalamus (VMH) (Wray, 2010). GnRH neurons project the majority of their axons into the median eminence (ME), in the vicinity of the capillaries of the hypothalamic-pituitary portal system (Duittoz and Prévot, 2014) (figure 5).

During oestrus, high concentrations of E2 induce a GnRH peak which leads to the pre-ovulatory peak of LH (Clarke *et al.*, 1989; Moenter *et al.*, 1991) but GnRH neurons do not express oestrogen receptor alpha involved in the positive retrocontrol, so this effect must be mediated by an intermediate system.

b. Role of kisspeptin in triggering spontaneous ovulation

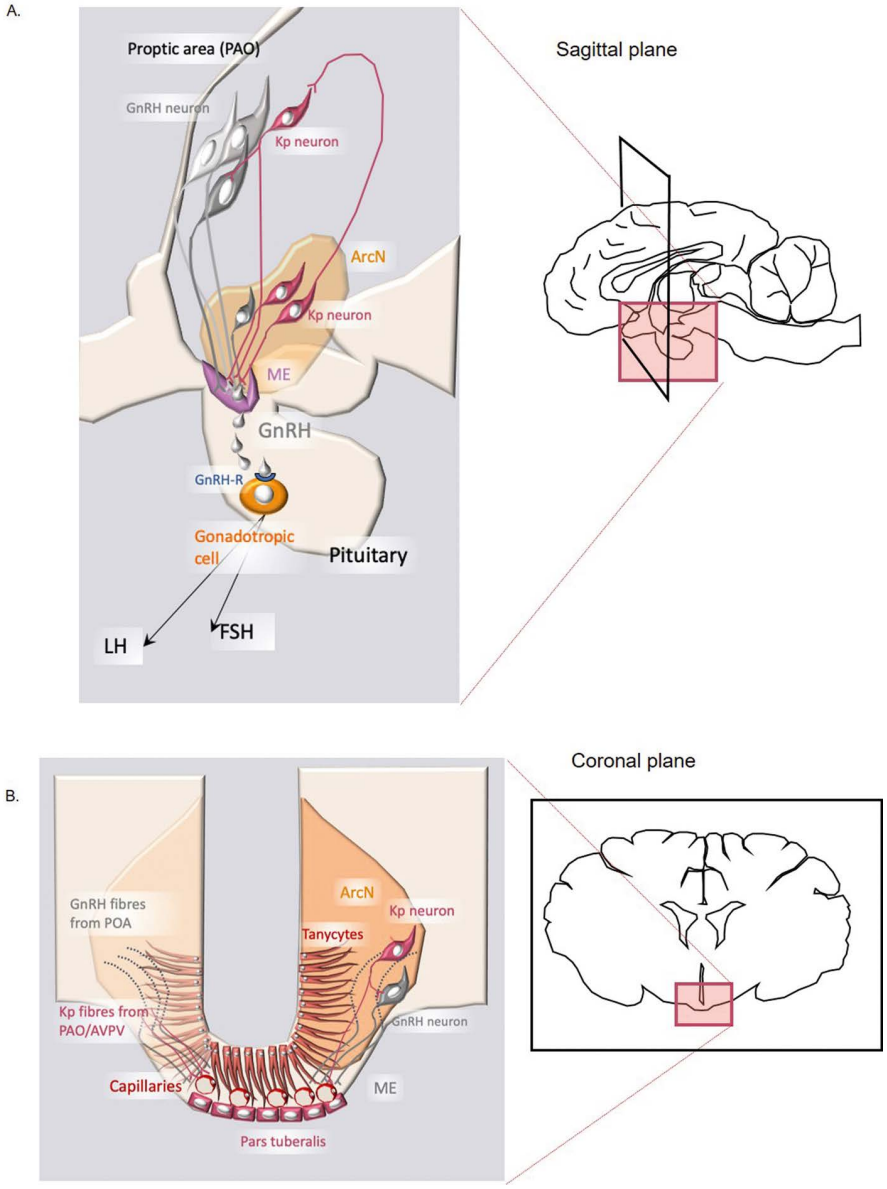
The expression of *KISS-1* gene results in a pre-pro-peptide, which is then cut into different peptides ranging from 54 amino acids for the longest, to 10 amino acids for the shortest. The set of these peptides is generally known as kisspeptin (Kp) (figure 6). The essential role of Kp in the control of reproduction was discovered through the study of mutations in the *KISS1R* gene encoding the GPR54 receptor, inducing a loss of function associated with hypogonadotropic hypogonadism in infertile patients (de Roux *et al.*, 2003). Administration of Kp in ewes results in the secretion of GnRH and the gonadotropins LH and FSH (Caraty *et al.*, 2007). Kp acts directly on GnRH neurons, the majority of which express the GPR54 receptor and produce an increase in their electrical activity (Messager *et al.*, 2005). Kp is secreted by two distinct populations of neurons located one in the arcuate nucleus (ArcN) and the other in the POA in ewes or the periventricular anteroventricular area (AVPV) in rodents. Kp neurons in POA/AVPV show a marked sexual dimorphism. In males, there are very few cell bodies in POA/AVPV while in females this region is rich in Kp expressing neurons. The somas of GnRH neurons are mostly contacted by POA Kp while GnRH neurons fibres located in ME are mostly contacted by ArcN Kp neurons (Caraty and de Roux, 2014). Both Kp neurons populations express the α receptor for estrogen, and thus could support the transmission of positive E2 feedback (Franceschini *et al.*, 2006; Smith, 2008).

c. Influence of photoperiod

One of the environmental signals exerting a major control on the secretion of GnRH is the photoperiod. Photoperiod refers to variations in day length over the course of a year. It underlies the alternation of sexual activity and sexual rest phases in so-called seasonal species (e.g. sheep, goat, horse). This temporal marker allows these species to anticipate the period of the year that is favourable for the development of offspring.

The light message associated with the photoperiod is picked up by the retina and transmitted to the pineal gland through a multisynaptic pathway. In the absence of light, the pineal gland releases melatonin, this secretion

Figure 5. ***Neuroanatomy of GnRH and Kisspeptin (Kp) neurons.***



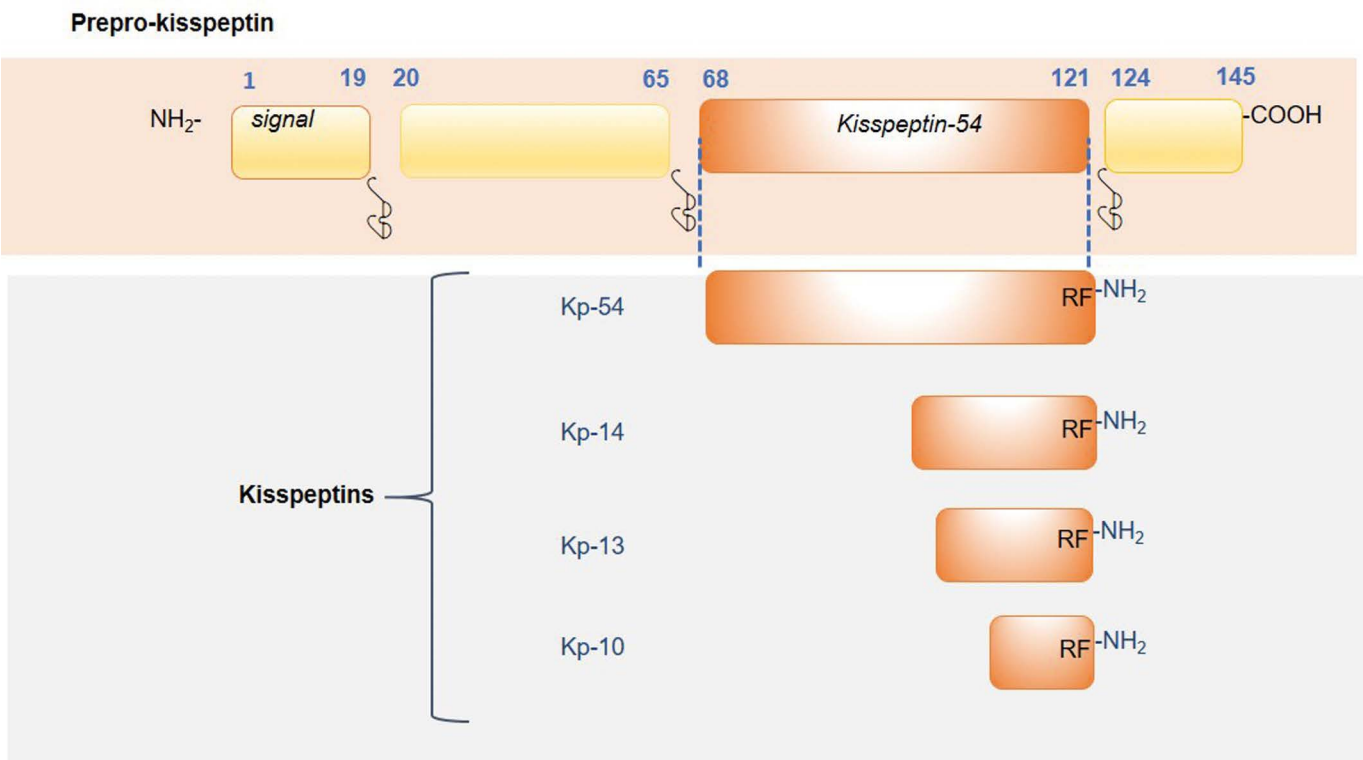
ArcN = arcuate nucleus.

is inhibited in the presence of light through the neural pathway from the retina. Melatonin does not act directly on GnRH neurons since they do not possess the type 1 melatonin receptor (MT1 involved in the control of reproductive activity on long days) (Migaud *et al.*, 2016). The precise mechanism of transmission of the melatonin message to GnRH neurons is not yet clearly established. Nevertheless, some crucial players seem to emerge. In particular, melatonin modulates the local concentrations of thyroid hormones via control of deiodinases, enzymes that convert thyroid hormone thyroxine (T4). Diodinases II (Dio II) and III (Dio III) are respectively responsible for the conversion of T4 into its active form triiodo thyronine (T3) or into inactive forms diiodo thyronine (T2) and reverse T3 (rT3) (Dardente *et al.*, 2014). However, the expressions of Dio II and Dio III vary according to the time of year. In ewes, Dio II is expressed more during the breeding season and Dio III during the sexual resting season. These variations in deiodinase expression are themselves dependent on variations in Thyroid stimulating hormone (TSH) expressed locally in the *pars tuberalis* of the pituitary gland. TSH secretion is known to be directly dependent on melatonin, whose MT1 receptor is strongly expressed in the *pars tuberalis*. However, two major questions remain unanswered. The first is what is the true physiological importance of this mechanism? Indeed, TSH/deiodinase/T3 pathway induced by long days is present not only in animals that breed on long days (e.g. hamster, quail...) but also in animals that breed on short days (e.g. sheep) and in non-seasonal animals (e.g. mice). The second question is how do local variations of T3 in the mediobasal hypothalamus control GnRH secretion? Several studies underline the role of populations of neurons expressing RF-amide type peptides: the arcuate nucleus with Kp neurons and the ventromedian and dorsomedian hypothalamus where neurons expressing RFRP-3 (also known as *gonadotropin-inhibitory hormone,* GnIH) are found (Dardente *et al.*, 2014). Kp neurons may thus represent a link between changes in thyroid hormone levels and reproductive axis function. T3 would act by blocking the estrogen/Kp dialogue essential for the secretion of GnRH (Dardente, 2011; figure 7).

d. Importance of the glial environment

GnRH neurons are closely associated with tanycytes, a particular type of glial cell that lines the third ventricle and the median eminence. Tanycytes undergo structural changes during the ovarian cycle, they retract their extensions or feet, allowing direct contact of GnRH neurons axonal endings onto blood vessels of the hypothalamic-pituitary portal system, which would facilitate the diffusion of GnRH into the blood (Prévot *et al.*, 1999). These changes in tanycyte morphology depend on the production of nitric oxide (NO)

Figure 6. ***Diagram of the synthesis of kisspeptin.***



by endothelial cells (de Seranno *et al.*, 2010). E2 leads to the retraction of tanycyte extensions notably by stimulating the expression of eNOS (endothelial nitrite oxide synthase) in endothelial cells. In addition, E2 also acts directly on tanycytes by stimulating the expression of COX-1 and COX-2 cyclooxygenases, stimulating the production and secretion of prostaglandin E2 (PGE2) which in turn, stimulates GnRH neurons electrical activity (Clasadonte *et al.*, 2011). Other factors such as neuregulins and Transforming Growth Factor α (TGF-α) via their erbB1 and erbB2 receptors present on tanycytes, stimulate the expression of COX and the production of PGE2 (Prévot *et al.*, 2010).

Glial cells forming the microenvironment of GnRH neurons are coupled together via gap junctions. Blocking communication through gap junctions causes a significant decrease in pulsatile GnRH secretion suggesting that in the basal state, communication via gap junctions between glial cells is essential for adequate GnRH secretion. (Pinet-Charvet *et al.*, 2016).

e. Role of the Beta Nerve Growth Factor in Ovulation

In mammals with induced ovulation such as llama, alpaca and dromedary, ovulation is triggered by a factor present in seminal plasma: OIF (see for review Adams *et al.,* 2016) . In alpacas, administration of OIF (1mg equivalent β-NGF) by intramuscular route causes ovulation by a mechanism involving the secretion of GnRH (Silva *et al.,* 2011). Other studies suggest that OIF is also present in spontaneously ovulating species. Indeed, intramuscular injection of seminal plasma from bulls, stallions and boars to female llama, provoke ovulation in 26%, 29% and 18% of cases respectively, suggesting that the seminal plasma of these species also contains molecules with OIF activity (Bogle *et al.,* 2011).

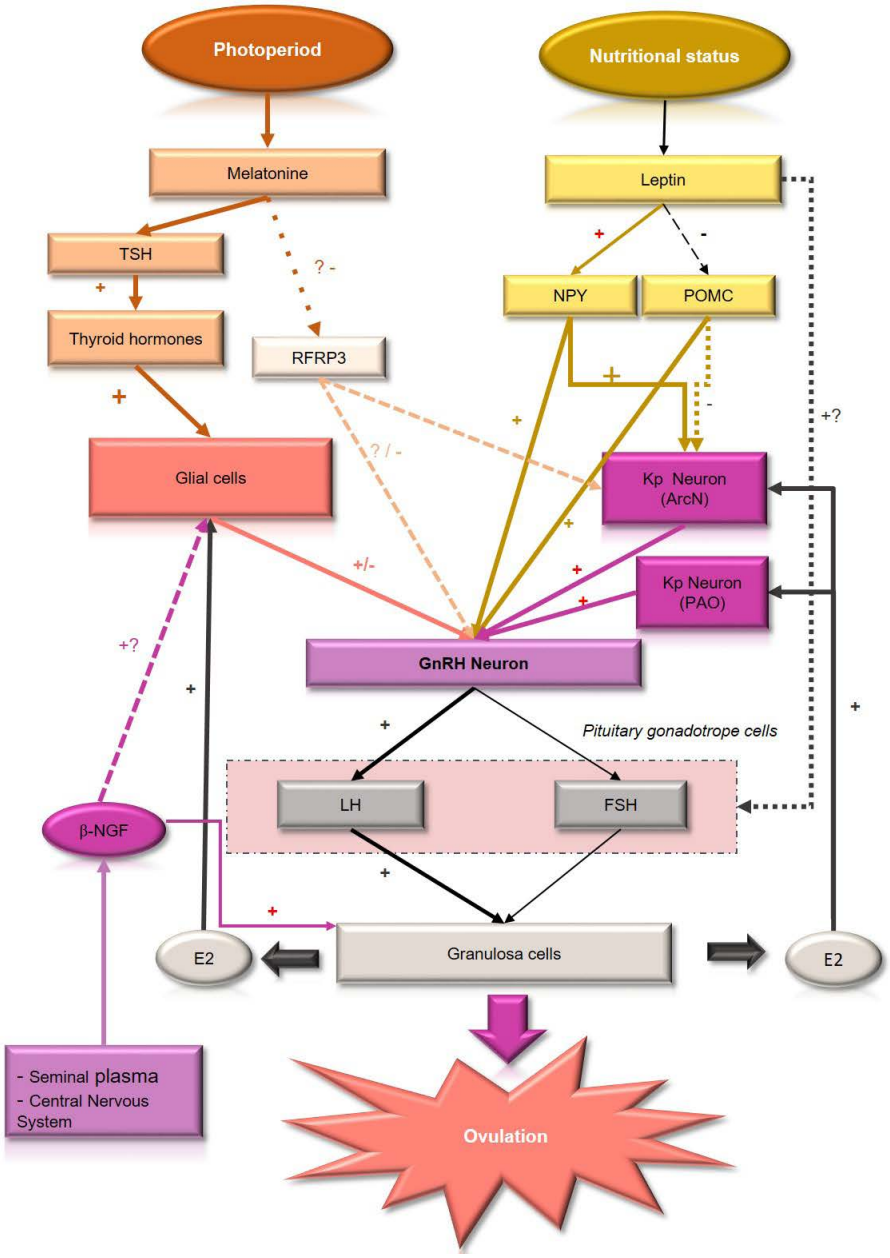
Proteomic analysis of alpaca seminal plasma allowed the identification of the chemical nature of OIF which corresponds to the "*β-Nerve Growth Factor*" (β-NGF) (Kershaw-Young *et al.*, 2012,Ratto *et al.*, 2012). β-NGF is a neurotrophin identified by Rita Levi-Montalcini (Nobel Prize in Physiology in 1986). β-NGF has two types of receptors, TrkA and P75NTR. β-NGF plays a role in the survival, proliferation and differentiation of neurons (for review Levi-Montalcini *et al.,* 1996).

Like Kp, β-NGF could play its role in ovulation induction by stimulating the secretion of GnRH. In support of this hypothesis, *in vitro* studies carried out on embryonic mouse GnRH neurons primary induces an increase in GnRH neurons electrical activity (Pinet-Charvet and Duittoz, 2015).

f. Role of leptin

Metabolic status also play a role in the regulation of GnRH secretion and in the induction of ovulation. Numerous studies show that fertility is closely linked to body condition and nutritional status (Dupont *et al.*, 2014). The link

Figure 7. ***General diagram of gonadotropic axis regulation.***



between weight and body condition and the onset of puberty in heifers or postpartum fertility in cows has been known for more than 50 years, for review (D'Occhio *et al.*, 2019). Numerous metabolic factors have been shown to influence either directly the ovarian follicles or indirectly via regulation of the hypothalamo-hypophysio-gonadal axis. Two main actors are hormonal in nature and have been discovered over the last 20 years, leptin, which is secreted by fat tissue, and ghrelin, which is secreted by the stomach.

Information on the state of an individual's lipid reserves is mediated primarily by leptin. This hormone is known to act on gonads and pituitary cells. It also seems to play a role in the secretion of GnRH, but indirectly. Since GnRH neurons do not express the leptin receptor, it could act via Kp neurons. Indeed, Kp neurons express leptin receptors (Backholer *et al.*, 2010; Hausman *et al.*, 2012). Other neurons may play this role, in sheep and rodents, ArcN Neuropeptide Y (NPY) neurons make synaptic contacts on GnRH neurons cell bodies (Norgren and Lehman, 1989; Tillet *et al.*, 1989). NPY may have either stimulating or inhibitory effects on the activity of GnRH neurons via the Y4 and Y1 receptors, respectively (Roa and Herbison, 2012). These NPY neurons express leptin receptors and are involved in the control of energy balance (Baskin *et al.*, 1999) (figure 7). Ghrelin is secreted by the stomach and acts on the hypothalamus; its secretion induces hunger feelings. Ghrelin acts directly on GnRH neurons by reducing their activity (Farkas *et al.*, 2013).

■ 2.4. Ovulation control

We will not deal here with the detection of the time of ovulation but will focus on the methods used for ovulation induction in different species and on ways of developing new paradigms.

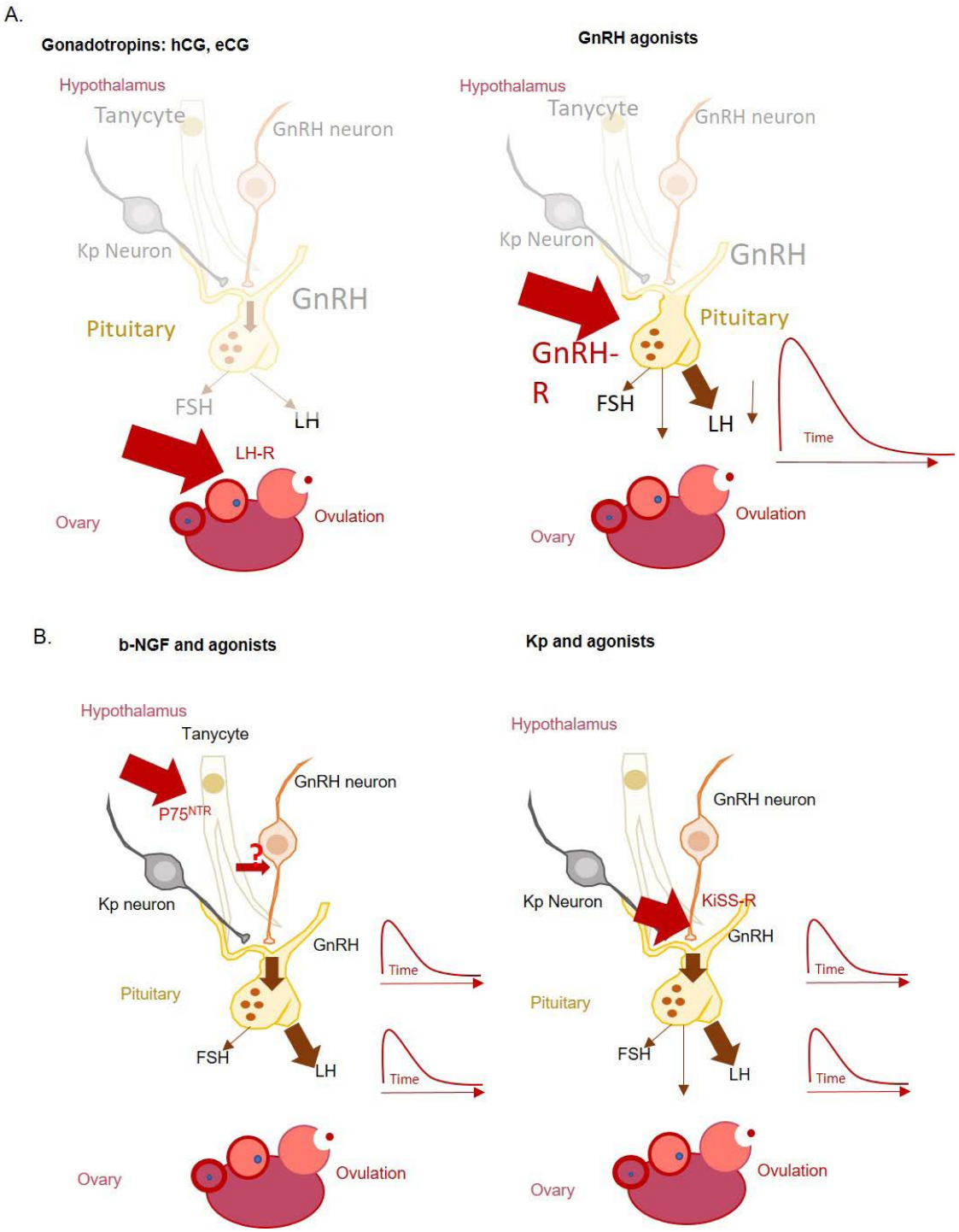
a. Current methods

Two main methods can be distinguished according to the target: ovary and hypothalamic-pituitary complex (figure 8).

b. Action at the ovarian level

Physiologically, LH causes ovulation by promoting the rupture of the follicle wall through the cellular and molecular mechanisms described above. However, LH has a short plasma half-life (less than two hours, except in mares). LH is therefore not used in practice. However, in the human and equine species, there are placental hormones with LH activity, hCG ("*human chorionic gonadotropin*") and eCG ("*equine chorionic Gonadotropin*"). hCG is produced by trophoblastic cells, its post-translational modifications give it a long half-life between 24 and 48 hours. Thus, hCG has been granted marketing authorization for the induction of ovulation in domestic mammals (equine, bovine, ovine, caprine, porcine, dog and cat) under the name Chorulon®.

Figure 8. ***Methods currently used for ovulation control (A) and new paradigms (B).***



A. Principles of current methods to induce ovulation. hCG or eCG gonadotropins act directly on the dominant follicle to induce ovulation. GnRH agonists act on the pituitary gland by stimulating the secretion of LH.
B. New paradigms. β-NGF acts at the hypothalamic level upstream of GnRH neurons by a still unknown mechanism. Kp acts at the hypothalamic level by stimulating GnRH neurons.

However, hCG aminoacids sequence is different from that of LH from other mammalian species and therefore, antibodies will be produced by the immune system and will block hCG activity. Furthermore, hCG can induce ovarian hyperstimulation. eCG has LH activity in the mare but has FSH-like activity in other species. It is used under its former name, PMSG ("*pregnant mare serum gonadotropin*", *e.g.* Chrono Gest®) to stimulate follicular growth after synchronisation by progestagens (fluorogestone e.g. Synchropart®). Ovulation occurs spontaneously following the development of pre-ovulatory follicles. This method is used for natural breeding or AI in fresh semen mainly in small ruminants. The

use of eCG poses the same problem as that of hCG, i.e. repeated injections favour the appearance of antibodies and the ineffectiveness of subsequent stimulation. In addition, the method of eCG production which involves bleeding of pregnant mares that are aborted, raises ethical issues that have recently been echoed in European media. hCG is purified from urine of pregnant women and does not pose any particular ethical problems.

c. Action at the hypothalamic-pituitary level

- *GnRH Agonists*

GnRH agonists will act on GnRH receptors expressed by gonadotropic cells and induce the secretion of LH and FSH. This stimulation can lead to a very important secretion of LH because the stocks of LH in the pituitary gland are much higher than the quantity of LH mobilized during a physiological stimulation by endogenous GnRH. GnRH agonists are peptides derivate from GnRH to prolong half-life. Of these compounds, only two have veterinary market authorisation in France, deslorelin (Ovuplant®) and buserelin (Receptal®). As the chemical structure of these agonists is based on the amino acid chain of GnRH, the probability that they induce an immune reaction is very low and to date, no such effect has been reported in domestic mamals. These compounds should be used with caution, because if their persitence is too long, the pituitary gland will desensitize and will no longer respond to endogenous GnRH. If, following induction of ovulation by means of a deslorelin implant, insemination is not followed by fertilisation, the next cycle will be delayed. Deslorelin in the form of an implant is used to induce an estrus state in heifers in order to synchronize them (Padula, 2005), but also in dogs and stallions to obtain chemical castration. In the human species, goserelin, triptorelin and leucoprorelin, three GnRH agonists are used orally or by injection to induce chemical castration and to lower circulating estrogen levels in the adjunctive treatment of estrogen-dependent tumours. Thus, the use of GnRH agonists to induce ovulation can be tricky and lead to contradictory.

- *Estrogen*

Administration of a high dose of E2 induces ovulation. The mechanism involves both hypothalamic and pituitary effects. High-dose E2 exerts positive feedback control on the hypothalamus and results in massive secretion of GnRH. It exerts positive feedback control on the pituitary gland by potentiating the secretion of LH. Unfortunately, the use of E2 or analogues poses many problems for the environment. Indeed, the analogues used are not very degradable in the organism and are eliminated in active form. They accumulate in surface waters and are not necessarily disposed of in water treatment plants. Estrogenic contamination of watercourses is a major environmental problem. The use of oestrogens for the control of reproduction has been banned in Europe since 2003 (Directive 2003/74/EC of the European Parliament). Nevertheless, their use outside Europe is developing, particularly in protocols without heat detection and programmed AI (Torres-Júnior *et al.,* 2014).

■ 2.5 New paradigms for ovulation control

Current ovulation induction methods use mechanisms downstream of the main ovulation control located in the hypothalamus. These methods, although effective, pose various problems that have been described above. In the search for new ovulation induction methods, the objectives to be achieved are: search for physiological stimulation avoiding ovarian and/or pituitary hyperstimulation, ease of use (single administration), robustness of the induction effect, absence of negative impacts on the environment, health and well-being of the animal. We will consider only two possibilities that seem particularly interesting to us and which target the hypothalamic level (figure 8).

a. Stimulation of GnRH secretion by Kp

In spontaneously ovulating species, ovulation is triggered by stimulation of GnRH neurons by Kp. In human *in vitro* fertilization protocols Kp54 has been successfully used to induce oocyte maturation when using a standard superovulation protocol (Jayasena *et al.*, 2014). Farm animal studies have so far focused on ewes with promising results. During anestrus season, Kp10 intra venous infusion lasting 24 hours or more is capable of reactivating the hypothalomo-hypophysis-gonad axis and triggering ovulation (Caraty *et al.*, 2007). More recently we have developed a Kp10 analogue, called C6, with an improved pharmacological profile (Beltramo *et al.*, 2015). A single intramuscular injection of C6 is capable, following pre-treatment with a progesterone analogue, of inducing pre-ovulatory peaks of LH and fertile ovulation in ewes (Decourt *et al.*, 2016a). These results confirm that it is possible to control reproduction by acting directly on GnRH neurons with Kp analogues.

b. Stimulation of GnRH secretion by β-NGF

In camelids, species with induced ovulation, β-NGF is the trigger for ovulation. However, it seems that the β-NGF is also capable of inducing ovulation in spontaneously ovulating mammals. Thus, administration of alpaca seminal plasma to prepubertal mice in an ovarian

stimulation protocol can induce ovulation (Bogle *et al.*, 2011). β-NGF increases LH secretion and improves corpus luteum function in cattle (Tribulo *et al.,* 2015). In another species with induced ovulation, the rabbit, recent work shows the coexistence of two mechanisms (Maranesi *et al.*, 2018). The first involves the β-NGF of the seminal plasma and the second involves the production of β-NGF by the uterus, the latter acting on the visceral sensory fibers that express the TrkA receptor, and sending a signal, as yet unidentified, to the hypothalamus. Although the mechanisms involved in the effects of β-NGF on ovulation have not yet been clarified, the results show the interest at deciphering the molecular mechanisms involved. Furthermore, an important point for further exploration of the potential of β-NGF to control reproduction in domestic mammals will be the creation of small synthetic agonists that will make the use of this strategy economically feasible. The first steps in this direction have been taken for example with the synthesis of small molecules such as LM11A-31 (Massa *et al.*, 2006) and BNN27 (Pediaditakis *et al.*, 2016).

Conclusion

The mechanisms triggering ovulation are universal in mammals, they involve the secretion of the neurohormone GnRH which will stimulate the secretion of LH leading to ovulation of the dominant follicle(s). However, the mechanisms that determine the timing of ovulation, i.e. those that control the increase in GnRH secretion, are complex and differ between species, particularly according to the mode of ovulation: spontaneous or induced. Current methods of ovulation induction act downstream of the hypothalamic stage and target either the ovary directly, as in the use of hCG or eCG, or the pituitary gland with the use of GnRH agonists. In addition to the problems of antibody development following the use of heterologous gonadotropins, gonadotropic hormones and GnRH agonists cause supra-physiological stimulation of the ovary or pituitary gland. In addition, in the case of eCG, the way in which this hormone is produced by pregnant mares raises ethical problems which have been relayed by animal protection associations and by the European *media*. The new paradigms for ovulation induction target the hypothalamic stage and the regulation of GnRH neuron activity, allowing the physiological induction of ovulation. C6, a Kp agonist, allows the induction of ovulation in small ruminants after a single administration. Several potential advantages would be linked to its use: absence of hyperstimulation, use of a low dose generating little or no residues released into the environment, lack of activity after ingestion, which is the classic route of contamination of endocrine disruptors. Regarding β-NGF, the scientific literature is less abundant, but it offers good prospects in species for which Kp is not or is less effective, as in the case of the mare.

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Abstract

The triggering of ovulation in mammals can be done according to two modes: spontaneous and provoked. Spontaneous ovulation occurs during the estrous cycle as a result of internal endocrine factors. Induced ovulation is triggered by mating. In both cases, it is the increase in GnRH secretion that leads to an increase in LH that causes ovulation. The factors involved in the stimulation of GnRH secretion are different according to the two modalities, mainly kisspeptin for spontaneous ovulators and β-NGF for provoked ovulators. Current protocols used to induce ovulation rely on a direct action on the ovary, through the use of heterologous gonadotropins, or by an action on the pituitary gland with GnRH agonists. These protocols present different disadvantages: loss of activity with time, supra-physiological stimulation, ethic questioning about the production of some of these products. New paradigms for triggering ovulation by targeting the hypothalamus, respectful of animal welfare and the environment are presented in this review. This review aims to introduce the reader to the cellular and molecular mechanisms involved in the regulation and triggering of ovulation as well as two new approaches that are being developed and that are respectful of the animal and of its environment.

Résumé

L'ovulation chez les mammifères

Le déclenchement de l'ovulation peut se faire selon deux modes chez les mammifères : spontané et provoqué. L'ovulation spontanée intervient au cours du cycle œstral sous l'effet de facteurs internes hormonaux. L'ovulation provoquée est déclenchée par l'accouplement avec un mâle. Dans les deux cas, c'est une augmentation de la sécrétion de GnRH qui conduit à l'augmentation de LH qui provoque l'ovulation. Les facteurs qui conduisent à l'augmentation de sécrétion de GnRH sont différents selon les deux modalités : principalement la kisspeptine (ovulation spontanée) et le β-NGF (ovulation provoquée). Les protocoles d'induction de l'ovulation actuels reposent sur une action directe sur l'ovaire, grâce à l'utilisation de gonadotropines hétérologues, ou bien par une action sur l'hypophyse grâce aux agonistes du GnRH. Ces protocoles présentent différents inconvénients : perte d'efficacité dans le temps, stimulation supra-physiologique qui peut être délétère, problème éthique posé par l'obtention de certaines molécules. De nouveaux paradigmes d'induction de l'ovulation ciblant l'hypothalamus, et favorisant un déclenchement physiologique de l'ovulation sans supplémentation en hormones sont en cours de développement. Cette revue a pour objectif de présenter au lecteur les mécanismes intimes impliqués dans la régulation et le déclenchement de l'ovulation ainsi que deux approches nouvelles respectueuses de la physiologie de l'animal et de l'environnement.

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