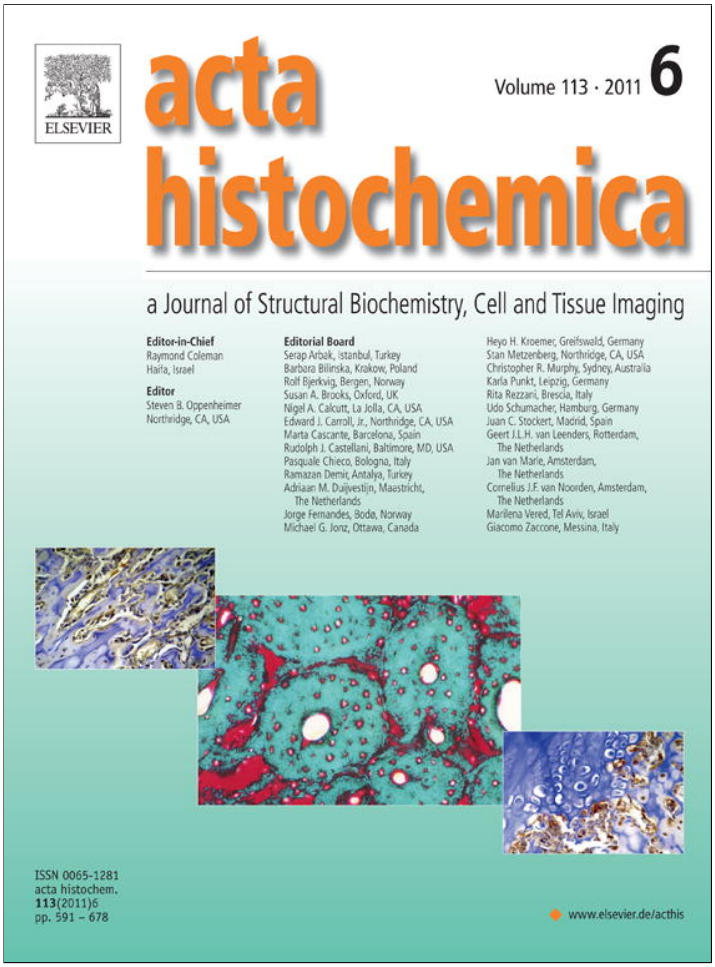

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Effect of the photoperiod and administration of melatonin on folliculostellate cells of the pituitary pars distalis of adult male viscacha (*Lagostomus maximus maximus*)

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ABSTRACT

Numerous reports have shown the effect of photoperiod and melatonin administration on the different hormone secreting cell types in the pituitary pars distalis. The viscacha (*Lagostomus maximus maximus*) is a rodent with photoperiod-dependent seasonal reproduction. The aim of this study was to examine the effect of photoperiod seasonal variations and melatonin administration on the folliculostellate cells in pituitary pars distalis of viscacha. Immunohistochemistry and image analysis were used to measure the percentage of S-100-positive area (total, cellular and colloidal) and the number of folliculostellate cells. The S-100 protein was immunolocalized at intracellular (folliculostellate cells) and extracellular (follicular colloid) levels. The morphometric parameters analyzed exhibited seasonal variations with highest values in the summer (long photoperiod) and lowest values in the winter (short photoperiod). The administration of melatonin caused a significant decrease of immunostaining. Results suggest that the natural photoperiod might be the most important environmental signal causing the decrease in folliculostellate cells immunostaining observed in the winter. These findings agree with seasonal changes previously reported in endocrine cells and suggest that folliculostellate cells may be involved in the paracrine regulation of the secretory activity of pituitary pars distalis through S-100 protein production.

Introduction

Pituitary folliculostellate cells (FSC) have been described in numerous species. Since the first descriptions (Cocchia and Miani, 1980; Nakajima et al., 1980), many authors have used the anti-S-100 protein antibody to study morphological and physiological aspects of the FSC. The S-100 protein is exclusively expressed in vertebrates and performs several intracellular and extracellular functions. Within cells, it regulates the phosphorylation of specific proteins, enzyme activities, calcium homeostasis, the assembly–disassembly of cytoskeleton proteins, and the transcription of various factors. In the extracellular space, it acts as a trophic and toxic agent, and probably also is involved in the regulation of inflammation and blood coagulation (Donato et al., 2009; Santamaría-Kisiel et al., 2006). The FSC release the S-100 protein to the extracellular space, modifying the cellular activity of pituitary hormone secreting cells (Allaerts and Denef, 1989; Allaerts and Vankelecom, 2005; Ishikawa et al., 1983; Lloyd and Mailloux, 1988; Marin et al., 1991). The many functions of the FSC include: support, phagocytosis, secretion of several cellular mediators and formation of a network of intercommunicated intercellular spaces (Acosta et al., 2010; Allaerts and Vankelecom, 2005; Devnath and Inoue, 2008). Many studies have demonstrated morphological changes of FSC in various conditions of pituitary hyperfunction, suggesting that they play a role in the regulation of the activities of endocrine cells through paracrine mechanisms (Dingemans and Feltkamp, 1972; Heinzlmann and Köves, 2008; Sbarbati et al., 1989; Yoshimura et al., 1977).

In most wild mammals, the natural photoperiod regulates biological cycles by means of the production of melatonin by the pineal gland (Goldman, 2001; Lincoln et al., 2006). Numerous reports have shown the effects of the photoperiod and melatonin on the different types of hormone secreting cells in the pituitary *pars distalis* (PD) (Baltaci et al., 2004; Console et al., 2002; Diaz-Rodriguez et al., 2001; Eagle and Tortonese, 2000; Singh and Krishna, 1997). The viscacha (*Lagostomus maximus maximus*) is a seasonal breeder of nocturnal habits. This rodent is the largest member of the Chinchillidae family and inhabits the southern hemisphere from Paraguay to the central region of Argentina (Jackson et al., 1996; Redford and Eisenberg, 1992). In its natural habitat the adult male viscacha exhibits an

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annual reproductive cycle synchronized by the photoperiod and modulated by the pineal gland and its main hormone, melatonin (Aguilera-Merlo et al., 2005; Dominguez et al., 1987; Filippa et al., 2005; Fuentes et al., 2003; Muñoz et al., 1997, 2001). The cellular activity of pinealocytes and the serum values of melatonin have been reported to be highest during the winter (short photoperiod) and lowest in the summer (long photoperiod) (Cernuda-Cernuda et al., 2003; Dominguez et al., 1987; Fuentes et al., 2003). In addition, numerous investigations have reported morphological and morphometric variations with season and melatonin administration in gonadotropes, corticotropes, somatotropes, thyrotropes and lactotropes of the pituitary PD of adult male viscacha. Seasonal changes in the activity of these cell populations have also been reported, with maximum values in the summer and minimum values in the winter (Filippa et al., 2005; Filippa and Mohamed, 2006a,b, 2008, 2010). Our previous studies of FSC in pituitary *pars intermedia* (PI) and PD have shown that these cells express the S-100 protein, form follicular structures with PAS-positive colloidal material inside, and exhibit a close association with the hormone secreting cell types (Acosta and Mohamed, 2009; Acosta et al., 2010). However, as far as we are aware, there is no information on the effects of the photoperiod and melatonin administration on the FSC in the pituitary PD.

The purpose of the present study was to examine the effects of photoperiod seasonal variations and melatonin administration on the FSC in pituitary PD of adult male viscacha.

Materials and methods

Sixteen adult male viscachas (body weight above 5 kg) were captured in their natural habitat near San Luis, Argentina (33°20′ south latitude, 760 m altitude), in the following periods: February to March (summer, long photoperiod), April to May (autumn), July to August (winter, short photoperiod) and November to December (spring).

After capture, animals were immediately taken to the laboratory, anesthetized with Nembutal (pentobarbital) and killed by decapitation. The brain was rapidly exposed and the pituitary gland was excised, sagittally sectioned, fixed in Bouin's fluid, processed for light microscopy, embedded in paraffin and serially sectioned in the horizontal plane. The experimental design was approved by the local ethics committee and was in agreement with the guidelines of the National Institutes of Health (Bethesda, MD, USA) for the use of experimental animals.

Melatonin administration

Eight adult male viscachas captured during February (summer) were used. The rodents were kept in indoor individual boxes with the following dimensions: 1 m × 1 m × 2 m (width, length and height, respectively), with solid concrete floor and walls. Animals were maintained under long photoperiods with a controlled lighting regimen (14L:10D), at 20 ± 2 °C and free access to water and food. The experimental group received two daily subcutaneous injections of melatonin (M5250, Sigma–Aldrich, St. Louis, MO, USA, 100 μg/kg body weight dissolved in olive oil) at 09:00 and 17:00 h for 9 weeks. The control group received two daily subcutaneous injections (at 09:00 and 17:00 h) of only the diluent (olive oil; Sigma–Aldrich cat. 75348). The experimental design was carried out according to protocols previously used in viscachas in our laboratory (Filippa et al., 2005; Filippa and Mohamed, 2006a,b, 2008, 2010; Mohamed et al., 2000; Muñoz, 1998; Perez-Romera et al., 2010; Scardapane et al., 1983). In addition, in both groups, the histological study of the testes was carried out to confirm the effect of melatonin on the reproductive status. In the melatonin treated viscachas, an inhibitory effect of this hormone on the spermatogenic activity was observed, which was in agreement with previously reported results (Muñoz, 1998).

Immunohistochemistry

The streptavidin–biotin immunoperoxidase method was used as described previously (Acosta and Mohamed, 2009; Acosta et al., 2010). The ready-to-use polyclonal anti-S-100 protein antibody (AR058-5R; BioGenex, San Ramon, CA, USA) and was incubated for 12 h in a moist chamber at 4 °C. The tissue sections were first deparaffinized with xylene and hydrated by decreasing concentrations of ethanol. Microwave pre-treatment (antigen retrieval) was performed by incubating the sections in 0.01 M citrate buffer (pH 6.0). After incubation for 20 min in a solution of 3% H_2O_2 in water in order to inhibit endogenous peroxidase activity, they were washed (3 × 10 min) in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). Non-specific binding sites for immunoglobulins were blocked by 15 min incubation with 0.25% casein in PBS, washed in PBS and incubated with the primary antibody. The slides were subsequently washed (3 × 10 min) in PBS. The immunohistochemical visualization was carried out using the Super Sensitive Ready-to-Use Immunostaining Kit (QD000-5L; BioGenex, San Ramon, CA, USA) at 20 °C. The sections were incubated for 30 min with biotinylated anti-IgG, and after being washed (3 × 5 min) in PBS, they were incubated for 30 min with horseradish peroxidase-conjugated streptavidin, and finally washed in PBS. The reaction site was revealed by 100 μl 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen solution in 2.5 ml PBS and 50 μl H_2O_2 substrate solution, resulting in a brown precipitate. The sections were counterstained with Harris' hematoxylin for 30 s, washed 10 min in running water, dehydrated in increasing graded ethanol, cleared in xylene and mounted with Entellan (Merck, Darmstadt, Germany). Labeling was assessed using an Olympus BX-40 light microscope.

To confirm the specificity of the immunoreactions the following control procedures were carried out: (1) replacement of primary antibody with normal goat serum, and (2) omission of primary antibody. No positive structures or cells were stained in these sections.

Morphometric analysis

Morphometric parameters were determined with a computer-assisted image analysis system consisting of an Olympus BX-40 binocular microscope, interfaced with a host computer, image processing and recording system. The images were captured by a Sony SSC-DC5OA camera and processed with Image Pro Plus 5.0 software (Media Cybernetics, Bethesda, MD, USA) under control of a Pentium IV computer. The software allowed the following processes: image acquisition, automatic analogous adjustment, thresholding, background subtraction, distance calibration, area measuring and diskette data logging. Before counting, a reference area of 3000 μm^2 (×100 objective) was defined on the color monitor, and distance calibration was performed using a slide with a micrometric scale for microscopy (Reichert, Austria). Eight regularly spaced serial sections (5 μm thick) were analyzed in every pituitary gland. All the microscopic fields captured with ×100 objective were analyzed in each section (340 microscopic fields per section), and 4 pituitary glands were studied in each group of animals. In total, 10,880 microscopic fields or measurements were carried out per group. The following morphometric parameters were determined:

- Percentage of S-100-positive total area (%S-100): calculated using the formula: %S-100 = ΣAi/ΣRA × 100, where ΣAi was the sum of the S-100-positive area and ΣRA was the sum of the PD area of every microscopic field.

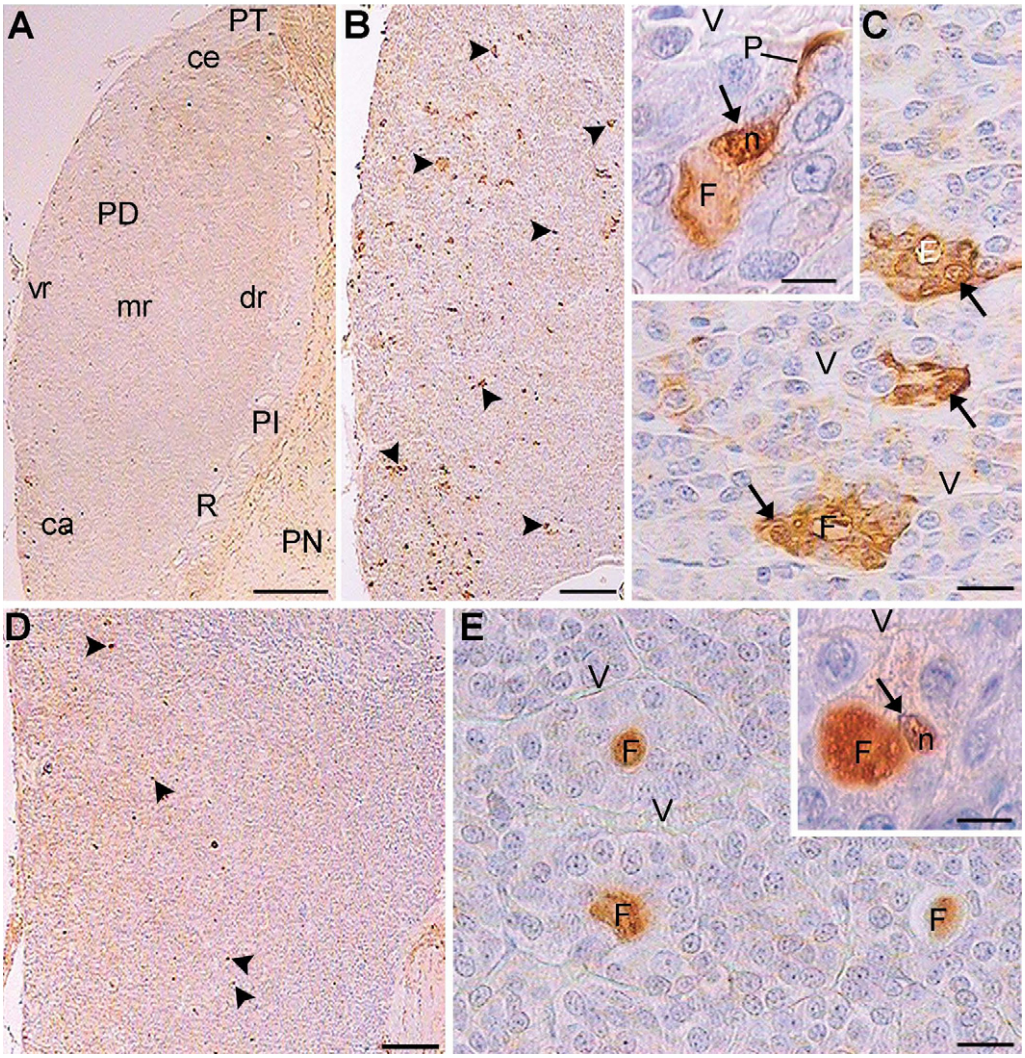

Fig. 1. Sections of the pituitary of adult male viscachas captured in summer (A–C) and autumn (D and E) immunostained with anti-S-100 protein. (A) The immunolabelling for this protein is observed in all pituitary zones: *pars distalis* (PD), *pars intermedia* (PI) and *pars nervosa* (PN). PD is separated from PI by Rathke's pouch (R). Three regions and two extremes are observed: a ventral one (vr, anterior), a dorsal one (dr, posterior or rostral) in contact with Rathke's pouch, and a medial one (mr); a cephalic extremity (ce, upper) extending to the *pars tuberalis* (PT), and a caudal one (ca, under). Scale bars = 250 μm. (B and C, and inset) The immunostaining for S-100 (arrowheads) is distributed throughout all the PD parenchyma and is mainly located in the FSC (arrows) and follicular colloid (F). These cells show immunostaining in the nucleus (n) and cytoplasm and exhibit cytoplasmic processes (P) that are in contact with adjacent cells or blood vessels (V). Scale bars: B = 125 μm; C = 12.5 μm; inset = 5 μm. (D and E, and inset) A lower immunostaining (arrowheads) for S-100 protein is observed in the PD of viscachas captured in autumn. This immunostaining is mainly located in the follicular colloid (F). The nucleus (n) of a FSC (arrow) is immunostained with anti-S-100 protein. V: blood vessels. Scale bars: D = 125 μm; E = 12.5 μm; inset = 5 μm.

- Percentage of S-100-positive cellular area (%S-100-cel): calculated using the formula: %S-100-cel = ΣAcel/ΣRA × 100, where ΣAcel was the sum of the area of S-100-positive cells and ΣRA was the sum of the PD area of every microscopic field.
- Percentage of S-100-positive colloidal area (%S-100-col): calculated using the formula: %S-100-col = ΣAcol/ΣRA × 100, where ΣAcol was the sum of the area of S-100-positive follicular colloids and ΣRA was the sum of the PD area of every microscopic field.
- The number of anti-S-100 immunostained FSC (FSC/RA) with a visible nucleus was counted in 340 microscopic fields per section. The results were expressed as number of FSC per reference area.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM) for all data sets. The different groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test. Differences between experimental and control groups were evaluated using Student's *t*-test. A probability of less than 0.05 was assumed to be significant.

Results

Seasonal study

Values of solar irradiation expressed as heliophany and seasonal mean values of precipitation and temperature recorded in San Luis, Argentina (Table 1) were provided by the Servicio Meteorológico Nacional San Luis (www.smn.gov.ar). The lowest values of heliophany, precipitation and temperature were observed in winter.

Table 1
Seasonal environmental conditions.

	Summer	Autumn	Winter	Spring
Heliophany (h)	9.38	7.09	6.82	9.09
Precipitation (mm)	90	27	11	58.5
Temperature (°C)	22	13	12	19.66

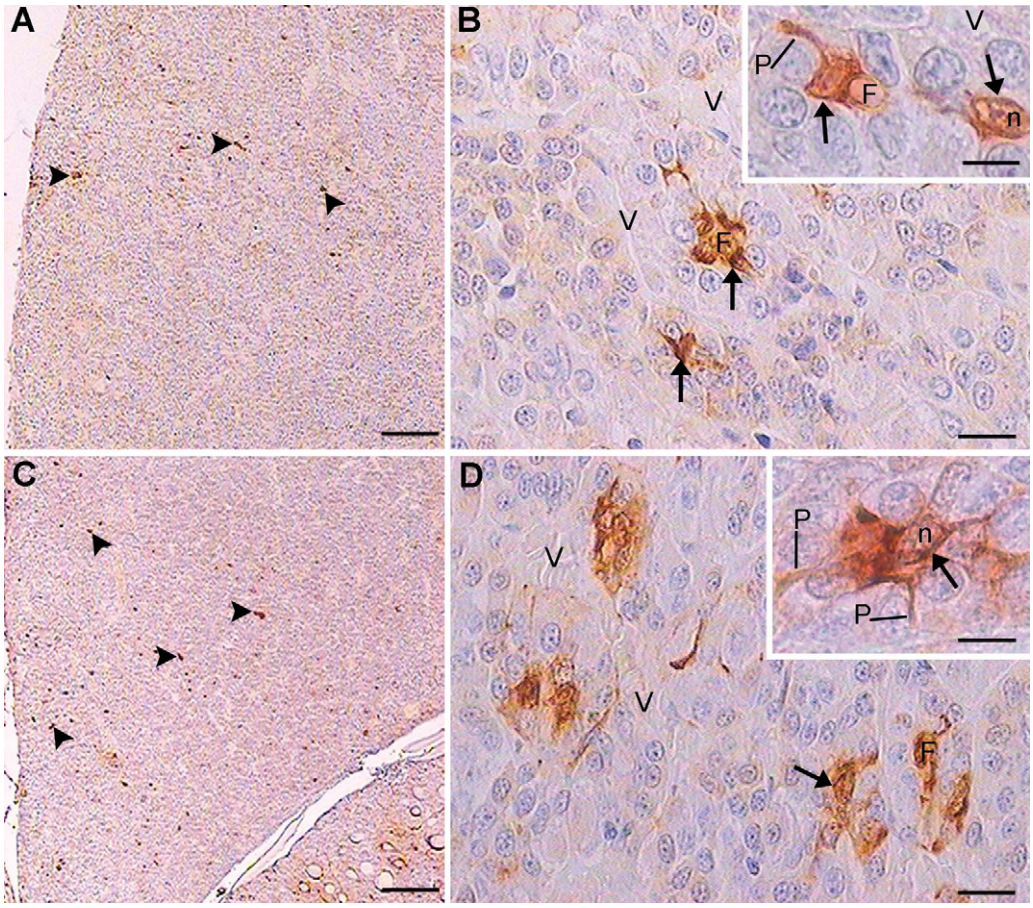

Fig. 2. Pituitary PD sections of the adult male viscachas captured in winter (A and B) and spring (C and D) immunostained with anti-S-100 protein. (A and B, and inset) During winter the lowest immunostaining (arrowheads) is observed for S-100. Arrows: FSC; F: follicular colloid; P: cytoplasmic process; V: blood vessels. Scale bars: A = 125 μm; B = 12.5 μm; inset = 5 μm. (C and D, and inset) A significant increase of the immunostaining (arrowheads) for this protein is observed in spring. The labeling is located into the follicular colloid (F) and nucleus (n), cytoplasm or both of the FSC (arrows). Scale bars: C = 125 μm; D = 12.5 μm; inset = 5 μm.

The PD, which is the most developed portion of the viscacha adenohypophysis, was separated from the PI by Rathke's pouch. Two extremes were distinguished: a cephalic one, extending to the *pars tuberalis* (PT), and a caudal one, where the PD becomes narrower and is communicated with the PI by blood vessels. Three regions were observed: an anterior one, a dorsal one in contact with Rathke's pouch, and a medial one (Fig. 1A). Scarce connective tissue and abundant irrigation were observed in the parenchyma, where the cells were organized in cords or around follicular structures.

In all the studied seasons, the FSC of pituitary PD originated follicles, and were isolated or in small groups. In addition, they exhibited an irregular nucleus, star-like shape and cytoplasmic processes which delimited follicular structures or contacted blood vessels or adjacent cells. The S-100 protein was localized both intracellularly (FSC) and extracellularly (follicular colloid). FSC were immunostained in the nucleus, cytoplasm, or both, while the follicular colloid exhibited a heterogeneous staining pattern. Colloidal immunostaining was intense, moderate, scarce or absent.

In the animals captured in the summer (long photoperiod), the morphometric study showed that all the parameters analyzed (%S-100, %S-100-cel, %S-100-col; FSC/RA) were highest and immunostaining was distributed throughout the PD parenchyma, mainly in the follicular colloid of the caudal extremity (Fig. 1A–C and Table 2). During the autumn, a significant decrease of these parameters was observed, with immunopositivity located in both pituitary extremities and along the anterior region. The FSC exhibited a few stained cytoplasmic processes (Fig. 1D and E, Table 2). During the winter (short photoperiod), the morphometric parameters were lowest and immunostaining was mainly localized in small follicles of the PD anterior region (Fig. 2A and B, Table 2). A significant increase in the values of the analyzed parameters was observed in the spring, with immunostaining distributed throughout all the PD parenchyma (Fig. 2C and D, Table 2).

Melatonin administration

The morphology and distribution of the pituitary FSC in animals that received melatonin and in controls were similar to that previously described in the seasonal study. However, all the morphometric parameters analyzed showed a significant decrease in

Table 2
Morphometric study of the pituitary PD folliculostellate cells in relation to seasonal variation of photoperiod.

Parameters	Summer	Autumn	Winter	Spring
%S-100	0.86 ± 0.04[a]	0.73 ± 0.05[c]	0.56 ± 0.02	0.79 ± 0.03[a]
%S-100-cel	0.44 ± 0.03[b]	0.35 ± 0.05	0.25 ± 0.02	0.40 ± 0.05
%S-100-col	0.46 ± 0.01[b]	0.33 ± 0.05	0.26 ± 0.02	0.41 ± 0.02[b]
FSC/RA	0.40 ± 0.04[a,c]	0.23 ± 0.08	0.19 ± 0.02	0.21 ± 0.02

The values are expressed as mean ± SEM ($n = 4$). %S-100: percentage of S-100-positive area; %S-100-cel: percentage of S-100-positive cellular area; %S-100-col: percentage of S-100-positive colloidal area; FSC/RA: number of FSC S-100-positive per reference area. Significant differences were determined by analysis of variance followed by Tukey–Kramer multiple comparison test.

[a] $p < 0.001$; Summer vs. Winter and Spring vs. Winter.
[b] $p < 0.01$; Summer vs. Winter and Spring vs. Winter.
[c] $p < 0.05$; Autumn vs. Winter and Summer vs. Spring.

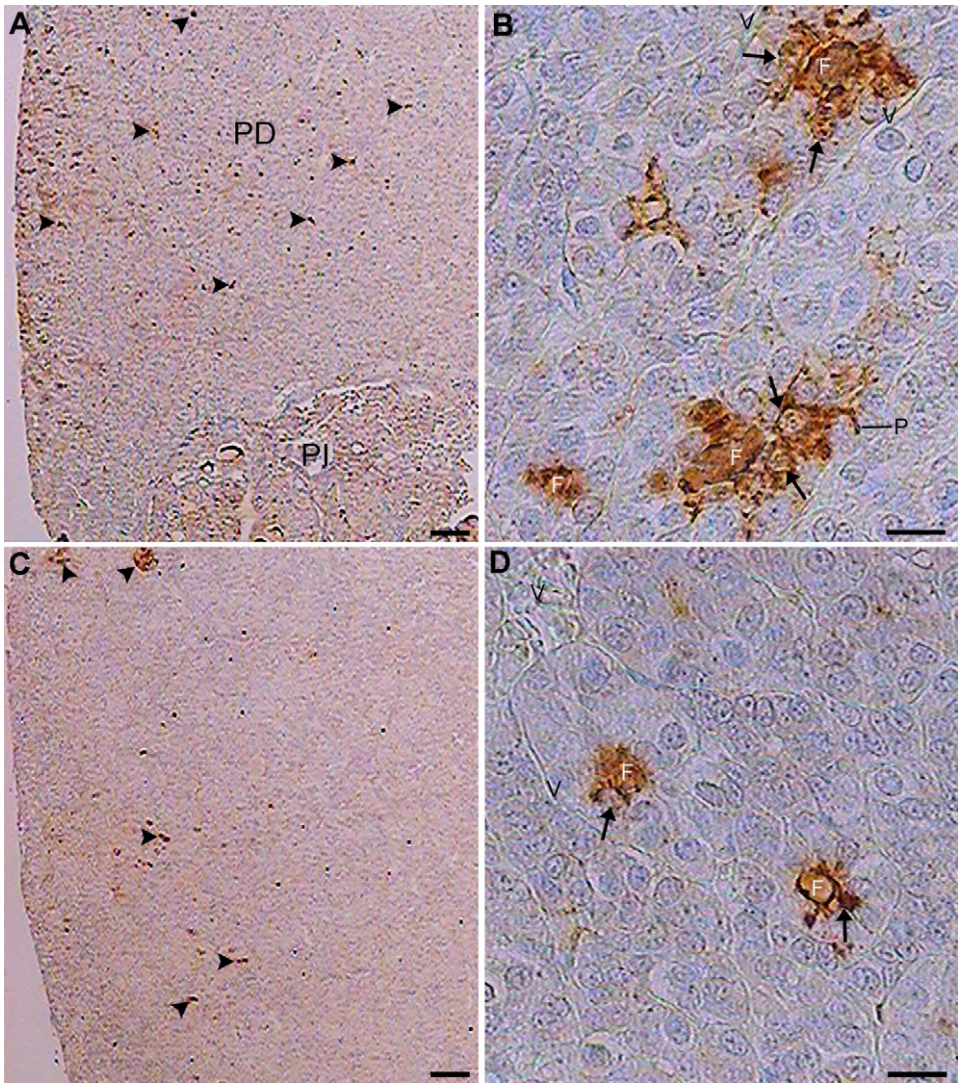

Fig. 3. Effect of the melatonin administration on the S-100 protein expression in pituitary PD of viscachas. (A and B) Pituitary PD of the control viscachas. The immunostaining (arrowheads) is widely distributed in the PD, and is observed in FSC (arrows) and follicular colloids (F). PI: *pars intermedia*; P: cytoplasmic process; V: blood vessels. (C and D) A significant decrease of the immunostaining for S-100 protein (arrowheads) in the viscachas administrated with melatonin is observed in FSC (arrows) and follicular colloids (F). V: blood vessels. Scale bars: A and C = 125 μm; B and D = 12.5 μm.

the animals that received melatonin as compared to the controls (Fig. 3 and Table 3).

Discussion

In wild animals, physiological conditions are modified by environmental factors such as photoperiod, temperature, water and food availability and social interactions. These environmental signals determine the beginning and end of specific seasonal adaptation, developing the endocrine adjustments necessary to ensure survival and reproductive success. In pituitary PD, the photoperiod and melatonin have effects on the hormone secreting cells and also on colloid extracellular accumulations (Baltaci et al., 2004; Console et al., 2002; Diaz-Rodriguez et al., 2001; Eagle and Tortonese, 2000; Nunez and Gershon, 1982; Singh and Krishna, 1997). Morphological variations of FSC during the reproductive cycle were determined in mink by Cardin et al. (2000).

In addition to the well-known control of pituitary secretion by hypothalamic factors and feedback signals from the peripheral endocrine glands, there is also an intrinsic pituitary control based on autocrine, juxtacrine and paracrine interactions (Denef et al., 1989). In this respect, the association between certain types of pituitary cells may partially account for the existence of these complex paracrine interactions (Jones et al., 1990; Schwartz et al., 1998). It has also been reported that FSC release the S-100 protein and other cellular mediators, which modulate the secretion of the pituitary hormones (Allaerts and Denef, 1989; Allaerts et al., 1990; Ishikawa et al., 1983; Lloyd and Mailloux, 1988).

Table 3
Effect of melatonin administration on the pituitary PD folliculostellate cells.

Parameters	Control	Melatonin
%S-100	1.08 ± 0.11	0.65 ± 0.05[a]
%S-100-cel	0.73 ± 0.06	0.47 ± 0.05[a]
%S-100-col	0.34 ± 0.04	0.20 ± 0.03[b]
FSC/RA	0.41 ± 0.02	0.30 ± 0.01[b]

The values are expressed as mean ± SEM ($n = 4$). %S-100: percentage of S-100-positive area; %S-100-cel: percentage of S-100-positive cellular area; %S-100-col: percentage of S-100-positive colloidal area; FSC/RA: number of FSC S-100-positive per reference area. Significant differences were determined by the Student's *t*-test.

[a] $p < 0.01$; Melatonin vs. Control.

[b] $p < 0.05$; Melatonin vs. Control.

In viscacha, the effect of the photoperiod and melatonin on the PD hormone secreting cells has been demonstrated by the analysis of various morphometric parameters considered as indicators of cellular activity. During the winter, the lowest values in gonadotropes, corticotropes, somatotropes, thyrotropes and lactotropes were observed, which could be associated with the low endocrine activity of the gland in this season (Filippa et al., 2005; Filippa and Mohamed, 2006a,b, 2008, 2010). On the other hand, variations in the number of the PAS-positive colloid with season and melatonin administration have been reported (Mohamed et al., 2000). We have recently demonstrated the expression of the S-100 protein in FSC and the follicular colloid of PD. A close association between FSC and hormone secreting cells has also been reported (Acosta et al., 2010). In the present study, the variations of the S-100 protein in the follicular colloid support the hypothesis that follicles are not static structures for the storage of proteic substances, but rather play a dynamic role in the gland secretory activity. The FSC that generate follicles might be in charge of formation and mobilization of the colloid and probably play an active role in the regulation and coordination of the pituitary secretory activity.

The photoperiod is the most predictable environmental signal and generates, through the production of melatonin by the pineal gland, several physiological responses in the organism (Goldman, 2001). To the best of our knowledge, this present study is the first to show that the expression of the S-100 protein at intracellular (FSC) and extracellular (follicular colloid) levels exhibits seasonal variations, with minimum values in the winter (short photoperiod). In addition, a decrease in the morphometric parameters analyzed by effect of melatonin administration was observed. Both results suggest that the natural photoperiod might be the most important environmental signal causing the decrease in FSC immunostaining observed in the winter. In this season, minimum serum levels of melatonin have been reported for viscacha (Fuentes et al., 2003). However, the influence of other factors such as temperature, water and food restriction should not be discarded. FSC probably participate in the processes of pituitary paracrine regulation through the production of S-100 protein, although further studies are necessary to elucidate the mechanisms involved.

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