

Differential endocrine response in rams to intracerebroventricular infusion of genistein

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The intracerebroventricular infusions of genistein (total 40 µg) were made in male sheep (November) to test its influence on melatonin, growth hormone (GH) and luteinizing hormone (LH) secretion. The analysis of the results encompassed 3 similar periods: before the infusion (afternoon hours), the first (evening hours), and the second (night hours) halves of the treatment. The night plasma concentration of melatonin in genistein-infused rams was significantly lower than that noted during the respective period in vehicle-infused rams. Plasma GH concentration increased significantly in both vehicle- and genistein-infused rams during the night hours, as compared with the concentrations noted during the afternoon and evening, however, genistein significantly stimulated the amplitude of GH pulses in these latter. The LH concentration was significantly lower during the second part of genistein treatment, than in vehicle-infused rams. The frequency and amplitude of LH pulses clearly tended to decrease following genistein infusion. In conclusion, genistein, acting at the central nervous system level in sexually active rams is able to reduce the secretion of melatonin and LH and has also a slight stimulatory effect on the amplitude of GH pulses.

Key words: genistein, melatonin, growth hormone, luteinizing hormone, sheep endocrinology

INTRODUCTION

The structure and function of many neuronal systems in the brain are under the influence of gonadal steroids. Certain genomic actions of steroids are mediated through nuclear receptors that are differentially expressed within the hypothalamic tissue, suggesting that they may have unique functions (Shughrue et al. 1996, 1997, Tilbrook and Clarke 2001). An immediate effect of endogenous steroids, especially of estrogens, via a nongenomic signaling pathway has also been described (Arreguin-Arevalo and Nett 2005). Phytoestrogens, which are found in many plants that are used in ruminant feeds, and their metabolites are structurally similar to endogenous estrogens and their mechanism of action may involve binding to estrogen receptors (ERs) α and β (Kuiper et al. 1997, Setchell and Adlercreutz 1988). It was reported that several

phytoestrogens, including genistein, have a higher binding affinity for ER β than for ER α (Kuiper et al. 1998), this is however an order of magnitude lower binding affinity than that of 17 β -estradiol (E₂) for both ER subtypes. Phytoestrogens may also produce their effects by acting indirectly, i.e. to modulate the concentrations of endogenous estrogens (Kao et al. 1998) or they may also induce effects that are not mediated through ERs (Kim et al. 1998). In this context, genistein is known as a broad-spectrum inhibitor of protein tyrosine kinases.

Following ingestion, phytoestrogens may reach circulating concentrations that, in some circumstances, exceed the amount of endogenous estrogens. Lundh and coworkers (1990) demonstrated that the total amounts of the plant estrogens and their metabolites in ovine blood plasma increased successively and reached the maximum level after about 3 h of intake. Moreover, ruminal microorganisms may take 6 to 10 days to adapt fully to these substrates, so genistein may produce effects during the first few days after introduction of the phytoestrogenic diet. Studies on rats showed that following

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Received 29 August 2007, accepted 7 November 2007

intraperitoneal administration, genistein rapidly appears in brain and then in microdialysate fluid from the corpus striata together with its metabolite *p*-ethyl-phenol (Chang et al. 2000). It was suggested that in ewes, phytoestrogens are able to interfere with the estrogen feedback mechanisms involved in the regulation of the pituitary trophic hormones (Mathieson and Kitts 1980) and recent data showed that genistein, infused directly into the third ventricle of the brain (IIIv), effectively inhibited luteinizing hormone (LH) and stimulated prolactin secretion in ovariectomized (OVX) ewes (Romanowicz et al. 2004a, Romanowicz and Misztal 2005). Genistein, as E_2 (Jansson et al. 1984, Martinoli et al. 1991), is also an effective stimulator of growth hormone (GH) secretion in OVX ewes by acting at the level of the central nervous system (CNS) (Misztal et al. 2007). Moreover, ERs and androgen receptors (ARs) are localized in the pineal gland, the main source of circadian melatonin secretion and direct *in vitro* and *in vivo* effects of E_2 and testosterone on pineal melatonin synthesis and release have been demonstrated in rats (Gupta et al. 1993, Martin et al. 1996, Okatani et al. 1998). Taking together a broad-spectrum of estrogens action in the brain, as well as the possible sexual differences in the regulation of the neuronal activity (Robinson et al. 2003), the aim of this work was to study the central influence of genistein on the secretion of melatonin, GH and LH – the hormones representing three different regulatory axes – in male sheep. In our experimental model the endocrine response was examined in conscious sexually active rams, following a several-hour-long infusions of genistein into the IIIv.

METHODS

Animals and management

All experimental procedures were approved by the Local Ethics Committee, according to the Polish Guide for the Care and Use of Animals (1997). The animals used in the study were one-year-old rams of the Blackface breed of sheep that show marked seasonal cycles in reproduction. They were maintained indoors under natural lighting conditions (52°N, 21°E) and fed a constant diet of commercial concentrates, with hay and water available *ad libitum*. The implantation of a stainless steel guide cannulae (1.2 mm o.d.) into the IIIv was performed under stereotaxic control (Traczyk and Przekop 1963), 1 month before the experiment (Misztal et al. 2007).

Experimental procedure

The experiment was performed during a period of short days, in November (sunset at 4:30 PM), when the rams reached their first sexual activity. Genistein (Sigma) was dissolved in ethanol and stored at -20°C as a stock solution (1 mg/500 μl) for no longer than 3 days. It was further diluted in saline before administration (4.8% of alcohol). A similar solution without genistein was prepared as a vehicle. The rams ($n=6$) were infused into the IIIv twice: once with vehicle (control) and once with genistein, 10 $\mu\text{g}/100\text{ }\mu\text{l/h}$ (total 40 μg), at 2-week intervals. The dose of genistein was selected according to our previous study on OVX ewes (Romanowicz et al. 2004a, Romanowicz and Misztal 2005), in which it evoked significant changes in the secretion of LH (decrease) and prolactin (increase). The treatments were made in a series of four 1-h infusions at 30-min intervals, from 4:30 PM to 10:00 PM. A BAS BeeTM microinjection pump (Bioanalytical Systems Inc., USA) and calibrated 1.0-ml gas-tight syringes were used. Plasma samples were collected for 8 h from 2:00 PM to 10:00 PM at 15-min intervals, through a catheter inserted into the jugular vein the day before the experiment. Blood volume taken each time was about 5 ml per sample, it means that about 3% of the total blood volume was withdrawn during the collection period. After centrifugation in heparinized tubes, plasma was stored at -20°C until hormone assays were performed. During the experiments, the rams were kept in comfortable cages where they could lie down and had unrestrained access to hay.

Analytical techniques

Concentrations of melatonin, GH, and LH in the blood plasma were measured by radioimmunoassays (RIA). Melatonin was assayed in unextracted plasma according to the method of Fraser and coworkers (1983), modified by Misztal and others (1996). Ovine anti-melatonin serum (AB/S/01, Stockgrand Ltd., UK), synthetic melatonin (Sigma) as a standard, and [O -methyl- ^3H]-melatonin (Amersham) as a tracer were used. Melatonin-free plasma for calibrated curves and blanks was obtained by early afternoon bleeds from sheep and striped from endogenous melatonin by activated charcoal Norit-A (Sigma). The range of the calibrated curve was from 15.6 to 1000 pg/ml and the working dilution of antibodies was 1:4000. Bound and

free tracer were separated after overnight incubation at 4°C by dextran-coated charcoal. Sensitivity of the assay was 16.8 ± 8.0 pg/ml and the intra- and interassay coefficients of variations were 10.5 and 13.2%, respectively.

Plasma GH and LH concentrations were assayed by double-antibody methods, according to Slaba and coauthors (1994) and Stupnicki and Madej (1976), respectively. The assay sensitivity for GH was 0.6 ng/ml, and the intra- and interassay coefficients of variation were 5.9 and 10.2%, respectively. The assay sensitivity for LH was 0.3 ng/ml, and the respective intra- and interassay coefficients of variation were 8.2 and 12.5%.

Statistics

To express precisely dynamics of changes in hormones secretion in response to genistein, the statistical analysis of the data included 3 equal periods: before the infusion (2:00–4:30 PM, afternoon hours), the first (4:45–7:15 PM, evening hours), and the second (7:30–10:00 PM, night hours) halves of the treatment. The effect of the treatment on the hormones concentrations was analyzed by the non-parametric equivalents of the analysis of variance: the ANOVA rank Kruskal-Wallis test (within groups) and the Freedman test (between groups).

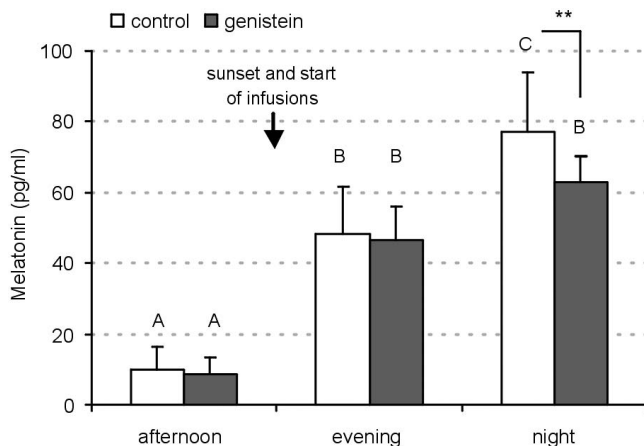


Fig. 1. Mean (\pm SEM) plasma concentrations of melatonin during the afternoon (2:00–4:30 PM), evening (4:45–7:15 PM) and night (7:30–10:00 PM) hours in vehicle- (white bars) and genistein-(dark bars) infused rams. Sunset and start of infusion at 4:30 PM. $A < B < C$ $P < 0.001$ (within groups); $**P < 0.01$ (between groups).

The frequency and amplitude of GH and LH pulses were determined by the PC-PULSAR computer program according to the method of Merriam and Wachter (1982) with cut-off G parameters: $G1 = 3.98$; $G2 = 2.40$; $G3 = 1.68$; $G4 = 1.24$ and $G5 = 0.93$. Analysis was performed individually for every ram and encompassed the entire sampling period. The frequency of GH and LH pulses was defined as the number of identified pulses per collecting period. The significance of differences in GH and LH pulse frequency between genistein and vehicle treatments was assessed by the non-parametric Mann-Whitney test. The significance of differences in the amplitude of GH and LH pulses between the treatments was assessed by the ANOVA rank Kruskal-Wallis test. Plasma melatonin, GH and LH concentrations, as well as the parameters of pulsatile GH and LH secretion (frequency and amplitude) are expressed as means \pm SEM.

RESULTS

As expected, in vehicle-infused rams, mean plasma melatonin concentrations increased gradually, but significantly, from the afternoon to the evening hours ($P < 0.001$) and next to the night hours ($P < 0.001$, Fig. 1). In genistein-infused rams, no significant effect on melatonin secretion was noted during the afternoon and evening, while during the night, mean concentration of melatonin was significantly ($P < 0.01$) lower than that noted during the respective period in vehicle-infused rams. Representative patterns of melatonin secretion in vehicle- and genistein infused rams are shown in Fig. 2.

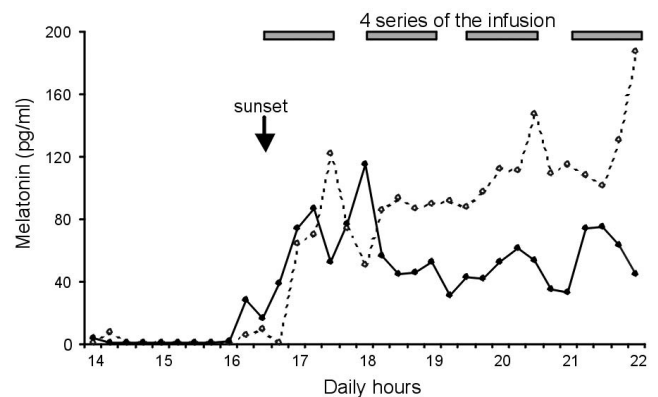


Fig. 2. Representative patterns of melatonin secretion in vehicle- (open) and genistein- ($10 \mu\text{g}/100 \mu\text{l}/\text{h}$, filled circles) infused rams from 4:30 PM to 10:00 PM. Plasma samples collected from 2:00 PM to 10:00 PM, at 15-min intervals.

The plasma GH concentration in vehicle-infused rams increased significantly ($P<0.05$) during the night hours as compared with the concentrations noted during the afternoon and evening (Fig. 3). A significant ($P<0.01$) increase in GH concentration during the night hours was also observed in genistein-infused rams, however, no significant differences in GH concentrations were noted during the studied periods between vehicle- and genistein-infused animals. The frequency and amplitude of GH pulses during the collecting peri-

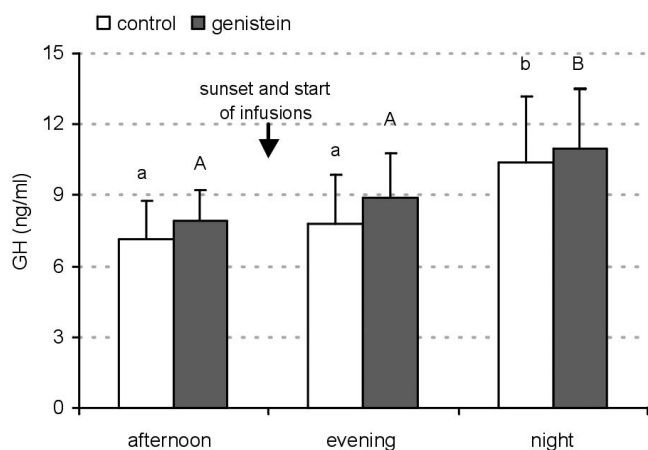


Fig. 3. Mean (\pm SEM) plasma concentrations of growth hormone (GH) during the afternoon (2:00–4:30 PM), evening (4:45–7:15 PM), and night (7:30–10:00 PM) hours in vehicle- (white bars) and genistein-infused (dark bars) rams. Sunset and start of infusion at 4:30 PM. ^{ab} $P<0.05$; ^{AB} $P<0.01$ (within groups).

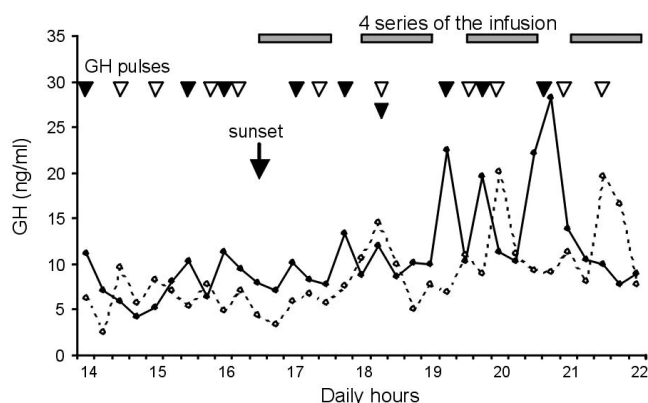


Fig. 4. Representative patterns of GH secretion in vehicle- (open) and genistein- (10 μ g/100 μ l/h, filled circles) infused rams from 4:30 PM to 10:00 PM. Plasma samples collected from 2:00 PM to 10:00 PM, at 15-min intervals. Black and white arrows indicate statistically significant pulses.

od in both vehicle- and genistein-infused rams are shown in Table I and representative patterns of pulsatile GH secretion in these rams are shown in Fig. 4.

A significant increase in LH concentrations occurred in vehicle-infused rams during the night hours, as compared with the concentrations noted during the afternoon ($P<0.001$) and evening ($P<0.05$, Fig. 5). In genistein-infused rams, LH secretion was sustained on a similar level during the afternoon and evening, while the concentration noted during the night

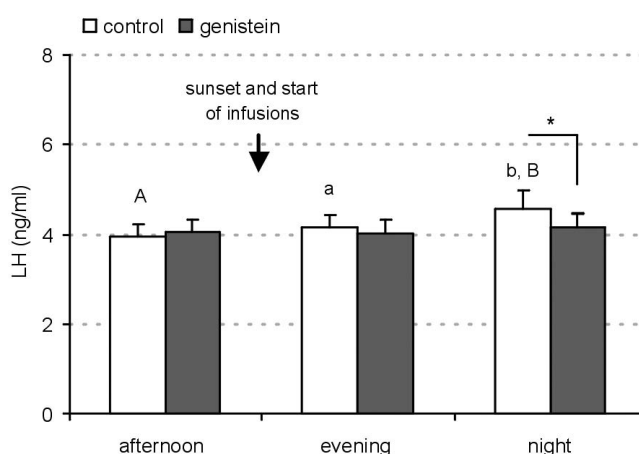


Fig. 5. Mean (\pm SEM) plasma concentrations of luteinizing hormone (LH) during the afternoon (2:00–4:30 PM), evening (4:45–7:15 PM) and night (7:30–10:00 PM) hours in vehicle- (white bars) and genistein-infused (dark bars) rams. Sunset and start of infusion at 4:30 PM. ^{ab} $P<0.05$; ^{AB} $P<0.01$ (within groups); * $P<0.05$ (between groups).

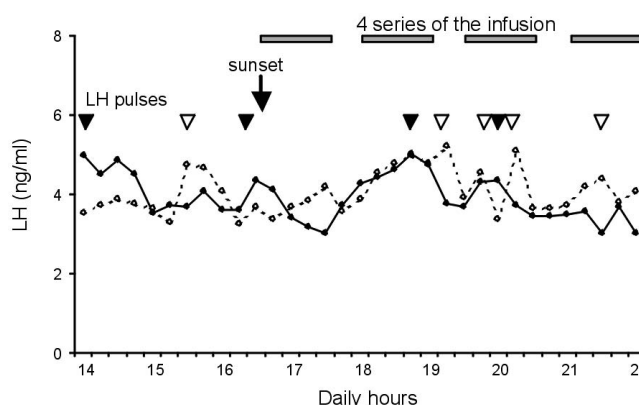


Fig. 6. Representative patterns of LH secretion in vehicle- (open) and genistein- (10 μ g/100 μ l/h, filled circles) infused rams from 4:30 PM to 10:00 PM. Plasma samples collected from 2:00 PM to 10:00 PM, at 15-min intervals. Black and white arrows indicate statistically significant pulses.

Table I

Mean (\pm SEM) growth hormone (GH) and luteinizing hormone (LH) pulse frequency (pulses/8 h) and amplitude (ng/ml) in vehicle- and genistein-infused rams during the experimental period		
	Vehicle-infused	Genistein-infused
GH		
Frequency	8.25 \pm 1.03	9.75 \pm 0.75
Amplitude	3.92 \pm 0.58 ^a	6.01 \pm 0.71 ^b
LH		
Frequency	4.50 \pm 0.29	3.75 \pm 0.25
Amplitude	1.30 \pm 0.15	1.09 \pm 0.09

^{a,b} $P < 0.05$

hours was significantly ($P < 0.05$) lower than during the respective period in vehicle-infused rams. The frequency and amplitude of LH pulses during the collecting period in both vehicle- and genistein-infused rams are shown in Table I and representative patterns of pulsatile LH secretion in these rams are shown in Fig. 6.

DISCUSSION

The results of the present study show that genistein, infused into the IIIv in sexually active rams, is able to reduce secretion of melatonin and LH, but in case of GH, it has a slight stimulatory effect on the amplitude of GH pulses. The changes observed in the secretion of these three hormones may suggest an action of genistein at the level of the CNS.

Melatonin is the principal hormonal product of the pineal gland and transfers photoperiodic information via the blood stream to the each cell of the body. Its characteristic circadian rhythm consists of a phase of high secretion during the night and a phase of low secretion during the day (Moore and Klein 1974, Rollag et al. 1978, Misztal et al. 1996). Here we have demonstrated that genistein, a plant-derived estrogen-like compound, was able to reduce nocturnal rise in melatonin, when administered into the CNS of rams. To our knowledge, no effects of any phytoestrogens on melatonin secretion *in vivo* have been reported to date, especially in sheep, in which melatonin drives the reproductive cycle (Bittman et al. 1983, Misztal et al.

2002). Evidence exists that ERs and ARs are located in the pineal gland of rodents and humans (Gupta et al. 1993, Luboshitzky et al. 1997) and that gonadal steroids may affect pineal melatonin release in both males and females (Cardinali et al. 1987, Martin et al. 1996, Okatani et al. 1998). An *in vitro* study by Martin and colleagues (1996) showed a marked increase in melatonin release by glands removed during the dark span, following perfusion with testosterone and E_2 . In contrast, ovariectomy in peripubertal female rats led to increases in the levels of melatonin and N-acetylserotonin and in the activity of N-acetyltransferase (NAT), the principal enzyme of the melatonin synthesis pathway (Okatani et al. 1998). The cited authors also demonstrated that E_2 replacement decreased the levels of these parameters to control levels and suggested that the inhibitory effect of estrogen on melatonin synthesis is mediated by the modulation of NAT activity (Okatani et al. 1998). Further studies revealed that estrogen might negatively affect the stimulation of pineal adenylate cyclase activity by noradrenaline (Hayashi and Okatani 1999). Following this aspect, observations in sheep indicated that gonadal steroids, androstenedione and E_2 , might regulate pineal β -adrenoceptor number and sensitivity (Foldes et al. 1985, Maxwell et al. 1989). The mechanism by which genistein influenced melatonin secretion in rams was not studied here, but our results prompt to further investigation of the noradrenaline signaling in this aspect.

The GH secretory profile in our rams seemed to be similar to that described earlier by Romanowicz and coworkers (2004b), as well as in males of other animal species, i.e., regular high-amplitude GH pulses with relatively low interpulse GH levels, especially after sunset (Tannenbaum and Martin 1976, Jansson et al. 1984). Gatford and coauthors (1997) found that mean and baseline plasma GH concentrations and GH pulse amplitude in growing rams were greater than in ewes. The relationship between the endogenous GH pulsatile pattern and gonadal steroids remains controversial, however, Painson and colleagues (1992) reported that short-term administration of E_2 to gonadectomized adult male rats feminized the male pattern of spontaneous and GHRH-stimulated GH secretion, that is, E_2 decreased the pulse amplitude and increased basal GH levels. In other studies, a significant decrease in GH mRNA levels was observed in adult male rats following castration (Gonzalez-Parra et al. 1996). In male

Shiba goats, postpubertal castration increased the amplitude of GH pulses, without affecting the inter-pulse interval and area under the curve of GH secretion (Mogi et al. 2002). These observations indicate that gonadal steroids affect GH secretion in males, but the effects may vary among species. Although in our experimental model changes in the patterns of GH secretion were observable in both groups of rams, they were more expressed in genistein-treated animals. In both groups, plasma GH concentrations increased during the evening hours, reaching the highest level during the night, but genistein markedly stimulated the amplitude of GH pulses. In this aspect, the action of a phytoestrogen might be opposite to that of E_2 observed in male rats (Painson et al. 1992) and directed at the mechanism generating GH pulses. Increased release of GH in response to genistein infusion, accompanied by diminution in the storage of GH by the pituitary somatotropes was demonstrated earlier by our lab in OVX ewes (Misztal et al. 2007).

It is generally accepted that GH synthesis and release from pituitary somatotropes is controlled by the opposing actions of the hypothalamic neuropeptides, GH-releasing hormone (GHRH), and somatostatin (SS). Recent studies on both male and female rats revealed that the majority of GHRH neurons in the hypothalamic arcuate nucleus (ARC) have $ER\alpha$, but not $ER\beta$, and only a few SS cells in the periventricular nucleus and ARC have $ER\alpha$ or $ER\beta$ (Kamegai et al. 2001, Shimizu et al. 2005). In contrast, GHRH neurons in ewes do not express $ER\alpha$, however, they are surrounded by many $ER\alpha$ -expressing cells (Scanlan and Skinner 2002), suggesting an indirect activation of GHRH neurons by estrogens in this species.

In addition to reported changes in melatonin and GH release, our present study showed that genistein inhibited LH secretion, confirming its possible interference with the gonadal steroid feedback mechanism involved in the release of this gonadotropin. In rams, secretion of LH is under the negative feedback regulation of testicular steroids that act predominantly within the CNS to suppress gonadotropin-releasing hormone (GnRH) secretion (Tilbrook and Clarke 2001). It is unclear to what extent these actions of testicular steroids result from the direct action of testosterone or its primary metabolites, E_2 or dihydrotestosterone. Existing evidence suggests that in rams, E_2 is able to mediate the feedback effects of testosterone (Sharma et al. 2000). Furthermore, ERs were found in the hypothalamus of

males (Scott et al. 2000). It is also unclear whether gonadal steroids affect GnRH/LH secretion acting directly on GnRH neurons or using an integrated neuronal network within the hypothalamus. Recently, Skinner and Dufourny (2005) showed that numerous $ER\beta$ -immunoreactive cells were located throughout the ovine hypothalamus and over 50% of the GnRH neurons were found to express immunoreactive $ER\beta$. Since isoflavones of plant origin poses high affinity for $ER\beta$ (Kuiper et al. 1998), GnRH cells seem to be a potential target for their action. However, this hypothesis requires further verification. A similar immediate decrease in LH release in response to genistein was also observed in OVX ewes during the anestrus and breeding seasons (Romanowicz et al. 2004a, Romanowicz and Misztal 2005). Although the organization of the neuronal control of GnRH/LH release differs in both sexes (Robinson et al. 2003), one can conclude that some reproductive abnormalities, resulting i.e. from feeding phytoestrogenic diets (Adams 1977), might be related to the action of phytoestrogens at the hypothalamic level.

CONCLUSION

Genistein, acting at the CNS level in sexually active rams is able to reduce the secretion of melatonin and LH and has also a slight stimulatory effect on the amplitude of GH pulses. Taking into account a possible mechanisms of genistein action, which are not mediated through ERs, the present data may set the course for further investigation on the phytoestrogens' action in the sheep brain.

ACKNOWLEDGEMENTS

The authors would like to express their thanks to veterinary surgeon J. Rutkowski for help in the brain surgery and to the other members of the Department of Endocrinology for technical assistance with the radioimmunoassays.

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