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Immunohistochemical Localization of Androgen, Meletonin and Glucocorticoid Receptors in the Testis of Indian Pygmy Field Mouse *Mus terricolor*

Sweta Arora[1,2], Poonam Singh[1], Priyoneel Basu[3] and Chandana Haldar[2*]

[1]Mahila Mahavidyalaya, Banaras Hindu University, Varanasi – 221005, Uttar Pradesh, India
[2]Pineal Research Laboratory, Department of Zoology – Centre of Advanced Study, Banaras Hindu University, Varanasi – 221005, Uttar Pradesh, India; chaldar2001@yahoo.com
[3]Behavioural Neuroscience Unit, Department of Psychology, Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada

Abstract

Testosterone, melatonin and glucocorticoids are known to mediate their actions through their respective receptors, i.e., Androgen Receptor (AR), Melatonin Receptor (MT1) and Glucocorticoid Receptor (GR), endowed on the target tissues. The gonads of *M. terricolor* responded positively to administration of these hormones, suggesting the possibility of a direct action of these hormones on the gonads through their respective receptors. The present study was, therefore, undertaken to localize the Androgen (AR), Melatonin (MT1) and Glucocorticoid (GR) receptors in the testis of this rodent adopting immunohistochemistry as the tool. The result indicated that AR is expressed in the Leydig cells and germ cells of the testis of *M. terricolor*. MT1 receptor is localized in the Leydig cells, peritubular myoid cells, and endothelial lining of blood vessels, all in the interstitial zone. The GR was localized in the nuclei of Leydig cells, macrophages and fibroblasts, and endothelial cells lining the blood vessels, all in the interstitium. The immunohistochemical localization of AR, MT1 and GR receptors in the testes of *M. terricolor* support the previous understanding that testis could be a target for the above-mentioned hormones in this rodent.

Keywords: Immunohistochemistry, *Mus terricolor,* Receptors, Seasonal Reproduction

1. Introduction

Mus terricolor is a seasonally breeding wild rodent[1]. Effects gonadal steroids[2,3], melatonin[2,4] and adrenal steroids[2,5] on the reproductive functions of the male *Mus terricolor* have been studied. The gonads of *M. terricolor* showed positive response to these hormones. In fact the actions of these hormones have been suggested to be mediated through their respective receptors present on the cells of the target organs. Therefore, the possibility of direct action of these hormones on the gonads through their respective receptors has been indicated. In this background the present study was undertaken to decipher the possibility of direct action of these hormones (testosterone, melatonin and glucocorticoids) by way of localizing Androgen Receptor (AR), Melatonin Receptor (MT1) and Glucocorticoid Receptor (GR) in the testes of this rodent adopting immunohistochemical approach.

Androgens regulate the functions of the testis as well as the male accessory sex organs via Androgen Receptors (AR). Androgens are well recognized for their role in the regulation of several secretory substances (Sex Steroid Binding Globulin, Sex Hormone Binding Protein, Androgen Binding Protein, etc.) that are associated with maintenance of spermatogenesis. The Androgen Receptor (AR), known as NR3C4 (nuclear receptor subfamily 3,

*Author for correspondence

group C, member 4), is a type of nuclear receptor that is activated by binding of either of the androgenic hormones testosterone or dihydrotestosterone in the cytoplasm and then translocating into the nucleus. AR has been earlier immunolocalized in the testis of domesticated mice and rats[6–8].

Membrane melatonin receptor is a G Protein-Coupled Receptor (GPCR) that binds with melatonin. Three types of melatonin receptors have been cloned. The MT_1 (Mel_{1A} or MTNR1A) and MT_2 (Mel_{1B} or MTNR1B) receptor subtypes are present in humans and other mammals, while an additional melatonin receptor subtype MT_3 (Mel_{1C} or MTNR1C) has been identified in amphibians and birds[9].

MT1 receptor is supposed to be involved in mediating the action of melatonin in the context of seasonal variations. The MT2 receptor does not seem to play a major role in regulating seasonal functions. MT1 receptor has been immunolocalized in the Leydig cells of the testis of rat *Rattus norvegicus* and Indian palm squirrel *Funambulus pennanti*[10–12]. These findings suggest that MT1 mediates the seasonal effect of melatonin on reproduction.

Most actions of glucocorticoids are mediated by the glucocorticoid receptor (GR, also known as nuclear receptor subfamily 3, group C, member 1 or NR3C1), a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors. After ligand binding, GR undergoes a conformational change, allowing the receptor to translocate into the nucleus, to recruit regulatory cofactor complexes, and to control target gene transcription[13,14]. Glucocorticoid Receptor (GR) has been localized in the testis of domesticated mice and rats[15,16].

However, to-date there has been no focused study to find the expression patterns of receptors for Androgen (AR), Melatonin (MT1) and Glucocorticoids (GR) and their relationship with the circulatory hormones in this wild rodent during reproductively active phase.

2. Materials and Methods

2.1 Maintenance of Animals

The revised Animal (Specific Procedure) Act of 2007 of Government of India on animal welfare was strictly followed. The mice were collected from paddy and wheat fields near Varanasi (Lat. 25°, 18' N; Long. 83°, 1'E), India, during the Reproductively Active Phase (RAP) and acclimated to the animal room conditions (27±2°C, with gentle ventilation) for 15 days. The mice were housed in polypropylene cages, and fed on commercially available wheat, rice, and mouse feed pellets and water *ad libitum*.

2.2 Sample Collection

The mice were anesthetized with mild dose of ketamine, the testes were dissected out on ice, cleaned of blood and extra tissue, fixed in neutral buffered formalin, embedded in paraffin, and sectioned at 6 µm thickness. The sections were mounted on gelatin (1%)-coated slides.

2.3 Preparation of Sections for Immunohistochemical Localization of Hormone Receptors

The sections were deparaffinized and rehydrated. The endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol at Room Temperature (RT) for half an hour. The slides were washed thrice with phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, and 2.7 mM KCl; pH 7.4.). The sections were pre-incubated with horse blocking serum (1:100 in PBS; PK-6200, Vector Laboratories, Burlinghame, CA) for 2 h, followed by incubation with primary antibody (AR, N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200) overnight at 4°C. The next day the slides were given three washes with PBS and added biotinylated secondary antibody (Vectastain ABC Universal kit; PK-6200, Vector Laboratories, Burlinghame, CA, dilution 1:50). The process of washing sections with PBS was repeated and a pre-formed ABC reagent was conjugated to the free biotin of the secondary antibody. The antigens were visualized using the 0.03% peroxidase substrate 3, 3-diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, USA) in 0.01 M Tris–Cl (pH 7.6) and 0.1% H_2O_2. After dehydration the sections were mounted with DPX mountant and observed and photographed in Leitz-MPV-3 microscope (Germany). The primary antibody was omitted in negative controls. The sections of negative controls were incubated with goat serum.

The following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200) were used: Androgen Receptor AR, N-20[17]; Melatonin Receptor Mel1aR; R-18 (anti-rat; goat raised)[12]; and Glucocorticoid Receptor GR, M-20, sc 1004[5].

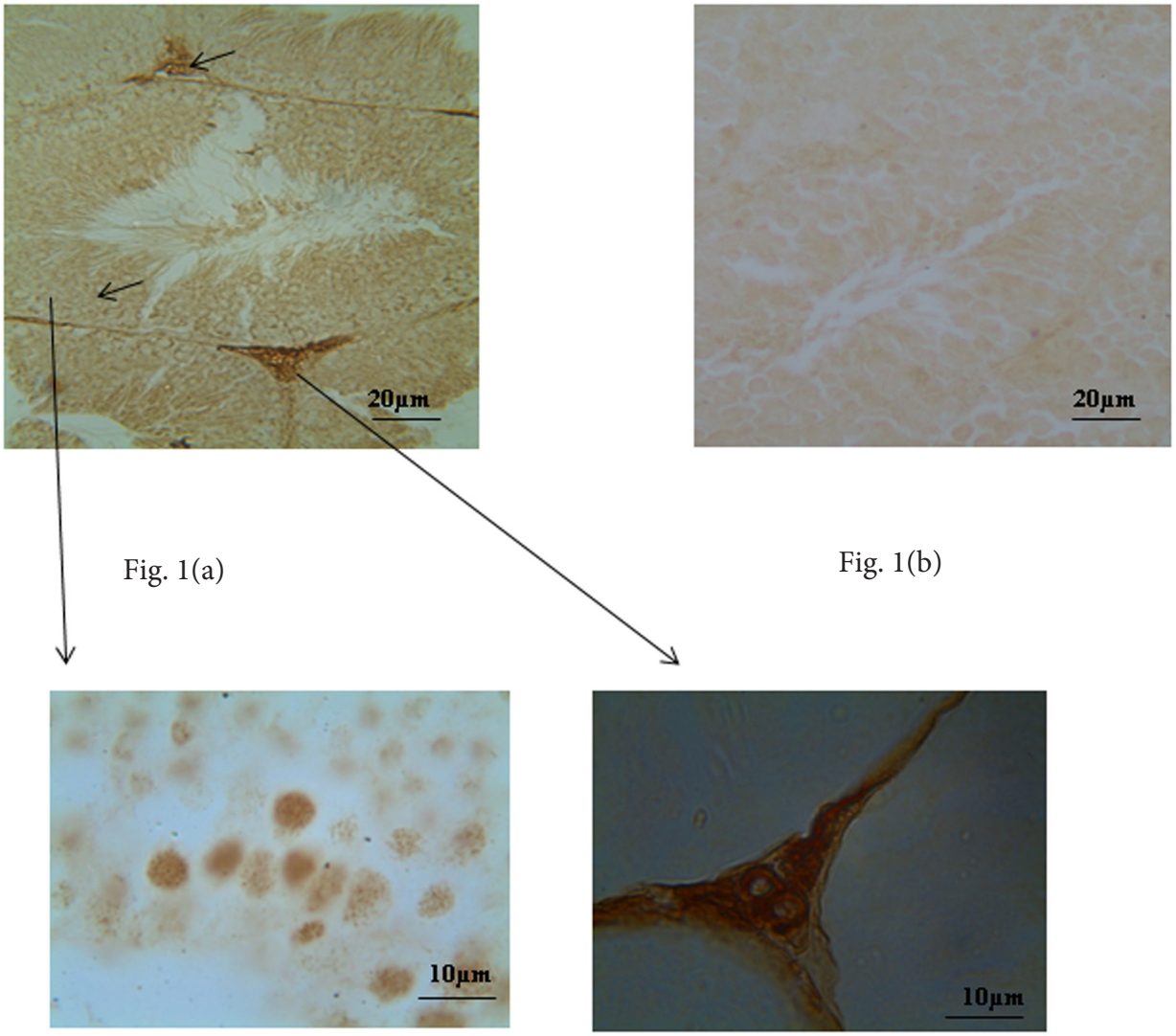


Figure 1(a). Immunolocalization of Androgen Receptor (AR) in the testis of *M. terricolor.* (b) Negative control showing no immunoreactivity for Androgen Receptor (AR) in the testis of *M. terricolor.*

3. Results

3.1 Immunolocalization of Androgen Receptor (AR)

AR was expressed in Leydig cells and germ cells of the testis (Figure 1(a)). However no immunostaining for AR was detected in the Leydig cells of the negative control (Figure 1(b)).

3.2 Immunolocalization of Melatonin Receptor (MT1)

The immunopositivity for MT1 receptor was detected in the Leydig cells, peritubular myoid cells and endothelial lining of blood vessels in the interstitial area of the testis (Figure 2(a)). However no immunostaining for MT1 receptor was detected in these regions of the testis of the negative control (Figure 2(b)).

3.3 Immunolocalization of Glucocorticoid Receptor (GR)

The nuclei of the interstitial cells such as Leydig cells, macrophages and fibroblasts, and endothelial cells of blood vessels of the testis were immunopositive for GR (Figure 3(a)). However no immunostaining was detected in these regions of the testicular sections of the negative control (Figure 3(b)).

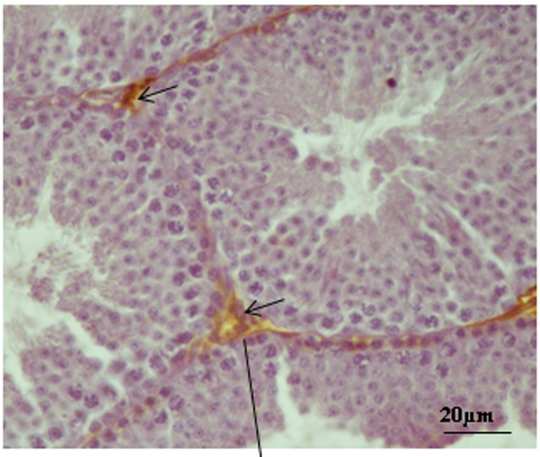
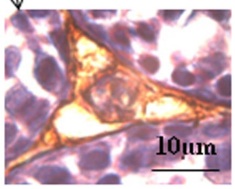


Fig. 2(a)



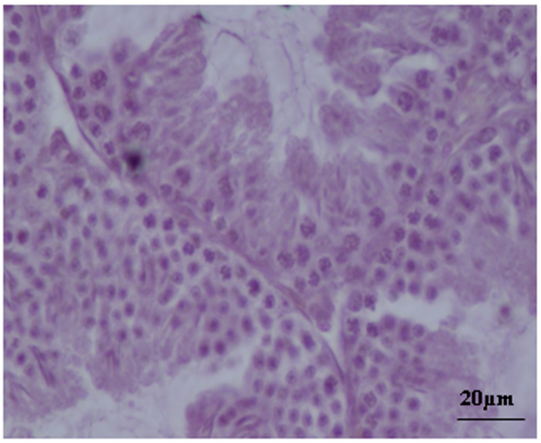


Fig. 2(b)

Figure 2(a). Immunolocalization of Melatonin Receptor (MT1) in the testis of *M .terricolor.* (b) Negative control showing no immunoreactivity for Melatonin Receptor (MT1) in the testis of *M. terricolor.*

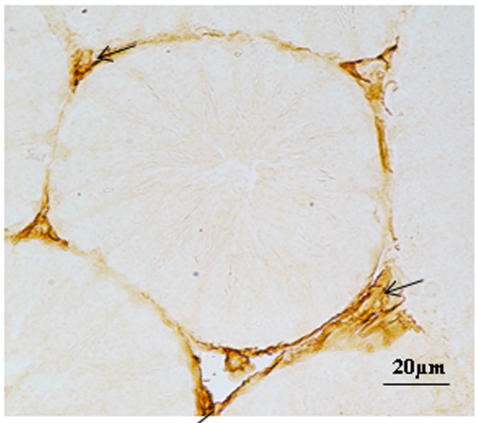
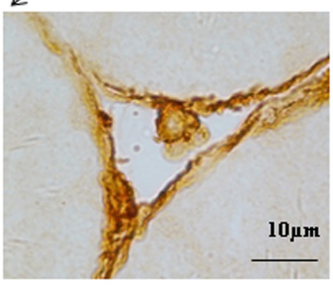


Fig. 3(a)



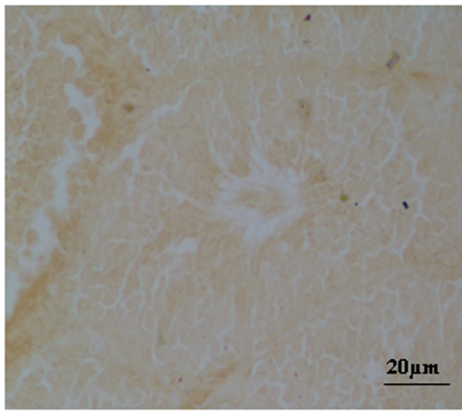


Fig. 3(b)

Figure 3(a). Immunolocalization of Glucocorticoid Receptor (GR) in the testis of *M. terricolor.* (b) Negative control showing no immunoreactivity for Glucocorticoid Receptor (GR) in the testis of *M. terricolor.*

4. Discussion

For the first time, Androgen Receptor (AR), Melatonin Receptor (MT1) and Glucocorticoid Receptor (GR) have been immunolocalized in the testis of seasonally breeding wild rodent *M. terricolor*.

AR was immunolocalized in the testis. It was expressed in the Leydig cells and germ cells. Androgens are considered to be the primary steroid hormones concerned with maintenance of male reproductive functions[18]. Thus, the presence of AR in all males would be expected. Because of the significance of nuclear steroid receptors to our understanding of reproductive biology, it was important to localize the androgen receptor in the gonad of this rodent. In the present study, the pattern of immunohistochemical expression was examined in this mouse showing AR positive Leydig cells and germ cells. Earlier, the presence of AR has been reported in the adult testis in many species[7,8,19–23].

MT1 receptor was immunolocalized and detected in the Leydig cells, peritubular myoid cells, and endothelial lining of blood vessels in the interstitial zone of the testis. Substantial evidence exists to support a direct action of melatonin on the gonads. Melatonin is reported to modulate the morphology, steroidogenesis or cGMP production of testicular tissues in *vitro.* The main target for melatonin action in the male reproductive system is believed to be Leydig cell[24]. The MT1 receptor is reported to be present in Leydig cells, peritubular myoid cells, endothelial lining of blood vessels in the interstitial zone of testis of rat and and squirrel[10-12]. It has been proposed that melatonin receptors are coupled to a pertussis toxin-sensitive G-protein and is responsible for the inhibition of forskolin and LH-induced testosterone secretion by the rat Leydig cells *in vitro*[11].

The immunopositivity for Glucocorticoid Receptor (GR) was detected in the nuclei of interstitial cells such as Leydig cells, macrophages and fibroblasts, and endothelial cells of blood vessels of the testis. The localization of GR in these regions suggests a role for this steroid in regulating the function of the testis. The results of the present study get support from the earlier studies that have suggested the role of glucocorticoids in the stress-induced inhibition of testicular steroidogenesis via glucocorticoid receptor in the interstitium of the testis i.e. in the nuclei of Leydig cells, macrophages, fibroblasts, smooth muscle cells and endothelial cells of blood vessels[15,16].

5. Conclusion

Since AR, MT1R, and GR are localized in the testis, male *M. terricolor* would show responses to the effects of different hormones such as gonadal steroids, melatonin and adrenal steroids. It was thus perceived that these hormones exert a direct action on the testis of this wild rodent through their respective receptors i.e., Androgen Receptor (AR), Melatonin Receptor (MT1) and Glucocorticoid Receptor (GR).

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