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ORIGINAL ARTICLE

Differential Expression of RFamide-Related Peptide, a Mammalian Gonadotrophin-Inhibitory Hormone Orthologue, and Kisspeptin in the Hypothalamus of Abadeh Ecotype Does During Breeding and Anoestrous Seasons

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Gonadotrophin-inhibitory hormone (GnIH) is a novel hypothalamic neuropeptide that was discovered in birds as an inhibitory factor for gonadotrophin release. RFamide-related peptide (RFRP) is a mammalian GnIH orthologue that inhibits gonadotrophin synthesis and release in mammals through actions on gonadotrophin-releasing hormone (GnRH) neurones and gonadotrophs, mediated via the GnIH receptor (GnIH-R), GPR147. On the other hand, hypothalamic kisspeptin provokes the release of GnRH from the hypothalamus. The present study aimed to compare the expression of RFRP in the dorsomedial hypothalamus and paraventricular nucleus (DMH/PVN) and that of kisspeptin in the arcuate nucleus (ARC) of the female goat hypothalamus during anoestrous and breeding seasons. Mature female Abadeh does were used during anoestrus, as well as the follicular and luteal phases of the cycle. The number of RFRP-immunoreactive (-IR) neurones in the follicular phase was lower than in the luteal and anoestrous stages. Irrespective of the ovarian stage, the number of RFRP-IR neurones in the rostral and middle regions of the DMH/PVN was higher than in the caudal region. By contrast, the number of kisspeptin-IR neurones in the follicular stage was greater than in the luteal stage and during the anoestrous stage. Irrespective of the stage of the ovarian cycle, the number of kisspeptin-IR neurones in the caudal region of the ARC was greater than in the middle and rostral regions. In conclusion, RFRP-IR cells were more abundant in the rostral region of the DMH/PVN nuclei of the hypothalamus, with a greater number being found during the luteal and anoestrous stages compared to the follicular stage. On the other hand, kisspeptin-IR neurones were more abundant in the caudal part of the ARC, with a greater number recorded in the follicular stage compared to the luteal and anoestrous stages.

Key words: RFamide-related peptide, kisspeptin, hypothalamus, breeding season, anoestrus, goat

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The neuroendocrine integration of environmental and internal signals controls reproduction across vertebrate species. The reproductive axis of vertebrates integrates the information from a wide range of systems via direct and indirect neurochemical inputs. Many of the neuropeptidergic pathways involved in the transduction of environmental stimuli into neuroendocrine signals have been well studied. It has been well established that a hypothalamic neuropeptide, gonadotrophin-releasing hormone (GnRH), the

primary signal regulating the secretion of gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone, acts as a key neurohormone to vertebrate reproduction. Recently, two opposing RFamide (Arg-Phe-NH_2) novel neuropeptides have emerged as pronounced regulators of the vertebrate reproductive axis. Gonadotrophin-inhibitory hormone [GnIH; also known as RFamide-related peptide (RFRPs) in mammals] and kisspeptin are key inhibitory and stimulatory regulators of the hypothalamic-pituitary-gonadal axis, respectively.

In 2000, Tsutsui and colleagues identified a novel hypothalamic neuropeptide (SIKPSAYLPLRFamide) in quails that directly acts on the pituitary to inhibit gonadotrophin release, and termed it GnIH (1). This was the first demonstration of a hypothalamic neuropeptide inhibiting gonadotrophin release in any vertebrate. This was further isolated as a mature peptide in starlings (2) and zebra finches (3).

The GnIH orthologues were subsequently identified in a number of other vertebrates, including mammals (4). The cDNAs identified from the mammalian brain encode three GnIH orthologues (known as RFRPs), RFRP-1, -2 and -3, in cattle and humans and two GnIH orthologues, RFRP-1 and -3, in rodents (5–7). As in birds, the mammalian GnIH orthologues, RFRP-1 and RFRP-3, act to inhibit gonadotrophin secretion in mammals. Taken together, GnIH and its orthologues appear to act similarly across vertebrate species to regulate reproduction, although exceptions do exist.

By contrast to GnIH (or RFRP), kisspeptin, a neuropeptide encoded by the *KiSS1* gene, acts as a pronounced stimulatory regulator of the GnRH system. *KiSS1* was originally identified as a human gene that suppressed metastases of human melanomas and breast carcinomas (8). In 2003, it was found that individuals with mutations in the gene *GPR54*, the receptor for kisspeptin, exhibited hypogonadotrophic hypogonadism and failed to enter puberty (9). Similar findings were reported in knockout mice lacking the *GPR54* gene (9). It is now considered that kisspeptin is a critical regulator of sexual differentiation and maturation, as well as normal, adult reproductive functioning, across mammalian species (10–14).

Hypothalamic kisspeptin stimulates gonadotrophin release in many mammalian species (15) including the goat (16). The effect of kisspeptin on LH secretion is via GnRH release because kisspeptin treatment in ewes, in which the hypothalamus and hypophysis were disconnected, did not change LH secretion (17). Kisspeptin-immunoreactive (-IR) cells are mostly located in sheep (18) and goat (19) arcuate nucleus (ARC).

Melatonin is considered as a key hormone regulating RFRP secretion in rodents and in seasonal species such as sheep (20). Melatonin receptors have been found in the ovine premammillary region, which contains the caudal portion of the ARC (21). The RFRP cell bodies were also localised to the dorsomedial hypothalamus (DMH) of Syrian hamsters, which is considered a key region with respect to mediating the effect of melatonin on gonadal regression (22). Neural pathways through which melatonin regulates seasonality have not been clearly identified; however, kisspeptin and RFRP may act as the mediators of melatonin action on GnRH secretion in seasonal breeders (21). An effect of melatonin on RFRP/GnIH was reported in hamsters (23) and quails (24,25).

Thus, the discovery of RFRP and kisspeptin has changed our understanding of the vertebrate reproductive axis despite the well accepted view that reproduction is mainly under the stimulatory action of GnRH. Seasonal breeders rely on environmental and internal cues to initiate and/or terminate their reproduction, and these signals need to be further translated and integrated to generate the appropriate neuroendocrine response. Goat, a short-day seasonal breeder (27), is an excellent animal model for understanding the interaction of RFRP and kisspeptin for optimal reproductive functioning.

Using immunohistochemial staining techniques, neurones expressing kisspeptin and RFRP were identified in the hypothalamus of many species; however, there appears to be no report on the expression of RFRP and kisspeptin neurones during the ovarian cycle of doe. The present study aimed to quantify the number of RFRP neurones in the DMH/paraventricular nucleus (PVN) and that of kisspeptin neurones in the ARC in Abadeh ecotype does during the breeding (follicular and luteal stages) and anoestrous seasons.

Materials and methods

Animals

Nine healthy multiparous native Abadeh does (an Iranian native goat ecotype; 3–5 years old) with an mean live weight of 40 kg were used. The does were acclimatised for 6 months at the Animal Research Station of the College of Agriculture, Shiraz University, Shiraz, Iran (latitude 29° 44′N; longitude 52° 37′E; 1810 m above sea level). During the first week of adaptation, physical examination was performed and the does were treated for worms and ectoparasites. The animals had free access to water and food (alfalfa hay and wheat straw) and were kept in an open shed barn with adequate space and air movement. All procedures were carried out in accordance with Shiraz University guidelines for animal handling and the project was approved by the Ethics Committee of Shiraz University (Grant Number: 91GCU3M148075).

Ovarian cycle determination

The experiment was carried out during the breeding (October to January) and anoestrous (August to September) seasons. During the breeding season, does in oestrus were identified by using a healthy sexually active aproned-buck, and observing the does for standing heat twice daily for 1 h in early morning and late afternoon. Six oestrous does were selected. Blood samples were collected from the jugular vein 2 weeks after standing heat to determine the serum concentrations of progesterone (P4). During the anoestrous season, blood samples were collected from six other does, on 10 occasions at a 1-day interval, for determination of the serum P4 concentration.

Blood serum was separated by centrifugation (10 min at 3000 **g**) and stored at −22 °C until assayed. Serum P4 concentration was determined using a validated commercial radioimmunoassay kit (Immunotech, Marseille, France). The intra- and inter-assay coefficients of variations were 5.8 and 9%, respectively. The assay sensitivity was 0.05 ng/ml, with a recovery rate of 85–110%. During the breeding season, the does with a serum P4 concentration of > 5 ng/ml were considered to be in the luteal phase of the ovarian cycle (27). During the anoestrous season, does with a serum P4 concentration < 1 ng/ml for 20 days were considered to be in anoestrus. Three does, identified to be in their luteal phase, were intramuscularly injected with 1.5 ml of $PGF_{2\alpha}$ (Lutalyse; Pfizer, Elsene, Belgium) and, when the serum P4 concentration reached < 1 ng/ml (30 h after injection), they

were considered to be in the follicular phase of the ovarian cycle. The remaining three does were allotted to the luteal phase group.

Sample preparations for immunohistochemistry

Does were humanely slaughtered and their heads were immediately separated. The brain was perfused with normal saline (2 l), containing 25 000 units of heparin (Rotexmedica, Trittau, Germany) via the carotid artery using a perfusion pump. Two litres of 10% buffered-formalin solution and 2 l of 10% buffered-formalin solution containing 20% sucrose were also infused to fix the brain. The brain was dissected out and the diencephalon was kept in buffered-formalin solution containing 30% sucrose overnight, and then in buffered 30% sucrose for 7 days. Diencephalons were then kept frozen at −80 °C. Using a freezing microtome (Slee, Mainz, Germany), the diencephalon was cut into 30-μm sections. Sections containing the rostral, midlle, and caudal regions (three sections per region) of the DMH/PVN. and ARC were used for staining (Fig. 1). Sections were selected based on Zuccolilli *et al.* (28).

Immunohistochemical staining of RFRP-IR neurones

Sections containing rostral, middle and caudal regions of the DMH/PVN were post-fixed for 10–20 min in 10% buffered-formalin and washed for 5 min in phosphate-buffered saline (PBS) three times. They were then incubated in a blocking solution (1% bovine serum albumin (BSA), 0.3% Triton X-100, 1% normal goat serum in 10 mM PBS] for 30 min. The sections were then incubated in rabbit anti-quail GnIH (SIKPSAYLPLRFamide) serum (supplied by K. Tsutsui, Waseda University, Tokyo, Japan) (1000-fold dilution in the blocking solution) overnight and washed for 10 min in PBS for three times. Then, the sections were incubated in fluorescein isothiocyanate-labelled goat anti-rabbit immunoglobulin G (Invitrogen, Carlsbad, CA, USA; 50-fold dilution in the blocking solution) for 60 min. The sections were washed for 10 min in PBS for three times and covered with the Dako fluorescence mounting medium (Dako, Glostrup, Denmark). Immunoreactive RFRP neurones were counted using a fluorescence microscope (Nikon, Tokyo, Japan).

Immunohistochemical staining of kisspeptin-IR neurones

Sections containing rostral, middle and caudal regions of ARC were washed three times with the PBS solution containing 0.3% Triton X100 for 5 min. The sections were placed in 3% hydrogen peroxide solution diluted in methanol and washed for 5 min in PBS three times. They were incubated in the blocking solution (1% BSA, 10% goat serum in 10 mM PBS) for 60 min. Then, they were incubated in rabbit anti-mouse kisspeptin (supplied by A. Caraty, Universite′ Tours, Nouzilly, France) (18) serum (AC#566; dilution 1 : 5000) overnight at 4 °C and washed in PBS. The sections were then incubated in EnVision + Dual Link System-HRP (Dako) for 30 min and washed in PBS. To visualise kisspeptin, diaminobenzidine (Dako) was used. Kisspeptin neurones were quantified using light microscopy (Zeiss, Oberkochen, Germany) and photographed (Eos, Kin, X4, BA-520; Canon, Tokyo, Japan).

Immunohistochemical controls included the omission of the primary antibody (negative control) and additional controls for the specificity of the primary antibody were carried out. The specificity of the primary antibody was assessed by adsorption tests of the antibody with synthetic quail GnIH in a saturating concentration (20 μg/ml), synthetic human NPFF (FLFQPQRFamide) at 10 μg/ml and synthetic human Kiss-10 (YNWNSFGLRFamide) at 10 μg/ml (supplied by K. Tsutsui, Waseda University, Tokyo, Japan) and kisspeptin-10 (supplied by I. J. Clarke, Monash University, Melbourne, Australia) in a saturating concentration (20 μg/ml).

Statistical analysis

Data with respect to the number of neurones expressing RFRP or kisspeptin were subjected to the test of normality. Data were analysed using PROC MIXED (SAS Institute, Cary, NC, USA) and a mean comparison was performed using the Tukey–Kramer test. The mean ± SE number of immunoreactive neurones is represented. $P < 0.01$ was considered statistically significant.

Results

Distribution of RFRP-IR neurones and changes in their numbers in the hypothalamus of does

The RFRP immunoreactive cell bodies were identified in the DMH/PVN during follicular phase, luteal phase and anoestrus (Fig. 2). The mean number of RFRP-IR neurones in follicular phase was lower than luteal phase and anoestrous stage ($P < 0.0001$; Fig. 3A). Irrespective of the ovarian stage, the number of RFRP-IR neurones in the rostral region of the DMH/PVN was higher than in the caudal region ($P < 0.0001$; Fig. 3B), being lower than in the middle region ($P = 0.001$).

During the follicular phase of the cycle, no difference was recorded in the distribution of the RFRP-IR neurones in three anatomical regions of the DMH/PVN ($P = 0.9$; Fig. 3C); however, during the luteal phase, the number of RFRP-IR neurones in the rostral region was greater than in the caudal region ($P < 0.0001$; Fig. 3C). During anoestrus, the number of RFRP-IR neurones in the rostral

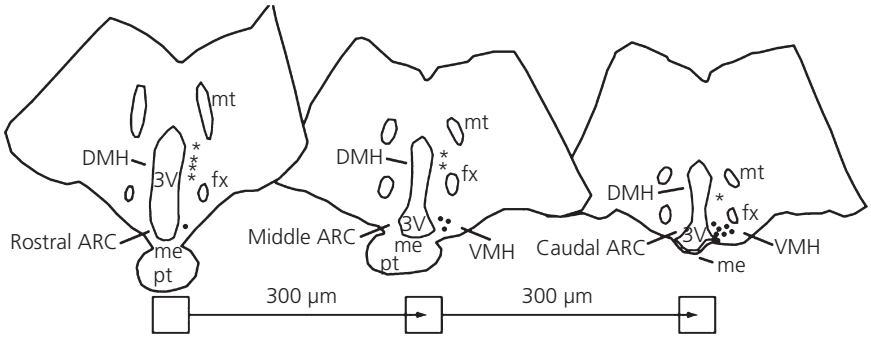

Fig. 1. A schematic drawing of three regions of the arcuate and dorsomedial nuclei of the goat hypothalamus. 3V, third ventricle; ARC, arcuate nucleus; DMH, dorsomedial nucleus; fx, fornix; me, median eminence; mt, mammillothalamic tract; pt, pituitary gland; VMH, ventromedial nucleus.

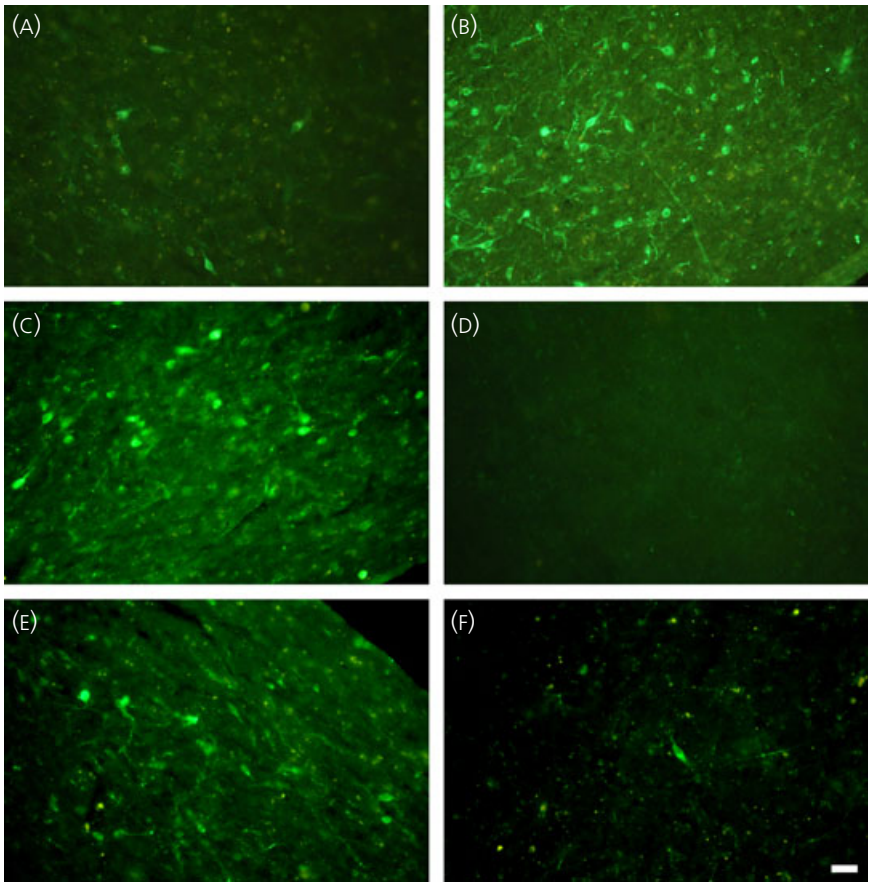

Fig. 2. Expression of RFamide-related peptide in neuronal cell bodies in the rostral region of the dorsomedial hypothalamus and paraventricular nucleus of the doe using immunohistochemistry. (A) Follicular phase, (B) luteal phase, (C) anoestrous stage and (D) immunohistochemistry using gonadotrophin-inhibitory hormone (GnIH) antibody incubated with synthetic quail GnIH at 20 μg/ml during the anoestrous phase, (E) immunohistochemistry using GnIH antibody incubated with synthetic human NPFF at 10 μg/ml during the anoestrous phase and (F) immunohistochemistry using GnIH antibody incubated with synthetic human KiSS-10 at 10 μg/ml during the anoestrous phase. Scale bar = 30 μm (images represent extremes for the observed differences).

region was greater than in the caudal region of the DMH/PVN ($P < 0.0001$; Fig. 3C). The number of RFRP-IR neurones in the rostral region of the DMH/PVN was lower during the follicular phase than during the luteal and anoestrous stages ($P < 0.0001$; Fig. 3C). The number of RFRP-IR neurones in the middle and rostral regions of the DMH/PVN was not affected by the stage of the ovarian cycle ($P > 0.01$).

Distribution of kisspeptin-IR neurones and changes in their numbers in the hypothalamus of does

Kisspeptin-IR cell bodies were identified in the ARC during the follicular phase, luteal phase and anoestrous stage (Fig. 4). The mean number of kisspeptin-IR neurones in the follicular phase was greater than in luteal and anoestrous stages ($P < 0.0001$; Fig. 5A). There was no difference between the number of kisspeptin-IR neurones in the luteal phase and anoestrous stage ($P = 0.30$ Fig. 5A). Irrespective of the ovarian stage, the number of kisspeptin-IR neurones in the caudal region of ARC was greater than in the middle and rostral regions ($P < 0.0001$; Fig. 5B). The number of kisspeptin-IR cells in the middle region was greater than in the rostral region ($P < 0.0001$ Fig. 5B).

At all stages studied, the number of kisspeptin-IR neurones in the caudal ARC was greater than in the middle and rostral ARC ($P < 0.0001$; Fig. 5C). More kisspeptin-IR neurones were found in the middle than in the rostral ARC ($P < 0.0001$; Fig. 5C). The number of kisspeptin-IR neurones in the rostral ARC was not affected by the stage of the ovarian cycle ($P > 0.01$) but, in the middle and caudal ARC, more kisspeptin-IR neurones were observed during the follicular phase than luteal phase and anoestrous stage ($P < 0.0001$; Fig. 5C).

Discussion

The present study describes the presence of RFRP-IR cell bodies in the DMH/PVN of the does. Clarke *et al.* (29) and Caraty *et al.* (30) previously reported the presence of RFRP in the ovine DMH/PVN. The RFRP-IR cells were also identified in the rodent DMH (31,32). In the present study, DMH/PVN sections were divided into rostral, middle and caudal regions. Irrespective of the ovarian stage, the

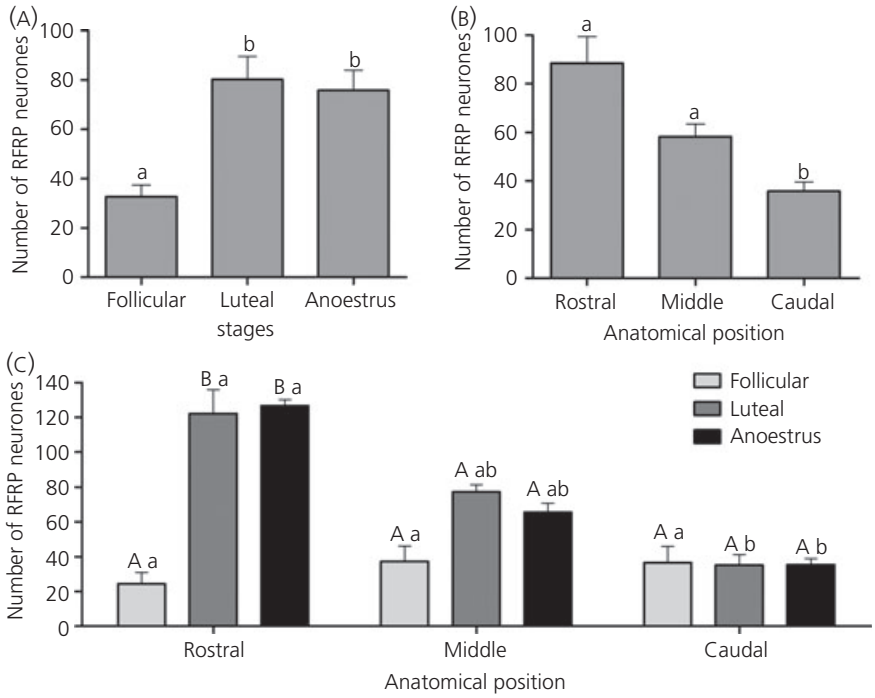

Fig. 3. Mean ± SE number of RFamide-related peptide (RFRP) neurones in the dorsomedial hypothalamus and paraventricular nucleus (DMH/PVN) of the doe ($n = 3$); at (A) during follicular, luteal and anoestrous stages; (B) in the rostral, middle and caudal regions of the DMH/PVN; and (C) in the rostral, middle and caudal regions of the DMH/PVN during different stages of the ovarian cycle. Different lowercase superscript letters indicate significant differences between the columns with similar pattern. Different uppercase superscript letters indicate significant differences between different reproductive stages in the same anatomical region.

number of RFRP-IR neurones in the rostral and middle regions of the DMH/PVN was greater than in the caudal region. It has been shown that RFRP fibres form close appositions with the GnRH neurones (33) and that RFRP has an inhibitory effect on GnRH neuronal system in mammals (34–36). GnRH neurones were identified in the sheep preoptic area (POA) (21). Because the POA is rostral to the DMH/PVN and some studies have shown that GnIH (or RFRP) neuronal terminals form close contacts with the GnRH neurones in poultry (37) and several mammals (6,7,20,38,39), it may be logical to find more RFRP-IR neurones in the rostral region of the DMH/PVN.

The number of RFRP-IR neurones in goat follicular phase was lower than in the luteal phase and the anoestrous stage. The number of RFRP neurones in sheep decreased during short days (21) and increased during long days (40). The levels of RFRP and RFRP receptor mRNA were lowest at oestrus and highest during dioestrus in sows (40). In the hamster, fewer RFRP-IR cell numbers were recorded during pro-oestrus compared to dioestrus (35). The level of RFRP-IR in the mouse ovary increased during late dioestrus (42). The lower number of RFRP-IR cells in the DMH/PVN of does during the follicular phase, compared to the luteal and anoestrous stages, is in line with the inhibitory effect of RFRP on GnRH secretion.

During the follicular phase, the number of RFRP-IR neurones in rostral, middle and caudal regions of DMH/PVN was the same; however, during the luteal phase and the anoestrous stage, the largest number of RFRP-IR neurones was recorded in the rostral region. On the other hand, the number of RFRP-IR neurones in the rostral region during the follicular phase was lower than in the luteal phase and the anoestrous stage. This is in line with findings of Clarke *et al.* (43), who noted that the expression of RFRP mRNA is reduced during the follicular phase of the oestrous cycle in ewes compared to the luteal phase. In sheep, RFRP-IR cell bodies were present in the PVN and DMH, with approximately 40% more cells being recorded during the nonbreeding season compared to the breeding season (21). Approximately 40% of RFRP neurones in the DMH of hamsters expressed oestradiol receptor α (ERα), with the axon terminals of the neurones projecting to GnRH neurones in mice, rats and hamsters (31). Furthermore, RFRP cells in rats were detected in the region between the DMH and ventromedial nucleus (44), with cells extending rostrally to the perifornical area (45). Based on retrograde tracing studies, neurones in the DMH/PVN do not project to the median eminence, where their terminals are co-localised with the GnRH neurones in the rat (44) and sparrow (46). In addition, GnIH neuronal terminals form close contacts with the GnRH neurones in poultry (37) and several mammals (6,7,20,38,39). Moreover, RFRP-IR terminals, forming contacts with more rostral GnRH neurones, were greater during the nonbreeding season in sheep (21). These findings suggest an effective contribution of rostral area of the DMH/PVN to convey the inhibitory signal to the POA during the luteal and anoestrous phases.

More kisspeptin neurones were found during the follicular phase compared to the anoestrous stage in the doe. Similarly, Smith *et al.* (21) reported a greater number of kisspeptin neurones during the

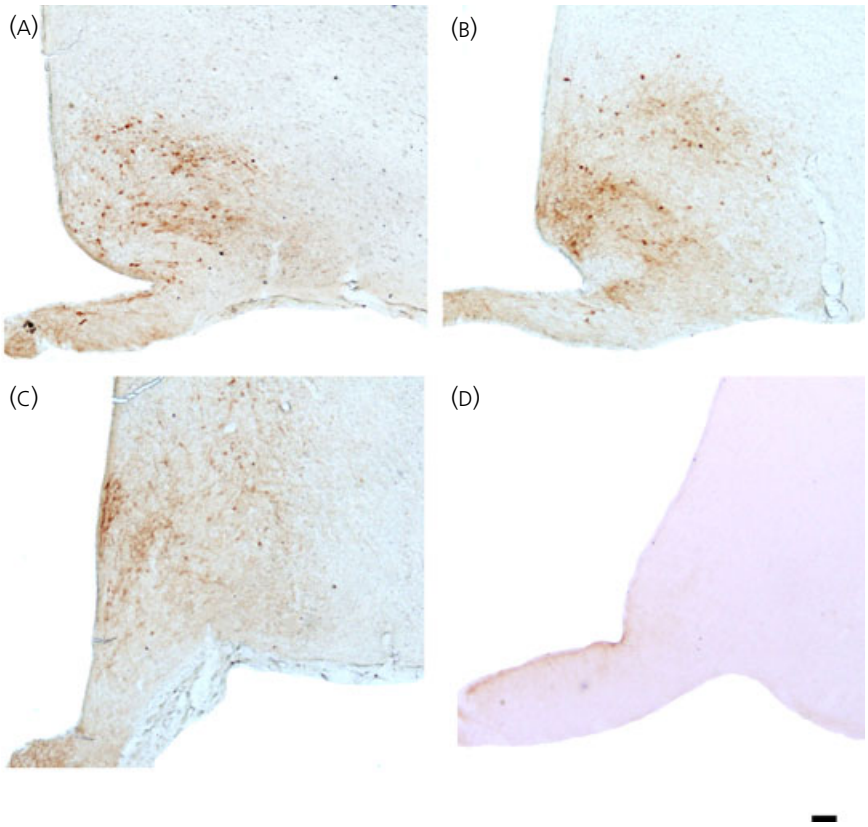

Fig. 4. Expression of kisspeptin in neurone cell bodies of the arcuate nucleus (ARC) of the hypothalamus in the doe, using immunohistochemistry. (A) Follicular phase, (B) luteal phase, (C) anoestrous stage and (D) immunohistochemistry using kisspeptin antibody incubated with synthetic mouse kisspeptin-10 at 20 μg/ml in the ARC. Scale bar = 100 μm.

breeding season in sheep compared to the anoestrous season, in line with the higher level of *KiSS1* mRNA in the ARC (47). These findings indicate a role for kisspeptin in the control of the breeding season. Wagner *et al.* (47) showed that *KiSS1* expression was three-fold higher during an LD 8 : 16 h photoperiod (identical to the breeding season) than during the longer photoperiods in ewes. More kisspeptin neurones were found in the ARC of ewes that were kept under LD 8 : 16 h photoperiods compared to long photoperiods (48). The expression of *KiSS1* mRNA in the ARC of ovariectomised ewes decreased during anoestrus but increased at the start of the breeding season (49). Moreover, in oestradiol-implanted ovariectomised ewes, with an oestradiol concentration similar to the luteal phase level, the number of kisspeptin neurones during the breeding season in the middle and caudal parts of the ARC was four-fold greater than in the anoestrous season (21). The expression of kisspeptin is probably mediated by photoperiod, being stimulated through melatonin (50). In addition to variations in the seasonal expression of kisspeptin in ewe, kisspeptin neuronal terminals formed a greater number of contacts with the GnRH neurones during the breeding season compared to the anoestrous season (21). On the other hand, treatment with kisspeptin during anoestrus induced ovulation in the ewe (51). Therefore, kisspeptin could also be a factor in activation of the hypothalamic-pituitary-ovarian axis in goats.

The mean number of kisspeptin-IR neurones in the doe ARC was similar during the luteal phase and anoestrus. P4 treatment of ovariectomised ewes decreased the number of cells expressing *KiSS1* mRNA (49). In the ewe, positive- and negative-feedback effects of oestrogen are transferred to the GnRH neurones via ARC neurones (52,53). The number of kisspeptin-IR neurones in the caudal ARC of the doe was higher than in the middle and rostral regions, consistent with the data obtained in the ewe showing that more than 90% of kisspeptin neurones in the caudal part of the ARC express ERα (18). During late follicular phase, *KiSS1* mRNA and kisspeptin-IR neurones increased in the middle and caudal parts of the ARC (54,55). The present findings in the doe support the data in sheep, indicating that kisspeptin neurones in the caudal part of the ARC may be involved in the positive-feedback effects of oestradiol on GnRH secretion.

In conclusion, the number of RFRP-IR cells during the luteal and anoestrous stages was greater than during the follicular phase in Abadeh does. These cells were more concentrated in the rostral part of the DMH/PVN. On the other hand, the number of kisspeptin-IR cells during the follicular phase was greater than during the luteal and anoestrous stages. The higher kisspeptin and lower RFRP expressions in the hypothalamus during the breeding season indicate a role for these peptides in the control of seasonal breeding in caprine.

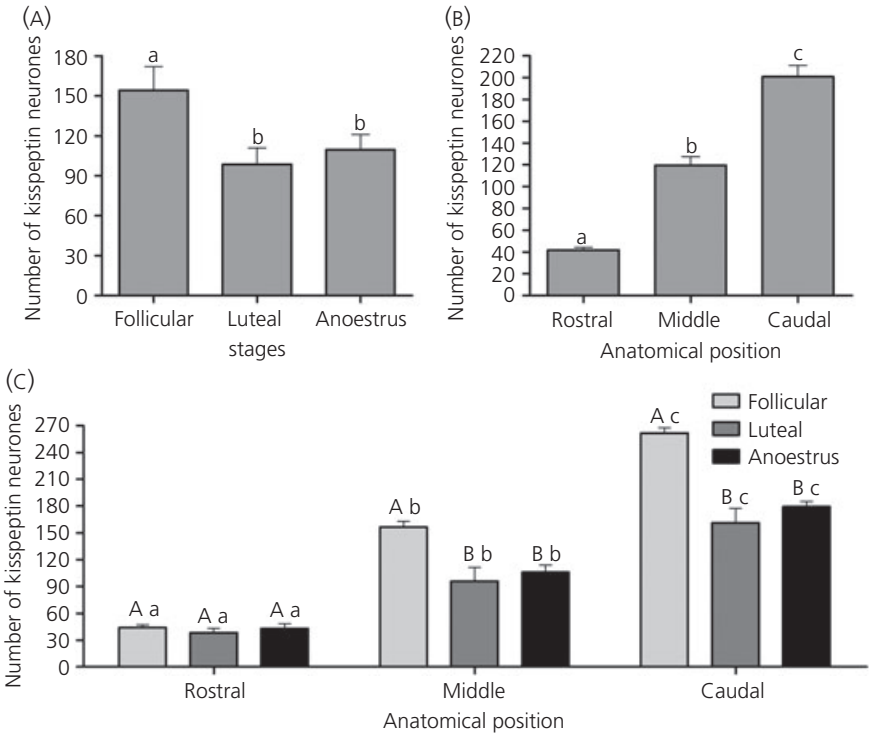

Fig. 5. Mean ± SE number of kisspeptin neurones in the arcuate nucleus (ARC) of goats (n = 3); (A) during the follicular, luteal and anoestrous stages; (B) in the rostral, middle and caudal regions of the ARC; and (C) in the rostral, middle and caudal regions of the ARC during the ovarian cycle. Different lowercase superscript letters indicate significant differences between columns with similar pattern. Different uppercase superscript letters indicate significant differences between different reproductive stages in the same anatomical region.

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