Kisspeptin activates the hypothalamic–adenohypophyseal–gonadal axis in prepubertal ewe lambs

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Abstract

The onset of puberty in mammals involves an increase in the pulsatile release of GNRH and LH. The *KISS1* gene is essential for pubertal development, and its product, kisspeptin, stimulates the release of LH. The objective of this study was to determine the effects of kisspeptin in the hypothalamic–adenohypophyseal–gonadal axis of prepubertal ewe lambs. Ewe lambs (28 weeks of age) were treated intravenously with saline (control, $n=6$) or kisspeptin (20 µg kisspeptin; $n=6$) every hour for 24 h. Kisspeptin stimulated pulse-like release of LH within 15 min following injections, and increased the frequency and amplitude of LH pulses, and mean circulating concentrations of LH and estradiol. A surge-like release of LH was observed in four kisspeptin-treated lambs beginning 17 h after the onset of treatment, and all four lambs had elevated circulating concentrations of progesterone within 5 days post-treatment. However, circulating concentrations of progesterone decreased within 2 days after the initial rise in three of the four ewe lambs, indicating that induced luteal activity was of short duration. The proportion of lambs that were pubertal (defined by circulating concentrations of progesterone above 1 ng/ml for at least 7 days) by 35 weeks of age (8/11) and the mean age at puberty (32 ± 1 weeks) for those reaching puberty within the experimental period did not differ between treatments. Results support a role for kisspeptin in the activation of the hypothalamic–adenohypophyseal axis leading to the onset of puberty in ewe lambs.

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Introduction

Maturation of the reproductive neuroendocrine axis in mammalian species is characterized by an increase in the frequency of episodic release of LH (Foster *et al.* 1975, Huffman *et al.* 1987, Ebling *et al.* 1990) in response to increased GNRH release from hypothalamic neurons (Watanabe & Terasawa 1989). Sustained elevation of circulating concentrations of LH supports the final stages of follicular development, steroidogenesis and ultimately, ovulation (Foster & Jackson 2006). Although pubertal activation of pulsatile GNRH release has been well characterized (Wildt *et al.* 1980, Kinder *et al.* 1995), the mechanisms and neuronal pathways leading to increased frequency of GNRH release during the peripubertal period remain unclear. Kisspeptin, a peptide with potent stimulatory effects on the release of GNRH and LH (Matsui *et al.* 2004, Messenger *et al.* 2005, Kadokawa *et al.* 2008, Lents *et al.* 2008, Magee *et al.* 2009, Smith *et al.* 2009b), plays a critical role. Kisspeptin (d'Anglemonet de Tassigny *et al.* 2007, Lapatto *et al.* 2007) and its receptor (de Roux *et al.* 2003, Seminara

et al. 2003) are essential for normal onset of puberty. Administration of kisspeptin to prepubertal rats advances the time of vaginal opening (Navarro *et al.* 2004b), suggesting that kisspeptin can hasten maturation of reproductive function. Moreover, kisspeptin prevents the delay in puberty caused by undernutrition in rats (Castellano *et al.* 2005).

The effects of kisspeptin on reproductive function seem to occur primarily by stimulation of GNRH secretion (Gottsch *et al.* 2004, Navarro *et al.* 2004a, Messenger *et al.* 2005), although direct effects at the level of the adenohypophysis have been demonstrated (Suzuki *et al.* 2008). In mature ewes, continuous infusion of kisspeptin sustains elevated circulating concentrations of LH for 24 h and stimulates ovulation during the anestrus season (Caraty *et al.* 2007). The ability of kisspeptin to stimulate LH release in prepubertal females (Navarro *et al.* 2004b, Kadokawa *et al.* 2008, Lents *et al.* 2008) indicates that exogenous kisspeptin can override the deficiency in LH release characteristic of immature states. In this study, we determined the effects of

Figure 1 Patterns of LH release in representative saline- (control) and kisspeptin-treated ewe lambs during period I (0–6 h) of the experiment. Asterisks indicate detected pulses of LH; arrows indicate the time of injections of saline (control) or kisspeptin. Note that in kisspeptin-treated lambs, a pulse of LH was observed following every injection of kisspeptin.

intermittent injections of kisspeptin on the release of LH in prepubertal lambs. We also determined whether repeated hourly administration of kisspeptin leads to synchronized hourly release of LH and stimulates follicular growth, ovulation, and initiation of regular reproductive cycles.

Results

Elevated concentrations of progesterone were observed in one control ewe on the first day of the experiment, and data obtained from her were eliminated from further analyses. Mean $\pm$ S.E.M. body weight at the onset of treatments was 42.5 ± 1.7 kg and did not differ among groups.

Mean concentrations of LH prior to the onset of treatments (period 0) did not differ between groups and averaged 1.9 ± 0.4 ng/ml. As expected, control ewes exhibited infrequent pulses of LH characteristic of prepubertal ewes (Fig. 1, Table 1). In kisspeptin-treated ewes, a pulse of LH was observed within 15 min after every injection (Fig. 1), resulting in an increased ($P < 0.02$) frequency of LH pulses compared with controls during the first 6 h after the onset of treatments (Table 1). Similarly, mean concentrations of LH and mean amplitude of LH pulses were greater ($P < 0.005$) in kisspeptin-treated lambs than those in controls during period I (Table 1).

During period III (18–24 h after the onset of treatments), the pattern of LH release in control ewe lambs remained similar to that of period I, with only infrequent episodes of LH release observed. In the kisspeptin-treated group, four of six ewe lambs were observed to exhibit a surge release of LH beginning ~ 20 h after the onset of kisspeptin injections (Fig. 2), while none of the saline-treated ewe lambs had such response ($P < 0.05$). The interval from the start of treatment to the onset of

LH surges averaged ($\pm$ S.E.M.) 21 ± 2.3 h. The mean maximum concentration of LH observed during the surge was 22 ± 13 ng/ml. The duration of the LH surge lasted ~ 12 h (Fig. 3).

Mean concentrations of estradiol (E_2) were low at the beginning of the treatments and did not differ between the two groups (Fig. 4). However, concentrations of E_2 increased in kisspeptin-treated ewes and were greater ($P < 0.02$) 18 h after initiation of injections than those in saline-treated ewes (Fig. 4). Ewes observed to exhibit a LH surge (four of six ewe lambs) had elevated concentrations of progesterone (above 1 ng/ml) 5 days after the onset of treatments, indicating that the surge of LH caused ovulation and/or follicle luteinization. During the experiment, two of the kisspeptin-treated ewe lambs did not exhibit surge release of LH, although they continued to respond to the kisspeptin injections with a pulse-like release of LH within 15 min after injections (Fig. 2) and had elevations in concentrations of circulating E_2 . None of the saline-treated ewe lambs were observed to have elevated progesterone in this period ($P < 0.05$).

In three of the four kisspeptin-treated lambs that exhibit luteal activity after treatments, concentrations of progesterone were elevated only briefly and decreased below 1 ng/ml 2–3 days after the initial rise. Circulating concentrations of progesterone remained elevated for an extended period in only one of the four kisspeptin-treated ewe lambs that had exhibited luteal activity. No control ewe lambs exhibited elevated concentrations of progesterone within 5 days following saline injections. The proportion of ewes determined pubertal in each group by 35 weeks of age, defined as ewes exhibiting circulating concentrations of progesterone above 1 ng/ml for at least three consecutive samples collected every 3 days, did not differ between groups. Overall, eight of eleven lambs were pubertal by the end of the experiment.

Discussion

The ability of kisspeptin to stimulate the release of LH in prepubertal female mammals has been demonstrated in rats (Navarro *et al.* 2004b), heifers (Kadokawa *et al.* 2008), and gilts (Lents *et al.* 2008). In this study, we

Table 1 Mean ($\pm$ S.E.M.) circulating concentrations of LH, and frequency and amplitude of LH pulses in saline- (control) and kisspeptin-treated ewe lambs during period I (0–6 h) of the experiment. Mean concentrations of LH as well as frequency and amplitude of LH pulses were greater in kisspeptin-treated ewes than those in controls.

	Control (n = 5)	Kisspeptin (n = 6)
Concentrations (ng/ml)	2.3 ± 0.1^a	4.5 ± 0.2^b
Frequency of pulses (pulses/6 h)	3.0 ± 0.7^c	6.0 ± 0.0^d
Amplitude of pulses (ng/ml)	3.1 ± 0.4^a	5.3 ± 0.4^b

^{a,b} Differs $P < 0.005$; ^{c,d} Differs $P < 0.02$.

Figure 2 Patterns of LH release in representative saline- (control) and kisspeptin-treated ewe lambs during period III (18–24 h) of the experiment. Asterisks indicate detected pulses of LH; arrows indicate the time of injections of saline (control) or kisspeptin. Note that Y axes for lambs 820 and 8124 differ from the others.

extended these findings by demonstrating that intermittent injections of kisspeptin elicit pulse-like release of LH in prepubertal ewe lambs. Moreover, hourly injections of kisspeptin administered for 24 h stimulated ovarian function in prepubertal lambs leading to enhanced ovarian steroidogenesis, stimulation of a preovulatory surge of LH and ovulation/luteal activity. The ability of kisspeptin to advance the onset of puberty has been suggested by observations that it induces early vaginal canalization in rats, a physiological event that precedes the first estrous cycle in female rats (Navarro *et al.* 2004b). Although in our studies kisspeptin treatment stimulated ovulation/luteinization in prepubertal ewes, duration of luteal activity was brief (2–3 days), and the age at establishment of regular estrous cycles was not advanced as hypothesized.

Kisspeptin is a potent stimulator of LH release in various mammalian and non-mammalian species (Caraty & Franceschini 2008, Clarkson & Herbison 2009, Elizur 2009). Although our studies did not distinguish the site of kisspeptin actions, the effects of kisspeptin on stimulating the release of LH in prepubertal ewe lambs are likely mediated by direct actions upon GnRH neurons. Kisspeptin receptor is present in GnRH neurons (Seminara *et al.* 2003, Messenger *et al.* 2005), and kisspeptin administered centrally (Messenger *et al.* 2005)

or intravenously (Caraty *et al.* 2007) stimulates the release of GnRH. Moreover, kisspeptin stimulation of LH release is blocked by GnRH antagonist (Gottsch *et al.* 2004, Navarro *et al.* 2004b, Plant *et al.* 2006) and by passive immunization with GnRH antiserum (Arreguin-Arevalo *et al.* 2007). However, direct effects of kisspeptin at the adenohypophysis to stimulate LH release cannot be discounted. Kisspeptin receptor is also expressed in various tissues, including the adenohypophysis (Gutierrez-Pascual *et al.* 2007), and *in vitro* studies demonstrate that kisspeptin induces LH release in primary culture of adenohypophyseal cells (Smith *et al.* 2008). Nevertheless, the physiological relevance for the direct effects of kisspeptin in the adenohypophysis remains controversial, because kisspeptin failed to stimulate the release of LH in hypothalamo–hypophyseal disconnected ewes (Smith *et al.* 2008).

In this study, repetitive hourly injections of kisspeptin in prepubertal ewe lambs elicited episodes of LH release similar to intermittent injections of kisspeptin in agonadal, male monkeys (Plant *et al.* 2006). In control-treated ewe lambs, spontaneous pulses of LH were observed to vary in frequency characteristic of ewe lambs at this age. Because all episodes of LH release observed in kisspeptin-treated lambs occurred immediately after kisspeptin injections and no additional

Figure 3 Mean ($\pm$ S.E.M.) concentrations of LH in saline- (control) and kisspeptin-treated ewe lambs. Arrows indicate hourly injections of saline (control) or kisspeptin.

Figure 4 Mean ($\pm$ S.E.M.) concentrations of estradiol in saline- (control) and kisspeptin-treated ewe lambs during the first 24 h of treatments. Asterisks indicate differences ($P < 0.02$) between kisspeptin and saline treatments.

secondary LH pulses between kisspeptin injections were detected, we speculate that kisspeptin may be involved in the neuronal pathways leading to synchronized episodic release of GnRH. There is evidence to indicate that GnRH neurons have an intrinsic ability to generate pulses (Wetsel *et al.* 1992, Terasawa *et al.* 1999); however, pulses of GnRH release may be entrained by actions of various neurochemicals, including neurotransmitters and glial-derived factors (Moenter *et al.* 2003, Ojeda *et al.* 2008). Kisspeptin has been shown to act directly on GnRH neurons, as well as via transsynaptic pathways involving GABAergic and glutamatergic afferents (Pielecka-Fortuna *et al.* 2008, Pielecka-Fortuna & Moenter 2010). Therefore, kisspeptin may play a role in synchronizing episodic release of GnRH by acting at multiple levels, including direct stimulation of GnRH neuronal activity, and by modulating GnRH neuron afferents.

The mode of kisspeptin treatment used in this study differs from other studies using constant infusion (Messenger *et al.* 2005, Shahab *et al.* 2005, Caraty *et al.* 2007). In monkeys, after an initial increase, concentrations of LH decreased with continuous infusion of kisspeptin and were not different than controls within 24 h (Seminara *et al.* 2006). Although a single bolus of kisspeptin failed to release LH during continuous administration of kisspeptin, *N*-methyl-D-aspartate (NMDA), a known GnRH secretagogue, and GnRH elicited LH release (Seminara *et al.* 2006). This observation indicates that the kisspeptin receptor is desensitized by continuous kisspeptin treatment. The mechanisms of kisspeptin receptor desensitization are not clear but seem to involve G-protein-coupled receptor (GPCR) kinase 2 (Pampillo *et al.* 2009). In sheep, concentrations of LH also decrease during continuous infusion of kisspeptin (Messenger *et al.* 2005); however, low doses of kisspeptin infused constantly increase concentrations of E_2 in circulation and stimulate ovulation in anestrus ewes (Caraty *et al.* 2007, Sebert *et al.* 2010). Moreover, in peripubertal female rats, circulating concentrations of LH remained elevated after 7 days of continuous intracerebral infusion of kisspeptin in peripubertal female rats, an effect not observed in cyclic rats (Roa *et al.* 2008). Therefore, within a specific range of concentrations and depending

on developmental stage, continuous infusion of kisspeptin for extended periods can stimulate secretion of GnRH. Nevertheless, endogenous kisspeptin release in the median eminence seems to be episodic (Keen *et al.* 2008), and this mode of action may be relevant for a fine control of GnRH neuronal activity.

It has been proposed that proper activation of the kisspeptin system is essential for normal pubertal onset (Seminara *et al.* 2003, Navarro *et al.* 2004a, Shahab *et al.* 2005). Increases in kisspeptin gene expression (Navarro *et al.* 2004a, Shahab *et al.* 2005) and peptide (Clarkson *et al.* 2009) have been associated with pubertal development. Recent studies using a kisspeptin antagonist support the requirement for kisspeptin signaling in timing puberty in female rats (Pineda *et al.* 2010). In ewe lambs, initiation of frequent pulsatile LH release is associated with increases in expression of *KISS1* in the arcuate nucleus (Amstalden *et al.* 2010). Therefore, kisspeptin stimulation of GnRH neuronal function may be critical for pubertal transition toward patterns of pulsatile LH release that support final stages of follicle development (~ 1 pulse/h in the sheep) and establishment of regular estrous cycles. The current experiment supports this hypothesis by demonstrating that episodic stimulation of LH release by kisspeptin is effective to induce gonadal activity in prepubertal ewe lambs.

LH stimulation is crucial for increased follicular E_2 synthesis and sustained elevated concentrations of E_2 that lead to the preovulatory surge of LH. The ability of E_2 to induce LH surge develops early during the juvenile period (Foster & Karsch 1975, Andrews *et al.* 1981). Thus, E_2 positive feedback is not the limiting factor in the initiation of regular ovulatory cycles. In 18-week-old ewes, hourly injections of LH for 48 h stimulated elevations in circulating E_2 , and surges of LH occurred between 23 and 55 h after initiation of LH injections in four of seven lambs (Foster *et al.* 1984). Ovulation was followed by increases in circulating concentrations of progesterone that remained elevated for a full normal-length luteal phase in only one lamb. Similar results were observed in this study using hourly injections of kisspeptin, in which $\sim 70\%$ of kisspeptin-treated ewe lambs exhibited luteal activity. Therefore, follicular development, increased steroidogenesis, and ovulation were achieved by intermittent kisspeptin injections. In contrast to the earlier study (Foster *et al.* 1984), establishment of full-length luteal phase and regular estrous cycles was not induced in this study, indicating a requirement for additional maturation of neuroendocrine functions upstream to GnRH neurons.

In this study, two ewe lambs had the onset of the LH surge during the second period of intensive blood sampling at the end of kisspeptin treatment. Interestingly, despite escalating concentrations of LH in circulation, clear elevations in LH were observed in association with kisspeptin injections. This pattern of secretion differs from the usual preovulatory surge of LH lacking clearly

detectable pulses (Moenter *et al.* 1991). Our observation in this study may reflect differential actions of kisspeptin in distinct populations of GNRH neurons. During the preovulatory LH surge, ~40% of GNRH neurons contain immunoreactive FOS (Lee *et al.* 1990, Moenter *et al.* 1993), indicating that only a subpopulation of GNRH neurons is activated. Moreover, in sheep, GNRH neurons dispersed throughout the preoptic area, and hypothalamus become activated during the LH surge (Moenter *et al.* 1993), whereas GNRH neurons residing mainly in the mediobasal hypothalamus are activated during the pulsatile release of LH (Boukhliq *et al.* 1999). Alternatively, exogenous kisspeptin may have exerted its actions on distinct cellular sites (e.g., cell bodies/dendrites or terminals) to stimulate the release of GNRH during the surge induced by E₂. It has been demonstrated that kisspeptin can stimulate GNRH release by a direct action at GNRH nerve terminals (d'Anglemont de Tassigny *et al.* 2008). Therefore, pulses of LH that appeared superimposed during the surge of LH secretion in the two lambs may reflect actions of exogenous kisspeptin at this level.

There is compelling evidence for a role of kisspeptin in mediating E₂ positive feedback on GNRH release. In rodents, the population of kisspeptin neurons in the anteroventral periventricular area (AVPV) seems to be particularly relevant. E₂ stimulates *Kiss1* expression in the AVPV (Smith *et al.* 2005), kisspeptin neurons in the AVPV express the immediate-early gene *Fos* (*cFos*) during LH surge (Smith *et al.* 2006), and immunoneutralization of kisspeptin in the preoptic area (POA) blocks the preovulatory surge of GNRH (Kinoshita *et al.* 2005). In sheep, the involvement of POA kisspeptin neurons in the E₂ positive feedback is less clear. Recent observations suggest that kisspeptin neurons in the caudal arcuate nucleus are involved in the E₂ positive feedback (Smith *et al.* 2009a). Nevertheless, kisspeptin neurons in the POA of sheep may also be involved in the preovulatory surge of GNRH/LH, because *KISS1* expression increases in the POA of ewes just before ovulation. Moreover, increase in FOS immunoreactivity was observed in POA kisspeptin neurons at the time of the surge (Hoffman *et al.* 2011).

Formation of the corpus luteum after ovulation and initial secretion of progesterone do not require hypothyseal hormones (Yuh *et al.* 1984). However, adequate LH stimulation is required for maintenance of normal luteal function (Hutchison *et al.* 1986). In the current experiment, ewes that ovulated following kisspeptin treatment had only a brief period of elevated progesterone. This short period of luteal activity may reflect insufficient release of LH after termination of kisspeptin treatment. Although short luteal phases are commonly observed in ewes undergoing pubertal transition, it is often followed immediately by another ovulation and a normal-length estrous cycle (Foster & Jackson 2006). The lack of immediate establishment of regular estrous cycles in ewe lambs ovulating following kisspeptin treatment in

this study indicates that the reproductive neuroendocrine axis was not yet fully mature. This observation corresponds to findings reported by Wildt *et al.* (1980) demonstrating that regular menstrual cycles induced by pulsatile GNRH in immature monkeys ceased with termination of GNRH treatments.

In summary, injection of kisspeptin stimulated the release of LH in prepubertal ewe lambs. Intermittent treatments with kisspeptin that create hourly pulses of LH stimulated folliculogenesis and steroidogenesis, and led to the development of the preovulatory LH surge and ovulation. However, luteal activity following induced ovulation was limited, and regular estrous cycles were not established immediately in the absence of kisspeptin treatments. Therefore, kisspeptin seems to play a major role in the maturation of the hypothalamic–gonadotropic–gonadal axis by stimulating GNRH neuronal activity and pulsatile release of LH during the onset of puberty in ewe lambs.

Materials and Methods

Animals and experimental procedures

Experimental procedures involving animals were performed at the Texas A&M University O.D. Buttler Animal Science Teaching and Research Complex, College Station, TX, USA. The Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System approved all procedures used in these studies.

A total of twelve intact, spring-born, Suffolk ewe lambs, weaned at 8 weeks of age, were used for this study. Ewe lambs were fed *ad libitum* a complete-mixed diet containing 16% CP and 2.8 MCal/kg formulated to promote weight gain of ~0.2–0.25 kg/day. Ewe lambs were housed in partially covered pens and had free access to water and a mineral supplement. At 26 weeks of age, lambs were brought into indoor pens and acclimated to experimental conditions (periodic loose restraint with halter) for 2 weeks. At 28 weeks of age, ewe lambs were assigned randomly to one of two groups: 1) control (*n*=6) or 2) kisspeptin (*n*=6). On the day before the onset of treatments, lambs were fitted with a jugular catheter. On the next day, ewe lambs were injected i.v. with either saline (solution containing 0.9% NaCl, w/v; control) or saline containing 20 µg of a decapeptide representing the carboxyl terminus of the ovine kisspeptin (kisspeptin; Arreguin-Arevalo *et al.* 2007) every 1 h for 24 h. Kisspeptin decapeptide was obtained by custom synthesis (American Peptide Company, Inc., Sunnyvale, CA, USA). Synthesized peptide was 96.5% pure based on HPLC analysis and had 1318.4 amu of molecular weight based on mass spectral analysis. The dose of kisspeptin used in this experiment was determined based on previous reports demonstrating the effectiveness of kisspeptin to stimulate the release of LH in sheep (Caraty *et al.* 2007).

Blood samples (5 ml) were collected from the jugular vein every 15 min starting 30 min before the first injection of saline or kisspeptin (period 0) and continued for 6 h at 15 min intervals (period I). Between 6 and 18 h from the onset of

treatments (period II), blood samples were collected just prior to each hourly injection. Collection of blood samples at 15 min intervals resumed 18 h after the start of treatments and continued for an additional 6 h (period III). Following the 24 h administration of treatments, blood samples were collected every 3 h for 36 h (period IV), daily for the next 15 days and then every 3 days until the ewe lambs were detected pubertal or reached 35 weeks of age.

Intensive (every 15 min), hourly, and 3-hourly blood samples were placed in tubes containing 50 µl solution of heparin (3000 U/ml) and 5% EDTA. Jugular catheters were flushed with physiological saline solution after every sampling and with heparinized saline every 1 h to prevent clotting of the catheter. Daily and 3-daily blood samples were collected by jugular venipuncture. Blood samples were chilled on ice until centrifugation at 1500 *g* for 20 min at 4°C, and plasma or serum was collected and stored at –20°C until processed for determination of concentrations of LH, E₂, and progesterone by RIA.

RIA

Concentrations of LH were determined by a double antibody RIA using rabbit anti-ovine LH (AFP192279; National Hormone and Peptide Program, Torrance, CA, USA) at a dilution of 1:1 250 000. A highly purified ovine LH (AFP8614B; National Hormone and Pituitary Program) was used for both as the iodinated tracer and the reference standard preparation. Sensitivity of the assay was 0.2 ng/ml, and the curve was linear to 50 ng/ml. The intra- and inter-assay coefficients of variation averaged 11 and 13.5% respectively.

Concentrations of E₂ were determined in plasma extracts using a double antibody RIA (Estradiol double antibody; Siemens, Los Angeles, CA, USA). Extraction was performed in duplicates of 1 ml plasma and reference samples using 4 ml methyl-tert-butyl ether (MTBE). Solvent extracts were dried using nitrogen gas, and the extracts were rehydrated with 200 µl '0' calibrator provided with RIA kit. The assay was performed as per the manufacturer's instructions with the addition of a 1 pg/ml standard. Concentrations of E₂ in samples were determined in a single assay, which yielded 43% binding at B/O and detection limit of 0.5 pg/tube at 90% binding. Extraction efficiency averaged 73%, and estimates of blank extracts averaged 99.8% binding. The intra-assay coefficient of variation for reference samples averaged 5%.

Concentrations of progesterone were determined in blood samples using a solid-phase RIA (Coat-A-Count; Siemens). The detection limit of the assay was 0.1 ng/ml. The intra- and interassay coefficients of variation for reference samples averaged 7.5 and 24% respectively.

Statistical analyses

Frequency and amplitude of LH pulses were determined using a pulse detection algorithm (Pulsefit 1.2; (Kushler & Brown 1991)). LH data were analyzed using the Proc Mixed procedures for repeated measures of SAS (SAS 9.1; SAS Institute, Cary, NC, USA). The main effects of treatment (saline or kisspeptin) on mean concentrations of LH and on the

frequency and amplitude of LH pulses were compared using period (I: 0–6 h and III: 18–24 h) as repeated variable and ewe (treatment) as the subject. The proportions of ewes in each treatment exhibiting a surge-like release of LH and elevated circulating progesterone 5 days after the onset of treatments were analyzed by Fisher's exact test procedure of SAS.

Declaration of interest

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

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