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17β-estradiol effect on testicular β-endorphin expression in *Psammomys obesus*

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Abstract

Introduction. Testicular function in the sand rodent *Psammomys obesus* is subjected to seasonal alternations with a trigger of spermatogenesis in winter and a total quiescence which extends from late spring to summer. The aim of this study was to investigate the distribution of β-endorphin in the testis at the period of winter sexual activity and at its summer regression, and assess the effect of 17β-estradiol treatment on testicular morphology and β-endorphin expression.

Material and methods. The adult males were grouped into 4 groups (rest group, sexually active group, rest treated with 17β-estradiol group and controls at sexual rest injected with olive oil, n = 5 in each group). Using anti-serum against β-endorphin, we studied its testicular expression by Western blot and cellular location by immunohistochemical (IHC) method, respectively.

Results. We detected by Western blot a peptide of 3.5 kDa molecular weight corresponding to β-endorphin only in sexually resting and control males. The 17β-estradiol treatment induced a clear reduction in the β-endorphin band expression compared with the latter. These results were confirmed by the IHC analysis since β-endorphin was only observed in the testis at sexual rest and in controls, in majority of seminiferous tubules at the level of germ cells. The intensity of IHC labeling was significantly different between spermatogonia and spermatocytes I or round spermatids which revealed the strongest labeling. The intense immunoreactivity was also located in Leydig cells and highly significantly varied compared to the germ cells. The 17β-estradiol treatment decreased significantly the β-endorphin signal in germ cells but not in Leydig cells.

Conclusion. The 17β-estradiol treatment induces a repressive effect on seasonal testicular endorphinergic system in *P. obesus* and this action targets exclusively the germ cells. *(Folia Histochem Cytobiol. 2016, Vol. 54, No. 2, 108–117)*.

Key words: *Psammomys obesus*; seasonal reproduction; testis; 17β-estradiol; β-endorphin; immunohistochemistry; Western blot

Introduction

Spermatogenesis is a cyclical and continuous process, organized into seminiferous epithelium, during which spermatogonia A and B proliferate and generate spermatozoa. This process is coordinated under the endocrine control of gonadotropins and steroid hormones, together with manifold local regulatory factors [1]. The Leydig cells are the main source of testosterone, leading to high testicular levels, which is essential for initiation and maintenance of spermatogenesis. The effect of testosterone on spermatogenesis is indirectly mediated through Sertoli cells and the peritubular cells [2]; it is very specific and occurs mainly in stages VII and VIII of the germinal cycle of mice [3].

The presence of estrogens has been documented in the testis for more than 50 years. Morphological and biochemical studies showed that in adult rodents all testicular cells, except peritubular cells, spermatogo-

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nia and early spermatocyte, express aromatase, the enzyme necessary for the synthesis of estrogens [4]. Until recently, estrogens were considered essentially as feminine hormones. However, growing evidence has demonstrated that estrogens play a crucial role in the regulation of spermatogenesis [5–7], acting in the testis through estrogen receptors α (ERα) and β (ERβ) [1]. In the rat, both ER forms were expressed in spermatocytes, elongating spermatids, Sertoli cells and Leydig cells [8]. Estradiol plays a regulatory role in rat spermatogenesis initiation [9] and induces apoptosis and drastic decline in round spermatids and spermatozoa [10]. Recent data show that the GPR30 is implicated in the apoptotic effects of estrogens at the pachytene spermatocytes I and round spermatids stages [11, 12].

In mammals, endorphinergic system has been shown to modulate reproduction and β-endorphin, a pro-opiomelanocortin (POMC)-derived peptide, has been found to influence sexual functions acting at multiple levels of the hypothalamus–pituitary–gonadal axis. Substantial research demonstrates that β-endorphin inhibits the secretion of gonadotropin releasing hormone (GnRH) *via* opioid receptors present in the hypothalamus [13]. Moreover, a paracrine action of β-endorphin in addition to its central role in the regulation of testis function has been suggested [14].

Recent data established that photoperiod modulates steroidogenesis and spermatogenesis through modulation of melatonin receptor 1 expression and local melatonin content in adult golden hamster *Mesocricetus auratus* testes which exhibit seasonal testicular activity [15]. Among additional mediators besides local melatonergic system in photoperiodic control of testicular steroidogenesis, β-endorphin and its receptor, μ-opioid receptor, was suggested in golden hamster. Indeed, an increase in the level of plasma β-endorphin [16] and a strong expression of its receptor in testes of *Mesocricetus auratus* [17] have been obtained under short day exposure.

Photoperiod seems to tightly regulate the timing of the onset and cessation of testicular functions characterized by dramatic alterations in steroidogenesis in seasonally breeding rodents such as the sand rat, *P. obesus*, which, compared to golden hamster, exhibits a summer sexual activity [18]. To further test this hypothesis, we attempted to establish the photoperiodic profile of testicular endorphinergic system in *P. obesus* and to assess the effects of 17μ-estradiol (17μE) treatment.

Material and methods

Animals and sampling. *P. obesus* is a diurnal rodent that displays a seasonal reproductive cycle with a breeding period from autumn through early spring and a resting phase from late spring through summer. The animals studied were adult males, whose body weight was 80–100 g [19]. They were captured in the region of Beni Abbes, situated at North-West of the Algerian Sahara (30°7' North latitude and 2°10' West longitude) at about 500 m of altitude, during the sexual rest (July end, 43–45°C) and activity (December, 16–18°C). Experimental groups, each composed of 5 animals, were constituted as follows: (i) males at sexual rest, (ii) males at sexual rest treated with 17βE, (iii) control males at sexual rest injected with olive oil, and (iv) males at sexual activity. Animals that came directly from the biotope were sacrificed on the same day from 8 am to 1 pm for the first and the last groups. By cons, the 17βE-treated and control animals were maintained in the laboratory under summer natural photoperiod and temperature (25°C the same temperature as in their burrows) during the treatment period.

All experiments were carried out in compliance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) following approval by the local Ethical Committee of Houari Boumediene University of Sciences and Technology, Algeria.

Hormonal treatment. The animals of group II were submitted to daily intraperitoneal injection of 17βE (75 μg *per* day dissolved in 40 μL olive oil *per* 100 g of body weight, BW) during 21 days. The third group was considered as control and animals were injected with 40 μL olive oil only *per* 100 g BW during 21 days.

Western blot analysis. Testes were dissected and frozen in liquid nitrogen and conserved at –80°C. The tissue homogenates were prepared by cold sonication in 0.1 N HCl. The homogenate was centrifuged (15,000 *g* for 30 min at 4°C) and supernatant was collected by the acid extraction for BCA protein assay. The β-endorphin in testis extracts was analyzed by Western blotting with a preparation of tissue samples for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and denaturing conditions with β-mercaptoethanol and sodium dodecyl sulfate, respectively. The samples were submitted to electrophoresis on 16% tricine gels according to the modified method of Hayder et al. [20]. Proteins were transferred electrophoretically to PVDF membranes (ISEQ00010 Immobilon-P^{SQ} Membrane, PVDF, 0.2 μm, Merck Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat dried milk and 1% bovine serum albumin TPBS buffer (phosphate-buffered saline, PBS, and 0.1% Tween 20) for 2h and incubated with the mouse monoclonal antibody (ABC117-1262, Eurobio AbCys, Les Ulis, Paris, France) or monoclonal rabbit anti-β-endorphin1-31 approved for Western blotting (ABfinity™ β-endorphin recombinant, Life Technologies, Carlsbad, CA, USA), respectively, at the final dilution of 1:500 and 1:250 in TPBS overnight. After

washing in TPBS and incubation with respective secondary antibody anti-mouse-horseradish peroxidase [IgG (H + L), Vector, Burlingame, CA, USA] and anti-rabbit horseradish peroxidase [IgG (H + L) 074-1506, KPL, Gaithersburg, MD, USA] for 2h at the final dilution of 1:30,000 and 1:20,000 followed by washing, the membrane was developed by enhanced chemiluminescence (Amersham™ ECL™ select Western blotting detection reagent according to the manufacturer's protocol (GE, Little Chalfont, UK).

A positive control was performed with the β-endorphin rat recombinant protein (E1142, Sigma-Aldrich, St. Louis, MO, USA) to test the specificity of the primary antibody.

Immunohistochemical staining. After anesthesia by an intraperitoneal injection of 25% urethane solution (0.4 mL per 100 g of body weight), testes were removed and fixed by immersion in Bouin-Holland's solution or in 10% formalin, dehydrated in increasing concentrations of ethanol (70%, 95% and 100%), cleared in toluene, and finally embedded in paraffin wax. 5 μm-thick paraffin sections were obtained and mounted on Superfrost® glass slides. The labeling was carried out by an indirect immunoenzymatic technique using the streptavidin biotin peroxidase detection system by amplification with the Vectastain kit (Vectastain Universal, Vector Laboratories). This technique enabled us to locate β-endorphin, using a mouse monoclonal antibody (ABC117-1262, Eurobio AbCys).

Sections were deparaffinized with cyclohexane, rehydrated with decreasing concentrations of ethanol and rinsed with PBS. Endogenous peroxidase was blocked by incubating the slides with H_2O_2 at 3% for 20 min followed by two baths in PBS. The blocking of nonspecific sites was performed by 10% of normal horse serum for 1 h at room temperature. Sections were then incubated overnight in a humid chamber at 4°C with the primary specific antibody diluted at 1/200 in PBS. Following two baths of rinse in PBS of 5 min, each section was incubated with prediluted secondary antibody, anti-mouse IgG (Vectastain kit) for 30 min at room temperature and then rinsed in PBS. The sections were incubated with streptaividine for 30 min at room temperature and rinsed in PBS. The labeling was revealed in brown by the chromogen 3,3'-diaminobenzidine (DAB) and the sections were counterstained with Mayer's hematoxylin.

Two methods of negative control were performed: the first one was by omitting the primary antibody to test the secondary antibody's specificity. The second one consisted in testing primary antibody's specificity by saturation with a homologous antigen; the β-endorphin antibody was pre-absorbed with an excess of rat β-endorphin recombinant protein (E1142, Sigma-Aldrich) at 37°C for 1 h.

Morphometry and labeling quantification. The diameter of seminiferous tubules was measured by photonic microscopy (Zeiss, Jena, Germany) at objective magnification ×40, with a number of 20 seminiferous tubules per section per slide for each animal.

A quantitative study of immunoreactivity was carried out using Fiji Win 32, a distribution of Image J software (NIH, Bethesda, USA) that measures the average darkness of the image due to DAB signal. The image was inverted to clean out the white noise, then the regions of interest were drawn to define the stain color, the background and the counterstaining with hematoxylin were subtracted. The labeling was measured for 20 cells of various cellular categories (spermatocytes I, round spermatids, spermatogonia, Leydig cells) (100 cells per animal group) using a ×40 objective magnification.

Statistical analysis. The results are expressed as means ± standard mean error (SEM). Comparisons were performed using a one-way variance analysis (ANOVA) followed by the Scheffe's *post hoc* test. P values lower than 0.05 ($p < 0.05$) were considered as indicative of a significant difference.

Results

Weight of testis and diameter of seminiferous tubuli

The weight of the testis (Figure 1A) and seminiferous tubules diameter (Figure 1B) varied depending on the physiological state of the animals. There were no statistically significant differences in the mean weight and seminiferous tubules diameter between males at sexual rest (138.0 ± 4 mg, 151.4 ± 3.73 μm, mean ± SEM) and control animals (137.6 ± 7 mg, 152.14 ± 3.54 μm). However, the difference was significant between sexual rest males and 17βE-treated males which exhibited the lowest testis weight and seminiferous tubules diameter (116.0 ± 6 mg, 135.9 ± 2.9 μm). The sexually active males exhibit the highest testis weight and seminiferous tubules diameter (305.8 ± 11 mg, 276.5 ± 3.04 μm), and the differences were significant compared with sexual rest males, 17βE-treated males and control animals (Figure 1A, B).

Western blot analysis

Western blot analysis revealed the expression of testicular β-endorphin only in quiescent and control groups of *P. obesus* corresponding to a low molecular weight band of 3.5 kDa. An important decrease in the expression of β-endorphin in testes was noted following 17βE administration as compared with control counterparts (Figure 2).

Localization of β-endorphin in testes of P. obesus

In *P. obesus* at the sexual rest period and control animals injected with olive, most of the seminiferous tubules presented a reduced lumen (Figure 3A). Sertoli cells were located at the base of seminiferous

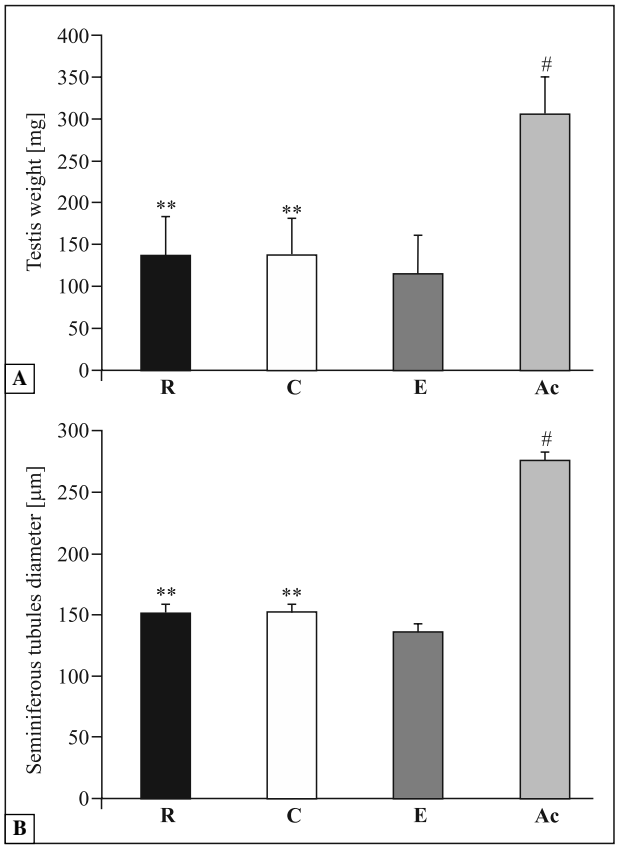

Figure 1. Testes weight and diameter of seminiferous tubules in *P. obesus*. **A.** Testis weight of *P. obesus* males at sexual rest (R), control males (C), males at sexual rest treated with 17β-E (E) and males in a period of sexual activity (Ac). Values were expressed as means, vertical bars show standard errors. **Significantly different from treated (R-E, C-E), $p < 0.05$, #highly significant difference *vs.* other groups, $p < 0.001$, $n = 5$ for each group; **B.** Seminiferous tubules diameter of *P. obesus*, symbols of animal groups as above. **Significantly different from treated (R-E, C-E), $p < 0.05$ and #highly significant differences *vs.* other groups, $p < 0.001$, $n = 5$ for each group.

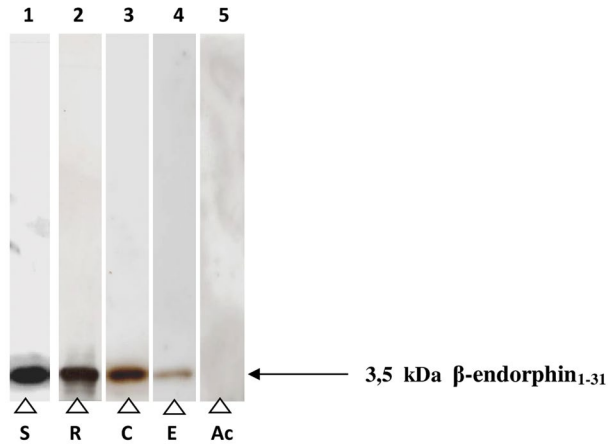

Figure 2. Western blot analysis of testicular β-endorphin presence after treatment of *P. obesus* with 17β-E for 21 days. The panel shows the blot with a standard β-endorphin, used as loading control which migrated as a 3.5 kDa band (S lane 1). The 3.5 kDa band corresponding to β-endorphin was expressed at sexual rest (R, lane 2) and in control animals (C, lane 3). Lack of β-endorphin expression in period of sexual activity (Ac, line 5), and the band is barely expressed after 17β-E treatment (E, lane 4).

epithelium in which spermatogonia, spermatocytes I and few round spermatids were visible. Spermatozoa were totally absent (Figure 3B, C) and numerous gonial divisions were observed (Figure 3C). In the majority of seminiferous tubules, immunohistochemical (IHC) method revealed a positive staining in the spermatogonia (Figure 3C), spermatocytes I and scarce round spermatids (Figure 3B, C). The immunoreactivity was particularly more intense in the spermatocytes I and round spermatids as well as in Leydig cells (Figure 3B, C). Moreover, some seminiferous tubules which exhibited a lot of mitotic spermatogonia were lacking β-endorphin. Any signal of β-endorphin was observed in both negative controls either by absorption in excess of β-endorphin or by primary anti-β-endorphin antibody omission (Figure 3D).

Prolonged administration of 17β-E to males at the rest period resulted in the reduction of the light of seminiferous tubules (Figure 4A). Moreover, the gonial divisions were more numerous and the spermatocytes I appeared less abundant compared with those observed in the testis of untreated animals and controls. The round spermatids were rare (Figure 4B). The immunolocalization of the β-endorphin revealed a decrease in the staining intensity as compared with untreated animals and controls at the level of the spermatogonia, spermatocytes I and round spermatids in all seminiferous tubules (Figure 4A); however, Leydig cells remained always highly immunoreactive (Figure 4B). The negative control did not show any β-endorphin labeling (Figure 4D).

In *P. obesus* studied at sexual active period, all seminiferous tubules presented a large lumen and contained a lot of spermatozoa and abundant round and elongated spermatids (Figure 5A, B). Spermatogonial divisions were common (Figure 5A). Interstitial tissue was well developed (Figure 5A). β-endorphin immunoreactive structures could be observed neither in the tubular nor the interstitial compartment (Figure 5).

The assessment of the intensity of β-endorphin immunoreactivity was performed only on the germinal and interstitial cells, *i.e.* in the following *P. obesus* groups: sexually resting, resting control and resting

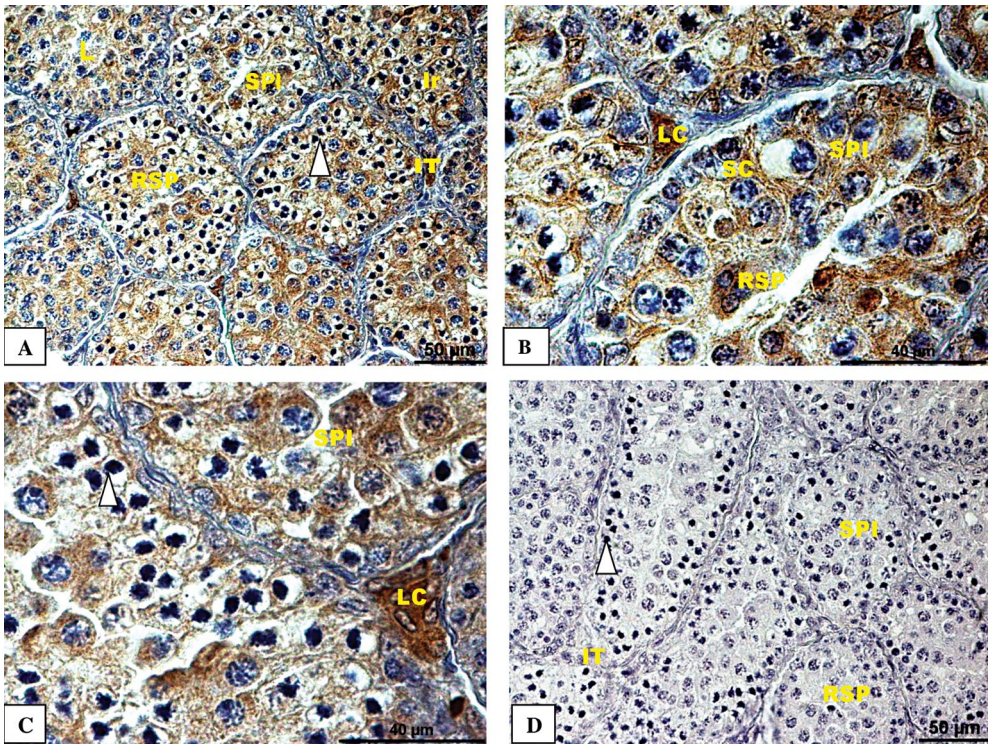

Figure 3. Localization of β-endorphin in testes of *P. obesus* at sexually rest period. **A.** Positive immunoreactivity (Ir) in germ cells; **B.** Strong β-endorphin signal was observed in spermatocytes I (SPI) and round spermatids (RSP). β-endorphin was totally absent in Sertoli cells (SC); **C.** Intense β-endorphin Ir was present in Leydig cells (LC); **D.** Negative control. Gonial divisions (arrowhead); interstitial tissue (IT). Sections of *P. obesus* testis were stained by immunohistochemistry (IHC) to detect β-endorphin as described in Material and methods. Bars: 60 μm (A), 40 μm (B, C), and 50 μm (C).

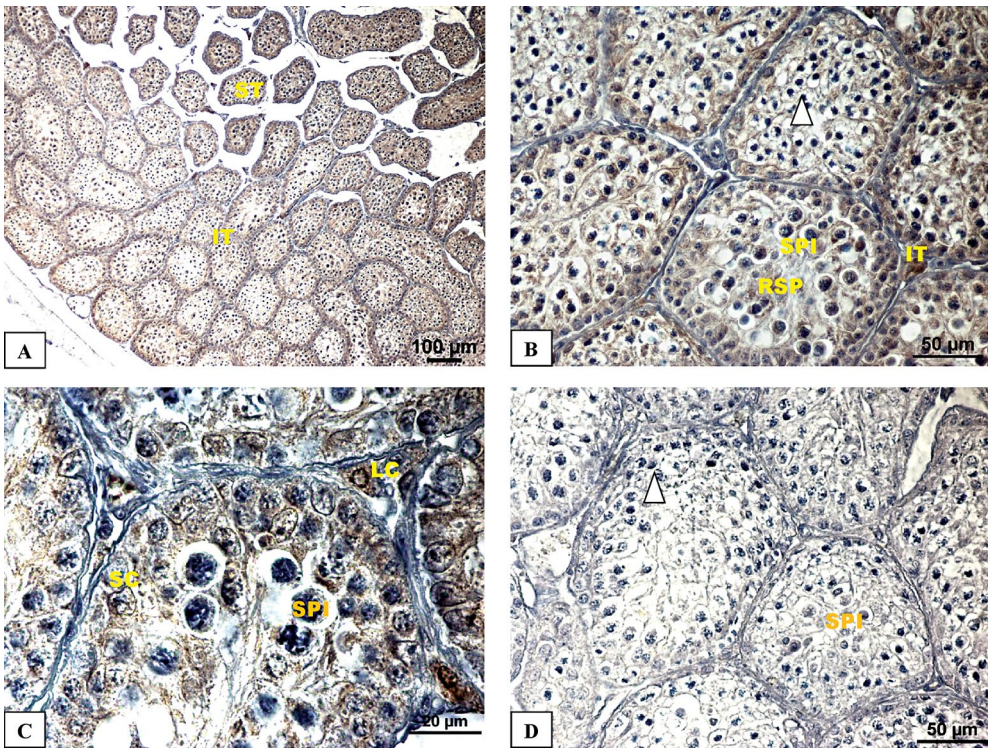

Figure 4. Testicular β-endorphin localization after treatment of *P. obesus* with 17βE for 21 days. **A, B.** Reduction of labeling in germ cells of all seminiferous tubules (ST) and increased divisions of spermatogonia (arrowhead); **C.** Leydig cells (LC) remained well labeled; **D.** Negative control. Sections of *P. obesus* testis were stained by IHC to detect β-endorphin as described in Material and methods. Bars: 100 μm (A), 50 μm (B, D) and 20 μm (C).

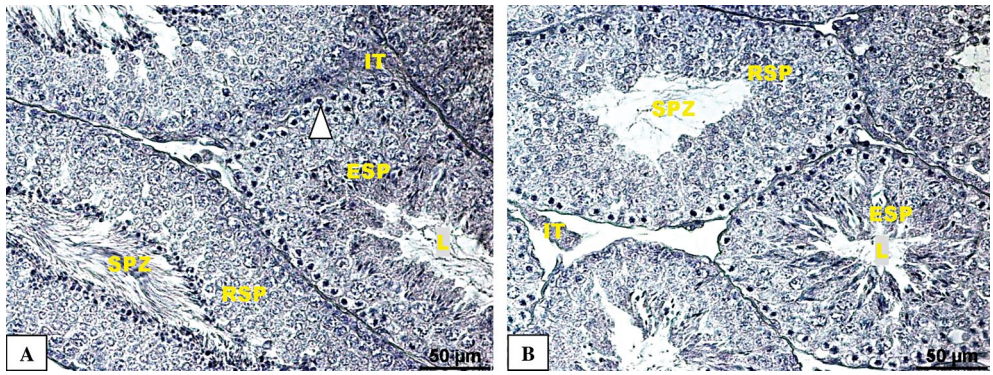

Figure 5. Localization of β-endorphin in testes of *P. obesus* at sexual activity. **A, B.** Absence of labeling in tubular and interstitial compartments. Gonial divisions were common (arrowhead), Sertoli cells (SC) with substantially triangular nucleus, interstitial tissue highly developed (IT), round spermatids (RSP) and elongated spermatids (ESP) were present and spermatozoa (SPZ) were abundant. Sections of *P. obesus* testis were stained by IHC to detect β-endorphin as described in Material and methods. Bars: 50 μm (A, B).

Table 1. β-endorphin immunoreactivity levels in *P. obesus* testis at sexual rest, in controls and after 17βE treatment

Cell types / Year cycle	Leydig cells	Spermatocytes I	Round spermatids	Spermatogonia
Group of males at sexual rest (July)	34.8 ± 2%	28.7 ± 1.25%	25.6 ± 1.7%	11.7 ± 0.74%
Group of control males at sexual rest injected with olive oil (July)	33.0 ± 2.58%	29.4 ± 2.09%	25.94 ± 1.5%	11.4 ± 0.82%
Group of males at sexual rest treated with 17βE (July)	33.2 ± 2.6%	157 ± 4.4%	14.8 ± 4.3%	8.14 ± 1.3%

The labeling was measured for 20 cells of various cellular categories (spermatocytes I, round spermatids, spermatogonia, Leydig cells) (100 cells per animal group). Values are expressed as mean ± SEM, n = 5 in each group.

treated with 17βE. The intensity of signal varied depending on the cellular categories of the testis and physiological state (Table 1). Indeed, the Leydig cells expressed higher β-endorphin immunoreactivity than the germline, and the difference was statistically highly significant in three groups (Table 1, Figure 6A). The intensity of β-endorphin immunoreactivity in Leydig cells was almost the same in the three compared groups (Table 1, Figure 6B). Spermatogonia were clearly less stained than spermatocytes I and round spermatids regardless of the animal group. Following the 17βE administration, the intensity of β-endorphin immunoreactivity of these three germ cell types decreased significantly compared with quiescent and control animal groups (Table 1, Figure 6B).

Discussion

In *P. obesus*, the β-endorphin was detected in Leydig cells and germ cells. This corroborates the limited results obtained in mammals which indicate the expression of the POMC mRNA in Leydig cells in rat and golden hamster [21], and spermatogonia and spermatocytes in mouse [22]. The presence of β-endorphin was only reported in the interstitial compartment in rat Leydig cells and macrophages [23–25] and human spermatozoa [26]. The results obtained in *P. obesus* demonstrate for the first time β-endorphin presence in the germinal compartment.

In *P. obesus*, a negative correlation was deduced between β-endorphin and testicular activity as this peptide was only expressed at sexual rest in spermatogonia, mainly in spermatocytes I, round spermatids and Leydig cells. We observed testicular β-endorphin expression in *P. obesus* in long photoperiod while in Syrian hamster *Mesocricetus auratus* [27], the level of POMC [28] and the β-endorphin immunoreactivity [28] were strong in short photoperiod. These data suggest that β-endorphin can play an important role in the regulation of seasonal gonadal activity depending on the species reproductive cycle. It was evident that β-endorphin was clearly involved in sexual quiescence. Based on our results we suggest that photoperiod differentially regulates the β-endorphin expression. A short photoperiod has an anti-gonadal effect for *Mesocricetus auratus* and pro-gonadal effect for *P. obesus.*

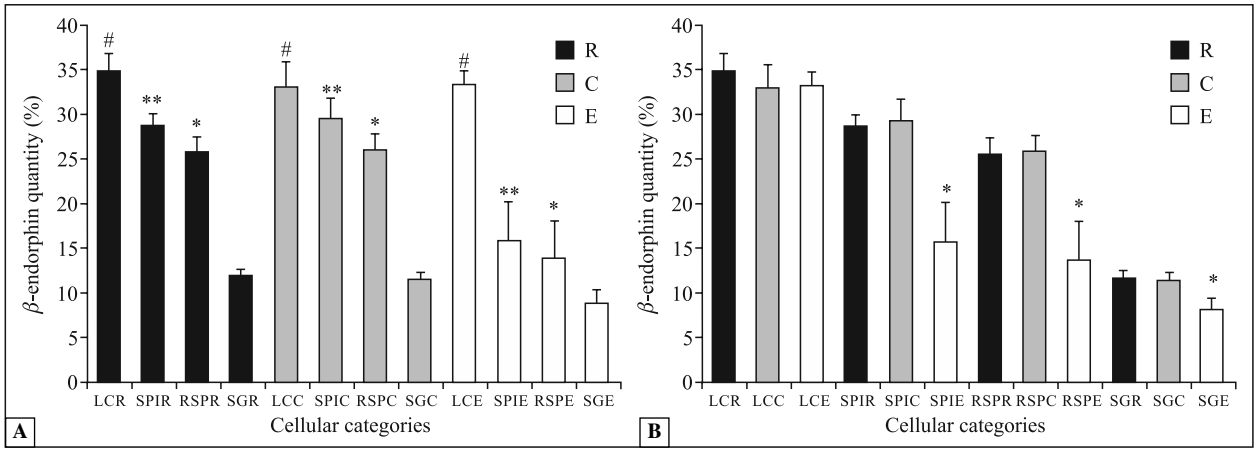

Figure 6. Histoscore of β-endorphin immunoreactive cells in the testis of *P. obesus* at sexual rest, in control and 17βE-treated animals. **A.** Expression levels of β-endorphin in different cellular categories within each group. Males at sexual rest (black bars: R), control males at sexual rest injected with olive oil (gray bars: C) and males at sexual rest treated with 17βE (white bars: E). Males at sexual rest: Leydig cells (LCR), spermatocytes I (SPIR), round spermatids (RSPR), spermatogonia (SGR). Control males: Leydig cells (LCC), spermatocytes I (SPIC), round spermatids (RSPC), spermatogonia (SGC). Males after 17βE treatment: Leydig cells (LCE), spermatocytes I (SPIE), round spermatids (RSPE), spermatogonia 17βE treatment (SGE). Values represent means and vertical bars show standard errors. *Expression levels of β-endorphin significantly different from spermatogonia (RSPR-SGR, RSPC-SGC, RSPE-SGE), $p < 0.05$. **Expression levels of β-endorphin significantly different from round spermatids (SPIR-RSPR, SPIC-RSPC, SPIE-RSPE), $p < 0.05$ and spermatogonia (SPIR-SGR, SIPC-SGC, SPIE-SGE), $p < 0.01$, respectively, in three studied groups. #Highly significant difference *vs.* other cellular categories of the same animal group, $p < 0.001$. Number of animals in each group, $n = 5$. Number of analyzed sections: 1 section per animal, 100 seminiferous tubuli in each animal group; **B.** Expression levels of β-endorphin variation between the same cellular categories of the different groups. Values represent means and vertical bars show standard errors, symbols of animal groups as above. *Significant difference between the same cellular categories of different animal groups: (SGE-SGC, SGE-SGR), (SPIE-SPIC, SPIE-SPIR) and (RSPE-RSPC, RSPE-RSPR), $p < 0.05$. Number of analyzed sections: 1 section per animal, 100 seminiferous tubuli in each animal group.

In *P. obesus*, β-endorphin detected in Leydig cells, spermatogonia, spermatocytes I and round spermatids could act in a paracrine way on spermatogenesis by regulating Sertoli cell function. Using reverse transcriptase polymerase chain reaction analysis, it was clearly demonstrated that rat cultured Sertoli cells expressed two β-endorphin receptors, more β form than μ one [29] allowing this peptide to inhibit the Sertoli cell functions. Specifically, β-endorphin was shown to inhibit the inhibin synthesis in rat [30] and the production of androgen-binding protein [28].

β-endorphin observed in the germ cells could also exert an autocrine regulation since some of the germ cell categories express a specific receptor. In rat, the pachytene spermatocyte I express μ and β receptors [29] and round spermatids express mainly μ form [29]. In *P. obesus*, the temporal coincidence between spermatocytes I abundance, mainly β-endorphin immunoreactive, and scarcity of round spermatids, suggests a possible regulatory effect of β-endorphin on spermatogenesis by inhibiting meiosis and spermiogenesis.

β-endorphin location in Leydig cells in *P. obesus* during sexual rest, argues in favor of its inhibitory autocrine effect on steroidogenesis since in rat β-endorphin inhibited testosterone release in Leydig cells [31].

The results of our recent study on the expression of GnRH I in the testis of *P. obesus* [32], suggest that β-endorphin and GnRH I act in *P. obesus* in an opposite way: GnRH I was present during sexual activity in the tubular as well as the interstitial compartment [32] whereas we observed no β-endorphin immunoreactivity in the period of sexual activity. In mammals, GnRH I seemed to exert a stimulatory action on androgen synthesis [33] inducing 3β-hydroxysteroid dehydrogenase expression [34].

Testicular β-endorphin synthesis was also inhibited by glucocorticoids in rat [35]. The plasma levels of glucocorticoids in *P. obesus* vary during the annual cycle, they decrease in summer [36] when β-endorphin expression appears. Since the classic glucocorticoid receptor was expressed in rat Leydig cells [37] and spermatocytes I at zygotene and early pachytene stages [38] it may be also involved in this inhibitory action in *P. obesus*.

It was suggested that β-endorphin biosynthesis was under androgen control [39]. In *P. obesus*, the plasma concentrations of testosterone and androstenedione decrease in summer [18].

17βE treatment caused a significant decrease in testis weight and a reduction of seminiferous tubules diameter in *P. obesus*. This atrophy may be explained by a relative reduction of the number of spermatocytes I and the low frequency of round spermatids. The decrease of testis weight after estrogen administration was reported in rat [40]. Moreover, chronic injection of aromatase inhibitor in adult rats reduced testicular estradiol level and increased testis weight [41]. Despite the abundance of data about the response of testis to treatment with estrogens, the results remain conflicting. In adult rat, estradiol seems to promote apoptosis of germ cells except spermatogonia A_2, spermatogonia B and metaphasic spermatocyte I and the most vulnerable to apoptosis were pachytene spermatocyte I [11, 42], spermatocyte II and round and elongated spermatids [42] which lead to a drastic decline in the number of spermatids and spermatozoa [10]. Similar observations were made in amphibia, *Rhinella arenarum*, where estradiol enhanced spermatocytes' apoptosis during the reproductive season [43]. The abundance of spermatogonial mitosis after the 17βE treatment in *P. obesus* may reflect its stimulatory effect on proliferation. It was shown *in vitro* that estradiol enhances the multiplication of rat gonocytes [44]. Recently, many studies have reported that estradiol may act as spermatogonial growth factor in rat [45], activate the spermatogonial cell line proliferation in mouse [46] and induce the renewal of spermatogonial stem cells in the Japanese eel *via* the expression of spermatogonial stem cell renewal factor by the Sertoli cells [47, 48]. However, a study conducted in male mice demonstrated that the endogenous estrogens can physiologically inhibit germ cell growth acting *via* ERβ receptor [49].

To our knowledge, no data was available concerning the 17βE effect on testicular endorphinic opioid system. The role of this steroid was studied in endometrial cell line showing that β-endorphin release was inhibited by estradiol [50]. Steroids were shown to influence POMC mRNA expression and β-endorphin synthesis at the hypothalamic level [51], and estradiol treatment of male rats decreased plasma and pituitary β-endorphin content [52]. The result obtained in *P. obesus* demonstrates for the first time that β-endorphin distribution in spermatogonia, spermatocytes I and round spermatids was under 17βE control. Evidence for the role of estrogens in photoperiod mediation on the germ cells' endorphinic system has been emerging in *P. obesus* besides their effects on proliferation and viability functions.

In *P. obesus,* the significant decrease of β-endorphin labeling and expression in seminiferous tubules after 17βE treatment without altering those of Leydig cells indicates that estrogen is not involved in the opioid peptide regulation in these cells. The net biologic effect of estradiol in Leydig cells appears to be the inhibition of steroidogenic function [53, 54] *via* ERα receptors [54]. If 17βE appears to be ineffective on β-endorphin expression in Leydig cells this may be due to the fact that both estradiol and β-endorphin inhibit the testosterone synthesis in adult rats [55, 56].

Besides finding of local effects of 17βE administration on β-endorphin expression in testis we cannot neglect its effects on the hypothalamic-pituitary axis which were reported by many authors. *E.g.* adult male rats that received increasing doses of estradiol showed significant decreases in circulating concentrations of FSH and LH, which lead to subsequent reductions in serum and testicular testosterone levels [57]. Surprisingly, the authors of this study also noted a stimulatory effect of low doses of estradiol on FSH, as was demonstrated by studies in the neonate [58], and in adult hypogonadal mice given physiological doses of estradiol [59]. Thus, estrogens can participate in both negative and positive effects on the pituitary in male rodents. Some of the effects of estrogen on FSH secretion may be mediated by its ability to promote changes of Sertoli cell production of inhibin B [60], which is an important mediator of FSH secretion in males. Interestingly, estrogens were also shown to increase the expression of the βB-subunit of inhibin B in breast cancer cells [61]. While estrogen treatment clearly causes a decrease in gonadotropin levels, an increase of FSH and LH concentrations was seen in rats after aromatase inhibitor administration [42]. An important study in human demonstrated that estrogen acts at the hypothalamus to decrease GnRH pulse frequency and at the pituitary to decrease responsiveness to GnRH [62].

Conclusions

We have demonstrated by the use of Western blot and immunohistochemical technique that the testicular expression of β-endorphin in *P. obesus* exhibits seasonal variations. The opioid peptide appeared exclusively during the sexual rest period in Leydig cells, spermatogonia, spermatocytes I and round spermatids suggesting a local repressor effect on steroidogenesis and spermatogenesis.

We also demonstrate for the first time that 17βE alters the expression of β-endorphin in these cell types. Further studies should clarify the role of endorphin and its interactions with estrogens in the local regulation of spermatogenesis in *P. obesus.*

Disclosure of interest

The authors declare they have no conflicts of interest concerning this article.

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