Ovulation-inducing factor/nerve growth factor (OIF/NGF): Immunohistochemical studies of the bovine ovary and the llama hypothalamus

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Saskatoon, Saskatchewan, Canada

By

Rodrigo Carrasco

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Department of Veterinary Biomedical Sciences

DEGREE: Master of Science

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ABSTRACT

The overall objective was to elucidate the mechanism of action of ovulation-inducing factor/nerve growth factor (OIF/NGF) in the reproductive function of spontaneous and induced ovulators, using cow and llama as models.

In Study 1, the dynamics of trkA, the high affinity receptor for OIF/NGF, were studied during periovulatory period in cows. Unilateral ovariectomies were performed by colpotomy on Days 2, 4 and 6 of the estrous cycle (Day 0= ovulation), and before and after LH administration. Ovarian samples were processed for immunofluorescent detection of trkA. The intensity and area of immuno-positive staining, and the proportion of immuno-positive cells in both the granulosa and theca layers were higher in dominant than in subordinate follicles ($P<0.05$). Dominant follicles displayed a different intracellular distribution of trkA from subordinate follicles. The number of positive cells was higher in the developing CL (Day 2 and 4) than in the mature or regressing CL (Day 6, Pre-LH, and Post-LH).

In Study 2, the distribution of GnRH neurons in the hypothalamus was examined in female llamas ($n = 4$). Hypothalamic samples were processed for immunohistochemistry for GnRH. The distribution of GnRH neurons had no evident accumulation in specific hypothalamic nuclei. The majority of GnRH neurons were detected in the anterior and medio-basal hypothalamus ($P<0.05$). The GnRH neuron fibers were detected primarily in the median eminence and in the medio-basal hypothalamus.

In Study 3, the relationship between trkA and GnRH neurons in the llama diencephalon was examined in llama brains ($n = 4$) obtained in Study 2. Samples were stained using double immunofluorescence. TrkA immuno-reactivity was present in most hypothalamic areas examined; the highest density was found in the diagonal band of Broca and the periventricular nuclei. A low percentage of GnRH cells (1%) showed immuno-reactivity to trkA. Close association between

immuno-reactive cells (i.e., GnRH and trkA in the same microscopic field) was detected rarely (3/160 GnRH neurons).

We concluded that: 1) the high affinity receptor for OIF/NGF is expressed in greater quantities in dominant than subordinate follicles and in the developing CL; 2) GnRH neurons of llamas are concentrated in the anterior and middle hypothalamus, in close relationship to the third ventricle; and, 3) expression of trkA receptors on GnRH neurons was rare, suggesting that the ovulatory effect of OIF/NGF is not via direct interaction with GnRH neurons.

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AC = anterior commissure

AHA = anterior hypothalamic area

ALHA = anterolateral hypothalamic area

ARC = arcuate nucleus

BSA = bovine serum albumin

CL = corpus luteum

DAB = 3,3'-diaminobenzidine

Dapi = diaminophenil indol

DBB = diagonal band of Broca

DH = dorsal hypothalamus

GnRH = gonadotropin-releasing hormone

HCl = hydrochloric acid

HRP = horseradish peroxidase

Im = intramuscular

IU = international units

Kg = kilograms

LH = luteinizing hormone

LHA = lateral hypothalamic area

LPO = lateral preoptic area

LS = lateral septum

MBH = medio-basal hypothalamus

ME = median eminence

Mg = milligrams

MHz = megahertz

MLH = mid-lateral hypothalamus

Mm = millimeters

MPO = medial preoptic area

MS = medial septum

NGF = nerve growth factor

OIF = ovulation-inducing factor

PBS = phosphate buffered saline

PVN = periventricular nucleus

pLH = porcine luteinizing hormone

Rx = retrochiasmatic area

SCH = suprachiasmatic nucleus

SON = supraoptic nucleus

trkA = tyrosine kinase A

ug = micrograms

Chapter 1. Introduction

The first evidence of a seminal component influencing female ovarian function came from a study in Bactrian camels (Chen et al., 1985). It was reported that ovulation occurred in 87% of female Bactrian camels after infusion of seminal plasma in the vagina. This surprising finding remained unexplored for years; however, a series of studies expanded the concept of this ovulation-inducing factor to a related group of induced ovulators, the South American camelids (Reviewed by Adams et al., 2013). Interestingly, the factor has been detected in the seminal plasma of both induced and spontaneous ovulators (Ratto et al., 2006; Bogle et al., 2011), establishing that seminal plasma is more than just the fluid accompanying sperm, and in fact, is capable of influencing ovarian function in the female.

1.1. Ovulation-inducing factor in seminal plasma

Mating is a prolonged process in camelids and involves intrauterine semen deposition (Bravo, 2002; Tibary et al., 2006). The mechanism by which OIF/NGF is absorbed by the endometrium and reaches the circulatory system is not fully understood. In an early study, no ovulations were detected among female alpacas treated with 1 ml of seminal plasma by intrauterine infusion whereas 93% of alpacas ovulated when treated intramuscularly (Adams et al., 2005). The role of uterine abrasion in facilitating absorption of seminal OIF/NGF was examined in a subsequent study (Ratto et al., 2005). Based on the observation that copulation in camelids results in transient inflammation of the endometrium (Bravo et al., 1996), alpacas were treated with 2 ml of seminal plasma intramuscularly or by intrauterine infusion with or without endometrial curettage. It was concluded that disruption of the endometrial mucosa by curettage facilitated absorption of OIF and increased the ovulatory effect of seminal plasma. An

increase in absorption, in effect, increased the dose of OIF in circulation. The dose-related effect of OIF was reflected in these 2 studies; the increased dose increased the ovulatory response in llamas. As well, an increased dose of OIF/NGF which mimicked physiological levels (up to 20 mg) in intrauterine infusion (Silva et al., 2015) resulted in an ovulatory response in 100% of llamas. Taken together, these findings support the idea that the intrauterine dose of OIF/NGF determines the amount of OIF/NGF absorbed and the ovulation rate.

In studies designed to elucidate the nature of OIF in seminal plasma, the llama was used as an in vivo bioassay to test the ovulation-inducing effect of various seminal plasma fractions (Ratto et al., 2010). Seminal plasma treated with heat (up to65°C), charcoal dextran or proteinase k mantained the ovulatory effect of seminal plasma in llamas. However, treatment with pronase did prevent the ovulatory effect, suggesting that the factor that triggered ovulation in seminal plasma was a protein. When fractions of seminal plasma were filtered with different molecular mass cut-offs ovulations were induced only in those animals treated with fractions ≥ 30 KDa. However, the ovulatory effect was retained even after proteinase K digestion which rendered all proteins less than 19 KDa (Ratto et al., 2010). These findings seemed contradictory at first and were explained only when ovulation-inducing factor was discovered to be identical to nerve growth factor, a homodimer (Ratto et al., 2012). It was concluded that the differences in protein profiles and ovulatory effect were due to sample preparation prior to SDS-PAGE. Mercaptoethanol reduced the bonds between the protein dimers, and given the retention of bioactivity, it was unlikely that proteinase K itself actually rendered seminal NGF into its monomers (Ratto et al., 2012).

1.2. Effect of OIF/NGF on ovarian function

Seminal plasma or purified OIF/NGF induced ovulation when administered intravenously, intramuscularly, or by intrauterine infusion in llamas and alpacas (Adams et al., 2005; Ratto et al., 2006; Berland et al., 2012). Given that the ovulatory effect is mediated by a massive LH release, it was infered that OIF/NGF is absorbed into the bloodstream and distributed systemically. Results of initial studies supported the hypothesis that the ovulatory effect of OIF/NGF was mediated directly via gonadotropes of the anterior pituitary which were involved in the OIF/NGF driven ovulatory response. OIF/NGF elicited LH secretion from anterior pituitary cell cultures of the rat (Paolicchi et al., 1998), cow and llama (Bogle et al., 2012). However, llamas treated with a GnRH receptor blocker (Cetrorelix) eliminated the LH and ovulatory response to OIF/NGF (Silva et al., 2011), suggesting that the site of action of OIF/NGF is primarily at the level of the hypothalamus.

A local ovarian effect cannot be disregarded in South American camelids. It is established that llamas and alpacas that ovulated due to OIF/NGF treatment exhibited a larger CL that produced more progesterone than control animals (Adams et al., 2005). In addition, Llamas treated with OIF/NGF had a greater vascularization in the preovulatory follicle and the developing CL than control animals (Ulloa-Leal et al., 2014). Subsequent studies documented the existence of OIF/NGF in the seminal plasma of other species, such as equine and porcine, using the llama as a bioassay (Bogle et al., 2011). In addition, nerve growth factor receptors have been detected in the ovaries of several mammalian species (Levanti et al., 2005; Ren et al., 2005). However, the hypothesis that OIF/NGF exerts a local effect in non-camelids species is supported by studies in farm animals that have reported effects of OIF/NGF in follicular and luteal dynamics (see below).

An initial study in cattle, reported that the seminal plasma of bulls was capable of inducing ovulation in alpacas, but at a lower rate than seminal plasma of llamas or alpacas (Ratto et al., 2006), this provided a basis to study the potential role of OIF/NGF in cows. However, OIF/NGF did not induced ovulation in heifers treated during the first follicular wave, it hastened the emergence of the second follicular wave (Tanco et al., 2012). Furthermore, OIF/NGF treatment in the preovulatory period favored synchronization of ovulation, producing a window of 4 hours where all treated cows ovulated, whereas the control group ovulated in a dispersed manner over time (Tribulo et al., 2015). Notwithstanding, a luteotrophic effect was observed in both studies, and cows treated with seminal plasma containing 250 ug of OIF/NGF had higher blood progesterone concentrations and longer lasting CL than the control group. These findings suggest that OIF/NGF receptors are present in the reproductive tract of cows and that they have functional properties.

This literature review will focus on basic and functional aspects related to the organization of the central nervous system and the function of the ovary.

1.3. Mechanism of ovulation in different species

Mammals have been classified as spontaneous or induced ovulators (Conaway, 1971). Spontaneous ovulators include animals in which high circulating estrogen concentrations have a positive feedback on the hypothalamus and trigger the preovulatory LH surge. Induced ovulators are the species that ovulate only after mating. The classical concept is that physical stimulation of coitus is responsible for triggering ovulation (Fernandez-baca et al., 1970; Wildt et al., 1980). However, the results of more

recent findings suggest that this classification is an oversimplification of the multiple factors influencing the complex cascade of ovulation.

As an example, rodents normally have an obvious estrous cycle punctuated by spontaneous ovulation every 5 days (mice; Jaiswal et al., 2009) or 4 days (rats; Mandl., 1951), but they can behave as induced ovulators under certain conditions. Female rats maintained under constant light for a period of 2 months and subsequently housed overnight with males ovulated consistently after penile intromission (Brown-Grant et al., 1973). Moreover, rats treated with barbiturates and allowed to mate with a male displayed either pseudopregnancy or aberrant follicular development (Everett, 1967). More recently, prepubertal mice were induced to ovulate by intraperitoneal administration of seminal plasma (Bogle et al., 2011). Taken together, the findings support the idea that rodents conserve neuronal pathways related to induced ovulation. Furthermore, social interaction facilitates or induces ovulation in some species, such as female-to-female contact in rabbits (Staples, 1967; Cervantes et al., 2015) or male-to-female contact in sheep (Gelez et al., 2004). Hence, the GnRH system in females is sensitive to stimulation from different physico-chemical sources (endogenous and exogenous), the existence of a well conserved ovulation inducing factor in seminal plasma raises questions about our current understanding of mechanisms controlling ovulation among species.

An early concept was that induced ovulation was a primitive trait which remained in some superior Orders (Conaway, 1971); however, both induced and spontaneous ovulation are present in many mammalian groups (Kauffman et al., 2005). Further, induced ovulation is not a common feature in lower non-mammalian Orders (Baker et al., 2000). The finding suggests that induced ovulation is a later adaptation of certain species and reflects a complex evolutionary specialization. It is important to

highlight that every mammalian Order has one or more species displaying induced ovulation, although the relative frequency of induced ovulation as a trait among the Orders needs to be studied further (Kaufman et al., 2005).

1.4. Anatomy of the GnRH system

The GnRH neurons are a scattered population of cells distributed along the midline of the brain in a rostro-caudal fashion (Herbison, 2005). Originated in the nasal placode, GnRH neurons migrate caudally during early embryonic development to differentiate into mature GnRH neurons (Whitlock, 2005). Evidence of this neuronal migration is the existence of hypogonadal mice, an accident of nature that is characterized by the lack of GnRH neurons in the brain, and accumulation of GnRH neurons in the nasal area (Gibson et al., 1997). The degree of rostro-caudal migration varies between species, for example in rodents a greater proportion of GnRH immuno-reactive cells are located in the preoptic area, but in mink the majority of GnRH neurons are located caudally in the mediobasal hypothalamus (Toumi et al., 1992). The causes and consequences of the differences in GnRH neuron distribution among species remains unknown.

The GnRH neurons that are involved in reproduction are situated in the septum, preoptic area and infundibular portions of the hypothalamus, i.e. the septo-preoptico-infundibular pathway (Silverman et al., 1994). The final output of this pathway occurs in the median eminence where the neurosecretory terminals release GnRH into the portal vessels of the pituitary gland. The median eminence is one of the circumventricular organs, i.e. structures adjacent to and whose function is dependent on the ventricles of the brain. (Rodriguez et al., 2010). Topographically, the median eminence forms the floor

of the third ventricle and is highly vascularized by the superior hypophysial arteries, branches of the internal carotid arteries. After these vessels form capillaries in the median eminence (primary plexus) they drain their content into the portal vessels that supply the anterior pituitary, forming a second capillary plexus allowing an efficient distribution within the anterior pituitary (Page, 2005). The existence of extra-hypothalamic GnRH neurons and pathways located in areas such as the hippocampus, septum and the pre-piriform cortex have been reported, but the role of these neurons in reproductive function, if any, remains to be established (Merchentaler et al., 1984).

1.5. Physiology of GnRH neurons

The GnRH system acts as a network of cells that secrete this decapeptide into the pituitary portal system in a coordinated fashion that changes relative to the stage of the estrous cycle. Using a model of portal cannulation in sheep, GnRH concentrations in portal blood remained basal during the luteal phase, displaying one pulse every 4 hours (Moenter et al., 1991). The frequency of GnRH pulses increased to 1 pulse per hour after luteolysis as circulating progesterone concentrations dropped. The majority (90%) of GnRH pulses in the portal system or the cerebrospinal fluid are accompanied by an LH pulse from the pituitary (Moenter et al., 1991; Yoshioka et al., 2000). The consequent rise in circulating estradiol concentrations induced an increase in GnRH pulse frequency and a decrease in pulse amplitude (Karsh et al., 1996), which culminated in a large preovulatory LH surge followed by ovulation. GnRH secretion remained low during early luteal development and was minimal during mid-diestrus (Yoshioka et al., 2000).

The existence of a so-called surge-center and a tonic-center of GnRH neurons in the hypothalamus and preoptic areas of the brain were originally hypothesized based on studies in which the afferents to the hypothalamus were surgically sectioned. Early studies (Wiegand et al., 1980) where lesions were placed in the medial preoptic area and suprachiasmatic nuclei of rats, resulted in signs of persistent estrous (i.e., vaginal cornification). It was inferred that the pathways that were controlling the preovulatory LH surge were located in the medial preoptic area of rats. Conversely, the tonic center has been suggested to exist somewhere in the medio-basal hypothalamus. However, the anatomical location of the tonic centre has been elusive and the concept of separate surge and tonic centers of GnRH neurons in the brain remains a hypothesis to test (Herbison, 1998).

Rather than GnRH 'centers', the GnRH system may be better characterized as a diffuse network (Herbison, 1998; 2005) that reacts in a coordinated manner despite a relatively widespread scattered cell population. During events leading up to ovulation, estradiol activates the neurons of the network or, more likely, activates neurons that synapse with the GnRH network. Using immunocytochemistry of mouse brain tissues sacrificed at the time of the expected LH surge with or without exposure to males, only a subset of the total GnRH neuronal population was activated (expressed c-Fos proto-oncogene, i.e., up to 40% of the total GnRH neurons in the preoptic area) (Wu et al., 1992). In sheep, there was a similar degree of GnRH neuron activation in ewes undergoing an induced preovulatory LH surge (Moenter et al., 1993). Curiously, there were non-GnRH neurons that were activated and GnRH neurons that were not involved in the GnRH surge.

1.6. GnRH afferents

The regulation of GnRH neurons has been a topic of intensive research since the discovery of GnRH itself in 1971(Clarke, 2011). Several molecules have been implicated in affecting GnRH function. In an early report, the injection of an epinephrine antagonist blocked ovulation in rabbits (Sawyer et al., 1947). Findings from recent studies suggest that norepinephrine can act as a permissive or repressive factor to ovulation (Herbison, 1997). Norepinephrine neurons are located in the brainstem and project cranially into the hypothalamus establishing synaptic contacts with some GnRH neurons, and about 40% of norepinephrine immuno-reactive cells in the brainstem display estradiol receptors (Temel et al., 2002).

GnRH neurons express kisspeptin receptors, suggesting that kisspeptin is a primary regulator of GnRH secretion (Messager et al., 2005). In addition, kisspeptin or kisspeptin receptor knock-out mice display an absence of puberty and under-developed gonads (d'Anglemont de Tassign et al., 2010). Treatment with exogenous kisspeptin induces LH secretion n most species studied to date (presumably in response to GnRH secretion), including both induced and spontaneous ovulators (Inoue et al., 2011; Caraty et al., 2007). The extent of the influence of kisspeptin on reproduction may also extend to sexual differentiation (Clarkson et al., 2014).

Glutamate is another excitatory neurotransmitter in the brain (Herbison, 2005) and has been shown to activate GnRH neurons in vitro (Spergel et al., 1999). GnRH neurons express all types of glutamate receptors (Herbison, 1998) and the glutamate synaptic inputs can modify GnRH neuron excitability (Iremonger et al., 2010). Conversely, gamma amino butyric acid (GABA) is the principal inhibitory neurotransmitter in the brain and there is general consensus about the inhibitory effects of GABA on GnRH neurons (Herbison et al., 1991). However, there are some reports about a stimulatory effect and

recently it has been suggested that some areas of the brain display excitatory or inhibitory actions on GnRH neurons depending on the conditions (Watanabe et al., 2014).

Currently, there is a lack of studies addressing the role of neurotropins on central regulation of reproduction, in contrast to the current information regarding their local role in ovarian function and development (see below). That OIF/NGF induces a preovulatory LH documents that neurotropins regulate ovarian and hypothalamic function in an endocrine manner, and potentially this effect can help in the understanding the regulation of reproduction.

1.7 NGF in the ovary

OIF/NGF has been detected in the ovaries of several mammalian species using immunohistochemistry or western blot analysis (Levanti et al., 2005; Dissen et al., 1996; Li et al., 2014; Jana et al., 2011). Principal receptors for OIF/NGF are the high-affinity tyrosine receptor kinase A (trkA), and the low-affinity receptor, p75 (Meakin et al., 1992). TrkA mediates most of NGF effects, such as survival of sympathetic neurons in the nervous system (Levi-Montalcini et al., 1960) and adrenal medulla outgrowth (Unsicker et al., 1978).

Nerve growth factor is a determinant factor regulating ovarian function in mature, but also, in developing ovaries. Studies from NGF knock-out mice have documented that ovaries from pups had lower quantities of primary and secondary follicles, as well as higher number of naked oocytes (lack of pre-follicular cells) than the wild-type mouse (Dissen et al., 2002), suggesting that NGF has a role in follicular assembly. In addition, 2-day old rat ovaries cultured with NGF induced the expression of

functional FSH receptors (Romero et al., 2002). However, this finding may not be only dependent on NGF, since neurotropin 4 induces also FSH receptors through interaction with tyrosine kinase B (Kerr et al., 2009).

Conversely, p75 is a low affinity receptor for NGF and also has the ability to bind to other neurotrophins (Underwood et al., 2008); hence, it has been referred to as the pan-neurotropic receptor (Dissen et al., 2000). This receptor is a member of the tumor necrosis factor superfamily and has been associated primarily with inducing cell death or apoptosis (Dechant et al., 2002). However, p75 activation has also been implicated in other processes including cell differentiation, inhibition of neurite outgrowth, and, in certain instances, enhancing trkA effects (Underwood et al., 2008)

1.8 NGF during ovulation.

Several neurotrophins have been identified in the mammalian ovary such as nerve growth factor, neurotrophin-3, brain derived neurotrophic factor and neurotrophin 4/5 (Ojeda et al., 1996). The majority of neurotrophins act in a paracrine or autocrine way mediating cellular differentiation and function. The most studied neurotrophin is nerve growth factor which has been identified to have a role in neuronal growth and survival. In the ovary, the expression of nerve growth factor increases during the gonadotropin surge at first ovulation and this increase is accompanied by an increase of the high affinity receptor, trkA (Dissen et al., 1996). The rise in the NGF/trkA system increases up to 100 fold after the LH surge and virtually disappears from the ovary afterwards (Dissen et al., 1996). The source of NGF and trkA is the cells of the theca layer, but it can be also detected in the granulosa layer in cows (Dissen et al., 2000).

Cows are an excellent model to study the local ovarian effects of OIF/NGF. Results from studies have shown that when cows are treated with OIF/NGF there is no preovulatory secretion of LH (Tanco et al., 2012; Tribulo et al., 2015), thus the confounding effect of LH is not present as in llamas (Ratto et al., 2005). In addition, the detailed knowledge of the follicular dynamics in this species allow us to track follicular populations, and evaluate temporal changes during follicular or luteal development under different physiological environments (see below).

Transrectal ultrasonography of cattle has been useful to describe final part folliculogenesis. During this stage, antral follicles grow in a wave-like fashion. Each follicular wave is characterized ultrasonically by the sudden apeareance of a group of 8 to 41 follicles of 3-4 mm (Adams et al, 2008). Initially, the follicles grow at a similar rate and when the largest growing follicle reaches 6 mm the rest of the cohort suffers inhibition of their growth and undergo regression (Ginther, 2000). The largest follicle, namely dominant follicle, continues to grow and can reach 15 to 20 mm in size to later regress. The dominant follicle can ovulate when it reaches 8 to 10 mm (Martinez et al, 1999) when the CL is nonfunctional and progesterone concentrations are low, this environment allows the hypothalamus to be sensitive to estrogen and the positive feedback.

1.9. Objectives and hypotheses

The overall objective of the research in the present thesis was to gain a better understanding of the route and mechanism by which seminal plasma (OIF/NGF) elicits its effects on ovarian function in both induced and spontaneous ovulators. We now know that the ovulation-inducing effect of OIF/NGF in camelids is mediated at the level of the brain, but the location in the brain and the cells involved in

triggering the preovulatory surge in circulating concentrations of LH remain unknown. Further, the site and mechanism of action of the luteotrophic effect of OIF/NGF (central or local) observed in both induced and spontaneous ovulators are also unknown.

The specific objective of Study 1 was to understand the role of OIF/NGF locally at the level of the ovary. Although the existence of the high-affinity receptor for OIF/NGF (trkA) in the ovary has been reported previously, its role and the dynamics of its expression in both induced and spontaneous ovulators are unknown. By characterizing the spatial and temporal distribution of trkA in ovarian follicles and the CL at known stages of development in the bovine model, we tested the hypothesis that the luteotrophic effect of OIF/NGF is mediated by an increase in trkA receptors in the ovulatory follicle and early CL.

To gain a better understanding of structural pathways involved in the mechanism of ovulation in camelids, the specific objectives of Study 2 were to identify the anatomical distribution of GnRH neurons in the llama hypothalamus and preoptic area and to characterize the cytological characteristics of GnRH neurons and their pathways throughout the hypothalamus.

Given the known involvement of GnRH in OIF/NGF-induced ovulation, Study 3 was designed to test the hypothesis that the ovulatory effect of OIF/NGF in camelids is produced by direct interaction with GnRH neurons. We examined the structural relationship between trkA and GnRH in the hypothalamus and preoptic areas of the llama brain.

Chapter 2. The dynamics of trkA expression in the bovine ovary are associated with a luteotrophic effect of OIF/NGF

R Carrasco, J Singh, GP Adams

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

2.1. Abstract

Ovulation-inducing factor in semen (OIF/NGF) influences ovulation and CL form and function in camelids and, remarkably, in cows. The ovulation-inducing effect in camelids is mediated at the level of the hypothalamo-pituitary axis, but the site of action of the luteotrophic effect is unknown and may be particularly important in understanding the effect of OIF/NGF in spontaneous ovulators. An experiment was designed to characterize the expression of the high affinity OIF/NGF receptor (trkA) in the ovary during the periovulatory period in cattle. Cows (n = 14) were examined daily by transrectal ultrasonography to determine the day of ovulation (Day 0), and were assigned randomly to be unilaterally ovariectomized on Day 2, 4, 6 or in the pre-ovulatory period just before or after exogenous LH treatment. After a complete interovulatory interval, the cows were re-assigned to a different day-group on which the remaining ovary was removed (n = 4 to 5 ovaries/day-group). Ovaries were fixed in paraformaldehyde, and 5 µm sections of ovarian tissue representing the dominant follicle, largest subordinate follicle, and the CL were processed for immunofluorescent detection of trkA receptor. A statistical interaction in the intensity of the immuno-reaction between follicle type and day-groups was detected (P = 0.004). The proportion of trkA immuno-positive cells was higher in dominant than

subordinate follicles in both the theca and granulosa layers ($P < 0.05$). The number of positive cells was higher in the developing CL (Day 2 and 4) than the mature-regressing CL (Day 6, Pre-LH, and Post-LH). Dominant follicles displayed a predominantly diffuse intracellular distribution of trkA immuno-reactivity in Day-group 2 and the Post-LH group when compared to subordinate follicles ($P < 0.05$). We concluded that the luteotrophic effect of OIF/NGF in cattle is driven by a direct interaction with its receptor in the theca and granulosa layer of dominant follicles and the early CL.

2.2. Introduction

Ovulation-inducing factor (OIF) is a protein in the seminal plasma that has been shown to elicit an ovulatory response in camelids when administered intramuscularly, intravenously or by intrauterine infusion (Chen et al., 1985, Adams et al., 2005; Ratto et al., 2006; Berland et al., 2012). The protein has subsequently been identified as beta nerve growth factor (β-NGF; Ratto et al., 2012), and is present in the seminal plasma of all species examined to-date (Bogle, 2015). The existence and effect of this seminal protein challenge the classic assumption that the physical stimulation of copulation is the principal factor involved in inducing ovulation in South American camelids (Fernandez-Baca et al., 1970). For the purposes of the present study, the abbreviation OIF/NGF will be used to indicate NGF of seminal plasma origin.

Beta nerve growth factor is a homodimer with a molecular mass of 26 – 28 KDa (Angeletti et al., 1971), and was discovered in abundance in mouse sarcomas (Levi-Montalcini et al., 1951), snake venom (Cohen et al., 1956), and mouse salivary glands (Cohen, 1960). The effects of NGF were initially thought to be restricted to nerve function and development, as indicated by a potent stimulatory effect

on dorsal root ganglia during embryonic limb development in chicks (Levi-Montalcini, 1987). More recently, NGF has been shown to play a role in a variety of non-neuronal systems such as in immune-related (Bonini et al., 2003), inflammatory (Leon et al., 1992), reproductive (Lara et al., 1990), and endothelial tissues (Cantarella et al., 2002). The biological actions of NGF are mediated by interaction with two different receptors. Tyrosine kinase receptor A (trkA) is a high affinity receptor for NGF and mediates its neurogenic effects (e.g., survival of dorsal root ganglia neurons in mice; Minichiello et al., 1995, or induction of neurite outgrowth in PC_{12} cells in vitro; Loeb et al., 1991). A non-specific low-affinity receptor (p75NTR) has been implicated in mediating trkA activation, increasing the affinity of trkA for NGF, and inducing apoptosis in cell culture (Yoon et al., 1998). The p75NTR receptor also has a low affinity interaction with other neurotrophins such as brain-derived neurotrophin factor and neurotrophin 3 (Underwood et al., 2008).

Nerve growth factor has been implicated as a local mediator at different stages of development of the reproductive system. In the infantile NGF knock-out mouse, primary and secondary follicle populations are lower than the wild type mouse (Dissen et al., 2001), suggesting that obliteration of the NGF signaling system has detrimental effects on fetal ovarian development. In prepubertal rats treated with pregnant mare serum gonadotropin, administration of anti-NGF or a trkA blocker into the ovarian bursa on the day of expected LH surge impaired ovarian prostaglandin E2 production and reduced the ovulatory response (Dissen et al., 1996). In addition, a role in the maintenance of follicular and luteal vasculature was reflected in vascular cell proliferation of neonatal rat ovaries cultured *in vitro* after treatment with NGF, either directly or through synthesis of vascular endothelial growth factor (Julio-Piper et al., 2008).

A novel endocrine effect of OIF/NGF was discovered in a series of studies on ovulation in species categorized as induced ovulators (reviewed by Adams et al., 2012). Intramuscular administration of seminal plasma (containing OIF/NGF) in llamas and alpacas elicited a surge in plasma LH concentrations, followed by ovulation in >90% of animals and was associated with enhanced CL development (Adams et al., 2005). It was concluded that the mechanism involves a central effect on the hypothalamus or pituitary gland via a systemic route (Ratto et al., 2005). However, the results of later studies in cattle (a spontaneous ovulator) provided rationale for the hypothesis that the luteotrophic effect of OIF/NGF is mediated by a local route. Although treatment with purified OIF/NGF did not induce ovulation in pre-pubertal heifers, treatment during the first follicular wave in post-pubertal heifers hastened the emergence of the following follicular wave and was luteotrophic (Tanco et al., 2012). In a subsequent study in cattle, the administration of bull seminal plasma (containing 250 ug of OIF/NGF) did not elicit an LH response or ovulation, but did enhance CL development (Tribulo et al., 2015). Plasma progesterone concentrations increased more rapidly and the CL lifespan was longer in the seminal plasma-treated group than in the control group. Surprisingly, ovulation occurred more synchronously in the seminal plasma-treated group (within a period of 4 hours) than in the control group (within a period of 18 hours; Tribulo et al., 2015). The mechanisms by which OIF/NGF induced the ovarian changes in cattle are unknown. Although NGF and its receptors have been detected in bovine and porcine ovaries (Levanti et al., 2005), their temporal expression in the ovary in relation to follicular dynamics, ovulation, and CL development have not been characterized.

To determine the role of OIF/NGF at the level of the ovary, the objective of the present study was to characterize the spatial and temporal distribution of trkA in ovarian follicles and CL at known stages

of the estrous cycle, and to test the hypothesis that the luteotrophic effect of OIF/NGF is mediated by an increase in trkA receptors in the ovulatory follicle and early CL.

2.3. Materials and Methods

2.3.1. Animals

Non-lactating Hereford-cross cows (n = 6) and sexually mature heifers (n = 8) from the research herd at the University of Saskatchewan Goodale Farm were used from August to October. The experimental protocol was approved by the University Committee on Animal Care and Supply and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.3.2. Experimental design

The ovaries were examined daily by transrectal ultrasonography to detect ovulation (Day 0). Animals were then assigned randomly in replicate to be unilaterally ovariectomized on Day 2, 4, 6, or in the pre-ovulatory period either just before, or just after, the LH surge. Animals assigned to pre-ovulatory groups were given a luteolytic dose of prostaglandin $F_{2\alpha}$ (500 ug cloprostenol im, Estroplan, Vétoquinol, Georges Lavaltrie, QC, Canada) during the luteal phase when the dominant follicle of the second follicular wave was ≥10 mm and growing. Animals assigned to the Pre-LH group were ovariectomized 24 hours after prostaglandin treatment. Animals assigned to the Post-LH group were given pLH (25 mg Luthropin im, Bioniche, Belleville, Ontario, Canada) 24 hours after prostaglandin treatment and were ovariectomized 18 hours later. After one complete interovulatory interval following

the first ovariectomy, animals were re-assigned randomly to a different day-group on which the remaining ovary was removed (n = 4 to 5 ovaries per day-group; Fig 2.1).

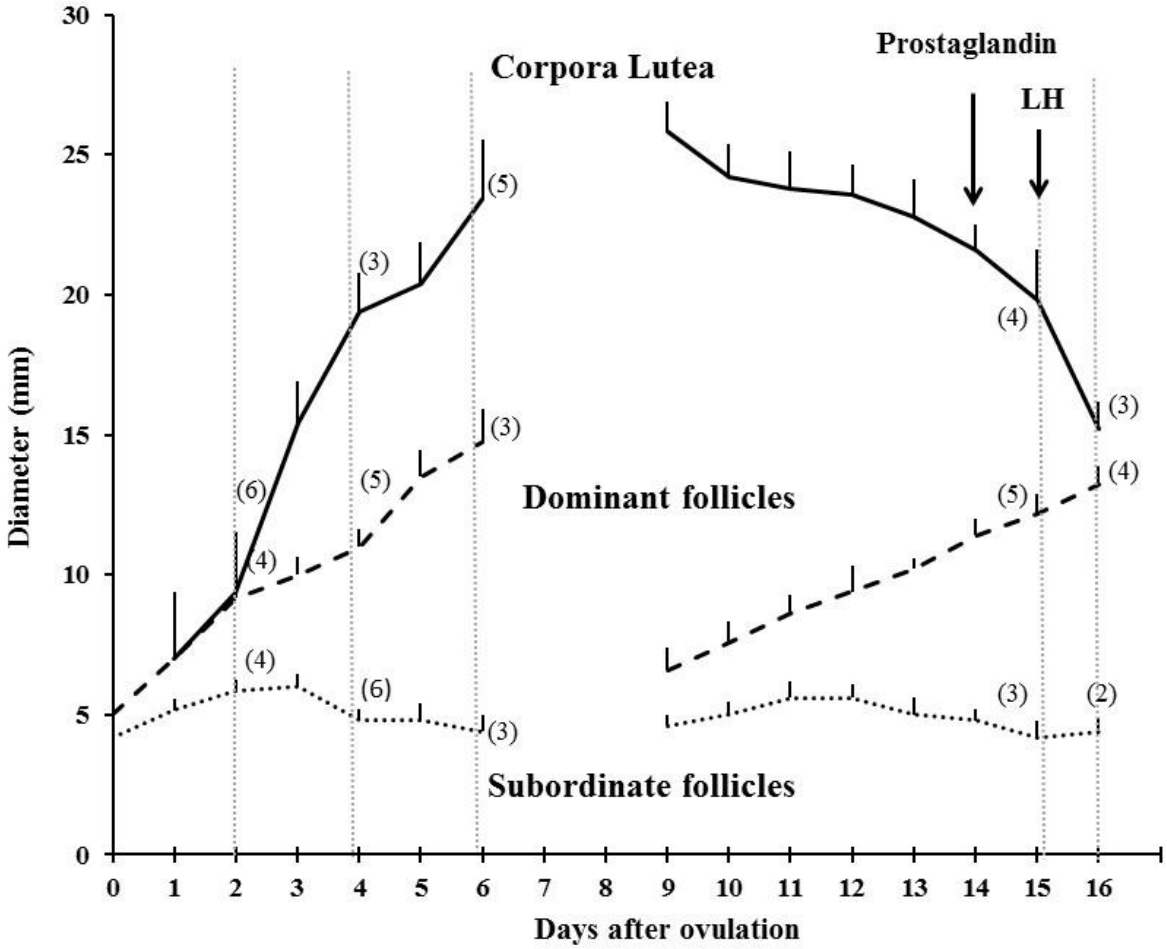

Figure 2.1. Experimental design showing follicle and CL diameter (mean ± SEM) profiles in cows preceding unilateral ovariectomy (vertical dashed lines) on Days 2, 4, 6, and the pre-ovulatory period just before and just after treatment with LH. The number in parentheses accompanying the vertical dashed lines reflects the number of the respective structures (i.e. follicle, CL) analyzed per time point. For illustration purposes, dominant follicle diameters of the second wave were normalized to the mean day of emergence of Wave 2.

2.3.3. Ultrasonographic monitoring

The ovaries were examined daily by transrectal ultrasonography using a 7.5 MHz linear-array probe (Mylab, Esaote Canada, Georgetown, Ontario, Canada). The CL and ovarian follicles ≥4 mm were individually identified and monitored from day-to-day to determine luteal and follicular wave status. Wave emergence was defined as the day on which the follicle destined to become dominant was first detected at a diameter of 4 – 5 mm. If the future dominant follicle was first detected at 6 mm, the previous day was taken as wave emergence (Ginther et al., 1989). Ovulation was defined as the sudden disappearance of a follicle ≥10 mm from one examination to the next (Knopf et al., 1989).

2.3.4. Ovariectomy and tissue handling

Unilateral ovariectomy was performed via colpotomy in the standing position under caudal epidural anesthesia using 2% (w/v) lidocaine HCl with 0.01mg/ml epinephrine (Singh et al., 1997). An incision was made in the dorsolateral aspect of the vaginal fornix and the peritoneum was manually punctured after blunt dissection through the adventitia. After manually compressing the mesovarium with a lidocaine-soaked gauze, the ovary containing the structure of interest was removed using a chain écraseur. Within a few minutes of collection, the ovarian artery was cannulated and perfused with 20 ml of cold phosphate buffered saline (PBS; pH = 7.4) followed by 20 ml of 4% paraformaldehyde in PBS (pH = 7.4). The ovary was then immersed in the same fixative for 24 hours at 4°C. After the fixation period, ovaries were rinsed in PBS (3 times for 15 min. each), and stored in PBS at 4ºC. Cows were treated post-operatively with procaine penicillin G (20,000 IU/kg;) im daily for 3 days.

2.3.5. Immunohistochemistry

The fixed ovaries were trimmed such that the structures of interest, previously identified by ultrasonography, were exposed for sectioning. The trimmed ovarian tissues were placed in plastic cassettes and dehydrated in graded ethanol solutions (50%, 70%, 90%, 95%, and 100%), cleared in xylene, and embedded in blocks of paraffin. The tissue blocks were sectioned at a thickness of 5 um and mounted on poly-L-lysine coated glass slides. Enzymatic antigen retrieval was performed using a concentration of 2 mg/ml of pepsin (Sigma, St Louis Missouri, USA) in a 0.01N HCl solution (pH= 1.5) for 20 minutes at room temperature. Slides were then washed in PBS, and incubated in blocking buffer (1% bovine serum albumin in PBS) for 1 hour. Slides were incubated overnight at 4° C with a primary antibody (rabbit anti-human trkA, Santa Cruz Biotechnologies, Santa Cruz, California, USA) diluted 1:200 in 1% BSA in PBS. The next day, slides were washed and incubated for 2 hours with a secondary antibody (goat anti-rabbit IgG, Alexa 488, Life Technologies, Burlington, Ontario, Canada) diluted 1:400 in 1% BSA in PBS. After washing, slides were counter-stained with DAPI, cover-slipped, and stored (≤1 week) for examination by confocal fluorescence microscopy (Leica LSM, Wetzlar, Germany). The specificity of the antibody was tested by pre-adsorbing the primary antibody with trkA peptide for 1 hour at room temperature or by omitting the primary antibody from the incubation process; either procedure prevented the detection of immunoreaction during assessment. Additionally, histologic sections from every ovary were stained with hematoxylin-eosin to assess and identify microscopic details, as described previously (Singh et al., 2000).

2.3.6. Image analysis

Confocal fluorescence images of the follicular wall and the CL were analyzed with ImageJ software (NIH, Bethesda, Maryland, USA). The proportion of positive cells, the intensity of the immuno-

reaction, the area stained, and the intracellular distribution of the immuno-reaction were estimated in the granulosa and theca layers of dominant and subordinate follicles. Two images per follicle were obtained and analyzed. Each image contained information corresponding to trkA immuno-reactivity (green channel, Alexa 488) and the nuclear counterstain (blue channel, DAPI). The basement membrane was used to differentiate between granulosa and theca layers, and was outlined manually using the aid of nuclear morphology a guide. The theca interna was defined as the region extending 100 um from the basement membrane into the ovarian stroma. The granulosa layer was defined as the area from the basement membrane into the follicular lumen. The follicular wall was considered as a composite of granulosa layer and theca interna. The proportion of cells that were immuno-positive was estimated from the total number of cells of the follicle wall, granulosa layer, or theca interna. The intensity of the immuno-reaction was estimated by creating a mask of the green channel (trkA reactive) using an algorithm to select immuno-reactive areas, and from those areas, the grayscale value per stained square micron was calculated. The immuno-reactive area (um^2) was calculated by creating a binary image (black = 0; white =256) of the green channel using a common threshold for all images; the area is expressed as a percentage of the total area of the follicular wall, the granulosa layer or the theca interna. For the CL, the number of cells that were immuno-reactive was estimated from the total number of cells counted per high-powered field (63x). The intensity of the immuno-reaction of CL was analyzed, as described above. Based on the degree of granularity, two patterns of intracellular distribution of trkA were apparent; diffuse or focal (Fig 3.6). A grid overlay was placed on each image of the follicles and CL. The cells counted and classified for granularity were those in which the nucleus was overlain by the intersection of orthogonal grid crosses.

2.3.7. Data analysis

Differences between follicle type and day-groups were compared by two-way analysis of variance. For the CL, a day-group effect was analyzed by one-way analysis of variance. When significant differences were detected, multiple comparisons were made using the method of least significant difference. Data are presented as the mean ± SEM, and significance was considered when $P<0.05$. Cell counts from corpora lutea were pooled into early (Day 2 and Day 4) and mature-regressing day-groups (Day 6, Pre-LH and Post-LH) and compared by t-test for unequal variance (Ruxton, 2006). The intracellular distribution of trkA immuno-reactivity of dominant and subordinate follicles is expressed as mean ± SEM of the diffuse:focal ratio and was compared as described for follicles above.

2.4. Results

The diameters of dominant and subordinate ovarian follicles and the CL at the time of ovariectomy are presented in Fig. 3.1 (n=2 to 5 per structure/ per day group). The diameter profile of the subordinate follicle in day-groups 2, 4 and 6 is that of the largest subordinate in the excised ovary, but not necessarily the largest subordinate of the follicular wave.

The fluorescence signal in ovarian tissues (Fig. 3.2) was restricted to follicles and the CL. No reaction was detected in stromal cells or blood vessels, and no signal was detected in regressing follicles or the regressing CL from the previous cycle. Various degrees of positive reaction were detected in the thecal layer of some small antral follicles ≤ 1mm (i.e., those not detected by ultrasonography). At the cellular level, immuno-reactivity was restricted to the cytoplasm; nuclear staining was not observed. No statistical difference was detected in trkA immuno-reactivity of the dominant follicles collected after

the first versus second unilateral ovariectomy; hence, data for all structures were analyzed regardless of been collected on the first or second ovariectomy.

2.4.1. Follicles

An interaction ($P < 0.01$) between follicle type (dominant versus subordinate) and day-group (Day 2, 4, 6, pre- and Post-LH) on the intensity of trkA immuno-reactivity was the result of greater intensity in dominant versus subordinate follicles in all day-groups except one, the Pre-LH group (Fig. 2.3.A). Among dominant follicles, the intensity of the immuno-reaction was greater on Day 2 than on Day 6, Pre-LH, or Post-LH ($P = 0.04$, $P = 0.01$, $P = 0.03$, respectively), The intensity of subordinate follicles remained constant among day-groups, except for the Pre-LH group that was greater and similar to the intensity of dominant follicles (Fig. 2.3A). A similar pattern was observed when the intensity of the immuno-reaction was analyzed with respect to the granulosa layer (Fig. 2.3.B) or the theca interna (Fig. 2.3.C) separately.

The area of immuno-reactivity of dominant follicles was greater than their subordinate counterparts on Day 4 ($P = 0.004$) and Post-LH ($P = 0.04$, Fig. 2.4A). An interaction between day-group and follicle type ($P < 0.001$) in the immuno-stained area of the theca layer was attributed to a dramatic increase in the subordinate follicles of the Pre-LH group compared to other groups (Fig. 2.4). In addition, a progressive reduction in the immuno-stained area of the theca layer of dominant follicles from Day 2 to Day 6 was detected ($P < 0.05$; Fig. 2.4B).

Dominant follicles had a greater proportion of immuno-positive cells than subordinates follicles in both the granulosa ($P < 0.001$) and theca ($P < 0.001$) layers, but no effect of day-group was detected (Fig. 2.4 B, D). The proportion of positive cells in the granulosa layer of dominant follicles was greater than subordinate follicles on Day 2 ($P = 0.001$), 4 ($P = 0.018$), and Post-LH ($P < 0.001$). The theca layer of dominant follicles had a greater proportion of immuno-reactive cells than subordinates follicles on Day 4 ($P < 0.001$), Day 6 ($P = 0.015$) and Post-LH ($P < 0.001$). The proportion of positive cells of the theca layer of subordinate follicles was similar among day-groups, except in the Post-LH group that was lower than the Day 2 group ($P = 0.02$). The follicular wall of dominant follicles displayed a greater diffuse to focal ratio of trkA immuno-reactivity when compared to subordinate follicles on day 2 and on day Post-LH ($P < 0.05$; Fig. 2.5).

2.4.2. Corpus luteum

In luteal cells, trkA immuno-fluorescent granules were distributed homogenously within the cytoplasm of immuno-positive cells in all day-groups, but the grayscale intensity values and number of immuno-positive cells tended to differ among day-groups ($P = 0.09$; Fig. 2.2 and 2.6). In a retrospective comparison, the number of immuno-positive cells was greater in early developing CL (Days 2 and 4 combined) than in mature or regressing CL (Day 6, Pre- and Post-LH combined; 41.1 ± 10.4 vs 9.7 ± 3.4 cells per high-powered field; $P = 0.01$).

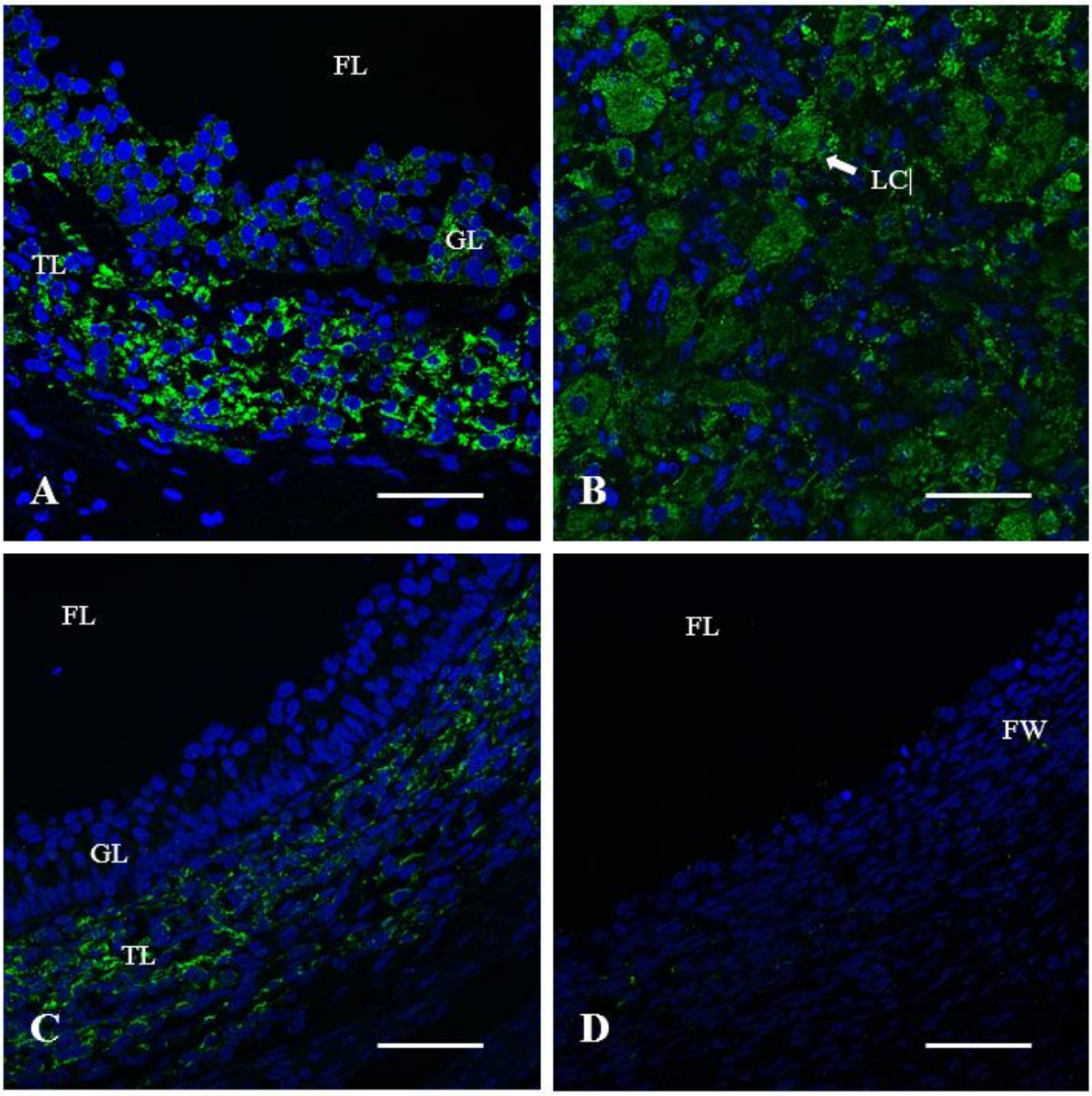

Figure 2.2. Immunofluorescence staining pattern of trkA in a dominant follicle (A), CL (B), subordinate follicle (C) and regressing follicle (D) in cattle. FL: Follicular lumen, GL: Granulosa layer, TL: Theca layer, FW: Follicular wall, LC: Luteal cell. Scale bar = 50 µm.

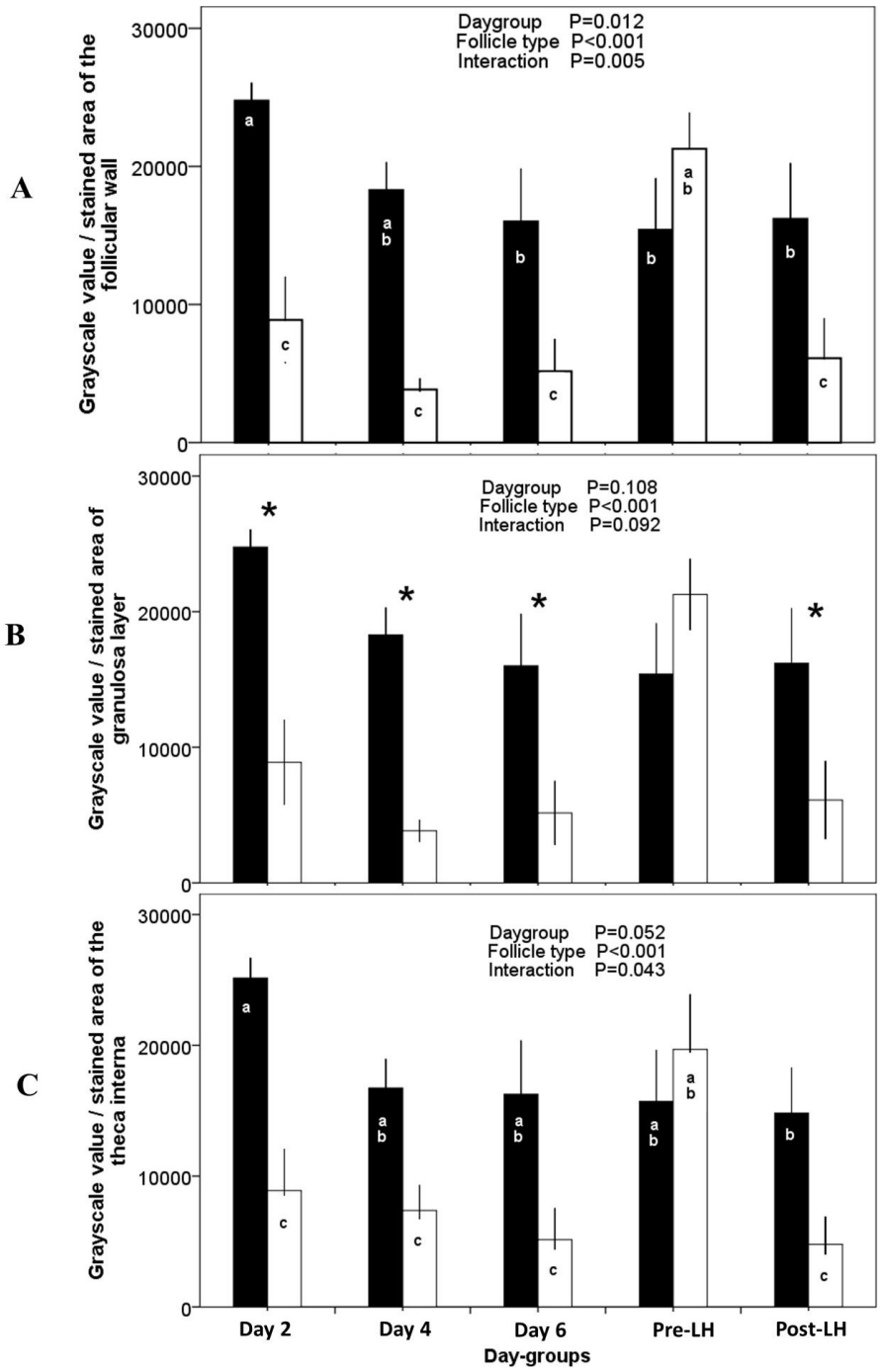

Figure 2.3. Grayscale intensity of pixels (mean ± SEM) representing the trkA immuno-positive area of dominant (black bars) and subordinate ovarian follicles (white bars) collected at the time of ovariectomy (Day-groups; Day 0 = ovulation) in cattle. Intensity values (0 = black; 65536 = white) of the follicular wall (A), the granulosa layer (B), and the theca interna (C). [abc] Values with no common superscript are different (P<0.05). *Difference between dominant and subordinate follicles (P<0.05).

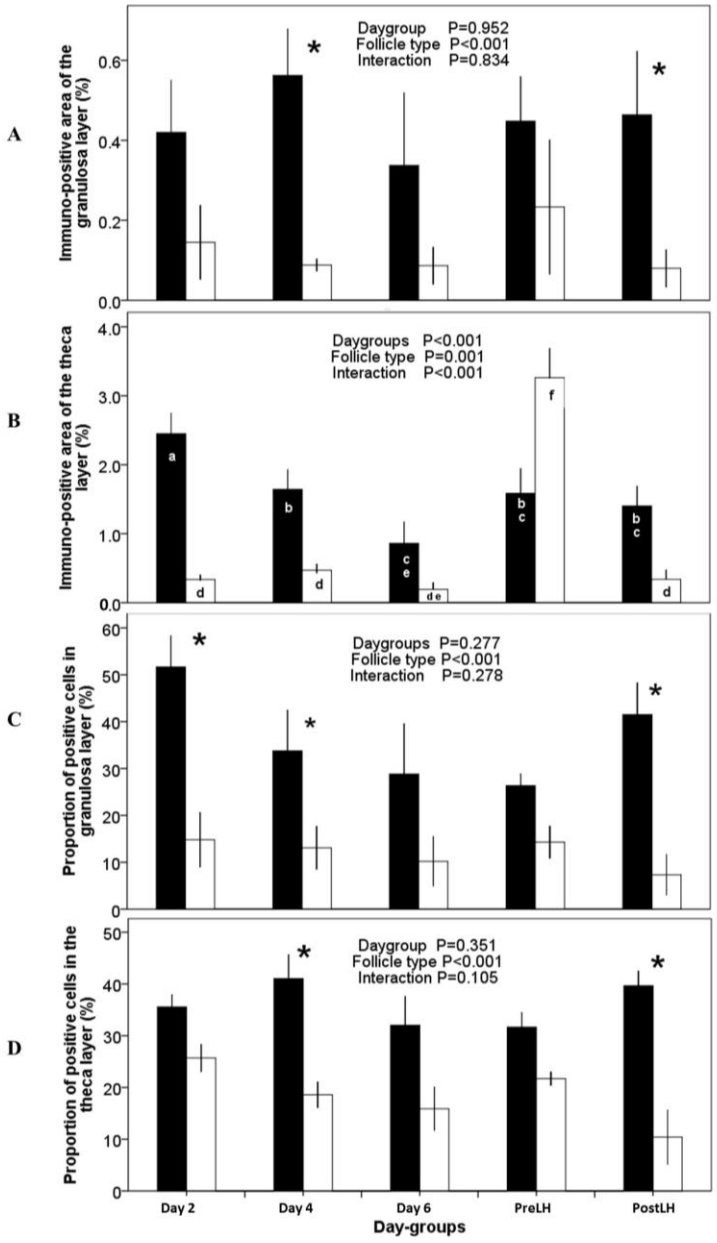

Figure 2.4. Comparison of immuno-reactivity of the follicular wall (granulosa and theca) to trkA in dominant (black bars) and subordinate follicles (white bars) taken during the periovulatory period in cattle (mean ± SEM; ovulation = Day 0). (A) Immuno-positive area of the granulosa layer (% of the total area of the granulosa). (B) Immuno-positive area of the theca layer (% of the total area of the theca). (C) Proportion of cells in the granulosa layer that are immuno-positive. (D) Proportion of cells in the theca layer that are immuno-positive. [abc] Values with no common superscripts are different (P<0.05).*Difference between dominant and subordinate follicles (P<0.05)

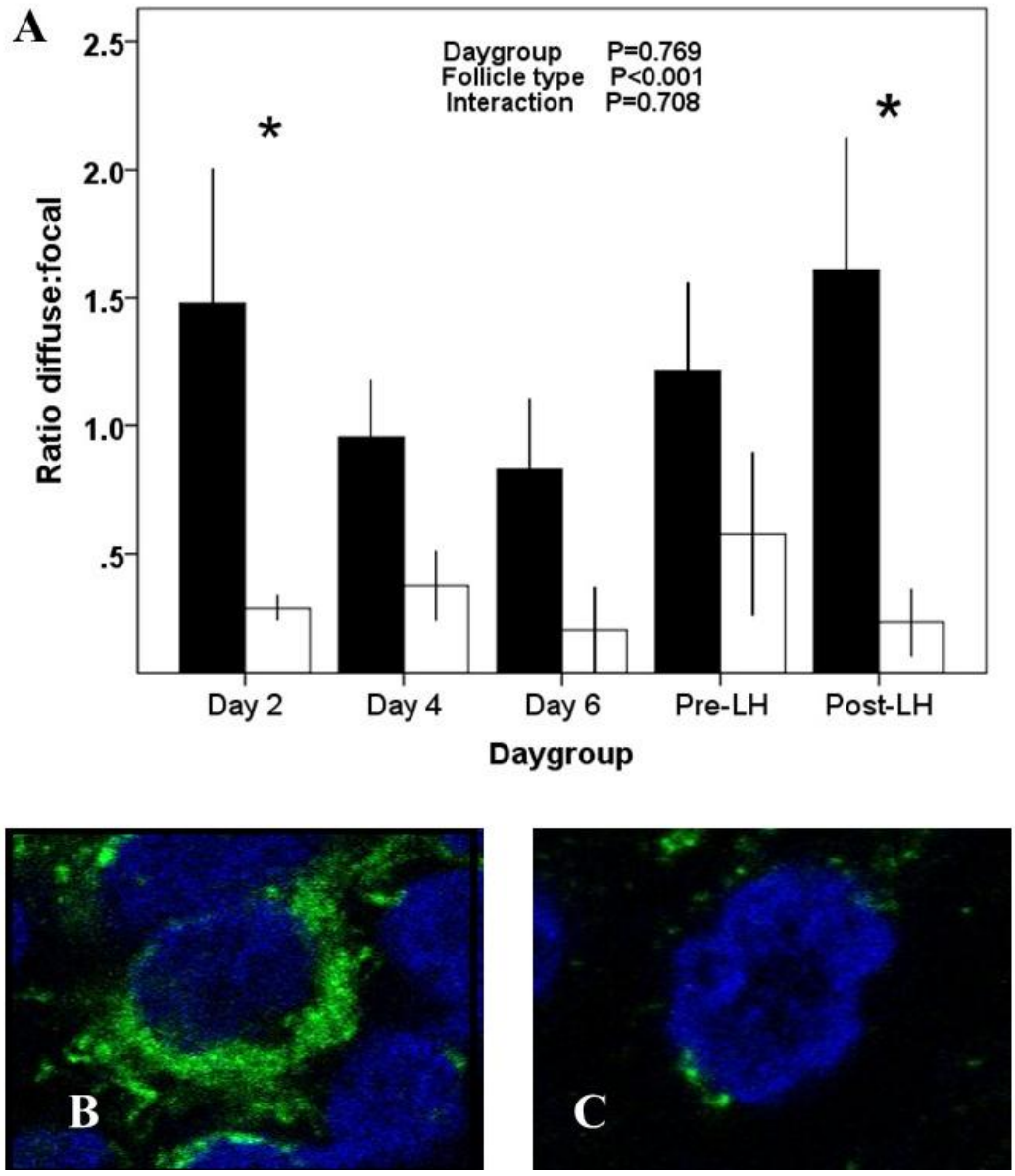

Figure 2.5. Patterns of intracellular distribution of trkA receptor in cells of the granulosa and theca layers of dominant and subordinate follicles in cattle, assessed by confocal microscopy. (A) The pattern of granularity is expressed as the ratio of diffuse versus focal distribution in dominant (black bars) and subordinate follicles (white bars) among day-groups. (B) Diffuse granularity. (C) Focal granularity.

*Difference between dominant and subordinate follicles ($P<0.05$).

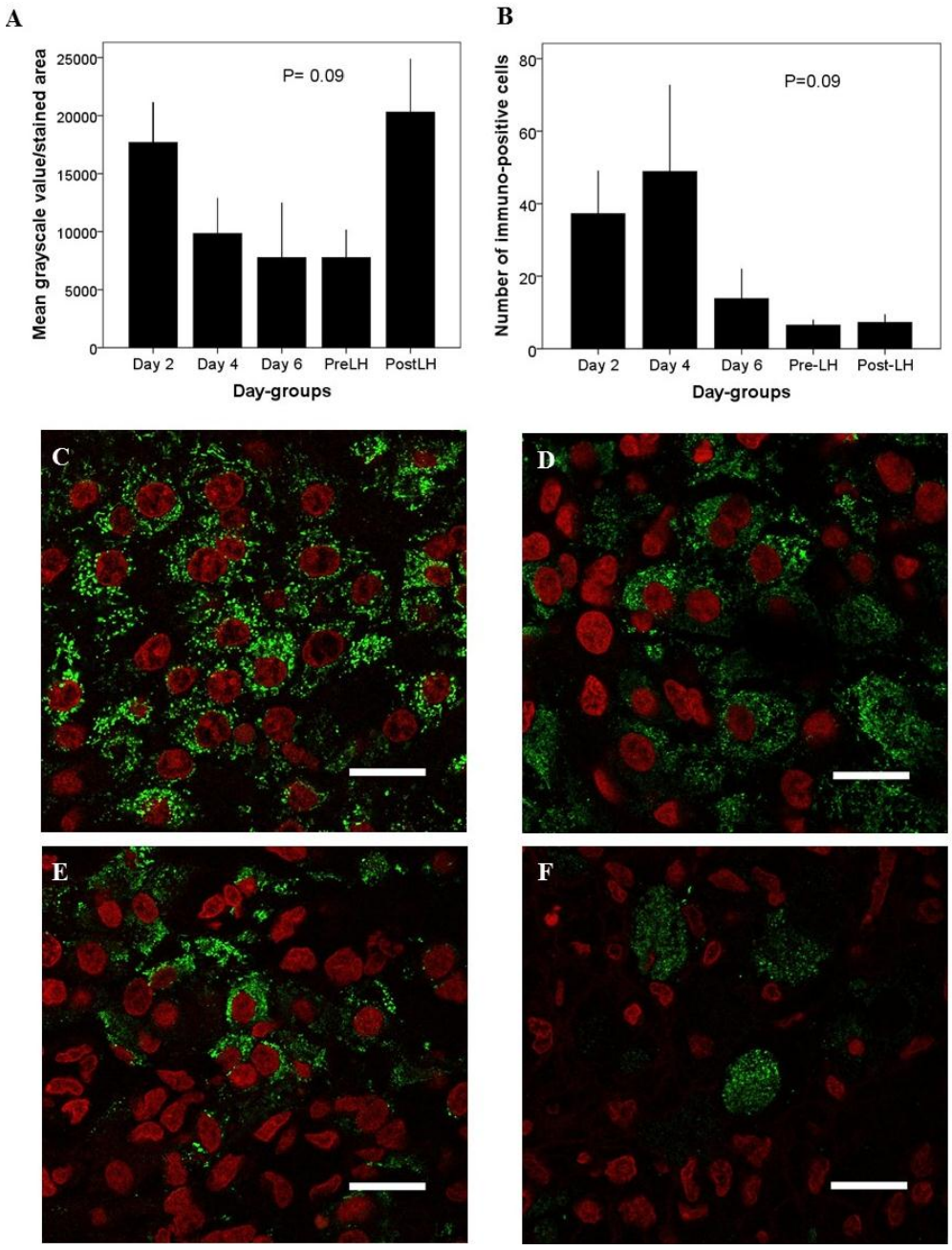

Figure 2.6. Anti-trkA staining pattern of the bovine CL collected in the periovulatory period, assessed by confocal microscopy. (A) Grayscale intensity values of immuno-reactive cells and (B) number of immuno-positive cells per high-powered field (mean ± SEM) in the CL among different day-groups (n=3-5 ovaries per group; Day 0 = ovulation). (C-F) Photomicrographs depicting anti-trkA immuno-fluorescence (green) in bovine CL on Day 2 (C), Day 4 (D), Day 6 (E) and Post-LH (F). Cell nuclei are shown in red (pseudo-color). Scale = 20 µm.

2.5. Discussion

The bovine model was used in the present study as a species representative of spontaneous ovulators and because of the ability to monitor ovarian events over time in relation to putative factors controlling ovarian function (Adams et al., 1995, 2008). Antral follicular dynamics during the estrous cycle in cattle and other species is a highly coordinated phenomenon characterized by two or more waves of follicle development. Each follicular wave consists of simultaneous growth of 8 to 40 follicles, detected initially at a diameter of ≥1 mm, one of which continues to grow (dominant follicle) while the others regress (subordinate follicles) (Ginther, 2000; Jaiswal et al., 2004; Adams et al., 2008). The dominant follicle during the luteal phase (i.e., elevated progesterone and low LH) will ultimately cease growth and undergo regression, whereas dominant follicle during lueolysis or in the absence of a CL (absence of the inhibitory effect of progesterone on LH release) will ovulate.

Results of the present study reveal abundant expression of the NGF-specific receptor, trkA, in ovarian follicles and the CL throughout the estrous cycle in cattle. TrkA has been isolated in theca and granulosa cells of bovine ovarian follicles of varying sizes in ovaries collected from the abattoir (Dissen et al., 2000) and in immuno-histochemical studies (Levanti et al., 2005), but the physiologic role of the NGF/trkA system in ovarian function in cattle was not examined. In the present study, the granulosa and theca layers of the dominant follicle of both anovulatory and ovulatory follicular waves of the estrous cycle expressed higher levels (intensity, area stained, and proportion of positive cells) of trkA receptors than that of subordinate follicles, suggesting a role of OIF/NGF during follicle selection and maturation. Results are consistent with those of a study involving in vitro culture of isolated ovarian follicles from sheep in which concentrations of NGF in the follicular fluid were greater in follicles ≥4 mm than in those ≤3 mm (Mattioli et al., 1999). The contrast in trkA expression between dominant and

subordinate follicles was most obvious during early CL development (Days 2 and 4) and after luteolysis (Post-LH); i.e., during periods of low progesterone and elevated LH, suggesting that trkA receptors may be induced by LH. In this regard, NGF and trkA were detected solely within 4 hours before the first preovulatory LH surge at the time of puberty in rats (Dissen et al., 1996). In addition, we found that early antral follicles (<1 mm in diameter) were immuno-reactive to trkA, similar to that previously reported in cattle (Levanti et al., 2005), reinforcing the idea that OIF/NGF is involved in follicular growth and maturation. Finally, in a recent report in cattle (Tanco et al., 2012), cows treated with OIF on Day 6 of the first follicular wave had earlier emergence of the next follicular wave than in the untreated control group. Taken together, the pattern of expression of trkA in the bovine ovary suggest that OIF/NGF has effects not only in mature stages as reported in other species, but also at multiple stages of folliculogenesis.

An unexpected finding in our study was the high expression (in intensity and area stained) of trkA receptors in subordinate follicles of the Pre-LH group. An earlier histomorphometric study of bovine follicular populations (Singh et al., 2000) described a thickening or hypertrophy (luteinization) of the theca interna of subordinate follicles in both ovulatory and non-ovulatory waves. In the present study, we found this thickening effect only in the Pre-LH group and not in the Day 6 subordinate follicles. We attributed this finding to be a consequence of luteolysis and the associated increase in LH pulse frequency as a result of decreasing plasma progesterone concentrations (Ireland et al., 1982, Goodman et al., 1980). The relative absence of trkA immuno-reactivity in the subordinate follicles of the Post-LH group was attributed to a more advanced state of atresia than in the Pre-LH group.

Analysis of the Cl revealed a tendency for a difference among day-groups in the number of immuno-positive cells. The difference was attributed to a greater number of immuno-positive cells during the early luteal phase (Days 2 and 4) than during mature and regressing phases (Day 6, Pre- and Post-LH). A greater number of trkA-responsive cells is consistent with the findings of a luteotrophic effect of OIF/NGF reported in cattle (Tanco et al., 2012; Tribulo et al., 2015). Perhaps trkA expression in the early CL is a carry-over of trkA immuno-positive cells of the theca and granulosa cells of the preovulatory follicle, since trkA and NGF have been implicated as regulators of cyto-differentiation at follicle rupture (Mayerhoffer et al., 1996). In gilts, tkrA and NGF were detected in the CL from Day 3 to Day 16 of the estrous cycle (as measured by immunofluorescence and western blot (Jana et al., 2011).

In conclusion, our data support the hypothesis that the luteotrophic effect of OIF/NGF is mediated, in whole or in part, by a rise in trkA receptor expression in the ovulatory follicle and early CL. Rather than an indirect effect via gonadotropin release, we infered that the follicular and luteogenic effects of OIF/NGF (endogenous or exogenous) in cattle (a spontaneous ovulator) is mediated directly at the level of the ovaries through interactions with trkA.

Chapter 3. Distribution of GnRH neurons in the hypothalamus and preoptic area of llamas (Lama glama)

R Carrasco, J Singh, GP Adams

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada

3.1. Abstract

Gonadotropin-releasing hormone (GnRH) is a decapeptide involved in the regulation of reproduction in all mammals studied to-date and its distribution within the brain shows wide variation across species. The objective of the present study was to characterize the number and distribution of GnRH neurons in the hypothalamus and preoptic area of llamas, an induced ovulator. The brains of female llamas (n = 4) were fixed, frozen and sectioned serially every 50 microns (coronal sections). Every 10^{th} section was prepared for immunohistochemical staining of GnRH. The immuno-reaction was revealed by *3,3′-diaminobenzidine* (DAB) and hydrogen peroxide. The number of GnRH immuno-reactive cells ranged from 220 to 250 cells per brain and were localized in the medio-basal hypothalamus (44.3%), anterior hypothalamus (27%), preoptic area (14.9%), diagonal band of Broca/medial septum (13.4%), and mammillary area (0.5%). The majority of immuno-reactive cells were not localized in specific hypothalamic nuclei, but rather appeared to be distributed diffusely. GnRH fibers were identified in most of the areas analyzed, including the posterior pituitary. The highest concentration of fibers ($P<0.05$) was detected in the median eminence. No discernible pattern was detected in the distribution of different immuno-reactive cell morphologies (monopolar, bipolar, multipolar) within the

hypothalamus. We concluded that GnRH neurons in llamas are concentrated in the anterior and medio-basal hypothalamus in close relationship to the third ventricle.

3.2. Introduction

Gonadotrophin-releasing hormone (GnRH) is a decapeptide that is fundamental in the regulation of reproduction (Gibson et al., 1997). It was originally isolated from the porcine hypothalamus (Schally et al., 1971) and later its presence was identified in the brains of many different species (King et al., 1985). To date, three different GnRH isoforms have been discovered across species, but the isoform involved mainly in reproductive processes is type 1 or mammalian GnRH (reviewed by Herbison, 2005).

During embryonic development, GnRH cells migrate caudally from the nasal placode into the brain in a mid-ventral direction toward the hypothalamus (Wray, 2010), but also to non-hypothalamic areas (Merchentaller et al., 1982). Consequently, the distribution of GnRH cells is not homogenous throughout the brain and varies markedly among species (reviewed by Silverman et al., 1994). Studies in rodents using immunocytochemistry and in situ hybridization have shown an accumulation of GnRH cells in the preoptic area surrounding the organum vasculosum of the lamina terminalis (reviewed by Silverman et al., 1994). In contrast, similar studies in sheep detected the presence of immuno-reactive cells in caudal and cranial portions of the hypothalamus (Lehman et al., 1986; Caldani et al., 1989). In a study in sheep designed to determine the areas responsible for the surge or tonic secretion of GnRH, implants of estradiol in the medio-basal hypothalamus induced LH secretion whereas implants in the preoptic area did not (Caraty et al., 1998). In contrast, rodents whose preoptic afferents to the

hypothalamus were surgically sectioned failed to ovulate (Wiegand et al., 1980). This discrepancy in the role of different neuron populations suggests anatomical specialization of GnRH neurons (Herbison, 1998), which is an important factor to consider when studying ovulatory processes in different animals.

Llamas are South American camelids classified as induced ovulators (Fernandez-Baca et al., 1970). Using transrectal ultrasonography it has been determined that these animals display continuous follicular waves. (Adams et al., 1990). Mating triggers a rise in circulating levels of luteinizing hormone (Bravo et al., 1990), which causes ovulation and corpus luteum formation (Adams et al, 1989, 1991). Based on information from other induced ovulators (Carrol et al., 1985; Greulich, 1934), it was initially assumed that ovulation in llamas was triggered by physical stimulation of the genitalia (Fernandez-Baca et al., 1970). Based on the discovery of an ovulation-inducing factor (OIF) in the seminal plasma of camelids (Adams et al., 2005), however, the results of recent studies demonstrate that systemic absorption of this seminal protein is the primary trigger for the ovulatory response in camelids (Ratto et al., 2005, 2006, 2010). Ovulation-inducing factor (OIF) was recently found to be identical in amino-acid sequence and structure to nerve growth factor (NGF) (Ratto et al., 2012), the mechanism by which this protein triggers ovulation involves the release of LH (Adams et al., 2005) induced by triggering, directly or indirectly, GnRH neuron secretion (Silva et al., 2011).

No information relating to the GnRH system in llamas was found in a search of the published literature, and the relationship between OIF and GnRH neurons therefore is unknown. The objective of the present study was to identify the anatomical distribution of GnRH neurons in the llama hypothalamus

and preoptic area, characterize the cytological characteristics of GnRH neurons, and identify the major GnRH pathways throughout the hypothalamus.

3.3. Materials and Methods

3.3.1. Animals and tissue collection

Female llamas (n = 4) from the llama herd of the University of Saskatchewan were euthanized with an overdose of pentobarbital following the guidelines of the Animal Care Committee of the University of Saskatchewan. The head was separated from the body, perfused with 2 liters of cold heparinized saline solution (10,000 IU Na heparin/L), followed by 2 liters of a cold 4% paraformaldehyde in phosphate buffered saline (PBS; pH = 7.4). The brain was extracted from the cranium, and the portion of mid-brain containing the preoptic area and hypothalamus was dissected and immersed in 4% paraformaldehyde overnight at 4°C (Fig 3.1). The next day, the sample was washed 3 times in PBS and immersed in 30% sucrose in PBS at 4°C until the tissues sank. Tissues were frozen at -80°C and sectioned every 50 um using a cryostat and sections were stored in cryoprotectant solution (30% sucrose, 30% ethylene glycol in PBS) at -20°C until immunohistochemistry was performed. In addition, the pituitary glands of 3 of the llamas were sectioned sagittally at 20 um increments, mounted on glass slides and stained for GnRH as described below

3.3.2. Immunohistochemistry

Immunohistochemical procedure was applied to unmounted (free-floating) sections to obtain optimal staining of thick sections. The cryoprotectant solution was removed by rinsing the sections 4 times in PBS for 15 minutes each. Endogenous peroxidase activity was blocked by incubating sections for 30 minutes in 30% hydrogen peroxide at room temperature, followed by 2 rinses in PBS. Antigen retrieval was performed by heating the samples to 93°C for 30 minutes in a sodium citrate solution (sodium citrate 0.1 M in distilled water; pH = 6.0). After cooling to room temperature and removing the antigen retrieval solution, non-specific binding was blocked by incubating sections with 1% BSA 0.3% triton X-100 in PBS for 3 hours. The primary antibody was diluted in 1% BSA, 0.3% triton x-100, 0.1% sodium azide in PBS (pH = 7.4) at a dilution of 1:5000 (mouse anti-GnRH; Stemberger Monoclonals, Cedarlane, Burlington, Ontario, Canada) and incubated with the sections for 24 hours at 4°C. Sections were washed 3 times for 15 minutes each with PBS and subsequently incubated with goat anti-mouse/HRP antibody (1:200; Dako, Burlington, Ontario, Canada) for 24 hours at 4°C. The immuno-reaction was revealed by incubating the sections in DAB for 30 minutes and all sections were rinsed in distilled water to stop the reaction (Hoffman et al., 2008). A set of sections were mounted in poly-L-lysine coated slides and counterstained with hematoxylin. In addition, adjacent sections were stained with Cresyl violet for assessment of anatomical detail. Antibody specificity was tested by omitting the primary antibody; specificity was confirmed when no reaction was detected. The primary antibody used in the present study has been tested previously in different species (rat and sheep; Egginger et al., 2011 and Tillet et al., 2012, respectively), and no cross-reactivity with other antigens has been reported.

The brain atlas of the *Lama glama*, from the collection of the University of Wisconsin-Madison (http://brainmuseum.org/) was used to determine the anatomical areas of the hypothalamus and

preoptic area. In addition, stereotaxic atlases of other mammals (Rabbit, Urban et al., 1972 and Girgis et al., 1981; Pig, Felix et al., 1999) were used for reference. Slides were assessed with a light microscope at magnifications of 5x, 10x and 20x to determine anatomical detail and cell distribution, and at 40x and 100X to examine GnRH cell projections. Only cells where the nucleus was identified were quantified. The number of positive cells and fibers per section, per nucleus and per area, as well as the number of cell to cell contacts, and the number of cell projections were recorded among sections.

3.3.3. Data analysis

From each brain, 1 in every 10 sections (10% of total sections) was stained against GnRH and examined using a light microscope. The first section of each brain to be stained was selected randomly from the first 10 sections. Data are presented as mean ± SEM number of GnRH neurons and proportion of total neurons that were identified as GnRH neurons in the respective hypothalamic areas and nuclei. The number of GnRH immuno-positive cells are expressed in two ways: total number of GnRH cells per anatomical structure and density of GnRH neurons per anatomical structure. The density of GnRH neurons was calculated by dividing the immuno-reactive cell number per hypothalamic area and nuclei by the number of sections containing the area or nuclei. Differences in total GnRH immuno-reactive cells or densities of GnRH neurons among areas were compared by one way-analysis of variance for repeated measures (that is, counts from one area of the brain for an individual were not independent of counts for another area). When significant differences were detected, post hoc analyses using the method of least significant difference were conducted. The distribution of cell morphologies (monopolar, bipolar or multipolar) were compared by a goodness-to-fit chi-square test. Significance was considered when $P<0.05$.

3.4. Results

The distance between the optic chiasma and the mammillary bodies corresponded to 17.5 mm (35 sections stained per brain x 10 sections between stained sections x 50 um thickness per section). Based on the total number of cells per animal (range 220 to 250) and that 10% of the sections per brain were stained and analyzed, we estimated that the total number of GnRH immuno-reactive cells within the preoptic area and hypothalamus in the llama ranges between 2200 and 2500 cells (mean ± SEM = 2390 ± 88 cells).

3.4.1. GnRH neuron cytology

Three different cell projection patterns were identified in immuno-reactive cells; monopolar, bipolar and multipolar (Fig 3.1 A-D). The overall prevalence of these three morphologies throughout the hypothalamus was similar (monopolar 33.3%, bipolar 38.5%, multipolar 28.3%). However, in the medio-basal hypothalamus, the bipolar type of cell was most frequent (40.5%), followed by the monopolar type (35.2%), and the multipolar type (24.2%, $P < 0.05$). Immuno-reactive cells displayed two distinct profiles; an irregular border with multiple spines and a smooth border (Fig. 3.1 A, C). Contact between adjacent GnRH immuno-reactive cells was observed in all brains examined, but at a low frequency (2 to 3 per brain; Figure 3.1 E).

3.4.2. GnRH immuno-reactive fibers

Fibers were detected in all sections analyzed but fiber density was not homogeneous among sections. The presence of enlargements or varicosities was detected along every fiber examined (Fig 3.1 F). Fibers were detected in close relation to the lateral ventricles or in close apposition to other GnRH

neurons. The median eminence was the structure of the brain with the highest quantity of fibers ($P < 0.05$; Fig 3.3 E). These fibers were present in the internal and external layers of the median eminence. The orientation of the fibers varied along the median eminence: in the rostral portion, axons had a rostro-caudal direction, and in the mid- and caudal parts of the median eminence the fibers were oriented in a latero-medial fashion. The medio-basal hypothalamus and the medial preoptic area displayed almost 20% of the total number of immuno-reactive fibers (Table 3.1).

3.4.3. Fiber projections in the posterior pituitary

GnRH fibers in the posterior portion of the pituitary gland were found in sagittal sections of pituitaries and transverse sections of the median eminence (Fig. 3.3). The fibers showed a caudo-ventral orientation within the pituitary and had a similar appearance to the fibers detected in the hypothalamus. Further, transverse sections of the caudal portion of the median eminence showed a dense presence of immuno-reactive fibers running in different directions (Fig. 3.3 E). These fibers were in proximity to the pituitary cleft with no penetration into the anterior pituitary. Immuno-reactive fibers were in close apposition to blood vessels but no contact was detected.

3.4.4. GnRH cell distribution

The number of GnRH immuno-reactive cells and fibers were not equally distributed among hypothalamic areas examined ($P < 0.001$ and $P = 0.03$, respectively; Table 3.1). While a relative accumulation of immuno-reactive cells and fibers in the middle portions of the hypothalamus (anterior and medio-basal hypothalamus) was observed (Fig 3.4), GnRH cells were scattered; that is, they were not aggregated in specific hypothalamic nuclei (Fig 3.2). The highest proportion ($P < 0.05$) of GnRH

cells was observed in the medio-basal hypothalamus and anterior hypothalamus (Table 3.1). The anterior hypothalamic areas displayed a similar number of GnRH cells to the lateral anterior and lateral hypothalamus, and both contained a greater number of cells than the lateral and medial septa, arcuate nuclei and periventricular nuclei ($P < 0.05$). No differences were detected in GnRH neuron density between the preoptic area, anterior hypothalamic area and medio-basal hypothalamus (Fig 3.4), but these regions had more GnRH cells than the diagonal band of Broca/medial septum or the mammillary area ($P < 0.05$). A representative drawing of the relative position of immuno-reactive neurons and fibers is shown in Fig 3.2.

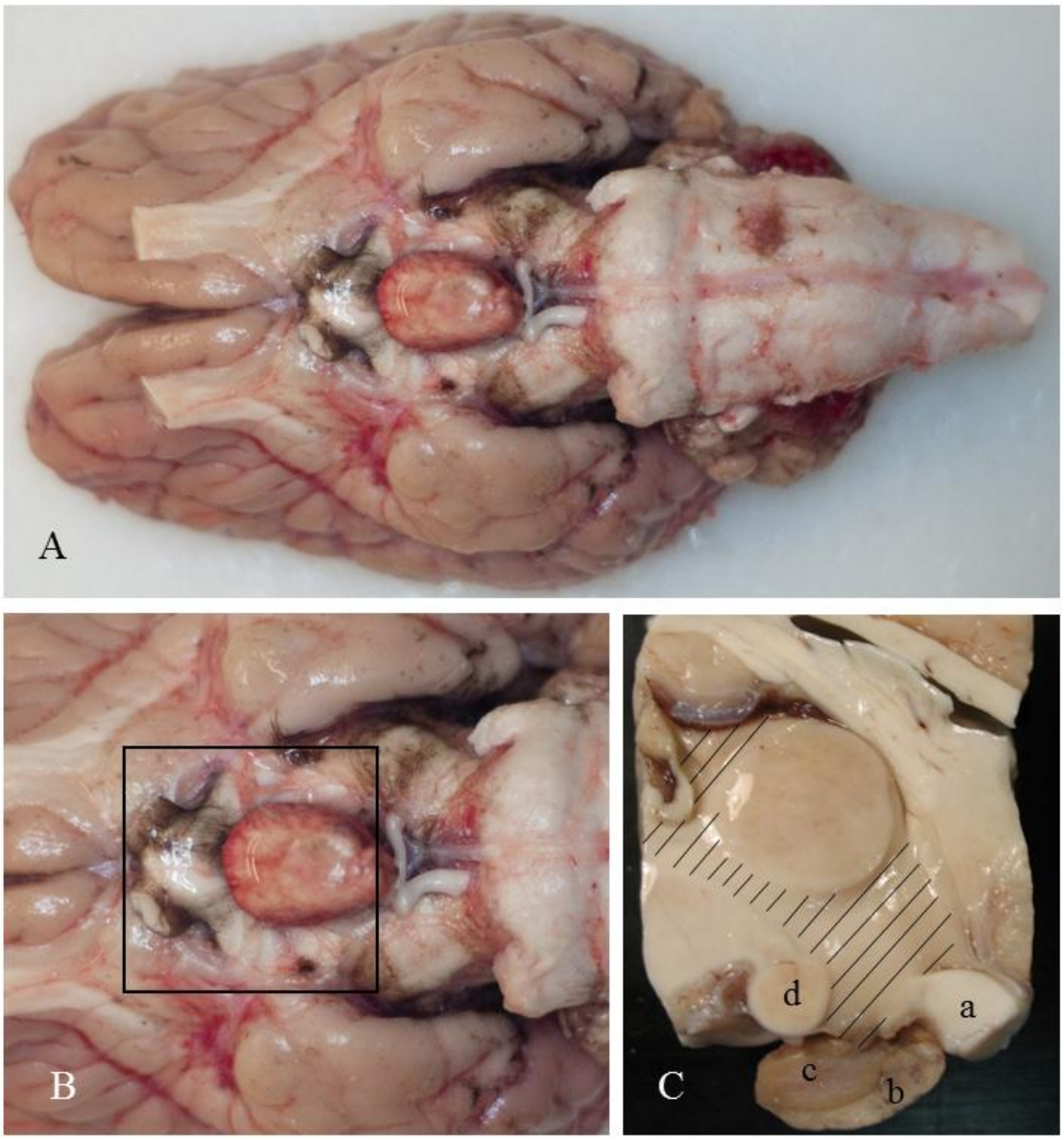

Figure 3.1. Example of the general organization of llama brain. A. Ventral view of the llama brain. B. Magnification of A, square box indicates the area that was used for immunohistochemistry. C. Llama hypothalamus sectioned in the midline to expose the third ventricle (parallel lines). a= optic chiasma, b= anterior pituitary, c= posterior pituitary, d= mammillary bodies.

Table 3.1. Distribution of GnRH neurons and fibers (mean ± SEM and percent of total) in major hypothalamic areas and nuclei of llama brains (n = 4). Statistical comparisons are summarized in the table below.

	Neurons		Fibers	
	Mean ± SEM	%	Mean ± SEM	%
Diagonal band of Broca	21.5 ± 1.4[a]	9.0	29.3 ± 6.6[a]	5.1
Medial septum	6.0 ± 1.0[b]	2.5	7.3 ± 3.3[b]	1.3
Lateral septum	2.0 ± 0.5[b]	0.8	14.0 ± 5.2[b]	2.5
Medial preoptic area	21.5 ± 1.3[a]	9.0	47.0 ± 10.7[a]	8.2
Lateral preoptic area	11.5 ± 3.7[a]	4.8	9.5 ± 4.9[b]	1.7
Anterior hypothalamic area	35.3 ± 9.3[ac]	14.7	32.8 ± 8.1[a]	5.7
Lateral anterior hypothalamic area	24.3 ± 3.1[a]	10.1	19.0 ± 7.5[b]	3.3
Retrochiasmatic area	5.3 ± 1.0[ab]	2.2	6.3 ± 1.3[c]	1.1
Arcuate nucleus	1.8 ± 0.6[b]	0.7	4.3 ± 3.3[c]	0.7
Median eminence	0[b]	0.0	309.8 ± 82.2[d]	54.2
Medio-basal hypothalamus	72.0 ± 9.3[c]	30.1	68.8 ± 31.5[ab]	12.0
Lateral hypothalamic area	25.8 ± 4.3[a]	10.8	14.8 ± 7.8[b]	2.6
Dorsal hypothalamus	6.8 ± 1.8[b]	2.8	2.8 ± 1.9[c]	0.5
Supraoptic nucleus	3.0 ± 1.8[b]	1.3	1.5 ± 1.5[c]	0.3
Suprachiasmatic nucleus	0[b]	0.0	0.8 ± 0.8[c]	0.1
Periventricular nucleus	1.5 ± 1.0[b]	0.6	0.8 ± 0.8[c]	0.1
Mammillary area	1.0 ± 0[b]	0.4	2.8 ± 2.8[c]	0.5
TOTAL*	239.0 ± 8.8	100%	571 ± 150	100%

* Total GnRH immuno-reactive neurons or fibers per llama

[abcd] Within rows, values with different superscripts are different, $P<0.05$

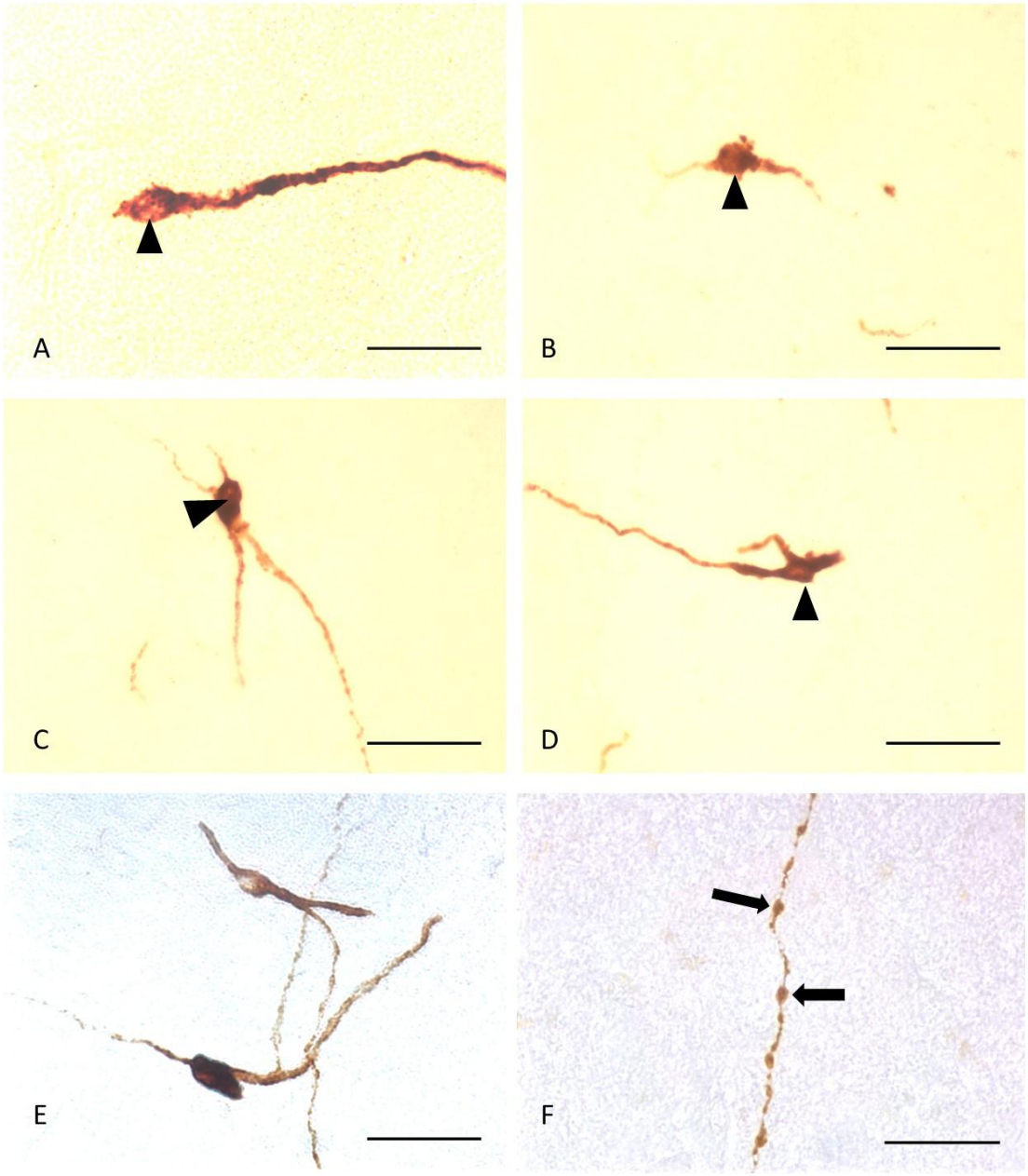

Figure 3.2. GnRH immuno-reactive cell profiles in llama hypothalamus. Micrographs showing the different neuronal shapes observed. (A) Monopolar neuron. (B, D) Bipolar neuron. (C) Multipolar neuron. (E) Cell to cell contact. (F) Appearance of GnRH fibers, arrows show varicosities. (A-D) Arrowheads show the nucleus. A-E scale bar 50 µm. F scale bar 20 µm.

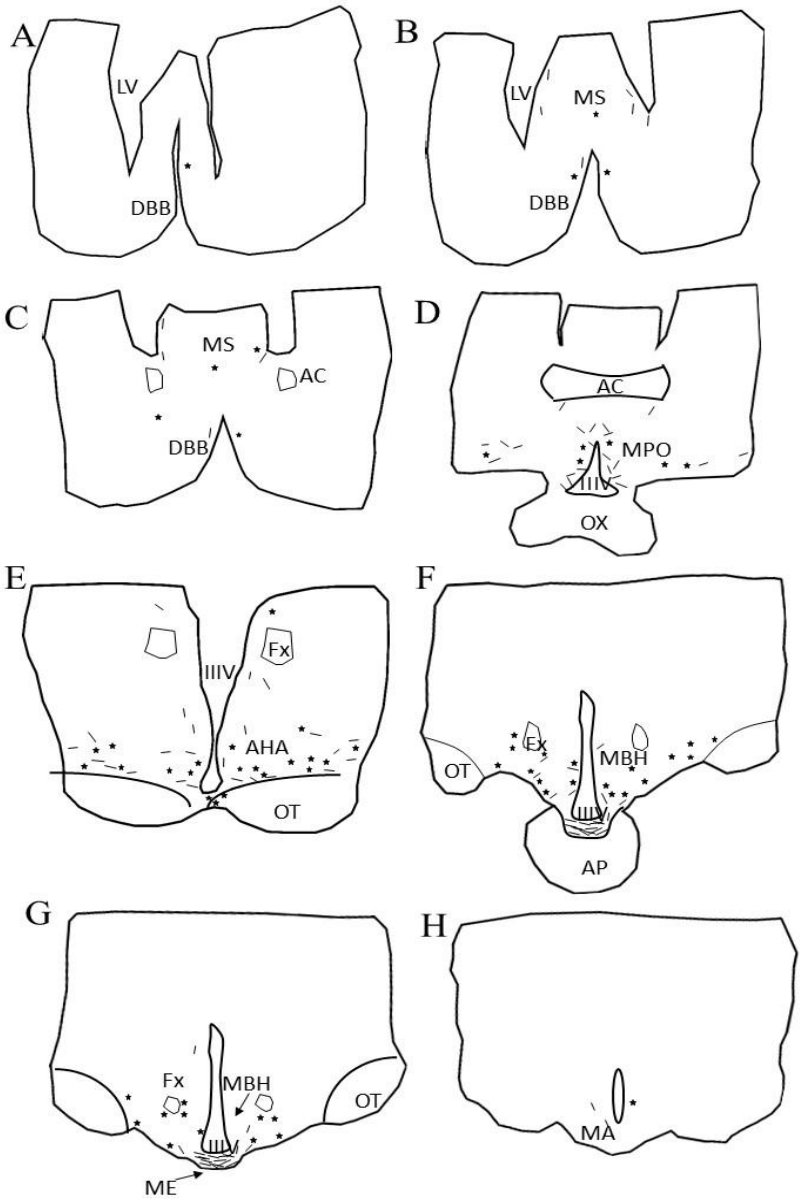

Figure 3.3. Schematic drawing of GnRH immuno-reactive cells (stars) and fibers (lines) distribution at different areas in the preoptic area and hypothalamus of llamas. Sections are 2000 to 3000 um apart and are arranged from rostral (left top; A) to caudal (right bottom; H). LV: Lateral ventricle, AC: Anterior commissure, AHA: Anterior hypothalamic area, MBH: Medio-basal hypothalamus, ME: Median eminence, IIIV: third ventricle, OT: Optic tract, Fx: fornix, DBB: Diagonal band of Broca, MS: Medial septum, MPO: Medial preoptic area, OX: Optic chiasma, AP: Anterior pituitary, MA: Mammillary area.

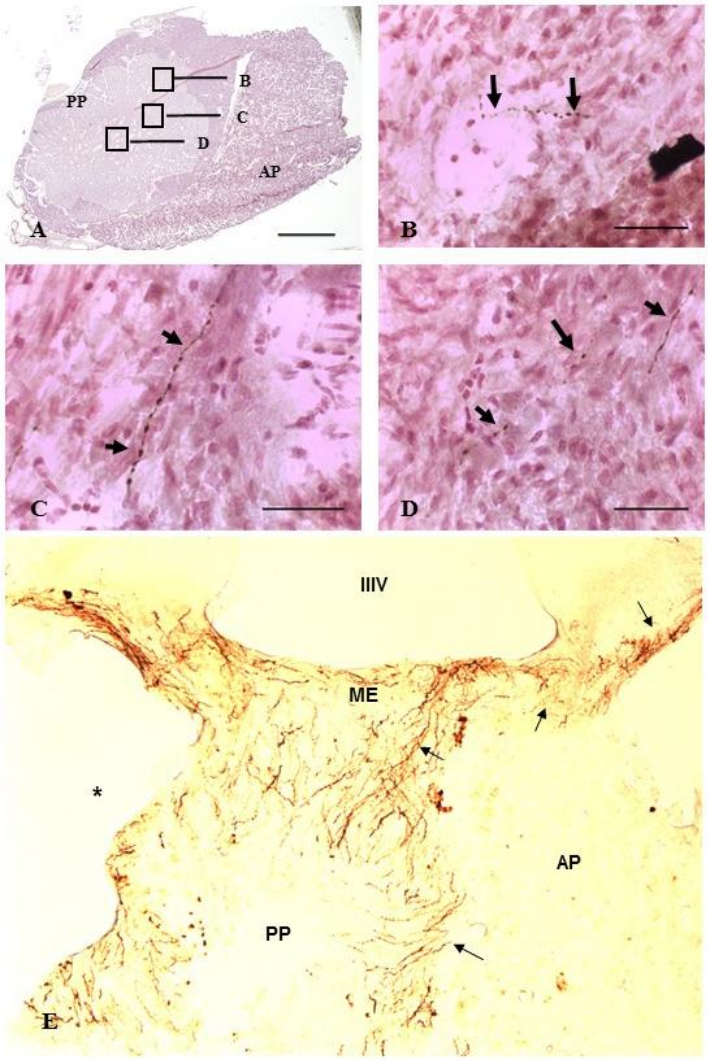

Figure 3.4. Distribution of GnRH fibers in the pituitary and median eminence of llamas. (A) Low magnification of a parasagittal section of posterior pituitary (PP) and anterior pituitary (AP; scale bar 2 mm). Squares are magnified in B-D. (B-D) High magnification of GnRH fibers (arrows) running through the caudal lobe of the pituitary, as shown in A. (E) Transverse section of the median eminence of llama stained against GnRH. Arrows show the immuno-reactive axons running through the median eminence and the posterior pituitary. Part of the anterior pituitary was lost (shown by asterisk) during processing. IIIV: Third ventricle, ME: Median eminence, AP: Anterior pituitary, PP: Posterior pituitary. (A–D) hematoxylin counterstain. Scale bar 50 um.

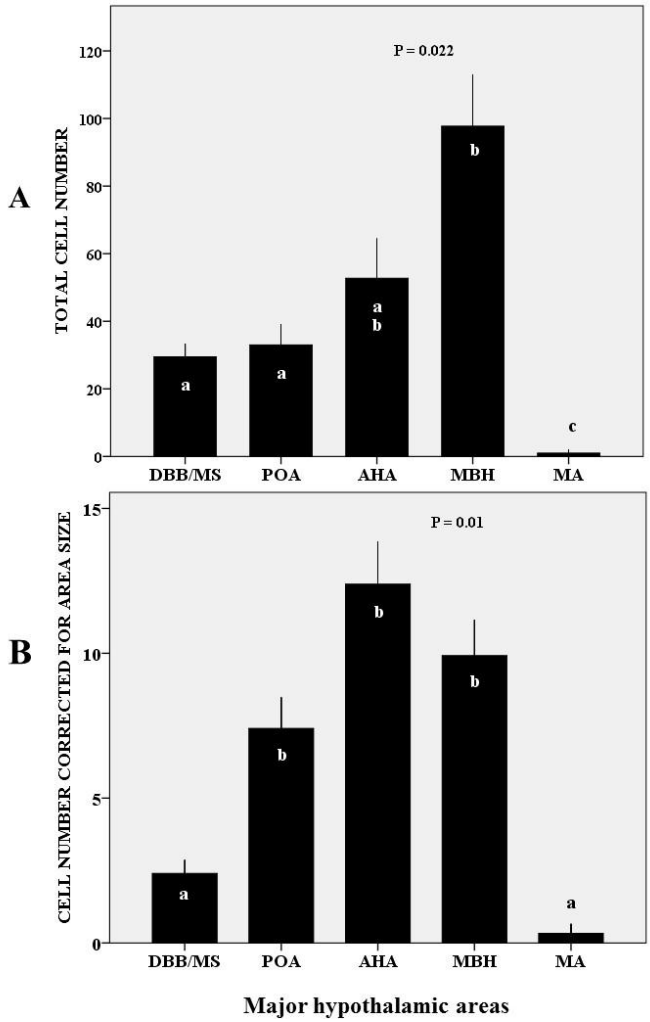

Figure 3.5. Number (mean ± SEM) of immuno-reactive cells in major areas throughout the hypothalamus and preoptic area of llamas (n = 4). (A) Total number of immuno-reactive cells among the hypothalamic areas examined in four animals. (B) GnRH immuno-reactive cell density in the hypothalamic areas examined, total cell number was corrected for the number of sections displaying the area. Areas are organized rostro-caudally (From DBB to MA). Bars with no common superscript show significant differences ($P<0.05$). DBB/MS: Diagonal band of Broca/medial septum/lateral septum, POA: Medial and lateral preoptic area, AHA: Medial and lateral anterior hypothalamic area, MBH: Medio-basal and lateral hypothalamus, MA: Mammillary area.

Table 3.4. Anatomical distribution of monopolar, bipolar and multipolar GnRH neurons in the llama hypothalamus (mean ± SEM; n = 4 brains).

Anatomical area	Monopolar	Bipolar	Multipolar
Diagonal band of Broca/Medial and Lateral Septa	12.8 ± 4.0 (39.8%)	11.3 ± 2.3 (35.2%)	8 ± 1.9 (25.0%)
Preoptic area	11.5 ± 3.4 (32.2%)	14.0 ± 2.9 (39.2%)	10.3 ± 1.3 (28.7%)
Anterior hypothalamus	17.3 ± 4.8 (27.8%)	22.8 ± 4.9 (36.7%)	22.0 ± 4.1 (35.6%)
Medio-basal Hypothalamus	35.3 ± 5.6 [a] (35.3%)	40.5 ± 13.9 [b] (40.5%)	24.3 ± 6.0 [c] (24.3%)
Mammillary Area	0.0 (0.0%)	0.3 ± 0.3 (25.0%)	30.8 ± 0.5 (75.0%)

[abc] Within rows, values with different superscripts are different, P<0.05.

*** Percentage of cell type within an area.**

3.5. Discussion

In the present study GnRH neurons and fibers had similar morphological features among mammalian species in terms of shape and staining patterns (King et al., 1985). Similar to sheep (Lehman et al., 1986; Silverman., 1994), and rat (Merchenthaler et al., 1984), GnRH neurons in llamas were not localized in specific hypothalamic nuclei and, in general, were scattered loosely among major hypothalamic areas.

GnRH neurons have been described previously in different species (mouse, rat, guinea pig, sheep, human; King et al., 1985) and the results have shown a wide variation among species in relation to their distribution. In the rat, the majority of GnRH neurons are located in the medial septal-preoptic regions and very few cells are located in the medio-basal hypothalamus (Merchentaler et al., 1984). A similar pattern was described in sheep (Lehman et al., 1986; Caldani et al., 1988), but showing a higher proportion (15-30 %) of cells present in the anterior hypothalamus. We found that in llamas 27% and 44% of the immuno-reactive cells were located in the anterior and medio-basal hypothalamus, respectively (Fig 3.4), and few cells were located in the preoptic area (lateral and medial; 13,8% of the total GnRH neurons). Our data is in agreement with observations from the mink, another induced ovulator, where 80% of GnRH cells are located in the medio-basal hypothalamus (Toumi et al., 1992) and close to 20% of cells are located in the preoptic area and anterior hypothalamus. The accumulation of cells in the medio-basal hypothalamus has been described, to a lesser degree, in humans and monkeys (King et al., 1985). There is a scarcity of this type of studies in induced ovulators, and it remains to be established as to whether this distribution of GnRH neurons is characteristic or not of induced ovulators.

Although GnRH neurons act in a pulsatile fashion throughout the estrous cycle there is evidence that there are subpopulations that are specialized in certain processes (i.e. ovulation). In rabbit, electrical damage of the area surrounding the organum vasculosum did not compromise reflex ovulation in does, and were able to develop hemorrhagic follicles 24 hours after copulation with a fertile buck (Lescure et al., 1978). Similarly, female ferrets that received penile intromission had a higher proportion of activated GnRH neurons in the medio-basal hypothalamus than other areas (Wersinger et al., 1997; Baker et al., 2001). In addition, in ewes that were implanted with estradiol, only those implanted in the medio-basal hypothalamus displayed an LH surge whereas those implanted in the preoptic area failed to ovulate (Caraty et al., 1998). Taken together, these findings suggest that the GnRH system contains specialized neuronal subpopulations associated to specific areas in the brain, and it remains to be established if any of the GnRH populations described in the present study is involved actively during ovulation in llamas.

An ovulation-inducing factor has been detected in Bactrian camelids (Chen et al., 1985) and South American camelids (Adams et al., 2005; Ratto et al., 2011). In llamas and alpacas, the factor has been found to be identical to nerve growth factor (OIF/NGF; Ratto et al., 2012). The distribution of OIF/NGF receptors have been predominately found in an area denominated as the basal forebrain (Gibbs et al., 1994), which includes the diagonal band of Broca and the lateral and medial septum. We found that the number of GnRH neurons in the areas corresponding to the basal forebrain is around 11% of the total of GnRH cells. We inferred that given this relative low density of immuno-reactive cells is unlikely that such a small population of immuno-reactive cells in the diagonal band of Broca are capable of inducing a preovulatory LH surge. Further, it has been estimated in mouse that at least 40% of GnRH neurons are activated during ovulation in mouse (Wu et al., 1992). It may be possible that

other neuronal components, including GnRH neurons, express one of the two receptors for OIF/NGF; the high affinity trkA and the low affinity p75.

The median eminence is the place where hypothalamic hormones are released to the portal vessels which later reach and stimulate the anterior pituitary (Page, 2005). An unexpected finding in our study was the presence of GnRH immuno-reactive projections in the neural lobe of the pituitary (Fig 3.3), the projections were in close relation to the pituitary cleft and occasionally distinguished within the neural lobe (Fig 3.3). Such GnRH projections have also been reported in a multispecies comparative study of the GnRH system (King et al., 1985), where bats, ferrets, and humans resembled the organization described in the present study. The functional consequences of this arrangement have not been elucidated, and whether they contribute to critical events such as the preovulatory LH surge remains to be established.

In summary, the GnRH system in llamas forms a continuum with an accumulation of cells in the anterior and medio-basal hypothalamus on the lateral aspects of the third ventricle. This proximity between the cerebral ventricle and the GnRH cells, suggest a potential route for OIF in the cerebrospinal fluid to stimulate directly or indirectly the preovulatory secretion of GnRH/LH.

Chapter 4. The relationship between gonadotropin releasing hormone and tyrosine receptor kinase A in the hypothalamus and preoptic area of the llama

Carrasco R, Singh J, Adams GP

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.

4.1. Abstract

A molecule identical to nerve growth factor, with ovulation-inducing properties has been discovered in the seminal plasma of South American camelids. The effect has been shown to be mediated, at the level of the hypothalamus, presumably by GnRH neurons. The objective of the present study was to establish a morphological relationship between GnRH neurons and OIF/NGF high affinity receptor, trkA. Mature llamas (n = 4) were euthanized and their brain tissue was fixed and processed for immunohistochemistry on free-floating sections. Ten equidistant sections per brain were stained using antibodies against trkA and GnRH for immunofluorescence and immunoperoxidase. Cells immuno-reactive to trkA were detected in most hypothalamic areas, especially in the diagonal band of Broca, the periventricular nucleus, and the lateral preoptic nucleus. A low proportion of GnRH neurons were immuno-reactive to trkA (1% of total GnRH cells). Some GnRH fibers were found occasionally, to be in proximity to trkA immuno-positive neurons. Results did not support the hypothesis that the effect of OIF/NGF is driven by its direct interaction with GnRH neurons.

4.2. Introduction

Llamas and alpacas ovulate in response to copulation; that is, they are induced ovulators (England et al., 1969; Fernandez-Baca et al., 1970). The physical stimulation of coitus, however, is not the primary trigger for ovulation, as initially proposed, but rather it is in response to a factor present in seminal plasma that induces a preovulatory LH surge (Adams et al., 2005; Ratto et al., 2012). The seminal ovulation-inducing factor (OIF) is a potent stimulator of LH release (Adams et al., 2005; Ratto et al., 2011), and is capable of inducing ovulation in llamas and alpacas at dose $1/100^{th}$ of that present in a normal ejaculate (Tanco et al., 2011). The factor was recently found to be identical to nerve growth factor (Ratto et al., 2012), and will be herein-after referred to as OIF/NGF. In a study designed to determine the mechanism by which OIF/NGF elicits LH release from the pituitary gland (Silva et al., 2011), llamas pretreated with a GnRH receptor antagonist and subsequently treated with OIF/NGF failed to have a preovulatory LH surge. Although there is evidence of OIF/NGF-induced LH release from pituitary gonadotrophs in vitro (Bogle et al., 2012), the main site of action of OIF/NGF in vivo appears to be at the level of the hypothalamus.

Ovarian follicular development in llamas and alpacas occurs in a wave-like pattern (Adams et al., 1990), as described in other farm animals (Adams, 1999; Draincourt, 2001). As a monotocous species, each follicular wave involves development of a single dominant follicle which, in llamas and alpacas, is capable of ovulating when it is $\geq$ 7 mm in diameter. In the absence of mating, ovulation does not occur, a CL does not develop, and successive follicular waves emerge at periodic intervals. This is a striking difference from spontaneous ovulators, where the corpus luteum is present during the majority of the estrous cycle, and progesterone plays and important role in follicle maturation and oocyte competence (Fair et al., 2011). In induced ovulators, ovarian estradiol is not associated with positive

feedback on the hypothalamic-pituitary axis to elicit the LH surge as in spontaneous ovulators, but it does modulate pituitary LH secretion in OIF/NGF treated llamas (Silva et al., 2012).

Nerve growth factor is a molecule with the special ability to maintain and enhance neuron survival (Levi-Montalcini, 1987), and is present in restricted areas of the central nervous system, such as the dorsal root ganglia and in cholinergic pathways in the ventral forebrain (Conner et al., 1994). Nerve growth factor mediates actions through interaction with two different receptors; trkA and p75. TrkA (also known as NTRK1) is a high affinity receptor and mediates most of the classical actions of NGF. Conversely, p75 (also termed NGFR) is a low affinity receptor that has the ability to bind other neurotropins. In vitro culture studies support the idea that p75 is involved in inducing cell death and, under certain conditions, is capable of mediating the effects of NGF (Yoon et al., 1998). Since pharmacological blockade of trkA eliminated most of the effects of NGF (Ohmichi et al., 1992), it is likely that the ovulation-inducing effect of OIF/NGF is driven by interaction with the high affinity receptor, trkA.

The NGF-trkA system has been described in several species and different tissues either by in situ hybridization (Gibbs et al., 1994; Dissen et al., 2000), immunohistochemistry (Gibbs et al., 1994; Ren et al., 2005; Badowska-Szalewska et al., 2006) or autoradiography (Richardson et al., 1986). In the rat brain, NGF receptors have been found in the diagonal band of Broca, caudal putamen, lateral preoptic area and globus palidus (Richardson et al., 1986). The relationship between the NGF/TrkA system and autonomous nervous system has been shown at central and peripheral level. TrkA immuno-reactive cells in the rat brain were also immuno-positive in a high proportion to choline acetyltransferase, an enzyme involved in the synthesis of acetylcholine. (Sobreviela et al., 1994). Conversely, an in vivo

study revealed that continuous injections of a NGF antiserum produced a depletion of sympathetic structures in newborn mice (Levi-Montalcini et al., 1960).

To test the hypothesis that OIF/NGF effects a response through direct interaction with GnRH neurons in llamas, the objectives were to determine if GnRH neurons or their neuronal terminals express trkA receptors.

4.3. Materials and Methods

4.3.1. Animals and tissue collection

Mature llamas (n = 4) were euthanized using an overdose of pentobarbital, and the head was separated and immediately perfused with 2 liters of cold saline heparinized solution (10,000 IU Na heparin/L), followed by 2 liters of a solution of 4% paraformaldehyde in phosphate buffered saline (PBS; pH= 7.4). After the brain was extracted from the cranium, the preoptic area and hypothalamus were dissected out and immersed in the same fixative overnight at 4°C. The next day, the tissues were washed 3 times in PBS and stored in PBS with 0.1% (w:v) sodium azide at 4°C until cryoprotection. Samples were immersed in cryoprotectant solution (30% sucrose in PBS) until the tissues sank, then were frozen at -80°C until sectioning. Tissues were sectioned transversely (coronal plane) at 50 um using a cryostat and each section was stored in a mixture of 30% sucrose and 30% ethylene glycol in PBS at -20°C until immunostaining. Animal procedures were approved by the University of Saskatchewan Committee on Animal Care in accordance with guidelines of the Canadian Council on Animal Care.

4.3.2. Immunohistochemistry

Immunofluorescence was carried out on unmounted (free floating) sections to optimize staining of thick sections. Ten equidistant sections per brain (one every 1500 um) were selected for double immunofluorescence labelling. After removing the cryoprotectant solution, sections were rinsed 4 times in PBS for 15 minutes each. Antigen retrieval was performed by heating the samples at 80° C for 35 minutes in sodium citrate solution (pH: 6.0). After cooling to room temperature, sections were blocked with 0.5 % BSA 0.5% triton X-100 in PBS for 3 hours. Sections were incubated with a cocktail of primary antibodies diluted in 0.5 % BSA, 0.5 % triton x-100, and 0.1% sodium azide in PBS for 48 hours at 4°C. Anti-GnRH antibody (mouse anti GnRH; Stemberger Monoclonals; Cedarlane, Burlington, Ontario, Canada) was used at a dilution of 1:10,000 and anti-trkA (rabbit anti-trkA, Santa Cruz biotechnologies; Dallas, Texas, USA) was used at a dilution of 1:500. Sections were washed 3 times with PBS and subsequently incubated with goat anti-rabbit antibody conjugated to biotin (1:500; Life Technologies; Burlington, Ontario, Canada) and goat anti-mouse/Alexa 546 antibody (1:500; Life Technologies; Burlington, Ontario, Canada) for 3 hours at 37°C in blocking buffer. After washing the secondary antibodies, samples were incubated with streptavidin conjugated to Alexa 488 (Life Technologies; Burlington, Ontario, Canada) diluted in blocking buffer to 1:200 for two hours at 37°C (Hoffman et al., 2008). Finally, sections were washed and mounted on poly-L-lysine coated slides, air dried, incubated 10 minutes in a solution of 0.3% sudan black in 70% ethanol, air dried again, covered with Vectashield mounting medium (Vectorlabs, Burlington, Ontario, Canada) containing DAPI, and a coverslip was applied.

An additional set of sections was stained by immune-peroxidase for trkA and GnRH using DAB and Nickel DAB as chromogens. Nickel DAB (blue-black product) and DAB (brown product) can be used

for immuno-detection of two different molecules in the same section. The immune-peroxidase procedure was carried as stated above.

A set of adjacent sections was stained with Cresyl violet for assessment of the structural and anatomical detail. Anatomical organization was determined using the aid of the *Lama glama* brain atlas from the brain atlas collection of the University of Wisconsin, Madison, and stereotaxic atlases of other mammals (Urban et al., 1972, Girgis et al., 198 and Felix et al., 1999).

4.3.3. Antibody controls

Preabsorption of the primary anti-trkA antibody with trkA immunogen (Santa Cruz Biotechnologies; Dallas, Texas, USA) was performed in a 1 to 5 ratio (weight:weight) with no resultant immuno-detection. In addition, llama dorsal root ganglia were used as a positive control (Fig 4.1). GnRH is highly conserved among species (Fernald et al., 1999), and use of the anti-GnRH antibody has been validated previously with different species (rat, Egginger et al., 2011; sheep, Tillet et al., 2012). Omission of the anti-GnRH or anti-trkA antibody eliminated immuno-detection in hypothalamic sections.

4.3.4. Data analysis

Cell numbers were counted manually by a single observer using a wide-field fluorescent microscope at 20x magnification (Zeiss, Axioskope 40; Thornwood, New York, USA). To avoid double counting, only cells with a distinguishable single nuclei were quantified. Confocal microscopy was performed

on a Leica LSM confocal microscope (Concord, Ontario, Canada) with lasers for excitation of Alexa 488 and Alexa 546. Cell number are expressed as mean number of cells per anatomical structure or area among animals. Data are expressed as mean ± SEM or as a percentage of the total number of cells displaying immuno-reactivity. Number of trkA and GnRH immuno-reactive cells within an area or nucleus were compared by paired t-tests. The number of trkA immune-positive cells along the hypothalamus and preoptic area was compared by analysis of variance for repeated measures. Differences were considered to be significant when the p-value was less than 0.05.

4.4. Results

4.4.1. General distribution of trkA immuno-reactive cells

Llama dorsal root ganglia stained against trkA receptor showed a strong immunoreaction (Fig 4.1 A). The signal was restricted to sensory neurons; no reaction was detected in satellite cells. When the antibody was pre-incubated with trkA, no reaction was detected (Fig 4.1 B), documenting the specificity of the antibody signal.

TrkA immuno-reactivity was present in all hypothalamic areas and nuclei examined, except in median eminence, dorsal hypothalamus and optic chiasma. Areas that were positive to trkA immunoreactivity had a wide variation in the number of positive cells (Table 4.1). The areas with highest density of trkA immuno-positive cells were the diagonal band of Broca and the periventricular nuclei ($P < 0.05$). Low quantities of trkA immuno-reactive cells were detected in the arcuate nucleus and retrochiasmatic area. The immuno-reactive signal was restricted to the cytoplasm surrounding the nuclei, no identifiable neuronal projections were detected.

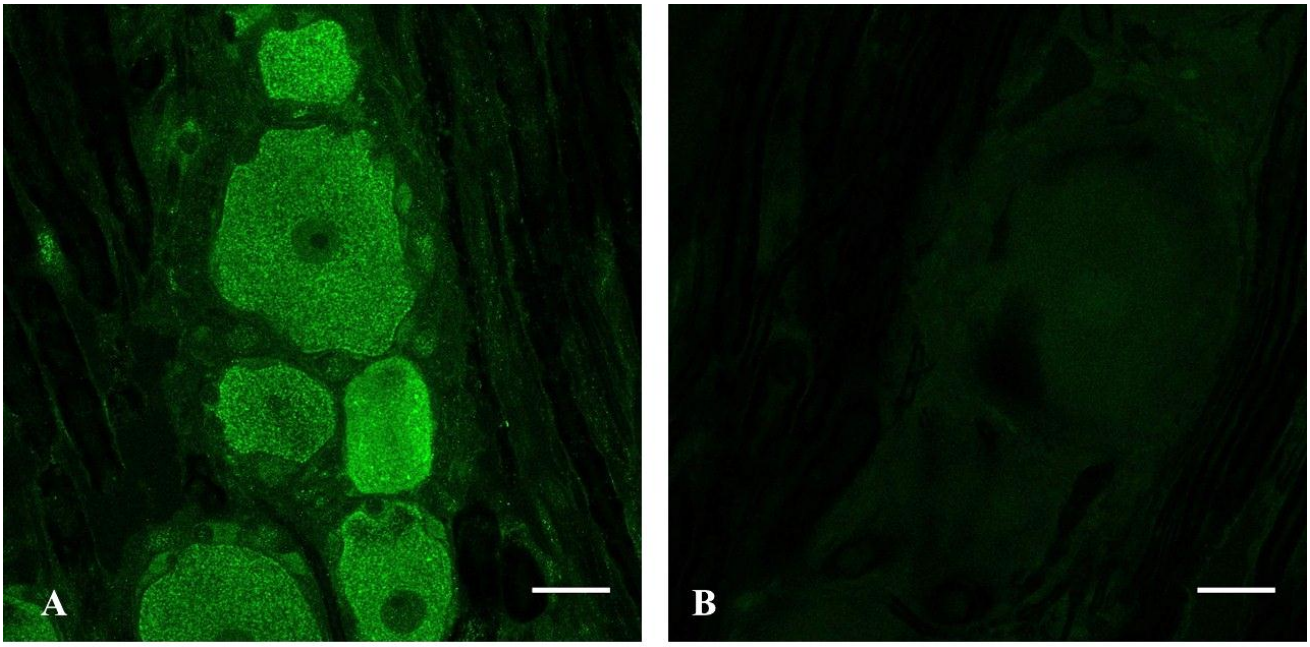

Figure 4.1. Immuno-reactivity (A) and negative control (B) for trkA in a dorsal root ganglium of a llama. The negative control section was stained with the antibody pre-absorbed with the immunogen. The same antibody dilutions were used in A and B. Scale bar = 30 um.

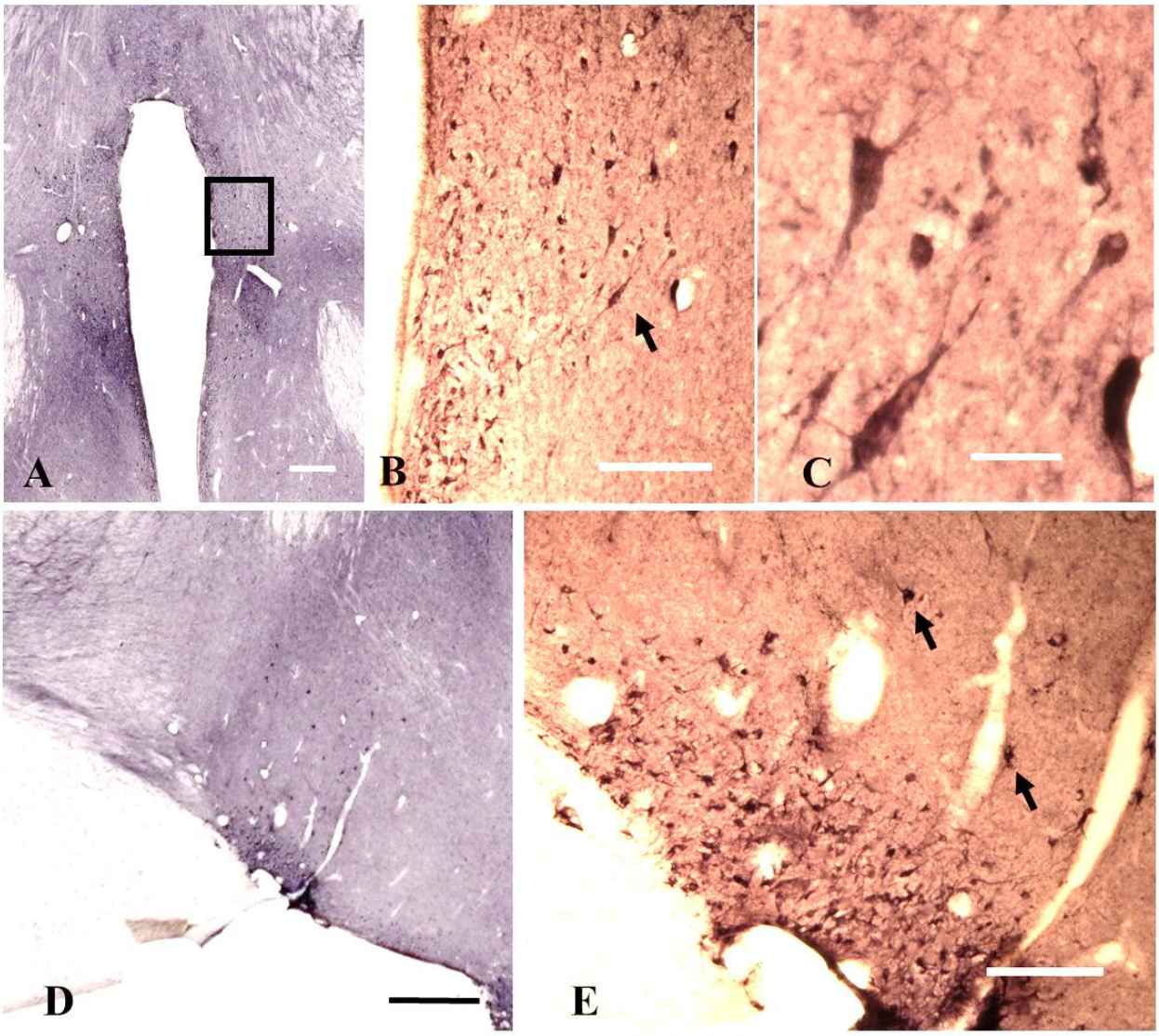

Figure 4.2. Immuno-reactivity to trkA receptor in the diencephalon of llamas. (A-C) Distribution of trkA positive cells in the periventricular area of llama brain at increasing magnifications. (B and C) Arrows show immuno-reactive neurons at high magnification. (D-E) Presence of trkA positive cells (arrows) in the supraoptic nucleus. Scale bars: A 1mm. B 150 µm. C 30 µm. D 2 mm. E 150 µm.

Table 4.1: Distribution of cells and cell fibres expressing GnRH and trkA immuno-reactivity (mean number ± SEM, and percentage of the total number) in the hypothalamus and preoptic area of llamas (n = 4 brains).

	GnRH			TrkA	
Area	Cells	%	Fibers	Cells	%
Diagonal band of Broca	1 ± 0.8*	3.1	8.8 ± 3.1	193 ± 17.5[a]	22.3
Medial septum	1 ± 0.3	1.2	2.5 ± 0.6	48 ± 15.5[b]	4.9
Lateral septum	0	0.0	0.0	0[c]	0
Medial preoptic	6 ± 3.7	14.8	14.3 ± 8.5	39 ± 15.6[b]	4.5
Lateral preoptic	3 ± 1.8	7.4	5.0 ± 2.4	101 ± 36.1[b]	11.7
Optic chiasma	0	0.0	0.0	0[c]	0.0
Suprachiasmatic nuclei	0	0.0	0.0	0[c]	0.0
Supraoptic nuclei	0*	0.0	0.0	56 ± 14.1[b]	6.5
Anterior hypothalamus	2 ± 0.6	4.3	6.5 ± 1.2	33 ± 17.4[b]	3.8
Lateral anterior hypothalamus	3 ± 0.4	7.4	4.8 ± 2.1	69 ± 28.8[b]	8.0
Periventricular hypothalamus	1 ± 0.3*	1.9	0.3 ± 0.3	174 ± 25.7[a]	20.2
Medio-basal hypothalamus	16 ± 3.2*	40.1	12.5 ± 3.0	52 ± 6.1[b]	6.0
Lateral hypothalamus	7 ± 2.2*	16.7	5.3 ± 1.8	47 ± 9.0[b]	5.4
Mammillary hypothalamus	0	0.0	0.0	36 ± 20.9[c]	4.1
Dorsal hypothalamus	0	0.0	0.0	0[c]	0.0
Retrochiasmatic area	1 ± 0.7	2.5	5.3 ± 2.8	12 ± 7.5[c]	1.4
Arcuate nucleus	0	0.0	8.3 ± 3.8	5 ± 4.3[c]	0.6
Total	40.5 ±7.3			2390 ± 131	

* Within rows, asterisk indicates differences between trkA and GnRH cells ($P < 0.05$)

[abc] Within columns, values with different superscripts are different ($P < 0.05$)

4.4.2. Morphological relationship between trkA and GnRH

The GnRH neuronal population was scarce in comparison to trkA immuno-positive cells (40.5 ± 7.3 vs 2390.2 ± 131 cells; $P<0.001$; table 4.1). Of the total number of cells in the hypothalamus and preoptic areas displaying immuno-reactivity to GnRH, 156/160 (99%) stained for GnRH alone and 4/160 (1%) stained for both GnRH and trkA. Of the number of cells in the hypothalamus and preoptic areas displaying immuno-reactivity to trkA, 9477/9481 (>99%) stained for trkA alone and 4/9481 (<1%) stained for both GnRH and trkA. Aside from the lack of co-localization, TrkA and GnRH neurons were not commonly visualized in the same anatomical plane, and on only three occasions appeared closely related (i.e. within the same microscopic field; Fig 4.3). In the few instances (three ocacions) where GnRH immuno-reactive fibers were found in close relationship to trkA immuno-reactive cells, there was no apparent contact (Fig 4.4).

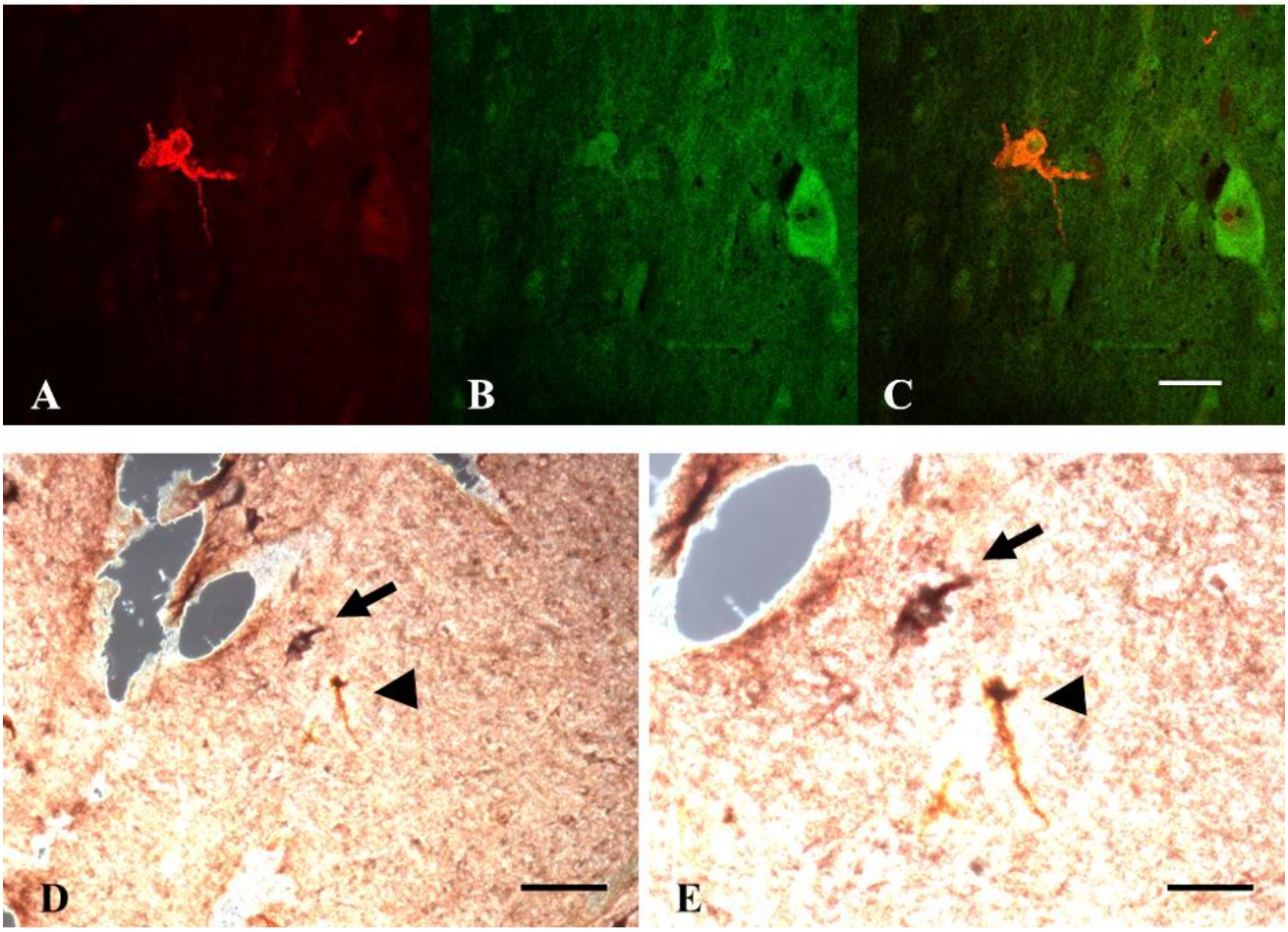

Figure 4.3. Immuno-reactivity to GnRH and trkA in llama hypothalamus, detected by double immunofluorescence (A-C) or double immunoperoxidase (D-E). (A-C) Top panel illustrate different cells displaying immuno-reactivity for GnRH (A; red), trkA (B; green) and both channels (C; merged) in a single microscopic field. (D-E) Darkfield micrographs showing immunoreactivity for GnRH (brown; arrow heads) and trkA (black; arrows) at different magnifications (D 10x; E 20x). Scale bars: A-C 30 µm. D: 120 µm. E: 60 µm.

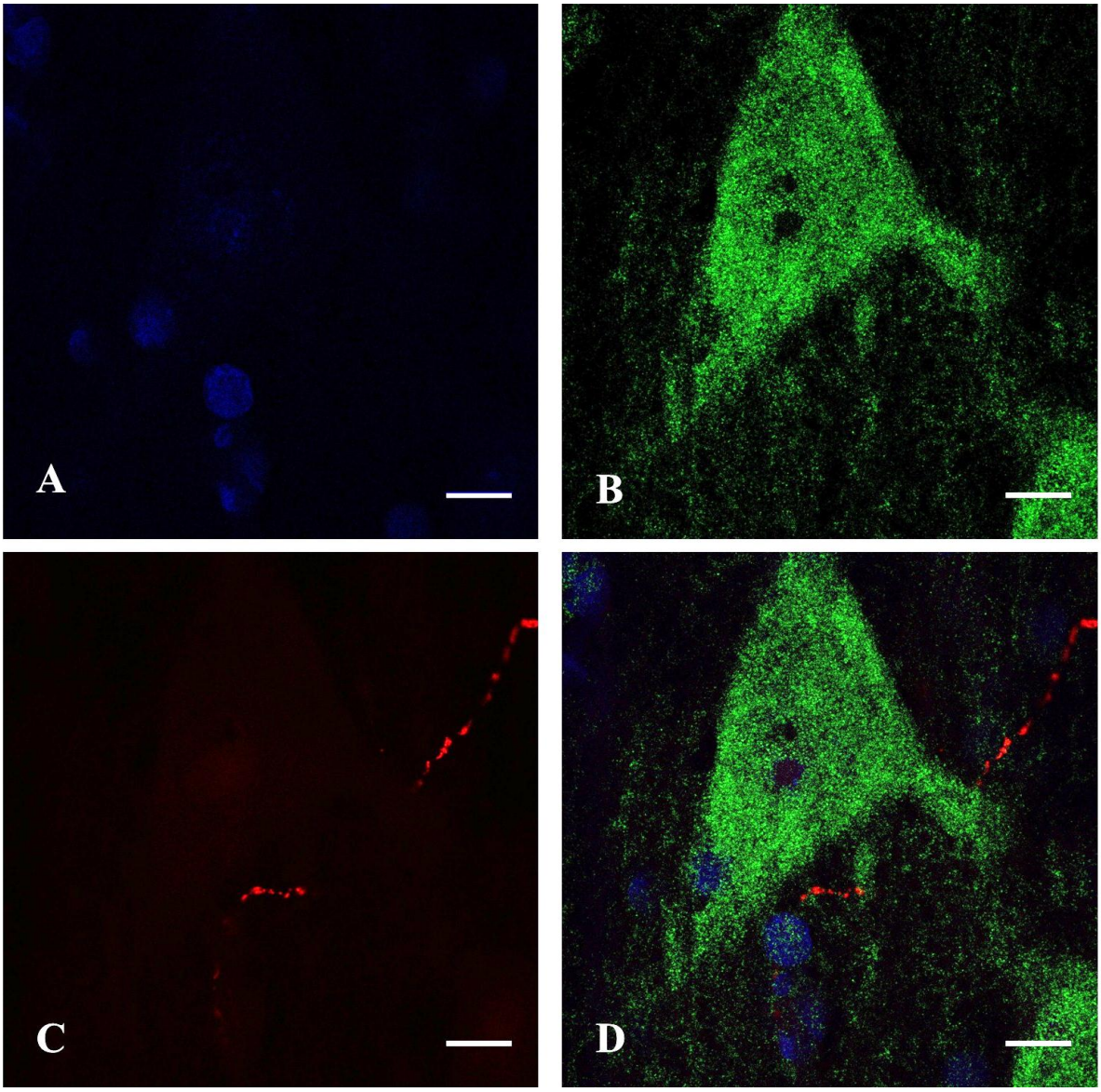

Figure 4.4: Relationship between trkA immuno-reactive cells (green) and GnRH fibres (Red) in the llama hypothalamus. Series of fluorescence micrographs of the same sample of the hypothalamus of a llama showing (A) cell nuclei (DAPI; blue), (B) immune-reaction against trkA cells (green), (C) immuno-reaction against GnRH fibers (red), (D) Merged channels. Scale bars 30 µm.

4.5. Discussion

Ovulation-inducing factor has been shown to induce ovulation in a high proportion of llamas and alpacas after parenteral administration. The effect is mediated via GnRH neurons (directly or indirectly) resulting in LH secretion from gonadotropes in the anterior pituitary (Silva et al., 2011). Results of the present study do not support the hypothesis that OIF/NGF effects a response through direct interaction with GnRH neurons in llamas, since the high affinity receptor for OIF/NGF was detected in ≤1% of GnRH neurons. It is unlikely that such a low proportion of GnRH neurons would drive the preovulatory GnRH and LH surge since, in mice at least, around 40% of GnRH neurons are activated during the LH surge, as measured by detection of the c-FOS proto-oncogene (an established marker of activation; Wu et al., 1992). Similar to the triggering factor for the LH surge in spontaneous ovulators (estradiol), that of induced ovulators like camelids (OIF/NGF) must involve an intermediate cell type to interact with GnRH neurons (e.g., kisspeptin cells, norepinephrine cells). Consequently, GnRH neurons act as a final output for a complex interplay between neurons (Herbison, 2005), and in llamas it appears that they do not interact with OIF/NGF via trkA receptor.

The role of NGF on reproductive tissues has been elucidated during the last 20 years. Early studies documented the presence and modulation of NGF content in the ovary after denervation in rodents (Lara et al, 1990). Further studies established that NGF and trkA increased their expression prior to first ovulation in the prepubertal mouse (Dissen et al., 1996). Despite these surprising findings, no evidence linking NGF to hypothalamic GnRH functions have been described. Studies have reported that other growth factors have an effect in the hypothalamic GnRH population. GnRH neuron secretion can be triggered by transforming growth factor alpha in the female rat hypothalamus in culture (Ojeda et al., 1990). Most notably, a role of glial cells on GnRH function has been shown in the mouse; this

effect appears to be mediated by an interaction of astrocytes, tanycytes and GnRH neurons at the median eminence (Prevot, 2002). Furthermore, intraventricular injection of NGF or NGF antibodies modified FSH levels in blood and breeding patterns in birds (Bentley et al., 1997). The finding suggests that disrupting or enhancing the NGF system at a central level affects reproduction.

Both the high molecular weight form (7s NGF) and the low molecular weight form (2.5s NGF) were detected in the central nervous system after intravenous administration of NGF in mice (Pan et al., 1998). There are two routes by which OIF/NGF can cross the blood-brain-barrier; 1) by interacting with the choroid plexus, or, 2) by crossing cerebral capillaries. If OIF/NGF crosses the blood-brain-barrier by interacting with the epithelium of the choroid plexus, the expression of a specific receptor may be required. In this regard, only one report has documented the expression of neurotropins and their receptors in the choroid plexus, showing undetectable levels of trkA mRNA in the rat choroid plexus in comparison to other neurotropin receptors (Timmursk et al., 1995). The low affinity NGF receptor has been described in primary culture of the rat choroid plexus (Spuch et al., 2011). Thus, it remains to be established if in South American camelids, OIF/NGF crosses the blood-brain-barrier.

Our study of the llama brain shows that trkA immuno-reactive cells were present in most areas of the hypothalamus, but were accumulated in two major areas: 1) the diagonal band of Broca; and, 2) the periventricular area. The large number of trkA immune-reactive cells in the periventricular area offers interesting insight for the OIF/NGF hypothesis; this area is in close contact with the third ventricle, suggesting that if OIF/NGF crosses the blood-brain-barrier and reaches the cerebrospinal fluid it may be available to interact with the trkA receptor in regions of the brain that influence GnRH neurons. Although, the ependymal epithelia lining the third ventricle is permeable to molecules, close to the

median eminence the ependymal cells are modified into tanycytes which regulate the passage of molecules between the cerebrospinal fluid and brain tissue (Rodriguez et al., 2010; Fanglet et al., 2013). Thus, it remains to be established if OIF/NGF is capable to diffuse within the brain tissue and interact with the trkA immune-reactive cells.

We concluded that the proportion of GnRH neurons that express trkA receptors in the llama hypothalamus is too low to support the hypothesis that OIF/NGF interacts directly with GnRH neurons to elicit ovulation. The neurochemical identity of the trkA immuno-reactive cells remains to be established.

Chapter 5. General Discussion

In three studies we examined aspects of the ovulatory and luteotrophic effect of OIF/NGF in cows and the anatomy of the GnRH system in llamas. The local and systemic aspects of the OIF/NGF theory reflect the different mechanisms by which OIF/NGF exerts an effect in induced and spontaneous ovulators. Studies designed to determine the effect of OIG/NGF on ovulation in cows have failed to show changes in luteinizing hormone concentrations in plasma after treatment with OIF/NGF. However, effects on follicular dynamics and CL development have been reported consistently (Tanco et al., 2012; Tribulo et al., 2015). In llamas, copulation and treatment with seminal plasma or purified OIF/NGF elicits an ovulatory response in a high proportion of females. These two examples illustrate the systemic or central effect (central nervous system in llamas), and the local or peripheral effect in different species (ovarian effect in cow or sheep).

The presence of trkA receptor in the follicular wall has been intensively studied in rodents (Lara et al., 1990; Dissen et al., 1996; Dissen et al., 2000; Dissen et al., 2001; Romero et al., 2002). In mouse, the expression of trkA receptor is restricted to the theca layer of preovulatory follicles in the hours prior to first ovulation (Dissen et al., 1996). The temporal interaction was not examined before in other species in relation to the estrous cycle. The results of the present study suggests that trkA receptor is expressed constantly either in the granulosa or theca layer of dominant follicles during the follicular wave in cattle. Perhaps, the difference in expression patterns of trkA may be related to a difference between mono-ovulatory and poly-ovulatory species (i.e. cow versus mice), suggesting that the differential pattern of expression in trkA or NGF may be the result of dramatic differences in ovarian and follicular dynamics.

Species differences may be reflected in whether or not OIF/NGF or endogenous NGF modifies ovarian function. It is well established that endogenous NGF is present in ovarian tissues (mouse and cow, Dissen et al., 2000; pig and cow Levanti et al., 2005; ground squirrel, Li et al., 2014) and follicular fluid (sheep, Barboni et al., 2002; human, Sadeu et al., 2012). Conversely, OIF/NGF can be detected in different sexual accessory glands in males of several species (Bogle, 2015). It is unclear if intrinsic factors in the female may favour the action of NGF of different origins (endogenous or seminal plasma). For example, an early report (Marion et al., 1950) showed that the time from the end of estrous to ovulation and the length of estrous are decreased when females are mated with a vasectomized bull. In addition, it was found that intrauterine treatment with seminal plasma tended to increase conception rates in dairy and beef cows (Odhiambo et al., 2009). Furthermore, bull semen contains OIF/NGF and is functional in the llama (Ratto et al., 2006) and cow (Tribulo et al., 2015). All these findings reflect the effects of OIF/NGF in the reproductive system of the cow. Perhaps the expression and secretion of endogenous NGF is repressed in the female, and OIF/NGF administration (or insemination) surpasses that regulatory system and induces effects otherwise not produced.

The mode of action of OIF/NGF during ovulation in the brain of llamas has not been examined to date, but presumably induces ovulation by influencing directly or indirectly GnRH neurons (Silva et al., 2011). If so, OIF/NGF would cross the blood-brain barrier and passage through the blood-brain barrier should happen in a rapid manner. In favor of this hypothesis there is evidence that β-NGF can cross the blood brain barrier in mice (Pan et al., 1998). Hypothetically, NGF can cross the blood brain barrier on two ways in llamas, through the choroid plexus or across cerebral capillaries. However, given the existence of tight junctions in cerebral capillaries and the blood brain barrier, is most likely that NGF reaches neural tissue via the choroid plexus. Studies have pointed the existence of leaky capillaries, expressing markers of fenestrations in the arcuate nucleus of rodents (Cioffi et al., 2009; Cioffi, 2011),

suggesting that this brain area may be exposed to the internal milieu. In order to reach the cerebrospinal fluid by the choroid plexus, one would expect the existence of receptors or transporters associated with NGF. However, to our knowledge this has not been reported in the literature (Timmusk et al., 1995), only the low affinity neurotropic receptor has been reported (Spuch et al., 2011). Injection of radiolabeled NGF into the lateral ventricle of rats labels the surrounding neural tissue (Ferguson et al., 1994), implying that OIF/NGF diffuses freely into cerebral tissue and neurons.

The cerebrospinal fluid at the level of the third ventricle offers a good opportunity for neurotransmission and signaling for three reasons. First, it provides a constant flow allowing downstream signaling to specific targets (Johansen et al., 2005). Second, the ventral aspect of the third ventricle displays low CSF flow, which may facilitate the diffusion of OIF into the adjacent areas. And third, other molecules such as leptin, t3, and prolactin have been shown to reach the CSF through the choroid plexus and exert effects at specific areas in the brain (Rodriguez et al., 2010). Our data shows that in llamas the anterior hypothalamus and the medio-basal hypothalamus display higher proportion of cells reactive to GnRH and trkA. These two areas are located surrounding the ventral aspect of the third ventricle, and given this location it is structurally feasible for OIF to reach GnRH neurons or other neurons that provide inputs to them.

If OIF/NGF does not cross the blood brain barrier in llamas, is possible that OIF interacts with some GnRH elements in circumventricular organs, such as the median eminence and organum vasculosum of the lamina terminalis. We found that the population of GnRH neurons around the organum vasculosum of the lamina terminalis is low, and there are no GnRH or trkA immuno-reactive cells in the median eminence besides GnRH immuno-positive fibers. However, recent evidence in mice has

shown that dendrites, the part of the neuron that is involved in reception of signals, are extremely long and branched in GnRH neurons, reaching up to 100 microns away from the cell body (Herde et al., 2011). In addition, these neuronal projections may be in contact with the interstitial fluid outside the blood-brain barrier, since tracers injected intraperitoneally can be colocalized in immuno-reactive GnRH neurons. Thus, GnRH neurons display specializations to sense endocrine changes in the bloodstream and in llamas this aspect must be clarified.

5.1. Conclusions.

The following conclusions were obtained from this work:

- TrkA receptor is expressed in dominant and subordinate follicles during the follicular wave. The degree of expression is higher in dominant compared to subordinate follicles;
- The corpus luteum of cattle expresses varying degrees of immuno-reactivity during formation and regression;
- The majority of GnRH cells in the brain of adult llamas are predominantly in the anterior and medio-basal hypothalamus;
- The majority of GnRH fibers are present in the median eminence and medio-basal hypothalamus;
- GnRH-like immuno-reactivity resembling fibers are detectable in the posterior pituitary;
- TrkA immuno-reactivity is detectable in the preoptic area and hypothalamus of llamas, principally in the diagonal band of Broca, periventricular nuclei and lateral preoptic area; and,
- A low proportion of GnRH neurons (about 1%) appeared immuno-reactive to trkA;

5.2. Future studies.

The complex arrangement of neural structures offers a challenge in understanding the biology of OIF/NGF. Given the wide array of neuropeptides that are involved in ovulation, we have to ask ourselves if molecules such as kisspeptin or norepinephrine mediate the ovulatory effect of OIF/NGF. Furthermore, are these neurons (kisspeptin, norepinephrine, or others) activated during ovulation? Our findings did not support the hypothesis that GnRH neurons express trkA receptor, however, it may be important to precise by which mechanism OIF/NGF crosses the blood brain barrier and which part of the brain OIF/NGF is capable of reaching. Since the onset of puberty is determined by the first ovulation, would treatment with OIF/NGF affect puberty in camelids? Or would sexual maturation be earlier in camelids? An additional question would be is the trkA/p75/NGF system any different in induced and spontaneous ovulators?

NGF has been implicated in a variety of ovarian processes. It seems that NGF is more than a molecule maintaining the innervation of organs and based on our results it may be involved in folliculogenesis, would a local source of OIF/NGF in the ovary enhance follicular or luteal development? Is OIF/NGF or any neural component involved in follicular dominance? In this regard, it has been suggested that the ovaries of ruminants have a higher density of adrenergic innervation than other species. Is it possible that the classical endocrine regulation of ovarian function may be modulated or complemented by neural imputs?

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