## GENERAL CONSIDERATIONS

The male reproductive system and the testis in particular have received scientific attention from very early times. Though the major structural and functional features were elucidated in the early half of the century, many aspects continued to remain enigmatic. Studies in the last few decades have provided exciting new findings and, the intricacies at the cellular and molecular level are gradually becoming amenable. These new findings have added new dimensions in the understanding of, what apparently appeared to be simple functions of mammalian reproductive system and, are providing a basis for the development of as yet elusive methods/agents for male contraception.

The role of the testis in normal male development and the effects of castration were known from the time of Aristotle. The experiment of Berthold (1849) involving castration of the cock and the observation that this resulted in the regression of the comb and that, it could be prevented by transplanting the testis to a new site provided compelling evidence for the humoral actions of testis. This was one of the pioneering experiments in endocrinology. It was during the third decade of this century, it was discovered that the functions of testis are not possible without the support of the pituitary gland (Greep *et al.*, 1936; Greep and Fevold, 1937). The two pituitary

hormones, Follicle stimulating hormone (FSH) and the Interstitial or Leydig cell stimulating hormone (ICSH or LH) were identified as the principal mediators of the pituitary support and, thereby designated as the gonadotropic hormones. It was during this time that Butenandt (1931) crystallised androsterone from urine and testosterone was soon isolated in crystalline form (David *et al.*, 1935). In the same year the synthesis of testosterone was achieved (Butenandt and Harrisch, 1935; Ruzicka and Wettstein, 1935). This enabled the experimental use of these hormones which resulted in considerable progress being made in elucidating the mechanism of action of androgens. The dual functions of testis in the reproductive life of the adult male that emerged right from the beginning were, the germinal function of providing gametes and, the endocrine function of secreting hormones. During the fetal development, the testis also controls the differentiation of the male genital track. It is by now clear that the two functions of testis are not divorced isolated functions but, are greatly interdependent. From the beginning itself it became evident that LH stimulates the Leydig cells to produce testosterone (T) which in turn stimulates the germinal epithelium (Greep *et al.*, 1936; Greep and Fevold, 1937; Greep, 1961; Albert, 1961; Clermont and Harvey, 1967). It was generally understood that FSH acts directly on the germinal epithelium though, the nature of this action was not entirely elucidated (Greep, 1961; Lostroh, 1963; Lostroh *et al.*, 1963; Clermont and Harvey, 1967).

The last three decades have witnessed exciting revelations regarding the regulation of gametogenic and endocrine functions of testis at the cellular, intercellular and molecular levels. Some of the investigations have highlighted the temporal changes in the hormonal *milieu* and differential sensitivities during the development of the male gonad from the fetal to the adult stage. Of far reaching implications and of great academic value, are the findings on the subtle fine tuning of the spermatogenic and steroidogenic functions at the local level by a plethora of growth factors, peptides and hormones. These studies have revealed an intricate network of intercellular communication between the various cell types in the interstitial compartment as well

as the tubular compartment of the testis or even at the interphase between them (Saez *et al.*, 1990; Verhoeven, 1992; Jegou, 1993; Avallet *et al.*, 1993; Pescovitz, 1994).

Extensive research on regulation of male reproductive functions led to the enunciation of the classical concept of hypothalamo-hypophysial-gonad axis represented by, the hypothalamic gonadotropin releasing hormones, the pituitary gonadotropins and, the gonadal steroids. This axis has been considered to be the regulatory mechanism of the reproductive system. Investigations carried out in the last two decades gradually gave evidences for other endocrine agents as well, in regulating gonadal functions either by modifying or modulating the axis or even, by acting directly or indirectly. Of the many other hormone candidates, the hormones of the pancreas, thyroid, adrenal and pineal were found to exert major effects. Reports on altered reproductive functions under disorders affecting one or the other of these endocrine glands made a justifiable cause for not ignoring the importance of these endocrine glands in normal reproductive functions.

From the time pineal was recognised as a legitimate member of the endocrine orchestra, the main functions of this endocrine gland that became apparent were with reference to reproduction (Tinley and Waren, 1919; Thieblot, 1960; Kinson, 1976; Reiter, 1980, 1981). The discovery of melatonin as the principal pineal hormone and, its cyclic nature of secretion, it was realised that the pineal is a neuroendocrine transducer which translates the environmental input of information into adaptive physiological responses. When they discovered the relation of this gland with rhythmic functions, its importance in regulating reproductive functions was greatly manifested and hence investigated in seasonal breeders, especially mammals. There is by now a vast amount of literature dealing with various aspects of pineal control of seasonal breeding activities of mammals as well as other vertebrates (Reiter, 1981; Ebling and Foster, 1989). Its importance in domesticated animals and continuous breeders was considered to be insignificant due greatly to the emancipation of these animals from environmental agents like light,

temperature and food and with lesser need for integration of and adaptation to these factors. Experiments on the laboratory rat, have principally contributed to the insignificant status of the pineal *vis-à-vis* reproductive functions in continuous breeders. But of late, the observed endocrine profile of various human subjects with manifested reproductive failures and dysfunctions associated with various types of disorders have caused doubts on the validity of the insignificant status of the pineal (Reiter *et al.*, 1985; Silman, 1991). At best, these findings suggest the need to look at the role of the pineal in humans and continuous breeders from a different angle. It is worth reflecting here, the comments of Binkly in a recent review on pineal in which the author comments that it is hard to think that the pineal of the rat which shows a robust rhythmic functioning in terms of melatonin secretion will have no important functional role (Binkly, 1983).

Most of the early studies on rat involved Px or melatonin administration. Both these were found to have no significant effect in the adult male. However, studies on pineal in the immature rat, have reported early pubescence and even slightly increased testis and accessory weights (Kinson, 1976). Experiments involving melatonin administration were also shown to be in general ineffective and, some of the recent studies have shown that melatonin is effective only during a critical period between 20 and 40 days, which again results only in a slightly delayed pubescence (Lang *et al.*, 1984).

The thyroid gland has been known to exert control overt metabolic functions of various organs through its hormones. Though the thyroid hormones have been functionally correlated with metabolic activity of various organs, from very early itself, the male gonad was considered to be insensitive to the metabolic actions of thyroid hormones (Barker and Klitgaard, 1952). This precluded studies on the influence of thyroid hormones on male reproductive functions and dampened the spirits of those interested in elucidating thyroid-testis interactions. Ironically, the impact of hypo or hyperthyroidism on female gonadal functions was getting clearly established

(Burrow, 1991; Stradtman, 1993); the same on male gonadal functions remained unelucidated due to the many controversial reports (Van Wyk and Grumbach, 1960; De la Balze *et al.*, 1962; Franks and Stempfel, 1963; Becker *et al.*, 1968; Laron *et al.*, 1970; Hemady *et al.*, 1978; Kugler and Huseman, 1983; Wortsman *et al.*, 1987). Even experimental investigations in animal models resulted in much controversy and differential effects on gonadotrophs, gonadotrophic hormones and testicular steroids were reported (Jannini *et al.*, 1995). Even though thyroid hormones were considered to be unimportant in the overall testicular physiology, one group of workers challenged this concept by their observations on the effect of thyroid hormone status on many metabolic parameters of rat testis (Aruldhas *et al.*, 1982 a, b; 1983; 1984; 1986 a, b).

A string of recent reports on the basis of precisely controlled experimental manipulations have shown that transient neonatal hypothyroidism induced during the preweanling period could result in significantly enlarged testis with increased germ cell numbers and Sertoli cells (Cooke and Meisami, 1991; Meisami, *et al.*, 1992; Kirby *et al.*, 1992; Van Haaster *et al.*, 1992; Hess *et al.*, 1993; de Franca *et al.*, 1995; Simorangkir *et al.*, 1995). Some of these reports even suggested neonatal hypothyroidism as a possible model for increased sperm productivity and regulation of male fertility. The reason for these observed effects has been accredited to the role of $T_3$ as a differentiation promotor of Sertoli cells. Apart from, $T_3$ inducing Sertoli cell differentiation, it also inhibits Sertoli cell proliferation. It is this dual action of the hormone that had been considered responsible for the normal course of differentiation in the immediate post-weanling days. Low titre of the hormone at this period is suggested to prolong the period of Sertoli cell proliferation and significantly increase their overall number per testis, resulting in greater sperm production (Cooke *et al.*, 1991; 1992; 1993; Kirby *et al.*, 1992; Van Haaster *et al.*, 1992; de Franca *et al.*, 1995). These results which were obtained principally in the Long-Evans and Sprague-Dawley strains of rat were also found to hold good for a strain of mouse (Joyce *et al.*, 1993). As a corollary, neonatal hyperthyroidism induced by $T_3$ administration in the Wistar strain of rat was shown to result in much smaller adult testis size (Van Haaster *et al.*, 1993). It

is these observations and the lack of any study evaluating the long term consequences of neonatal Px coupled with, some observations of neonatal chemical Px or melatonin administration showing effects on growth of reproductive organs and on some metabolic features, that kindled the idea of studying the possible long term influence of neonatal hypo- or hyper-thyroidism in neonatally pinealectomised animals.

Surprisingly, intact rats rendered neonatally hypothyroidic taken essentially as a control for observing the hypothyroidic effects in pinealectomised animals, showed a stunningly opposite response from those obtained by other workers. Neonatal hypothyroidism in the present study resulted in retarded testicular growth ultimately leading to a testis size at 90 days which is only 65% of the controls. This was in contrast with that of other workers who showed 35% heavier testis at the same time (Cooke and Meisami, 1991; Kirby *et al.*, 1992; Meisami *et al.*, 1992; Hess *et al.*, 1993). Evaluations of histological and histochemical profile as well as the hormone profile gave evidence to the fact, FSH to be the prime inducer of Sertoli cell proliferation, unlike in the previous studies where $T_3$ was shown to be responsible. The observations nevertheless provided compelling rationale for the need of $T_3$ as a permissive factor for FSH induced Sertoli cell differentiation. A reasonable conclusion that was drawn is that reduced FSH levels due to hypothyroidism decreased Sertoli cell proliferation and, the subsequent simultaneous acquisition of optimal levels of thyroid hormones and FSH, prompted Sertoli cell differentiation thereby resulting in overall reduced Sertoli cells per testis. This was validated by the observed smaller size of testis and the reduced population of germ cells. Since, these observations were reproduced consistently for at least in every one of the five repetitions, a harder look at the paradoxical situation was necessitated. The only tangible and plausible explanation that emerged from the critical considerations is that there ought to be a strain difference. The present study employed the Charles foster strain of rat which has been perpetuated over generations from an original stock at the Haffkine's Institute, Bombay. Apparently most of the white rats that have been used for experimental purposes in laboratories all over India, masquerading as the Wistar

strain are, actually the members of Charles foster strain. From the present observations it is clear that this strain has significant genetic difference compared to Long-Evans, Sprague-Dawley or even Wistar in their hormonal make up as well as responses. The results documented in Chapter 1 shows not only growth retardation of the testis but also of the accessory sex organs. Though this appeared to be due to the lack of thyroid hormone primed growth effects in the early stages, the later study involving Px, dispelled this notion. Since, the pinealectomised animals though also showed a hypothyroidic effect in the prepubertal period, the weights of the accessory sex organs were however significantly greater at 90 days compared to the controls. These two observations put together led to a natural inference that the observed growth retardation in intact hypothyroid animals is due to the potentiated growth retarding influence of melatonin. Since, the pinealectomised animals recorded increased growth response of the accessory sex organs despite being hypothyroidic during the prepubertal period, the only rational explanation is that melatonin has an inhibitory influence on the growth of the accessory sex organs in the prepubertal period and that the thyroid hormones effectively resist the same. Clearly, in the absence of thyroid hormones, pineal probably through its hormone melatonin manifests its inhibitory influence in its full potency.

Interestingly, neonatal Px in the Charles foster strain resulted in significantly increased testis size at 90 days. There was an apparent increase in the number of Sertoli and germ cells as well as early induction of steroidogenesis which suggests a possible inhibitory influence of melatonin in intact animals. Another explanation is the prolonged or increased degree of Sertoli cell proliferation as a consequence of elevated levels of FSH in pinealectomised animals. Obviously the degree of Sertoli cell proliferation is controlled by the level and duration of the exposure to FSH in Charles foster strain rather than the negative influence of thyroid hormones as in the other strains (chapter 1). The results obtained in the Px animals are similar to hemicastration and FSH induced effects (chapter 2). In both hypothyroid as well as pinealectomised rats, Sertoli cell differentiation occurs only with the elevation in thyroid hormone

levels. This clearly indicates a need for thyroid hormones as a permissive factor in FSH induced Sertoli cell differentiation. The fact that the pinealectomised rats, despite having enlarged testis shows sluggishness in forming sperms indicates some sort of impediment in the completion of spermiogenesis. This needs to be evaluated in detail and no rationale justification could be provided at this juncture.

The retardatory influence on testis growth caused by neonatal hypothyroidism was completely nullified by Px as, the HPOT + Px rats had similar testes weights as compared to the controls at 90 days. Mixed effects of hypothyroidism and Px were visible, in the form of increased germ cell numbers and reduced tubular diameter respectively. The present study clearly reveals that the final tubular diameter is influenced by the permissive influence of the thyroid hormones in the early stages as, both HPOT and HPOT + Px rats had reduced tubular diameter. This is in contrast to the observations of Hess *et al.* (1993) and Mesiami *et al.* (1994) and, is again an indication of strain difference. The increased germ cells found in HPOT + Px rats is accredited to the hyperproliferative effect of FSH on Sertoli cells in the early period, clearly a Px effect. A delay in the progression of spermatogenesis beyond the meiotic stages in HPOT, Px and HPOT + Px animals can be related to the delayed attainment of normal $T_4$ and $T_3$ levels. Predictably, the induction of optimum number of $T_3$ receptors on Sertoli cells (Jannini *et al.*, 1990; Palmero *et al.*, 1993) and the subsequent action of $T_3$ in permitting FSH induced Sertoli cell differentiation are delayed and, the time period of progression of spermatogenesis through meiotic stages in the three experimental groups show correspondence with the period of attainment of normal thyroid hormone levels. Based on the observation of Leydig cells in both Px and HPOT + Px animals, it is inferred that melatonin has a possible regulatory inhibitory influence on Leydig cell proliferation in the immature stages. This assumption is justified by the recently reported presence of melatonin receptors in the Leydig cells (Valladares *et al.*, 1992; Ayre and Pang, 1994).

Corollary experiments involving neonatal hyperthyroidism also paradoxically resulted in reduced testis size and germ cell number. The reduced testis size and body weight of HPRT animals are a consequence of reduced GH secretion and a lowering of the set-point of pituitary-thyroid ax is (Dussault *et al.*, 1982).The reduced number of germ cells in the HPRT animals is a consequence of a delayed and decreased period of Sertoli cell proliferation as, neonatal hyperthyroidism decreases the FSH level. Due to the temporal closeness of the attainment of optimal FSH and thyroid hormone levels only a short period is available for Sertoli cell proliferation and in presence of thyroid hormone, FSH induces Sertoli cell differentiation. This observation on HPRT animals fully supports the contention that in the Charles foster strain, FSH is the stimulator of Sertoli cell proliferation and thyroid hormone is required for inducing Sertoli cell differentiation. However, if the hyperthyroid animals are neonatally pinealectomised, the testis size is normalised and there is also increased number of germ cells and presence of mature sperms in the tubules. These are again due to the hyperproliferative effect of FSH, a consequence of Px, and delayed Sertoli cell maturation and differentiation due to the delayed attainment of optimal thyroid hormone levels. Apparently, both HPRT and HPRT + Px can increase germ cell number and sperm production but, the HPRT + Px condition exerts a much better qualitative effect.

The morphometric and histological observations on the development of the sex accessory organs suggest that melatonin has a growth retardatory influence as, in all the pinealectomised groups of animals (Px, HPOT + Px and HPRT + Px), the accessory sex organs showed increased growth in the mature stages. The observations on HPOT and HPOT + Px animals put together suggest that the thyroid hormone can resist the growth retardatory influence of melatonin in the mature stages as, in intact thyroidectomised animals, the accessory organs showed severe growth retardation. This apparently indicates that, the growth retardatory action of melatonin is greatly accentuated in the absence of thyroid hormone. Based on the observations of control and experimental animals, it is also surmised that, the pubertal and

postpubertal growth and maturation of the accessory organs are influenced by T and PRL. One aspect that is less studied in literature, is the growth of epididymis, while that of seminal vesicles and prostate are better studied. The observations made during the course of the present study have given some indication for a differential hormone requirement for the growth of epididymis. It is likely that the epididymis is more dependent on GH and thyroid hormones for its growth in the juvenile and prepubertal periods. Investigations on these lines are warranted.

The evaluation regarding alterations in the carbohydrate metabolism under the various experimental paradigms have revealed a unique feature of early attainment of adult type carbohydrate homeostasis. A common consequence of all the experimental manipulations is the decreased thyroid hormone levels in the juvenile and the prepubertal periods. This has been taken to its logical conclusion suggesting a retardatory action of thyroxine in increasing the inuslin:glucagon molar ratio, an adult characteristic. In fact, a still earlier attainment of the adult type carbohydrate homeostasis in Px animals, suggests a resisting action of melatonin as well, on the attainment of high inuslin:glucagon molar ratio. Expectedly, under conditions of lowered threshold levels of $T_4$ and melatonin, there is a facilitatory influence on the attainment of higher inuslin:glucagon ratio. Further experimental evaluations on these lines could be fruitful. Another important aspect that has been brought out is the dependence of the first wave of spermatogenesis on the testicular store of glycogen, though subsequently, blood glucose becomes the choice source. This is accredited to the requirement for the establishment of glucose transporting and concentrating mechanisms, a property of differentiated Sertoli cells. The dependence on testis glycogen during the first wave of spermatogenesis is clearly reflected in the observed correspondence in the time periods of glycogen depletion and the full establishment of spermiogenesis. In the various experimental groups, though the establishment of full spermatogenesis occurred at different time periods, there was nevertheless, a strict temporal correspondence between glycogen depletion and the occurrence of spermiogenesis.

Overall, the present study has thrown up a number of novel observations and has also opened new avenues for future investigations.