DISSERTATION

HYPOTHALAMIC CONCENTRATION OF KISSPEPTIN AND GnRH DURING BREEDING SEASON (BS) AND NON BREEDING SEASON (NBS) IN SHEEP

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ABSTRACT

HYPOTHALAMIC CONCENTRATION OF KISSPEPTIN AND GnRH DURING BREEDING SEASON (BS) AND NON BREEDING SEASON (NBS) IN SHEEP

The kisspeptin system has emerged as an important regulator of mammalian reproduction. In ewes, kisspeptin neurons are located in specific hypothalamic regions such as the preoptic area (POA) and medio-basal hypothalamus (MBH) which include the arcuate nucleus (ARC). A specific radioimmunoassay (RIA) for the quantification of hypothalamic kisspeptin was developed to test the hypothesis that estradiol decreases the production of kisspeptin during the NBS in the MBH in addition to other forebrain areas that harbor kisspeptin neurons and/or axons such as the POA, the anterior hypothalamic area (AHA), and the median eminence (ME). The kisspeptin RIA results indicated that the concentrations of kisspeptin per milligram of tissue were decreased during NBS in the MBH and the POA with a tendency for lower kisspeptin concentrations observed in the AHA. Likewise, the total content of kisspeptin was decreased in the MBH and POA during the NBS, with a similar tendency for lower content of kisspeptin observed in the AHA during the NBS. Supporting the notion that kisspeptin modulates secretion of GnRH at the level of the ME, a positive correlation between these neuropeptides was observed during the BS in this region. It may be, that kisspeptin neurons are relevant for the seasonal regulation of GnRH and LH secretion exerted by estradiol, since the GnRH neurons do not express estrogen receptor alpha (ERα) which is the relevant ER subtype for the regulation of the hypothalamic pituitary gonadal axis (GnRH/LH pulsatility). We investigated if the negative feedback exerted by estradiol during the NBS, promoting a decrease in the concentrations of kisspeptin in the ARC, can be blocked by the

intracerebral ventricular (ICV) administration of an estradiol antagonist (ICI) to promote an increase in LH pulsatility. As expected, in ewes that received the ICI treatment an increase in the average number of LH pulses was observed. The increased frequency in LH pulsatility was probably a consequence of eliminating estradiol inhibitory actions over ARC kisspeptin neurons which send axonal projections to the ME and promote the release of GnRH, and thus LH. Interestingly, the ME is a circumventricular organ (CVO) located outside of the blood brain barrier (BBB). Ovariectomized ewes were immunized against kisspeptin and antiserum to kisspeptin generated. The antibodies to kisspeptin were intended to eliminate kisspeptin release from the ME and consequently block kisspeptin input to GnRH axon terminals. The blockade of kisspeptin input to GnRH axon terminals was intended to inhibit the release of GnRH and hence LH. When compared to controls, ewes immunized against kisspeptin tended to have lower average and basal secretion of LH. The lack of significant decrease observed in immunized ewes suggests that higher titers to kisspeptin could be needed to fully suppress GnRH and hence LH pulsatility. Still, the tendency for lower levels of LH observed in immunized ewes suggest that kisspeptin release from the ME is relevant for the modulation of the pulsatile secretion of GnRH and thus LH. Likewise, delayed onset of the preovulatory like surge of LH in immunized ewes suggests a partial inhibition of the massive release of kisspeptin/GnRH was obtained during the start of the surge. However the possibility that the preovulatory surge of kisspeptin/GnRH is also regulated inside the BBB or that kisspeptin independent or indirect mechanisms play an important role in the generation of the GnRH/LH surge in ewes cannot be ruled out.

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INTRODUCTION

Expression of the *Kiss1* gene is highly modulated in the hypothalamus (Kitahashi and Parhar, 2013) with some metabolic hormones such as leptin (Backholer et al., 2010), insulin like growth factor 1 (IGF-1) (Hiney et al., 2010) and adiponectin (Wen et al., 2012) regulating its expression. Reports in mice show that estradiol controls *Kiss1* gene expression through an estrogen responsive element (ERE) independent pathway in the ARC while in the antero ventrally located periventricular nucleus (AVPV) it is regulated through an ERE dependent or "classical" pathway (Gottsch et al., 2009). Additionally, estradiol promotes acetylation of the *Kiss1* promoter region in the AVPV and its deacetylation in the ARC (Tomikawa et al., 2012).

At present, it is known that the production of kisspeptin is highly modulated in different hypothalamic regions in rodents, these regions include the ARC and the AVPV (Clarkson and Herbison, 2006). In ovine, kisspeptin is highly expressed in the ARC and POA where the POA is the homologous region to the AVPV found in rodents (Franceschini et al., 2006).

During the non-breeding season (NBS) in ewes, the hypersensitivity to the negative feedback effects of estradiol is translated by a reduction in the production of kisspeptin at the level of the ARC which is located in the MBH (Smith et al., 2008). It is not clear whether the hypersensitivity to the negative feedback of estradiol affects the production of kisspeptin in other hypothalamic regions like the AHA, POA or ME. Due to the relevant role that kisspeptin might play in GnRH production and release, we decided to develop a specific RIA for the quantification of kisspeptin in different hypothalamic regions that harbor a considerable number of kisspeptin/GnRH neurons and/or kisspeptin/GnRH axons. We hypothesized that during NBS in addition to the MBH, estradiol promotes a reduction in the concentrations and content of kisspeptin in hypothalamic regions including the AHA, POA and ME.

Additionally, it was investigated if the blockade of estradiol by the intra-cerebral administration of an estrogen antagonist (ICI182 780) can promote an increase LH secretion during the late NBS. Finally the blockade of kisspeptin outside of the brain blood barrier, and its effects on LH pulsatility and preovulatory like surge of LH were investigated by inducing the production of antisera to kisspeptin thus blocking kisspeptin release from the ME. The objective of the last experiment was to eliminate the input of kisspeptin at the GnRH axon terminal via blockade of kisspeptin axonal terminals present at the level of the ME.

CHAPTER 1: LITERATURE REVIEW

The kisspeptin system

The discovery of the kisspeptin system occurred in the field of cancer biology in 1996 when it was found that the expression of *Kiss1* mRNA was linked to melanoma cells that had low metastatic activity (Lee et al., 1996). The K*iss1* gene is expressed in several tissues that include the placenta, brain, kidney, pancreas, lung, liver, small intestine, testicles, ovaries and colon (Ohtaki et al., 2001). The highest levels of *Kiss1 a*re detected in the placenta (Muir et al., 2001), but its expression is found to be wide spread, suggesting that the kisspeptin system is involved in multiple physiological processes. Some of these physiological conditions include the regulation of brain sexual differentiation in rats (Kauffman et al., 2007) and sheep (Cheng et al., 2010).

Kisspeptin is involved in pubertal development in humans (Teles et al., 2008), and mice (Seminara et al., 2003) as well as in implantation/placentation during pregnancy (Bilban et al., 2004; Hiden et al., 2007). The neuropeptide kisspeptin is involved in thermoregulation (Csabafi et al., 2013) and prolactin release (Szawka et al., 2010). The kisspeptin system serves as a connection between the nutritional status and reproductive activity/fertility (Castellano et al., 2005), and it is also implicated in glucose homeostasis (Hauge-Evans et al., 2006). Kisspeptin possess anti-oxidant effects (Aydin et al., 2010) as well as having anti-metastatic properties (Mitchell et al., 2006a).

In humans, the *Kiss1* gene is located in chromosome one (West et al., 1998) and is comprised of two coding exons placed next to a non-coding exon (d'Anglemont et al., 2010). The gene is close to the "*Golta"* (Golgi transport 1 homolog A) gene and according to d'Anglemont et al. (2010), the kisspeptin promoter (p1) contains a TATA box for transcription start as well as numerous potential sites for the binding of transcription factors like specificity protein 1 (SP1).

SP1 facilitates the binding of activator protein 2α (AP-2α) promoting the formation of the AP-2α /SP1 complex (Mitchell et al., 2006b). The activity of SP1 is also regulated by a coactivator protein called DRiP-130 that stimulates the basal expression of the kisspeptin gene through the DRiP-130/Sp1 complex (Mitchell et al., 2007).

In vivo studies in rodents have shown that silencers of the polycomb group (PcG) repress *Kiss1* gene expression with two PcGs genes, Eed and Cbx7, play an important role in *Kiss1* gene expression. For instance, puberty onset was accompanied by methylation of *Eed and Cbx7* promoters allowing an indirect increase in *Kiss1* expression to take place. The increase in *Kiss1* gene expression resulted from loss of Eed from the *Kiss1* promoter and an increase in histone H3 modifications (Lomniczi et al., 2013).

There are multiple physiological conditions where expression of the *Kiss1* gene is modulated. For instance, during lactation *Kiss1* transcription is reduced, probably as a consequence of the high levels of prolactin and neuropeptide Y that can exert a negative impact over the kisspeptin system (Sonigo et al., 2012; Yamada et al., 2007). Other hormones like melanocortins (Backholer et al., 2009) and melatonin also modulate the levels of *Kiss1 mRNA* in the hypothalamus, particularly in seasonally breeding species like Syrian Hamsters (Ansel et al., 2010), Siberian Hamsters (Mason et al., 2007) and sheep (Smith et al., 2007).

Likewise, an important regulation of *Kiss1* gene expression is promoted by gonadal steroids, i.e. progesterone, testosterone and estradiol (Smith et al., 2007). For instance estradiol regulates *Kiss1* gene expression through two SP1 binding sites located in close proximity to the transcriptional regulator site of the *Kiss1* gene where estradiol promotes the formation of the Sp1-ERα complex and facilitates the transcription of *Kiss1* gene (Li et al., 2007).

The synthesis of kisspeptin protein/peptide

In humans, transcription and translation of the *Kiss1* gene gives rise to a kisspeptin preprohormone that is 145 amino acids long. This preprohormone contains a 19 aminoacid secretion signal (Nash and Welch, 2006). The preprohormone is cleaved at amino acid number 67 and 124. It is cleaved one more time at amino acid 121, and it is further amidated, resulting in a 54 amino acid polypeptide (kisspeptin-54). Kisspetpin-54 has the capacity to bind to the kisspeptin receptor, a G protein coupled receptor, GPR54 (Muir et al., 2001). Kisspeptin-54 is further cleaved to produce smaller sequences that retain biological activity and have an identical C-terminal region (Kotani et al., 2001). The processing of the preprohormone to kisspeptin peptide is thought to be carried out by a family of protein convertase enzymes and according to Harihar et al. (2014), furin is the major convertase implicated in the production of the shortest kisspeptin isoform (kisspeptin-10). Kisspeptin-10 has an amino acid structure that has a high percent of homology between different species studied including human, macaque, sheep, mouse, frog and zebrafish (d'Anglemont de Tassigny et al., 2010; Topaloglu et al., 2012).

In contrast to humans (kisspeptin-54), in rats and mice the longest isoform with biological activity is 52 amino acids in length (Terao et al., 2004; Desroziers et al., 2010) and in sheep it is 53 amino acids long (Caraty, 2012). There are several isoforms of the hormone present in the hypothalamus of ewes and their prevalence has been estimated as follows: kisspeptin-53 = 15%, kisspeptin-16 = 42%, kisspeptin-14 = 8%, kisspeptin-13 = 33%, kisspeptin-10 = 2% (Caraty, 2012). There are no studies comparing the bioactivity of all the kisspeptin isoforms found in the brain (Franceschini et al., 2013), but it is suggested that kisspeptin 52 is more potent than kisspeptin 10 in terms of LH secretion in rats (Tovar et al., 2006). However, studies in humans

have found no difference in terms of the LH stimulatory effects between kisspeptin 54 and kisspeptin 10 (Jayasena et al., 2015).

Hypothalamic expression pattern of *Kiss1* and *GPR54*

The expression pattern of *Kiss1 mRNA* in the hypothalamus was first described in mouse using in situ hybridization (ISH) with *Kiss1* being highly expressed in the AVPV, ARC, the antero-dorsal POA, medial amygdala and the bed nucleus of the stria terminalis (Gottsch et al., 2004). A similar pattern in the expression of *Kiss1* mRNA has been detected in the hypothalamus of other rodent species like rats (Adachi et al., 2007) and hamsters (Greives et al., 2007). In sheep, *Kiss1* mRNA is highly expressed in the medio basal hypothalamus (MBH) and ARC, with a pattern similar to that reported in the ARC of rodents (Smith et al., 2007; Smith et al., 2008). The POA is another area where the *Kiss1* gene is highly expressed in sheep (Smith et al., 2007) and this area is the analogous region to the AVPV in rodents (Gottsch et al., 2004). Likewise, humans and non-human primates show high levels of *Kiss1* in the ARC and POA (Rometo et al., 2007; Ramaswamy et al., 2008). With regard to the reproductive relevance of kisspeptin, it has been noted that the hypothalamic expression of *Kiss1* is highly localized in the AVPV (Han et al., 2005) where most GnRH neurons also reside in rodents.

Relative to the hypothalamic expression of *GPR54* mRNA, it has been reported in whole hypothalamic fractions (Roa et al., 2006), being predominantly expressed in the AVPV/POA where most GnRH neurons reside in rodents (Herbison et al., 2010), in addition to the ARC and ME where GnRH fibers are abundant (Kinoshita et al., 2005; Yamada et al., 2007). In sheep, *GPR54* is highly expressed in the POA but its expression has also been reported in the ARC (Smith et al., 2011).

The effects of kisspeptin on reproduction

In 2003, researchers discovered that the kisspeptin system had a dramatic impact over the reproductive physiology of humans (de Roux et al., 2003) by characterizing a type of hypogonadotropic hypogonadism that was associated with a mutation of the *GPR54* gene (Seminara et al., 2003). Interestingly, the same phenotype that was detected in humans has been replicated in animals that have been engineered to lack a functional *GPR54* gene (Funes et al., 2003; Seminara et al., 2003). In the *GPR54* knock out models described by Seminara et al., 2003, it was found that migration of the GnRH neurons as well as synthesis of GnRH was maintained. In addition, the responsiveness to GnRH stimulation in terms of LH secretion was maintained suggesting that the limiting factor was the upstream regulation of GnRH release (Messager, 2005). It was confirmed that kisspeptin stimulated the release of GnRH in mice (Messager et al., 2005) and sheep (Smith et al., 2011) and further evidence supporting the relevance of kisspeptin as a GnRH secretagogue came from the study of d'Anglemont de Tassigny et al. (2008) who reported that *GPR54* knock out animals were not able to secrete GnRH after kisspeptin administration.

There are multiple studies supporting the notion that kisspeptin is an important modulator of the release of GnRH, and hence LH. For instance, it has been reported that the administration of either a kisspeptin antagonist (Pineda et al., 2010; Roseweir et al., 2009) or kisspeptin to GnRH antagonist pretreated rats (Irwig et al., 2004) eliminates the secretion of LH (suggesting that the kisspeptin/GnRH axis needs to be functional for LH secretion to take place). The direct action of kisspeptin on GnRH neurons was further supported by studies in which an increase in the expression of c-Fos was detected in GnRH neurons after the administration of kisspeptin (Matsui et al., 2004; Irwig et al., 2004) as well as by studies demonstrating depolarization of GnRH neurons after kisspeptin administration (Pielecka-Fortuna et al., 2008). Currently, it is known that by

stimulating GnRH secretion, kisspeptin indirectly promotes the release of LH/FSH in rats (Kinsey-Jones et al., 2008), mice (Gottsch et al., 2004), monkeys (Keen et al., 2008), humans (George, Anderson and Millar., 2012) and sheep (Caraty et al., 2013) as suggested by the intracerebroventricular or systemic kisspeptin administration which were able to stimulate LH release (Navarro et al., 2004; Navarro et al., 2005; Arreguin-Arevalo et al., 2007).

Additionally, it seems that kisspeptin has the capacity to cross the brain blood barrier when administered peripherally (intra vascular) (Irwig et al., 2004; Matsui et al., 2004) and/or to act outside of the brain blood barrier at the level of the median eminence (ME) (Smith et al., 2011; Caraty et al., 2013).

Kisspeptin production during the BS and NBS in ewes

The reproductive physiology of mammals is highly modulated by the hypothalamic expression of different genes (Lincoln et al., 2002), and in turn some of these hypothalamic genes can regulate the production of several other hormones like melatonin, whose production/secretion is inhibited by light (Lincoln et al., 2003). Furthermore, melatonin mediates the effects for differential production of kisspeptin during the BS and NBS (Simonneaux et al., 2009). It has been shown that melatonin (Boyce and Kennaway, 1987) and estradiol (Karsch et al., 1993) are important for the regulation of seasonal reproductive physiology. For example, in sheep different conditions of light/dark exposure that are present during the BS (Fall and Winter) or during the NBS (Spring and Summer) differentially regulate the synthesis of melatonin at the level of the pineal gland. During the BS melatonin synthesis occurs for longer periods since the night time is longer during Fall/Winter when compared to the Spring/Summer during the NBS (Alila Johansson et al., 2001).

As previously mentioned, melatonin mediates the effects of photoperiod over the reproductive physiology (Goldman, 2001); the longer periods of melatonin synthesis that take place during the BS matches the augmentation of the number of pulses of GnRH/LH that occur per day (Barrell et al., 1992; Goodman et al., 1982). In contrast, when compared to the BS, synthesis of melatonin during the NBS occurs for shorter periods of time and this is accompanied by a reduction in the number of pulses of GnRH that occur every 24 hours (Barrell et al., 1992), and accordingly a decreased LH pulsatility is detected during the NBS in the serum of ewes (Legan et al., 1977). The change in frequency of the pulsatile secretion of GnRH/LH between the BS and NBS is a consequence of a shift in the sensitivity to the negative feedback effects of estradiol (Goodman et al., 1982; Karsch et al., 1993; Robinson et al., 1985). The sensitivity to the negative feedback effects of estradiol is high (hypersensitivity) during the NBS and low (reduced sensitivity) during the BS.

Relative to the participation of kisspeptin during the NBS and BS, it has been noted that the onset of the BS in ewes is linked to an increment in the levels of *Kiss1* mRNA (Wagner et al., 2008) as well as an increase in the kisspeptin protein/peptide expression that takes place at the level of the ARC (Smith, Pereira and Clarke, 2009). The increment in kisspeptin production has been linked to a higher kisspeptin input to GnRH neurons (Clarke et al., 2009) that can result in a higher frequency in the secretion of GnRH, and consequently LH pulses (Karsch et al., 1993).

Seasonal and steroidal conditions that affect kisspeptin production in the ARC of sheep

To some extent, there is an effect on the production of kisspeptin at the level of the hypothalamus that is dependent on seasonal changes in melatonin production, but independent on estradiol input. This idea is based on evidence provided by studies in sheep where the input of

estradiol has been removed from the system by ovariectomy. In this type of experimental model (ovariectomized ewes), an increase in the levels of *Kiss1* mRNA takes place in the ARC during the BS compared to the NBS (Smith et al., 2007). Even though a reduction in the levels of *Kiss1* mRNA in the ARC of ovariectomized sheep seems to take place during the NBS when compared to the BS, the latter phenomenon does not seems to impact the number of pulses of GnRH and LH in a dramatic fashion since the LH pulsatility is not affected between seasons in this model (Legan et al., 1977; Karsch et al., 1980).

On the other hand, when estradiol is present (in the model of ovariectomized ewes implanted with estradiol), it dramatically decreases the number of GnRH/LH pulses during the NBS when compared to the BS (Karsch et al., 1993), and this decrease seems to be directly related to a reduction in the levels of *Kiss1* mRNA detected by ISH in the ARC nucleus (Smith et al., 2007). Estradiol, besides affecting the levels of *Kiss1* mRNA in the ARC detected by ISH, also reduces the number of neurons that express kisspeptin protein in this hypothalamic nucleus, as evidenced by IHC (Smith et al., 2008). There is no data related with quantitative changes in hypothalamic *Kiss1* gene expression obtained by PCR analysis during the BS or NBS in ewes.

It is likely that the marked change in kisspeptin neuron number, which occurs under different seasonal conditions and under the influence of estradiol, reflects to some extent a differential input of the kisspeptin hormone over the GnRH system as directly demonstrated by Smith et al. (2008). In this study, it was shown that the percent of kisspeptin fibers that make contact with GnRH neurons decreased during the NBS when compared to the BS in ewes. Therefore, it can be speculated that by modulating the hypothalamic production of kisspeptin, estradiol can control kisspeptin/GnRH release, increasing it during the BS and decreasing it during the NBS (Chalivoix et al., 2010; Barrel et al., 1992).

The effect of season and estradiol on the hypothalamic production of kisspeptin in the ARC and POA

A better understanding about the effects of season and estradiol on the hypothalamic modulation of kisspeptin production in the ARC and POA of ewes, both during the BS and NBS, was obtained by the use of IHC (kisspeptin protein) and ISH (*Kiss1* mRNA) (Smith et al., 2008). It is known that estradiol dramatically impacts the production of kisspeptin in the ARC and POA of sheep allowing the modulation of the GnRH system by kisspeptin during the BS and NBS (Smith et al., 2008). In this case, the general consensus is that during the NBS kisspeptin production is decreased while during the BS it is increased at the level of the ARC (in the model of the ovariectomized ewes implanted with estradiol).

Contrary to the regulation of kisspeptin production exerted by estradiol at the level of the ARC, in the POA the synthesis of kisspeptin does not seem to be as greatly affected by season, but rather to be positively correlated with the input of estradiol. The previous assumption about the regulation of kisspeptin production by estradiol comes from studies in ewes, where estradiol withdrawal reduces *Kiss1*mRNA (grains per neuron - ISH) and kisspeptin protein (based on number of kisspeptin neurons - IHC) in the POA while estradiol treatment increases *Kiss1* mRNA and kisspeptin protein irrespective of the season (Smith et al., 2008). This phenomenon has led to the speculation that in general, estradiol only exerts a positive regulation over the production of *Kiss1* mRNA/kisspeptin protein at the level of the POA (Smith et al., 2007; Smith et al., 2008). There is a lack of information related with the quantification of hypothalamic kisspeptin under different physiological processes and further studies are needed in sheep to properly quantify small changes in kisspeptin production during the BS and NBS, not only at the level of the ARC and POA, but also in other hypothalamic regions such as the AHA and ME.

Activation of estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), and the hypothalamic expression of the *Kiss1* gene

As mentioned before, it is well known that estradiol treatment inhibits expression of the *Kiss1* gene in the ARC equally in female and male mice (Smith et al., 2005a; Smith et al., 2005b) with a similar decrease in *Kiss1* gene expression taking place in the ARC of sheep under the influence of estradiol during the NBS (Smith et al., 2008). Again, contrary to the negative regulation of the *Kiss1* gene exerted by estradiol in the ARC, in the AVPV of female mice (Smith et al., 2005a) and POA of sheep, estradiol promotes an increase in the levels of *Kiss1* mRNA (Smith et al., 2008). The negative and positive modulation of hypothalamic *Kiss1* expression exerted by estradiol in the ARC and AVPV respectively, might occur through the activation of ERα (Smith et al., 2005a) since most kisspeptin neurons in these hypothalamic regions express ERα in mice (Cravo et al., 2011) and rats (Smith et al., 2006).

The hypothesis that ERα plays an important role in the modulation of kisspeptin production ewes is supported by the fact that a high percent of kisspeptin neurons in both the POA and ARC express ERα in sheep (Franceschini et al., 2006). Further supporting the notion that ERα is important in the regulation of *Kiss1* gene expression comes from studies in ERα knock out animals. Smith et al., (2005a) demonstrated that ERα knock out mice were unable to modulate the expression of *Kiss1* gene under the influence of estradiol. In these genetically modified, ovariectomized mice, estradiol replacement had no effect on *Kiss1* gene expression, while in ovariectomized wild type animals estradiol replacement reduced *Kiss1* gene expression. Extra evidence for the relevance of ERα activation on *Kiss1* expression comes from studies in rats, in which only the agonist of ERα (PPT), but not the agonist of ERβ (DPN), suppressed *Kiss1* mRNA levels in the hypothalamus (Navarro et al., 2004).

On the other hand, ERβ does not dramatically impact the expression of the *Kiss1* gene in the hypothalamus of either female or male rodents, even though a certain percent of kisspeptin neurons located in the ARC and AVPV coexpress ERβ in this species (Smith et al., 2005a; Smith et al., 2005b). For instance, in ERβ knockout mice, ovariectomy increases *Kiss1* mRNA expression, whereas estradiol replacement decreases its expression, as normally occurs in wild type animals, suggesting that the negative feedback effects of estradiol remains intact in ERβ knockout mice and occurs through ERα activation (Smith et al., 2005a).

Transcriptional regulation of the *Kiss1* gene through estrogen response element (ERE) dependent and ERE independent pathways

Although kisspeptin neurons located in the ARC and AVPV/POA express nuclear ERα, each area seems to predominantly activate one specific estradiol signaling pathway that ultimately allows a differential regulation of kisspeptin production. For instance, the negative regulation of kisspeptin production exerted by estradiol in the ARC of rodents occurs through activation of an ERE independent pathway (Gottsch et al., 2009). Estradiol promotes histone deacetylation of the *Kiss1* promoter (Tomikawa et al., 2012) negatively impacting the expression of *Kiss1*, and therefore kisspeptin production in the ARC. It has been observed, that estradiol can down regulate the expression of the *Kiss1* gene by inhibiting the binding of RNA polymerase II to the proximal promoter, and by modulating the activity of the RNA polymerase present in the gene (Huijbregts & de Roux., 2010).

Contrary to the negative regulation of kisspeptin production exerted by estradiol in the ARC, in the AVPV estradiol promotes *Kiss1* and Dynorphin (Dyn) gene expression through an ERE dependent pathway (Gottsch et al., 2009). The up regulation of the *Kiss1* gene promoted by

estradiol is thought to occur by activation of Sp1 sites through the interaction of ERα and Sp1 protein complexes to guanine-cytosine rich motifs (Li et al., 2007).

In the AVPV of mouse, the *Kiss1* promoter is acetylated and the ERα is recruited at this level under the conditions of positive feedback exerted by estradiol during the generation of the surge of GnRH/LH (Tomikawa et al., 2012). The exact pathway by which estradiol regulates *Kiss1* gene expression remains unknown. However studies in cell lines done by Safe and Kim (2008) have led to the suggestion that regulation of the *Kiss1* gene expression occurs in a similar fashion to the regulation of the *VEGFR2* gene which seems to require the interaction of ERα and Sp1.

Moreover, the epigenetic regulation of the *Kiss1* gene exerted by estradiol in the AVPV has also been reported in mice. It was observed that histones located upstream to the *Kiss1* gene in neurons of the AVPV were highly acetylated, and that estradiol altered the acetylation status resulting in up regulation of *Kiss1* by promoting formation of a chromatin loop (Tomikawa et al., 2012).

Kisspeptin stimulation of GnRH release and desensitization/inactivation of GPR54

In studies using hypothalamic explants, kisspeptin administration promotes GnRH release (Messager et al., 2005; Seminara et al., 2006; Nazian, 2006; d'Anglemont de Tassigny et al., 2008; Choe et al., 2013). In this regard, kisspeptin can depolarize and excite most GnRH neurons (75 to 90% of them) coinciding with the high level of GPR54 expression found in these neurons (Han et al., 2005; Liu et al., 2008; Zhang et al., 2008). Kisspeptin activates GPR54 by stimulating the alpha subunit of the trimeric G proteins found in the cytoplasmatic compartment (Stafford et al., 2002); the hormone binds to its receptor promoting guanosine diphosphate (GDP) release from the Gα protein which is normally bound to the β and γ dimer. The GDP release promotes the binding of

the α subunit to guanosine triphosphate (GTP) and promotes its separation from the β and γ dimer. Then the GTP that is bound to the α subunit separates from the receptor promoting the enzymatic activity of phospholipase c beta (PLCβ). This in turn causes the hydrolysis of inositol 4,5-bis phosphate (PIP2) resulting in the production of diacyl-glycerol (DAG) as well as the production of inositol 1,4,5-tri phosphate (IP3). DAG activates the Ca^{++} dependent protein kinase C (PKC), leading to the activation of ERK 1/2 (extracellular regulated kinase 1/2) as well as p-38-MAPK (p38-mitogen activated protein kinase) (Muir et al., 2001; Ohtaki et al., 2001; Kotani et al., 2001).

The blockade of either PLC or 1,4,5-tri phosphate receptor (IP3R) are effective in reducing the percent of GnRH neurons that respond to kisspeptin stimulation (Liu et al., 2008) since the binding of IP3 to its receptor (IP3R) promotes the release of calcium (Ca^{++}) from the endoplasmic reticulum (ER) into the cytoplasm and allows an increase in Ca^{++} leading to the secretion of GnRH. In addition to Ca^{++} mobilization, secretion of GnRH induced by kisspeptin seems to require ERK and p-38-MAPK activation (Castellano et al., 2006).

Similar to other GPCRs, the continuous stimulation of GPR54 leads to its desensitization and inactivation. Desensitization of GPR54 by continuous kisspeptin input has been reported by d'Anglemont de Tassigny et al. (2008). They observed that MBH explants continuously treated with kisspeptin resulted in a sustained release of GnRH for four hours followed by a decrease in GnRH secretion despite continuous kisspeptin administration. A similar pattern of desensitization was reported in juvenile rhesus monkeys by Seminara et al., (2006) who observed an initial rise in LH secretion followed by a dramatic drop in LH despite continuous intravascular administration of kisspeptin.

It seems that GPR54 undergoes internalization by a process that require the binding of arrestin to the phosphorylated receptor thus preventing any further receptor signaling (Ferguson,

2001). Another important mediator of GPR54 desensitization and internalization seems to be phosphorylation of G protein receptor kinase-2 (GRK2). Over expression of GRK2 inhibits G protein signaling after kisspeptin activation of the receptor while over expression of a catalytically inactive GRK2 mutant (k220R-GRK2) results in an increment in the signaling of GPR54 after kisspeptin stimulation (Pampillo et al., 2009). Interestingly, Bianco et al., (2011) observed that after its internalization, GPR54 could either be degraded or recycled back to the plasma membrane with most internalized receptor being recycled back to the plasma membrane.

The kisspeptin regulation of GnRH synthesis

In male rats, subcutaneous kisspeptin administration induces expression of c-Fos in GnRH neurons located in the POA, as well as in GnRH neurons located in the MBH (Matsui et al., 2004). More recent studies related to the synthesis and secretion of GnRH have used hypothalamic organotypic slice culture (Choe et al., 2013), or transgenic animals (Novaira et al., 2012). In these studies (Novaira et al., 2012; Choe et al., 2013), it was demonstrated that kisspeptin administration induced GnRH secretion, as well as *GnRH* mRNA synthesis. It was suggested that stimulation of *GnRH* mRNA synthesis, occurred through the phosphorylation of ERK1/2 and PI3K (Novaira et al., 2009). Additionally, it was shown that kisspeptin stimulated synthesis *OTX-2* mRNA and the binding of OTX to a *Kiss1* response element (KsRE) located within -3446 and -2806 base pairs on the mouse *GnRH* gene (Novaira et al., 2012).

GnRH neuron depolarization by kisspeptin

As previously mentioned, brain slice preparations in which the kisspeptin/GPR54 pathway is activated results in initiation of a PLC-IP3K-calcium cascade allowing the modulation of both

potassium (K+) and non-selective cation channels (NSCC), and the initialization of depolarization of GnRH neurons (Liu et al., 2008). For example, blockade of either the NSCC or K+ channels in GnRH neurons reduces the GnRH neuronal response to kisspeptin (Liu et al., 2008).

Kisspeptin can also promote depolarization of GnRH neurons by a mechanism that involves presynaptic kisspeptin stimulation of GABA-A and Glutamate input while promoting a direct inhibition of inwardly rectifying K + currents (Kir 6.2 and GIRK) (Liu et al., 2008; Pielecka-Fortuna et al., 2008; Pielecka-Fortuna et al., 2011). Inhibition of K+ channels is thought to be important since they are found in GnRH neurons (K_{ATP} and GIRK channels) and hold GnRH neurons in a hyperpolarized state (Zhang et al., 2007; Zhang et al., 2009). Additionally, activation of non-selective transient receptor potential channels (TRPC) may be part of the mechanism by which kisspeptin depolarizes GnRH neurons and promotes GnRH neuronal firing augmenting GnRH secretion under conditions of positive feedback of estradiol (Zhang et al., 2013b; Ronnekleiv and Kelly, 2013).

Kisspeptin induces GnRH neuron depolarization by binding to GPR54, activating the Gαq-phospholipase C β (PLC) and diacylglycerol (DAG) pathway. This leads to activation of the TRPC allowing entrance of Na and Ca^{++} into the neuron (Zhang et al., 2008; Liu et al., 2008; Constantin et al., 2009; Kroll et al., 2011). TRPC are highly expressed in the brain of mammals (Venkatachalam and Montell, 2007) and kisspeptin activates TRPC by depletion of PIP2 and activation of proto-oncogene tyrosine-protein kinase Src (c-Src) resulting in the excitation of GnRH neurons (Zhang et al., 2013b).

Hypothalamic regulation of the *GPR54* gene expression

As previously mentioned the hypothalamic expression of *GPR54* mRNA has been reported in whole hypothalamic fractions (Roa et al., 2006), but it is predominantly expressed in the AVPV/POA of rodents (Herbison et al., 2010), as well as in the ARC and ME where GnRH fibers are abundant (Kinoshita et al., 2005; Yamada et al., 2007). In sheep, *GPR54* mRNA is abundant in the POA and it is also expressed in the ARC (Smith et al., 2011).

Studies in rats have determined that *GPR54* mRNA expression reaches its highest levels at puberty, both in female and male rats (Navarro et al., 2004). In mice approximately 40% of GnRH neurons express *GPR54* at birth and as animals approach puberty that expression is increased to 70% (Herbison et al., 2010). Likewise, studies in primates have reported an increase in *GpR54* in the ARC of intact female monkey as it approaches puberty (Shahab et al., 2005).

On the other hand, in rats the hypothalamic expression of *GPR54* mRNA is not affected by the administration of either estradiol and/or progesterone when are compared controls (Roa et al., 2006). Also, no difference in *GPR54* mRNA expression has been detected across the estrous cycle in sheep (Smith et al., 2011). Consequently, it can be inferred that the hypothalamic expression of *GpR54* mRNA is not changed across the estrous cycle in species like sheep and rodents (Roa et al., 2006; Smith et al., 2011). Whether the lack of change in hypothalamic *GpR54* mRNA across the estrous cycle detected by Smith and colleagues (2011) could reflect a lack of change in GpR54 receptor (protein) is not known. Future studies using the radio-receptor assay approach could provide an insight into the possible changes in the number of GpR54 receptors.

Relative to possible changes in *GPR54* mRNA during anestrous conditions, studies in rats during lactational anestrus demonstrated that the expression of *GPR54* was reduced at the level of the AVPV when compared to non-lactating controls (Yamada et al., 2007).

Another factor involved in the regulation of hypothalamic *GPR54* mRNA expression is the receptor's ligand itself (kisspeptin). For example, during the NBS in gonadal intact ewes there was a rise in *GPR54* mRNA expression compared to gonadal intact animals during the BS (Li et al., 2012). Besides, the administration of kisspeptin during the NBS also results in a decrease in *GPR54* mRNA expression compared to NBS control animals (Li et al., 2012). From these findings it has been suggested that during the BS the expression of *GPR54* mRNA was reduced due to a higher kisspeptin input into GnRH neurons reflecting a negative modulation of the *GPR54* gene by kisspeptin (Li et al., 2012). Whether the higher kisspeptin input during the BS compared to NBS promotes a down regulation of hypothalamic *GPR54* gene expression that could be associated with higher internalization rates of GPR54 receptor (protein) is a phenomenon that requires investigation.

The GnRH and kisspeptin hypothalamic system in ewes

In ewes, most GnRH neurons reside in the medial POA, close to the organum vasculosum of the lamina terminalis and in close association with the anterior commissure (Caldani et al., 1988). These GnRH neurons are spread rostrally into the diagonal band of Brocca and are also present ventro-laterally in the anterior hypothalamus as well as in the lateral hypothalamus, with only a small percent (1-15%) being found in the ARC or its vicinity (Caldani et al., 1988; Lehman et al., 1986).

Rostral (POA) GnRH neurons send projections to the tuberoinfundibular sulcus of the median eminence through two pathways in sheep. The major route seems to be the ventrolateral pathway that runs above the optic tract in the AHA. The other route, the lateral hypothalamic pathway, is a less prominent and runs lateral to the third ventricle (Lehman et al., 1986).

For the kisspeptin neuronal system to regulate GnRH synthesis and release, it needs to be in close contact with the neurons and axonal terminals of GnRH. IHC studies in sheep have suggested that some kisspeptin axonal projections have their origin in the medial POA, run dorsally and caudally relative to the optic chiasm and in a periventricular pathway reaching the ARC and ME where a large number of kisspeptin axons terminals are found. Likewise, kisspeptin neurons are also present in the ARC and send projections to the external medial eminence (Franceschini et al., 2006).

Kisspeptin immunoreactivity (IHC) has been reported in the same hypothalamic regions where a large number of GnRH neurons/axons have been detected (POA, AHA and the ARC), supporting the perception of an important interplay or contact between kisspeptin neurons and GnRH neurons, (Lehman et al., 1986; Caldani et al., 1988; Smith et al., 2008). The origin of the kisspeptin axonal projections that make close apposition with GnRH neurons in the POA seems to be the kisspeptin neurons present within the POA itself, with no substantial input from the kisspeptin neurons of the ARC (Backholer et al., 2009).

The indirect regulation of the GnRH system by estradiol through kisspeptin neurons in the ARC has been implied since most kisspeptin neurons of the ARC express ERα (Franceschini et al., 2006). It has been inferred that kisspeptin neurons with their origin in the ARC send axonal projections toward the ME where they control GnRH release through kisspeptin axonal - GnRH axonal communication. This assumption comes from in vitro studies where the administration of kisspeptin resulted in the release of GnRH from the GnRH axon terminals of the mediobasal hypothalamus (MBH) (d'Anglemont de Tassigny et al., 2008) and in isolated ovine ME containing GnRH axon terminals (Smith et al., 2011).

Presynaptic stimulation of GnRH neurons by kisspeptin and the hyperpolarizing effects of estradiol

Zhang et al. (2009) observed that kisspeptin counters the negative effect exerted by estradiol which activates GIRK and promotes GnRH neuronal hyperpolarization through the presynaptic activation of $GABA_B$ receptor (Lagrange et al., 1995). It has been reported that kisspeptin counters the hyperpolarizing effects of Ba2+ sensitive inwardly rectifying K channels (Zhang et al., 2008). Zhang and Spergel, (2012) reported that kisspeptin can inhibit high voltage-activated (HVA) Ca2+ (e.g., L-type) channels promoting further depolarization of GnRH neurons, and hence induction of GnRH secretion. The increase in Ca2+ that promoted the secretion of GnRH also leads to the inactivation of voltage gated calcium channels (VGCC) through a Ca2+ - calmodulin (CaM) pathway (Zhang and Spergel, 2012).

On the other hand, the T-type calcium channels (Zhang et al., 2009) and hyperpolarized-activated, cyclic nucleotide-gated channels (HCN) are highly expressed in GnRH neurons (Bosch et al., 2013), and it has been proposed that the activation of these channels is dependent on membrane hyperpolarization (Kelly and Wagner, 2002).

Estradiol hyperpolarizes GnRH neurons and recruits calcium channels (T-type and HCN) which are important for setting a phasic burst firing rate in GnRH neurons (Zhang et al., 2013a). As previously mentioned, kisspeptin probably attenuates the hyperpolarization states of GnRH neurons by inhibiting IRK channels and activating TRPC to cause depolarization and firing (Pielecka-Fortuna et al., 2008; Liu et al., 2008; Zhang et al., 2008; Pielecka-Fortuna et al., 2011). Additionally, Lee et al. (2010) observed that calcium activates small conductance, calcium-activated K+, SK+ after-hyperpolarization currents that can serve to repolarize the plasma

membrane of GnRH neurons and allow for an oscillation in the patterns of burst firing in GnRH neurons.

Most information available in the literature related with seasonal changes in the kisspeptin system involves detection of *Kiss1* mRNA (ISH) or kisspeptin neuronal immunoreactivity (IHC) in the POA and MBH (ARC) only (Smith et al., 2007; Smith et al., 2008). Kisspeptin protein has not been quantified in the hypothalamus of sheep and it is currently unknown whether changes in concentrations and content of kisspeptin take place in hypothalamic regions other than the ARC like the POA, AHA and ME. In the first study, kisspeptin and GnRH were quantified in different hypothalamic regions important for the estrogenic modulation of kisspeptin production.

Estradiol exerts an increased inhibitory feedback during the NBS and decreases kisspeptin neuronal immunoreactivity in the hypothalamic ARC, which suggest that the content of kisspeptin might be lower during the NBS when compared to the BS (Smith et al., 2007; Smith et al., 2008; Chalivoix et al., 2010). The presumed decrease in kisspeptin content that takes place during the NBS might be translated in a decrease in the kisspeptin input over the GnRH system and thus in a reduction in GnRH/LH pulsatility (Barrel et al., 1992; Karsch et al., 1993). In the second study, due to the increased inhibitory effects that estradiol exerts in the hypothalamus during the NBS, the intracerebroventricular administration of an estrogen antagonist (ICI) and its effects in LH secretion was investigated. ICI administration was intended to promote an increase LH pulsatility.

In the third study, due to the presence of kisspeptin axonal terminals in the ME which presumably stimulates GnRH secretion from GnRH axonal terminals present outside of the brain blood barrier (BBB); the blockade of the kisspeptin system was investigated by the induction of antiserum to kisspeptin. Kisspeptin antiserum was intended to block kisspeptin axon terminals in the ME, decrease the kisspeptin input to GnRH axon terminals in the ME, and thus block the

pulsatile secretion and preovulatory like surge of GnRH. Finally, by blocking GnRH secretion a reduction in LH release was expected.

CHAPTER 2. HYPOTHALAMIC CONCENTRATION OF KISSPEPTIN AND GnRH DURING BREEDING SEASON (BS) AND NON BREEDING SEASON (NBS) IN SHEEP.

Summary:

Our understanding of the influence of estradiol and season on the expression of kisspeptin protein in the hypothalamus is based mainly on immunohistochemical (IHC) studies. Under the influence of low circulating levels of estradiol, the number of neurons staining positive for kisspeptin is reduced in ewes during the NBS compared to ewes during BS. This change in kisspeptin has been consistently reported in the arcuate nucleus (ARC) located in the medial basal hypothalamus (MBH); however, the influence of estradiol and season on the expression of kisspeptin in other hypothalamic areas is less clear. To date quantitative methods to measure kisspeptin in biological samples have not been available. To this end, a radioimmunoassay (RIA) for kisspeptin was developed. The specificity of kisspeptin antiserum was confirmed by examining cross-reactivity to several hypothalamic peptides and by the absence of immunostaining in hypothalamic sections after pre-absorption of the antiserum with kisspeptin. Further, the hypothalamic staining pattern observed with our kisspeptin antiserum matched that obtained with ovine kisspeptin antiserum generated by Dr. Alain Caraty. We hypothesized that the use of more sensitive method like the RIA would reveal other hypothalamic regions as potential targets of the seasonal negative feedback effects of estradiol on kisspeptin protein expression. Ovariectomized (OVX) ewes, carrying a subcutaneous implant of estradiol, were euthanized during the BS (n = 4) and the NBS (n = 3). Coronal sections of the preoptic area (POA), anterior hypothalamic area (AHA), and MBH were collected, as well as the median eminence (ME), cortex, brain stem and cerebellum. Kisspeptin concentrations were lower during NBS compared to BS in the MBH (4.5

± 1.2 vs 12.9 ± 1.3 pg/mg tissue, $p < 0.01$); similarly, the content of kisspeptin was also reduced in this region during the NBS (695.3 ± 189.6 vs 1803 ± 150.4 pg, $p< 0.01$). Concentrations and content of kisspeptin were less during the NBS in the POA (3.9 ± 0.8 vs 7.3 ± 0.7 pg/mg tissue, $p < 0.05$) and (252 ± 16.7 vs 543 ± 39.28 pg, $p < 0.01$) respectively. Likewise, there was a tendency for a lower concentrations of kisspeptin in the AHA during NBS compared to BS (5.5 ± 0.4 vs 13.2 ± 3.2 pg/mg tissue, $p = 0.1$) and a strong tendency for a reduced content of kisspeptin was detected during the NBS (463.3 ± 27.8 pg vs 1034 ± 195 pg, $p = 0.059$). No difference was detected in the concentrations or content of kisspeptin between seasons in the ME, but a positive correlation between kisspeptin and GnRH was observed during the BS in this region ($r= 0.97$ $p = 0.05$). On the other hand, concentrations and content of GnRH were similar in the POA, AHA, MBH and ME between seasons. Although kisspeptin was detected in tissue adjacent to the POA and the MBH, concentrations of this neuropeptide were lower than in the POA and MBH and were not affected by season. Similarly, low concentrations of kisspeptin were detected in the cortex, brain stem and cerebellum, and did not differ between seasons. The function of kisspeptin in these extra hypothalamic tissues is yet to be determined. By using a sensitive and quantitative method, as the RIA, we found that in addition to the MBH, the POA and AHA appear to be involved in the seasonal negative feedback effect of estradiol on kisspeptin expression.

Introduction

GnRH neurons do not express estrogen receptor α (ERα) (Herbison & Theodosis, 1992), the receptor of physiological relevance for the control of the hypothalamic pituitary gonadal axis (Glidewell-Kenney et al., 2007; Roa et al., 2008). Therefore, an intermediary is needed for estradiol to modulate the hypothalamic pituitary axis and a good candidate are the kisspeptin

neurons which do express ERα (Franceschini et al., 2006). Additionally kisspeptin production is highly modulated by estradiol (Smith et al., 2005; Smith et al., 2008).

By regulating production of kisspeptin, estradiol modulates the input of kisspeptin to the GnRH neuronal system since most GnRH neurons express the kisspeptin receptor *Gpr54* (Smith et al., 2011; Li et al., 2012). Additionally, production of *GnRH* mRNA (Novaira et al., 2009) and secretion of GnRH (d'Anglemont de Tassigny et al., 2008) are highly modulated by kisspeptin.

Regulation of kisspeptin by estradiol has been studied in several animal models including sheep (Estrada et al., 2006; Smith et al., 2007a). During the NBS estradiol exerts an enhanced inhibitory effect over the production of kisspeptin at the level of the ARC, which is located in the MBH, by reducing the amount of *Kiss1* mRNA detectable by in situ hybridization (ISH) as well as decreasing the number of kisspeptin neurons detected by IHC (Smith et al., 2007b; Smith et al., 2008). It is not known to what extent estradiol regulates the content of kisspeptin in other hypothalamic areas.

The POA is a hypothalamic region that also possesses a considerable number of kisspeptin neurons (Franceschini et al., 2006) but the role of this region in seasonal reproduction is less clear. For instance, Smith et al., (2008) reported no change between the BS and NBS in the number of kisspeptin neurons detected by IHC in the POA, but Chalivoix et al. (2010) detected a higher number of kisspeptin neurons in the POA during the BS when compared to the NBS.

This lack of consistency in kisspeptin neuron number observed at the level of the POA could be due to the use of semi-quantitative methodologies like IHC that are not sensitive enough to detect small changes in the concentrations of kisspeptin. Similarly, since only a few sections are usually analyzed per hypothalamic region when using IHC (from 3 to 6 per region) (Smith et al., 2007; Smith et al., 2008; Smith et al., 2009; Smith et al., 2011; Merkley et al., 2012), a more subtle

change in kisspeptin content could be overlooked due to the limited number of sections that are analyzed.

Besides the POA, other hypothalamic regions like the AHA might be relevant for regulation of GnRH by kisspeptin in ewes. Smith et al. (2008) reported that the number of contacts of kisspeptin neurons by GnRH neurons at this hypothalamic level is increased during the BS compared to the NBS. Most studies examining changes in kisspeptin production across seasons have relied on changes in *Kiss1* mRNA measured by ISH or the number of kisspeptin neurons determined by IHC (Smith et al., 2007b; Smith et al., 2008; Chalivoix et al., 2010), but neither the content nor the concentration of kisspeptin in various hypothalamic areas have been quantified.

The aim of this study was to determine if the increased sensitivity to the negative feedback effects of estradiol that occur during the NBS in ewes (Legan et al., 1977) promotes a decrease in the hypothalamic concentration of kisspeptin and/or GnRH in different hypothalamic regions. It was hypothesized that in addition to the decrease in kisspeptin expression induced by estradiol at the level of the MBH (ARC) during the NBS, estradiol would also decrease amounts of kisspeptin in other hypothalamic areas like the POA, AHA, and ME.

Material and methods

All experiments were approved by the Colorado State University Animal Care and Use Committee and complied with the guidelines of the National Institutes of Health (NIH).

Animals (rabbits)

Antisera against kisspeptin were produced, to enhance their antigenicity a modified ovine (NH_2-CYNWNSFGLRY-Amide) or a modified equine kisspeptin (NH_2-CYRWNSFGLRY-

Amide) were conjugated to maleimide activated bovine serum albumin (BSA) as described by Walker et al. (2006). Conjugation efficiency was estimated to be about 80 and 90%; therefore, 25 to 30 molecules of kisspeptin were attached to each BSA molecule. Three rabbits each were immunized intradermally with 1.0 mg of either ovine or equine kisspeptin conjugate in complete Freund's adjuvant and boosted at monthly intervals with 0.5 mg of conjugate in incomplete adjuvant. Until acceptable titers were obtained, serum was collected every two weeks and checked for the binding to radiolabeled ^{125}I – ovine kisspeptin 10. In order to develop a highly sensitive RIA for the quantification of kisspeptin two different hormones (equine kisspeptin and ovine kisspeptin) were used as antigens and the sensitivity of the two antisera were compared by RIA.

Animals (sheep)

Mature western-range ewes were housed under natural light at Colorado State University (Animal Reproduction and Biotechnology Laboratory). All ewes were ovariectomized and estradiol implants were inserted into the axillary region for at least four weeks before hypothalamic tissue was collected (Karsch and Foster., 1975; Goodman et al., 1982). Estradiol implants were intended to maintain physiological levels of estradiol (3-5 pg/mL) to simulate the levels found during the luteal phase of the estrous cycle. SILASTIC brand tubing (inner and outer diameter of 3.35 and 4.65 mm, Dow Corning Corp., Midland, MI) was packed with 3 cm crystalline estradiol 17-β (Sigma Chemical Co., St Louis, MO) and soaked overnight in 0.1M phosphate buffered saline (PBS) prior to placement to avoid an initial post insertion peak of estradiol. Experiments were carried out during the NBS (March to August) and BS (September to January).

Immunohistochemical detection of kisspeptin and GnRH in the ovine hypothalamus

To test the specificity of our equine kisspeptin antiserum and compare it with the immunoreactivity patterns of the antiserum against ovine kisspeptin (kindly provided by Dr. Alain Caraty), three sheep were euthanized during the NBS with an overdose of sodium pentobarbital. Heads were perfused bilaterally via the carotid artery with 6 liters of 4% paraformaldehyde in phosphate buffer (PB 0.1M) (pH 7.4), heparin (10U/ml), and 15% picric acid (Goodman et al., 2007). The skull was opened, the brain removed and the hypothalamus was dissected. The segment removed was bounded anteriorly by the anterior portion of the optic chiasm (Diagonal band of Brocca), posteriorly by the mammillary body and laterally by the hypothalamic fissures (Glass et al., 1986). Hypothalami were post-fixed for an additional 24 hours at 4° C with 4% paraformaldehyde in 0.1M PBS (pH 7.4). Hypothalami were immersed in 0.1M PBS (pH 7.4) with 20% sucrose until the tissue sunk (Smith et al., 2009). Coronal sections (40um) extending rostral from the mediobasal POA to the caudal ARC were cut using a cryostat. Sections were then placed in a cryoprotectant solution containing 30% ethylene glycol and 20% glycerol in 0.1M PBS, and stored at -20 ° C until immunostaining (Watson et al., 1986).

Floating sections were washed overnight with 0.1M PBS buffer (pH 7.4) to remove cryoprotectant, and incubated in nanopure water containing 3% H_2O_2 for 1 hour to quench endogenous peroxidase activity. Hypothalamic sections were then placed in blocking buffer [0.1M phosphate buffered saline, 5% normal goat serum (NGS) and 0.1% Triton X-100] for 60 minutes. Sections were incubated for 72 hours with primary antibody previously diluted in 0.1% Triton-X and 5% NGS at a pH 7.4, 1:20,000 anti-GnRH, 1:20,000 anti-equine kisspeptin or 1:40,000 anti-ovine kisspeptin (kindly provided by Dr. Alain Caraty). Sections were incubated with a 1:500 biotinylated goat anti-rabbit globulin (2^{nd} antibody) for 1 hour. Tissue was incubated with a 1:400

Avidin-Biotin complex (ABC) for 60 minutes and then for 30 seconds with diaminobenzidine (DAB Vector) to produce a brown color. Floating sections were mounted with 0.1 M PB buffer (pH 7.4) into positively charged microscope slides (Superfrost-Thermo Scientific Portsmouth, New Hampshire). Mounted sections were washed in nanopure water and then counterstained for 20 minutes in 0.1% cresyl violet. Tissue sections were dehydrated through 70, 95 and 100% ethanol (20 dips per ethanol concentration) followed by two incubations (5 minutes each) with 100% ethanol. Sections were cleared in xylene for 10 minutes and air dried at room temperature. Hypothalamic sections were mounted with permanent mounting medium (VectaMount-Vector Burlingame, CA) and covered with micro cover glass (VWR). Covered slides were warmed at 70°C for 20 minutes and then at 50°C four one hour to eliminate bubbles. Slides were left at room temperature for 24 hours before visualization of kisspeptin or GnRH neuron and/or axon fiber terminals under brightfield microscopy (Nikon Eclipse E800). The intensity of kisspeptin neuron/fiber immunostaining was subjectively classified as low (1) medium (3) and high (5) and compared between anti-equine and anti-ovine kisspeptin.

Sections representing the ARC were used for examining the specificity of the immunostaining. Antisera (1:20,000, anti-equine kisspeptin; 1:40,000, anti-ovine kisspeptin, Dr. Caraty) or 1:20,000 anti-GnRH (previously validated for IHC by Glass et al., 1986) were preincubated for 24 hours at 4^{o} C with a solution of 0.1% Tris buffered saline (TBS) containing 1 ug/mL of kisspeptin or GnRH. Sections were incubated for 72 hours with the preadsorbed antiserum and processed for the immunodetection of kisspeptin or GnRH following the ABC-DAB protocol as described above.

Preparation of radioiodinated ovine kisspeptin-10.

The Chloramine T radioiodination method (Greenwood et al., 1963) was used to incorporate oxidized iodine into tyrosine in the ovine kisspeptin-10. Two point five ug of ovine kisspeptin-10 were diluted in 50 uL of 0.5 M phosphate buffer (pH 7.5) and 1.5 millicuries of Na-^{125}I added. The reaction was started by adding 7.5 uL of chloramine T (15 ug) for 30 seconds at room temperature. The reaction was then stopped with 39 uL of sodium metabisulfite (62.5 ug). One hundred uL of 0.002 M ammonium acetate (AA) buffer were added to the mixture and the solution loaded into the separation column. A commercial CM Sepharose Fast Flow matrix with a weak cation exchanger (GE Healthcare, Uppsala, Sweden) was set to pH 4.6 by washing it with 4 liters of a 0.002 M AA buffer (pH of 4.6). A serological glass pipet (10 mL VWR international West Chester, PA. USA) was loaded with ten centimeters of the CM Sepharose matrix. The ovine ^{125}I-kisspeptin-10 was separated from the radioiodination mixture by using buffers with different pH (modified from Arimura et al., 1973).

Briefly, three fractions (one mL) were first collected using a 0.002 M ammonium acetate (AA) buffer (pH = 4.6). After the third fraction a 0.4M AA solution (pH = 4.6) was used to collect sixteen fractions. From fractions 20-29, 10% acetonitrile was added to a 0.4M AA solution (pH = 4.85). From fraction 30-39, 20% acetonitrile was added to the 0.4M AA solution (pH = 5.0). From fraction 40-48, 30% acetonitrile was added to the 0.4M AA buffer (pH = 5.19). From fraction 49-64. 40% acetonitrile was added to the 0.4M AA buffer (pH = 5.27). Finally, from fraction 65-69, 50% acetonitrile was added to the 0.4M AA buffer (pH = 5.77). Two independent CM sepharose columns were used to determine where radiolabeled ^{125}I-kisspeptin-10 was obtained relative to free^{125}I and to non-radiolabeled kisspeptin-10. The first column was loaded with the radioiodinated mixture (free ^{125}I, non-radiolabeled kisspeptin-10 and^{125}I-kisspeptin-10); the second column was

loaded with 5 ug non radioiodinated ovine kisspeptin-10 only (control) and the kisspeptin in each fraction was quantified by RIA. Fractions from column one and column two were compared and fractions containing free ^{125}I, non-radiolabeled kisspeptin-10 and radiolabeled ^{125}I-kisspeptin-10 determined.

Validation of the kisspeptin RIA

The sensitivity of the homologous RIA systems (ovine kisspeptin standard with anti-ovine kisspeptin; equine kisspeptin standard with anti-equine kisspeptin) and heterologous RIA systems (ovine kisspeptin standard with anti-equine kisspeptin; equine kisspeptin standard with anti-ovine kisspeptin) were compared (Figure 2.9, Supplementary data). Due to greater sensitivity of the anti-equine kisspeptin to immuno-detect ovine kisspeptin, this antiserum was used for the rest of this study (supplemental material, figure 2.11). To determine if the quantity of tissue affected extraction efficiency, different amounts of brain cortex were spiked with 250 pg of ovine kisspeptin. The linearity of the kisspeptin RIA was analyzed by spiking 4 ng of kisspeptin into neuronal tissue (cortex), extracting the spiked samples and assaying different volumes (50, 100 and 200 uL). The precision of the kisspeptin RIA was 20.38 % for high values (n=10, 1114.6 ± 233.7 pgs), 16.7 % for medium values (n=13, 216.8 ± 36.2 pgs) and 24.2 % for low values (n=12, 73.9 ± 17.9 pgs). The precision for quantification of GnRH was 9.9 % for high values (n=7, 83.1 ± 8.3 pgs), 7.4 % for medium values (n=7, 25.1 ± 1.9) and 13.1 % for low values (n=7, 8.1 ± 1.1 pgs). The specific immunodetection of kisspeptin by the anti-equine kisspeptin was further established by examining the ability of different kisspeptin analogs to inhibit the binding of ^{125}I-ovine kisspeptin-10. Competitive inhibition curves used to corroborate specificity of the anti-kisspeptin included hypothalamic hormones like oxytocin, GnRH, Gonadotropin inhibitory

hormone related peptide (GnIH-RP), neurokinin B, ovine kisspeptin-10 (okp) and ovine kisspeptin-16 (okp-16). The content of kisspeptin detected by RIA in the POA, AHA, MBH, ME, cortex and cerebellum were compared using the anti-equine kisspeptin and the anti-ovine kisspeptin obtained by Dr. Alain Caraty (Table 1). The specificity of the RIA for GnRH was reported previously (Nett et al., 1973).

Tissue collection

RIA. To quantify amounts of kisspeptin and GnRH in the hypothalamus by RIA, ovariectomized ewes were implanted with estradiol and processed during the NBS (n=3) and the BS (n=4). Sheep were euthanized with an overdose of sodium pentobarbital and hypothalami were collected in 40% glycerol in 0.1 M PBS at pH 7.4 and kept at -20° C for the subsequent extraction of kisspeptin and GnRH. Coronal sections averaging 1.27 ± 0.14 mm were obtained starting 1 section prior to the optic chiasm (Diagonal band of Brocca) and continued until the mammillary body just posterior to the caudal ARC. Gross hypothalamic sections were first cut and kept on ice in 0.1M PBS (pH 7.4). The hypothalamic section was further cut to limit the POA, AHA, MBH or ME and the obtained region was kept on ice at all times in tubes containing a 3mL of acidified methanol. The right and left region of the POA, AHA, MBH and ME were processed separately to confirm the uniformity in selection of each sample. The samples comprising the preoptic area (POA) were limited dorsally by the anterior commissure (AC) and ventrally by optic chiasm (OC). The POA was selected due to the large number of kisspeptin and GnRH neurons present in this area in sheep (Franceschini et al., 2006; Lehman et al., 1986). The AHA was selected for the study of the kisspeptin system, due to the presence of kisspeptin and GnRH axons (Smith et al., 2008;

Lehman et al., 1986). Samples of the anterior hypothalamic area (AHA) were limited dorsally by the fornix (FX) and ventrally by the optic tract (OT).

Samples representative of the medial basal hypothalamus (MBH) contained the arcuate nucleus (ARC) and were limited dorsolateral by the presence of the mammillothalamic tract (MT) and ventromedial by the presence of the fornix. Due to the presence of kisspeptin neurons and axons present in MBH region in ewes (Franceschini et al., 2006), the rostral, middle and caudal regions of the ARC as well as the lateral wall of the third ventricle were included in samples designated as the MBH. Regions adjacent to the POA (POA-R) and MBH (MBH-R) were processed due to the low levels of kisspeptin and GnRH expected in this areas.

Extraction of kisspeptin and GnRH from neuronal tissue

Neuronal tissue was homogenized in acidified methanol using 3 bursts of 10 seconds each in a Polytron. Homogenates were centrifuged at 3000 *g* for 15 minutes at -15° C to pellet white fat. The supernatant was incubated at -80 ° C for 4 hours and centrifuged as above. This step was repeated three times to precipitate remaining fat. The supernatant was dried in a heating block under nitrogen flow. Samples were reconstituted in 2 mL of PBS-gel (0.1%), vortexed and left at 4° C overnight. Samples in PBS-gel were vortexed for 2 minutes, centrifuged at 2000 *g* for 30 minutes at 4° C and supernatants stored at -20° C.

Hypothalamic quantification of kisspeptin and GnRH by RIA

Standard curves with 11 points that ranged from 13 pg to 20 ng/mL (kisspeptin) and standard curves with 8 points ranging from 5.59 pg to 200 pg (GnRH) were included for the kisspeptin and GnRH RIA assays, respectively. All standards and samples were assayed in

triplicate (Kisspeptin) and duplicate (GnRH). Standards/samples were incubated with primary antibody (anti-equine kisspeptin 1:100,000 or anti-GnRH 1:80,000) for 24 h at 4º C. Twenty-four hours after addition of primary antisera, 100uL of ^{125}I-kisspeptin or ^{125}I-GnRH were added for 24 hours. Twenty-four hours after the addition of ^{125}I-kisspeptin or ^{125}I-GnRH, a second antibody (goat anti-rabbit gamma globulin) was added and incubated at 4º C for a period of 72 hours. The hormone-antibody complexes were precipitated by adding 3 mL of cold PBS per tube and centrifuging at 2500 rpm for 25 minutes. Supernatants were decanted and ^{125}I in the precipitates was quantified in a gamma spectrometer (Adapted from Nett et al., 1973).

Statistical analysis

Concentrations of kisspeptin or GnRH between the BS and NBS (Mean ± SEM) were compared using a t-test (SAS: SAS User's Guide SC. NC: Statistical Analysis System Institute, Inc.; 1987). A paired t test was used to determine if the concentrations of kisspeptin or GnRH were similar between the POA or MBH and their adjacent zones (POA-R and MBH-R), or to determine if the concentrations of kisspeptin or GnRH in the POA or MBH were different from the concentrations of kisspeptin in the brain stem, cortex and cerebellum. A regression analysis between kisspeptin and GnRH was performed within each hypothalamic region (POA, AHA, MBH and ME). Data are presented as mean ± standard error of the mean, and statistical differences with p values equal to or less than 0.05 were considered significant.

Results

Hypothalamic sections representative of the POA are presented in Figures 2.1.A, sections of the AHA are shown in Figures 2.1.B, while sections of the MBH are presented in Figures 2.1.C.

Kisspeptin and GnRH were also quantified in regions lateral to the POA or MBH as well as in extra hypothalamic tissue like the cortex, brain stem and cerebellum. Kisspeptin neurons and axon terminals were observed in the ARC of the MBH sections by the use of the anti-equine kisspeptin (Figure 2.2.A). A similar pattern of kisspeptin immunoreactivity for neurons and axons was observed in the ARC of sections when using the anti-ovine kisspeptin (provided by Dr Alain Caraty, Figure 2.3.A). GnRH axons were abundantly detected in the ARC when using the antiserum raised against GnRH (Figure 2.4.A). Pre-absorption of anti-equine kisspeptin (Figure 2.2.B) or anti-ovine kisspeptin (Figure 2.3.B) with ovine kisspeptin or anti-GnRH (Figure 2.4.B) with GnRH, eliminated the immuno-reactivity of kisspeptin or GnRH in hypothalamic sections containing the ARC nucleus. A low intensity pattern of kisspeptin immunostaining was obtained in the different hypothalamic regions analyzed since ewes were processed during the NBS. Intensity of kisspeptin immunoreactivity was similar in the POA, AHA and ARC when the two antisera were compared (Table 2.1).

A pure ^{125}I-ovine kisspeptin-10 ligand was obtained using weak cation exchange column chromatography. Free ^{125}I was eluted in fractions 9 to 13. Non-radioiodinated kisspeptin was eluted in fractions 46 to 52 while ^{125}I-kisspeptin-10 was eluted in fractions 53 to 63 (Figure 2.5). The specific activity of ^{125}I-ovine kisspeptin-10 was 1580 curies/mmole.

Initially, the specificity and sensitivity of anti-ovine kisspeptin and anti-equine kisspeptin were compared by the use of homologous (anti ovine kisspeptin with ^{125}I-ovine kisspeptin-10 ligand) and heterologous systems (anti equine kisspeptin with ^{125}I-ovine kisspeptin-10 ligand). The anti-equine kisspeptin resulted in a more sensitive assay than the anti-ovine kisspeptin (supplemental material, figure 2.11) so all subsequent studies were completed using the anti-equine kisspeptin. When using anti-equine kisspeptin, ovine kisspeptin-10 and ovine kisspeptin-

16 reacted equally. Hypothalamic hormones other than kisspeptin did not cross react with the antiserum (Figure 2.6).

Concentration of kisspeptin recovered after spiking 250 pg of kisspeptin into cerebral cortex was not affected by the amount of tissue processed (5 mg = 259 ± 37 pg/total, 10 mg = 236 ± 82 pg/total, 25 mg = 222 ± 19 pg/total, 50 mg = 277 ± 89 pg/total, and 100 mg = 251 ± 29 pg/total). Extraction efficiency of kisspeptin averaged 98.6 ± 3. 8 % across all tissue concentrations. When different volumes of sample (extracted cortex spiked with 4 ng of kisspeptin) were assayed, the inhibition curved obtained was parallel to the standard curve (50 uL= 4.2 ± 0.2 ng/mL, 100 uL= 4.2 ± 0.4 ng/mL 200 uL= 3.9 ± 0.4 ng/mL, Figure 2.7). A positive correlation (R = 0.96) was observed between the amount of kisspeptin added (x axis) and the concentration of kisspeptin quantified (y axis); the slope indicated that for every pg of kisspeptin added 1.007 pgs of kisspeptin were quantified (slope, Figure 2.8.A). The amounts of GnRH added was highly correlated with the amount quantified (R = 0.98) since on average for every pg of GnRH added to the sample 1.129 pg of GnRH were quantified by RIA as indicated by the slope in Figure 2.8.B.

The sensitivity for the kisspeptin RIA was 16.4 ± 1.4 pg/tube (n = 5). The intra-assay coefficient of variation was 15 ± 6 % for high values, 18 ± 3 % for medium values and 26 ± 4% for low values (n = 5). The inter-assay coefficient of variation was 16.6, 5.7 and 18.3 % for high, medium and low values, respectively. The average inter-assay coefficient of variation was 13.5 ± 3.9 % (n = 5). The assay sensitivity for GnRH was 367 ± 51 fg/tube (n = 4). The intra-assay coefficient of variation was 2 ± 1 % for high values, 5 ± 2 % for medium values and 18 ± 7 % for low values. The inter-assay coefficient of variation was 10.4 % for high values, 8.4 % for medium and 9.90 % for low values, with an average inter-assay coefficient of variation of 9.5 ± 0.6 % (n =

4). Additionally, the content of kisspeptin in the hypothalamus measured by the anti-equine kisspeptin was compared to that measured using Caraty's antibody (this antiserum was used as reference since it is the only antiserum in sheep that has been validated for IHC). As shown in table 2.2, the content of kisspeptin quantified in different hypothalamic regions was similar ($p = 0.36$) between antisera.

During the NBS concentrations of kisspeptin were lower than during BS in the MBH (4.5 ± 1.2 vs 12.9 ± 2.6 pg/mg, $p < 0.01$, Figure 2.9.A). Similarly, the content of kisspeptin was lower during the NBS in this region (695.3 ± 189.6 vs 1803 ± 150.4 pg, $p < 0.01$, Figure 2.9.B). In the POA, kisspeptin concentrations were lower during the NBS compared to the BS (3.9 ± 0.8 vs 7.3 ± 1.7 pg/mg, $p < 0.04$, Figure 2.9.A). Likewise, the content of kisspeptin in the POA was lower during the NBS compare to the BS (252 ± 16.7 vs 543 ± 39.28 pg, $p < 0.01$, Figure 2.9.B). For the AHA, there was a tendency for concentrations of kisspeptin to be lower during the NBS compared to the BS (5.5 ± 0.4 vs 13.2 ± 3.2 pg/mg, $p = 0.097$, Figure 2.9.A). Also, AHA kisspeptin content tended to be lower during the NBS (463.3 ± 27.8 pg vs 1034 ± 195 pg, $p = 0.059$, Figure 2.9.B). Concentration and content of kisspeptin in the ME were similar between seasons (Figures 2.9.A and 2.9.B).

Low levels of kisspeptin were detected in regions adjacent to the POA and MBH (POA-R and MBH-R, Figure 2.10.A). However, the concentrations of kisspeptin in the POA-R and MBH-R did not change between seasons Figure 10A). Similarly, low levels of kisspeptin were detected in the cortex, brain stem and cerebellum, and their concentrations were not affected by season (Figure 2.10.B).

Neither concentration (Figure 2.11.A) nor content (Figure 2.11.B) of GnRH was affected by season in any hypothalamic region studied. The concentrations of kisspeptin and GnRH in the

ME were correlated during the BS (r= .97 P≤ 0.05, Figure 2.12.A) but not during the NBS (Figure 2.12.B). No correlation was observed between these hormones in any other hypothalamic structure regardless season.

Discussion

An antiserum was produced and validated to quantify kisspeptin in neuronal tissue. The RIA was sensitive enough to quantify fentomolar concentrations of endogenous kisspeptin in ovine neural tissue and the specificity of our kisspeptin antiserum was demonstrated by showing that other neuropeptides did not displace binding of ^{125}I-ovine kisspeptin from the antiserum. Further, kisspeptin immunoreactivity was observed histochemically in the ARC nucleus but absent after preadsorption of the antiserum with ovine kisspeptin. Additionally, the amounts of kisspeptin quantified by RIA with the anti-equine kisspeptin in the POA, AHA, MBH and ME were similar to those obtained with Caraty's antiserum. Likewise, patterns of kisspeptin immunoreactivity in the POA, AHA and ARC were similar between those observed with the anti-equine and Caraty's anti-ovine antiserum.

Seasonal changes in amounts of kisspeptin expression in different hypothalamic regions of the ewe were quantified by RIA. During the NBS concentrations and content of kisspeptin in the MBH (rostral-middle-caudal ARC and the ventral section of the wall of the third ventricle) and the POA were lower than during the BS; similarly, in the AHA the concentrations and content of kisspeptin tended to be lower in the NBS compared to the BS.

Kisspeptin might modulate GnRH neuronal activity in the MBH since the *Gpr54* mRNA is expressed in this hypothalamic region in ewes (Smith, Pereira & Clarke; 2009; Smith et al., 2011) and the MBH is the purported site of the GnRH pulse generator in sheep and goat (Boukhliq

et al., 1999; Goodman et al., 2007; Lehman et al., 2010; Wakabashi et al., 2010). The lower concentrations and content of kisspeptin detected by RIA in the MBH during the NBS in sheep are consistent with a lower number of immunoreactive kisspeptin neurons (indicative of a lower protein content) and *Kiss1* mRNA (ISH) in the ARC during the NBS (Smith et al., 2008). The results reported herein and those of Smith et al. (2008) agree with the negative feedback effect of estradiol on kisspeptin expression in the MBH (ARC).

In sheep the largest populations of GnRH neurons reside in rostral hypothalamic regions, particularly the POA (Jansen et al., 1997; Lehman et al., 1986; Caldani et al., 1988). Kisspeptin likely regulates the GnRH neuronal activity in the POA (Chalivoix et al., 2010) since these neurons also express *Gpr54* mRNA (Smith et al., 2009; Smith et al., 2011). Contrary to the consistency in the reports on the effects of estradiol on kisspeptin expression in the MBH, there is a lack of agreement among authors relative to the effects of estradiol on kisspeptin expression in the POA between seasons. By using the kisspeptin RIA, lower concentrations and content of kisspeptin in the POA were detected during the NBS. Using IHC Chalivoix et al. (2010) reported a lower number of kisspeptin immunoreactive neurons in the POA during the NBS. In contrast, Smith et al. (2008) did not detect differences in numbers of kisspeptin neurons between seasons in the POA. The inconsistency between histological studies may have arisen due to differences in the area within the POA selected for the quantification of kisspeptin neurons. The fact that we used the whole POA to quantify content of kisspeptin may overcome the limitation of selecting only a small area for histological examination.

The AHA might be an additional hypothalamic site where kisspeptin regulates the GnRH system since a considerable number of GnRH neurons and GnRH axons reside in this region in ewes (Jansen et al., 1997). In this regard, Smith et al. (2008) observed a higher number of

kisspeptin axons contacting GnRH neurons in the AHA during the BS when compared to the NBS. This is the first study to quantify changes in concentrations and content of kisspeptin between seasons in the AHA, and interestingly, a strong tendency ($P = 0.059$) for a lower content of kisspeptin was observed during the NBS in this region.

Since there is a large number of GnRH and kisspeptin axon terminals in the ME of ewes (Lehman et al., 1986; Franceschini et al., 2006), this region may represent another site where kisspeptin could regulate GnRH secretion. For example, Smith et al. (2011) showed that kisspeptin stimulated GnRH secretion from isolated ME. However, when quantifying kisspeptin by RIA there was no effect of season on the amounts of kisspeptin in the ME, but concentrations of kisspeptin and GnRH were correlated during the BS. This correlation was not present during the NBS. This is the first time that concentrations of both kisspeptin and GnRH were quantified in the ME of ewes during the BS and NBS.

Kiss1 RNA is expressed in regions outside of the hypothalamus and in rodents the expression of the *Kiss1* gene has been detected in the cerebral cortex, neocortex, lateral septum, olfactory bulb and amygdala (Cravo et al., 2011). The functions of kisspeptin in these extra hypothalamic tissue are currently unknown. In agreement with Cravo et al. (2011), who reported low levels of *Kiss1* RNA in the brain stem, cortex and cerebellum when compared to the hypothalamus in female rodents, low levels of kisspeptin were quantified by RIA in the cortex, brain stem and cerebellum when compared to the hypothalamus of sheep in the present study.

In contrast to the seasonal changes in kisspeptin expression, concentrations of GnRH did not change with season. This is in agreement with studies of Nett (1983) who reported no change in the hypothalamic concentrations of GnRH between seasons in ewes. Similar results have been

reported in rodents housed under controlled conditions of photoperiod (Glass, 1986; Brown et al., 2001).

The RIA results reported here support the notion that there is a higher kisspeptin input to the GnRH neuronal system during the BS, thus promoting an increase in GnRH secretion. In contrast, during the NBS the reduced kisspeptin input exerted on GnRH neurons results in a decreased rate of GnRH secretion.

Interestingly, other studies have provided data supporting the notion that kisspeptin has an important role in the secretion of GnRH, but GnRH synthesis may be to some extent constitutive. For instance, *Kiss1* null mice have an apparent normal hypothalamic content of GnRH when compared to wild type mice (d'Anglemont de Tassigny et al., 2007). Likewise, the GnRH system in *Kiss1* null mice appears normal since their GnRH neurons project toward the ME (Messager et al., 2005; d'Anglemont de Tassigny et al., 2007) and these knockout rodents have a normal response to kisspeptin administration by secreting GnRH similar to wild type rodents (d'Anglemont de Tassigny et al. (2007).

Conclusions

A RIA was validated for quantification of kisspeptin. The only other antiserum available for the immuno detection of kisspeptin in sheep was produced in France by Dr. Alain Caraty. When used for IHC our antiserum (anti-equine kisspeptin) produced a similar pattern of immunoreactivity in the MBH as Caraty's antiserum. Likewise, the amounts of kisspeptin quantified by RIA with the anti-equine or the anti-ovine kisspeptin (generated by Dr. Caraty) were similar in the different hypothalamic nuclei analyzed. Using the RIA, less kisspeptin was measured in the ovine hypothalamus during the NBS compared to the BS. The evidence provided herein

supports the notion that a reduction in the concentrations and content of kisspeptin occurs in the POA in sheep during the NBS. A tendency for less kisspeptin during the NBS was observed for the first time in the AHA. Kisspeptin was detected in regions surrounding the POA and MBH as well as in other extra-hypothalamic regions but its function in these regions is unknown.

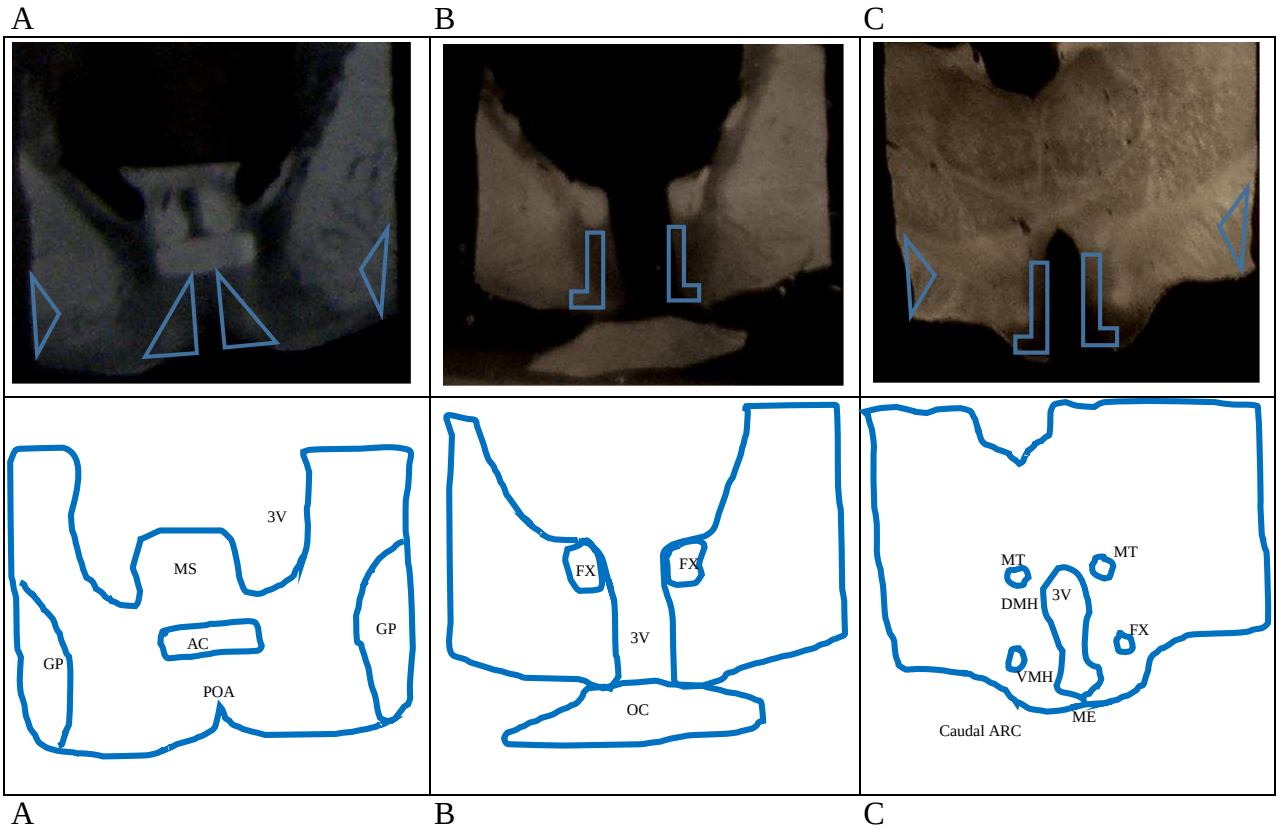


Figure 2.1. Hypothalamic pictures (top) and drawings (bottom) of sections representative of the POA (A), AHA (B) and MBH (C). Regions that were processed for the quantification of kisspeptin and GnRH by RIA are limited by a blue line. Zones adjacent to the POA and MBH (left and right triangle), were selected due to the lower concentrations of kisspeptin and GnRH expected in these regions.

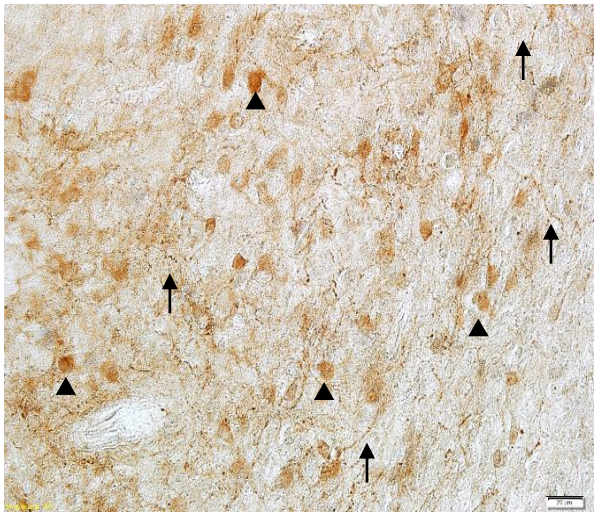
A  B 

Figure 2.2. A. Kisspeptin neuron (arrow head) and axon (arrow) immunoreactivity in the caudal ARC obtained using the antiserum (1: 20,000) raised against equine kisspeptin (20X magnification). B. Lack of kisspeptin immunoreactivity in the caudal ARC after the preadsorption of the antiserum with 1 ug ovine kisspeptin-10 (10X magnification).

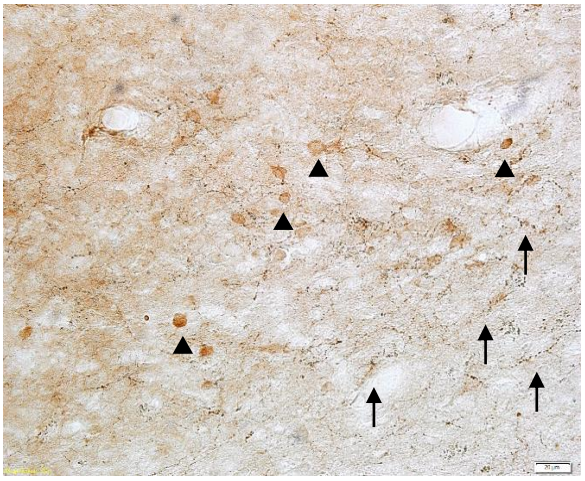
A  B 

Figure 2.3. A. Immunoreactivity of kisspeptin neuron (arrow head) and axon (arrow) in the caudal ARC (20X magnification) when using antiserum (1:40,000) against ovine kisspeptin, kindly provided by Dr Caraty. B. 10X amplification. Kisspeptin neuronal immunoreactivity is absent when using the antiserum that has been previously preadsorbed with 1 ug ovine kisspeptin-10 (10X magnification).

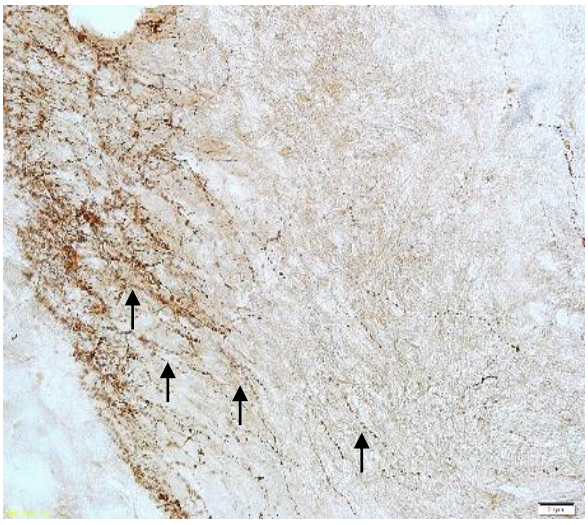
A  B 

Figure 2.4. A. Immunoreactivity in axon terminals (arrow) in the caudal ARC using anti-GnRH (1:20,000, 20X magnification). B. Lack of GnRH immunoreactivity was observed when using the antiserum preadsorbed with 1 ug GnRH (10X magnification).

Table 2.1. Comparison of the intensity of immuno-reactivity for kisspeptin neurons and axons detected by IHC when using an anti-equine kisspeptin or anti-ovine kisspeptin antiserum (3 ewes).

	Immuno-reactivity	POA neurons	POA axons	AHA neurons	AHA axons	ARC neurons	ARC axons
Anti-equine kisspeptin	Intensity	+++	+	++	+	++	++
Caraty's antiserum	Intensity	+++	+	+++	++	+++	+++

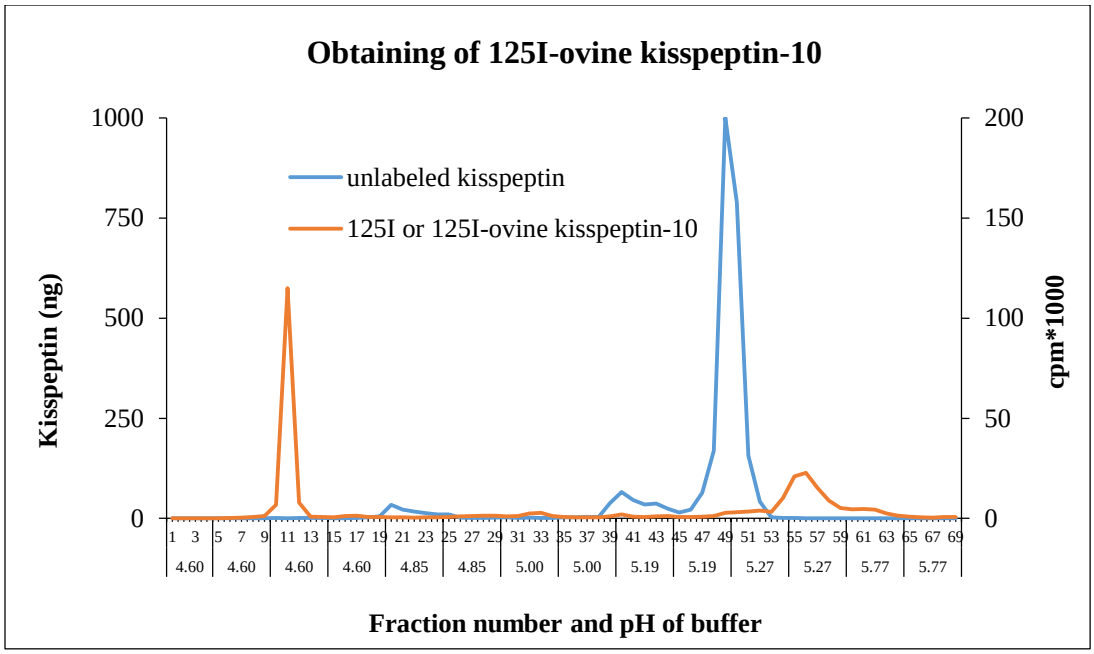


Figure 2.5. Purification of ^{125}I-ovine kisspeptin using a weak cation exchange column and several buffers with different ionic strength.

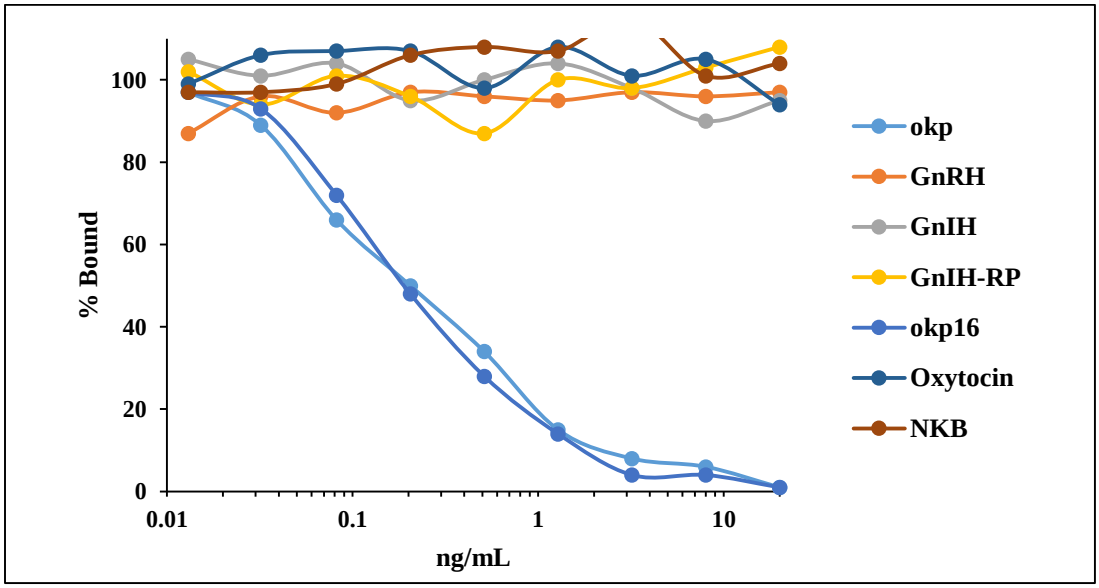


Figure 2.6. Cross reactivity of oxytocin, gonadotropin releasing hormone (GnRH), gonadotropin inhibitory hormone (GnIH), gonadotropin inhibitory hormone related peptide (GnIH-RP), Neurokinin B (NKB2), ovine kisspeptin-10 (okp) or ovine kisspeptin-16 (okp 16) in the kisspeptin radioimmunoassay.

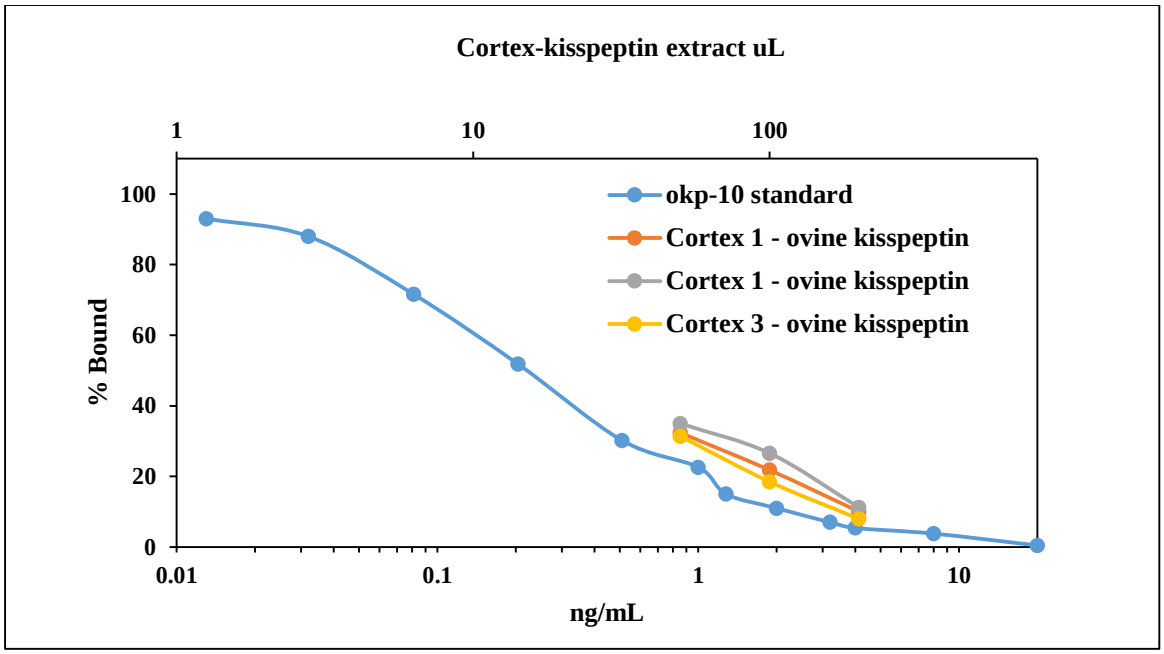
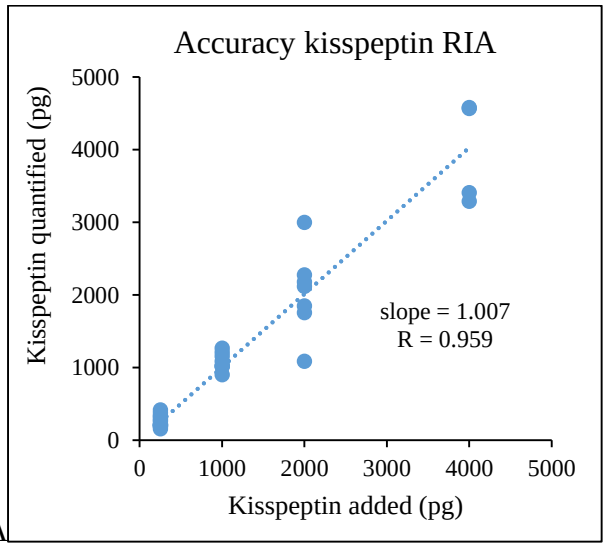


Figure 2.7. Parallelism of the kisspeptin RIA. Similar amounts of neuronal cortex (three sets) were spiked with 4 ng of kisspeptin and 50, 100 or 200 uL of each sample was assayed after extraction.

A



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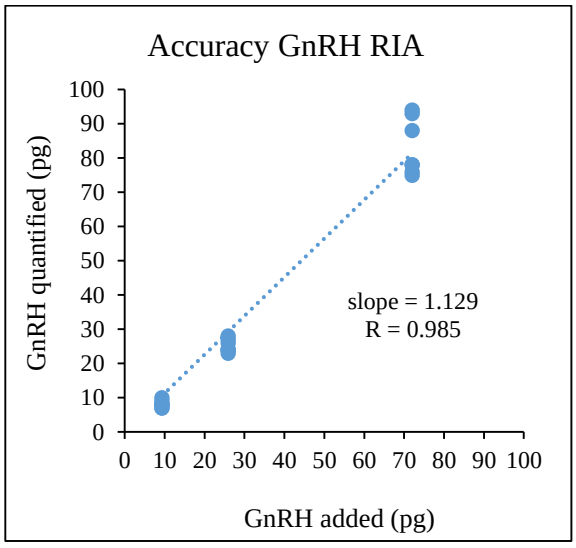


Figure 2.8. Accuracy of the quantification of kisspeptin (A), and GnRH (B). A positive linear relationship between data was observed for both RIAs (R).Different concentrations of hormone were added (*x axis*) and compared to the concentration of hormone quantified (*y axis*) by RIA.

Table 2.2. Two independent RIA's were performed, one using the antiserum raised against equine kisspeptin and the other one by using an antiserum raised against ovine kisspeptin-10. The content of kisspeptin (total) detected in different regions with either antiserum was not statistically different (p = 0.3636).

Region	Equine's kisspeptin antiserum (pg kisspeptin)	Ovine's antiserum (Dr Caraty) (pg kisspeptin)
POA	93	87
AHA	351	335
MBH (ARC)	135	126
ME	419	396
CORTEX	17	35
CEREBELUM	18	19

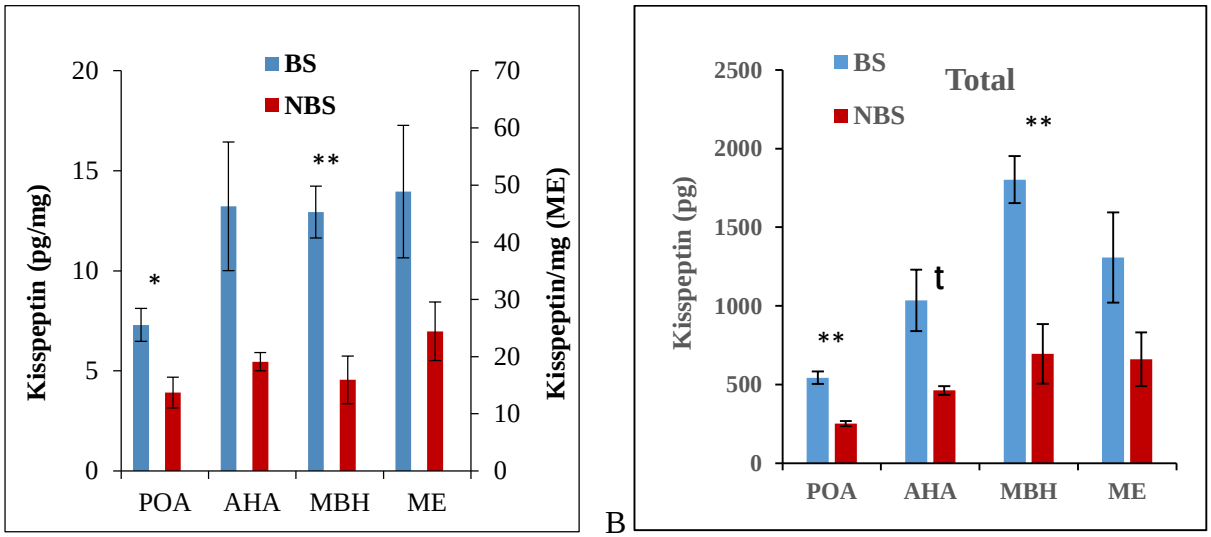
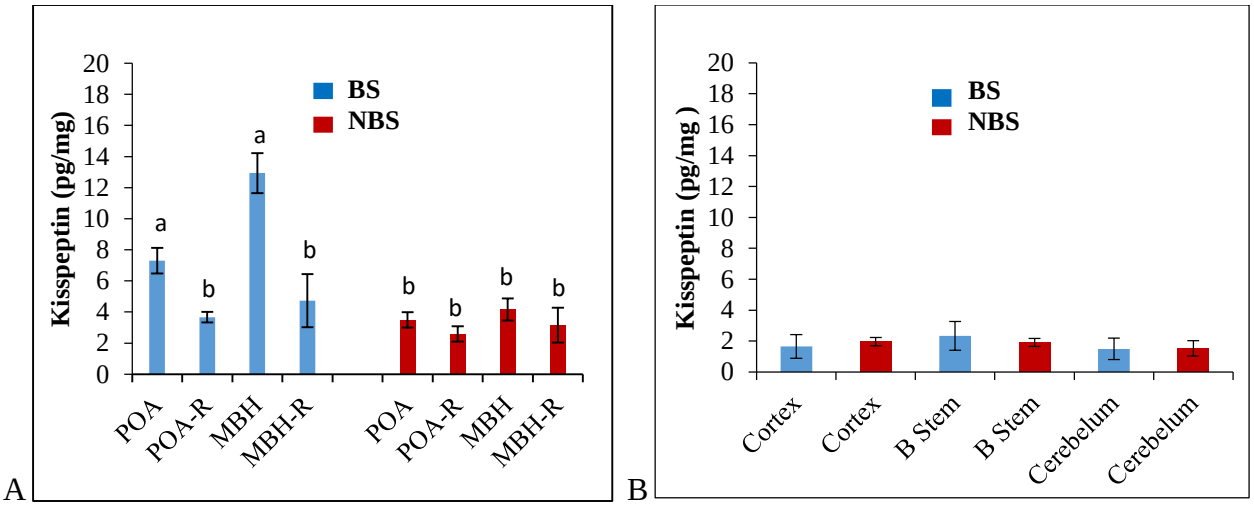


Figure 2.9. Differences in the hypothalamic concentrations (A) of kisspeptin per region, during the BS and NBS. Values are expressed as pg kisspeptin per milligram of tissue. * = $p < 0.05$, ** = $p < 0.01$. AHA= $p \leq 0.097$. Differences in the content (B) of kisspeptin in different hypothalamic regions BS (blue) and NBS (red). ** = $p < 0.01$, ţ p = 0.059.



Figures 2.10. A. Difference in the concentrations of kisspeptin during the BS (blue) and NBS (red) in the POA and MBH, as well as in areas adjacent to the POA or MBH (A). Within each season the concentrations of kisspeptin were compared between the POA and the regions adjacent to it (POA-R), or between the MBH and its adjacent region (MBH-R). B. A comparison between the BS (left) and NBS (right) was also made for the concentrations of kisspeptin in extra hypothalamic regions like the cortex, brain stem (B Stem) and cerebellum.

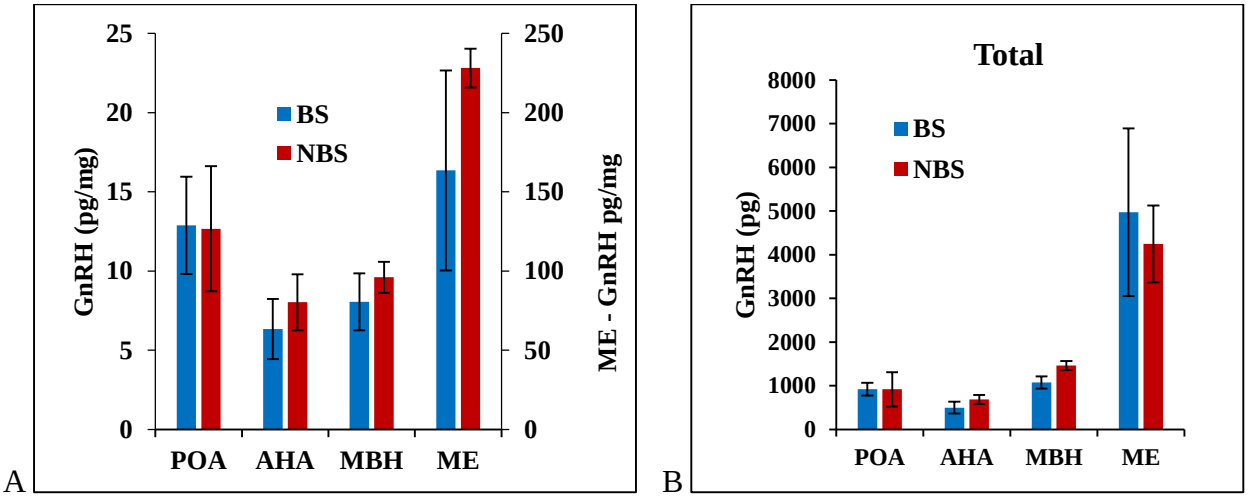
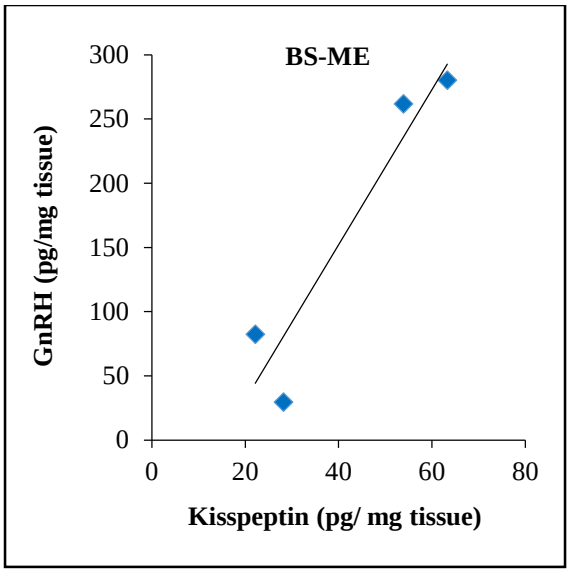


Figure 2.11. A. Comparison of the concentrations of GnRH (pg/mg tissue) quantified by RIA in different hypothalamic regions between the BS (blue) and NBS (red). B**.** Differences in the total **c**ontent of GnRH in different regions during the BS (blue) and NBS (red). POA (p =0.60), AHA (p=0.97), MBH (p=0.97) and ME (p=0.42).

A 

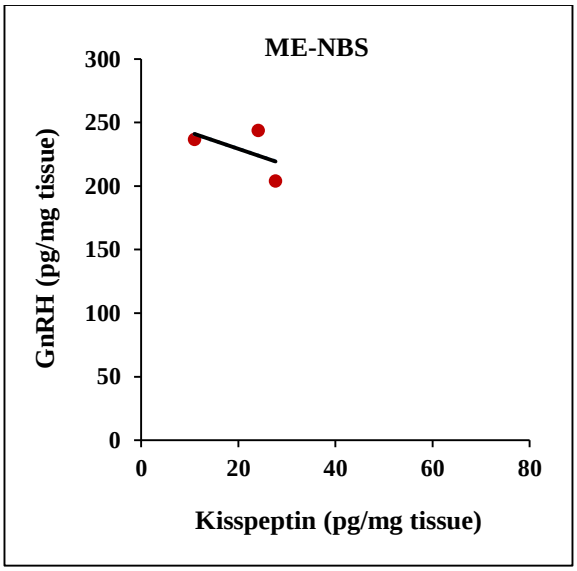
B 

Figure 2.12. Correlation between the concentrations of kisspeptin and GnRH in the ME during the BS (r=.97 p=0.05) (A) and NBS (r= -.53 p=0.64) (B).

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CHAPTER 3. THE INTRACEREBROVENTRICULAR ADMINISTRATION OF ESTRADIOL ANTAGONIST AND ITS EFFECTS ON LH PULSE FREQUENCY DURING THE TRANSITION PERIOD OF THE LATE NON BREEDING SEASON (NBS) IN EWES

Summary:

During the NBS the sensitivity to the negative feedback effects of estradiol is dramatically increased which leads to a reduction in GnRH and LH secretion when compared to the BS. However a direct action over GnRH neurons is not probable since GnRH neurons do not express the ERα which is the receptor subtype of relevance for the modulation of GnRH and LH secretion. In ewes hypothalamic kisspeptin neurons express ERα and are located in regions like the MBH where the pulse generator mechanism is known to reside. Then it is probable that by inhibiting the production of kisspeptin, estradiol reduces the kisspeptin input over the GnRH system indirectly inhibiting GnRH and LH release during the NBS. It was hypothesized that the intracerebral administration of an estrogen antagonist, ICI 182 780 (ICI) can block the estrogen receptors in the hypothalamus, leading to an increase in the frequency of GnRH secretion that is reflected in a higher frequency of LH pulses during the NBS. In order to test this hypothesis ICI was administered into the cerebral lateral ventricle for 27 days and its effects in LH secretion evaluated. Blood samples were collected for a period of 6 hours one day prior to ICI (treated) or saline (control) and 27 after ICI or saline administration. The intracerebral administration of a saline solution for 27 days resulted in no change in the number of pulses, amplitude or mean LH during the NBS when compared to the day before its administration (before vs after; pulses = 1.00 ± 0.3 vs 1.2 ± 0.5), amplitude (1.07 ± 0.39 vs 1.78 ± 0.87 ng/mL) and mean (0.66 ± 0.57 vs 0.79 ± 0.49

ng/mL). On the other hand, the administration of ICI for 27 days increased the frequency of LH pulses when compared to the day before its administration (before vs after = 3.2 ± 0.58 vs 5.4 ± 0.59, $p < 0.02$), with a tendency for a higher amplitude also observed (0.69 ± 0.23 vs 1.2 ± 0.24 ng/mL, $p = 0.15$). No difference was observed in the mean LH secretion with the administration of ICI (0.59 ± 0.12 vs 0.75 ± 0.17 ng/mL). The observed increase in pulse frequency and the tendency for higher amplitude in LH suggests that the intracerebral blockade of estradiol resulted in the stimulation of kisspeptin production which promoted a higher kisspeptin input over the GnRH system, leading to an increase in GnRH and thus LH secretion during the NBS.

Introduction

In sheep, the frequency of LH pulses is affected by season (Goodman et al., 1982). The NBS is characterized by a hypersensitivity to the negative feedback effects of estradiol that is translated into a reduction in the frequency of LH pulses (Karsch et al., 1980). Some evidence suggest that estradiol indirectly inhibits the frequency of LH pulses by modulating the GnRH system (Barrel et al., 1992; Karsch et al., 1993). It has been proposed that estradiol needs an intermediary to regulate the GnRH neuronal activity since the GnRH neurons do not express ERα, the ER subtype of relevance for the regulation of LH secretion (Lindzey et al., 2006). Kisspeptin neurons potentially can serve as the intermediary neurons for the seasonal modulation of GnRH, and hence LH secretion exerted by estradiol, since kisspeptin neuronal input into GnRH neurons is reduced during the NBS and increased during the BS (Smith et al., 2008) and kisspeptin neurons express ERα (Franceschini et al., 2006) while GnRH neurons express *GpR54* mRNA (Li et al., 2012).

There are two important populations of kisspeptin neurons in the hypothalamus of sheep, one located in the POA and the other is present in the ARC of the MBH. About 95 and 50 % of the kisspeptin neurons in the ARC and POA, respectively, express ERα (Franceschini et al., 2006). Expression of kisspeptin mRNA is highly modulated by estradiol in a seasonal fashion (Smith et al., 2007; Smith et al., 2008) and kisspeptin is a potent stimulator of GnRH secretion in ewes (Caraty et al., 2013).

Estradiol, by differential regulation on the expression of kisspeptin during the BS and NBS, might indirectly modulate seasonal changes in GnRH and LH secretion in ewes. For instance, estradiol reduces the number of kisspeptin neurons that can be detected by IHC during the NBS (Smith et al., 2008) and the latter might be associated with a decrease in the kisspeptin input over the GnRH system. The decreased kisspeptin input on GnRH neurons during the NBS is likely related with a lower frequency of GnRH pulses/secretion (Barrell et al., 1992) and hence, a lower pulsatility of LH (Karsch et al., 1993; Karsch et al., 1980). Due to the importance that estradiol plays during the NBS in the induction of a negative feedback modulation over the kisspeptin/GnRH system (Smith et al., 2008) and consequently LH secretion (Legan et al., 1977), the hypothesis that by blocking the hypothalamic negative feedback effects of estradiol during the NBS, an increase in LH pulsatility would occur was tested. It is important to mention that ICI 182 780 (ICI) is considered one of the best antiestrogens available (Howell et al., 2000, Labrie et al., 2001; Varshochi et al., 2005) even though it has been shown to possess weak agonist activities in some experimental models studied (Robertson et al., 2001; Zhao et al., 2006). Being that ICI the best anti-estrogenic currently available, and due to the lack of information related with the cerebral administration of this compound in ewes, its effects in LH pulsatility during the NBS was tested.

Material and Methods

The procedures that involved animals in the present experiment were done according to the Colorado State University Animal Care and Use Committee and complied with the guidelines of the National Institutes of Health (NIH). Mature western-range ewes with ages ranging from 3-5 years and similar body weight were housed under natural light at Colorado State University (Animal Reproduction and Biotechnology Laboratory facilities). The experiment was carried out during the late NBS (July and August, 2014).

Experimental designs

The purpose of this experiment was to block the hypothalamic negative feedback effects of estradiol. Bilateral ovariectomy was performed in nine ewes via a mid-ventral incision. At the time of surgery, each animal received a subcutaneous estradiol implant (SILASTIC brand tubing (Dow Corning Corp., Midland, MI) filled with 3 cm estradiol 17-β (estradiol - Sigma Chemical Co., St Louis, MO)) which was inserted in the axillary region (May). The estradiol implant had an inner and outer diameter of 3.35 and 4.65 mm, respectively. Implants were soaked overnight in 0.1M PBS buffer prior placement to avoid an initial post-insertion peak of the hormone and were intended to produce levels of 3-5 pg of estradiol /mL of serum simulating the levels found during the luteal phase of the ovine estrous cycle (Goodman et al., 1982). The purpose of estradiol implantation was to reduce the frequency of pulsatile LH secretion during the NBS (Karsch et al., 1980). The intracerebroventricular administration of the estrogen antagonist ICI was intended to reverse the hypothalamic negative feedback effects of estradiol during the NBS with the intention of promoting an increase in the frequency of LH pulses.

Intracerebroventricular administration of an estrogen antagonist (ICI)

A small cannula was inserted into the lateral cerebral ventricle of each ewe and connected to an osmotic pump to deliver an intracerebroventricular (ICV) saline solution or the estrogen antagonist ICI. An incision through the skin was made at the top of the head. The skull was exposed at a level anterior to the lateral cerebral bregma, and a small hole (about 2 mm diameter) drilled (Figure 3.1.1). A 16 G needle was inserted through the hole and the dura to reach the lateral ventricle. Micro-Rethane tubing (25 G) was passed through the needle and the proper localization of the tubing in the third ventricle was confirmed by the flow of cerebrospinal fluid out of the tubing. Once the tubing was in the proper position, the needle was removed and the tubing anchored to the skull using Dracon mesh and super glue (Figure 3.2.1). The external end of the tubing was attached to an Alzet mini osmotic pump (Figure 3-3.1). The pump was anchored subcutaneously and the skin sutured (Figure 3.3.2). The estrogen antagonist ICI was diluted in 50% DMSO and 50% sterile saline solution and treated ewes received an ICV dose of 0.64 mg per day for 27 days.

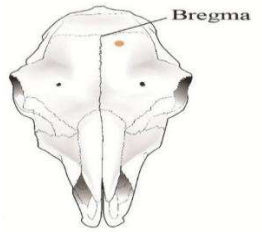


Figure 3.1

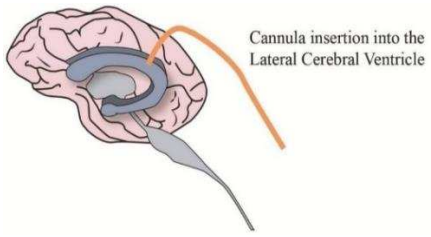


Figure 3.2

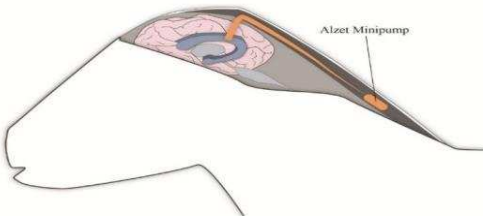


Figure 3.3

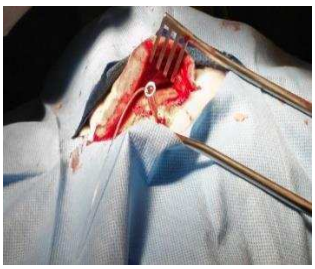


Figure 3.1.1

Figure 3.2.1

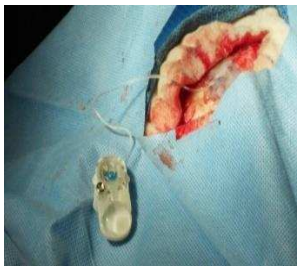


Figure 3.3.1

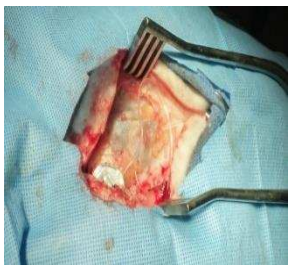


Figure 3.3.2

Determination of LH concentrations in serum

Each ewe received a jugular cannula 12 hours prior to the sampling period starting time in order to facilitate blood collection. Blood samples were collected every 15 minutes for 6 hours, one day prior to ICI treatment and at day 27 after starting the intracerebroventricular administration of ICI. Blood was allowed to clot for 6 hours at room temperature (20° C) and then stored overnight at 4° C. Serum was obtained by centrifuging samples at 2500 rpm (1500 *g*) for 15 minutes and then stored at -20C until quantified for LH by RIA (Niswender et al., 1969). The reference standard used for LH was NIH-oLHS24. Three standard curves were included in each assay and duplicate samples were analyzed by adding 50 µL of serum per tube. The intra-assay coefficient of variation was 3.1 ± 1 % and 7.6 ± 4 % for medium and high values respectively. The inter-assay coefficient of variation observed was 9.63 % for medium values and 25.95 % for high values. The assay sensitivity averaged 13.74 ± 1.63 pg/tube. Parameters evaluated were: basal LH, defined as the five lowest concentrations of LH within each six hour period for each ewe, mean LH for the six hour period, and the number of pulses. A pulse of LH was defined as an increase in the concentration of LH greater than the mean concentration for the six hour period plus two standard deviations that were preceded and followed by at least one lower concentration (adapted from Clark and Cummins, 1984 and Clark et al., 1988)..

Data Analysis

Data were analyzed by a paired t-test using SAS (SAS: SAS User's Guide SC. NC: Statistical Analysis System Institute, Inc.; 1987). The following parameters were analyzed: basal LH, number and frequency of LH pulses, and mean LH. The frequency of LH pulses (categorical variable) was subjected to arcsine transformation because this parameter does not follow a normal

distribution. Data are presented as Mean ± SEM. Statistical differences with p values equal to or less than 0.05 were considered significant.

Results

The number of pulses were 1.00 ± 0.3 the day before saline administration, while the amplitude of LH pulses was 1.07 ± 0.39 ng/mL. Mean LH levels were 0.66 ± 0.57 ng/mL, Figure 3.4. Administration of saline solution for 27 days resulted in a frequency of 1.2 ± 0.5 pulses of LH, an amplitude of 1.78 ± 0.87 ng/mL and a mean LH concentration of 0.79 ± 0.49 ng/ml, Figure 3.4. No statistical difference was observed in frequency, amplitude or mean LH between the day before and 27 days after saline administration (Figure 3.4). Relative to the ICI treated group, the number of pulses detected the day before ICI administration were 3.2 ± 0.58, whereas the amplitude of LH secretion was 0.69 ± 0.23 ng/mL. The mean concentrations of LH were 0.59 ± 0.12 ng/mL, Figure 3.5. After the administration of ICI for a period of 27 days, the frequency of LH pulses were 5.4 ± 0.59 while the amplitude was 1.2 ± 0.24 ng/mL. Mean concentration of LH after ICI administration was 0.75 ± 0.17 ng/ml, Figure 3.5. When comparing LH parameters in ewes between the day before and 27 days after ICI administration, the frequency of pulses was increased with the ICI treatment (before = 3.2 ± 0.58 pulses vs after = 5.4 ± 0.59 pulses, $p < 0.02$, Figure 3.5).

Discussion

In ewes, regardless of season, ovariectomy removes the negative feedback effects of estradiol leading to an increase in the secretion of LH (Montgomery et al., 1985). Conversely, in

ovariectomized ewes, chronic low doses of estradiol reduces the frequency of LH pulses during the NBS (Legan et al., 1977).

Regardless of season, each pulse of LH is correlated with a pulse of GnRH (Karsch et al., 1993); but since hypothalamic concentrations of GnRH do not change between seasons (Nett et al., 1983; Urias-Castro 2015 cf, previous chapter), another mechanism must be involved in regulating GnRH secretion during the NBS and BS. It is possible that kisspeptin differentially regulates the frequency of GnRH/LH secretion by decreasing its frequency during the NBS, and increasing it during the BS (Karsch et al., 1993; Karsch et al., 1980); however, there are no studies demonstrating a differential and direct effect of kisspeptin on GnRH secretion by season. Supporting the hypothesis that kisspeptin might modulate LH secretion is the study of Wakabayashi et al. (2010) who observed that ARC-kisspeptin neurons modulate LH pulse frequency. The involvement of kisspeptin system as a mediator of seasonal modulation of GnRH, and hence LH secretion, is a potential mechanism by which estradiol indirectly modulates LH secretion. For instance, during the NBS and compared to the BS, estradiol exerts an increased inhibitory feedback in the kisspeptin system by: 1) reducing *Kiss1* mRNA (Smith et al., 2007; Wagner et al., 2008, Smith et al., 2008), 2) kisspeptin neuron number (Smith et al., 2008), and 3) decreasing content of kisspeptin in the hypothalamus (see chapter 2).

It is probable that the increase in the frequency of LH pulses observed at day 27 after ICI treatment, was consequence at least in part, of blocking estradiol negative feedback over the kisspeptin/GnRH system. The latter may have resulted in an increase in kisspeptin production and then a higher kisspeptin input over the GnRH neurons promoting an increase in the secretion of GnRH and thus LH during the NBS. The notion that kisspeptin is relevant for the differential

control of GnRH, and hence LH secretion, during the NBS and BS exerted by estradiol, is based on the fact that GnRH neurons do not express ERα (Herbison & Theodosis, 1992).

ERα is the ER subtype of physiological relevance for the modulation of the hypothalamic-pituitary axis (Dorling et al., 2003, Smith et al., 2005; Lindzey et al., 2006; Glidewell-Kenney et al., 2007; Roa et al., 2008). Moreover, hypothalamic kisspeptin neurons express the ERα (Franceschini et al., 2006) and it has been observed that the activation of the ERα by chronic administration of estradiol promotes a decrease in the expression of c-Fos in the ARC kisspeptin neurons during the NBS suggestive of a direct negative feedback by estradiol on the kisspeptin system in ewes (Smith et al., 2009).

In this study, it was hypothesized that by blocking the effects of estradiol, kisspeptin neurons can be released from the chronic negative feedback effects of the steroid hormone leading to a restoration in the hypothalamic concentrations of kisspeptin, and consequently an increase in the number of GnRH/LH pulses. To test this hypothesis, we used an estradiol antagonist (ICI 172 780), considered the best antiestrogen available (Howell et al., 2000; Labrie et al., 2001).

As mentioned previously, activation of ERα is involved in the modulation of the hypothalamic pituitary axis (Smith et al., 2005a; Roa et al., 2008b). For example, in ERα knock out mice there is an increase in the levels of the *Kiss1* mRNA at the level of the ARC compared to ERβ knock out mice, and it is probable that the increase in *Kiss1* mRNA in ERα knock mice is due to the lack of a negative feedback of estradiol at the level of the kisspeptin neurons (Smith et al., 2005a). Additionally, E_2 replacement in ERα knock out rodents has no impact on the hypothalamic expression of kisspeptin because it does not reduce *Kiss1* mRNA as it does in wild type mice. Also, it appears that activation of ERα, but not ERβ, regulates expression of *Kiss1* (Smith et al., 2005a). Activation of ERα by estradiol is crucial for the hypothalamic modulation of GnRH, and

hence secretion of LH. For example, Dorling et al. (2003) observed that ERα knock out mice had an increased content of GnRH mRNA compared to ERβ knock out mice and an increased secretion of LH suggestive of a lack of negative feedback by estradiol at the level of the hypothalamus (Dorling et al., 2003).

On the other hand, the receptor for kisspeptin (GPR54) is essential for modulating effects of kisspeptin on GnRH/LH secretion, and in this regard, Roseweir et al., (2009) observed that the post ovariectomy rise in LH secretion in ewes can be blocked by administration of kisspeptin antagonist. Moreover, the increase in secretion of LH observed after ovariectomy in wild type mice is not detected in Gpr54 knock out animals (Dungan et al., 2007). Thus, it seems likely that the blockade of estradiol by ICI in the present study led to a higher number of LH pulses by promoting the production of kisspeptin mRNA and kisspeptin protein. Increased concentrations of kisspeptin measured by RIA during the BS when the inhibitory actions of estradiol are reduced support this supposition (cf. previous chapter).

It is well known that estradiol also acts directly at the pituitary level and exert an inhibitory action on LH secretion (Arreguin-Arevalo and Nett, 2006). However, the stimulatory actions of ICI in the hypothalamus might promote a higher kisspeptin input over the GnRH system resulting in an increased pulsatile secretion of GnRH. A higher rate of hypothalamic GnRH secretion might override the inhibitory actions of estradiol at the level of the pituitary which inhibits LH secretion for short periods of time (hours).

Modulation of hypothalamic kisspeptin production by estradiol is well conserved across species (Mice: Smith et al., 2005a; Rats: Adachi et al., 2007; sheep: Smith et al., 2008; primates: Rometo et al., 2007). In this regard, estradiol treatment reduces kisspeptin expression in the ARC

of rodents (Smith et al., 2005a; Smith et al., 2005b) and a similar reduction is exerted by estradiol in the ARC of sheep (Smith et al., 2008; Smith et al., 2009).

ERα is expressed in virtually all the kisspeptin neurons of the ARC in mice and ewes (Smith et al., 2005a; Franceschini et al., 2006), however ERα expression in kisspeptin neurons of the infundibular regions (ARC) has not been directly proven in primates (Shorupskaite et al., 2014). In rodents, the inhibition of *Kiss1* mRNA expression in the ARC involves a non-classical estrogen pathway, that does not require the binding of estradiol-ERα complex to the ERE (Gottsch et al., 2009). Thus, it appears that reduction of *Kiss1* mRNA and likewise kisspeptin protein content in the ARC of ewes by estradiol, may occur via the activation of an ERE independent or non-classical pathways.

Most studies examining *Kiss1* gene expression involve experiments performed in mice. In this rodent species, estradiol causes deacetylation of the kisspeptin promoter in the ARC (Tomikawa et al., 2012). Likewise in rats, silencers of the polycomb group (PcG) repress *Kiss1* gene expression with two PcGs genes, Eed and Cbx7 playing important roles prior to puberty; further, removal of Eed from the *Kiss1* promoter increases *Kiss1 gene* (Lomniczi et al., 2013). However, the role of the polycomb group in the modulation of hypothalamic *Kiss1* in periods of enhanced sensitivity to the negative feedback effects of estradiol (prepuberal, lactational, or seasonal anestrous) has not been studied in sheep.

As previously mentioned, the BS in ewes is characterized by a loss in the sensitivity to the negative feedback effects of estradiol, while the NBS is characterized by a hypersensitivity similar to what occurs in prepuberal rodents. The increase in kisspeptin production detected by RIA (see previous chapter) during the BS compared to the NBS was probably due to an escape from the negative feedback effects of estradiol. In ewes, an increase in the methylation of *Eed and Cbx7*

promoters (as observed by Lomniczi et al. (2013) in prepuberal rats) might result in an increase in the expression of *Kiss1 mRNA* during the BS when compared to the NBS.

Studies involving the MDA-MB-231 cancer cell lines have shown that inhibition of *Kiss1* expression by estradiol involves a reduction in the activity of the RNAPII present in the *Kiss1* promoter by a mechanism that does not require binding of the ERα to DNA (Huijbregts & de Roux., 2010). There are several possible non-classical pathways by which estradiol can modulate the expression of *Kiss1*; for example, estradiol-ERα can bind to SP1 and this complex then interacts with the *Kiss1* promoter enhancing *Kiss1* expression (Li et al., 2007).

There are multiple examples corroborating the activation of non-classical pathways by estradiol. For instance, binding of estradiol to ERα can lead to activation of a fast membrane initiated signaling pathway (Levin et al., 2005) and hypothalamic non-genomic actions have been reported in GnRH neurons, where estradiol increases the phosphorylation of cAMP response element binding protein (CREB) (Abraham et al., 2003), potassium currents (Abe et al., 2008) and calcium oscillations (Zhang et al., 2007). Additionally, ERα has been detected in the plasma membrane of the GnRH neuronal cell line GT1-7 (Morales et al., 2007) and rapid actions of estradiol have been observed in hypothalamic neurons where this steroid activates protein kinase C pathways (Qui et al., 2003).

Besides ERα, other putative estrogen receptors may also be activated in the hypothalamus, more specifically in kisspeptin neurons. Estradiol can act through the activation of G protein coupled receptor (GPR30) which is present in membranes of the endoplasmic reticulum, the Golgi apparatus and nucleus where it is activated by estradiol and other estrogenic agonists (Filardo et al., 2002; Brailoiu et al., 2007; Prossnitz et al., 2008). GPR30 is present in GnRH neurons of mouse and primates (Sun et al., 2010; Noel et al., 2009) and that estradiol-BSA can induce rapid effects

on GnRH neuronal activity suggestive of a plasma membrane localization for GPR30 (Noel et al., 2009). Receptors for estradiol other than ERα, ERβ and GPR30 reside in the plasma membrane of hypothalamic neurons and are activated by estradiol through G proteins (Qiu et al., 2008).

More studies are needed to determine the possible involvement of different estrogen receptors (GPR30, ERα, ERβ and others) in the negative feedback effects of estradiol on kisspeptin neurons of the ARC and/or POA, due to the importance that these hypothalamic regions play in seasonal reproduction. There are limited data examining changes in number of estrogen receptors in specific hypothalamic regions during the BS and NBS, but previous studies examining the whole hypothalamus have demonstrated that there is an increase in the number of cytosolic ERs during the NBS in ewes (Clarke et al., 1981). Others have reported an increase in receptors for estradiol during the BS in the POA, with no changes detected in ventromedial nucleus or ARC of ewes (Skinner & Herbison, 1997). Thus, it is likely that the increased sensitivity to the negative feedback effects of estradiol during the NBS may involve an increase in the number of ER in the hypothalamus, but studies related with seasonal changes in specific estrogen receptor subtypes (ERα-ERβ) in different hypothalamic regions like the POA, AHA, ARC and ME are not available for the ewe.

Interestingly, the increased sensitivity to the negative feedback effects of estradiol that takes place during the NBS in ewes (Legan et al., 1977; Goodman et al., 1982), also occurs during pre-pubertal development in ewes (Foster et al., 1979), heifers and bull calves (Foster and Ryan, 1979; Day et al., 1984; Amann et al., 1986; Kinder et al., 1995) and during postpartum anestrus in ewes and cows (Acosta et al., 1983; Wise et al., 1986). A decrease in the number of estrogen receptors in the anterior and posterior hypothalamus was observed in pre-pubertal bull calves and heifers as the animals approach the onset of puberty and escape from the increased sensitivity to

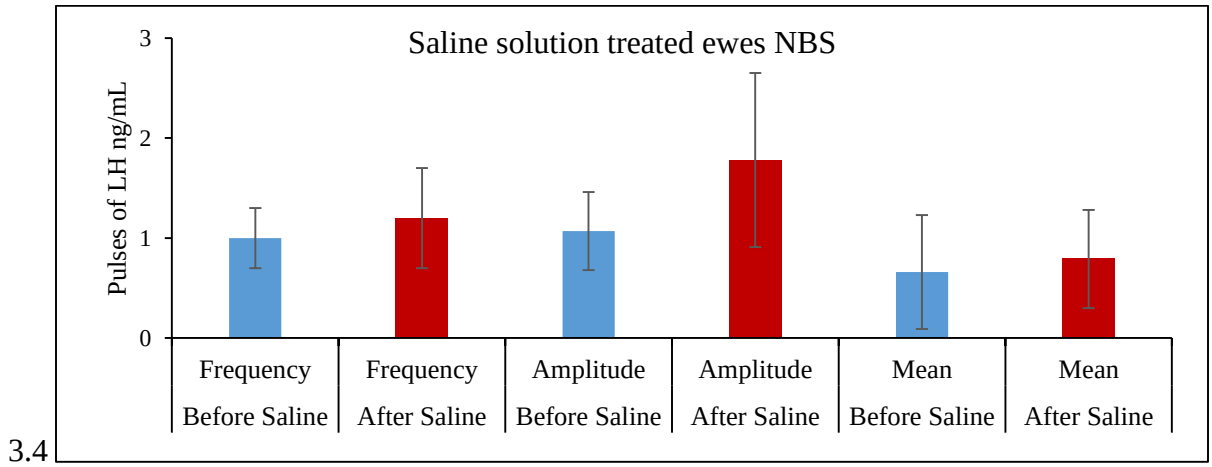
the negative feedback effects of estradiol (Amann et al., 1986; Day et al., 1987). The decrease in concentrations of ERs observed in the anterior and posterior hypothalamus was correlated with an increase in the number of LH pulses as those animals approached puberty (Amann et al., 1986; Day et al., 1987).

Along with changes in the number of ERs between seasons, changes in the number of kisspeptin receptors (GpR54) may also occur between the BS and NBS in ewes. Similar to ewes, prepuberal rodents and girls are very sensitive to the negative feedback effects of estradiol, and this sensitivity is reduced or lost as these species approach puberty (Foster et al., 1979; Navarro et al., 2004; Winter and Faiman, 1973; Messinis, 2006). The decreased sensitivity to the negative feedback effects of estradiol may be associated with an increase in GpR54 since mice, rats and primates have increased *GpR54* levels as they approach puberty (Herbison et al., 2010; Navarro et al., 2004; Shahab et al., 2005).

However, and contrary to most reports showing an increase in *GpR54* expression as rodents and primates approach puberty, in ewes hypothalamic expression of *GpR54* mRNA decreases during the BS (when ewes have a reduced sensitivity to estradiol) compared to the NBS (Li et al., 2012). At least in ewes, the decrease in the expression of *GpR54* observed during the BS appears to be caused by a mechanism involving an increase in kisspeptin input. For example, administration of kisspeptin during the NBS to ovariectomized ewes decreases *GpR54* gene expression (Li et al., 2012).

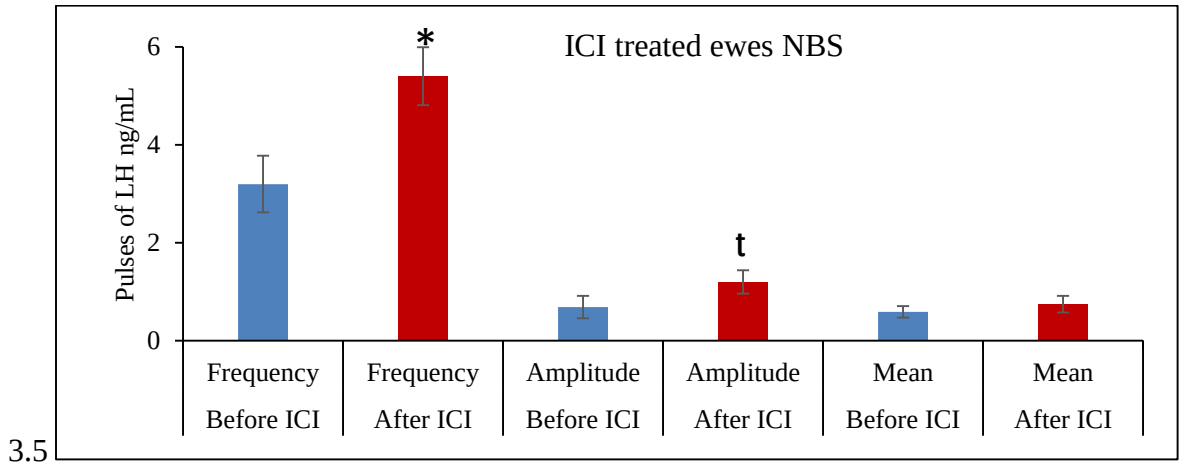
As formerly mentioned, the increased LH pulsatility observed in ICI treated ewes was probably the consequence of a higher kisspeptin input to the GnRH neuronal system. Whether seasonal changes in the degree of kisspeptin input to GnRH neurons leads to a decrease (BS) or an

increase (NBS) in the number of GpR54 due to a higher internalization rate requires further investigation.



3.4

Figure 3.4. Comparison of frequency, amplitude and mean LH before saline administration (blue bars) and at day 27 after saline solution administration (red bars). No statistical difference was observed in the frequency, amplitude or mean LH between groups.



3.5

Figure 3.5. Comparison of frequency, amplitude and mean LH before ICI treatment (blue bars) and at day 27 after the administration of ICI (red bars). The frequency of pulses before ICI were 3.2 ± 0.58 compared to 5.4 ± 0.59 after ICI administration. The ICI treatment increased the number LH pulses ($P < 0.02$) and tended to increase the amplitude of LH pulses ($p = 0.15$). No change was observed in the amplitude or mean LH with ICI treatment.

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CHAPTER 4. THE BLOCKADE OF THE KISSPEPTIN SYSTEM OUTSIDE OF THE BRAIN BLOOD BARRIER IN SHEEP AND ITS EFFECTS ON LH PULSATILITY/PREOVULATORY LIKE SURGE

Summary:

Kisspeptin administration promotes the release of GnRH and LH in ewes and the stimulatory actions of kisspeptin over the GnRH system can be exerted at different hypothalamic regions as the POA, AHA, MBH and ME. In the ovine species *GpR54* mRNA expression has been confirmed in the POA and MBH and the presence of GpR54 protein is suggested in the POA, MBH and ME. However GpR54 protein has not been confirmed in any hypothalamic region and there are not current studies related with the immuno neutralization of kisspeptin or the blockade of its receptor in specific hypothalamic regions. It is currently unknown whether the central i.e. POA, AHA, MBH or peripheral i.e. ME is sufficient to block kisspeptin release and thus GnRH and LH secretion. The ME is a circumventricular organ (CVO) that allows the exit of polypeptide hormones from the brain without affecting the blood brain barrier (BBB) and antibodies to kisspeptin might have access to the ME allowing the blockade of the kisspeptin system at this level. It was hypothesized that antibodies to kisspeptin will block kisspeptin axon terminals in the ME reducing the input of kisspeptin over the GnRH axon terminals in this region thus lowering the release of GnRH and hence LH secretion. In order to test this hypothesis, four ovariectomized ewes were immunized, antiserum to kisspeptin produced and its effects of LH secretion (for inference GnRH) studied during the BS. Blood samples were collected for a period of four hours and the parameters related with LH pulsatility evaluated in immunized and control ewes. A preovulatory like surge of LH was induced with the intra muscular injection of 50 ug of estradiol

and the amplitude and area under the curve (total LH) compared between groups. When compared to controls, immunized sheep had a dramatic tendency for lower average (controls vs immunized = 7.26 ± 1.23 vs 2.25 ± 0.47 ng/mL, p = 0.06) and basal (5.62 ± 0.97 vs 2.69 ± 0.81 ng/mL, p = 0.07) concentrations of LH. Immunized ewes had a reduced average (p = 0.04), a tendency for a lower amplitude (p = 0.07) and total LH (p = 0.1) during the first 14 hours of the preovulatory like surge sampling period when compared to controls. The overall strong tendency for lower LH levels during the pulsatile mode of secretion and the delayed onset of the preovulatory like surge of LH in immunized ewes suggest that the immunological blockade of the kisspeptin system at the level of the ME might be of high relevance in the regulation of GnRH and thus LH secretion in ewes.

Introduction

In mammals there are a number of brain structures denoted as circumventricular organs (CVOs), the median eminence (ME), the organum vasculosum of the lamina terminalis (OVLT), adenohypophysis, area postrema (AP) and subfornical organ (SFO) (Ganong, 2000). Others considered as CVOs (Duvernoy et al., 2007) are the subcommissural organ (SCO) and the pineal gland. Some of these CVOs, like the ME allow the exit of polypeptides hormones from the hypothalamus without affecting the integrity of the blood brain barrier (BBB).

The CVOs localized around the third and fourth ventricles of the brain are characterized by having an arrangement of fenestrated capillaries with endothelial cells that are highly permeable (Duvernoy et al., 2007; Petrov et al., 1994). It has been proposed that the ME is in close communication with the portal blood since intravenous administration of tracers like trypan blue, Evans blue, ferritin and horseradish peroxidase can escape from the portal system and reach the

intercellular space in the ME (Brightman et al., 1975; Broadwell et al., 1987; Mullier et al., 2010; Rodriguez et al., 2005; Reviewed by Rodriguez et al., 2010).

A CVO involved in the regulation of gonadotropin releasing hormone (GnRH) secretion by kisspeptin is the ME. In sheep, kisspeptin axonal projections, originating in the arcuate nucleus (ARC) reach the external ME (Franceschini et al., 2006); most GnRH neurons, which are located in the preoptic area (POA) (Lehman et al., 1986; Caldani et al., 1988) send GnRH axonal projections to the ME, primarily to its internal zone.

Studies in non-human primates have demonstrated that each pulse of GnRH is accompanied by a pulse of kisspeptin at the level of the ME (Keen et al., 2008). Likewise, kisspeptin stimulates release of GnRH from ovine ME incubated in vitro (Smith et al., 2011) and studies in rodents using medial basal hypothalamus (MBH) explants treated with kisspeptin respond by secreting GnRH (d'Anglemont de Tassigny et al., 2008). Interestingly, the stimulatory effects of kisspeptin on MBH explants when paired with administration of Na^+ channel blocking tetradotoxin did not prevent the secretion of GnRH suggesting a direct action of kisspeptin on GnRH axon terminals (d'Anglemont de Tassigny et al., 2008).

The evidence provided in non-human primates by Keen and colleagues (2008), in rodents by d'Anglemont de Tassigny et al (2008), and in sheep by Smith et al (2011) suggest that kisspeptin at the ME might be sufficient to promote the secretion of GnRH from the GnRH axon terminals. The reviewed literature led us to hypothesize that antiserum to kisspeptin could block the release of kisspeptin from the kisspeptin axon terminals at the ME outside the BBB. The binding of antiserum to kisspeptin released from axon terminals was intended to eliminate kisspeptin secretion and kisspeptin input over the GnRH axon terminals and the latter was expected to block/reduce GnRH release from the ME and consequently eliminate/reduce LH secretion. With

this purpose four sheep were immunized against kisspeptin to stimulate the production of antisera to this neuropeptide and neutralize kisspeptin.

Material and Methods

Animals and Experiments

Mature western-range ewes with ages ranging from 3-5 years old with similar body weight were housed under natural light conditions at Colorado State University (Animal Reproduction and Biotechnology Laboratory facilities). All experiments were performed according to the Colorado State University Animal Care and Use Committee and complied with the guidelines of the National Institutes of Health (NIH). The experiments were carried out during the breeding season (BS) in September 2015.

Antisera production against kisspeptin and serum titer

Antisera against kisspeptin were produced with a modified ovine kisspeptin peptide (NH_2-CYNWNSFGLRY-Amide) conjugated to maleimide activated bovine serum albumin (BSA) (Walker et al., 2006). Estimated conjugation efficiency was close to 100 %. Therefore, 30 to 35 molecules of kisspeptin were attached to each BSA molecule. Four ewes were immunized at multiple intradermal sites with 1.0 mg of kisspeptin conjugate in complete Freund's adjuvant and boosted subcutaneously at monthly intervals with 0.5 mg of conjugate in incomplete adjuvant. Serum was obtained every two weeks and checked for the binding to the radioiodinated ligand, ^{125}I – ovine kisspeptin-10. To corroborate the specific immunodetection of kisspeptin, antisera were incubated with different hypothalamic hormones (inhibition curves) that included oxytocin, gonadotropin inhibitory hormone (GnIH), gonadotropin inhibitory hormone related peptide

(GnIH-RP), gonadotropin releasing hormone (GnRH), neurokinin B (NKB), ovine kisspeptin-10 and ovine kisspeptin-16.

Experimental designs

The experimental objective was to eliminate the input of kisspeptin to GnRH axon terminals and thus eliminate or dramatically reduce the pulsatile secretion of GnRH and consequently LH release during the BS (September). With this purpose four ewes were immunized in April 2015. Serum was obtained every two weeks and checked for the binding to radiolabeled ^{125}I – ovine kisspeptin 10. Six ewes were used as controls and their LH secretory profiles compared to ewes immunized against kisspeptin.

Ewes were ovariectomized for at least three weeks before the blood sampling period. The experiment was performed in September 2015. The right jugular vein of each ewe was fitted with a cannula (Angiocath; Becton Dickson) 12 hours prior to blood withdrawal. Blood samples were collected every 15 minutes for a period of 4 hours (8 AM- 12 PM) to quantify the number of LH pulses. Basal LH was defined as the five lowest concentrations of LH within each four hour period for each ewe. Mean LH was determined as the average concentration of LH within the four hour period. A pulse of LH was defined as a concentration greater the average detected during the four hour period plus two standard deviations and was followed by at least one descending concentration. Ten hours later (10 PM) animals were administered 50 ug of estradiol i.m. Ten hours after estradiol (8 AM) blood samples were collected every two hours for 16 hours and parameters associated with the preovulatory like surge of LH quantified. The effects of immunization on the preovulatory-like surge of LH that were evaluated included amplitude,

defined as the highest concentration of LH detected for each animal during the preovulatory like surge of LH, and area under the curve (AUC) or total LH during the 16 hours collection period.

A preovulatory-like surge of LH was defined as the increase in LH secretion preceded by at least two ascending concentrations and followed by at least one descending concentration (adapted from Clark and Cummins, 1984 and Clark et al., 1988).

Determination of LH concentrations in serum

All blood samples were allowed to clot for six hours before placing them at 4°C overnight. Serum was obtained by centrifugation at 2500 rpm (1500 *g*) for 15 minutes and stored at -20º C for subsequent quantification of LH (Niswender et al., 1969). The reference standard for LH was oLH-S24. Three standard curves were included per assay and samples were analyzed in duplicate.

The intra-assay coefficient of variation was 7.3 % for high values (8 ng/mL of LH), which usually results in about 20% binding (relative to total binding or B0). The intra-assay coefficient of variation was 11.03 % for low values (600 pg/mL of LH) which resulted in about 95% binding (relative to total binding or B0). The limit of LH detection was 9.0 pg/tube.

Data Analysis

Data were evaluated by ANOVA using the general linear model of SAS (SAS: SAS User's Guide SC. NC: Statistical Analysis System Institute, Inc.; 1987). The following parameters were analyzed: basal LH, number of pulses, amplitude and mean LH. The number of LH pulses (which does not have normal distribution and due to the great difference in standard deviations between groups) was subject to arcsine transformation. A completely randomized design was used to

evaluate the parameters of the preovulatory-like surge of LH. Data are presented as Mean ± SEM and statistical differences with p values equal to or less than 0.05 were considered significant.

Results

Antisera from each were checked for the specific immunodetection of ovine kisspetpin-10 (okp) and ovine kisspeptin-16 (okp-16). From the four ewes immunized against kisspeptin, three produced antibodies to this neuropeptide with titers ≥ 1:1000 (i.e. bound about 50 % of 50,000 cpm of ^{125}I-ovine kisspeptin). The antisera obtained from ewe number 284, 295, and 299 cross reacted with ovine kisspeptin 10 and ovine kisspeptin 16 only and their cross reactivity was close to 100 % and these antisera did not bind RF-amide related peptides (GnIH, GnIH-RP), oxytocin, GnRH or neurokinin B (Figure 4.1, Figure 4.2 and 4.3).

The pulses of LH detected during the four hour period in ewes immunized against kisspeptin are shown in Figure 4.4. The average concentration of LH in immunized ewes was 3.49 ± 0.94 ng/mL while the basal concentration was 2.69 ± 0.81 ng/mL (Figure 4.6). Ewes immunized against kisspeptin had an average of 2.25 ± 0.47 pulses during the four hour period. The amplitude of LH secretion was 7.91 ± 2.37 ng/mL (data is presented as Mean ± SEM), Figure 4.6. Control ewes had an average LH concentration of 7.26 ± 1.23 ng/mL and a basal LH of 5.62 ± 0.97 ng/mL. The number of pulses averaged 2.67 ± 0.21 (frequency) whereas the amplitude was 11.64 ± 2.19 ng/mL, Figure 4.6. Average concentrations of LH tended to be lower in ewes immunized against kisspeptin ($p = 0.059$, Figure 4.6). Similarly, the basal concentration of LH tended to be lower in immunized ewes ($p = 0.065$). No significant reduction was detected in the frequency of LH pulses in immunized ewes ($p = 0.39$). The average LH amplitude was not significantly different between groups ($p = 0.29$), Figure 4.6.

Preovulatory-like surges of LH in the group of ewes immunized against kisspeptin are represented in Figures 4.7. LH surges in controls are illustrated in Figure 4.8. In immunized ewes, the amplitude was 129.91 ± 34.04 ng/mL, Figure 4.9. The AUC of LH (total) in immunized animals averaged 303.67 ± 62.51 ng. In control ewes the amplitude was 110.24 ± 27.60 ng/mL and the AUC averaged 295.48 ± 70.91 ng (Figure 4.9). No differences were detected neither in the amplitude ($p = 0.66$) nor in the AUC of the LH surge ($p = 0.93$) between immunized and control ewes, Figure 4.9. The onset of the preovulatory like surge of LH was delayed, a decrease in the average LH ($p < 0.05$), a tendency for lower amplitude ($p = 0.07$) and total LH ($p = 0.1$) was observed in immunized ewes when compared to controls during the first 14 hours.

Discussion

There are multiple pathways by which kisspeptin might regulate the GnRH system and hence, secretion of LH. Some pathways may include the regulation of GnRH neurons in the POA by kisspeptin. For instance kisspeptin neurons in the POA are important for the generation of the LH surge in ewes, as evidenced by Hoffman et al. (2011), who observed an increase in c-Fos expression in kisspeptin neurons of the POA during the GnRH/LH surge. GnRH neuronal activity may also be modulated at the level of the AHA by kisspeptin neurons originating in the POA and/or ARC (Smith et al., 2008a). Additionally, GnRH neurons and neuronal axon terminals are found in the MBH, especially in the ARC and ME (Caldani et al., 1988; Lehman et al., 1986; Uenoyama et al., 2011) and these hypothalamic regions might be relevant for the modulation of GnRH and thus LH secretion by kisspeptin since they possess a large number of kisspeptin neurons and axons (Lehman et al., 2010; Franceschini et al., 2006; Smith et al., 2011). In this regard, Wakabashi et

al. (2010) observed that multiple-unit activity spikes in the medio basal hypothalamus accompanied the pulsatile secretion of LH in goats.

Interestingly, we are beginning to understand how kisspeptin neurons in the AVPV/POA and ARC connect with the GnRH system (Smith et al., 2008a; Yip et al., 2015), but it is currently unknown as to what percent of kisspeptin neurons in the POA and ARC connect to the GnRH system in any species.

The profiles of LH observed during the pulsatile mode of secretion in immunized ewes suggest that a considerable amount of kisspeptin might have access to the outside of the BBB since a tendency for lower average ($p = 0.059$), and basal LH ($p = 0.065$) was observed (Figure 5.8) despite the low anti-kisspeptin titers. These data (lower LH) support the notion that kisspeptin input in the ME could be sufficient to modulate pulsatile GnRH and thus LH secretion.

Relevant to the present experimental design (immunization of ewes against kisspeptin designed to promote the production of antibodies to kisspeptin and block the secretion of this hormone from the ME) are the results of Smith and colleagues (2011) who found that ME explants treated with kisspeptin released GnRH *in vitro* further supporting the hypothesis that the blockade of kisspeptin at the level of the ME could dramatically affect GnRH secretion. Interestingly, even though it has been shown that the administration of kisspeptin to ME explants promotes the release of GnRH (Smith et al., 2001), whether a complete blockade of kisspeptin axonal terminals outside of the BBB in the ME is sufficient to eliminate kisspeptin input to GnRH axonal terminals and hence eliminate GnRH/LH secretion has not been proved.

The lack of a significant reduction of LH in ewes immunized against kisspeptin led us to speculate as to potential causes. One possible explanation is that it is more difficult to efficiently block the kisspeptin input to GnRH axons with low titered antiserum (1:1000). For instance, in the

study of Adams and Adams, (1986) it was reported that after actively immunizing ovx ewes against GnRH all animals (n=25) had titers of antibodies that exceeded 1:5000. Interestingly in the latter study, it was observed that basal LH secretion as well as the preovulatory like surge of LH were dramatically suppressed.

The immunological blockade of GnRH have produced interesting information in ewes. For instance, Adams and Adams, (1986) reported a dramatic reduction of the preovulatory like surge of LH in ovx ewes actively immunized against GnRH with titers of 1:5000, while Narayana and colleagues (1979) were not able to uniformly block the LH surge in intact ewes that were passively immunized against GnRH (utilizing 30 mL of antiserum with a 1:2000 titer). The low titered antiserum used in the study by Narayana and colleagues (1979) and low efficiency observed in the present study (ewes immunized against kisspeptin) supports the hypothesis that high titers are required to efficiently block generation of the kisspeptin/GnRH surge.

Likewise, in the study of Adams and Adams, (1986) the blockade of GnRH was efficient, potentially because once GnRH is secreted into the portal system it has to travel a short distance in the blood where it was exposed to the GnRH antibodies. Contrary to GnRH, which in theory is properly exposed to antibodies when secreted into the portal blood, it is possible that kisspeptin does not significantly reach the portal system and/or that kisspeptin mainly travels from the kisspeptin axon terminal to the GnRH axon terminal, both of which are present in the ME.

It is unclear as to whether kisspeptin has access to the portal blood during each pulse of GnRH or during the generation of the preovulatory like surge of GnRH. Studies in non-human primates have reported that each pulse of GnRH detected by pull-push perfusion in the ME is accompanied by a pulse of kisspeptin-54 (Keen et al., 2008). Whether this pulsatile input of kisspeptin at the level of the ME is accompanied by a pulsatile secretion of this neuropeptide to

the portal system has not been reported in any species studied to date. For instance, studies in ewes have not been able to detect an increase in kisspeptin in the portal system during the GnRH surge (Smith et al., 2008b) suggesting that no significant increase in portal kisspeptin accompanies the generation of the preovulatory surge of GnRH/LH.

It is also important to remember that in ewes an intramuscular injection with a high dose of estradiol (50 ug) promotes an acute inhibitory feedback at the level of the hypothalamus (Karsch et al., 1987; Caraty et al., 1998) by decreasing GnRH secretion and at the same time promoting a reduction in the secretion of LH from the pituitary (Arreguin-Arevalo et al., 2010). The acute inhibitory actions of estradiol are followed about 12 hours later by an increase in the hypothalamic secretion of GnRH that leads to a massive surge secretion of LH from the pituitary (Arreguin-Aervalo and Nett, 2006).

Before promoting an increase in the release of GnRH from the hypothalamus, estradiol induces an increase in the number of GnRH receptors in the pituitary (Nett et al., 1984; Crowder and Nett, 1984; Clarke et el., 1988) and it seems that by promoting an increase in GnRHR number prior to the massive release of GnRH, estradiol enhances the responsiveness of the gonadotropes to the stimulatory input of GnRH (Clarke, 1995). It is therefore probable that the increased sensitivity to the input of GnRH induced by estradiol makes the phenomenon of the preovulatory surge of LH pretty efficient. Then it is probable that low doses of kisspeptin may be sufficient to promote a GnRH/LH surge. The studies of Bowen and colleagues (1995) indirectly supported this hypothesis. In that study, it was observed that the massive amount of GnRH that promotes the generation of the LH surge exceeds that required for the production of a normal LH surge in ewes.

Additionally, the findings of Dungan and colleagues (2007) further support the hypothesis that low kisspeptin is sufficient for the generation of the GnRH/LH surge. These researchers

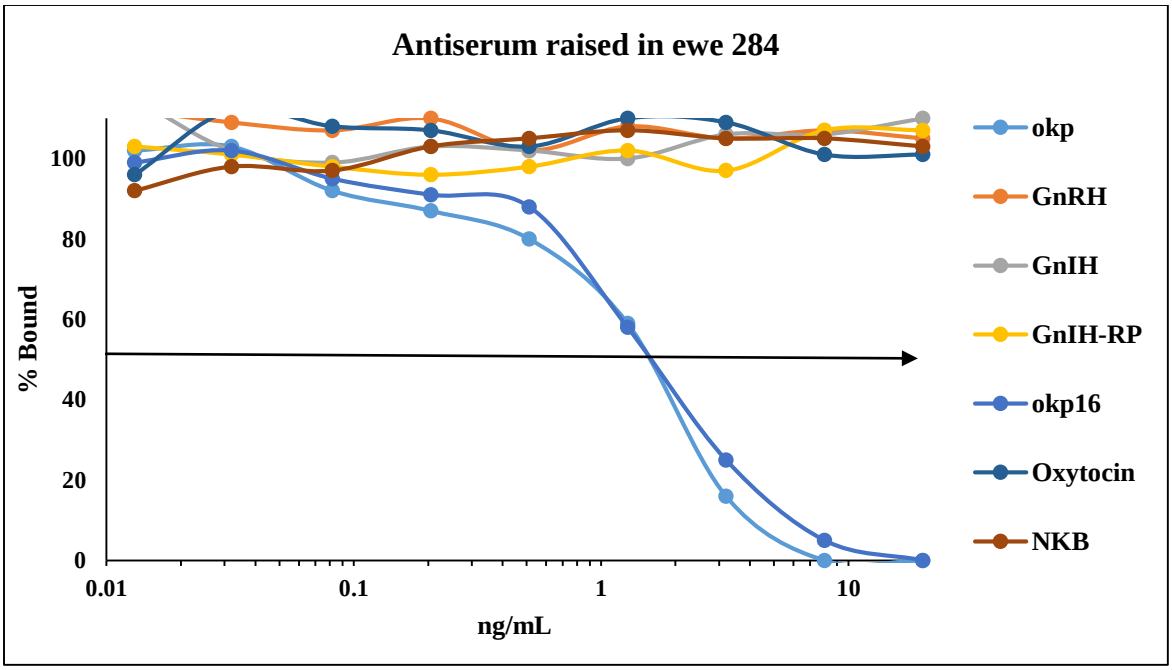
reported that GPR54 knock out mice were able to mount a surge of LH after estradiol administration. Interestingly, Dungan and colleagues concluded that activation of GPR54 was necessary for the pulsatile secretion of LH but not for the generation of the preovulatory surge of GnRH/LH in mice (Dungan et al., 2007). Whether the findings of Dungan reflected an incomplete knock out of the *GpR54* gene allowing for the generation of the preovulatory surge of GnRH/LH in mice requires further investigation.

Experiments by Popa and colleagues support the notion that low amounts of kisspeptin are sufficient for normal secretion of GnRH/LH (Popa et al., 2013). They observed that about 5% of kisspeptin production is needed for normal fertility in mice and suggested that females require higher levels of kisspeptin input for reproductive success than males, potentially because kisspeptin/GnRH in females is required for the induction of the LH surge. Moreover, Mayer et al. (2011) showed that in female mice that had the *KiSS1* gene ablated during development reached puberty between post-natal day 30 and 40 and they become fertile.

Alternatively, a possible cause for a lack of a significant reduction in LH secretion in kisspeptin immunized ewes is that not all GnRH neurons respond to kisspeptin, and some have a kisspeptin independent or kisspeptin indirect GnRH secretion mechanism. For instance, Dumalska and colleagues (2008) reported the existence of two groups of GnRH neurons in mice, one responsive to kisspeptin only, and another that responds only to glutamate receptor agonists. In this regard, Pielecka-Fortuna et al. (2008) observed that kisspeptin stimulates GnRH neuronal activity by transsynaptic mechanisms in ovariectomized mice treated with estradiol. The evidence provided by Pielecka-Fortuna and colleagues supports the notion that the blockade of kisspeptin outside of the BBB may not be sufficient to eliminate the input of kisspeptin over the GnRH system. Additional evidence for an indirect modulation of GnRH neuronal activity comes from the

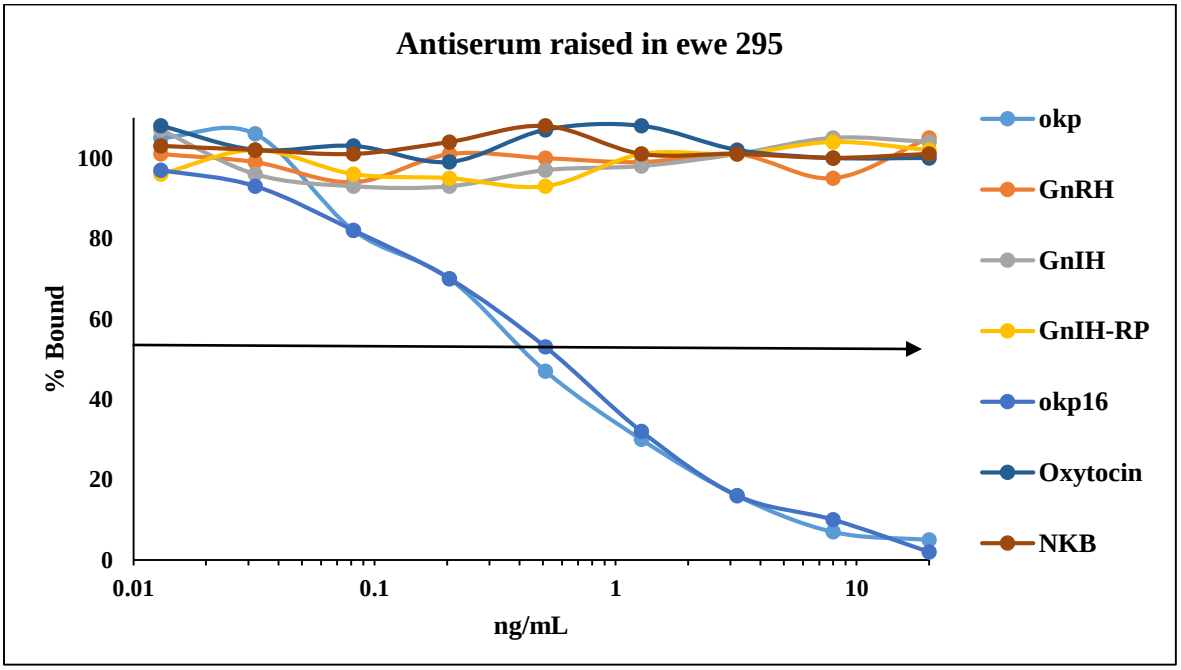
studies of Hanchate et al. (2012) who reported that kisspeptin stimulation of nitric oxide (NO) production and release in the vicinity of GnRH neurons was linked with the positive feedback effects of estradiol corroborating the importance of indirect mechanisms activated by kisspeptin during the generation of the GnRH/LH surge.

The input of kisspeptin in the ME might be relevant for the pulsatile GnRH and thus LH secretion. This hypothesis is supported by the tendency of lower average and basal LH observed during the pulsatile mode of secretion in immunized ewes. On the other hand, the induction of higher anti-kisspeptin titers could permit testing of the hypothesis of whether a complete blockade of kisspeptin in the ME is sufficient to significantly reduce or eliminate kisspeptin input over GnRH axon terminals, thus efficiently blocking pulses and/or a surge of GnRH/LH. Likewise, it will be relevant to block kisspeptin without interfering with other physiological condition regulated by this neuropeptide. The development of agonists or antagonists that can specifically act outside of the BBB could make it possible to directly block the portion of the kisspeptin involved in reproduction.



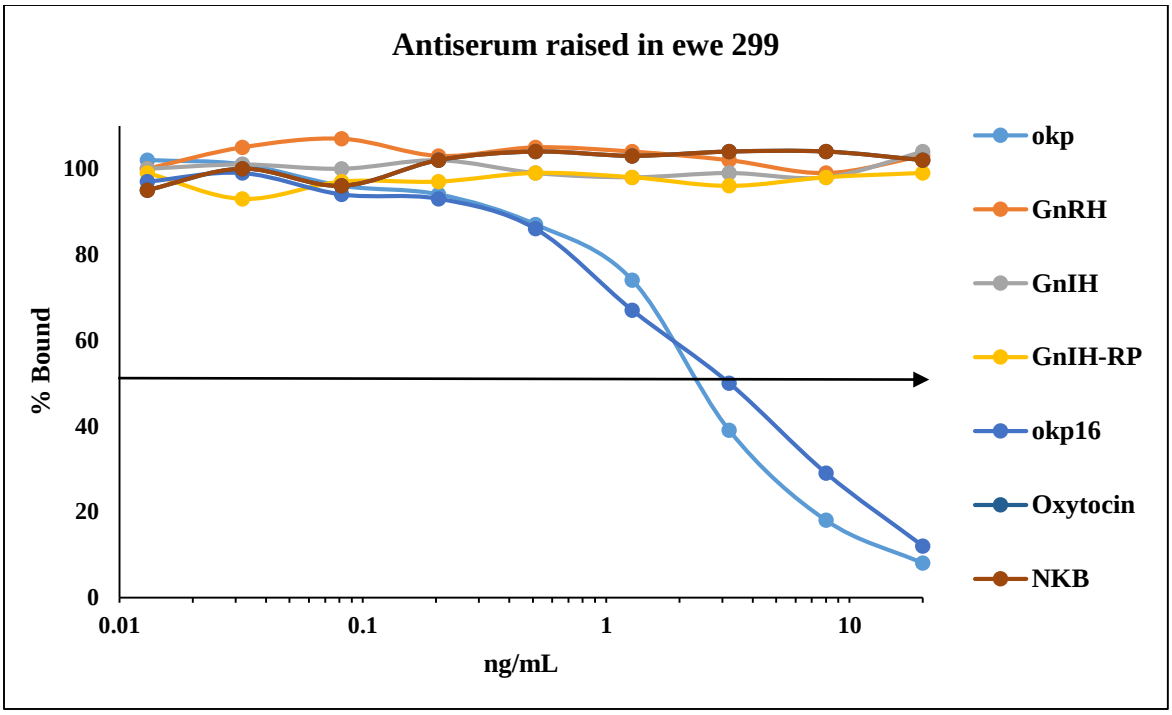
4.1

Figure 4.1. Cross reactivity of the antiserum raised against ovine kisspeptin obtained from ewe number 284. Inhibition curves obtained with several hypothalamic neuropeptides. Black line arrow depicting the amount of hormone required to inhibit the binding of ^{125}I - okp-10, 50,000 (cpm) to 50 %.



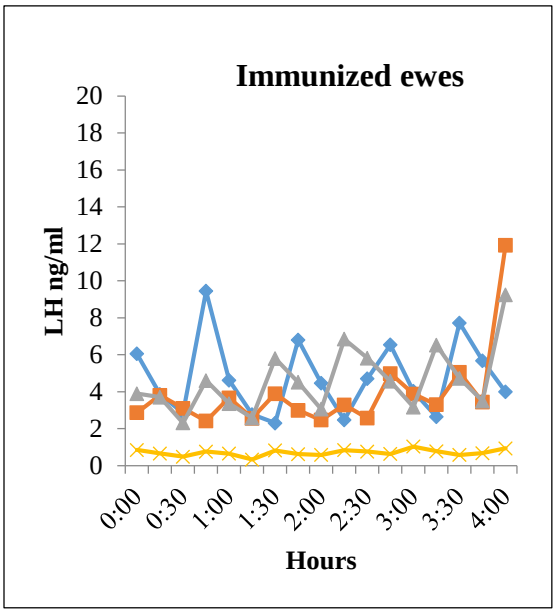
4.2

Figure 4.2. Cross reactivity of the antiserum raised in ewe number 295.

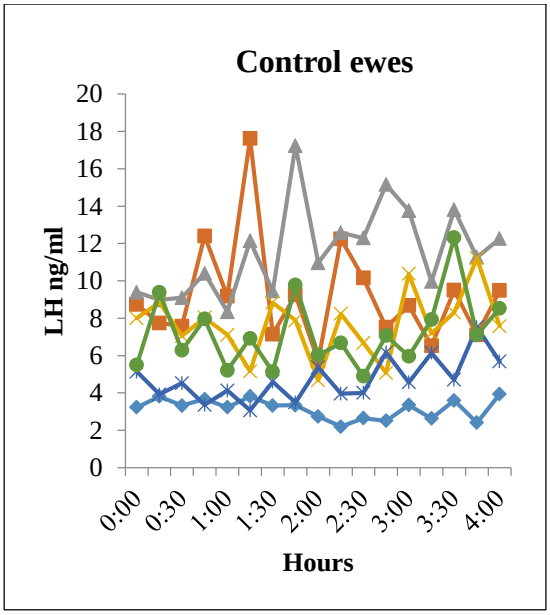


4.3

Figure 4.3. Cross reactivity of the antiserum raised in ewe number 299.

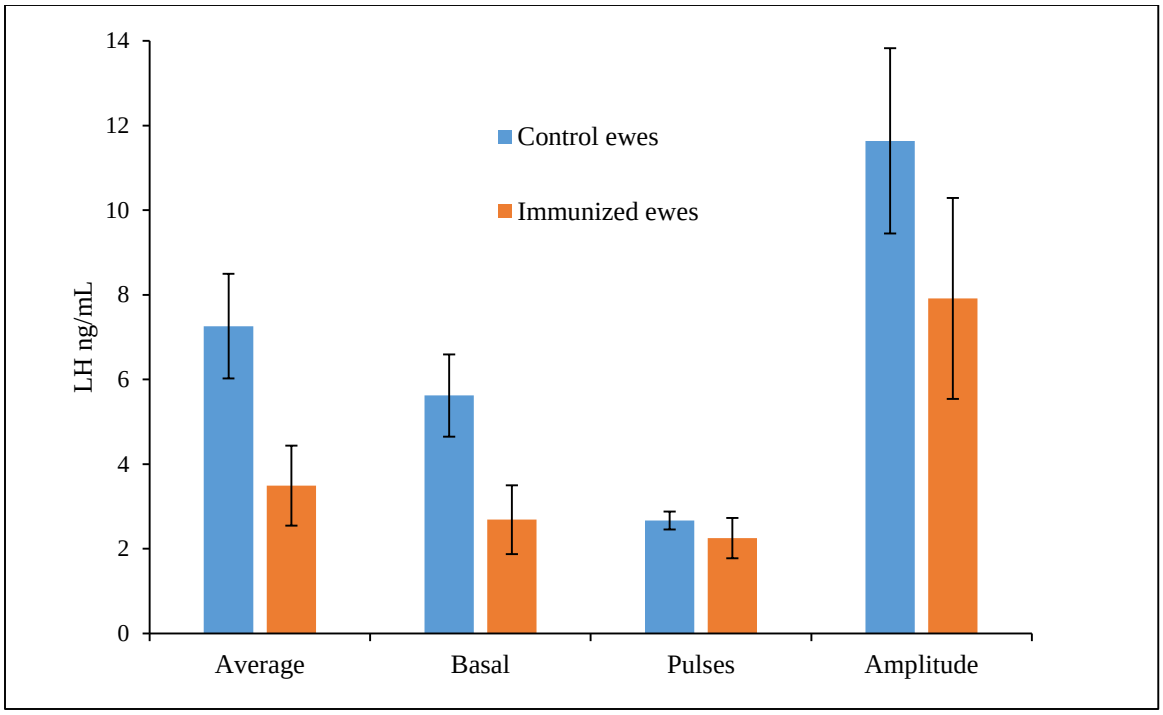


4.4



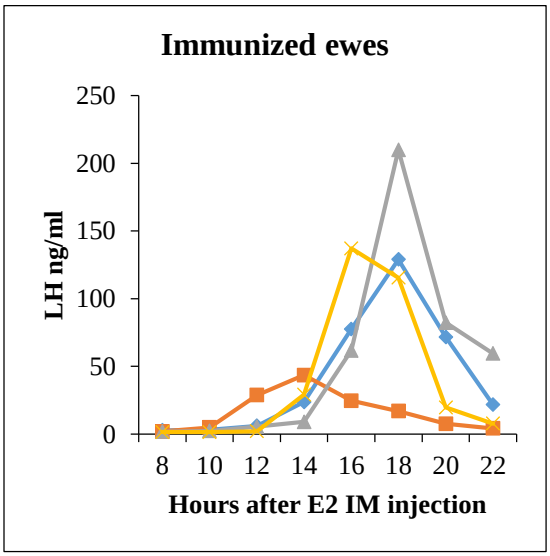
4.5

Figure 4.4. LH profiles during the BS of four ewes immunized against kisspeptin. LH profiles of control sheep during the BS, Figure 4.5.

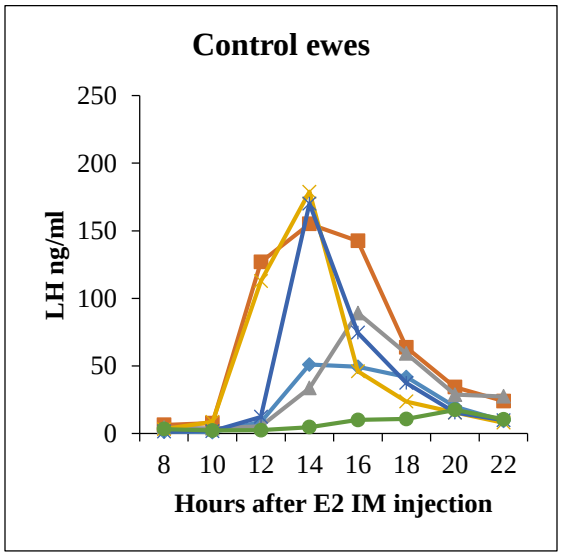


4.6

Figure 4.6. Comparison of LH parameters during a four hour sampling period between controls and immunized ewes. The immunization against kisspeptin resulted in a tendency for lower average (p = 0.059) and basal concentration (p = 0.065) of LH. No significant reduction in the number of pulses (p = 0.39) or LH amplitude was observed between groups (p = 0.29).

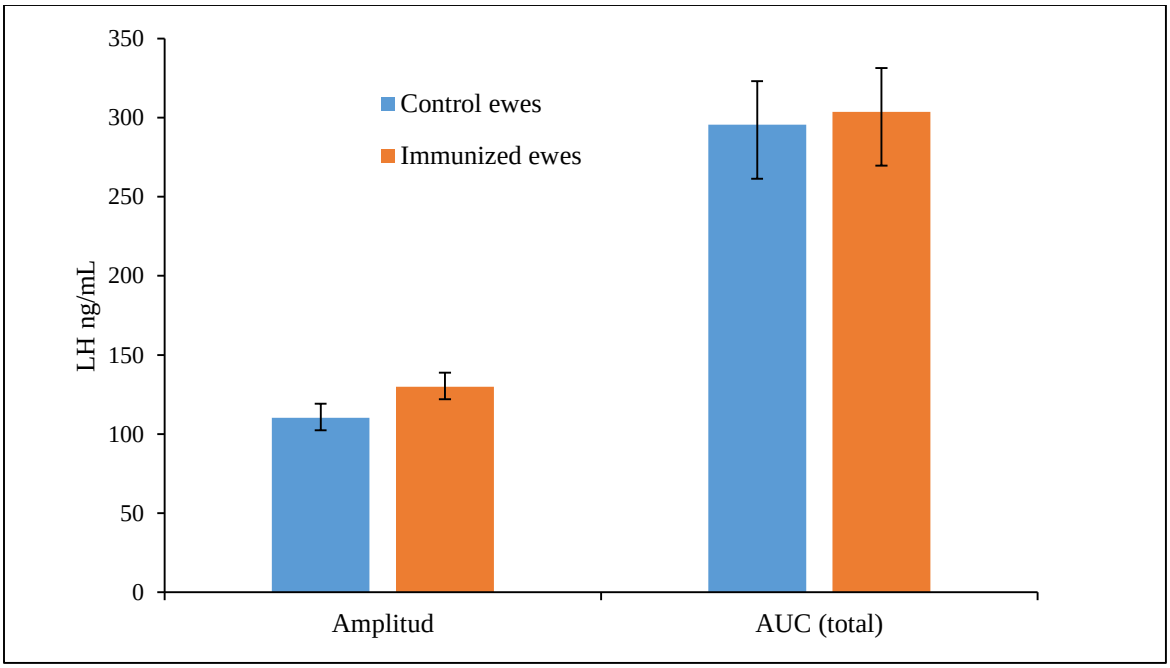


4.7



4.8

Figure 4.7. Preovulatory like surges of LH in ewes immunized with kisspeptin. Surges of LH of non-immunized ovx control sheep, Figure 4.8.



4.9

Figure 4.9. Parameters related with the preovulatory like surge of LH in ewes immunized against kisspeptin (orange bar) and controls (blue bar). No reduction was observed in amplitude ($p = 0.66$) and AUC (total) of LH ($p = 0.93$) with the immunization against kisspeptin.

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GENERAL DISSCUSSION

This dissertation was intended to advance the knowledge in regards to the way kisspeptin modulates the secretion of GnRH, and therefore LH. The goal of the first study was to determine if there are changes in the content and concentrations of kisspeptin in different hypothalamic regions between the BS and NBS of ewes. To this end, a specific RIA was developed validated, and used to quantify the concentrations and content of kisspeptin during the BS and NBS in hypothalamic regions that included the POA, AHA, MBH and ME. Through utilization of ovariectomized ewes implanted with estradiol, it was possible to determine that the content (total) and concentration (per mg tissue) of kisspeptin in the MBH was less during the NBS compared to the BS. These findings support the hypothesis that during the NBS estradiol exerts an increased negative feedback effect over the kisspeptin neurons located in the MBH which includes the ARC.

Additionally, evidence that estradiol modulates the production of kisspeptin in hypothalamic regions in addition to the MBH was provided, since a reduction in the concentrations and content of kisspeptin was detected at the level of the POA during the NBS. This finding is highly relevant to sheep as the largest population of GnRH neurons reside in the POA (Jansen et al., 1997; Lehman et al., 1986; Caldani et al., 1988) and kisspeptin might differentially modulate the GnRH neuronal activity by season in this hypothalamic area as indirectly suggested by the findings of Chalivoix et al. (2010). The decreased concentration and content of kisspeptin quantified in the MBH and POA provide strong evidence that a reduced kisspeptin input on GnRH neurons leads to a drop in GnRH and thus LH pulsatility during the NBS (Karsch et al., 1980).

Likewise, there was a strong tendency for a lower content and concentration of kisspeptin in the AHA during the NBS. The kisspeptin RIA results suggested that the AHA might be an

additional hypothalamic region where kisspeptin differentially modulates the GnRH neuronal system by season.

Quantitative methodologies like the kisspeptin RIA advance the current knowledge concerning the regulation of kisspeptin production by estradiol by allowing quantification of kisspeptin protein in different hypothalamic regions for the first time. Moreover, the high sensitivity of the kisspeptin RIA permitted detection of low concentrations of kisspeptin in extra-hypothalamic regions like the cortex, brain stem and cerebellum where the function of this neuropeptide is still unknown. Determining a role for kisspeptin in these areas requires further investigation.

The second study was intended to provide information related with the increased sensitivity to the negative feedback effects of estradiol during the NBS in ewes. The goal was to block the central inhibition exerted by estradiol during the NBS by using the estrogen antagonist (ICI 182 780) in order to promote an increase in LH secretion. To this end, ovariectomized ewes were implanted with estradiol and subjected to cranial surgery to insert a small cannula into the lateral cerebral ventricle. The cannula was connected to an osmotic pump and the antiestrogen ICI 182 780 (ICI) administered intracerebroventricularly for 27 days.

Interestingly, an increase in LH pulse frequency 27 days after the intracerebroventricular administration of ICI was observed during the NBS. It is probable that ICI increased LH pulsatility by blocking ERα present in kisspeptin neurons of the ARC, as this receptor subtype regulates the hypothalamic pituitary axis (Franceschini et al., 2006; Dorling et al., 2003, Smith et al., 2005; Lindzey et al., 2006; Glidewell-Kenney et al., 2007; Roa et al., 2008) and is expressed in virtually all the kisspeptin neurons of the ARC in mice and ewes (Smith et al., 2005a; Franceschini et al., 2006).

The increase in LH pulse frequency in the second study led us to speculate that ICI blocked hypothalamic ERα resulting in an increased kisspeptin production at the level of the ARC, that was translated in an increase in kisspeptin input over the GnRH neuronal system, enhancing GnRH release and thus LH secretion. This speculation is indirectly supported by the fact that there is an increase in *Kiss1* mRNA levels in the ARC of ERα knock out mice while ERβ knock out mice have normal *Kiss1* levels in this hypothalamic nucleus (Smith et al., 2005a). The increase in *Kiss1* mRNA observed by Smith and colleagues (2005a) in the ARC of ERα knock out mice was probably due to the lack of a negative feedback by estradiol on the kisspeptin neurons. Supporting the hypothesis that ICI promoted an increase in kisspeptin production that resulted in a higher kisspeptin input to the GnRH system and enhanced LH secretion are the studies of Dungan et al. (2007). In that study, ovariectomy resulted in an increase in LH secretion in wild type mice but not in Gpr54 knock outs animals (Dungan et al., 2007).

As previously mentioned the reduction of kisspeptin content in the ARC of ewes could involve the activation of ERE independent (non-classical) pathways by estradiol, but studies related with the modulation of hypothalamic kisspeptin through the activation of ERα or ERβ and are needed in sheep.

Studies related with the central administration of estrogen agonists and/or antagonists specific for ERα, ERβ and GPR30, may help us to determine whether the activation/inactivation of different ERs play important roles in modulating of seasonal reproduction. Changes in the number of specific ERs in the hypothalamus could also be of physiological relevance during the BS and NBS in sheep. In addition to changes in the number of ERs, it will be relevant to quantify hypothalamic kisspeptin receptors (GPR54) between seasons in sheep and determine if changes in *GPR54* mRNA (Herbison et al., 2010; Navarro et al., 2004; Shahab et al., 2005; Li et al., 2012)

follow the same pattern as changes in GPR54 protein. Interestingly, there are no studies comparing changes in GPR54 protein during the BS or NBS in hypothalamic regions as the POA and ARC, and further research is needed to determine if an increase/decrease in GPR54 receptor occurs between seasons.

The increased LH pulsatility observed in ewes treated with ICI in the second study of this dissertation set a background for a better understanding of the mechanism involved in seasonal anestrus. The intracerebral administration of ICI and the increased LH pulsatility observed in ewes during anestrous set the bases for developing treatments to decrease the duration of the NBS (advance the BS onset and/or delay the NBS onset). Likewise, the etiology of prepuberal and postpartum/lactational anestrus are also characterized by an enhanced sensitivity to the negative feedback effects of estradiol. Then the increased LH pulsatility detected in ICI treated ewes suggest that by preventing the hypothalamic actions of estradiol it could be possible to advance puberty onset or reduce the postpartum anestrus.

The purpose of the third experiment was to investigate whether the blockade of the kisspeptin system outside of the BBB was sufficient to eliminate kisspeptin input to the GnRH axon terminals in the ME. To achieve the blockade of the kisspeptin axon terminal, ewes were immunized with a kisspeptin conjugate to stimulate the production of antisera to kisspeptin.

Based on the tendency for lower average and basal levels of LH observed in immunized ewes during the pulsatile mode of secretion, it was speculated that a higher anti-kisspeptin titer could result in a more efficient blockade of kisspeptin, reducing kisspeptin input to GnRH axon terminal and thus the pulsatile GnRH/LH secretion. Another possible explanation for the non-significant reduction of LH secretion in immunized ewes is that the secretion of GnRH, but not kisspeptin, can be efficiently immuno neutralized (outside of the BBB). Future studies allowing

the complete blockade of kisspeptin axon terminals in the ME will help to better understand the modulation of GnRH secretion by kisspeptin at this level, since is currently unknown whether the kisspeptin input to GnRH axon terminals in the ME is sufficient to drive the pulsatile and/or preovulatory surge of GnRH.

Studies involving the inhibition/stimulation of the kisspeptin system outside of the BBB might allow development of techniques specifically enhancing/blocking the kisspeptin system in the ME, and in this manner GnRH and LH secretion, without affecting other central functions of kisspeptin. For example, specific inhibition of kisspeptin at the level of the ME might help to indirectly (by reducing GnRH and thus LH) control steroid responsive cancers and/or facilitate the development of contraceptives. Conversely, kisspeptin agonist specifically acting outside of the BBB can be useful in situations where reproduction is to be enhanced, like in reproductive assisted techniques requiring higher GnRH secretion to promote LH/FSH secretion and induction of superovulation for embryo transfer programs.

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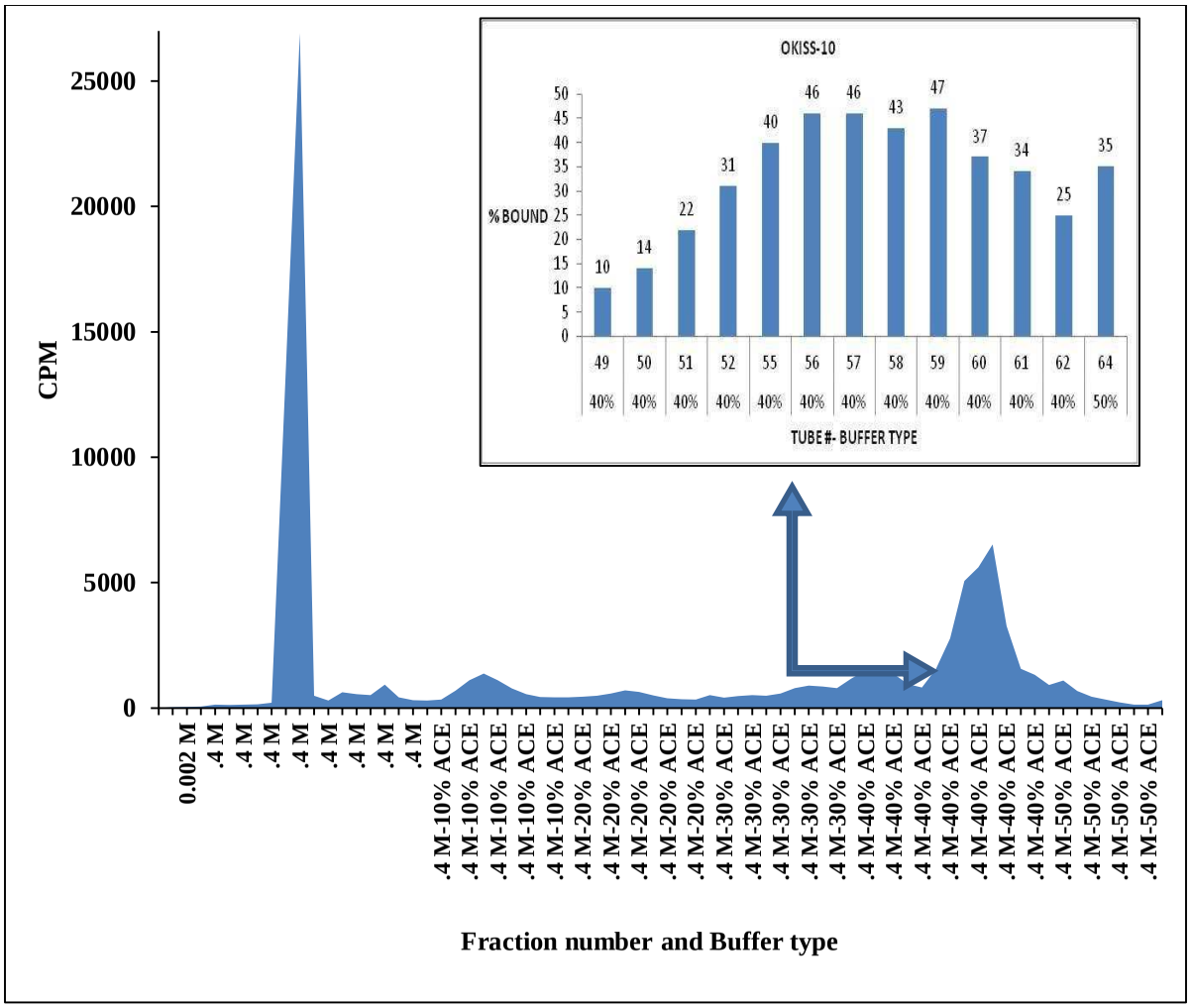
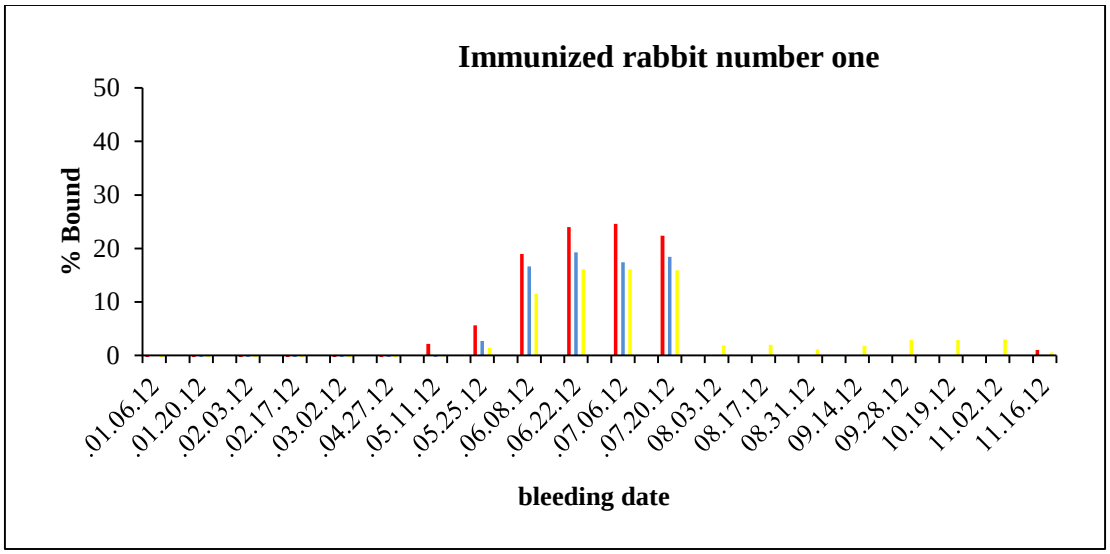
APPENDIX I

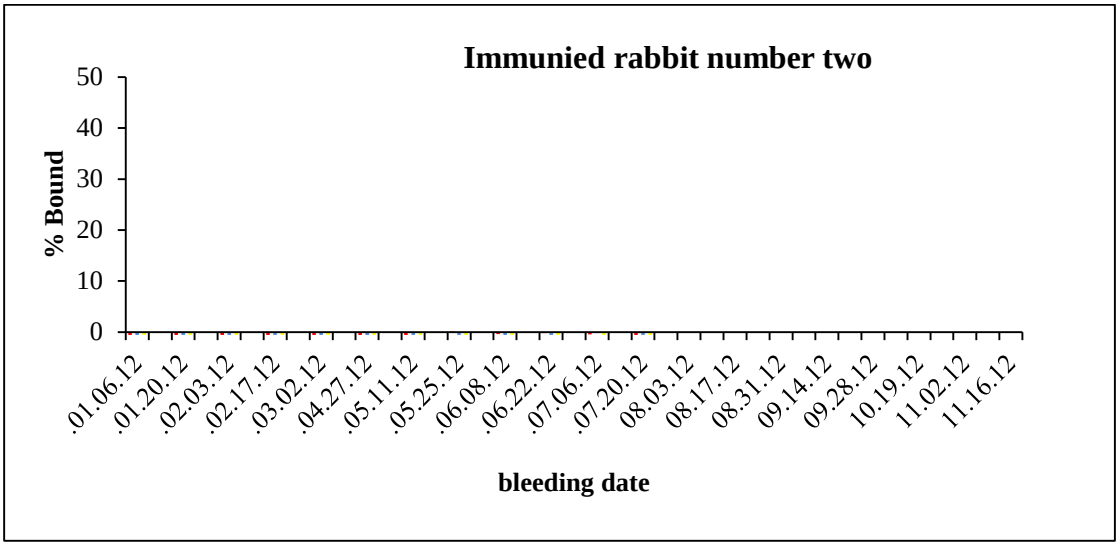
Supplemental material Chapter 2: *Hypothalamic concentration of kisspeptin and GnRH during breeding season (BS) and non-breeding season (NBS) in Sheep.*



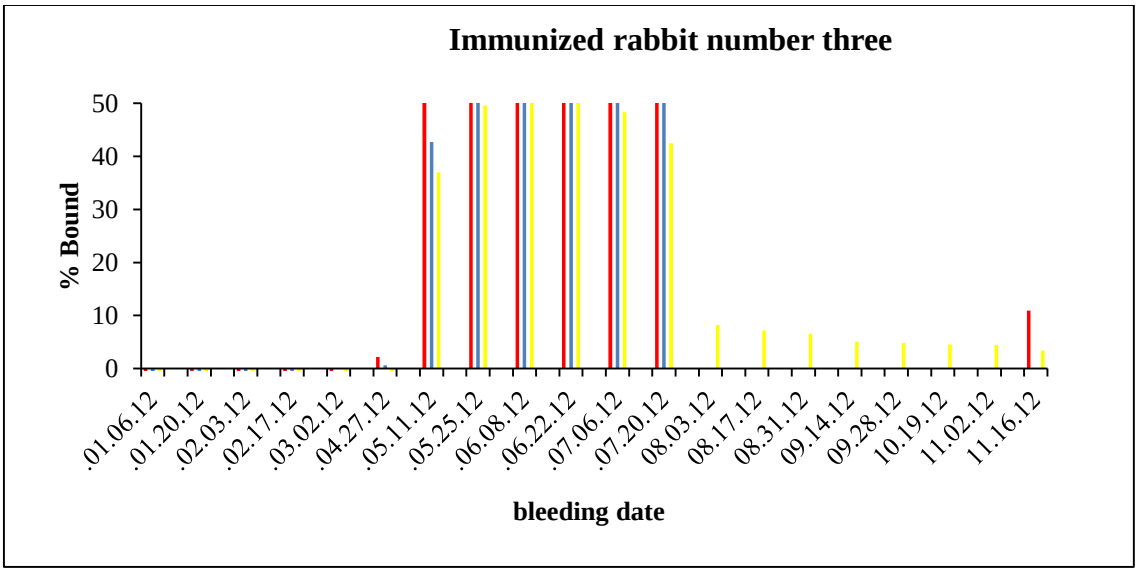
Supplemental figure 2.1. Purification of radio iodinated ovine kisspeptin-10.



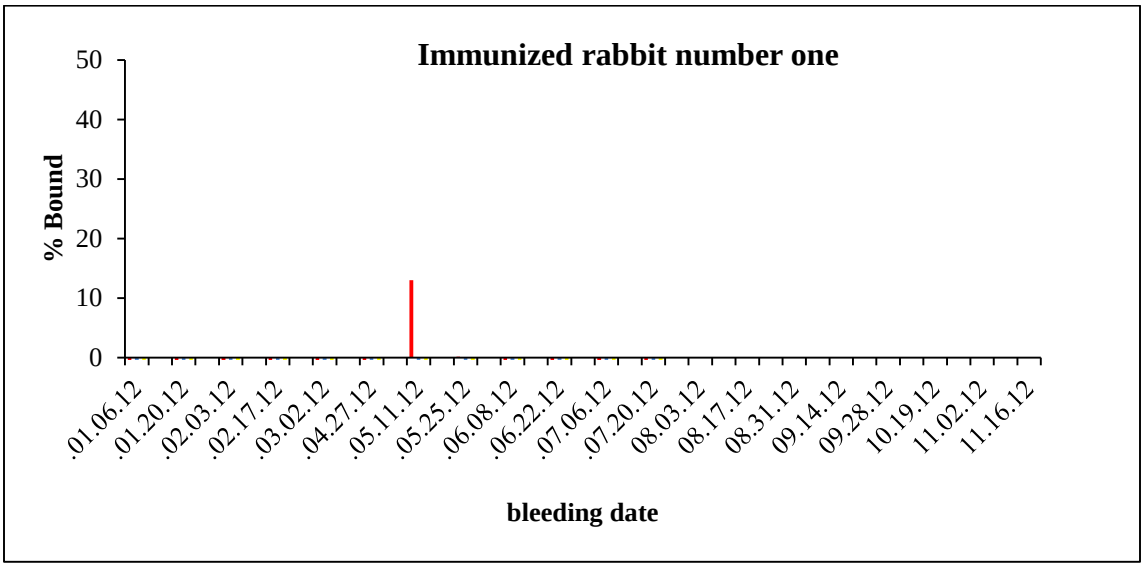
Supplemental figure 2.2. Antisera production to equine kisspeptin and binding check against ovine ^{125}I-kisspeptin-10. Immunized rabbit number one. Antisera dilution; 1:20,000 (red), 1:40,000 (blue) and 1:60:000 (yellow).



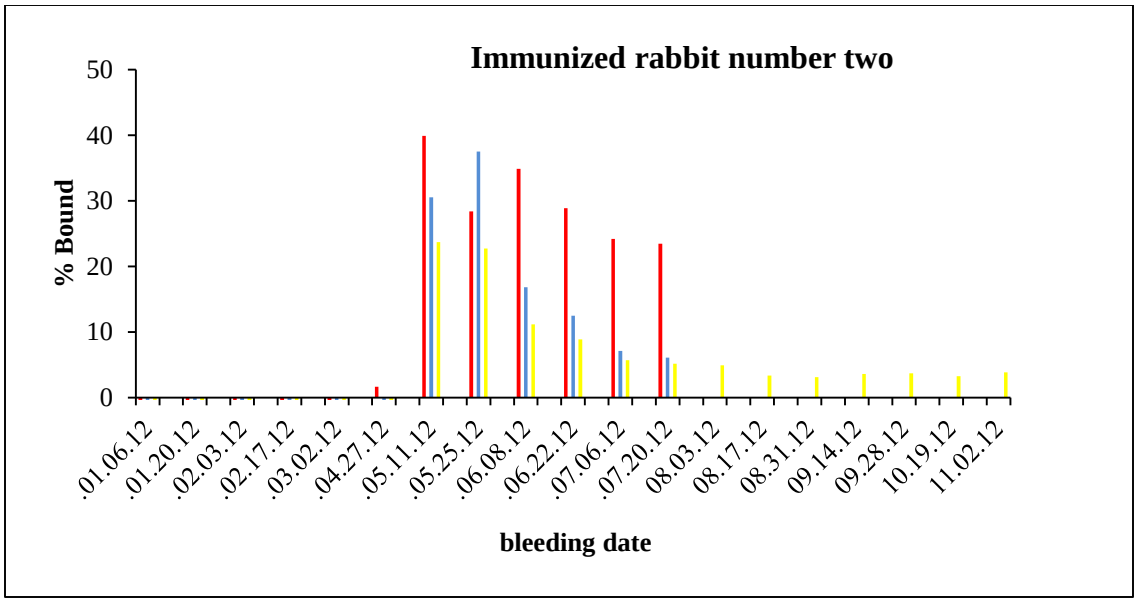
Supplemental figure 2.3. Antisera production to equine kisspeptin and binding check against ovine ^{125}I-kisspeptin-10. Immunized rabbit number two. Antisera dilution; 1:20,000 (red), 1:40,000 (blue) and 1:60:000 (yellow).



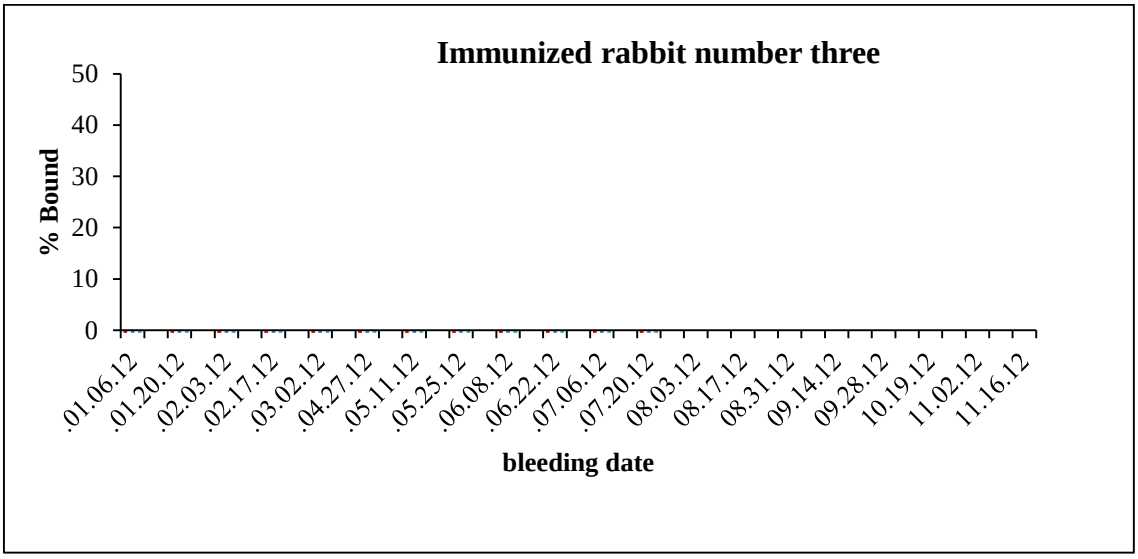
Supplemental figure 2.4. Antisera production to equine kisspeptin and binding check against ovine ^{125}I-kisspeptin-10. Immunized rabbit number three. Antisera dilution; 1:20,000 (red), 1:40,000 (blue) and 1:60:000 (yellow).



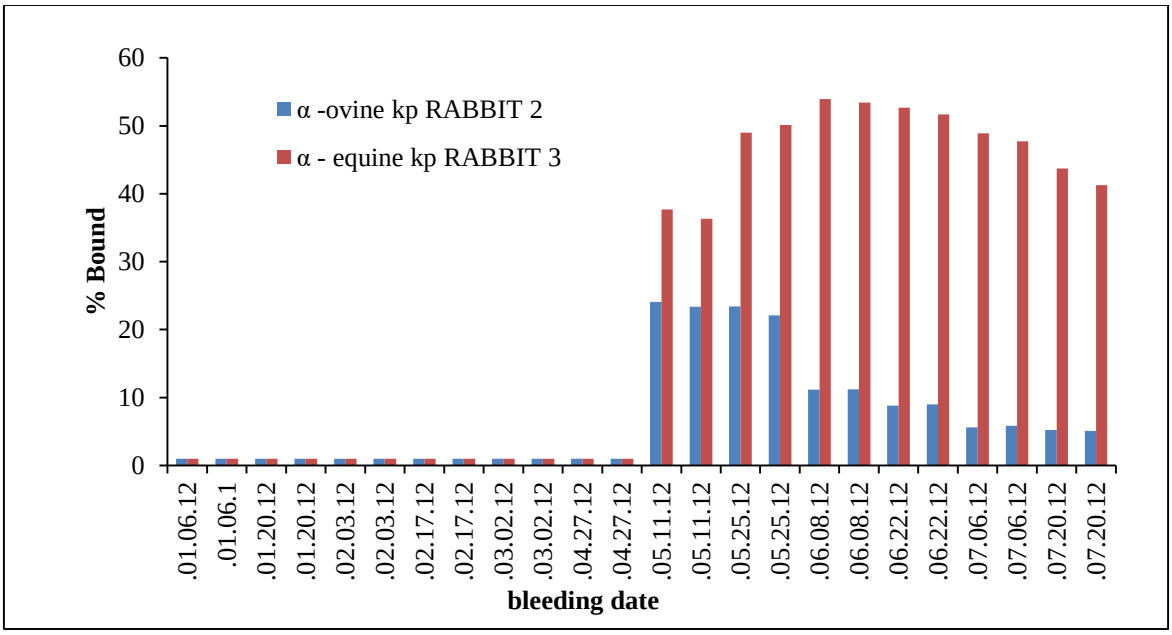
Supplemental figure 2.5. Antisera production to ovine kisspeptin and binding check against ovine ^{125}I-kisspeptin-10. Immunized rabbit number one. Antisera dilution; 1:20,000 (red), 1:40,000 (blue) and 1:60:000 (yellow).



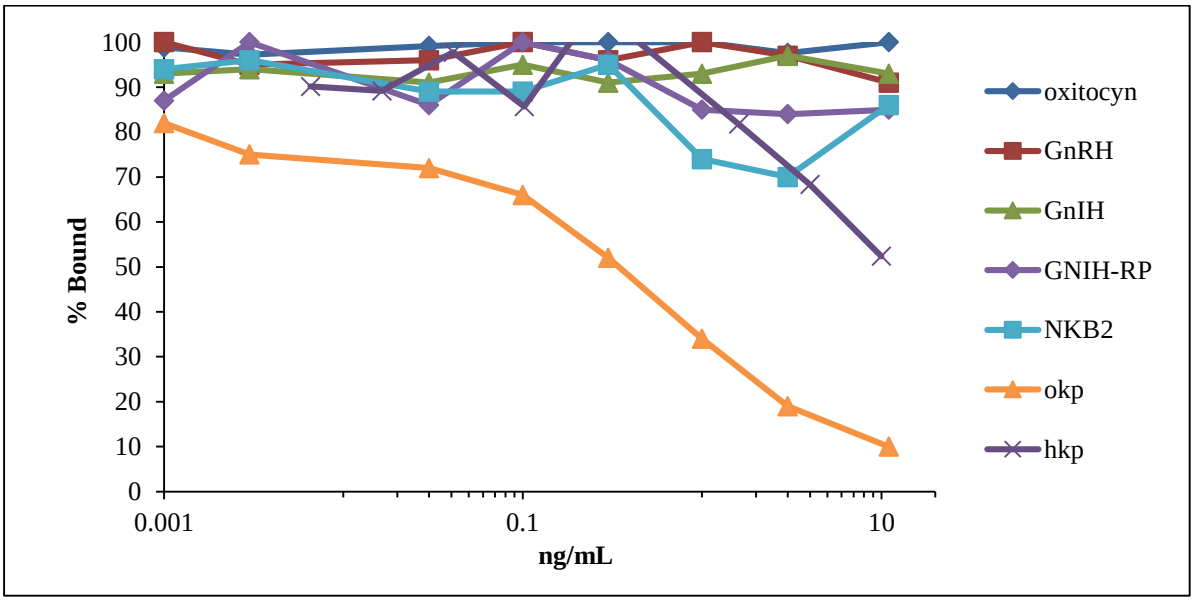
Supplemental figure 2.6. Antisera production to ovine kisspeptin and binding check against ovine ^{125}I-kisspeptin-10. Immunized rabbit number two. Antisera dilution; 1:20,000 (red), 1:40,000 (blue) and 1:60:000 (yellow).



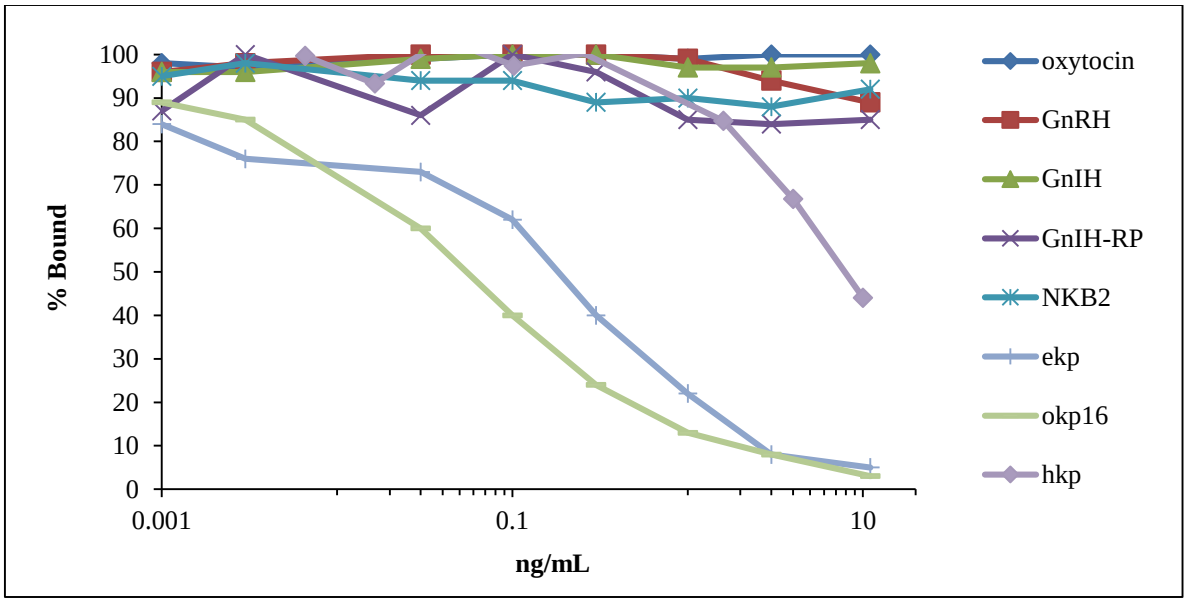
Supplemental figure 2.7. Antisera production to ovine kisspeptin and binding check against ovine ^{125}I-kisspeptin-10. Immunized rabbit number three. Antisera dilution; 1:20,000 (red), 1:40,000 (blue) and 1:60:000 (yellow).



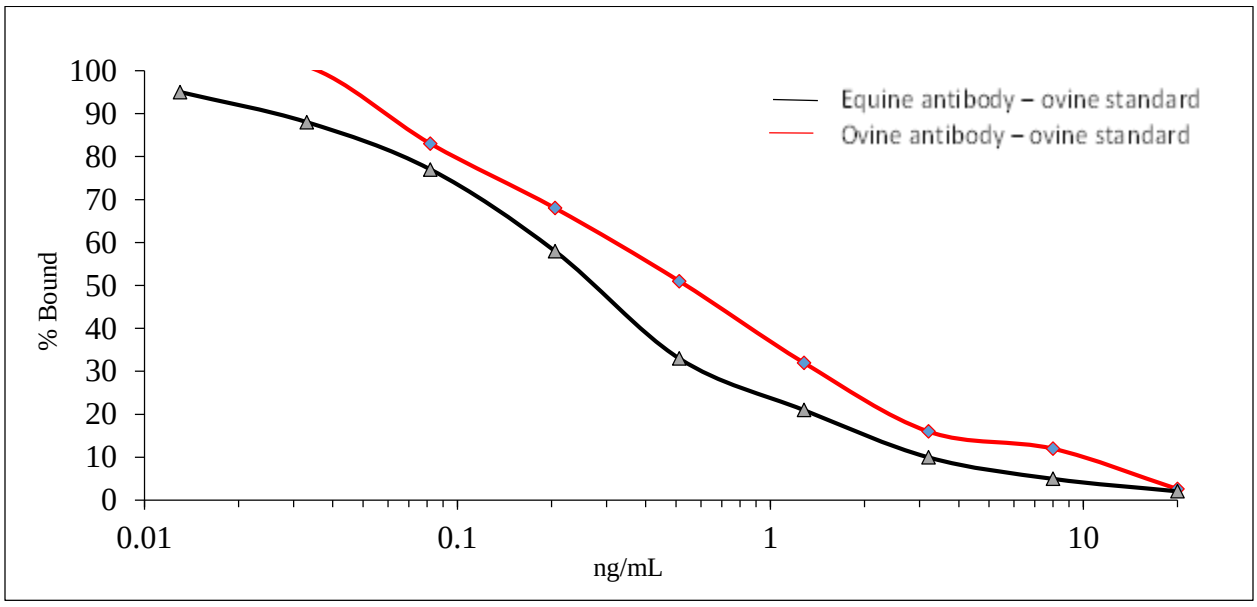
Supplemental figure 2.8. Binding of ovine ^{125}I-kisspeptin-10 to antisera raised against ovine kisspeptin (rabbit number two) at a dilution of 1:80,000 when compared to the binding of ovine ^{125}I-kisspeptin-10 to the antisera raised against equine kisspeptin at a dilution of 1:80,000 (rabbit number three).



Supplemental figure 2.9. Inhibition curves obtained in a radioimmunoassay (antiserum to ovine kisspeptin) by incubating increasing amounts of different hypothalamic neuropeptides. Oxytocin, gonadotropin releasing hormone (GnRH), gonadotropin inhibitory hormone (GnIH), gonadotropin inhibitory hormone related peptide (GnIH-RP), Neurokinin B (NKB2), equine kisspeptin (ekp), ovine kisspeptin-10 (okp) or human kisspeptin (hkp).



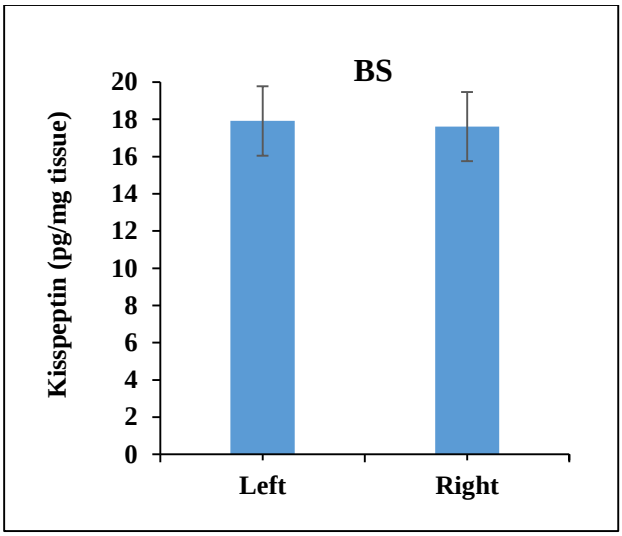
Supplemental figure 2.10. Inhibition curves obtained in a radioimmunoassay (antiserum to equine kisspeptin) by incubating increasing amounts of different hypothalamic neuropeptides. Oxytocin, gonadotropin releasing hormone (GnRH), gonadotropin inhibitory hormone (GnIH), gonadotropin inhibitory hormone related peptide (GnIH-RP), Neurokinin B (NKB2), equine kisspeptin (ekp), ovine kisspeptin-16 (okp 16) or human kisspeptin (hkp).



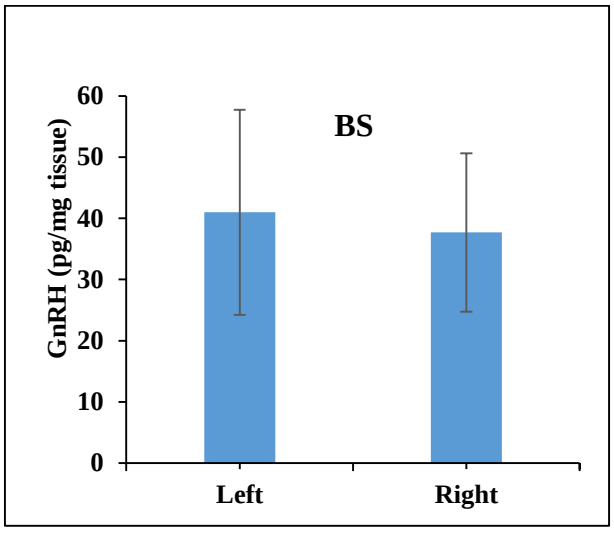
Supplemental figure 2.11 Comparison of the homologs and heterologous systems in terms of sensitivity.

Supplementary Table 2.1. A high sensitivity in the immuno detection of kisspeptin was achieved with the use of either the homologous or heterologous kisspeptin RIA systems.

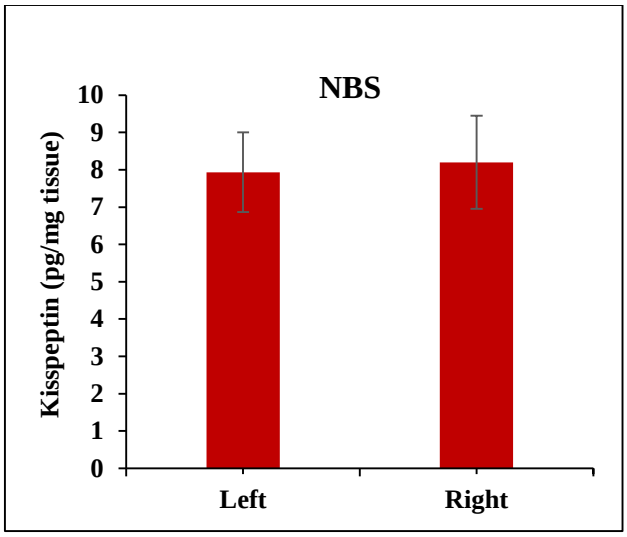
	Sensitivity
Equine standard with Equine antibody	12 pg
Ovine standard with Equine antibody	89 fg
Equine standard with Ovine antibody	9 pg
Ovine standard with Ovine antibody	388 fg



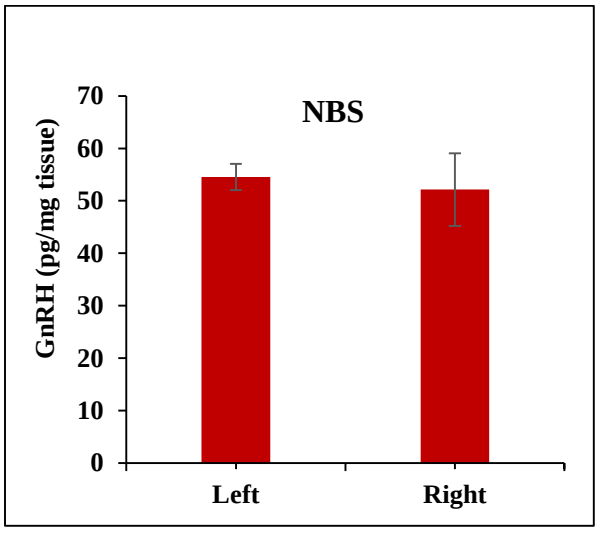
Supplemental figure 2.12. Differences in the content of kisspeptin in the right and left side of the hypothalamic samples (POA, AHA, MBH and ME) during the BS. No statistical difference was observed (n = 4, p = 0.63).



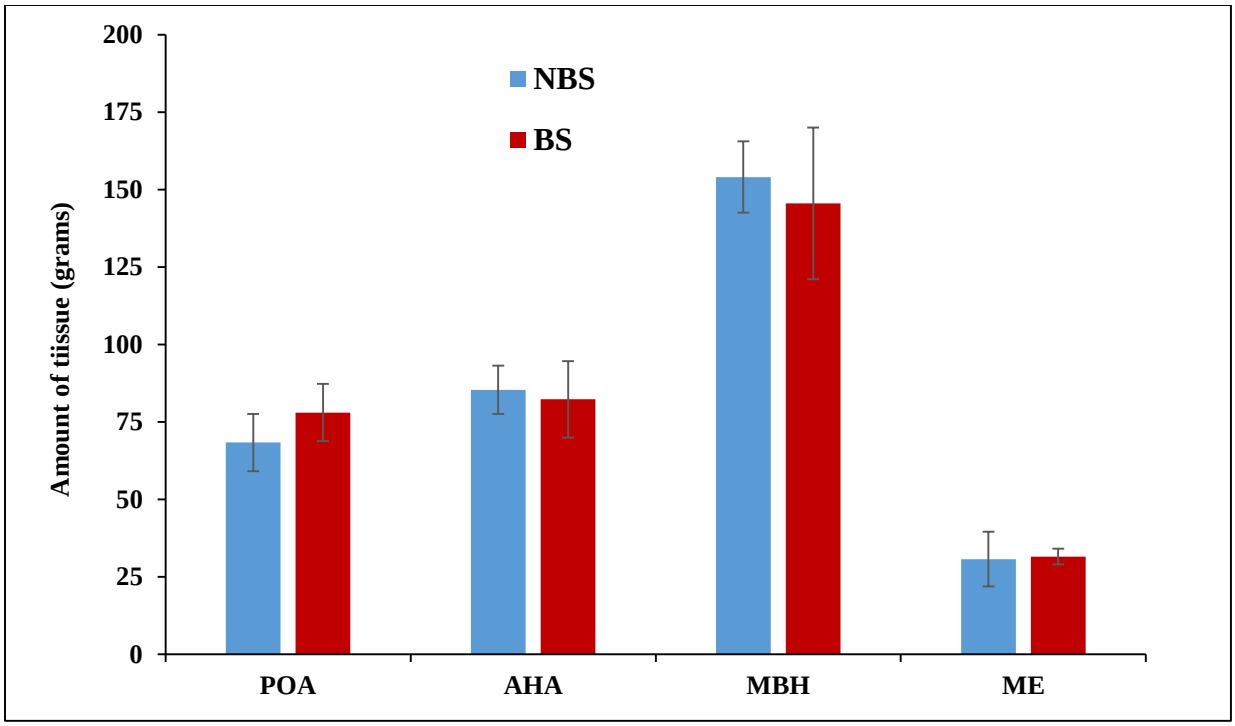
Supplemental figure 2.13. Differences in the content of GnRH between the right and left side of the hypothalamic samples selected for RIA (POA, AHA, MBH and ME) during the BS. No statistical difference was observed (n = 4, p=0.75).



Supplemental figure 2.14. Differences in the content of kisspeptin in the right and left side of the hypothalamic samples (POA, AHA, MBH and ME) during the NBS. No statistical difference was observed (n = 3, p = 0.87).



Supplemental figure 2.15. Differences in the content of GnRH between the right and left side of the hypothalamic samples selected for RIA (POA, AHA, MBH and ME) during the NBS. No statistical difference was observed (n = 3, p=0.76).



Supplemental figure 2.16. Differences in the amount of hypothalamic tissue processed during the NBS (n = 3) and BS (n = 4) in the POA (p=0.50), AHA (p=0.85), MBH (p=0.79), ME (p=0.92).