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The effect of nutrition on the neural mechanisms potentially involved in melatonin-stimulated LH secretion in female Mediterranean goats

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Abstract

This research examines which neural mechanisms among the endogenous opioid, dopaminergic, serotonergic and excitatory amino acid systems are involved in the stimulation of LH secretion by melatonin implantation and their modulation by nutritional level. Female goats were distributed to two experimental groups that received either 1·1 (group H; $n=24$) or 0·7 (group L; $n=24$) times their nutritional maintenance requirements. Half of each group was implanted with melatonin after a long-day period. Plasma LH concentrations were measured twice per week. The effects of i.v. injections of naloxone, pimozide, cyproheptadine and *N*-methyl-D,L-aspartate (NMDA) on LH secretion were assessed the day before melatonin implantation and again on days 30 and 45. The functioning of all but the dopaminergic systems was clearly modified by the level of nutrition, melatonin implantation and time elapsed since

implantation. Thirty days after implantation, naloxone increased LH concentrations irrespective of the level of nutrition ($P<0\cdot05$), similar to NMDA in the melatonin-implanted H goats (HM; $P<0\cdot01$). On day 45, naloxone increased LH concentrations in the HM animals ($P<0\cdot05$), similar to cyproheptadine in both the non-implanted H (HC) and the HM animals ($P<0\cdot01$). Finally, at 45 days, NMDA increased the LH concentration in all subgroups ($P<0\cdot01$). These results provide evidence that the effects of different neural systems on LH secretion are modified by nutritional level and melatonin implantation. Endogenous opioids seem to be most strongly involved in the inhibition of LH secretion on days 30 and 45 after melatonin implantation. However, the serotonergic mechanism appears to be most influenced by nutritional level.

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Introduction

Nutrition is thought to be an important factor affecting the reproductive function in domestic ruminants, influencing the onset of ovarian cyclicity in goats (Walkden-Brown *et al.* 1994, Zarazaga *et al.* 2005). Low levels of food intake or poor body condition are associated with enhanced hypothalamic sensitivity to oestradiol (Rhind *et al.* 1991, Rhind 1992). In Mediterranean goats, nutrition can modulate the effect of photoperiod on the length and depth of seasonal anoestrus (Zarazaga *et al.* 2005) and undernutrition induces lower LH concentrations during short and long days (Zarazaga *et al.* 2011*a,b*). But in undernourished sheep, or those with low body condition scores (BCS), melatonin implantation allows a high ovulation rate (Forcada *et al.* 1995, Rondón *et al.* 1996) and a large number of healthy cleaved oocytes and blastocyst rate to be obtained (Vázquez *et al.* 2009, 2010).

Melatonin regulates seasonal reproduction through changes in the pulsatile secretion of GNRH and LH (Bittman *et al.* 1985, Viguié *et al.* 1995*a*). Its action on the activity of GNRH neurones appears to be indirect. A number of studies have suggested that different neurotransmitters are involved in the

regulation of LH secretion by melatonin or photoperiod, especially in ewes (Meyer & Goodman 1985, 1986, Le Corre & Chemineau 1993, Forcada *et al.* 1997). The neuroendocrine mechanism downstream of the action of melatonin, leading to the regulation of LH secretion, appears to involve several dopaminergic structures in the hypothalamus, such as the A14 and A15 nuclei of the lateral reticulohypothalamic area (Havern *et al.* 1994) and the arcuate nucleus–median eminence region (Anderson *et al.* 1997). In sheep, serotonin receptors are thought to be involved in the neural mechanisms that regulate the release of LH associated with the photoperiod. In this species, the determination of the brain-binding sites of the tritiated 5-hydroxytryptamine receptor₂ (5HT₂) antagonist ketanserin led to the identification of a specific ventral hypothalamic zone in which the binding density parallels the change in LH pulsatility (Le Corre *et al.* 1994). In goats, it has recently been shown that inhibitory pathways, such as the endogenous opioid pathways, regulate LH secretion during the early inhibition of LH secretion by long days and during LH stimulation by short days. Further, the dopaminergic inhibitory pathways are responsible for a reduction in LH pulse frequency during the

onset of LH inhibition by long days and during the inhibition of LH secretion by long days. Finally, the serotonergic system is involved during the onset of LH inhibition by long days, in the inhibition of LH secretion by long days, and in the onset of LH stimulation by short days, but always in well-nourished females (Zarazaga *et al.* 2011a).

The excitatory amino acids (EAA), glutamate and aspartate, are major stimulatory neurotransmitters in the mammalian nervous system. Acting through *N*-methyl-D,L-aspartate (NMDA) receptors, in sheep, these amino acids modulate the secretion of LH (Estienne *et al.* 1990, Lincoln & Wu 1991). In support of this, the administration of NMDA, a glutamatergic receptor agonist, acutely stimulates LH secretion (Viguié *et al.* 1995b). This stimulatory effect is larger during the period of photoinhibition of LH secretion than during the photostimulation, suggesting that EAA are involved in the photoperiodic regulation of LH secretion (Lincoln & Wu 1991). In bucks, the stimulatory effect of NMDA on LH secretion is more pronounced when subjected to long days than short days (Gazal *et al.* 2002). However, the information available on the effect of neuroexcitatory amino acid receptors in LH secretion in melatonin-implanted goats, or goats subjected to different levels of nutrition, is lacking.

Continuing our previous research (Zarazaga *et al.* 2011a), the present research assesses the interaction between the nutritional level and melatonin implants on the regulation of LH secretion by the endogenous opioid, dopaminergic, serotonergic and NMDA systems in melatonin-implanted Mediterranean goats maintained under a long-day photoperiod.

Materials and Methods

Study conditions

All procedures were performed by trained personnel in strict accordance with Spanish guidelines for the protection of experimental animals (RD 1201/2005) and in agreement with European Union Directive 86/609. The study was conducted at the University of Huelva experimental farm (latitude 37° 15'N), which meets the requirements of the European Community Commission for Scientific Procedure Establishments (1986).

Animals, nutrition, photoperiod and melatonin treatments

The study animals were 48 adult female goats. All were ovariectomised 1 month before beginning the experiment and simultaneously implanted subcutaneously with a 3.0 cm Silastic implant (internal diameter 3.3 mm and external diameter 4.6 mm; Karsch *et al.* 1973) containing crystalline oestradiol (Sigma–Aldrich Chemical Co.). These implants were soaked in physiological serum before insertion to prevent an initial peak of steroid release.

The experiment had a 2×2 factorial design (two nutrition levels, plus implantation – or not – of exogenous melatonin). Doses assigned to two experimental groups were based on the provision of nutrition balanced for live weight (LW) and BCS (Hervieu *et al.* 1991). The members of the high-nutrition group (group H; *n*=24) were fed 700 g concentrate and 500 g barley straw each day, while the members of the low-nutrition group (group L; *n*=24) received 350 g concentrate and 500 g barley straw. These amounts correspond to a daily intake of 0.78 milk fodder units (UFL) and 100 g digestible protein (group H) and 0.49 UFL and 50 g digestible protein (group L), providing 1.1 and 0.7 times the maintenance requirements of a goat with an LW of 50 kg at the beginning of the experiment, according to the INRA standards (Morand-Fehr & Sauvant 1988). The concentrate was offered once a day, distributed individually; the barley straw was distributed loosely to each group. The concentrate was a commercial mixture of maize (26.3%), beans (20%), oats (14.1%), cotton-seed (13.7%), peas (13.4%), lupin (7.3%), barley (0.2%), wheat (0.2%), sunflower seeds (0.2%) and a commercial mineral–vitamin complement (4.6%). All the animals had free access to water and mineral blocks containing trace elements and vitamins.

Both groups were housed in a lightproof building under artificial lighting where they were exposed for 9 months to alternations between 3 months of long days (16 h of light/day; lights-on: 0700 h, lights-off: 2300 h) and 3 months of short days (8 h of light/day; lights-on: 0700 h, lights-off: 1500 h). The first 3 months of controlled photoperiod (long days, started on September 14th) was necessary for the animals to adapt to the experimental conditions. The next 3 months (short days) were deemed necessary to ensure that the animals were able to respond to a stimulatory photoperiod prior to melatonin insertion. The final phase of long days was required before melatonin could be implanted.

At the end of the second period of long days (June 14th), each nutritional group was divided into two subgroups, i.e. those that received a melatonin implantation (18 mg; Melovine, CEVA Salud Animal, Barcelona, Spain) at the base of the left ear (M) and those that did not (C): HM (*n*=12) and HC (*n*=12), and LM (*n*=12) and LC (*n*=12). After the implantation procedure, all animals were maintained under a long-day photoperiod to ensure that the positive effect of the melatonin implants on LH secretion was due to exogenous melatonin.

Sampling and measurements

The effect of photoperiod treatments on neuroendocrine LH activity was assessed through monitoring long-term plasma LH variations, collecting blood samples twice per week by jugular venipuncture at 0930 h. The samples were immediately centrifuged for 30 min (3000 g) and the plasma stored at –20 °C until use. The time of onset of LH secretion after melatonin implantation was determined for each animal as differing from the baseline by more than 3 s.d. The baseline

was defined as the mean value of the samples obtained from May 2 until June 13 – the period with the lowest LH concentrations (Fig. 1). The LW and BCS of all animals were recorded weekly.

To evaluate the melatonin concentrations induced by the implants, five weekly blood samples were taken from each goat during the light phase, starting 1 week after melatonin implantation.

Drugs

Antagonists to opioid receptors (naloxone), dopaminergic₂ receptors (pimozide), serotonin 5HT₂ receptors (cyproheptadine) and an agonist of neuroexcitatory amino acid receptors (NMDA) were administered at different time points during the stimulation of LH secretion by the melatonin implantation. Naloxone hydrochloride (Sigma–Aldrich Chemical Co.) was dissolved in 0.9% (w/v) sterile saline at a concentration of 4.8 mg/ml. Pimozide (Sigma–Aldrich Chemical Co.) was dissolved in 0.1 M tartaric acid at a

concentration of 3 mg/ml. Cyproheptadine hydrochloride sesquihydrate (Sigma–Aldrich Chemical Co.) was dissolved in 50% ethanol–propanediol at a concentration of 25 mg/ml. NMDA, comprising a mixture of D and L isomers (Sigma–Aldrich Chemical Co.), was dissolved in 0.9% sterile and apyrogenic saline at a concentration of 5 mg/ml. Each drug was dissolved <3 h before i.v. injection and stored at room temperature until use. All drugs were administered as a bolus into the jugular vein through a catheter.

Drug challenges

Preliminary experiment: NMDA dose–response curve A dose–response curve for the effect of NMDA on LH secretion was created in a preliminary experiment to determine the dosage to be used in the main experiment. This preliminary work was performed on three animals of group H (before randomisation for implantation). LH secretion in these animals was inhibited by exposure to 70 long days (16 h light:8 h darkness cycle) during the second period of long days. Three doses were tested at 48 h intervals on the same animals (1, 2 and 4 mg/kg BW) in increasing order. LH secretion was assessed in serial samples of jugular blood obtained every 10 min during the hour preceding the injection, and every 10 min for 2 h thereafter.

For the other drugs, doses similar to those used by Zarazaga *et al.* (2011a) were given: 2 mg/kg, and originally 0.6 and 0.6 mg/kg LW for naloxone, pimozide and cyproheptadine respectively. The latter two doses were reduced by 0.15 mg/kg since ataxia and akinesia symptoms were seen in 20% of the animals 3 h after the 0.6 mg/kg pimozide injection, and respiratory depression was observed in about 40% of animals after the 0.6 mg/kg cyproheptadine injection. No correlation was seen between these side effects and the LH responses to drug treatments.

Main experiment: the effects of naloxone, pimozide, cyproheptadine and NMDA on LH release in goats at different time points following melatonin implantation

The first drug challenge was given during the inhibition of LH secretion by long days before the melatonin implantation (day –1). The second challenge was given before the onset of LH increase, on day 30. The third challenge was given when LH secretion was stimulated by the melatonin implantation, on day 45. At each time, all goats of each subgroup (HC, HM, LC and LM) were distributed in balanced ‘drug groups’ according to the LW and BCS. Each animal, from each ‘drug group’, received naloxone and then NMDA after 5 days ($n=6$), or cyproheptadine and then pimozide after 5 days (using the other six females), the 5-day period allowing for washout of the previously administered drug. Drugs were always administered in the same order. As a consequence, each goat of each subgroup received two drugs, naloxone and NMDA or cyproheptadine and pimozide. During all drug challenges, blood samples were collected via jugular catheter at 10 min intervals 3 h before (control period)

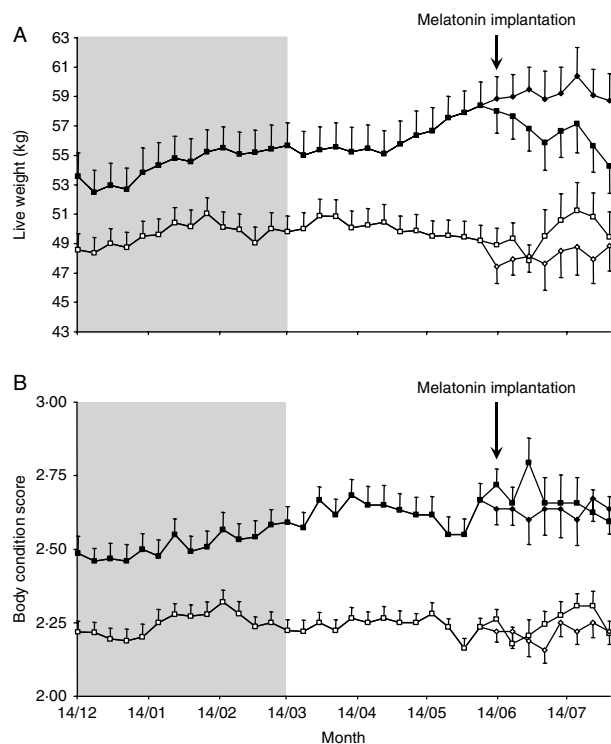


Figure 1 Changes (mean \pm S.E.M.) in live weight (A) and body condition score (B) in Mediterranean female goats subjected to alternations of 3 months of short days and 3 months of long days that received 1.1 (solid symbols, $n=24$) or 0.7 (open symbols, $n=24$) times their maintenance requirements, respectively, and that were implanted (filled square, $n=12$; open square, $n=12$) or not (filled diamond, $n=12$; open diamond, $n=12$) with melatonin (14/06). Shaded areas indicate the months when animals were exposed to short days.

and 3 h after (drug period) the i.v. injection of the drug or corresponding vehicle (controls). The sampling periods started at 0900 h and finished at 1500 h.

Hormone assays and LH pulse identification

Plasma LH concentrations were determined using double antibody ELISA, as described by Faure *et al.* (2005). The sensitivity of the assay was 0.1 ng/ml. The intra- and inter-assay coefficients of variation (CV) in the control were 6.5 and 3.0% respectively. All samples from the same challenge were measured in the same assay.

LH pulses were defined according to Baird *et al.* (1981) as the point with two consecutive values higher than the two preceding ones, with the highest value (pulse amplitude) exceeding the mean baseline value by at least four times the CV of the assay.

Plasma melatonin concentrations were measured by RIA in duplicate aliquots of 100 µl blood plasma (Fraser *et al.* 1983), using the antibody first raised by Tillet *et al.* (1986). The sensitivity of the assay was 4 pg/ml. The intra-assay CV was 5.83%.

All hormonal analyses were conducted at the hormone analysis laboratory at the Centre INRA de Tours, Nouzilly, France.

Statistical analysis

The data obtained during the first 3 months of the study (September 14th to December 14th) were excluded from the statistical analysis since they referred to the period needed by the animals to adapt to the sheds and nutritional treatments. The effects of nutrition on LW and BCS (measured weekly) and LH concentrations (measured twice weekly) were analysed using one-way ANOVA (nutritional treatments: groups H or L) with time as a repeated measure. Two-way ANOVA was used to determine the effect of nutrition plus melatonin treatment on LH secretion, with time as a repeated measure.

The mean LH concentrations and mean LH pulse frequencies and amplitudes in the drug treatments were calculated for each pre- and post-administration period. Due to the non-parametric nature of the LH pulse frequency, a Wilcoxon test was performed for between-dose comparisons of the effect of NMDA on the number of LH pulses. The effects of the treatments on mean LH concentration were analysed using a paired *t*-test (pre- vs post-injection periods).

Mean LH concentrations during the pre- and post-injection periods at each time point in the stimulation of the LH secretion by the melatonin treatment (−1, 30 and 45 day after melatonin insertion) were compared using ANOVA, with drug (naloxone, pimozi, cyproheptadine or NMDA), nutrition level (H or L), melatonin implantation (or not) and time as repeated measures. The Tukey test was performed when differences between drug treatments were significant. After ANOVA, the effect of the drug treatments

on mean LH concentration were analysed using a paired *t*-test (pre- vs post-injection periods) at each time point before and after melatonin implantation, and for each nutritional group.

Due to the non-parametric nature of LH pulse frequency, the Kruskal–Wallis ANOVA test was used to examine the differences between the HL, HM, LC and LM subgroups before drug injection. After ANOVA, the effects of drug treatments on the number of LH pulses were analysed using the Wilcoxon test (pre- vs post-injection periods) for each time point studied before and after melatonin implantation, and for each drug before and after its administration within each nutritional subgroup.

Significance was set at $P < 0.05$. All calculations were performed using the SPSS package (Statistical Package for the Social Sciences 2008).

Results

LW and BCS

Before melatonin implantation, mean LW and BCS varied substantially over time in both group H and group L animals ($P < 0.05$; Fig. 1), but with mean LW and BCS higher in the former (55.9 ± 0.3 kg and 2.59 ± 0.01 vs 49.5 ± 0.3 kg and 2.23 ± 0.01). After melatonin implantation, LW, but not BCS, varied substantially over time in all groups (HC, HM, LC and LM; $P < 0.01$), although no effect of melatonin treatment on LW or BCS was seen, nor was any interaction between sources of variation and time observed ($P > 0.05$).

LH concentration profiles

Nutrition had a significant effect on LH secretion prior to melatonin implantation (0.65 ± 0.02 vs 0.45 ± 0.01 ng/ml for the H and L groups respectively; $P < 0.01$; Fig. 2).

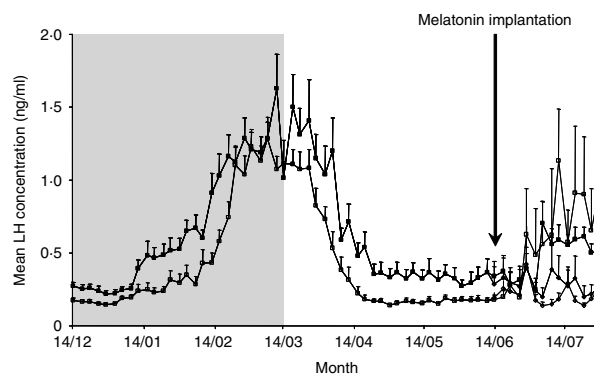


Figure 2 Changes (mean \pm S.E.M.) in plasma LH concentration (ng/ml) in Mediterranean female goats subjected to alternations of 3 months of short days and 3 months of long days that received 1.1 (solid symbols, $n = 24$) or 0.7 (open symbols, $n = 24$) times their maintenance requirements, respectively, and that were implanted (filled square, $n = 12$; open square, $n = 12$) or not (filled diamond, $n = 12$; open diamond, $n = 12$) with melatonin (14/06). Shaded areas indicate the months when animals were exposed to short days.

After melatonin implantation, LH concentrations increased ($P < 0.05$; Fig. 2). An interaction between nutrition and melatonin implantation ($P < 0.05$) affecting LH secretion was also observed; the LH concentration of the LM animals was clearly higher than that of the HM animals (Fig. 2).

The mean interval between melatonin implantation and the stimulation of LH secretion was 31.3 ± 3.4 days, with no differences seen between HM and LM animals. Only three goats from the HC and LC groups, which were maintained under long days, showed a slight increase in LH concentration.

Melatonin concentrations

Melatonin implantation had a clear effect on plasma melatonin concentration (227.1 ± 28.1 vs 4.2 ± 0.1 pg/ml for implanted and non-implanted animals respectively; $P < 0.01$). Neither nutrition nor the melatonin implantation by nutrition interaction had any effect on melatonin concentration ($P > 0.05$).

LH secretion response to NMDA dose (preliminary experiment)

The injection of NMDA caused an acute increase in LH secretion, characterised by a sustained and prolonged rise in plasma LH levels. This response was dose dependent ($P < 0.01$), with mean LH concentrations peaking at 1.1, 1.8 and 2.1 ng/ml for NMDA doses of 1, 2 and 4 mg/kg BW respectively. After injection of the highest dosage (4 mg/kg BW), LH secretion remained above baseline for more than 2 h. This dose was therefore used for the main experiment.

The effect of nutrition and drug treatment on LH secretion on day -1 before melatonin implantation

Figure 3 shows the differences between the pre- and post-injection periods for each nutritional treatment and drug.

Nutrition had a significant effect on mean LH concentration during the 3 h prior to drug injection (0.32 ± 0.01 vs 0.20 ± 0.01 ng/ml for the H and L groups respectively; $P < 0.05$) with no variations in concentration during this period irrespective of the drug vehicle injected as a control ($P > 0.05$). LH pulsatility was not modified by nutritional level during the pre-injection period ($P > 0.05$).

The NMDA treatment induced higher LH concentrations ($P < 0.001$; 0.73 ± 0.05 ng/ml) and pulsatility ($P < 0.05$) in both the L and H groups, especially in the former. Cyproheptadine increased LH concentration ($P < 0.01$) and pulsatility ($P < 0.05$) only in the H group animals.

The effect of nutrition, melatonin and drug treatment on LH secretion on day 30 after melatonin implantation

Figure 4 shows the differences in LH secretion between the pre- and post-injection periods for each nutritional subgroup (HM, HC, LM and LC) and drug.

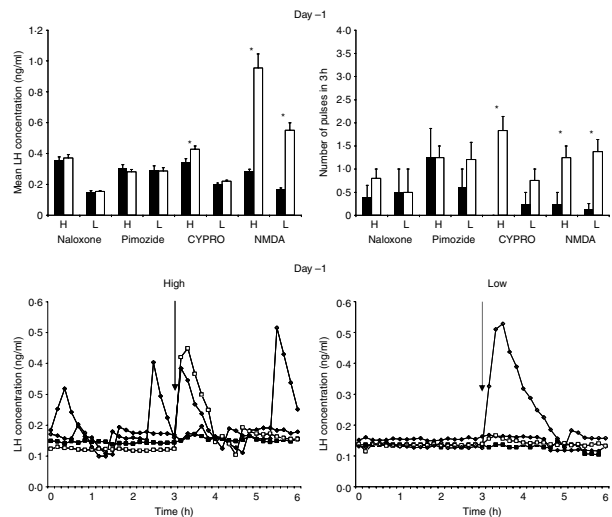


Figure 3 Effect of naloxone, pimoziide, cyproheptadine and NMDA on mean plasma LH concentrations (\pm s.e.m., top left panel) and on the mean number of LH pulses in the 3 h (\pm s.e.m., top right panel) period before (filled square) and after (open square) injection, on the day before melatonin insertion (day -1), into female Mediterranean goats that received 1.1 (H group, $n = 24$) or 0.7 times their maintenance requirements (L group, $n = 24$). Six goats per drug were used for each experimental group. A representative profile of an ovariectomised oestradiol-treated dose-administered naloxone (filled square), pimoziide (filled diamond), cyproheptadine (open square) or NMDA (open diamond) is shown at the bottom. The arrows indicate the time of the drug injection. * $P < 0.05$.

Prior to drug injection, no effect of nutrition was seen on LH secretion (a difference between the HC and LC groups was no longer observed due to the melatonin implantation by nutrition interaction). At this time, neither the melatonin implantation nor the drug vehicles, injected as controls, had any effect on LH secretion ($P > 0.05$). However, LH pulsatility was affected by nutritional level (0.52 ± 0.12 vs 1.23 ± 0.27 pulses for the H and L groups respectively; $P < 0.05$), but not by melatonin.

During the post-injection period, naloxone increased the LH concentration in the HM ($P < 0.01$) and LM animals ($P < 0.05$), and NMDA increased the LH concentrations in the HM and HC subgroups ($P < 0.01$). The injection of both naloxone and NMDA increased the mean number of LH pulses in the HM animals ($P < 0.05$).

The effect of nutrition, melatonin and drug treatments on LH secretion in goats on day 45 after melatonin insertion

Figure 5 shows the differences in LH secretion between the pre- and post-injection periods for each nutritional subgroup (HM, HC, LM and LC) and drug.

During the 3 h period prior to drug injection, no effect of nutrition on LH secretion was observed (again probably due to the melatonin implantation by nutrition interaction; $P > 0.05$), although both melatonin implantation (0.52 ± 0.02 vs 0.26 ± 0.01 ng/ml for the M and C groups

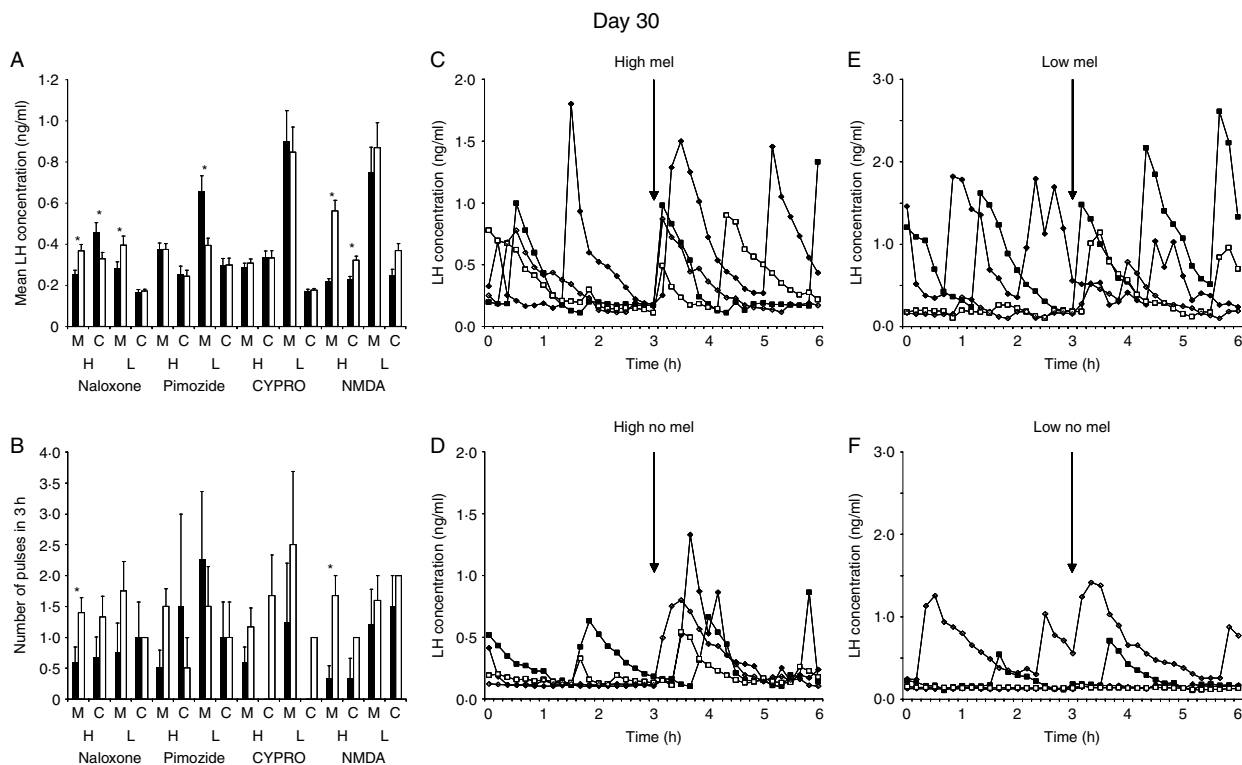


Figure 4 Effect of naloxone, pimozide, cyproheptadine and NMDA at 30 d after melatonin insertion on mean plasma LH concentration (\pm S.E.M., A), and on the mean number of LH pulses, in the 3 h (\pm S.E.M., B) period before (filled square) and after (open square) injection into female Mediterranean goats that received 1.1 (H group, $n=24$) or 0.7 times their maintenance requirements (L group, $n=24$), and that were implanted (M) or not (C) with melatonin. Six goats per drug were used for each experimental subgroup. A representative profile of an ovariectomised oestradiol-treated dose-administered naloxone (filled square), pimozide (filled diamond), cyproheptadine (open square) or NMDA (open diamond) is shown in C, D, E and F. The arrows indicate the time of the drug injection. * $P<0.05$.

respectively; $P<0.05$) and the melatonin implantation by nutrition interaction (0.32 ± 0.01 , 0.34 ± 0.02 , 0.70 ± 0.04 and 0.21 ± 0.01 ng/ml for the HM, HC, LM and LC groups respectively; $P<0.01$) had a significant effect. The different drug vehicles injected during this period as controls had no effect on LH secretion either ($P>0.05$). However, LH pulsatility was affected by the level of nutrition (0.80 ± 0.15 vs 1.33 ± 0.18 pulses for the H and L groups respectively; $P<0.05$) and melatonin implantation (1.30 ± 0.16 vs 0.70 ± 0.15 pulses for the M and C groups respectively; $P<0.05$).

Naloxone only increased the LH concentration in the HM group ($P<0.05$). Cyproheptadine increased LH concentrations in the HM and HC groups ($P<0.01$). NMDA clearly increased LH concentrations in all experimental groups ($P<0.01$). However, LH pulsatility was only increased by cyproheptadine in the HM and HC groups ($P<0.05$).

Discussion

In this study, before the implantation of melatonin, the lower level of nutrition induced a reduction in the mean LH concentration independent of the photoperiod, as reported in

previous work by our group (Zarazaga *et al.* 2011a). These results help explain earlier findings that a low BCS induced by a low level of nutrition (De Santiago-Miramontes *et al.* 2009), or a maintenance level of nutrition compared to a higher level of nutrition (Zarazaga *et al.* 2005), induced a longer seasonal anoestrus as a result of an earlier onset of the anovulatory period. The reason for this may be that, as seen in this study, hypothalamic–pituitary activity is diminished in animals with reduced levels of nutrition. This reduction in LH associated with a lower nutritional level contrasts with that reported by Meza-Herrera *et al.* (2008), who suggest that the increased ovarian activity observed with undegradable protein supplementation, and in goats with better body conditions, is not the result of changes in LH or GH. Rather these authors suggest an effect at local level caused by changes in insulin in a non-GNRH-gonadotrophin-dependent manner. The reason for this discrepancy could be that these authors studied LH concentrations only around the perioviulatory period. Similarly, Kouakou *et al.* (2008), working with dairy bucks subjected to underfeeding and refeeding, observed that neither underfeeding nor the transition to *ad libitum* feeding affected LH concentrations. Moreover, in this study, the LH concentrations observed before melatonin implantation were

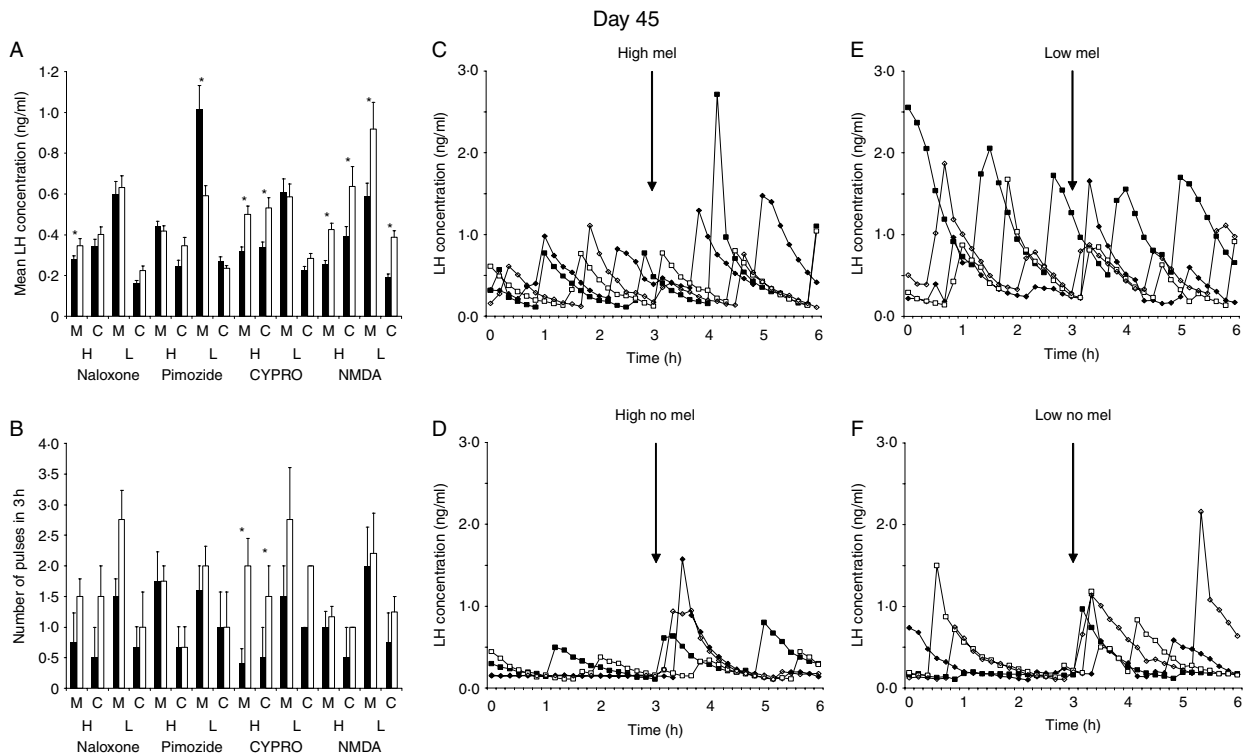


Figure 5 Effect of naloxone, pimozide, cyproheptadine and NMDA at 45 d after melatonin insertion on mean plasma LH concentration (\pm S.E.M., A), and on the mean number of LH pulses, in the 3 h (\pm S.E.M., B) period before (filled square) and after (open square) injection into female Mediterranean goats that received 1.1 (H group, $n=24$) or 0.7 times their maintenance requirements (L group, $n=24$), and that were implanted (M) or not (C) with melatonin. Six goats per drug were used for each experimental subgroup. A representative profile of an ovariectomised oestradiol-treated dose-administered naloxone (filled square), pimozide (filled diamond), cyproheptadine (open square) or NMDA (open diamond) is shown in C, D, E and F. The arrows indicate the time of the drug injection. * $P<0.05$.

similar to those described in OVX + E Mediterranean goats (Zarazaga *et al.* 2011a), confirming that, in caprines at this latitude, the photoperiod is the main environmental factor controlling pituitary activity but that nutrition is an important modulator of LH secretion.

After melatonin implantation, the LH concentration increased and the onset of pituitary activity was earlier than in non-implanted females. The period between implantation and the activation of pituitary activity (31.3 ± 3.4 days) was similar to that recorded in previous work when melatonin was implanted at the spring equinox (30.33 ± 10.17 days; Zarazaga *et al.* 2009) or after a long-day photoperiod treatment (29.7 ± 10.2 days; Zarazaga, Gatica, Celi, Guzmán unpublished data), but shorter than that described by Chemineau *et al.* (1986) who observed no ovulation after a period of 70 short days. This discrepancy is probably due to breed differences and/or lactation influences. The present results also contrast with those obtained in sheep, in which melatonin implantation around the spring equinox does not induce reproductive activity during seasonal anoestrus in OVX + E females that have no contact with males (Forcada *et al.* 2002), or in intact females in contact with males (Forcada *et al.* 1995).

The melatonin implantation by nutrition interaction had a significant effect on LH secretion. To our knowledge, this is the first report of such an observation in goats. However, in sheep, this interaction (Robinson *et al.* 1991, Forcada *et al.* 1995), and the BCS by melatonin implantation interaction (Rondón *et al.* 1996) have been reported to affect ovulation rate. All these authors report the enhancing effect of melatonin implantation on the ovulation rate to be more pronounced in ewes with a low, rather than a high, feed intake. The practical implications of this interaction could be important in induced reproductive activity during seasonal anoestrus in goats that have suffered reduced food availability.

On the day before melatonin implantation, during the inhibition of LH secretion by the long-day photoperiod, differences were seen in response to the drug challenges compared to that previously reported by our group (Zarazaga *et al.* 2011a). In our earlier study, pimozide and cyproheptadine increased the LH concentration during the inhibition of LH by long days, but in this study, pimozide had no such effect. The reason for this discrepancy might be that, in the present experiment, the injected doses of both pimozide and cyproheptadine were smaller than those in the previous study by 0.15 mg/kg.

The response to the naloxone injection before melatonin implantation suggests that endogenous opioids are not involved in the inhibition of LH secretion by long days since no response was observed, as previously reported (Zarazaga *et al.* 2011a). The injection of NMDA increased the LH concentration in both the H and L groups, indicating that nutritional level does not modify the response to EAA and that this mechanism is involved in LH secretion during the period of its photo-induced inhibition (Lincoln & Wu 1991).

On day 30 after melatonin implantation, naloxone increased LH secretion irrespective of the level of nutrition, and on day 45 in the HM subgroup. In a previous study (Zarazaga *et al.* 2011a), in goats exposed to alternations of 3 months of long days and 3 months of short days, increases in LH were also seen, although the drug challenge was given at 55 days after the onset of short days. The present results suggest that stimulation by the endogenous opioid system might occur earlier in well-nourished females or that melatonin implantation more strongly represses the opioid mechanism that inhibits LH secretion than that achieved by artificial short days. In support of this, in ewes, Misztal & Romanowicz (2005) observed that the stimulatory effect of melatonin or naloxone on LH secretion was more effective when they were infused intracerebroventricularly in combination. A possible explanation is that the secretion of GNRH/LH in response to the exclusion of the inhibitory tone of endogenous opioids becomes intensified by melatonin at the cellular level. Certainly, studies on the pars tuberalis cells have revealed that cAMP is dramatically elevated following simultaneous treatment with adenylate cyclase activators and melatonin (Hazlerigg *et al.* 1994, Barrett *et al.* 2003). Melatonin can induce a sensitised response to adenylate cyclase in ovine *pars tuberalis* cells, in which the receptor for melatonin is endogenously expressed (Barrett *et al.* 2003). This mechanism is, however, not fully understood. Nevertheless, the increase in the release of LH, evoked by melatonin under the conditions of exclusion of inhibitory opioid tone, reflects the potent induction of gonadotrophic axis activity. Nevertheless, Yang *et al.* (1989) failed to elevate LH concentration after the injection of WIN-3, an opioid antagonist, into melatonin-implanted or non-implanted control sheep at 5, 15 or 25 days after melatonin implantation. Indeed, at 60 days, they only achieved significantly enhanced LH secretion in three melatonin-implanted ewes showing ovarian cyclicity. These authors suggest that melatonin *per se*, at least at a level sufficient to advance the breeding season, is unlikely to induce reproductive cyclicity by initially switching LH control to an opioid-mediated system.

The lack of effect of pimozide at days 30 and 45 after melatonin implantation agrees with that described in goats exposed to alternations of 3 months of long days and 3 months of short days (Zarazaga *et al.* 2011a). These results contrast with previous findings by Forcada *et al.* (2002), who observed an increase in LH pulsatility in both high- and low-nutrition OVX + E ewes treated with melatonin. This might indicate differences between sheep and goats in relation to the

role of the dopaminergic system on regulation of the LH secretion in melatonin-implanted females.

The positive effect of cyproheptadine on day 45 after melatonin implantation on the HM and HC subgroups indicates that the negative effect of serotonin mechanism on LH secretion is reduced when stimulation of LH secretion by good nutrition levels occurs, independent of melatonin implantation. No evidence was found to support the hypothesis that the influence of melatonin on advancing the breeding season might occur via a serotonergic pathway. Moreover, these results agree with those previously reported by our group (Zarazaga *et al.* 2011a) when cyproheptadine was injected 55 days after the onset of short days. This procedure was only effective at inducing an increase in LH secretion in well-nourished animals. This suggests that the lower hypothalamic sensitivity to the negative feedback effects of oestradiol in goats receiving higher levels of nutrition is mediated, at least during the above 45 and 55 short day periods, by the serotonergic system.

The injection of NMDA acutely stimulated LH secretion in both the HC and the HM dose 30 days after melatonin implantation, and in all subgroups at 45 days. This suggests that, contrary to that described in sheep (Lincoln & Wu 1991, Vigiúé *et al.* 1995b), EAA are involved in LH secretion during periods of both photostimulation and photoinhibition. In fact, NMDA had a significant effect each time it was administered. The origin of this discrepancy between goats and sheep may be double. First, this mechanism may act in a different manner in ewes and goats, and secondly, Vigiúé *et al.* (1995b) studied the effect of NMDA in OVX + E ewes at 39 and 74 days after melatonin implantation – later than in the present experiment. In bucks, Meza-Herrera *et al.* (2007a) observed no effect on LH secretion of injecting L-glutamine three times per week during increasingly long days. In pre-pubertal goats, it has been shown that the injection of L-glutamine induces the earlier onset of puberty (Meza-Herrera *et al.* 2007b). According to the present results, the absence of any effect of NMDA on LH secretion in either the LM and the LC animals 30 days after melatonin implantation suggests that these EAA neurotransmitters are strongly inhibited by nutrition at this time. Finally, the positive effect observed at 30 days in both the HM and the HC animals, and at 45 days in all subgroups, provides no evidence to support the hypothesis that the influence of melatonin in advancing the breeding season may occur via a glutamatergic pathways.

Conclusions

To our knowledge, this is the first study to report results on the role of nutrition and the different neural mechanisms involved in the stimulation of LH secretion by exogenous melatonin in goats. The results provide evidence of a clear interaction between nutrition and melatonin and throw light on how the potential neural systems involved in the stimulation of LH secretion by melatonin are modulated by

nutritional level. Endogenous opioid receptors seem to be involved in this since the injection of naloxone increased LH concentrations only in melatonin-implanted animals. However, the serotonergic mechanism, as evidenced by the results of the cyproheptadine injection, appears to be most influenced by nutritional level since a clear response was observed 45 days after the moment of melatonin implantation in those animals that received 1.1 times their nutritional maintenance requirements, irrespective of whether they received melatonin or not. Further, the role of dopaminergic mechanisms would seem to be very small when LH is stimulated by exogenous melatonin. Finally, the stimulation of LH secretion by NMDA is strong in goats implanted with melatonin and maintained under photoperiod conditions that inhibit LH secretion. No evidence was found to support the hypothesis that the influence of melatonin in advancing the breeding season may occur via a glutamatergic pathway.

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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Author contribution statement

J L G and B M contributed actively in valuable discussion, drafted the paper and critically read the manuscript. I C contributed to the handling of the animals and recovering data. L A Z designed the study, analysed the data and wrote the paper.

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