Temporal phase relation of circadian neural oscillations as the basis of testicular maturation in mice: A test of a coincidence model

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To study the underlying mechanism of gonadal growth during the attainment of puberty and to test a coincidence model, 7 experimental groups of 2-week-old male mice, *Mus musculus*, were administered the serotonin precursor, 5-hydroxytryptophan, followed by the dopamine precursor, L-dihydroxyphenylalanine at hourly intervals of 6, 7, 8, 9, 10, 11 and 12 h (5 mg/100 g body weight per day for 13 days). At 11 days post-treatment, a suppression of gonadal activity was seen in the 7-h mice and a maximum suppression in the 8-h mice, along with a significantly increased degree of gonadal development in the 12-h mice, as compared with the controls. In addition to its known regulation of seasonal gonadal cycles, the relative position of two circadian neural oscillations may also affect the rate of gonadal development during the attainment of puberty in mice. Moreover, the present study provides an experimental paradigm to test the coincidence model of circadian oscillations.

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1. Introduction

Circadian rhythms play a fundamental role in the effective functioning of complex organisms by allowing them to anticipate changing environments in ways that enhance their survival (Pittendrigh 1993). Timing and rhythms are important during reproduction and development (Johnson and Day 2000; Day *et al*. 2001; Johnson 2001). For example, circadian rhythms and the suprachiasmatic nucleus (SCN) are intimately involved in the timing of the luteinizing hormone (LH) surge during the estrous cycle (Alleva *et al*. 1971; Turek *et al*. 1984; Gerhold *et al*. 2005), the initiation of fecundity at puberty (Kriegsfeld *et al*. 2002), and they influence, via the pineal, seasonal changes in fertility (Nuesslein-Hildesheim *et al*. 2000; Lincoln *et al*. 2003). In vertebrates, the existence of circadian variations in endocrine secretions, hypothalamic factors and neurotransmitters is well documented (Pittendrigh 1981a). As these regulatory agents have different activities as a function of the time of the day and the season of the year, it is not surprising that the temporal phase relation of hormonal as well as neural rhythms change seasonally.

Several studies have reported that the temporal phase relation of serotonergic and dopaminergic circadian oscillations affects gonadal development in many seasonally breeding birds and mammals (Miller and Meier 1983; Wilson and Meier 1989; Chaturvedi and Bhatt 1990; Chaturvedi and Jaiwal 1990). In general, the administration of serotonin followed by dopamine precursors (5-hydroxytryptophan [5-HTP] and L-dihydroxyphenylalanine [L-DOPA]) at an interval of 12 h (a 12-h temporal relation) induces not only the reproductive development/condition in these species but also the advancement of puberty in Japanese quail (Phillips and Chaturvedi 1995). On the other hand, the 8-h relation inhibits gonadal growth and sometimes inducing gonadal suppression and non-breeding conditions. Recently, for mice we reported that administering an 8-h temporal relation of 5-HTP and L-DOPA suppresses gonadal growth and a 12-h

Keywords. Coincidence model; 5-HTP; L-DOPA; mice; neural oscillations; temporal synergism; testis

Abbreviations used: FSH, follicle-stimulating hormone; 5-HTP, 5-hydroxytryptophan; L-DOPA, L-dihydroxyphenylalanine; LH, luteinizing hormone; P, Parkes; RIA, radioimmunoassay; SCN, suprachiasmatic nucleus

relation accelerates gonadal growth, whereas other relations (0-, 4-, 16- and 20-h) were ineffective (Sethi and Chaturvedi 2009). Further, the inhibitory effects of an 8-h phase relation and the stimulatory effects of a 12-h phase relation of neural oscillations on the gonadal growth of mice are inversely correlated with levels of RFRP-3, a mammalian ortholog of avian gonadotropin-inhibitory hormone (Sethi *et al*. 2010). On the basis of numerous other reports, it is evidently not the amount of the serotonergic or dopaminergic drug that is injected which is most important in triggering either gonadal stimulation or inhibition, but, rather, it is the temporal phase relation between the two neural oscillations, as entrained by their precursor drugs, 5-HTP and L-DOPA, injected at specific intervals. Although qualitatively similar at all times, the neurotransmitter precursor drugs appear to have different quantitative effects on gonadal growth depending on the phase relation between their injections within 24 h.

Various models have been proposed to describe the system of circadian organization in the post-transitional modification (PTM) of seasonally breeding animals, including external and internal coincidence models. In the external coincidence model, the coincidence of light with the photoinducible phase (*ø*i) of the photosensitive rhythm leads to a photoperiodic response under long days, whereas the non-stimulation under short days is due to non-coincidence of light with *ø*i (Bünning 1936). In the internal coincidence model, photoperiodic stimulation under long days occurs due to a change in the phase relationship (and thereby coincidence) between the two circadian neural oscillators and non-stimulation is due to a different relationship between two or more circadian oscillators (Pittendrigh and Minis 1964). This model assumes that the photoperiodic clock depends on the internal coincidence of two (or more) circadian rhythms whose relative phase relationships alter with the annual changes in day-length. The attractiveness of the Pittendrigh model is that it is based on entrainment theory (Pittendrigh 1981b) and does not require a special photoinducible oscillator.

The strongest physiological evidence for an internal coincidence device comes from Meier and his colleagues (Follet 1973) and later from numerous studies on many seasonally breeding avian and mammalian species, including the Japanese quail, as investigated by Chaturvedi and her colleagues. These workers find that seasonally breeding species possess daily rhythms in the secretion of hormones (corticosterone and prolactin) and neurotransmitters (serotonin and dopamine) and that when these factors exist in a particular phase relationship to each other, it initiates or terminates seasonal breeding and related events. Moreover, a different phase relationship has been reported in the circadian hypothalamic serotonin and dopamine rhythms of breeding versus non-breeding quail under both control and simulated conditions (Tiwari *et al*. 2006; Kumar *et al*. 2009) and in spawning and non-spawning fish (Senthilkumaran and Joy 1994).

Despite numerous studies, it is unclear at what the phase angle is when gonadal growth starts changing from having no effect to having an effect (e.g. 0- and 4-h with no effect versus the 8-h inhibitory effect) or from an inhibitory to a stimulatory effect (e.g. the 8-h inhibitory effect versus the 12-h stimulatory effect). Hence, the present study was undertaken to pinpoint the specific phase relation between the two injections which determines this change in gonadal response. To address the putative regulatory role of circadian oscillations in the reproductive development of laboratory mice and to pinpoint the exact temporal phase relation of these neural oscillations, the two drugs were injected at hourly intervals of 6, 7, 8, 9, 10, 11 and 12 h. The aim was to find out the specific phase relation that triggers gonado-inhibitory or gonado-stimulatory responses, leading to maximum activity or inactivity of the gonadal axis.

2. Materials and methods

2.1 *Animals*

Male laboratory mice (*Mus musculus*) of the Parkes (P) strain were obtained from our colony. The mice were housed under hygienic conditions in a well-ventilated, photoperiodically controlled (L:D = 12:12) room and were provided with commercial food (Pashu Aahar Kendra, Varanasi, India) and tap water *ad libitum*. All the experiments were conducted in accordance with institutional practices and within the framework of the revised Animals (Scientific Procedures) Act of 2002 of the Government of India.

2.2 *Experimental design*

The 2-week-old prepubertal mice (6–7 g), acclimatized to a continuous dim light (LL dim) for 2 days, were weighed and randomly divided into 8 groups (5 mice per group). The two precursor drugs, 5-HTP and L-DOPA (Sigma-Aldrich, St. Louis, MO, USA), were prepared in normal saline (0.9% NaCl) and injected intraperitoneally (5 mg/100 g body weight/day) in 0.1 ml injections over a period of 13 days. Mice in the 7 experimental groups were injected with 5-HTP (serotonin precursor) at 0800, followed by injections of L-DOPA (dopamine precursor) at different times in the different groups, i.e. at 1400, 1500, 1600, 1700, 1800, 1900 and 2000, so as to establish between the two injections 6-, 7-, 8-, 9-, 10-, 11- and 12-h phase relations. Mice in the control group received two daily injections of normal saline (0800 and 1600).

The doses used of 5-HTP and L-DOPA are reported to increase brain serotonin and dopamine, respectively, in rats (Ternaux *et al*. 1976; Jimenez *et al*. 1978). During the treatment period, the mice were maintained under continuous light (LL dim) to avoid any possible

photoperiodic interference from the light–dark cycle during the entrainment of the neural oscillations by the drug injections. After an injection period of 13 days, all the groups were returned to a 12L:12D photoperiod (lights on at 0800 and off at 2000 by an automatic timer) to allow the mice to mature under these conditions. The mice were weighed weekly. Eleven days after the last injection, when the mice were 38 days old, they were anesthesized with ether and then sacrificed. Blood was collected from the heart into a heparinized tube and centrifuged at 4000 rpm for 20 min at 4°C to separate the plasma. The length and width of the left testis was measured *in situ* with dial calipers. Both testes were excised and weighed. The testicular volume was calculated using Bissonett's formula $4/3\pi ab^2$ (a = ½ the long axis; b = ½ the short axis) (Jaiwal and Chaturvedi 1991; Chaturvedi *et al*. 1993).

2.3 *Histological preparations*

The right testis and its cauda epididymis were fixed in Bouin's fluid. Twenty-four hours after fixation, the tissues were dehydrated in an ascending series of alcohol, treated with xylene and then embedded in paraffin wax. The 6-μm thick sections were cut by a Weswox rotary microtome (Western Electric and Scientific Works, Ambala Cantt, India), and stained with hematoxylin-eosin. Histological sections of the testis were viewed under a microscope (Axioskop 2 Plus; Carl Zeiss AG, Oberkochen, Germany) and images were captured with a digital camera. The diameter of the seminiferous tubules was determined in 10 sections from each mouse testis by using the image analyser software Motic Images 2000, version 1.3.

To determine the percentage of affected seminiferous tubules, all the tubules in a randomly selected section of the testis from five mice of each group were counted (Sethi and Chaturvedi 2009). The seminiferous tubules were considered affected if they showed any of the following characteristics: intraepithelial vacuolation, exfoliation of germ cells, degenerated appearance of germ cells, loosening of germinal epithelium, presence of spermatids of different stages of the spermatogenic cycle in the same tubule, marginal condensation of chromatin in round spermatids or tubules lined with only Sertoli cells or lined with Sertoli cells and rare germ cells.

There are 12 designated cell associations or stages of spermatogenesis in mice. Particular cell associations or stages have a constant germ cell composition. To quantify any changes in spermatogenesis induced by the treatment, germ cell associations or stages were divided into three main stages: early stage (I–VI), middle stage (VII–VIII) and late stage (IX–XII). In the early stage, two generations of spermatids – round and elongating. The elongating spermatids are within deep crypts of the Sertoli cells. In the middle stage (just before sperm release), in addition of round spermatids, the elongated are also present but these spermatids come to the line the surface of the epithelium. At the late stage, there is only one generation of spermatids and these are elongating. Observations on stage VII–VIII were emphasized because its duration is the longest, the percentage of cells in this stage in the seminiferous tubules is the highest, and this stage features spermatogonia A, preleptotene spermatocytes, pachytene spermatocytes and spermatids. One hundred seminiferous tubules in a randomly selected section of the testis from each mouse were categorized with respect to the stages of spermatogenesis as per the criteria of Russell *et al*. (1990). An accurate identification of stages was not always possible because of occasional severe damage. The tubules were grouped as follows: I–VI, VII–VIII, IX–XII or unidentified. Finally, the frequency of the various stages of the spermatogenic cycle as categorized above was calculated.

2.4 *Sperm analysis*

At autopsy, spermatozoa were obtained from the cauda epididymis removed from each mouse and put in physiological saline maintained at 37°C. The motility, viability and number of spermatozoa were assessed by the method of Singh and Chakravarty (2003), as further described by Sethi and Chaturvedi (2009).

2.5 *Analysis of fructose content*

The concentration of fructose in the seminal vesicle was determined by the method of Lindner and Mann (1960).

2.6 *Testosterone assay*

A radioimmunoassay (RIA) of plasma testosterone was performed using a commercial RIA kit (Immunotech, Marseille, France) according to the manufacturer's instructions. The antiserum used in the assay was specific for testosterone; the cross-reactivity was less than 0.03% with estradiol, 0.03% with progesterone, 0.01% with dehydroepiandrosterone, and 0.6% with androstenedione. The sensitivity of the assay was 0.025 ng/ml. The intra- and inter-assay coefficients of variation were 14.8% and 15%, respectively.

2.7 *Statistical analysis*

All the numerical data were analysed by one-way analysis of variance (ANOVA), followed by the Dunnett test for the comparison of group means. Significance was assumed at the level of $P<0.05$.

3. Results

At the termination of the study, the 8-h mice had lower body weights as compared with the control group (figure 1A), although the weights of testes did not show any statistically significant variation among different groups (figure 1B). There was also a decrease in the testicular volume of 8-h mice, but the other experimental groups were not different from the controls (figure 1C). The plasma testosterone concentration decreased in 7-, 8-, 9- and 10-h mice but increased significantly in the 12-h mice (figure 1D), as compared with the controls. Furthermore, the levels of fructose in the seminal vesicles of mice decreased only in the 8-h mice (figure 2A). However, the sperm count, motility and viability decreased in the mice of many more groups (7-, 8-, 9- and 10-h) and increased in 12-h mice as compared with the control group (figures 2B, C and D).

Histologically, when observed at 11 days post-treatment, the testes of the control and all other groups, except the 7-, 8- and 9-h groups, showed more or less active spermatogenesis, with bunches of spermatozoa attached to spermatids and/or in the lumen of the seminiferous tubules. In contrast, the testes of 7-, 8- and 9-h mice showed degeneration, i.e. the seminiferous tubules contained layers of only spermatogonial cells, with few primary spermatocytes. Most of these tubules exhibited a marked depletion and exfoliation of the germ cells, intraepithelial vacuolation due to a degeneration of the Sertoli cells and a loosening of the germ cells, along with the formation of giant cells in some tubules. The spermatids appeared to be the most affected cells in the germinal epithelium of the testes undergoing degeneration. These cells disappeared more readily from the epithelium by exfoliation and a maximum atrophy was evident in 8-h mice, although a degenerative process was also evident in the 7-, 8- and 9-h groups (figure 3).

When measured, a significant reduction of the seminiferous tubule diameter was noted in the 7- and 8-h mice as compared with the control mice (in contrast to an increase in the 12-h mice) (figure 4A). The number of affected tubules was increased in the testes of 6-, 7-, 8- and 9-h mice as compared with the controls, with a highly significant effect ($P<0.001$) in the 8-h mice (figure 4B). Analysis of histological observations (stages of germ cells) indicates that the frequency of the middle stage (Stage VII–VIII) was reduced in the seminiferous tubules of the testes of 7-h ($P<0.01$), 8-h ($P<0.001$) and 9-h ($P<0.05$) mice and increased in the 12-h mice ($P<0.05$), whereas the frequency of the middle stage in the other groups was more or less similar to that in the controls (table 1).

4. Discussion

Our results indicate that the administration in 2-week-old male mice of 5-HTP followed by L-DOPA at different time

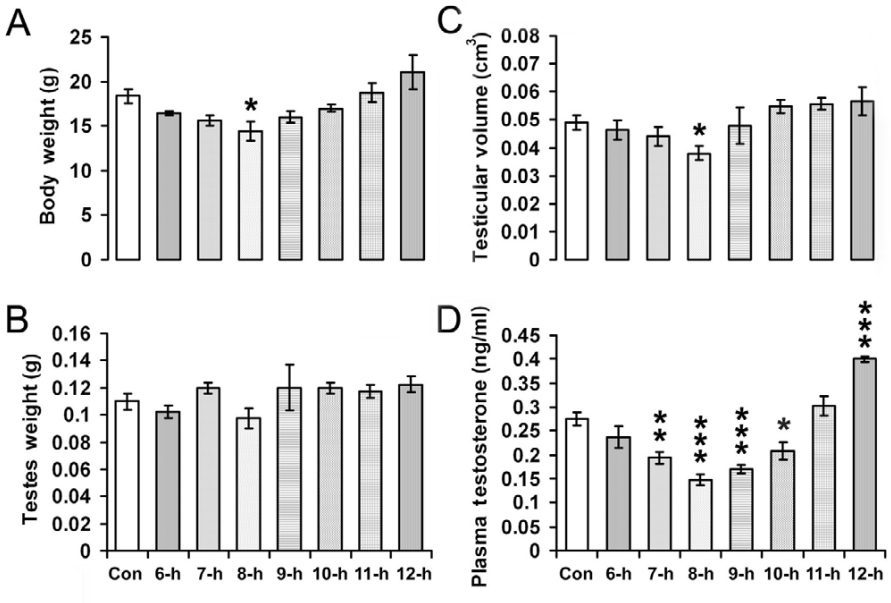

Figure 1. Effect of 5-HTP and L-DOPA injections given at different time intervals on **(A)** body weight, **(B)** testes weight, **(C)** testicular volume and **(D)** plasma testosterone levels in mice. 5-HTP was injected in all the experimental groups at 0800 and L-DOPA was injected in 7 different groups at different time intervals, i.e. at 1400, 1500, 1600, 1700, 1800, 1900 and 2000 establishing 6-, 7-, 8, 9-, 10-, 11- and 12-h phase relations, respectively, between the two injections. Mice in the control group received two daily injections of normal saline. Values are mean ± SE; the asterisks indicate the level of significant difference from the control group (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

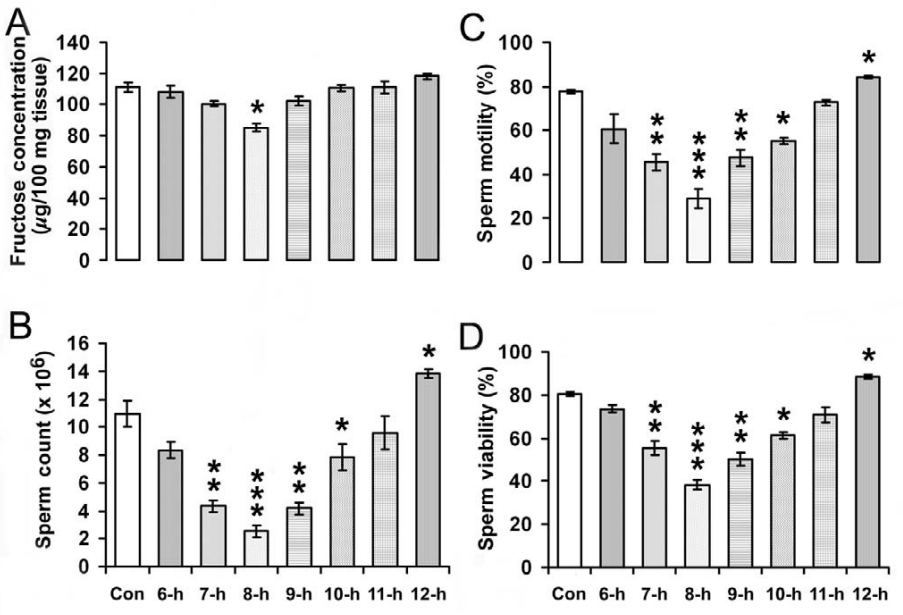

Figure 2. Effect of different temporal relationships of 5-HTP and L-DOPA injections on **(A)** fructose concentration, **(B)** sperm count, **(C)** sperm motility and **(D)** sperm viability (For details see figure 1). Values are mean ± SE; the asterisks indicate the level of significant difference from the control group (**P*<0.05, ***P*<0.01, ****P*<0.001).

Table 1. Effect of 5-HTP and L-DOPA administered at different time intervals on the frequency (%) of different stages of germ cells in the seminiferous tubules of mice

	Stages of spermatogenesis			
Groups	Early stage (I-VI)	Middle stage (VII-VIII)	Late stage (IX-XII)	Unidentified stage
Control	37.278 ± 3.17	40.731 ± 1.52	15.378 ± 2.99	6.613 ± 0.81
6-h	38.521 ± 1.37	37.475 ± 1.49	20.747 ± 2.03	3.257 ± 1.98
7-h	33.598 ± 1.93	30.189 ± 1.89**	20.580 ± 1.58	15.633 ± 1.01
8-h	31.147 ± 2.58	26.484 ± 1.78***	20.368 ± 1.46	22.001 ± 3.69
9-h	32.640 ± 1.53	33.453 ± 1.53*	16.642 ± 1.34	17.265 ± 2.06
10-h	34.754 ± 1.49	40.744 ± 3.75	22.634 ± 3.63	1.868 ± 0.43
11-h	32.643 ± 2.64	44.546 ± 2.54	21.346 ± 2.64	1.465 ± 0.74
12-h	30.453 ± 3.64	48.353 ± 3.64*	19.435 ± 1.86	1.759 ± 0.34

Values are mean ± SE (n = 5); controls received two daily injections of normal saline.
The asterisks indicate significant differences from the control group (**P*<0.05, ***P*<0.01, ****P*<0.001).

intervals, which evidently induced a different phase relation between the two circadian neural oscillations, had no effect on their body weight, except in the 8-h mice, which had lower body weights as compared with the control group. The results of the present study – the response to the injections of 5-HTP and L-DOPA given in numerous phase relationships differing by 1 h (6, 7, 8, 9, 10, 11 and 12 h) combined with those of an earlier study (Sethi and Chaturvedi 2009) – where the two injections were given at phase relationships differing by the interval of 4 h (i.e. 0, 4, 8, 12, 16 and 20 h) – are presented in figure 5. These combined results indicate that the suppression of the testicular activity and the degenerative changes in the seminiferous tubules were initiated in the 6- or 7-h mice, with a maximum suppression in the 8-h mice. Furthermore, an increase in the testicular activity was observed in the 9-h mice, with increased gonadal activity in the 12-h mice, as compared with controls. Obviously, it is not the amount of the neurotransmitters (serotonin and dopamine) that is important but the specific circadian phase relation between the two neural oscillations that appears to modulate gonadal development. Although neurotransmitter precursors administered (5 mg/100 g body weight) are qualitatively similar in all the groups, they appear to have different quantitative effects on gonadal growth, depending on the phase relation between the two drugs injected on a circadian basis.

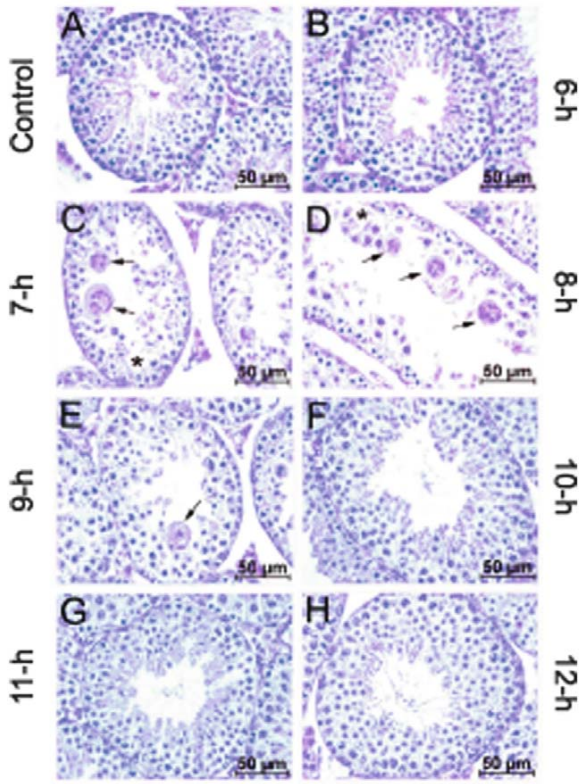

Figure 3. Haematoxylin-eosin-stained sections of testes of mice injected with 5-HTP and L-DOPA at different time intervals (6, 7, 8, 9, 10, 11 and 12 h = **B, C, D, E, F, G** and **H**). Note, the degenerative/regressive changes (depletion and exfoliation of germ cells) in the testes of 7-, 8- and 9-h mice **(C, D** and **E)** compared with developing condition in all other groups including control **(A)** that are showing successive stages of spermatogenesis in the seminiferous tubules. Note, the testes of 7-, 8- and 9-h group exhibiting degenerative changes in the seminiferous tubule showing exfoliation and vacuolation (indicated by asterisks) of germ cells along with the formation of giant cells (indicated by arrows) compared with the control group, whereas the 12-h group shows a full breeding condition **(H)**. (Scale bar = 50 μm)

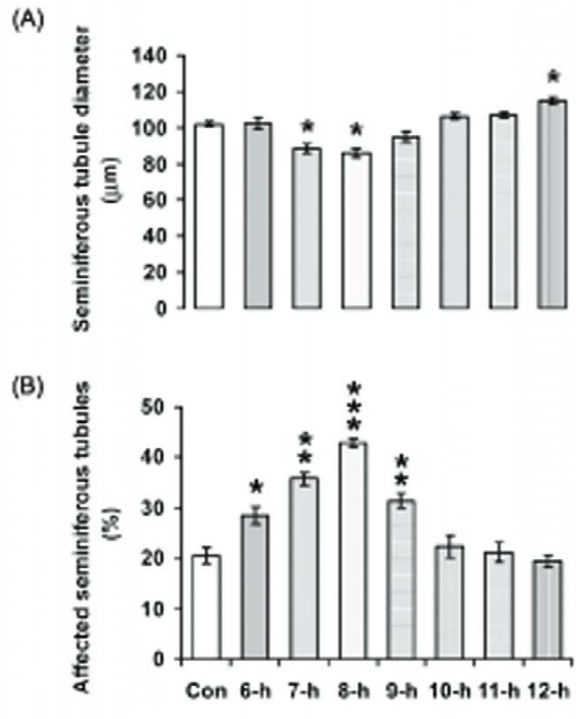

Figure 4. Effect of different temporal relationships of 5-HTP and L-DOPA injections on the seminiferous tubule diameter **(A)** and on the percentage of affected tubules in the testis **(B)**. Values are mean ± SE; the asterisks indicate the level of significant difference from the control group (**P*<0.05, ***P*<0.01, ****P*<0.001).

Further, the treatment did not cause any alteration in the weight of the testes, in spite of degenerative changes observed in the testes of 7-, 8- and 9-h mice, although the changes were not uniform. Previous reports have shown marked degenerative changes in the seminiferous tubules without a change in testis weight, for example, as seen after vasectomy (Singh and Dominic 1981) and treatment with flutamide (Singh 1984) in the musk shrew. The present results also show that 5-HTP and L-DOPA had variable effect on the testes, as both affected and normal seminiferous tubules were observed in the same sections. Such a variable response of the testis was seen in P mice after treatment with an aqueous leaf extract of *Azadirachta indica* (Mishra and Singh 2005), gossypol tetra-acetic acid (Singh and Rath 1990), STS 557 (Chakravarty and Singh 1998) or SC 12937 (Singh and Chakravarty 2003). In the present study, the frequency of affected seminiferous tubules in the testis of the 8-h mice was 42.9%, and it was 35.7% and 31.4% in the 7-h and 9-h groups, respectively, and close to 20% in the control and the other groups. In addition, we observed marked reductions in the frequencies of stage VII–VIII in the 7-, 8- and 9-h mice, which suggests that the administration of 5-HTP and L-DOPA at specific intervals (7 to 9 h) adversely affects spermatogenesis in P mice. Moreover, 5-HTP and L-DOPA in the 7-, 8- and 9-h mice also had adverse effects on the motility, viability and number of spermatozoa in the *cauda epididymis*, probably because of a suppressive effect on spermatogenesis. Interestingly, the sperm number returned to control levels in the 10- and the 11-h mice but increased in the 12-h mice, showing the stimulatory effect of this specific phase relationship (12-h) of the neurotransmitters. Our results showed that treatment with 5-HTP and L-DOPA at an interval of 8 h caused a marked reduction in the level of fructose in the seminal vesicles, suggesting an adverse effect of this specific phase relation of the neurotransmitter circadian rhythms on the secretory function of the accessory sex glands.

Our results also indicated that the daily injections of serotonergic and dopaminergic precursor drugs given in

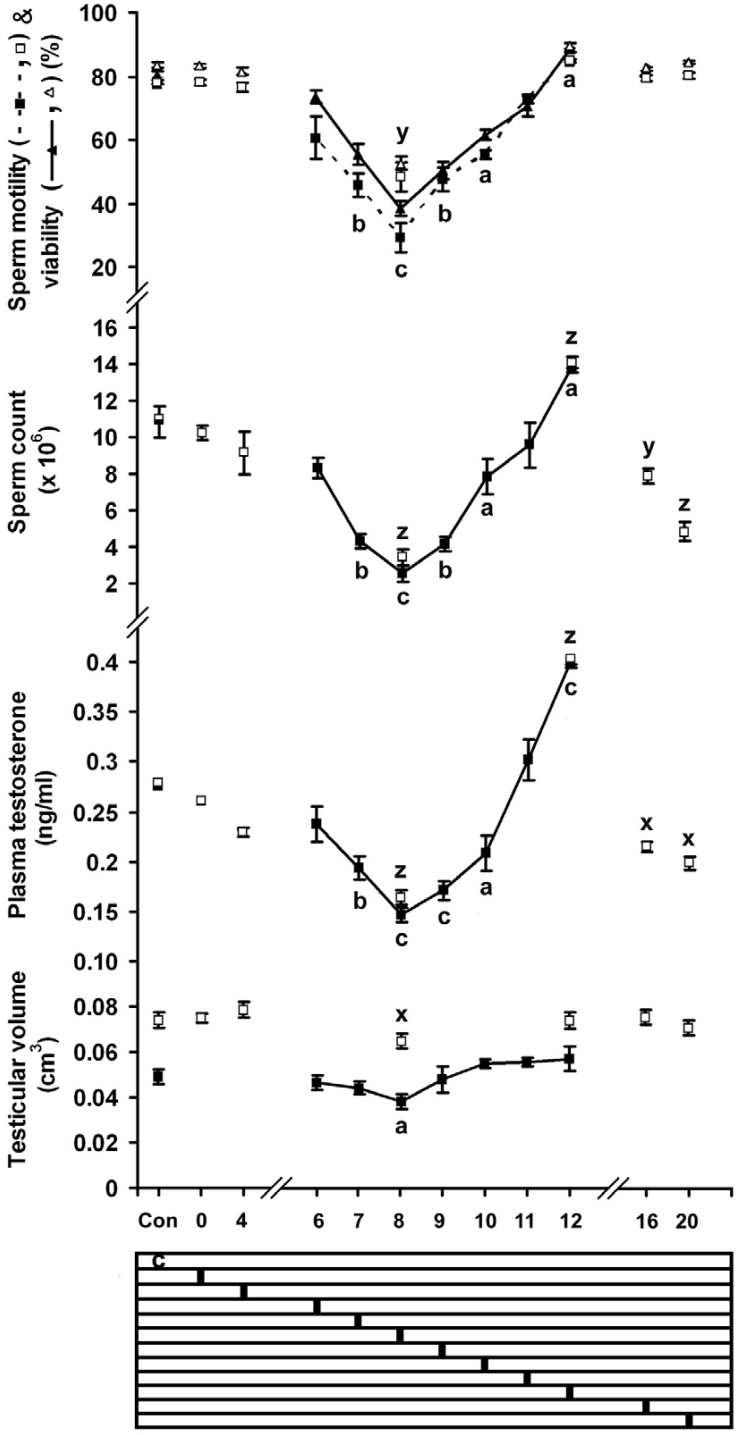

Figure 5. Testicular response of mice receiving 5-HTP and L-DOPA at various intervals (6, 7, 8, 9, 10, 11 and 12 h) – the present study (■, ▲) – and at the intervals of 4 h (0, 4, 8, 12, 16 and 20 h) – the earlier study (□, Δ). The injections were given in sexually immature mice for a period of 13 days under LL. After the treatment, the mice were maintained under 12L:12D and monitored for testicular activity at the age of 38 days (11 days post-treatment in the present study) and at the age of 58 days (24 days post-treatment in the earlier study). The time (h) in the abscissa represent the interval between the two daily injections, which are expected to initiate or entrain the two neural oscillations of the specific circadian phase relation. Con (c) represents the control mice receiving two daily injections of normal saline. The letters a, b, c and x, y, z indicate significant differences from the respective control groups (a/x, $P<0.05$; b/y, $P<0.01$; c/z, $P<0.001$).

a particular temporal relation altered the reproductive development and age-dependent body weight gain in mice at an age of about 5 weeks, i.e. 38 days. Spermatogenesis was somewhat suppressed in the 7-h mice, with maximum suppression in the 8-h mice, accompanied by a low testosterone level and highly reduced sperm counts, motility and viability. Although the 9-h mice also displayed suppression of testicular growth as compared with controls, the degree of suppression was less. In general, most of the other groups showed normal gonadal development that was no different from that in controls (figure 5).

The histological changes observed in the 7-, 8- and 9-h mice testes were similar to those that occurred after treatment with anti-spermatogenic agents (Singh 1999, 2006). The decrease in the gonadal activity and the reduction in the number of spermatozoa is the result of a suppressive effect on the neuroendocrine–gonadal axis, whereas alterations in other sperm parameters, such as motility and viability, might have resulted from disturbances in the epididymal function (Rajalakshmi 1992). It is well known that much of the control of germ cell development is mediated by hormones acting on Sertoli cells via their receptors for both follicle-stimulating hormone (FSH) and testosterone (Steinberger 1971; Orth and Christensen 1977; Sanborn *et al*. 1977). Moreover, LH has an indirect effect on spermatogenesis via a stimulation of the Leydig cells, which in turn synthesize and release testosterone. The lack of an assay of LH and FSH levels was one of the limitations of our study. Such data might have revealed more fully the mechanism by which phase relations impact the neuroendocrine control of gonadal development.

Our results show that 13 daily injections of 5-HTP followed by L-DOPA 7 h later started a suppression of spermatogenesis and the effect was also seen in the 9-h mice, but the maximum suppression occurred in the 8-h mice. Further, in the testes of the 7, 8 and 9-h mice, intraepithelial vacuoles were often observed in the seminiferous tubules. Hoffer (1983) reported that such vacuoles occurred primarily in the Sertoli cells of the seminiferous tubules in rats after gossypol treatment. It is logical to assume that the 8-h phase relation caused maximum suppression of both FSH and LH, resulting in the inhibition of both gametogenesis and endocrine functions such as testosterone secretion in the mouse testis. Contrarily, the 12-h phase relation of serotonergic and dopaminergic circadian oscillations, when established in mice at an early stage of development, increased the sperm count, motility, viability and the plasma testosterone levels, as compared with controls. This suggests a stimulatory effect of this relationship on the rate of reproduction/fertility. However, testis growth/volume and spermatogenic activity in 12-h mice (unlike the plasma testosterone level and the sperm index) were not significantly different from the controls, in contrast to previous reports on various other species (Wilson and Meier 1989; Chaturvedi and Bhatt 1990; Chaturvedi and Prasad 1991; Jaiwal and Chaturvedi 1991; Phillips and Chaturvedi 1995; Kumar and Chaturvedi 2009). By the end of the study, the control mice attained nearly full gonadal development; but, a stimulatory effect of the 12-h phase relation on all the parameters of testicular function was not evident, in spite of a significantly increased degree of gonadal activity as compared with the controls.

In accordance with the hypothesis of temporal synergism, the administration of 5-HTP followed by L-DOPA at an interval of 8 h suppressed the activity of the neuroendocrine–gonadal axis during the course of development and the attainment of puberty in mice. Obviously, these neural oscillations would have modulated GnRH neurons directly or indirectly and negatively or positively (by 8-h and 12-h relation, respectively) as a function of their temporal phase relation, which may transmit or transduce this information to the pituitary–gonadal axis. Hence, it was worthwhile to investigate further how just a 4-h difference in the phase relation may cause contrasting effects, i.e., an 8-h gonado-inhibitory relation as contrasted with a 12-h gonado-stimulatory relation. To more accurately determine the effectiveness of various phase relations, phase relations differing in lengths by hour intervals from 6 to 12 h were used, rather than the phase relations differing at intervals of 4 h as previously. This approach is somewhat analogous to Follet's (1973) in which 15-min light pulses were used at hourly intervals through the night to pinpoint more carefully the time of maximum light sensitivity.

The present study suggests a model explaining the attainment of gonadal development/puberty as a function of the circadian organization not only in seasonal breeders but also in continuous breeders. In Japanese quail, the functional maturation of the gonadal axis is modulated by this treatment when started in day-old chicks (Phillips and Chaturvedi 1995). The injection of L-DOPA at different hours after the administration of 5-HTP, to alter the relative phase relation of two neurotransmitter rhythms modulates quail gonadal development. The establishment of a 12-h relation between the two neural oscillations accelerates gonadal growth, whereas that of an 8-h relation leads to gonadal suppression, while most of the other temporal relations appear to be reproductively redundant.

This model based on the specific phase angle between circadian neural oscillations appears valid as well as useful (similar to the value of the internal coincidence model in understanding photoperiodic time measurement in birds). Circadian oscillators/oscillations change seasonally with the increasing/increased and decreasing/decreased day length as well as with different physiological/reproductive conditions. According to the internal coincidence model, the relative position of two cycles (which change with different day lengths) determines the induction of gonadal growth.

In our model, similarly, as the relative position or phase angle of two cycles changes, it may induce or suppress gonadal growth, as a function of the specific phase relation. Although these two analogous models induce similar effects/functions, the entrainment of the cycles is initiated by different means. In the internal coincidence model, light entrains the cycles; whereas, in our model, the cycles are experimentally entrained by the drug injections given at specific time intervals. But, in both the cases, the sum of the effects, gonadal induction or suppression is the outcome of coincidence or non-coincidence of the specific phase angle of two cycles. Unifying the two mechanisms, it is suggested that the specific response of the gonadal axis depends on a unique phase relationship developed between two or more physiological rhythms, which may be altered by either changing the day length (short or long) or by adminsitering different hormones or the neurotransmitter precursors at different intervals on a circadian basis. In physiological terms, these mechanisms require a temporal interaction of circadian cycles (hormonal, neural, or others) to suppress or facilitate the expression of complex seasonal cycles.

It is stressed that the recurrent activation of the gonadal axis in seasonal breeders and its stimulation during the attainment of puberty in non-seasonal breeders, although superficially appearing to be two different phenomena, depends on a unifying mechanism. In view of the central role of neurotransmitters (serotonin and dopamine) and the number of experimental evidences in seasonally breeding birds and mammalian species demonstrating a role of specific phase relations of serotonergic and dopaminergic oscillations in simulating reproductive conditions, it is not surprising that same principle/model may underlie the functional maturation of the gonadal axis in mice, as was previously observed in quail (Phillips and Chaturvedi 1995). Moreover, it is reasonable to suggest that the basic mechanism that switches on the quiescent gonadal axis for reproductive performance may be similar, whether for the first time in the life cycle, i.e. during the attainment of puberty, or repeatedly every year during the breeding phase.

Our model is similar to the internal coincidence model in which the relative position of two cycles, which changes with the different photoperiods (short or long day length) determines whether or not there is an induction of gonadal growth. In our model, the relative position of the two circadian cycles of neurotransmitters (and hence the specific phase angle between the two circadian rhythms) induces gonadal stimulation or suppression, as a function of the phase relation. The phase angles also may be changed by injections of neurotransmitter precursor given at specific time intervals. The evidence that all hormones, neurohormones and neurotransmitters do not follow the same circadian pattern, and that the pattern may vary seasonally or with various physiological conditions, supports our hypothesis. Injections of 5-HTP followed by L-DOPA administered at different time intervals simulated not only breeding/photosensitive conditions (by a 12-h relation) or non-breeding/photorefractory conditions (by an 8-h relation) but also a temporal correlation of hypothalamic neurotransmitters (serotonin and dopamine) that is also reported to differ in breeding and non-breeding mammalian (Wilson and Meier 1989), avian (Tiwari *et al*. 2006) and a piscine species (Senthilkumaran and Joy 1994). Further, the effects of simulated hypo- and hyper-reproductive conditions on the characteristics of the circadian rhythms in the hypothalamic concentration of serotonin, dopamine and plasma levels of T4, T3 and testosterone strongly supports the present hypothesis (Kumar *et al*. 2009).

These findings indicate that the temporal phase relation of neural circadian oscillations may influence reproductive development in prepubertal mice, and they further suggest an influence of circadian organization in the development of the neuroendocrine–gonadal axis. On combining our earlier reports with the present observations, it is obvious that the gonadal axis can differentiate between the phase angles of two oscillations with temporal phase relations that differ only slightly, i.e. an hour or so. Further studies, including those with LH measurements, are required to strengthen this proposition. However, on the basis of a number of earlier reports on seasonally breeding birds and mammals and recent reports on mice, it is concluded that (i) in addition to the existence of circadian rhythms of individual hormones or neurotransmitters, the specific temporal relation between the two circadian oscillations (as in an internal coincidence model) is required to facilitate or suppress physiological phenomena and (ii) in spite of different physiological bases, the utilization of circadian rhythms (and their coincidence and/or temporal phase relation) appears to be a common mechanism underlying gonadal growth during seasonal cycles and the attainment of puberty.

In summary, this study demonstrates an association of circadian rhythms and their temporal relations in regulating gonadal development in mice. The present work opens a new avenue of research that demonstrates the role of the circadian system in the regulation of testicular development during the maturation of the gonadal axis in mice, which are non-seasonal breeders. In addition, this study provides an experimental paradigm by which to test the internal coincidence model.

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