New insights of the role of β-NGF in the ovulation mechanism of induced ovulating species

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Abstract

The type of stimuli triggering GnRH secretion has been used to classify mammalian species into two categories: spontaneous or induced ovulators. In the former, ovarian steroids produced by a mature follicle elicit the release of GnRH from the hypothalamus, but in the latter, GnRH secretion requires coital stimulation. However, the mechanism responsible for eliciting the preovulatory LH surge in induced ovulators is still not well understood and seems to vary among species. The main goal of this review is to offer new information regarding the mechanism that regulates coitus-induced ovulation. Analysis of several studies documenting the discovery of β-NGF in seminal plasma and its role in the control of ovulation in the llama and rabbit will be described. We also propose a working hypothesis regarding the sites of action of β-NGF in the llama hypothalamus. Finally, we described the presence of β-NGF in the semen of species categorized as spontaneous ovulators, mainly cattle, and its potential role in ovarian function. The discovery of this seminal molecule and its ovulatory effect in induced ovulators challenges previous concepts about the neuroendocrinology of reflex ovulation and has provided a new opportunity to examine the mechanism(s) involved in the cascade of events leading to ovulation. The presence of the factor in the semen of induced as well as spontaneous ovulators highlights the importance of understanding its signaling pathways and mechanism of action and may have broad implications in mammalian fertility.

Introduction

Ovulation in mammals involves the release of GnRH from the hypothalamus into the hypophyseal portal system followed by the release of LH from the pituitary gland into the systemic circulation (Karsch 1987, Karsch et al. 1997). Mammals have been classified as either spontaneous or induced (reflex) ovulators. In spontaneously ovulating females (e.g., woman, sheep, cow, mare, sow), increasing systemic concentrations of estradiol, under basal levels of progesterone, induces release of GnRH from the hypothalamus (Jaffe & Keys 1974, Knobil 1980, Kelly et al. 1988, Turzillo & Nett 1999). Hence, a luteal phase in these species is induced spontaneously and ovulation occurs at regular intervals. By contrast, in induced ovulators (e.g., rabbits, ferrets, cats, camelids), copulation is responsible for triggering GnRH secretion from the hypothalamus, followed by a pre-ovulatory release of LH from the pituitary (Bakker & Baum 2000). Therefore, females from these species do not display an estrous cycle per se, as observed in spontaneous ovulators.

Reasons for the evolution of a species-specific mechanism of ovulation among mammals remain unknown. Current concepts of phylogeny do not explain the presence of one type of ovulatory mechanism over the other, and the evolutionary drive toward an induced or spontaneous ovulation mechanism may be associated with social or environmental cues for a given species. Although the LH surge is a mandatory event for triggering ovulation in both types of ovulators, the specific signaling pathway to elicit the GnRH/LH surge is still poorly understood in induced ovulators.

The main goal of this review is to offer new information regarding the mechanism that regulates coitus-induced ovulation. Analysis of several studies documenting the discovery of β-NGF in seminal plasma and its role in the ovulatory mechanism in the llama and rabbit will be described. We also propose a working hypothesis regarding the site of action of β-NGF in the llama hypothalamus. Undoubtedly, gaps remain in our understanding of the role of β-NGF in the ovulation mechanism, but the results of recent research on the presence and effect of this seminal factor in both spontaneous and induced ovulators are exciting and may have a profound impact on fertility management in domestic animals and humans.
General aspects of ovulation induction in induced ovulatory species

The first evidence of coitus-induced ovulation was documented more than 100 years ago by Heape (1905) who described the phenomenon in rabbits. In some induced ovulators, ovulation is triggered by penile intromission (mink and ferret; Carroll et al. 1985, Sundquist et al. 1988), mounting with or without copulation (rabbit; Ramirez & Soufi 1994), single copulation (camelids; Fernandez-Baca et al. 1970, England et al. 1971, Marie & Anouassi 1986) or multiple copulations (cat; Concannon et al. 1980). Although circulating estradiol concentration was positively correlated with increasing size of the dominant ovarian follicle in llamas and alpacas, the physiological increase did not result in ovulation in these species (Bravo et al. 1990a,b, 1991, 1992). Similarly, neither endogenous nor exogenous estradiol induced LH secretion in rabbits or ferrets (Sawyer & Markee 1959, Baum et al. 1990). However, estradiol in induced ovulators was reported to act on the female hypothalamus to induce the behaviors characteristic of the proceptive and receptive sexual stages (Baum & Schretlen 1978).

The rabbit is the only induced ovulator in which GnRH has been measured and was found to be released in a pulsatile manner using the push–pull perfusion technique (Pau et al. 1986, Ramirez et al. 1986). Coitus was associated with a dramatic increase in GnRH pulse-frequency from the medial basal hypothalamus (MBH) followed by an increase in circulating LH concentrations within 20–60 min (Pau et al. 1986, Ramirez et al. 1986). Neurotransmitter and peptidergic systems have been shown to affect GnRH secretion and ovulation in mammalian females (reviewed in McEwen & Parsons 1982). An increase in norepinephrine after coitus was deemed responsible for regulating GnRH secretion and subsequent ovulation in rabbits (reviewed in Kaufman & Rissman 2006). Norepinephrine neurons, A1, A2, A5 and A6 groups are located in several loci of the brainstem, including the medulla oblongata, solitary tract, lateral tegumentum of the pons and the locus coeruleus. The effects of neuropeptide Y and acetylcholine have also been reported to modulate ovulation in the rabbit (Ramirez & Soufi 1994).

Recent findings in camelids (llamas, alpacas, camels) offer new insight into the mechanism of induced ovulation (reviewed in Adams & Ratto 2013, Adams et al. 2016). An ovulation-inducing factor (OIF) in the seminal plasma of camelids has been isolated and identified as nerve growth factor (β-NFG; Kershaw-Young et al. 2012, Ratto et al. 2012, Kumar et al. 2013). Moreover, we have developed a llama model to document that genital-somatic sensory stimuli of copulation is not the principal ovulation-inducing signal in this species (Berland et al. 2016). Whether or not the same mechanism is present in other species of induced ovulators is yet unknown.

The discovery of β-NFG in the semen of camelids and its role in ovulation induction

Seminal plasma is produced by the male accessory sex glands and its composition may vary among species according to the type of glands present. Seminal plasma has been considered primarily a vehicle for sperm transport; however, it contains proteins, growth factors, hormones and cytokines that may regulate important functions in the spermatozoa and in the female reproductive system (Bromfield 2016).

The first study to document a direct role of semen on the ovulatory mechanism was reported by Chen et al. (1985) where ovulation was observed after intra-vaginal semen deposition in Bactrian camels without any physical contact with the male. Also, seminal plasma was found to stimulate LH secretion from rat pituitary cells cultured in vitro (Paolicchi et al. 1999). The first study to document the association between systemic seminal plasma administration and LH secretion and ovulation in alpacas and llamas was reported by Adams et al. (2005); results demonstrated the presence of an ovulation inducing-factor (OIF) in the seminal plasma of both species. Intramuscular administration of seminal plasma induced ovulation in >90% of females by eliciting a sustained preovulatory LH surge.

The effect of seminal plasma on ovulation may not be unique to induced ovulatory species. For instance, the preovulatory LH surge was advanced in cattle when mating was conducted within the first 6–8 h of behavioral estrus (Joehle 1975). Similarly, intrauterine infusion of boar seminal plasma accelerated ovulation in gilts (Waberski et al. 1995). Also, studies in humans suggested the presence of molecules with GnRH-like activity in semen (Izumi et al. 1985, Sokol et al. 1985).

Aside from the potent ovulatory effect of llama β-NFG, this molecule has a marked luteotrophic effect after intramuscular or intrauterine infusion in llamas and alpacas (Adams et al. 2005, Ratto et al. 2006). The CL that developed after ovulations induced by seminal plasma treatment tended to be larger and regress later than the CL resulting from GnRH treatment, and progesterone secretion was higher than that in GnRH-treated females (Adams et al. 2005). The magnitude of LH release and subsequent CL function in female llamas treated with seminal plasma suggest that the luteotropic effect of β-NFG is mediated by LH. In this regard, the luteotropic effects of seminal plasma has been confirmed in other studies using purified NGF from the seminal plasma of llamas (Ratto et al. 2011, Silva et al. 2011a,b, 2014, Tanco et al. 2012). Moreover, an increase in the vascular area of the CL was highly correlated to progesterone production in females treated with β-NFG compared to the GnRH-treated group (Ulloa-Leal et al. 2014).
Potential mechanism of seminal plasma β-NGF in ovulation induction in llamas

The circuits and basic mechanisms of stimulation and signaling for GnRH release and LH discharge have not been intensively investigated in induced ovulators. Although different stimuli such as olfactory, tactile, visual and auditory have been indicated as facilitators or modulators of ovulation in reflex ovulators, penile intromission during copulation has been considered the main factor associated with the preovulatory discharge of LH and subsequent ovulation in these species (Bakker & Baum 2000, Kauffman & Rissman 2006). However, more recent studies in llamas and alpacas have provided important and conclusive evidence to challenge the notion that physical stimulation of the genital tract is a necessary requirement to induce ovulation in these species (Adams & Ratto 2013, Adams et al. 2016, Berland et al. 2016).

Systemic administration of purified NGF from llama seminal plasma stimulates secretion of LH in both intact and ovariectomized llamas, and the magnitude of LH secretion was modulated by estradiol (Silva et al. 2012). In addition, the effect of OIF on LH secretion was inhibited in llamas pretreated with a GnRH antagonist (Silva et al. 2011a). Results document that NGF elicits a preovulatory LH surge by a direct or indirect action on GnRH neurons.

In a recent study (Berland et al. 2016), an urethrostomized male was used to discriminate between the effects of seminal plasma NGF and penile stimulation as the main stimulus to induce ovulation in llamas. Females mated with the urethrostomized male did not ovulate (0/6), but ovulation was observed in females mated with an intact male (6/7) or those given an intrauterine infusion of seminal plasma (5/6). Results indicated that intrauterine administration of seminal plasma was sufficient to elicit an LH surge and that circulating LH concentrations were positively correlated with a rapid systemic increase in β-NGF observed during the first hour after treatment. The results of this and previous studies (Ratto et al. 2005, Silva et al. 2011a, 2012, Berland et al. 2016) suggest that β-NGF from the seminal plasma is absorbed from the endometrium after copulation and enters systemic circulation to elicit GnRH secretion from the hypothalamus. However, the signaling pathway by which β-NGF elicits GnRH secretion remains unknown.

How then does β-NGF gain access at the central level to trigger GnRH secretion? What are the potential sites of action or binding of NGF in the hypothalamus? Is it necessary for NGF to cross the blood–brain barrier to reach the hypothalamus? Does NGF act directly or indirectly on GnRH neurons? There are still many questions to answer.

Gonadotrophin-releasing hormone is produced by hypothalamic neurons and transported to the terminal axons located at the median eminence (ME; Fink 1988). In mammals, GnRH nerve fibers are concentrated mainly in the lateral and medial post-infundibular regions, where their axon terminals are housed in the pericapillary space of the hypophyseal portal system in the ME (Baker et al. 1975, Barry & Dubois 1976, Rodríguez et al. 2010). As well, GnRH fibers located in the floor of the third ventricle are closely associated with highly specialized glial cells, the tanyocytes, which have also been ascribed a role in the direct release of GnRH into the hypophyseal portal system (reviewed in Ojeda et al. 2008, 2010, Rodríguez et al. 2010).

In a recent study (Carrasco et al. 2018a), GnRH neurons in llamas were not equally distributed among hypothalamic areas and were not aggregated in hypothalamic nuclei, but rather had a more diffuse distribution. A high number of GnRH neurons were located in the medio-basal hypothalamus, twice as many as the second most-populated region, the anterior hypothalamus. A lower population of GnRH neurons was located in the diagonal band of Brocca and the arcuate and periventricular nuclei (Carrasco et al. 2018a).

Another component of the cellular network of GnRH release includes the neurons that produce Kisspeptin. In rodents, kisspeptin has been described as the most potent activator of LH secretion and the majority of GnRH neurons express receptors for kisspeptin (GPR54 or KISS1R; Herbison 2015). In rodents, the neurons that synthesize kisspeptin form clusters and are distributed in close association with GnRH neurons in the preoptic area and basal hypothalamus (Herbison 2015). Associations among GnRH neurons, kisspeptin neurons and hypothalamic tanyocytes have been implicated in the preovulatory LH surge (Clarkson et al. 2010, Ojeda et al. 2010, Rodríguez et al. 2010).

The entry of peripheral peptides/proteins into the brain is regulated by the blood–brain barrier, a unique uninterrupted barrier that is located at the level of the endothelial cells of the cerebral capillaries (Rodríguez et al. 2010). However, this barrier is displaced at the circumventricular organs to specialized ependymal cells (Hawkins & Davis 2005, Rodriguez et al. 2010). The circumventricular organs have been described as windows that allow neural cells to sense the blood and exchange certain substances between blood and nerve tissue, which cannot otherwise cross due to the presence of the blood–brain barrier (reviewed in Prevot et al. 2010, Rodríguez et al. 2010, Spuch & Navarro 2010).

Recent immunocytochemical studies of nerve tissue from llamas treated with seminal plasma (Berland 2017) revealed strong immunoreactivity for trkA (high-affinity receptor) and its ligand β-NGF in the choroid plexus at the roof of the third ventricle (Fig. 1). An interesting aspect of this finding is that the expression of the receptor in the choroid plexus has not been described in any species previously. In rats, Timmusk et al. (1995) reported the presence of NGF, NT-4, NT-3 and BDNF mRNAs in the

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choroid plexus, as well as high mRNA levels for trkB, undetectable levels of trkA and trkC mRNA. Although the presence of β-NGF and its specific receptor in the choroid plexus does not necessarily indicate that β-NGF is transported from circulation to the CSF by this route, this feature may represent a unique particularity associated with induced ovulating species. Transport of other growth factors, peptides, neurotrophins and hormones through their corresponding specific receptors at the choroid plexus have been demonstrated (Redzic & Segal 2004, Rodríguez et al. 2010), suggesting that this route may be a potential window for NGF to access the brain via cerebrospinal fluid.

We have also identified β-NGF immunoreactivity in the tanycytes of the median eminence in llamas (Fig. 2), where GnRH nerve terminals are concentrated. Tanycytes in the median eminence contribute to the development of an anatomical link between ventricular CSF and portal blood (Rodríguez et al. 2005). This anatomical arrangement allows bidirectional passage of macromolecules between the portal blood and CSF (Rodríguez et al. 2005, 2010, Prevot et al. 2010) potentially providing signals to the GnRH or kisspeptin neurons. In rats, removal of tanycytes prevented GnRH release into the portal blood, the peak of luteinizing hormone and ovulation in rats (Rodríguez et al. 1979). The mechanism of tanycyte in leptin transport from the portal blood into the ventricular CSF has been demonstrated (Balland et al. 2014). In addition, a greater relative abundance of kisspeptin fibers was detected in association with GnRH fibers in the median eminence region (Fig. 3).

Therefore, we propose two potential pathways by which β-NGF from seminal plasma may signal at the level of the hypothalamic region (Fig. 4): (1) by entering into the cerebrospinal fluid of the third ventricle through the choroid plexus or via tanycyte transport from portal capillaries at the median eminence or (2) by acting directly or indirectly on GnRH neurons present at the level of median eminence without entering into the third ventricle.

In this context, another potential site of signaling of NGF for GnRH release, may be the organum vasculosum laminae terminalis (OVLT), a structure where a significant population of GnRH neurons have been found in rodents (Silverman et al. 1994, Herbison 2006, Herde et al. 2011). Although it is not yet known if neurons of the OVLT are involved in the control of the hypothalamic–hypophyseal axis, we cannot rule out that systemic β-NGF may transduce its signal at this site to initiate the ovulatory cascade in llamas. Herde et al. (2011) showed that GnRH neurons at the OVLT...
also suggested nearly a decade ago (Niño 2010, Silva et al. 2011b). Niño (2010) documented the expression of β-NGF in rabbit semen by western blot, and when female llamas were used in an in vivo bioassay, intramuscular administration of rabbit seminal plasma induced and LH surge and ovulation (Silva et al. 2011b). Curiously, however, neither the administration of rabbit nor the administration of llama seminal plasma induced LH release or ovulation in rabbits (Silva et al. 2011b, Cervantes et al. 2015), suggesting that a species-specific response exists between these two induced ovulators.

Recently, several studies (Maranesi et al. 2015, Casares-Crespo et al. 2016, García-García et al. 2017, Maranesi et al. 2018) have confirmed the presence of NGF in rabbit semen. The molecule was found at relatively high concentrations in rabbit seminal plasma (about 1894 ± 277 pg/mL and 151.9 ± 9.25 μg/mL; Maranesi et al. 2015, 2018); however, concentrations were lower than those described in camelids (Tanco et al. 2012). Moreover, different authors have suggested that the prostate gland is the main source of this neurotrophin in rabbit semen (Maranesi et al. 2015, García-García et al. 2017), similar to reports in llamas (Bogle et al. 2013, 2018).

Despite the abundance of NGF in rabbit semen, the ovulatory responses after treatment with seminal plasma or NGF have not been as consistent as in camelids. A recent study (García-García et al. 2017) reported that only 1 of 6 female rabbits treated intramuscularly with 24 μg of murine β-NGF, ovulated, and no preovulatory elevation in LH was detected. In the same study, mechanical stimulation of the vagina increased the ovulation rate to 2/4 in β-NGF-treated females (García-García et al. 2017), suggesting that physical stimulation may be required to complement the effect of ovulation-inducing factor in this species. In contrast, Rebollar et al. (2012) reported 75% ovulation rate in rabbits does artificially inseminated with raw semen delivered by intravaginal deposition, but again, no increase in plasma LH concentration was observed. In the same study, the use of lumbar anesthesia blocked ovulation in the inseminated female rabbits. Similarly, Maranesi et al. (2018) reported ovulation rates of 67% (4/6) and 17% (1/6) in female rabbits inseminated with raw semen in non-anesthetized and lumbar-aneasthetized females, respectively, and ovulations were preceded by an increase in plasma LH concentration during the 2 h after artificial insemination. These last studies highlight the role of efferent nervous pathways in the induction of ovulation in rabbits.

Strikingly similar ovulation rates and changes in blood concentrations of β-NGF and LH were described in a recent report in rabbits (Maranesi et al. 2018) as in an earlier report in llamas (Berland et al. 2016) after natural mating or artificial insemination with raw semen. However, in rabbits, administration of lumbar anesthesia or COX inhibitors before artificial insemination blocked

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**Potential mechanism of seminal plasma β-NGF on ovulation induction in rabbits**

Similar to camels, the presence of a factor capable of inducing ovulation in the seminal plasma of rabbits was...
the increase in plasma LH concentration, attenuated the systemic rise of β-NGF and reduced the ovulation rate. Based on these findings, the authors proposed that in rabbits (a) semen-derived NGF stimulates de novo synthesis of NGF in the uterine wall, (b) both seminal NGF and uterine NGF are absorbed into the bloodstream and act directly on the ovary rather than on the pituitary or hypothalamus and (c) semen-derived and locally synthesized NGF stimulate uterine/cervix sensory neurons which trigger GnRH neurons in the hypothalamus.

The local effect of NGF in the rabbit ovary was based on immunodetection of NGF and its high- (trkA) and low- (p75) affinity receptors in the oocyte, and the granulosa and theca cells of ovarian follicles at different stages of development (Maranesi et al. 2018). Interestingly, in rabbits, i.m. treatment with homologous seminal plasma (Silva et al. 2011b) or with murine β-NGF (García-García et al. 2017) resulted in a significant increase in the number of large hemorrhagic anovulatory follicles. Similar hemorrhagic follicles have been observed after superovulation of does with eCG (García-Ximénez & Vicente 1990, Mehaisen et al. 2005) or treatment with high doses of hCG (Bomsel-Helmreich et al. 1989), suggesting a direct local effect of these molecules at the follicular wall. Whether the formation of large hemorrhagic follicles observed after treatment with seminal plasma (Silva et al. 2011b) or NGF (García-García et al. 2017) is due to a direct action of NGF on its own receptors or by means of an indirect action through LH receptors at the follicular wall remains to be investigated.

Taken together, results of recent studies of the effects of seminal plasma lead us to re-evaluate the mechanism of reflex ovulation stated many years ago. For instance, β-NGF in camelid semen is the main driver of ovulation, and mechanical stimulation of the female genitalia appears to have little or no role in this species. Conversely, although the presence of β-NGF may be involved in the ovulatory mechanism in rabbits, it appears that physical stimulation is required to induce a high rate of ovulation.

### Potential mechanism of seminal plasma β-NGF in spontaneous ovulators

Several studies confirmed the presence of β-NGF in seminal plasma of spontaneous ovulators (e.g., bovine, equine and porcine; Ratto et al. 2012, Bogle et al. 2018). It was also documented that the administration of llama seminal plasma or purified β-NGF influenced ovarian function in spontaneous ovulators. For instance, llama seminal plasma induced ovulation in a pre-pubertal mouse model (Bogle et al. 2011), and systemic administration of purified β-NGF from llama seminal plasma altered ovarian follicular growth and had a luteotrophic effect in cattle (Tanco et al. 2012). In the last study, intramuscular administration of 4–5 mg/animal of purified β-NGF induced neither a preovulatory LH surge nor ovulation in prepubertal heifers, but increased FSH secretion and hastened the emergence of the next follicular wave. Also, the same authors found that llama β-NGF treatment induced a greater growth of the CL resulting in higher plasma progesterone concentration compared to GnRH-treated post-pubertal heifers. The high-affinity NGF receptor, trkA, has also been detected in bovine granulosa cells of preovulatory follicles and in luteal cells of bovine CL (Carrasco et al. 2016), and the preovulatory LH surge seems to upregulate the expression of trkA receptor in these cells. In a further study, the administration of bovine seminal plasma in heifers pre-treated with LH induced a more synchronous interval from treatment to ovulation than in non-pretreated heifers, and plasma progesterone concentrations tended to be higher than those of the control group, reinforcing the notion of a potential luteotrophic effect of NGF in cattle (Tribulo et al. 2015). In a recent bovine study (Stewart et al. 2018a), administration of purified β-NGF from bovine seminal plasma intramuscularly at the time of artificial insemination increased pregnancy rate up to 16%. Treated females displayed a greater plasma progesterone concentration, improving conceptus development and ultimately conception rate. In addition, concentration of NGF in bovine seminal plasma has been correlated with fertility in dairy and beef bulls (Stewart et al. 2018b).

### Conclusions

Among induced ovulators, there appears to be a differential species-specific mechanism that, contrary to the established notion that mechanical stimulation from copulation is the pivotal stimulus to trigger ovulation, relies on the presence of seminal factors that chemically induce endocrine changes that ultimately induce the ovulatory response. The discovery of β-NGF in the seminal plasma of camelids, and recently in rabbits, and its central role as the main chemical compound inducing ovulation in these species without the need of copulation, allow us to challenge the notion that reflex ovulation depends merely upon physical stimulation of the female genital tract. Surprisingly, β-NGF appears to act on different target tissues in these two species, suggesting different evolutionary adaptations to arrive as present-day induced ovulators. Evidence documented in cattle suggests that β-NGF may have a role as a luteotrophic factor in spontaneous ovulators.

### Supplementary data

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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