Introduction

Melatonin, the principal indoleamine secreted from the pineal gland of vertebrates, synchronizes the seasonal and circadian physiological functions, including reproduction (Reiter, 1975; Arendt, 1998). It has been established that melatonin regulates reproduction in phylogenetically distant seasonal breeders in a pro- or anti-gonadotropic manner (Chemineau et al., 2010; Frungieri et al., 2005) depending on the species. Being exposed to different photoperiodic and climatic conditions throughout the year, seasonal breeders breed at a particular time of the year to avoid infant mortality arising due to adverse climatic conditions. Herein, melatonin acts as a principal transducer of the photoperiodic stimuli and provides the animal with the ‘time-of-year signal’, restricting the delivery of the offspring to the most favourable period of the year (Reiter, 1993), which helps in minimizing the maternal cost to benefit ratio and maximizes the chances of offspring survival (Dardente, 2012). Melatonin modulates seasonality in reproductive status by influencing the hypothalamo-hypophysial-gonadal (HPG) axis (Aleandri et al., 1996). Photoperiodically modulated peripheral melatonin via MT1R influences the gonadotropin releasing hormone (GnRH) secreting neurons in the hypothalamus and pars tuberalis of the pituitary, thereby inhibiting the secretion of the GnRH from the hypothalamus (Roy and Belsham, 2002) and the gonadotropins (LH and FSH) from the gonadotrophs of the anterior pituitary (Filippa et al., 2005). Till date, two high affinity G-protein coupled melatonin membrane receptors (MT1R and MT2R) have been cloned and characterized and are reported to be expressed ubiquitously in mammalian tissues (Reppert et al., 1994; Dubocovich and Markowska, 2005). Despite the presence of both the receptor subtypes in the testis (Izzo et al., 2010), the major points of cross-talk of the melatonergic system with the reproductive system are mainly mediated via the MT1R subtype (Yasuo et al., 2009; Prendergast, 2010). The presence of two nonsense mutations in the coding frame of the MT2R subtype renders it inactive in the transduction of photoperiodic stimuli in hamster (Weaver et al., 1996; Jin et al., 2003) to an extent that transduction of photoperiodic stimuli occurs principally via the MT1R (Yasuo et al., 2009). The sexual cycle of the golden hamster,
Mesocricetus auratus, depends on the environmental photoperiod, wherein, the durations of the dark and the light periods affect its sexual activity (Hoffman and Reiter, 1965). Being a long-day breeder, it remains sexually active throughout the year when exposed to ≥ 12.5 hours of light (critical photoperiod) under laboratory conditions (Steger et al., 1985). The presence of MT1R has been reported in rat Leydig cells (Valenti et al., 1997) and it has been suggested that melatonin may have a local inhibitory action on the testicular steroidogenic pathway (Frungieri et al., 2005). Previous studies established that, exposure to inhibitory short photoperiods (Frungieri et al., 2005) or exogenous administration of melatonin (Reiter et al., 1977) inhibits testicular testosterone production. With the aforementioned reports, it is imperative that photoperiodic stimulus is an important modulator of the testicular steroidogenic pathway. Since, melatonin is the principal transducer of such stimuli it can be presumed that the local melatonergic system may have substantial cross-talk with the testicular steroidogenic pathway, via the MT1R. Based on the above hypothesis, we attempted to explicate the photoperiodic regulation of the expression of MT1R and its interaction with testicular steroidogenic pathways in adult male golden hamsters, M. auratus.

Experimental plan

Golden hamsters were procured from Central Drug Research Institute (CDRI), Lucknow, India and colony was developed and maintained in departmental animal house facility. Hamsters were kept under constant temperature (25±2 °C) and light/dark cycle (Critical photoperiod; 12.5 h light, 11.5 h dark; i.e. lights on at 07:00 a.m. and lights off at 7:30 p.m.). Animals were maintained in polypropylene cages of equal sizes and provided with commercial rodent pellet and tap-water ad libitum. Adult male golden hamsters (average weight 125 g, 90-100 days old) were randomly selected and divided into three experimental groups (n=5/group) and exposed to different photoperiodic regimes for 10 weeks. In Group I hamsters were kept under critical photoperiod (CP; 12.5 L: 11.5 D) whereas in Group II and Group III hamsters were exposed to Short-day (SD; 8L: 16D) and Long-day photoperiod (LD; 16L: 8D) respectively. Hamsters
were exposed to photostimulatory long day condition and photoinhibitory short day condition for 10 weeks to achieve maximum testicular growth and regression respectively. The additional group of hamsters exposed to the critical photoperiod, served as control.

**Sample collection**

At the end of the treatment, hamsters were weighed and sacrificed under deep ether anaesthesia. Right and left testes were immediately removed, blotted dry and weighed. Testes were then either quickly fixed in 10% neutral formalin for histological and immunohistochemical analyses or kept at -80°C for biochemical estimations and western blot analyses. For Arylakylamine N-acetyltransferase (AA-NAT) activity assay testes were dissected out under red light and quickly frozen in liquid nitrogen and kept at -80°C.

**Histological analyses**

The testes tissues were fixed in 10% neutral formalin for 48 h, dehydrated and embedded in paraffin. 5µm sections were stretched on slides pre-coated with 1% gelatine and stained with hematoxylin and eosin (H&E) and finally observed and photographed under research microscope (Nikon, E 200, Tokyo, Japan). Seminiferous tubular diameter and area were measured under research microscope (Nikon, E 200, Tokyo, Japan) 100 round seminiferous tubules from the observed cross-sections.

**Immunohistochemical localization of MT1R and AR in testes**

Immunohistochemical localization was performed following the method described in detail in the materials and methods section. The polyclonal primary antibodies against MT1R (MT1R, R-18, sc-13186, Lot # L1812, Santa Cruz Biotech, USA, dilution 1:100) and AR (AR, N-20, sc-816, Lot # E0412, Santa Cruz Biotech, USA, dilution 1:50) were used for immunolocalization of the same.

**Western blot analyses for AR, StAR, P450\textsubscript{scC}, LH-R and MT1R**

The aliquots containing 100µg of protein was resolved on 12% SDS-polyacrylamide gel (PAGE) for MT1R, P450\textsubscript{scC} and StAR and 10% SDS-PAGE
for AR and LH-R. Immunodetection was carried out by using specific antibodies against AR (AR, N-20, sc-816, Lot # E0412, Santa Cruz Biotech, USA, dilution 1:250); StAR (StAR, Rabbit anti-mouse, dilution 1:250); P450\textsubscript{SCC} (CYP11A1, H-165, sc-292456, Lot # J2511, Santa Cruz Biotech, USA, dilution 1:250) and LH-R (LH-R, Rabbit anti-human, dilution 1:100), MT1R (MT1R, R-18, sc-13186, Lot # L1812, Santa Cruz Biotech, USA, dilution 1:200) and \(\beta\)-actin (A-2228, Sigma-Aldrich Chemicals, St. Louis, USA, diluted 1:5000). Antibodies against StAR and LH-receptor were kind gifts from D.M. Stocco (Texas Tech University, Health Sciences Center, Lubbock, TX, USA) and Craig S. Atwood (William S. Middleton Memorial Veterans Hospital, Madison, WI, USA) respectively. Further, horseradish peroxidase conjugated secondary antibodies (donkey anti-goat IgG, for MT1R; donkey anti-rabbit IgG for AR, StAR, P450\textsubscript{SCC} and LH-R, dilution 1:1000 and donkey anti-mouse IgG for \(\beta\)-actin, dilution 1:10,000) were used. The details of the procedure have been provided in material and methods section.

**Testicular enzyme assay**

Testicular \(\Delta 5-3\beta\)-Hydroxysteroid Dehydrogenase (3\(\beta\)-HSD) and 17\(\beta\)-Hydroxysteroid Dehydrogenase (17\(\beta\)-HSD) activity were measured according to the method of Talalay (1962) and Jarabek et al. (1962). The specific details of the enzyme assay have been provided in material and methods section.

**Hormonal estimations**

The level of testosterone in serum was measured by using a highly sensitive and specific commercial ELISA kit (DiaMetra, Italy-DKO002) as per the manufacturer’s instructions. Plasma and testicular melatonin concentration was determined by using a commercial melatonin ELISA kit (RE54021, IBL, Hamburg, Germany) as per manufacturer’s instruction. Details of the assay procedure have been provided in materials and methods section.

**Testicular AA-NAT activity assay**

Testicular AA-NAT activity was measured following the radioisotopic method of Chae et al., (1999). The details of the procedure have been provided in the material and methods section.
Statistical analyses

Statistical analysis of the data was performed using SPSS 17.0 (SPSS Corp., USA) with one-way ANOVA followed by Tukey’s multiple range tests for multiple comparisons. The differences were considered statistically significant when \( p \leq 0.05 \).

Results

Effect of different photoperiodic regimes on body weight and relative testes weight

Variations were observed in body weight and relative testes weight in the hamsters exposed to different photoperiodic conditions. A significant \( p<0.01 \) increase in body weight (Fig. 1) with a significant \( p<0.01 \) decrease in relative testes weight (Fig. 1) was recorded in hamsters exposed to SD as compared to CP, whereas a significant \( p<0.01 \) decrease in body weight with a concomitant increase in paired testes weight was observed under LD condition when compared with SD exposed hamsters.

Effect of different photoperiod on morphometry and testicular histoarchitecture

Morphometric analyses of seminiferous tubules revealed a significant \( p<0.01 \) decrease in seminiferous tubular diameter (Fig. 2) and seminiferous tubular area (Fig. 2) under SD condition when compared with CP, whereas significant \( p<0.01 \) increase in diameter and area of seminiferous tubules was noted in LD group as compared to CP and SD exposed hamsters. Marked differences in testicular histoarchitecture were noted in hamsters exposed to SD and LD photoperiodic conditions. However, no such alterations were observed when comparison was made between CP and LD group (Data not shown). Under LD condition, normal histoarchitecture of the testis with sequential arrangement of germ cells at different stages of development and lumen filled with sperm was observed (Fig. 3a). Histologically, the testis of SD exposed hamsters showed signs of atrophy with vacuolization and presence of large number of giant cells in the seminiferous tubules with the lumen being completely devoid of sperm (Fig. 3b).
Immunohistochemical localization of MT1R and AR in testis

The product of immune reactions for MT1R was observed in the interstitial compartment, in general and specifically on the Leydig cells and peritubular cells. Weak immunoreactivity for MT1R was observed in testis under LD condition (Fig. 3c) as compared to strong immunoreactivity under SD condition (Fig. 3d). Strong nuclear immunoreactivity for AR was observed in the Leydig cells and Sertoli cells of hamsters exposed to LD (Fig. 3e) as compared to weak nuclear immunoreactivity for AR in the Leydig cells under SD condition (Fig. 3f).

Effect of different photoperiod on testicular steroidogenesis

Photoperiodic modulation of testicular steroidogenesis was assessed through western blot analyses of key regulators i.e. LH-R (Luteinizing hormone receptor), StAR (Steroidogenic acute regulatory protein), P450_{SCC} (P450 Side-chain cleavage) enzyme, AR and estimations of activity for the key enzymes i.e. 3β-HSD and 17β- HSD of steroidogenesis. Western blot analyses revealed a significant \((p<0.01)\) decrease in the expression of AR (Fig. 4a), StAR (Fig. 4a), P450_{SCC} (Fig. 4b) and LH-R (Fig. 4b) in hamsters exposed to SD condition as compared to CP and LD hamsters. However, a significant \((p<0.01)\) increase in the expression of all the key regulators of steroidogenesis was noted in hamsters exposed to LD condition when compared to CP and SD groups. Estimations of enzyme activities for 3β-HSD and 17β- HSD followed the same pattern with a significantly \((p<0.01)\) decreased enzyme activity under SD condition and vice-versa (Fig. 4c). Significant \((p<0.01)\) decrease in plasma level of testosterone was noted in SD exposed hamsters as compared to CP and LD groups (Fig. 4a). Taken together, an inhibitory action of SD exposure and a stimulatory action of LD exposure on testicular steroidogenesis might be suggested.

Effect of different photoperiod on plasma melatonin level and testicular MT1R expression

Marked alterations in plasma levels of melatonin and testicular MT1R expression was observed in hamsters exposed to different photoperiodic conditions. A significant \((p<0.01)\) elevation in the plasma level of melatonin was
observed under SD condition as compared to CP and LD exposed hamsters (Fig. 5). Western blot analysis for MT1R expression in testis showed a significant \((p<0.01)\) increase under SD condition as compared to CP and LD hamsters with a significantly \((p<0.01)\) decreased expression under LD exposure when compared with SD and CP exposed hamsters (Fig. 5).

**Effect of different photoperiod on testicular melatonin synthesis**

Photoperiodic variation in testicular melatonin production was assessed through measurement of enzyme activity for the AA-NAT, the rate-limiting enzyme for melatonin biosynthesis, and testicular melatonin level. A significant \((p<0.01)\) increase in AA-NAT enzyme activity (Fig. 6) and testicular melatonin concentration (Fig. 6) was recorded in hamsters exposed to SD condition as compared to CP and LD groups. LD exposure significantly \((p<0.01)\) abolished the AA-NAT enzyme activity and testicular melatonin concentration when compared with CP and SD hamsters.

**Discussion**

The essential role played by melatonin in regulation of seasonal reproductive rhythms has been known for quite some time (Reiter, 1981, Reiter et al., 2009]. The golden hamster, *M. auratus* is a long-day seasonal breeder with its neuroendocrine axis being highly sensitive to the changing photoperiod (Reiter, 1981). It is an excellent animal model for the study of photoperiodic modulation of reproduction (Bartke, 1985) and immunity (Vishwas and Haldar, 2013). Testis is the prime source of circulatory level of testosterone and the unique site for spermatogenesis in mammals. Hence, detailed understanding of the photoperiodic modulation of testicular steroidogenesis is of great significance. The question of whether photoperiodic stimuli influence testicular steroidogenic pathways *via* modulation of the melatonin-induced MT1R has never been explored.

In the present study, a significant increase in body mass was noted in hamsters exposed to SD condition along with a concomitant decrease in paired testes weight. The increased body weight in the hamsters is due to the increased carcass lipid content, adapted as a means of successful hibernation strategy
(Bartness and Wade, 1985). Further, the decreased testes weight in hamsters exposed to SD photoperiod may be attributed to the inhibitory effect of SD on testicular growth which thereby minimizes reproductive efforts under photoinhibitory SD condition (Reiter, 1975). The decrease in testicular weight and in the diameter and area of seminiferous tubules was accompanied by the presence of giant cells and intra-epithelial vacuolization in the seminiferous tubules with lumen being completely devoid of spermatozoa as evidenced from the histological analyses of testis under SD photoperiod. Our findings receive substantial support from previous studies (Hance et al., 2009) suggesting that the testicular regression is due to androgen deficiency under SD condition. Further, the presence of intraepithelial vacuoles and giant cells in the tubules can be regarded as a hallmark of decreased plasma levels of testosterone as observed during the normal process of ageing (Morales et al., 2004) or during exposure to immunosuppressive drugs (Caneguim et al., 2009). The significant decrease in the level of plasma testosterone under SD condition complies with the observed histological alterations in the seminiferous tubules. Therefore, it can be suggested that a trade-off relationship exists between testosterone and melatonin to provide the best possible way for the seasonal adjustment of reproduction (Ahmad and Haldar, 2009; 2010b).

The present study provides evidence for the photoperiodic variation in MT1R expression in testis, being higher during the SD condition and lower during LD condition in golden hamster, *M. auratus*. Till now, reports suggesting the photoperiodic regulation of MT1R expression in testis are unavailable for any seasonal mammal, in general, and specially for golden hamster. Melatonin is classically known to inhibit testicular androgen biosynthesis by acting at multiple levels of the H-P-G axis through MT1R (Aleandri et al., 1996). In the present study, an elevated level of peripheral melatonin was observed under SD condition, implying that the increased melatonin titre inhibits testicular steroidogenesis by up-regulating MT1R in the Leydig cells.

Further, immunohistochemical (IHC) studies revealed the presence of MT1R in the interstitial Leydig cells with strong immunoreactivity under SD
condition and weak immunoreactivity under LD condition suggesting a direct action of photoperiodically modulated peripheral melatonin on Leydig cell steroidogenesis. To confirm our IHC data, we quantitatively assessed the expression of MT1R through western blot analyses. Our immunoblot analyses revealed highest expression of MT1R under SD condition and lowest under the LD condition. The high circulatory level of melatonin paralleled the increased expression of MT1R under SD condition whereas decreased expression of MT1R was accompanied by low circulatory level of melatonin. Our results are in agreement with earlier reports demonstrating increased expression of MT1R coincident with high circulatory level of melatonin (Ahmad and Haldar, 2010a). Analyses of AR expression showed an opposite pattern as compared to MT1R expression i.e. minimal expression of AR under SD exposure and vice versa. Thus, decreased AR expression in testis parallels the decreased plasma level of testosterone. Our results mimic earlier reports in the bank vole (Tahka et al., 1997) implying that the stabilization and function of AR protein is dependent on prolonged receptor occupancy with androgen (Zhou et al., 1995). Thus, under LD condition the decreased level of melatonin and MT1R expression coincide with the increased level of testosterone and AR expression and vice versa suggesting an antagonistic relationship between melatonin and testosterone and their receptors.

To further elucidate the mechanism of decreased androgen biosynthesis under SD exposure, we delved into the testicular steroid biosynthetic pathway. Although many enzymes are involved, it is believed that the rate-limiting step in the steroidogenesis is the movement of cholesterol across the mitochondrial membrane so that it can be acted upon by the cytochrome P450 side chain cleavage enzyme (P450\textsubscript{SCC}; Payne and Hales, 2004). In mammals, the movement has been shown to be mediated by the steroidogenic acute regulatory protein (StAR; Stocco, 2001). A significant decrease in StAR, P450\textsubscript{SCC} and LH-R expression with a simultaneous decrease in 3β- and 17β-HSD enzyme activity was observed under SD condition as compared to LD and CP exposed hamsters, proposing an inhibitory action of SD condition on testicular steroidogenic machinery. The increased levels of StAR P450\textsubscript{SCC} and LH-R expression and
enzyme activities of 3β- and 17β-HSD under LD condition concord with the elevated plasma level of testosterone and are supported by earlier studies in golden hamster (Frungieri et al., 2005). LH is known to up-regulate the expression of StAR and P450<sub>SCC</sub> in a cAMP mediated pathway and thereby stimulate the synthesis of testosterone (Payne and Hales, 2004; Ascoli et al., 2002). On the other hand, binding of melatonin to MT1R decreases the cAMP level (von Gall et al., 2002) and thus might down-regulate LH-R signaling in Leydig cells and decreases the expression of steroidogenic genes. The decreased expression LH-R is concurrent to the decreased expression of StAR, P450<sub>SCC</sub> and plasma level of testosterone and is in accordance to the previous reports (Frungieri et al., 2005). Further, evidences suggest local synthesis of melatonin in rat testis (Tijmes et al., 1996). To check, whether melatonin is being produced in hamster testis, we measured the enzyme activity for AA-NAT-rate limiting enzyme in melatonin synthesis. Our observations confirmed testicular synthesis of melatonin and also in the variation of testicular melatonin concentration in a photoperiod dependent manner. Therefore, an elevated AA-NAT enzyme activity and testicular melatonin level under SD condition suggested a local inhibitory action of melatonin on testicular steroidogenesis. In conclusion, the present study demonstrates that the changing photoperiod causes alterations not only in the peripheral levels of melatonin and androgen but also in the expression pattern of MT1R and AR in the testis of a long-day breeder, *M. auratus*. Photoperiodic modulation of plasma level of melatonin and expression of MT1R demonstrate a reciprocal relationship with the plasma level of testosterone and expression of AR implying a trade-off relationship between melatonin and testosterone in the seasonal regulation of testicular steroidogenesis. Further, alterations in the AA-NAT activity and local melatonin level in hamster testis suggest a direct effect of photoperiodically modulated peripheral and local melatonin levels on testicular steroidogenesis establishing a local microcircuit of this indoleamine in the regulation of testicular androgen biosynthesis. In the light of the aforementioned, it can be concluded that seasonality in reproductive status in golden hamster might be modulated by photoperiodic moderation of the local melatonergic system in the testis.