ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol Exp 2005, 65: 1-9

Effective stimulation of daily LH secretion by the combined treatment with melatonin and naloxone in luteal-phase ewes

Tomasz Misztal and **Katarzyna Romanowicz**

Department of Endocrinology, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna n/Warsaw, Poland

Abstract. This study was designed to test the hypothesis that melatonin would intensify daily LH release after central blockade of the opiate receptors in sexually active ewes. The intracerebroventricular infusions of vehicle (control), melatonin, naloxone and melatonin in combination with naloxone were made in ewes in the luteal phase of the estrous cycle, from 2:00 P.M. to 5:00 P.M. Blood samples were collected from 11:00 A.M. to 8:00 P.M. at 10-min intervals. The mean plasma LH concentrations were measured before, during and after the infusions. The frequency and amplitude of LH pulses were determined during the whole experimental period. The LH concentrations recorded during melatonin or naloxone infusions were significantly higher than the concomitant concentration in vehicle-infused animals. The mean LH pulse amplitude in melatonin- and naloxone-treated ewes was also significantly higher than in controls. The LH concentration measured during the combined infusion of melatonin and naloxone was significantly higher than that during vehicle infusion. The LH concentration recorded in turn after the treatment was significantly higher than the concomitant concentrations in vehicle-, melatonin- and naloxone-infused animals. The mean LH pulse amplitude in this group was significantly higher than in the vehicle-infused group. These results indicate that blockade of the opiate receptors within the CNS facilitated effective stimulation of daily LH secretion by exogenous melatonin. In conclusion, a relationship between melatonin and endogenous opioid peptides may be crucial in enabling melatonin to exhibit stimulatory action on LH secretion during the luteal phase of the estrous cycle in ewes.

The correspondence should be addressed to T. Misztal, Email: t.misztal@ifzz.pan.pl

Key words: LH, melatonin, naloxone, endogenous opioid peptides, luteal phase, sheep endocrinology

INTRODUCTION

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone synthesized in the pineal gland and released in a precise diurnal pattern with low levels during the day (light hours) and high during the night time (dark period). The duration and magnitude of melatonin secretion is directly related to the length of the darkness period thus, its fundamental function is the transduction of photoperiodic information either to the central nervous system or other tissues of the organism (Reiter 1991). For years, melatonin has been associated with the reproductive activity of seasonal animal species (Goldman 1999, Lincoln 1991). In sheep, changes in melatonin secretion constitute a signal to the neural structures controlling the secretion of gonadotropins from the pituitary gland that a long duration is stimulatory and a short duration is inhibitory (Lincoln 1991). Melatonin can induce an early ovulatory period when given to ewes at a suitable time during anestrus (Arendt et al. 1983, Bittman et al. 1983). Activation of the gonadotropic system in anestrous ewes, leading to the onset of estrus, requires, however, several weeks of exposure to melatonin, i.e., in implants (Viguie et al. 1995). In the last decade it has been shown that the action of melatonin on reproductive function in sheep takes place in the premammillary hypothalamic area (PMH) (Malpaux et al. 1998).

We have recently demonstrated that melatonin can also modulate the daily rhythm of LH secretion, following its direct, short-term infusion into the central nervous system (Misztal et al. 2002). However, the response of this gonadotropin to melatonin is related to the animal's reproductive stage. In our previous study, an infusion of melatonin for 4 hours into the third brain ventricle of sexually active ewes induced a brief increase in the secretion of LH comprised of several LH pulses of enhanced amplitude during the time of the treatment (Misztal et al. 2002). In contrast, a similar, short-term infusion of melatonin into the third ventricle or into the medial basal hypothalamus (MBH) was not effective in stimulating GnRH or LH secretion in anestrous ewes (Romanowicz et al. 2001). Since melatonin receptors have not been found in the ewe's GnRH-LH system, this response of LH related to the season could result from melatonin's relationship to other neurotransmitter networks that are involved in the regulation of GnRH/LH secretion throughout the year.

The seasonal interactions between the different hypothalamic neurotransmitter networks, which mediate gonadal steroid feedback on the GnRH-LH axis, determine the course of the reproductive cycle in sheep (Gallegos-Sanchez et al. 1998, Tortonese 1999). It has been shown that the activity of endogenous opioid peptides (EOP) dominates during the breeding season and that administration of naloxone, an EOP antagonist, markedly stimulated pulsatile LH secretion in sexually active ewes and rams (Brooks et al. 1986, Meyer and Goodman 1985, Whisnant et al. 1991). Moreover, the LH elevation in response to naloxone was greater in sheep during the luteal than follicular phase of the estrous cycle (Brooks et al. 1986). Whisnant and coauthors (1991) demonstrated, in turn, multiple sites within the hypothalamus of the ewe where EOP could inhibit LH secretion and suggested that these different sites regulated different aspects of pulsatile LH secretion, that is LH pulse amplitude and frequency, during the follicular and luteal phases of the cycle. There is much evidence that primarily β-endorphin (β-END) tonically inhibits GnRH/LH secretion (Goodman 1994), however, the more recent reports indicate for dynorphin (DYN) as a major EOP mediating progesterone negative feedback on GnRH neurons in luteal-phase sheep (Foradori et al. 2002, Goodman et al. 2004).

Taking into account the somewhat weak properties of melatonin in modulating daily LH secretion in ewes (Misztal et al. 2002) and the involvement of EOP in the regulation of gonadotropic axis activity during the breeding season (Brooks et al. 1986, Foradori et al. 2002, Goodman et al. 2004, Meyer and Goodman 1985, Whisnant et al. 1991), the aim of the present study was to test a hypothesis that exclusion of the inhibitory opioid tone would facilitate the stimulation of LH by melatonin. In our experimental model, ewes in the luteal phase of the estrous cycle were subjected to a 3-hour infusion of melatonin into the third brain ventricle in the conditions of opiate receptors blockade by naloxone.

METHODS

Animals, management and experimental procedure

The experimental procedures were approved by the Local Ethics Committee, according to the Polish Guide of Care and Use of Animals (August 21, 1997). Fifteen adult Blackface ewes were used in the experiment. They were maintained indoors under natural lighting condi-

tions (52°N, 21°E) and fed a constant diet of commercial concentrates, with hay and water available *ad libitum*.

Stainless steel guide cannulae (1.2 mm OD) were implanted into the third ventricle of the brain under general anesthesia, following the stereotaxic procedure described by Traczyk and Przekop (1963). The guide cannula was fixed to the skull with stainless steel screws and dental cement. The external opening to the canal was closed with a stainless steel cap. The placement of the guide cannula was confirmed by the outflow of a small amount of cerebro-spinal fluid during the surgery and after slaughtering by the infusion of a small volume of blue ink.

The course of the estrous cycle was followed in all ewes by exposure to a vasectomized ram. Estrous behavior (acceptance of a ram and mounting by a ram) was used as a determinant of the GnRH/LH surge (Goodman 1994). Additionally, plasma progesterone concentration was measured every third day. The obtained data were recorded as a graph, individually for each ewe. The course of each curve obtained allowed proper determination of the days of the individual estrous cycle. In follicular-phase ewes, the concentration of progesterone was close to 0, and during the luteal phase, progesterone values between 2 and 4 ng/ml were recorded. At least two estrous cycles were completed in every ewe before the start of the experiment.

The experiment was performed during a period of decreasing day length, from October to the end of December, corresponding in this breed to high sexual activity. Ewes in the luteal phase of the estrous cycle (day 9–10) were infused intracerebroventricularly (i.c.v.) with the vehicle (VEH, control group, n=5), melatonin (MEL, 100 μg/100 μl/h, total 300 μg, n=5) or naloxone (NAL, 100 μg/100 μl/h, total 300 μg, n=5). Five randomly selected ewes were infused with melatonin in combination with naloxone (MEL+NAL) during consecutive cycles. The infusions were performed in the afternoon from 2:00 P.M. to 5:00 P.M., therefore, they started at least 2–3 h before sunset. Blood samples were collected from 11:00 A.M. to 8:00 P.M. at 10-min intervals, through a catheter inserted into the jugular vein a day before the experiment. After centrifugation in heparinized tubes, plasma was stored at -20°C until LH assay.

The drugs, MEL and NAL (EOP antagonist), were purchased from Sigma-Aldrich Co. Ltd. MEL was dissolved in ethanol and stored at -20°C as a stock solution (50 mg/500μl) for no longer than 3 days. Immediately before the infusion it was dissolved in saline. A similar solution, without MEL, was prepared as the control vehicle. NAL was dissolved in saline on the day of infusion. All infusions were done with calibrated 1.0-ml gas-tight syringes and a BAS Bee™ microinjection pump (Bioanalytical Systems Inc., USA). During the experiment the ewes were kept in comfortable cages where they could lie down and had unrestrained access to hay.

Analytical techniques

The plasma LH concentration was assayed by a radioimmunoassay (RIA) double-antibody method (Stupnicki and Madej 1976), using antiovine-LH and antirabbit-gammaglobulin antisera and bovine LH standard (NIH-LH-B6). The assay sensitivity was 0.3 ng/ml, and the intra- and interassay coefficients of variation were 8.2 and 12.5%, respectively.

Progesterone concentration, used to monitor the estrous cycle, was assayed by a direct RIA method, routinely used in our laboratory (Stupnicki and Kula 1982), with a sensitivity of 6.2 pg/sample.

Statistics

Plasma LH concentrations and the parameters of pulsatile LH secretion (pulse frequency and amplitude) are expressed as a mean ± SEM. The effects of the treatments on LH concentrations were analyzed by one-way analysis of variance followed by the RIR Tukey test (STATISTICA™). The number and amplitude of LH pulses were determined by the PC-PULSAR computer program according to the method of Merriam and Wachter (1982) with G parameters: G1 = 3.98; G2 = 2.40; G3 = 1.68; G4 = 1.24 and G5 = 0.93. Analysis was performed individually for every ewe and included the entire sampling period. The frequency of LH pulses was defined as the number of identified pulses per collecting period. The significance of differences in the frequency and amplitude of LH pulses between groups was assayed by the nonparametric Mann-Whitney and ANOVA rank Kruskal-Wallis tests, respectively (STATISTICA™).

RESULTS

In the VEH-infused group, LH secretion increased gradually from the morning to the evening hours, 6.71 ± 0.08, 6.97 ± 0.09 and 7.26 ± 0.13 ng/ml (± SEM), before, during and after the infusion, respectively (Fig. 1).

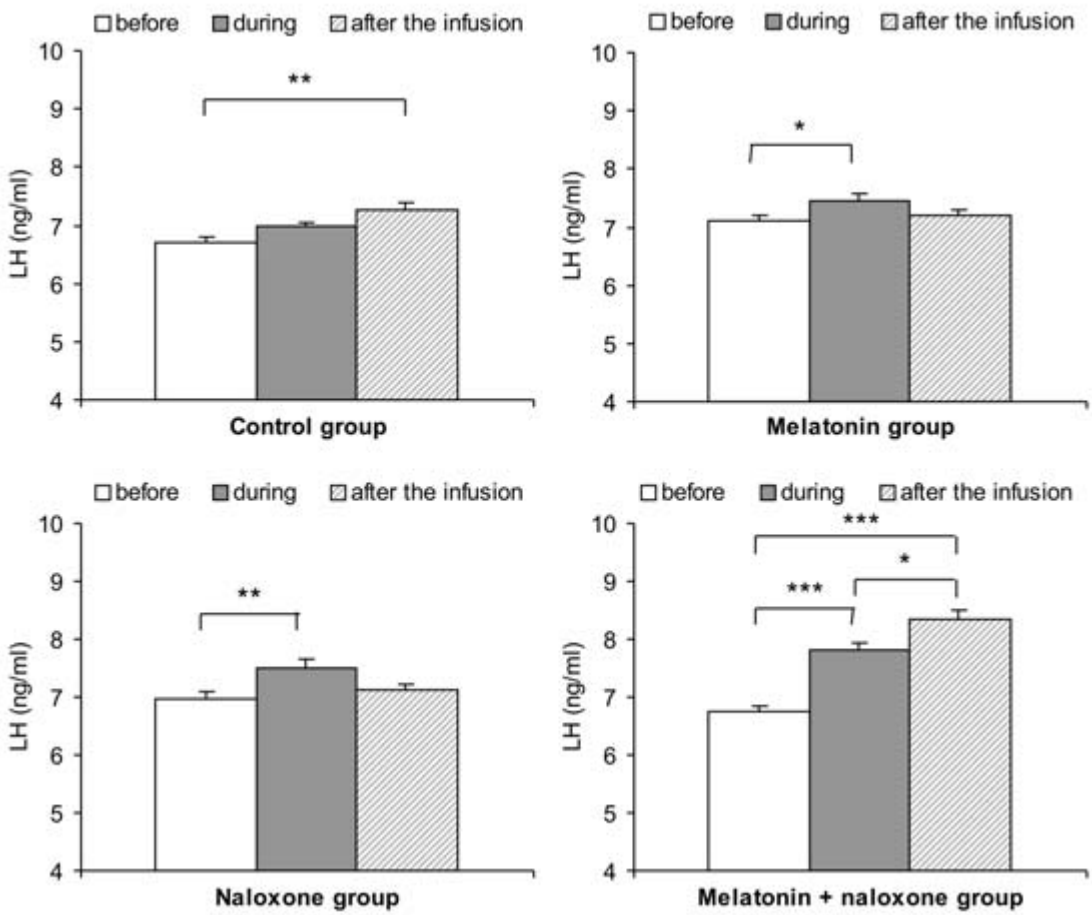

Fig. 1. Mean (± SEM) plasma LH concentrations in ewes (n=5) before, during and after intracerebroventricular infusions of a vehicle (control), melatonin, naloxone and melatonin in combination with naloxone from 2:00 P.M. to 5:00 P.M. (*P<0.05, **P<0.01, ***P<0.001). See the text for additional statistical analysis.

The mean plasma LH concentration measured after the infusion was significantly higher than that before (P<0.01). The mean frequency and amplitude of LH pulses during the collecting period was 4.0 ± 0.55 pulses/9 h and 1.21 ± 0.15 ng/ml, respectively.

In the MEL-treated group, mean LH concentrations measured before, during and after the infusion were 7.11 ± 0.10, 7.46 ± 0.10 and 7.19 ± 0.09 ng/ml, respectively (Fig. 1). The LH concentration recorded during MEL infusion was significantly (P<0.05) higher than that before. There was a significant (P<0.05) difference also between the LH concentrations measured during MEL and VEH infusion. The mean frequency and amplitude of LH pulses recorded during the collecting period was 5.2 ± 1.24 pulses/9 h and 1.60 ± 0.13 ng/ml, respectively. The LH pulse amplitude was significantly (P<0.05) higher in this group, compared to the VEH-infused group, showing a marked increase during MEL infusion.

In the NAL-treated group, the mean LH concentrations measured before, during and after the infusion were 6.97 ± 0.11, 7.50 ± 0.15 and 7.11 ± 0.12 ng/ml, respectively (Fig. 1). The LH concentration recorded during NAL infusion was significantly (P<0.01) higher than that before. The LH concentration measured during NAL infusion was also significantly (P<0.01) higher than that during VEH infusion. The mean frequency and amplitude of LH pulses recorded in the NAL-treated group during the collecting period was 6.00 ± 0.84 pulses/9 h and 1.92 ± 0.16 ng/ml, respectively. While the LH pulse amplitude was significantly (P<0.01) higher in this group than in the VEH-infused one, the LH pulse frequency showed only a tendency to increase (P<0.07).

In the MEL+NAL-treated group, the mean LH concentrations measured before, during and after the infusion were 6.74 ± 0.11, 7.81 ± 0.14 and 8.34 ± 0.17 ng/ml, respectively (Fig. 1). The LH concentration re-

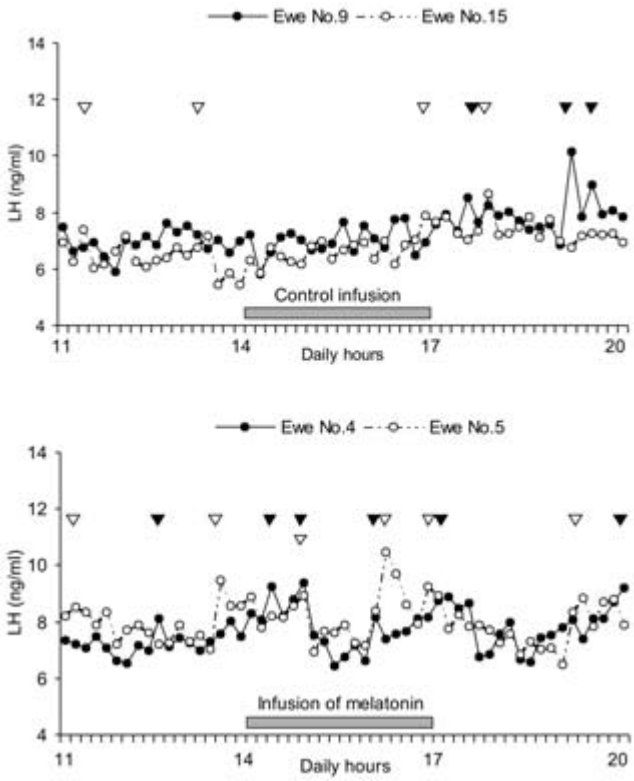

Fig. 2. Representative patterns of LH secretion in two ewes infused with a vehicle (control) and in two ewes infused with melatonin (100 μg/100 μl/h) from 2:00 P.M. to 5:00 P.M. Black and white arrows indicate statistically significant pulses.

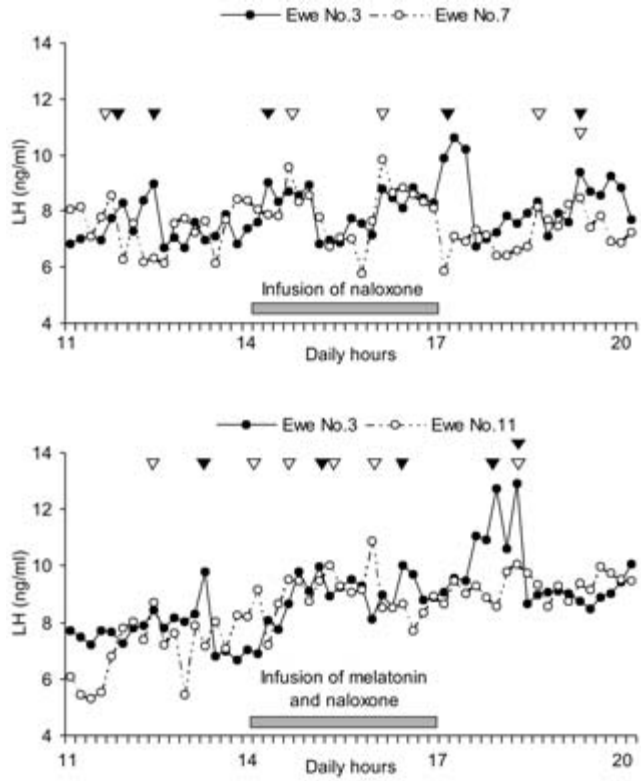

Fig. 3. Representative patterns of LH secretion in two ewes infused with naloxone (100 μg/100 μl/h) and in two ewes infused with melatonin in combination with naloxone (100 μg + 100 μg/100 μl/h) from 2:00 P.M. to 5:00 P.M. Black and white arrows indicate statistically significant pulses.

corded during MEL+NAL infusion was significantly (P<0.001) higher than that before. The secretion of LH subsequently continued to increase and the concentration measured after MEL+NAL treatment was significantly higher than both before (P<0.001) and during (P<0.05) the infusion. In addition, the LH concentration measured during MEL+NAL infusion was significantly (P<0.001) higher than that during VEH infusion. The LH concentration recorded in turn after MEL+NAL treatment was significantly (P<0.001) higher than the concomitant concentrations in VEH-, MEL- and NAL-infused animals. The mean frequency and amplitude of LH pulses recorded in the MEL+NAL group during the collecting period was 5.4 ± 0.81 pulses/9 h and 1.81 ± 0.17 ng/ml, respectively. The LH pulse amplitude in this group was significantly (P<0.01) higher than in the VEH-infused group, showing a marked increase during and after MEL+NAL infusion. Representative patterns of LH secretion in individual animals from all groups are shown in Figures 2 and 3.

DISCUSSION

The presented results reveal a complex relationship between melatonin and the opioidergic system in the modulation of daily LH secretion in ewes during the luteal phase of the estrous cycle. While melatonin and naloxone alone evoked a temporal enhancement in LH secretion, treatment with these drugs in combination evoked the durable stimulation of this gonadotropin. Such an effective action of melatonin on daily LH secretion in sheep, after the exclusion of opioidergic input, has been demonstrated here for the first time.

Our results confirm that the daily changes in LH secretion, with a marked increase in the concentration during the evening hours, occur in luteal-phase ewes and that intracerebroventricular infusion of exogenous melatonin is able to stimulate daily LH secretion in these ewes (Misztal et al. 2002). In this experiment melatonin induced several LH pulses of increased amplitude, which confirmed also the hypothesis that the

stimulatory properties of melatonin on daily LH secretion were maintained only during the time of treatment (Misztal et al. 2002). The precise mechanism of this action of melatonin is not clear at present. In the sheep, a high density of membrane-bound melatonin receptors has been found in the pars tuberalis (PT) of the pituitary gland (Morgan et al. 1994) as well in the PMH (Malpaux et al. 1998); both express the Mel-1a melatonin receptor subtype, also known as MT1 (Barrett et al. 1997, Migaud et al. 2005). The PMH is an important target for melatonin in regulating the seasonal reproductive activity in ewes (Malpaux et al. 1998). The principal effect of melatonin on PT cells is an inhibition of the adenylate cyclase enzyme to reduce the intracellular levels of cyclic adenosine 3',5'-monophosphate (cAMP) and inhibit cellular processes regulated by this second messenger (Morgan et al. 1992, 1994, 1996). It was shown that in sheep PT secretes LH and that this secretion is enhanced in response to GnRH (Skinner and Robinson 1997). Importantly, melatonin attenuated the GnRH-induced increase in LH secretion from the ovine PT, but not from the gonadotrophs of the pars distalis (PD) (Skinner and Robinson 1997). The subpopulation of gonadotrophs in the PT is probably too small to have a noticeable effect on circulating LH concentration. It is possible, however, that in some physiological stages, "melatonin-sensitive" LH, secreted by the PT, may act back on the brain to influence the reproductive neuroendocrine axis *via* a short-loop feedback system. Although long-acting melatonin micro-implants placed in the PT did not affect the seasonal LH cycle (Malpaux et al. 1995), it is reasonable to suggest that melatonin may affect the circadian rhythm of this gonadotropin by modulating LH release from the PT. Thus, the short-term inhibition of the PT signal by infused melatonin would induce the LH release from the PD gonadotrophs. The observed increase in LH release could represent a phase-shifted rise in LH secretion as was found in cycling ewes after sunset when melatonin secretion increased (Currie et al. 1993, Misztal et al. 2002). The slight decrease in plasma LH concentration observed in turn after melatonin infusion may reflect the restored PT signal, i.e., following desensitization to melatonin. It has been reported that the total number of melatonin receptors in the ovine PT cell membrane may be reduced following exposure to melatonin (Hazlerigg et al. 1993). It is noteworthy that plasma melatonin concentration, monitored during i.c.v. infusion of this hormone during the previous study (Misztal et al. 1997), was 3- to 4-fold higher than that during the winter night (Misztal et al. 1996a). The other possibility is that melatonin attenuates the action of any inhibitory system within the hypothalamus, whose activity renews after hormone administration.

The inhibitory tone of the opioidergic system dominates during the breeding season in sheep and the stimulatory effect of EOP antagonists on GnRH/LH secretion has been widely described (Brooks et al. 1986, Meyer and Goodman 1985, Whisnant et al. 1991). In our experiment, naloxone enhanced primarily the amplitude of LH pulses. According to Whisnant and coauthors (1991), during the luteal phase, when progesterone is the dominant inhibitory steroid, WIN 44,441-3 (an EOP antagonist with receptor-binding profiles similar to naloxone) administration to both the preoptic area (POA) and MBH increased LH pulse frequency. The lack of significant differences in the frequency of LH pulses in our study may result from the high individual variability and/or a too small number of animals within groups. On the other hand, studies on the pattern of GnRH release into the hypophyseal portal blood of ovariectomized (OVX) and estradiol-treated OVX ewes showed that naloxone increased GnRH pulse amplitude and also prolonged the duration of GnRH release during pulse that may render into a shape of LH pulses (Goodman et al. 1995).

Evidence from published studies supports primarily a role for β-END in transmitting information about the steroidal milieu to GnRH neurons (Goodman 1994). In sheep, the pituitary gland is the main source of β-END (Ebling et al. 1987, Smith and Funder 1988) and the existence of counter current transfer of β-END from the hypophyseal venous blood to the arterial blood supplying the brain was reported (Krzymowski et al. 1992, Skipor et al. 1997). Moreover, it was demonstrated that counter current transfer of β-END in ewes took place during the luteal phase of the estrous cycle (Skipor et al. 1997). The subsequent hypothalamic source of EOP in ewes is located in the arcuate nucleus and around the mammillary recess of the third ventricle (Whisnant et al. 1992). Immunocytochemical evidence has shown that β-END-containing processes are found in the ME, thus providing opportunities for synaptic interactions with GnRH neuronal terminals in this species (Conover et al. 1993). Recent evidence indicates in turn that a major role in mediating progesterone negative feedback on GnRH neurons in luteal-phase sheep may play the other EOP, dynorphin (DYN), acting through the κ opioid re-

ceptor (Foradori et al. 2002, Goodman et al. 2004). It has been shown that almost all GnRH neurons in the MBH of the sheep brain receive synaptic inputs from DYN-containing terminals (Goodman et al. 1999) and a large majority of DYN cells in the ovine POA and MBH co-express nuclear progesterone receptors (Foradori et al. 2002). Moreover, the latest study of Śliwowska and coauthors (2004) has revealed that neurons immunoreactive for DYN are also present in the ovine PMH, an area sensitive to melatonin. Since naloxone binds to all types of the EOP receptors, our observation that naloxone infusion increased both LH pulse amplitude and LH pulse frequency (visually) confirms multiple sites of EOP inhibition of GnRH/LH secretion (Foradori et al. 2002, Goodman et al. 1995, 2004, Whisnant et al. 1991). Interestingly, the LH response to naloxone treatment was similar to that of melatonin. This might suggest that melatonin indeed exerted its effect through inhibition of opioidergic system activity within the hypothalamus. Such a possibility could be supported by the results of our earlier work, demonstrating that an i.c.v. infusion of melatonin suppressed the plasma concentration of β-END in sexually active ewes (Misztal et al. 1996b). Infused melatonin might interfere with progesterone signaling in the opioidergic cells, which are involved in the control of GnRH/LH release during the luteal phase (Foradori et al. 2002, Goodman et al. 1995, 2004, Whisnant et al. 1991), by impairing progesterone receptor pathways. It has been demonstrated that melatonin acts as an anti-estrogen by preventing the estrogen-dependent transcriptional activation in MCF-7 cells through destabilization of the estradiol--estrogen receptor complex from binding to DNA, and it has been proposed that calmodulin is a potential candidate for mediating the anti-estrogenic effects of melatonin (Rato et al. 1999, del Rio et al. 2004). At present, we have no evidence that such a mechanism may be active *in vivo*, especially in the case of estrogen or progesterone receptors, during the estrous cycle of sheep.

The stimulatory effect of melatonin or naloxone on LH secretion was more effective when the drugs were infused i.c.v. in combination. Since their individual effects were less pronounced, that is, each of them stimulated LH secretion only during the time of infusion, the combined effect could result not only from the attenuation of the opioidergic input. The drugs infused into the third ventricle would act on the different hypothalamic areas and/or the pituitary gland, thus the presented effect could result from the accumulation of the single one. The secretion of GnRH/LH in response to the exclusion of the inhibitory tone of EOP might be intensified by melatonin on the cellular level, since studies on the PT cells have revealed that cAMP was dramatically elevated following simultaneous treatment with adenylate cyclase activators and melatonin (Barrett et al. 2003, Hazlerigg et al. 1994). Melatonin can induce a sensitized response of adenylate cyclase in ovine PT cells where the receptor for melatonin is endogenously expressed (Barrett et al. 2003). This mechanism is, however, not fully understood. Taking into consideration that the melatonin receptor has not been found on both the GnRH neurons and pituitary gonadotrophs (Morgan et al. 1994), it is not possible to indicate unequivocally the precise site of melatonin action. Its lipophilic nature may enable melatonin to interact with specific intracellular proteins or with nuclear receptor sites (Cardinali and Pevet 1998). Nevertheless, a continued increase in the release of LH, evoked by melatonin in the conditions of exclusion of the inhibitory opioid tone, reflects a potent induction of the gonadotropic axis activity. Therefore our results raise an important issue that the functional relationship between melatonin and the EOP, in control of GnRH/LH secretion, exists in luteal-phase ewes and may set the course for further investigation on the role of melatonin in sheep reproduction.

CONCLUSION

A relationship between melatonin and the endogenous opioid peptides may be crucial in enabling melatonin to exhibit stimulatory action on LH secretion during the luteal phase of the estrous cycle in ewes.

ACKNOWLEDGENENTS

We wish to thank Józef Rutkowski for help in brain surgery and the other members of the Department of Endocrinology for help in the collection of blood samples and technical assistance with the radioimmunoassays. This study was supported by the State Committee for Scientific Research (KBN, Poland).

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Received 25 June 2004, accepted 15 December 2004