INTRODUCTION

Virtually all organisms that have been studied exhibit circadian rhythms in physiology and behaviour. These rhythms have period lengths close to, but not exactly 24h when the organism is maintained in a time-free environment i.e. an environment that provides no cues for the time-of-day (Pittendrigh, 1993; Goldman, 1999; Hastings *et al.*, 2003). Many mammalian species use change in photoperiod to cue seasonal adjustments in reproduction (Reiter, 1975). Both vertebrates and invertebrate species of animals have adapted mechanisms to sense the changes in day length to time seasonal events, such as reproduction to assure the most favourable time of year for survival of the offsprings and parents (Bronson and Heideman, 1994).

Reproduction is potentially a process controlled by many different environmental variables as well as endogenous mechanisms. Under natural terms, only photoperiod cycles precisely occur each year; all other environmental factors are far less precise. In addition to photoperiod, animals are exposed to multiple environmental cues such as temperature, humidity, food availability etc. In the process of regulation of these exogenous and endogenous rhythms, "the pineal acts as an intermediary between the environment and

endocrine system and melatonin act as a chemical link". Heubner was first to study the physiological role of the pineal gland at the beginning of 20th century; He presented the case of three girls with pineal tumors and precocious puberty. He concluded that the destruction of the pineal by the tumor had prevented the normal production of an antigonadotropic pineal hormone and raised the hypothesis that the pineal may control the onset of puberty. Thus the link between the "pineal gland and reproduction" was established (Simonneaux and Ribelayga, 2003). Lerner *et al.* (1958a, b), isolated melatonin from the pineal extracts and described the structure of melatonin (Lerner *et al.*, 1959) as a pale yellow crystalline organic compound (N-acetyl-5-methoxytryptamine).

Link between the light/dark cycle and metabolism of the pineal gland was a milestone in the history of understanding the endocrine function of the pineal gland. Today, the target tissues and mechanisms of action of melatonin on the reproductive axis are still not totally understood. In addition, recent investigations have revealed that melatonin has differential effects in the organism for e.g. on the hypothalamic circadian clock, the immune system, in the retina, its antioxidant properties and, its ability to modulate neurotransmission show less specific and ubiquitous effects (Simonneaux and Ribelayga, 2003).

Photoperiodism is a process whereby organisms are able to use both absolute measures of day length and the direction of day length change as a basis for regulating seasonal changes in physiology and behaviour. The day/night organization of physiological process such as reproduction relies on endogenous rhythm clock(s) that generate rhythm and are capable of being entrained to cyclic environmental factors (e.g. light/dark); such clocks convey circadian information to the rest of the organism in nervous and/or endocrine pathways (Goldman, 2001). In many seasonally breeding species, the timing of initial reproductive development (puberty) is strongly influenced by day lengths experienced during the postnatal period. This shows that mammalian pineal gland acts as a neurotransducer converting neural signals, specifically ambient lighting conditions and environmental cues, into endocrine message leading to synthesis and release of melatonin from the pineal gland (Stetson and Watson, 1984; Reiter, 1993; Ganguly *et al.*, 2002; Stehle *et al.*, 2002). Melatonin secretion pattern is modulated by the photoperiod and thereby provides both a daily and seasonal endocrine message, and affects the reproductive activity by regulating the activity of hypothalamic neuroendocrine circuitry.

The pineal hormone "Melatonin" is known to regulate two major physiological functions, regulation of circadian rhythms and control of seasonal reproduction (Malpaux *et al.*, 2001; Pevet *et al.*, 2002).

The physiological role of melatonin in regulating the seasonal reproduction in mammals has been well established (Karsch *et al.*, 1984; Underwood and Goldman, 1987; Foster *et al.*, 1989, Bartness *et al.*, 1993; Weaver, 2000). Melatonin (MT) synthesis is restricted to dark hours and its duration proportionates to the length of night, thus reflecting annual photoperiodic changes (Klein *et al.*, 1997; Ardent, 1995). Other than pineal, melatonin is synthesized by a number of extra-pineal organs (non-endocrine), such as retina, lens, bone marrow cells, gut, skin, harderain gland, cerebellum, kidney, adrenals, thymus, thyroid, pancreas, placenta, endometrium, ovary (Kventnoy, 1999; Stefulj *et al.*, 2001) and testis (Tijmes, 1996). It has been also localized in non-neuroendocrine cells such as mast cells, natural killer cells, eosinophils, leukocytes, platelets and endothelial cells (Kventnoy, 1999; Slominski *et al.*, 2002; Tan *et al.*, 2002).

Laboratory conditions are different where only one potential cue is usually varied and the others remain constant. Multiple cues may interact to produce a reproductive response that is greater than the reproductive response produced by any single environmental cue alone (Stetson and Watson-Whitmyre, 1984). Laboratory rats have been considered the classical model of an opportunist, non-seasonal breeder, responding almost entirely too immediate conditions to regulate reproduction and physiology (Shoemaker and Heideman, 2002). In rodents, maternal melatonin crosses placenta and provides

day-length information to fetuses which along with photoperiod information that is obtained after birth, influence juvenile development. Studies have shown that rodents exposed to laboratory conditions such as short photoperiods cause regression of the reproductive organs in adults and delay reproductive development in juveniles, while long photoperiods maintains reproductive organ function in adults and stimulate rapid reproductive development in juveniles (Stetson and Watson-Whitmyre, 1984).

Melatonin, the main hormone of pineal gland, acts on the neuroendocrine-reproductive axis exerting an antigonadotropic effect (Tamarkin *et al.*, 1985; Glass and Dolan, 1988). However, in some experimental conditions, MT has been shown to induce stimulatory hypophyseal-gonadal axis (Amadar *et al.*, 1986). Short photoperiod induced regressed testes size, ceased spermatogenesis and atrophy of accessory reproductive organs have been well documented in adult male rats whereas long photoperiods significantly shows increased testicular weights, diameters of seminiferous tubules and serum testosterone levels (Kus *et al.*, 2003). Siberian Hamsters maintained from birth under long photoperiod shows enlarged and functional testes at 35-40 days of age whereas at short photoperiods testicular development is delayed up to 150 days of age (Gaston and Menaker, 1967; Bernard *et al.*, 1997)). Juvenile

Siberian Hamsters show increased testes size to that of adults by 35 days of age on long photoperiods and small and underdeveloped testes at short photoperiods (Gunduz and Stetson, 1994). Exposure to bright light, suppressing the concentration of melatonin in circulation, is hypothesized to be useful in treatment of both male and female infertility in couples with abnormal melatonin metabolism (Partonen, 1999).

Pinealectomy attenuates and reveres the effects of food and sensory deprivation; it can accelerate pubertal maturation in neonatally healthy rats and is also known to increase Prolactin (PRL) secretion in some mammals. Pinealectomy (Px) has been known to prevent the seasonal reduction in gonadotropin secretion and gonadal regression brought about by experimental or natural short photoperiod and also shown to increase reproductive activity in mammals and has shown hyperactivity of Leydig cells (Reiter, 1973, 1980; Reiter *et al.*, 1983; Kus *et al.*, 2000). It has been shown that chronic melatonin treatment in the afternoon inhibits testes development in immature rats and also delays sexual maturation when given between 20-40 days of age (Lang *et al.*, 1983, 1984). Exogenous melatonin administration reveres the effect of Px on gonadotropin secretion and causes gonadal regression. Plasma melatonin concentration is found to be almost nil (undetectable) after pinealectomy. Previous study from our laboratory has shown

that neonatal pinealectomy in the rat results in increased adult testes size and accessory sex organs (Sharma, 1996). It is shown that melatonin can alter the morphology, steroidogenesis or cGMP production of testicular tissues, Leydig cells (Ellis, 1977; Ng and Lo, 1988; Persengiev and Kehajova, 1991; Niedziela *et al.*, 1995; Valenti, *et al.*, 1997) and of corpus luteum and granulosa cells *in vitro* (Mac Phee *et al.*, 1975; Fiske *et al.*, 1984; Baratta and Tamanini, 1992; Marayama *et al.*, 1997). Recently it has been shown that there is increased incidence of spontaneous malformations in pups and increased litter size from pinealectomised dams (Takashi *et al.*, 2004). Pinealectomy suppresses responses to both long and short photoperiod, and melatonin, depending on its specific pattern, reinstates both these responses. Thus, role of melatonin is to provide an endocrine clock for day length. The effect of exogenous melatonin on circadian rhythm of T3, T4, corticosterone and testosterone secretion suggest that exogenous melatonin has the suppressive activity on diurnal secretion of T3, T4 and testosterone in pinealectomized rats but stimulates the rhythmical corticosterone secretion. Melatonin is found to have a general inhibitory effect on thyroid hormones (Wright *et al.*, 1996; Lewinski, 2002; Mogulkoc and Baltaci, 2002). It is known that melatonin administration inhibits thyroid growth, it is stimulated by pinealectomy (Px) and that MT

supplementation reverses this condition in pinealectomized rats (Wajs and Lewinski, 1992).

Pinealectomy is known to induce abortion in pregnant rats (Guerra *et al.,* 1973) while at the same time Nir et al. (1979) reported that pinealectomy affects maternal serum LH and PRL levels during late pregnancy, but does not affects the fetal survival. These findings suggest the direct action of melatonin on the testis and ovary. Melatonin is known to have differential influence on male and female reproductive activity. A higher concentration of MT is detected in the human follicular fluids than in serum (Brzezinski *et al.,* 1987). There are varied reports of melatonin action on female reproductive system in rats. Melatonin treatment during gestation in the rat produced delayed sexual maturation and vaginal opening in female offsprings (Colmenero *et al.,* 1991). Notable effects of exogenous melatonin in adult female rats include disruption of normal estrous cycles and reduced fertility (Villanua *et al.,* 1989; Hastuta *et al.,* 2004). Pinealectomy leads to morphological alterations of rat ovaries with functional changes in steroidogenesis and a decrease in progesterone receptor expression (Soares *et al.,* 2003). Functional pinealectomy by continuous light in Indian desert gerbil showed regression of all stages of follicular growth and also reduced number of small antral follicles (Sinhasane and Joshi, 1997).

Other than gonadal hormones, thyroid hormones too have definite influence on functions like reproduction, growth, differentiation and metabolism. The two basic biological functions of thyroid hormones (T3 and T4) are (1) effect on cellular differentiation and development, and (2) effect on metabolic pathways that are interconnected. Thyroid hormone modulates oxygen consumption, and basal metabolic rate (BMR), also called calorigenic or thermogenic nucleic acid, lipid, carbohydrate and, protein metabolism. In hypothyroid individuals, BMR can fall from 35-40 Kcal/m^2 body surface/hr to 20-25 Kcal/m^2/hr, whereas in thyrotoxicosis or hyperthyroidism, it may rise to as much as 60-65 Kcal/m^2/hr (Lagu, 2001).

The majority of effects of thyroid hormones are now believed to be mediated through interactions of thyroxine with its nuclear receptors, so as to bring about changes in gene expression (Palmero et al., 1988, 1995). The thyroid receptor is classified as a member of the estrogen-vitamin D receptor- thyroid subfamily. These receptors all share the same consensus sequence preference of AGGTCA in DNA binding domain. These receptors preferentially bind to this so called half site when it is present as a single or multiple copies in the promoters of genes that are subjected to regulation. A wide array of biological systems like intermediary metabolic enzymes (malic enzyme, phosphoenolpyruvate carboxykinase, 6-phosphogluconate

dehydrogenase), cholesterol biosynthesis (HMG-CoA reductase), hormone systems (growth hormone, oxytocin, nerve growth factor) oxygen metabolism (cytochrome oxidase), ion transport, and energy metabolism (Ca^{+2} ATPase, Na^{+}, K^{+} ATPase) are all influenced by thyroid hormones (Lagu, 2001). Thyroid hormone enhances effects produced by other hormones and therefore alters nitrogen balance and is either protein anabolic or catabolic.

Thus, co-operative role of thyroid hormones, produce changes in target tissues allow these tissues to be more responsive to another hormone, to neural stimulation or possibly to certain environmental stimuli such as light. Thyroid hormones deficiency (hypothyroidism) may result in a wide variety of clinical and physiological disturbances in virtually every organ system. Involvement of thyroid axis and reproduction are the two most important endocrine functions that have been often linked through positive or negative thyroid-gonadal interrelationship and in most species studied thyroid activity follows an annual cycle closely related with sexual cycle. Thyroid hormones also have documented actions on the secretion of hormones involved in reproduction and maintenance of pregnancy. Most of the previous reports are with reference to the relationship between thyroid status and gonadal function in adult male and female rats. The lack of thyroid hormones has been known to produce reproductive abnormalities

including irregular menstrual cycle, amenorrhea and galactorrhea accompanied by increased prolactin secretion in women (Onishi *et al.*, 1977; Honbo *et al.*, 1978; Shahshahani and Wong, 1978).

The role of thyroid hormones in testicular physiology is not yet totally understood but their effects on female reproductive system are well established (Stardtman, 1993; Longscope, 1996). Neonatal transient hypothyroidism induces precocious puberty and macroorchidism associated with an increase in daily sperm production and, in Sertoli, Leydig and germ cell numbers (Anasti *et al.*, 1995; Bruder *et al.*, 1995; Cooke *et al.*, 1991, 1992; Cooke and Meisami, 1991; Hardy *et al.*, 1993, 1996; Hess *et al.*, 1996, Van Haaster *et al.*, 1992; Ramachandran *et al.*, 2004). However, recent findings by Buzzard et al. (2000), demonstrate the thyroid hormone receptor expression in germ cells undergoing spermatogenic differentiation, which suggests a possible role for thyroid hormone even in the adult rat testis. Also, demonstrations of T_3 receptor mRNA in Leydig cells of immature and mature rats (Hardy *et al.*, 1996) give further idea about thyroid-testis relationship. The impact of hypothyroidism varies depending upon the age of affected subjects, species as well as age of induction, sex, photoperiod, temperature, duration and severity of the disease. This can be correlated with higher concentration of thyroid hormone receptors during pre-pubertal and pubertal period as the number of receptors decreases in testes after

puberty. Hypothyroidism in the prepubertal period results in abnormalities in spermatogenesis, maturation of cells and reduced motility of spermatozoa (Karimov *et al.*, 2003). Recent findings have shown that thyroid hormones have significant functional relationship with testis, in particular with Leydig cell differentiation, proliferation and stimulation of Leydig cell steroidogenic function (Handagam-Mendis and Ariyaratne, 2004), direct effects on the development of prepubertal testes and the regulation of FSH R and ABP gene expression in Sertoli cells, as well as the LHR mRNA levels in Leydig cells; all of which show that thyroid hormones have modulating effects on gonadotropin control of testis functions (Rao *et al.*, 2003). There have been various reports on relationship between thyroid and gonadotropin decreased concentration of FSH and LH in hypothyroid rats (Vilchez-Martinez, 1973; Amin and Sheikh, 1977; Valle *et al.*, 1985). However, some reports show either increase or no change in FSH and LH levels (Kalland *et al.*, 1978). These variations can be attributed to age, species, duration, and experimental conditions like photoperiod and temperature.

In adult rats, hypothyroidism influences normal follicular maturation of the ovary and gonadotropin secretion, resulting in irregular estrous cycles as a result of hypersecrtion of progesterone due to increase in prolactin levels which causes prolong periods of diestrous, suppression of LH secretion and follicular maturation

while T3 treatment restores normal estrous cycle and ovarian functions (Ortega *et al.*, 1990; Mattheji *et al.*, 1995; Armada-Dias *et al.*, 2001; Tohei, 2004). Hypothyroidism in pre-pubertal rats showed disturbed folliculogenesis, that hampers the differentiation and not the proliferation of granulosa cells (Dijkstra *et al.*, 1996). Thyroxine administration to pre-pubertal rats (day 21-29) showed improved follicular development but does not effect the gonadotrophin secretion.

Stress induced adverse effects on reproduction is known to be primarily mediated through higher concentration of glucocorticoids (GC) (Cooke *et al.*, 2004). Influence of GCs on mammalian reproduction is complex and known to be dose-dependent. Physiological levels of GC are usually beneficial and necessary for normal functioning of the reproductive system at various levels or stages of development. Excess of GC on the pituitary-gonadal axis of males and females as well as the developing fetus may be detrimental (Cooke *et al.*, 2004). GC exerts various deleterious effects on the interstitial Leydig cell of the testis, including direct inhibition of testosterone biosynthesis, suppression of LH receptor expression, testicular germ cell apoptosis and induction of Leydig cell apoptosis (Bambino and Hsueh, 1981; Monder *et al.*, 1994; Yazawa *et al.*, 2000; Sasagawa *et al.*, 2001; Gao *et al.*, 2002). Stress, is known to affect testicular function and steroidogenesis by inducing

GC increase (Collu *et al.*, 1984; Orr *et al.*, 1994; Maric *et al.*, 1996; Chatterton *et al.*, 1997) more so in the developmental stages. The foetal environment is the key determinant of the adult phenotype, being linked to timing of puberty. Corticosterone excess has been shown to inhibit testosterone production in adult males in vivo (Saplosky, 1985; Bambino and Hsheh, 1981; Mann *et al.*, 1987) and in vitro. Adrenalectomy of adult male rats elevates serum testosterone levels. Recent data also reveals that adreno-cortical-testicular interaction in the rat is influenced by age and stress type (Cooke *et al.*, 2004). Cold stress produced a decrease in serum testosterone, whereas restraint stress increases the serum T level in 60-day old rats (late puberty) and such a stress quality related differences were not observed in 40 day old rats (early puberty) (Gomez and Dallman, 2001; Gomez *et al.*, 2002). Basal GC favours development of female reproductive system. Excess endogenous maternal glucocorticoids in foetus delays puberty in female offsprings, whereas an experimental reduction in fetal glucocorticoid advances puberty in male offsprings (Smith and Waddell, 2000). Adrenalectomy delays puberty in the female rat, which is restored by corticosterone administration. A physiological level of GC at gestation produced by embryo participates in maturation of fetal organs and organ systems, initiation of parturition and fetal well-being post partum (Cooke *et al.*, 2004). Foetal GC exposure may be

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an important for postnatal reproductive development because puberty onset is delayed in the offspring of mothers subjected to stress (Politch and Henerkohl, 1984) or treated with ACTH during pregnancy (Harvey and Chevins, 1987). The major effect of GC seems to be a reduction in Leydig cell sensitivity to LH stimulation of testosterone synthesis as presence of GC receptors in Leydig cells is dominant (Evain *et al.,* 1976; Stalker *et al.,* 1989). Recently, high affinity cytosolic glucocorticoid receptors (GR) are isolated from Sertoli and peritubular cells of rat testis (Levy *et al.,* 1989). These findings suggest that circulating glucocorticoids can directly affect spermatogenesis.

Prenatal stress produces long-term changes in the HPA axis in the offspring (Henry *et al.,* 1994). The activity of HPA axis in adult offspring is mediated at least in part by a reduction in corticosteroid receptors at specific time of day. This indicates that prenatal stressed rats exhibit an altered temporal functioning of the HPA axis, which is not only age specific (Henry *et al.,* 1994) but also gender specific (Handa *et al.,* 1994; Koehl *et al.,* 1999; King and Edwards, 1999).

The effect of functional manipulations of thyroid or pineal individually has not been uniform and even more confusing and ill defined are the interactions between these endocrine glands and their role on reproductive functions. Variable effects of pineal on

adrenal activity with respect to reproductive functions have been reported in several mammalian species (Motta *et al.*, 1971; Vaughan *et al.*, 1972; Ogle and Kitay, 1976; Nir, 1978).

The disparate and often contradictory reports on the functional ability of these endocrine glands under photoperiodic manipulations and their mechanism of action in combination with each other have decipher the necessary impetus for the present work.

Most of the studies reviewed above are on adult animals, though there are some studies on immature rats with different experimental schedules. Whereas most of the hormonal manipulations in the adult would be transient and more of the nature of "activational", manipulation in the neonatal stages are likely to be long lasting and of the nature of "organizational" which can result in alterations and resetting of hormonal axes and adult physiology. Study on neonatal hypermelatonemia by melatonin administration revealed increased number of germ cells but loss of advanced germ cells by sloughing off. Neonatal hypermelatonemia also permanently lowered HHG axis but elevated the HHT axis though T4 levels were reduced in adults (Ramachandran *et al.*, 2004). Neonatal hypo/hyperthyroidism showed differential effects on adult testis functions with increased testes size and germ and Sertoli cell numbers and, decreased adult testes size, Sertoli cell number and sperm mass respectively (Lagu,

2001). Studies on neonatal exposure to glucocorticoids have shown dose and time dependent long-term effects on adult testis function and neuroendocrine axes. Optimum levels of corticosterone excess during neonatal period produced favourable influence on adult spermatogenic functions whereas hypocorticalism showed decreased germ cell number and sperm density in the adult testis (Bhavsar, 2001). Another study from also showed that neonatal pinealectomy results in increased adult testes size and accessory sex organs (Sharma, 1996).

Overall, results from our laboratory have suggested that neonatal hormonal disturbances can bring about possible favourable/unfavourable influences, which bring about permanent long-term consequences on the developmental neuroendocrine axis. There are no studies on alterations in melatonin, thyroid or corticosterone levels in relation to photoperiodic manipulations in the neonatal period on male adult reproductive functions. Hence, it was thought pertinent to study the long-term effects of experimental alterations in melatonin, thyroid and corticosterone status on growth, attainment of maturation and function of the testes at prepubertal, pubertal and adult stages along with hormonal profiles. Also, the long-term consequences of melatonin and thyroid interactions have been extended to female reproductive system to understand whether MT and thyroid hormones have possible

favourable/unfavourable influence on adult ovarian structure and function.

On this presumption, studies were undertaken from this laboratory to assess the impact of neonatal manipulation involving thyroid, pineal and adrenal hormones on adult testes functions.