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Effect of Different Phases of Estrous Cycle on Kisspeptin Expression in the Arcuate Nucleus of the Ewe

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Abstract: Kisspeptin (KP) is a prodct of prepro-kisspeptin. KP is a key regulator of GnRH and LH secretion in the ewe. In this study, Effect of different phases of estrous cycle on kisspeptin expression in the arcuate nucleus of the ewe examined. Because of no difference between phases of cycle, we suggested that perhaps KP neurons involved in mediating of both positive and negative feedback effects of steroids to GnRH neurons.

Key words: Kisspeptin, estrous cycle, arcuate nucleusm ewe, GnRH

INTRODUCTION

KP is a key regulator in LH and GnRH secretion (Dungan *et al*., 2006; Smith and Clarke, 2007; Roa and Tena-Sempere, 2007) and causes GnRH and LH releasing in rodent (Gottsch *et al*., 2004; Thompson *et al*., 2004; Navarro *et al*., 2004, 2005) sheep (Messager *et al*., 2005) monkey (Plant *et al*., 2006) and human (Dhillo *et al*., 2005). Mutation in GPR54 put off puberty beginning in human (De Roux *et al*., 2003) and mouse (Seminara *et al*., 2003; Funes *et al*., 2003). KP has been involved in feedback functions of ovary steroids. In sheep, KP exerts positive feedback actions in medio-basal hypothalamus which result in preovulatory surge of LH (Caraty *et al*., 1998; Couse and Korach, 1999). Effects of sexual steroids convey to GnRH neurons by different systems of cells. GnRH neurons do not express estrogen receptor-α (ER-α) or progesterone receptor (PR) (Herbison and Pape, 2001) but they express estrogen receptor-ß (ER-ß) (Dorling *et al*., 2003). Regarding steroid feedback effects on GnRH/LH, however, ER-ß appears to play a slight role in the regulation of reproduction. Strong evidences show that effects of steroid feedback on GnRH/LH results from cells which express ER-α (Goubillon *et al*., 1999). Possibly, stimulating effects on GnRH surge are influenced by ER-α (Couse and Korach, 1999). Most of ER-α located in arcuate nucleus (ARC). So, other sensitive neurons to steroid, rather than GnRH, should mediate effects of sexual steroids on GnRH secretion. Because KP producing cells in the ARC are ab-undant in sheep (Franceschini *et al*., 2006; Pompolo *et al*., 2006) and also these cells include ER-α, perhaps KP cells convey positive feedback of steroids to GnRH neurons.

This research aimed to test expression levels of KP neurons in estrous cycle. We proposed that levels of expression of KP in the follicular phase may be more than other phases.

MATERIALS AND METHODS

Animals: Nine Corriedale ewes with similar age and weight were maintained under natural pasture situation. Research was performed during the breeding season. Estrous cycle controlled by using rams in the group. Estrous cycles were synchronized with Cloprostenol. Three groups of animals (n = 3 in each phase) were used, indicating the luteal phase (day 10 of cycle), the follicular phase and estrous phase. Follicular and luteal phases animals were killed 24 h and 10 days after the injection of Cloprostenol, respectively. Estrous animals were killed 1-2 h after the onset of the Standing heat (about 48 h after Cloprostenol injection). Blood samples from the estrous group of ewes were collected prior to the onset of estrous and just before slaughtering of the animals (1-2 h after the onset of standing heat). For the follicular and luteal phase groups, the blood samples were taken just prior to killing the animals. Both ovaries of each animal were also examined for the presence/absence of corpora lutea. Levels of the plasma hormone in these sheep have been explained previously (Estrada *et al*., 2006). Progesterone levels in luteal phase's ewes were 3.2±0.5 ng mL^{-1} (MEAN±SEM). In other phases, levels of Progesterone

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were lower. Plasma LH levels were <1.0 ng mL^{-1} in ewes during the luteal phase. These levels in late-follicular and across estrus phase's ewes were 1.4-6.9 and 51.4±4.2 ng mL^{-1}, respectively.

Tissue collection: Tissue was collected and processed as previously described (Foradori *et al*., 2006; Pompolo *et al*., 2006). Briefly, ewes were killed with an overdose of sodium pentobarbital and their heads were perfused with 2 L of heparinized saline (12.5 U mL^{-1}) followed by 2 L of 4% paraformaldehyde plus 15% picric acid in PB and then 1 L of the same fixative containing 20% sucrose. Brains were removed, tissue containing the hypothalamus dissected out, infiltrated with 30% sucrose and frozen coronal sections (40 μm thick) cut and stored at -20°C in cryoprotectant.

Immunohistochemistry: Rabbit's antisera against kisspeptin were provided by Dr. Caraty. Test of Immunohistochemical characteristics of the rabbit polyclonal antibody against kisspeptin -10, in previous experiment has been explained (24), comparing it with a commercially available (Phoenix Pharmaceuticals, Inc) rabbit polyclonal antibody against kisspeptin-10 (human sequence). Three sections/sheep were selected for analysis to represent the rostral, middle and caudal regions of the ARC in each phase of estrous cycle. Sections were washed in 0.05 M PBS, mounted on slides and dried overnight. Kisspeptin-containing cells were labelled using antigen retrieval and Caraty kisspeptin antibody (1: 2000) was used. After incubation of slides with 1: 500 goat's anti-rabbit sera conjugated to Alexa488, they were washed 3 times with PBS and then counterstained with 0.3% Sudan Black B. After further washing, coverslips were applied with fluorescence mounting medium (Dako, Carpinteria, CA). A positive control (no antibody pre-absorption) and a negative control (antibody pre-absorbed with kisspeptin) were used. Images of immunoreactive cell bodies were captured with the proper excitation for Alexa 488. A single viewer then counted the total number of immunopositive cell bodies.

Statistical analysis: Data were analyzed by general linear model of SPSS software (version 11.5). The mean number of cells within areas was compared by 2-way ANOVA (main effects of cycle stages and anatomical area). Duncan's multiple range used as a post hoc test. And $p<0.05$ was considered as a significance level.

RESULTS

No effects of phase cycle within area of ARC were observed in cell number ($p>0.05$), except in luteal phase

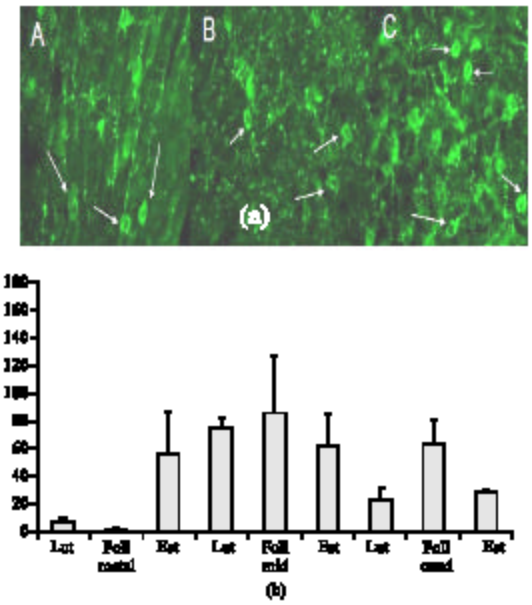

Fig. 1: Top panel: Fluorescence images of rostral (A) middle (B) and caudal ARC immunostained for kisspeptin. Arrows indicate examples of kisspeptin neurons. Bottom panel: Mean (±SEM) number of kiss cells in rostral, middle and caudal of ARC within estrous cycle

that it's cells from middle area were higher than 2 others area ($p<0.05$). Number of cells in the estrous phase of rostral area also were higher with respect to follicular and luteal phase in this area (Fig. 1).

DISCUSSION

This study showed that there is no significant difference between phases of cycle in areas of ARC. In contrast, in previous work (Estrada *et al*., 2006), up-regulation of KP in the rostral ARC during estrous phase has been reported. Exact details of this experiment whether which kind of antibody used, unknown. We used caraty antibody with high validity that previously demonstrated uncross reactivity of it with other RF-amides (Goodman *et al*., 2007). Because no difference between phases of cycle, several lines of evidence inferred that perhaps KP neurons involved in mediating of both positive and negative feedback effects of steroids to GnRH neurons. First of all, KP neurons in the ovine contain PR (Smith *et al*., 2007). We previously showed that KP neurons overlap with Dynorphin neurons in the ARC (Goodman *et al*., 2007). Dynorphin neurons contain PR (Foradori *et al*., 2002) and an antagonist to the Dynorphin receptor stimulates episodic LH secretion in luteal phase ewes (Goodman *et al*., 2004). Because

progesterone has negative feedback effects on GnRH neurons which have no PR, then our data suggest that KP neurons may relay progesterone negative feedback effects to GnRH neurons. Second, KP neurons abundant in the ARC of sheep (Franceschini *et al*., 2006; Herbison and Pape, 2001) and these neurons contain ER-α (Franceschini *et al*., 2006), we can suggest that KP neurons in the ARC transmit the positive feedback effect to the GnRH cells which do not own ER-α.

CONCLUSION

KP neurons may implicated in conveying of positive and negative feedback effects of ovarian steroids to GnRH neurons in preoptic area that have no PR or ER-α. Attempts are ongoing to apply the knowledge of the functions of KP to therapeutic uses including under fertility and over fertility.

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