Melatonin regulates delayed embryonic development in the short-nosed fruit bat, *Cynopterus sphinx*

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Abstract

The aim of the present study was to evaluate the seasonal variation in serum melatonin levels and their relationship to the changes in the serum progesterone level, ovarian steroidogenesis, and embryonic development during two successive pregnancies of *Cynopterus sphinx*. Circulating melatonin concentrations showed two peaks; one coincided with the period of low progesterone synthesis and delayed embryonic development, whereas the second peak coincided with regressing corpus luteum. This finding suggests that increased serum melatonin level during November–December may be responsible for delayed embryonic development by suppressing progesterone synthesis. The study showed increased melatonin receptors (MTNR1A and MTNR1B) in the corpus luteum and in the utero–embryonic unit during the period of delayed embryonic development. The *in vitro* study showed that a high dose of melatonin suppressed progesterone synthesis, whereas a lower dose of melatonin increased progesterone synthesis by the ovary. The effects of melatonin on ovarian steroidogenesis are mediated through changes in the expression of peripheral-type benzodiazepine receptor, P450 side chain cleavage enzyme, and LH receptor proteins. This study further showed a suppressive impact of melatonin on the progesterone receptor (PGR) in the utero–embryonic unit; this effect might contribute to delayed embryonic development in *C. sphinx*. The results of the present study thus suggest that a high circulating melatonin level has a dual contribution in retarding embryonic development in *C. sphinx* by impairing progesterone synthesis as well as by inhibiting progesterone action by reducing expression of PGR in the utero–embryonic unit.

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Introduction

Postimplantation delay (or retardation) in development is the least common form of embryonic delay and is observed only in the order Chiroptera (Krishna 1999). This was first reported in the phyllostomid bat, *Macrotus californicus* (Bradshaw 1962). Later, Bleier (1975) showed 8 months long gestation period of *M. californicus*, of which 4.5 months was of very slow embryonic growth. Delayed development has also been described in *Artibeus jamaicensis* (Flemming 1971) and was subsequently shown to occur in several other species of bats (Bernerd & Meester 1982, Krishna & Dominic 1982, 1983, Heideman 1989, Rasweiler & Badwaik 1997, Heideman & Powell 1998). Although delayed development has now been described in many bat species, the cause and control of delayed development have not yet been extensively investigated.

The mechanism and cause(s) of delayed embryonic development are under detailed investigations in the short-nosed fruit bat, *Cynopterus sphinx* (Meenakumari & Krishna 2005, Banerjee *et al.* 2007, Meenakumari *et al.* 2009). The bat breeds twice a year in quick

succession at Varanasi, India (Krishna & Dominic 1983). This species exhibits delayed embryonic development during the first (winter) pregnancy (October–March). The developmental delay (or arrest) occurs at the gastrula stage of the embryo in *C. sphinx* during November and December (Meenakumari & Krishna 2005). On the contrary, during the second (summer) pregnancy (April–July), no embryonic delay is noted in April, which corresponds to the gastrula stage of development. The developmental process proceeds relatively faster during the second pregnancy than during the first pregnancy (Meenakumari & Krishna 2005). A recent study on *C. sphinx* showed significantly lower concentrations of circulating progesterone and 17 β -estradiol (E₂) during the period of delayed development (Meenakumari *et al.* 2009). The cause of low progesterone synthesis during the period of delayed development in November–December needs further investigation.

Involvement of melatonin in the embryonic diapause has previously been described in carnivores (Mead 1993). Exogenous melatonin treatment was highly effective in lengthening the duration of the preimplantation

period in mink and spotted skunk (May & Mead 1986). Although the role of melatonin in bat reproduction is well demonstrated (Singh & Krishna 1995, Abhilasha & Krishna 1999), its role in delayed embryonic development has not yet been studied.

The objective of the present study, therefore, was first to determine the annual variation in the circulating melatonin level and correlate it with the serum progesterone level during different months of the two pregnancies; secondly to evaluate the role of melatonin on ovarian steroidogenic activity *in vivo* and *in vitro*; and thirdly to demonstrate whether melatonin directly acts at the utero-embryonic unit thus controlling delayed embryonic development in *C. sphinx*.

Results

In vivo study

Circulating level of melatonin and its correlation with serum progesterone level

The circulating melatonin level of *C. sphinx* varied significantly ($P < 0.01$) during different calendar months (Fig. 1a). The serum melatonin levels increased gradually from October to attain a highest concentration during November–December, coinciding with the period of delayed embryonic development. Melatonin levels then declined significantly ($P < 0.01$) during January and attained the lowest level in February–April. Circulating melatonin level rose again during May to attain a second peak in June, which coincided with regressing corpus luteum in the ovary. Melatonin levels remained high in July.

Changes in circulating progesterone (Fig. 1a) in *C. sphinx* have been described earlier in detail (Meenakumari *et al.* 2009). Serum melatonin level correlated with the circulating progesterone level differently during the two pregnancies. The serum melatonin level showed significant negative correlation ($r = -0.93$) with circulating progesterone level during the first pregnancy from November to March, but positively ($r = 0.95$) from April to July.

Circadian changes in circulating melatonin level

Circadian changes in circulating melatonin level in *C. sphinx* showed nocturnal cycle with a significantly ($P < 0.01$) low point found in the middle of the day and a high point at late night (Fig. 1b).

Changes in the melatonin receptors 1a and 1b in the ovary during early embryonic development during the two pregnancies

Immunoblot analysis of MTNR1A and MTNR1B in ovary

Immunoblot analysis of melatonin receptor 1a (MTNR1A) in the ovary showed a single band between 35 and 40 kDa

Figure 1 (a) Monthly changes in the circulating melatonin and progesterone concentration of *C. sphinx*. Values are given as the mean $\pm$ S.E.M. and showed significant variation ($P < 0.01$) by ANOVA. In case of serum melatonin, values are significantly different by Duncan's test as compared with a, February; b, March; c, April; d, June, and e, December. (b) Circadian changes in circulating melatonin level in *C. sphinx*. Circulating melatonin level showed significant ($P < 0.01$) circadian variation. Each point represents the mean $\pm$ S.E.M. for four animals. *Values are significantly high during dark/night phase as compared to light/day phase.

(Fig. 2a), which precisely corresponds to the predicted molecular mass of the receptor. There was greater expression of MTNR1A protein in the ovary during delayed phase than in the early phase of second pregnancy.

Immunoblot analysis of melatonin receptor 1b (MTNR1B) in the ovary showed a major band approximately between 40 and 45 kDa (Fig. 2c). There exists a significant ($P < 0.01$) difference in the expression of the 40–45 kDa band of MTNR1B in the ovary between delayed and normal gastrula phases of both the pregnancies, being high during delay. Both the receptors of melatonin showed a similar pattern of expression during the delayed embryonic development phase in November–December and in normal gastrula phase in April.

Immunohistochemistry

Immunohistochemical localization was performed in ovary for MTNR1B only. Localization of MTNR1B in the ovary during the early stage of two successive

Figure 2 (a) Immunoblot analysis of melatonin receptor 1a (MTNR1A) protein in the ovary of *C. sphinx* during the early phase of the two pregnancies. Immunoblot analysis of MTNR1A in the ovary showed a major band between 35 and 40 kDa. Values are mean $\pm$ S.E.M. Delay value (*) is significantly different ($P < 0.01$) from normal. (b) Immunoblot analysis of MTNR1A protein in the utero-embryonic unit of *C. sphinx* during the early phase of the two pregnancies. Immunoblot analysis of MTNR1A in the ovary showed a major band between 35 and 40 kDa. Values are mean $\pm$ S.E.M. Delay value (*) is significantly different ($P < 0.01$) from normal. (c) Immunoblot analysis of melatonin receptor 1b (MTNR1B) protein in the ovary of *C. sphinx* during the early phase of the two pregnancies. Immunoblot analysis of MTNR1B in the ovary showed a major band approximately between 40 and 45 kDa. Values are mean $\pm$ S.E.M. Delay value (*) is significantly different ($P < 0.01$) from normal. (d) Immunoblot analysis of MTNR1B protein in the utero-embryonic unit of *C. sphinx* during the early phase of the two pregnancies. Immunoblot analysis of MTNR1B in the utero-embryonic showed a major band approximately between 40 and 45 kDa. Values are mean $\pm$ S.E.M. Delay value (*) is significantly different ($P < 0.01$) from normal.

pregnancies is shown in the Fig. 3. Relatively intense immunostaining was observed mainly in the corpus luteum during the period of delayed embryonic development in November–December. The immunostaining for MTNR1B was very mild in the corpus luteum of second pregnancy in April. The MTNR1B protein in corpus luteum was located in the cytoplasm along with plasma membrane.

Figure 3 Immunolocalization of MTNR1B in the corpus luteum of *C. sphinx* during November–December (a) and April (b). Relatively intense immunostaining was observed mainly in the corpus luteum during the period of delayed embryonic development in November–December. The immunostaining for MTNR1B was very mild in the corpus luteum of second pregnancy in April. The MTNR1B protein in corpus luteum was located in the cytoplasm along with plasma membrane.

Immunoblot analyses of melatonin receptors (MTNR1A and B) and progesterone receptor in the utero-embryonic unit during the early stage of development of both the pregnancies of *C. sphinx*

Immunoblot analyses of MTNR1A showed a single immunoreactive band between 35 and 40 kDa (Fig. 2b). There was greater ($P < 0.01$) expression of MTNR1A protein in the utero-embryonic unit during delayed phase than in the early phase of second pregnancy.

Immunoblot analyses of MTNR1B in the utero-embryonic unit (Fig. 2d) revealed a major immunoreactive band approximately between 40 and 45 kDa respectively. Two other nonspecific immunoreactive bands were also approximately obtained between 29 and 40 kDa respectively. There was significant variation in the intensity of the major band approximately between 40 and 45 kDa in the utero-embryonic unit between

Figure 4 Immunoblot analysis of progesterone receptor (PGR) protein in the utero-embryonic unit of *C. sphinx* during the early phase of the two pregnancies. Immunoblot analysis of progesterone receptor in the utero-embryonic unit showed two immunoreactive bands at ~ 85 kDa (PGRA) and ~ 120 kDa (PGRB). Values are mean $\pm$ S.E.M. Delay value (*) is significantly different ($P < 0.01$) from normal.

Figure 5 Effect of low (100 ng/ml) and high (1 µg/ml) doses of melatonin on progesterone production by the ovaries of *C. sphinx* *in vitro*. Values ($n=3$) are mean $\pm$ S.E.M. *Values are significantly ($P<0.01$) different versus control.

delayed and normal phase, being higher during the delayed period.

Immunoblot analyses of progesterone receptor (PGR) in the utero-embryonic unit of *C. sphinx* showed two immunoreactive bands at ~85 kDa (PGRA) and ~120 kDa (PGRB; Fig. 4). Densitometric analysis of the immunoblot of PGR during the early phase (gastrula stage) of development of the two successive pregnancies showed significantly ($P<0.01$) lower intensity during the period of delayed embryonic development in November–December in the utero-embryonic unit of *C. sphinx*.

In vitro study

Effects of melatonin on ovarian steroidogenesis and expression of peripheral-type benzodiazepine receptor, P450 side chain cleavage enzyme, and LH receptors in vitro study

The effects of low and high doses of melatonin on ovarian progesterone synthesis *in vitro* by *C. sphinx*

Figure 6 Immunoblot analyses of TSPO, CYP11A1, and LHCGR proteins in the ovaries of *C. sphinx* treated *in vitro* with melatonin. The different lanes in the above-mentioned blots represent: (1) control, (2) low dose melatonin (100 ng/ml), and (3) high dose melatonin (1 µg/ml). Values ($n=3$) are mean $\pm$ S.E.M. *Values are significantly ($P<0.01$) different versus control.

Figure 7 Immunoblot analyses of progesterone receptor protein in the utero-embryonic unit of *C. sphinx* treated *in vitro* with high dose (1 µg/ml) melatonin. Melatonin treatment to *C. sphinx* utero-embryonic unit *in vitro* during early pregnancy in November–December caused significant decline in the PGRB subunit of progesterone receptor in the utero-embryonic unit as compared to its expression in the control group. Values ($n=5$) are mean $\pm$ S.E.M. *Values are significantly ($P<0.01$) different versus control.

ovaries are shown in Fig. 5. The low dose of melatonin significantly enhanced progesterone synthesis, but the higher dose of melatonin significantly suppressed the progesterone synthesis *in vitro* by the ovaries.

Similarly, the low dose of melatonin significantly enhanced ($P<0.01$) the ovarian expression of peripheral-type benzodiazepine receptor (TSPO), P450 side chain cleavage enzyme (CYP11A1), and LH receptor (LHCGR) proteins, whereas the high dose significantly suppressed ($P<0.01$) the expression of TSPO, CYP11A1, and LHCGR proteins in the ovaries of *C. sphinx* (Fig. 6).

Effects of melatonin treatment on the expression of PGR in the utero-embryonic unit in vitro study

Melatonin treatment *in vitro* of the utero-embryonic unit of *C. sphinx* during early pregnancy in November caused significant decline in the expression of PGRB subunit of PGR as compared with its expression in the utero-embryonic unit of the control group (Fig. 7).

Discussion

The results of the present study showed significant variation in the circulating melatonin levels during two successive pregnancies in *C. sphinx*. Circulating melatonin concentration showed two peaks during the breeding cycle of *C. sphinx*. The first peak of circulating melatonin concentration during November–December coincided with the period of delayed embryonic development in *C. sphinx*. This period of high melatonin also coincided with the period of low progesterone synthesis by the corpus luteum in *C. sphinx*. The second peak of serum melatonin level in June coincided with the regressing corpus luteum during the second pregnancy (Meenakumari *et al.* 2009). The two periods of nadirs in circulating melatonin concentration closely coincided

with the two periods of ovarian recrudescence and preovulatory follicular development in *C. sphinx*. These observations suggest that in *C. sphinx*, ovarian activity declines during the period of increased melatonin concentration. Thus, the present study suggests antigonadotropic action of melatonin in *C. sphinx* as demonstrated in several mammals (Reiter 1980).

Circadian changes in circulating melatonin level in *C. sphinx* showed nocturnal cycle with the low point found in the middle of the day, and the high point at late night. This is consistent with the findings demonstrated in mammals (McConnell & Hinds 1985, Gündüz 2002). A recent study suggests that the switch that governs the action of melatonin is usually set at dawn and dusk, regardless of the maximum concentration reached by melatonin during the night (Lincoln *et al.* 2003). Circulating melatonin level assay in this study from the serum collected during evening hours (between 1600 and 1900), thus may be physiological relevant. Melatonin concentration during night from *C. sphinx* has recently been demonstrated by Haldar *et al.* (2006). The values of melatonin though reported by Haldar *et al.* (2006) are much higher than the values described in the present study, though both demonstrated similar trend in the seasonal variation in serum melatonin level in *C. sphinx*. Seasonal variation in serum melatonin level noted in the present study is not caused by the shift in the time of dusk, as killing of bat adjusted with the shift in the timing of the dusk.

It has recently been well demonstrated that low circulating progesterone may be responsible for the delayed embryonic development in *C. sphinx* (Meenakumari *et al.* 2009). The majority of the bat species showing delayed embryonic development in which serum progesterone levels have been investigated also showed low levels of serum progesterone during the period of delayed embryonic development as compared to the period of normal embryogenesis (Krishna 1999). *M. californicus* showed low levels of plasma progesterone during the period of delayed development, and its level increased significantly on resumption of normal embryonic development (Crichton *et al.* 1990). The studies on *Miniopterus schreibersii* showed low circulating progesterone level during delayed implantation (van der Merwe & van Aarde 1989, Bernard *et al.* 1991). Progesterone levels are lowered during developmental delay in spotted skunk, *Mephitis mephitis* (Wade-Smith *et al.* 1980). Significant negative correlation between circulating melatonin and progesterone concentrations during the first pregnancy suggests that increased melatonin level during the delayed embryonic development may be responsible for suppressed progesterone synthesis in *C. sphinx*. This finding thus suggests that increased melatonin level during November–December may be responsible for delayed embryonic development by suppressing progesterone synthesis.

Previous studies showing involvement of melatonin in embryonic diapause have been described in carnivores (Mead 1989, 1993) and marsupials (McConnell & Hinds 1985, Oates *et al.* 2004). Melatonin treatment was highly effective in lengthening the duration of the preimplantation period in mink and spotted skunk (Duby *et al.* 1972, May & Mead 1986), while melatonin administration in marsupials terminates diapauses (Hinds & den Ottolander 1983, McConnell & Tyndale-Biscoe 1985). In mink and spotted skunk, melatonin blocks implantation by suppressing prolactin (PRL) release (Berria *et al.* 1989, Kaplan *et al.* 1991). Unfortunately, circulating PRL could not be assayed in *C. sphinx*. Two species of marsupials, the tammar wallaby (*Macropus eugenii*) and Bennett's Wallaby (*Macropus rufogriseus rufogriseus*), exhibit embryonic diapauses after the winter solstice, when the corpus luteum and embryo remain in quiescence in spite of the presence of a pouch young (Curlewis *et al.* 1987). Contrary to the mink and spotted skunk, treatment with exogenous melatonin during seasonal quiescence results in termination of diapauses in both the wallaby (Hinds & den Ottolander 1983, McConnell & Tyndale-Biscoe 1985). Bilateral removal of the superior cervical ganglion in female tammar eliminates seasonal embryonic diapause (Renfree *et al.* 1981).

Melatonin may regulate progesterone synthesis by acting in one or a variety of organs such as the gonad (Abhilasha & Krishna 1999), pituitary (Martin & Saltler 1979), hypothalamus (Glass & Lynch 1981, Malpoux *et al.* 2001), and CN (Glass & Lynch 1982). Most studies investigating the mechanism by which melatonin regulates reproduction have been focused in the hypothalamus and pituitary as target tissues (Malpoux *et al.* 2001) with little attention directed to the role of melatonin in the ovary itself.

In the present study, both immunohistochemistry (IHC) and immunoblot analysis revealed the presence of MTNR1A and MTNR1B mainly in the corpus luteum of the ovary during pregnancy. This is consistent with the earlier reports on human and rat demonstrating the presence of MTNR1A and MTNR1B in granulosa cells and luteal cells (Soares *et al.* 2003). The presence of high levels of melatonin in follicular fluid in human and binding sites in the ovary (Brzezinski *et al.* 1987, Ronnberg *et al.* 1990, Niles *et al.* 1999) and, now, the presence of MTNR1A and MTNR1B in ovary suggest that melatonin directly acts as a modulator of ovarian functions.

Immunoblot analysis of MTNR1B antibody revealed a major band at about 40–45 kDa. This is similar to *Xenopus* tectal cells, which showed the expression of a glycosylated monomer at 45 kDa, although a dimeric form of 85 kDa was also reported in these cells (Prada *et al.* 2005). The cloned MTNR1B (Reppert *et al.* 1995) has a predicted molecular weight of 40 kDa, not including posttranslational modification, which is similar to that reported for the deglycosylated receptor

(38 kDa; Prada *et al.* 2005) suggesting that the bands in the present study, which were found between 40 and 45 kDa, correspond to MTNR1B. The present IHC study further demonstrated that MTNR1B protein in corpus luteum is located in the cytoplasm together with plasma membrane. This is in agreement with earlier study suggesting that the melatonin receptors in membrane and cytosolic ovarian fractions mediate distinct functions (Cohen *et al.* 1978).

Interestingly, two different doses of melatonin caused different effects on ovarian progesterone synthesis *in vitro* of *C. sphinx*. A low dose of melatonin caused stimulatory, whereas a high dose caused inhibitory effects on ovarian progesterone synthesis *in vitro*. This finding thus supports the earlier concept that melatonin is an essential mediator of seasonal reproduction and should not be considered as either an anti- or a pro- gonadotropic factor, although under given circumstances it can have either effect (Reiter 1993). This dose-dependent effect suggests direct (primary) action of melatonin on progesterone synthesis in the ovary of *C. sphinx*. This effect could not simply be 'zeitgeber' changes. This confirmed our *in vivo* study showing that decreased progesterone concentration coincided with increased melatonin level during the period of delayed embryonic development in November–December, whereas increased progesterone concentration coincided with decrease melatonin level during the normal embryonic development. Similar differential response of ovary to melatonin was demonstrated earlier in *Scotophilus heathi* (Abhilasha & Krishna 1999). Earlier studies showed both stimulatory and inhibitory effects of melatonin on ovarian steroidogenesis *in vitro* during different phases of the reproductive cycle (Abhilasha & Krishna 1999). Melatonin inhibited LH-induced testosterone and progesterone synthesis by the ovary of *S. heathi* during winter dormancy and preovulatory phase. Whether increased melatonin level suppresses progesterone synthesis by inhibiting LH is not known. Although exogenous LH treatment in *C. sphinx* during the period of delayed development failed to increase the progesterone circulation (Meenakumari 2004). Batmanabane & Ramesh (1996) showed that melatonin along with hCG significantly increased the E₂ synthesis during recrudescence, an effect that may suggest its role in initiating the breeding season, as shown in sheep (Karsch *et al.* 1984). The present study thus suggests that variation in circulating melatonin is a key factor responsible for the changes in the circulating progesterone level in *C. sphinx*, which in turn affects the rate of embryonic development. The effects of melatonin in the corpus luteum *in vitro* are intriguing. It would be interesting to find out whether treatment with melatonin inhibitor would increase progesterone synthesis during delayed embryonic development.

Little is known about the molecular events that mediate the effect of melatonin on ovarian

steroidogenesis. Our previous investigation showed a direct effect of melatonin on steroidogenic enzymes such as 17 β -hydroxysteroid dehydrogenase (Singh & Krishna 1995). The effects of melatonin on steroidogenic enzymes have been shown in rat (Ng & Lo 1988, Mandal *et al.* 1990), and it appears that melatonin may have different effects on different steroidogenic enzymes. Our present study on *C. sphinx* suggests that the effect of melatonin on ovarian steroidogenesis is mediated by changes in the expression of TSPO, CYP11A1, and LHCGR proteins. The study showed marked variation in the expression of TSPO, CYP11A1, and LHCGR proteins in the ovary in response to different doses of melatonin treatment. Expression of TSPO, CYP11A1, and LHCGR decreases in response to treatment with higher dose of melatonin, whereas their expression decreased in response to lower dose. These findings thus suggest that high circulating concentration of melatonin during November–December may be responsible for suppressing progesterone level by decreasing expression of TSPO, CYP11A1, and LHCGR in the ovary of *C. sphinx*. This in turn may be responsible for the delayed embryonic development. It is possible that melatonin may be involved in maintaining a critical level of steroidogenic enzymes and LHCGR expression for ovarian function.

Since in an earlier study, treatment with progesterone failed to increase the rate of embryonic development in *C. sphinx* during the delayed period (Meenakumari & Krishna 2005), an attempt was also made to find out whether melatonin suppressed progesterone action by acting directly at the utero–embryonic unit through its receptor. This study showed the presence of melatonin receptor in the utero–embryonic unit, and its level was significantly higher during the delayed phase than in April. Expression of MTNR1B receptor in the utero–embryonic unit of bat is consistent with the earlier study showing *MTNR1A* and *MTNR1B* mRNA in human placenta during first trimester (Iwasaki *et al.* 2005) and human choriocarcinoma (JEG-2) cell lines (Lanoix *et al.* 2006). Our study further showed significant decline in PGR of utero–embryonic unit treated with melatonin *in vitro*.

In brief, our results suggest an association between increased serum melatonin level and decrease in serum progesterone level and delayed embryonic development during November–December in *C. sphinx*. This study also showed the increased concentration of melatonin receptors (MTNR1A and B) in the corpus luteum, which coincided with increased circulating melatonin level during November–December. This increased effects of melatonin on corpus luteum caused suppressed progesterone synthesis. This suppressive effect of melatonin on progesterone synthesis was found to be mediated through decreased expression of TSPO, CYP11A1, and LHCGR proteins in the ovary. This study further demonstrated the increased concentration of both the

melatonin receptors (MTNR1A and MTNR1B) in the utero-embryonic unit during November–December, and melatonin treatment or increased melatonin level during this period caused decreased expression of PGR protein. These findings thus suggest that increased melatonin level during November–December suppressed progesterone synthesis in the corpus luteum as well as suppressing the effect of progesterone on utero-embryonic unit thereby causing delayed embryonic development in *C. sphinx*.

Materials and Methods

All experiments were conducted in accordance with the principles and procedures approved by Banaras Hindu University, Departmental Research Committee. The female bats (*C. sphinx*) utilized in this study were captured alive from Ramnagar, Varanasi (25°N, 83°E), India, from October to July, 2005–2008. Bats were then transported to the laboratory immediately. Body weight of bats was recorded as soon as they were brought to the laboratory (within 2 h of capture). Females weighing 43 g or more and having wingspan exceeding 46 cm were sexually mature (Krishna & Dominic 1983). Pregnant female bats ($n=30$) were euthanized between 1600 and 1900 h with an overdose of ether in a glass jar. To check daily changes in peripheral melatonin level, killing was done in the month of December at an interval of 4 h in a 24-h cycle. Night killing was done in dim red light. Blood serum collected was saved and stored at -20°C until assayed for melatonin (seasonal and diurnal) and progesterone. Utero-embryonic unit of pregnant females in November–December (delayed gastrula phase) and in April (normal gastrula phase) was dissected out and kept at -20°C for immunoblot analysis. One side of the ovary of each bat was dissected out, cleaned, and fixed in Bouin's fixative for 24 h at room temperature, dehydrated in ethanol, cleared in xylene, embedded in paraffin, and sectioned at 6 μm . The ovary of the other side of the female genital tract was stored at -20°C for immunoblot analysis.

In vitro study

Effect of melatonin on ovarian steroidogenesis

In order to study the direct effect of melatonin on ovarian steroidogenesis, the ovaries ($n=9$) of *C. sphinx* were collected during delayed embryonic development in November–December. Effect of two doses of melatonin (100 ng/ml, low doses and 1 $\mu\text{g}/\text{ml}$, high doses) treatment on *in vitro* ovarian steroidogenesis (progesterone secretion) and on ovarian expression of TSPO, CYP11A1, and LHCGR proteins was studied. Changes in progesterone synthesis and ovarian expression of TSPO, CYP11A1, and LHCGR proteins are taken as parameters to study the changes in ovarian steroidogenesis. Female *C. sphinx* were killed by decapitation as soon as they were brought to the laboratory. Their ovaries were quickly taken out and cleaned of any adhered fat tissue and oviduct in DMEM (Himedia, Mumbai, India) containing 250 U/ml penicillin and 250 $\mu\text{g}/\text{ml}$ streptomycin sulfate. Ovaries were cultured by the method as

described previously (Srivastava & Krishna 2007) with some modifications. Culture medium was a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12 (1:1; v/v) (Himedia) containing 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.1% BSA (Sigma). After initial incubation for 2 h at 37°C , culture medium was discarded, and ovaries (one per tube) were finally cultured in 1 ml medium in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 48 h at 37°C with two doses of melatonin: high dose (1 $\mu\text{g}/\text{ml}$) or low dose (100 ng/ml). Each treatment group was run in triplicate. Ovaries cultured under these conditions appear healthy and do not show any sign of necrosis. Ovaries were collected at the end of culture, washed several times with PBS, and kept frozen at -20°C for immunoblot study. Media was saved at -20°C until assayed for progesterone.

Effect of melatonin on utero-embryonic unit

The utero-embryonic unit, the swollen part of the uterus during pregnancy, was dissected out from the bats during delayed embryonic development in November–December. The utero-embryonic unit containing the embryo in gastrula stage ($n=10$) was immediately kept in medium DMEM (Himedia) containing 250 U/ml penicillin and 250 $\mu\text{g}/\text{ml}$ streptomycin sulfate. Both the sides of the utero-embryonic unit were open, so that the culture media could freely pass across the entire unit during the *in vitro* culture. Then the entire unit was transferred to plastic culture dishes containing 1 ml medium, which was a mixture of DMEM (with sodium pyruvate and L-glutamine) and Ham's F-12 (1:1; v/v) (Himedia) containing 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.1% BSA (Sigma). The utero-embryonic unit was divided between control and melatonin- (1 $\mu\text{g}/\text{ml}$) treated groups. The plastic culture dishes having the utero-embryonic unit in medium were maintained in a humidified atmosphere with 95% air and 5% CO_2 to maintain pH 7.4 for 48 h at 37°C . Control and treatment groups were run in triplicate. Utero-embryonic unit cultured under these conditions appears healthy and does not show any sign of necrosis. After 6 h of culture, the tissue was viable and there was no sign of necrosis cross checked with histology and trypan blue staining of the sections. Tissues were collected after 6 h, washed several times with PBS, and kept frozen at -20°C for immunoblot study.

Validation of melatonin receptor (MTNR1A and MTNR1B) antibody

MTNR1A antibody was purchased from Santa Cruz, Biotech (MTNR1A, R-18, Santa Cruz, CA, USA). A polyclonal antibody for MTNR1B directed against a 13 amino acid peptide (VKSEFKPRMQSDF), corresponding to a region of an intracellular loop of the *Xenopus laevis* MTNR1B receptor (Reppert *et al.* 1995), was generated in rabbits (Invitrogen).

Antibodies against melatonin receptor (MTNR1A and MTNR1B) were validated for the use in the bat ovary and uterus by immunoblotting. Immunoblot was performed by comparing MTNR1A and B immunoreactivity in the bat ovary and uterus with the immunostaining of these antibodies in the rat brain as positive control. Immunoblot analysis for MTNR1A in bat ovary and uterus showed a single band between 35 and 40 kDa, corresponding to a similar band obtained in rat brain.

Immunoblot analysis for MTNR1B protein showed a single major band approximately between 40 and 45 kDa in the bat ovary and uterus, the position of which corresponded to the band obtained in rat brain used as positive control. However, two very weak nonspecific bands were also approximately found between 29 and 40 kDa in the uterus (data not shown).

Immunohistochemistry

Ovarian sections were processed through a standard protocol of IHC. After deparaffinization and rehydration, endogenous peroxidase was quenched with 0.3% H₂O₂, equilibrated in 0.05 mol/l Tris-Cl and 0.15 mol/l NaCl (Tris-buffered saline (TBS), pH 7.3). Background blocking was performed with normal horse serum. The tissue sections were incubated for 1 h at room temperature with rabbit polyclonal antibody against amphibian MTNR1B in TBS. The detection system used was ABC staining kit from Vector Laboratories, Novo Castra (UK). The peroxidase activity was revealed in 0.03% 3,3'-diaminobenzidine tetra-dihydrochloride (Sigma) in 0.01 M Tris-Cl (pH 7.6) and 0.1% H₂O₂. Nucleus was counterstained with Elrich's hematoxylin. The negative controls were obtained by omitting the primary antibody and incubating the ovarian section with rabbit serum.

Immunoblot

The utero-embryonic unit and ovaries (three pooled) were homogenized to produce 10% homogenate. Furthermore, protein extractions and immunoblotting were performed as described previously (Srivastava & Krishna 2007). Equal amount of proteins (50 µg) as determined by Folin's method was loaded on to SDS-PAGE (10%) for electrophoresis. Thereafter, proteins were transferred electrophoretically to nitrocellulose membranes (Sigma-Aldrich) overnight at 4 °C. Nitrocellulose membranes were blocked for 60 min with TBS (Tris 50 mM (pH 7.5) and NaCl 150 mM) containing 5% fat-free dry milk and incubated with melatonin receptor antibodies (MTNR1A at a dilution of 1:200; and MTNR1B at a dilution of 1:250), rabbit anti-human TSPO antibody (1:500), rabbit anti-human CYP11A1 antibody (1:1000), and rabbit anti-human LHCGR antibody (1:1000) for 1 h at room temperature. Antibodies against LHCGR and P450 CYP11A1 enzyme were generously supplied by Craig S Atwood (William S Middleton Memorial Veterans Hospital, Madison, WI, USA) and Michael J Soares (Ralph L Smith Mental Retardation Research Center, University of Kansas, Kansas, USA) respectively. Membranes were then washed with three changes of TBS over 10 min. Immunodetection was performed with anti-rabbit IgG-HRP conjugate (1:2000). Finally, blot was washed thrice with TBS and developed with ECL detection system (Bio-Rad). Similarly, a blot was developed for β-actin (Santa Cruz) at dilution 1:1000 as loading control. The densitometric analysis of the blots was performed by scanning and quantifying the bands for density value by using computer-assisted image analysis (Image J 1, 38×, NIH, Bethesda, MD, USA). Experiments were repeated thrice with the same result. The densitometric data were presented as the mean of the integrated density value ± S.E.M.

RIA

Melatonin RIA

Circulating melatonin level was measured using RIA kit (Cat. # RA608/100) purchased from DLD Diagnostika, Hamburg, Germany. The RIA kit was validated for melatonin measurement in *C. sphinx*. All the reagents were brought to room temperature before assay. Standards and bat serum samples (100 µl) were added to the respective tubes. Then 25 µl enzyme solution was added to all the tubes except the total count, mixed well, and centrifuged for 1 min at 500 g. The tubes were then incubated for 1 h at room temperature (~20 °C). Following incubation, 50 µl assay buffer, 50 µl ¹²⁵I tracer, and 50 µl antiserum (rabbit anti-melatonin) were added to all the tubes (no antiserum in total and non specific binding (NSB)) in the sequence as described above with gentle mixing after each addition. All the tubes were centrifuged again at 500 g for 1 min and incubated at room temperature for 20 h. After incubation, 500 µl cold precipitating antiserum (goat-anti-rabbit IgG in polyethylene glycol buffer) was added to all the tubes except total, and mixed thoroughly and incubated for 15 min at 4 °C. Again all the tubes were centrifuged at 4 °C for 15 min at 3000 g. Following centrifugation, supernatant was aspirated from each assay tube and the tubes were counted in a Beckman gamma counter. The coefficient of intra-assay variation (CV) was <5%. The inter-assay CV of the melatonin kit was <9.0%. The kit was validated for use in *C. sphinx*. Blood serum sample from bats (n=6) was pooled and serially diluted with assay buffer and treated against the standard curve. Dilution of bat serum ran parallel to the standard curve indicating the suitability and sensitivity of the assay in *C. sphinx* (data not shown).

Progesterone assay

Serum progesterone was assayed by RIA kit from ICN Biomedicals Inc., Costa Mesa, CA, USA. First, 25 µl of each standard and sample were added to the anti-progesterone coated tubes. Progesterone ¹²⁵I (1 ml) was then added to each tube. The tubes were incubated at 37 °C for 60 min. After incubation, the tubes were decanted and empty tubes were checked in a gamma counter (Beckman, Geneva, Switzerland). The concentration of progesterone in the samples was deduced by extrapolation from the standard curve. All the tubes for progesterone were assayed together and intra-assay variation was <10%. Steroid assays for bat samples have been previously validated (Abhilasha & Krishna 1996). Progesterone for the *in vitro* study was measured by using 100 µl of the culture media and the same RIA kit.

Statistical analysis

Data are expressed as mean ± S.E.M. The significance of the differences in melatonin and progesterone concentrations between groups was determined by one-way ANOVA followed by Duncan's multiple range test or a *t*-test. The data were considered significant if *P* < 0.01.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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