Introduction

The perpetuation of species is the result of successful reproduction which has emerged as one of the most important strategies of living organisms. The divergent evolution of life from a simple unicellular organism to complex multicellular forms has witnessed a gradual change in reproductive pattern from asexual to sexual mode, concomitant with the emergence of reproductive tissues and sexual dimorphism. Since its birth, the world’s climate has changed drastically from hot to cold and wet to dry. Mammals, the most evolved creature in the animal kingdom, appeared on earth around 250 million years ago. The Earth then, consisted of only one landmass—the supercontinent of Pangaea. Some parts of Pangaea experienced robust changes in climate and therefore food availability while others didn’t (Crowley, 1994). Therefore, some of the initial mammals showed year-round-variation in reproductive status to cope with the fluctuation in climate and energy availability while others reproduced throughout the year. Thus, seasonality in mammals developed as a consequence of selection pressure for survival. These adaptive mechanisms therefore evolved as ‘Seasonal Reproduction’. The accurate temporal orientation of reproduction is a target of natural selection and has played a major role in the evolution of seasonality (Prendergast et al., 2009). On the basis of the breeding pattern, mammals can be categorized into three classes:

(A) Seasonal breeders

Animal species that successfully breed only during the favourable time of the year. Example, golden hamster (*Mesocricetus auratus*), Indian palm squirrel (*Funambulus pennanti*), etc.

(i) Long day breeders are those species that breed under long photoperiodic conditions. Example: ferrets, minks, hedgehogs, white-footed mice, raccoons and mares etc.

(ii) Short day breeders are those animals that breed under short photoperiod when the length of the day shortens, Example: sheep, deer and goat are well known short day breeders.
(B) Aseasonal or Continuous breeders

Animal species that successfully breed throughout the year. Example: human (*Homo sapiens*).

(C) Opportunistic breeder

Animal species that successfully breed depending upon immediate climatic and nutritional conditions. As the optimum conditions are transient, this strategy is effective only for species that can produce offspring rapidly and therefore can complete lactation before the end of favourable conditions. (Heideman and Bronson, 1992). Example: tree kangaroo (*Dendrolagus* sp.).

Seasonality and seasonal reproduction

Most animal species experience marked variations in environmental conditions throughout the year. To maximize reproductive success, many animals restrict breeding to a given season when conditions are favourable (e.g. abundant food, low thermoregulatory demands) for successful rearing of offspring (Baker, 1938; Bronson, 1989) and curtail breeding during unfavourable conditions enabling greater energetic investment directed towards individual survival (e.g. cellular maintenance, thermoregulation or immune function; Demas and Nelson 1998; Demas, 2004). Many mammalian species are able to respond to the annual variation in day-length by adaptive alterations in physiological as well as behavioural processes in anticipation of the upcoming season (Reiter, 1975; Reiter, 1993). The switch on and off of the reproductive function during the annual breeding cycle is the most striking example of such a photoperiodically induced process (Reiter, 1980). Reproduction is an extremely complex and energetically demanding process and its physiological cost is primarily borne by the female (Wade and Schneider, 1992; Schneider, 2004). However, males of many species use considerable energy to mate as well as to prevent other males from mating with its partner. Hence, the mating of many species is restricted to a specific mating season as an adaptation to cope with this high energy cost (Prendergast, 2005; Simonneaux et al., 2013). Moreover, the precise timing of the reproduction minimizes maternal ‘cost-to benefit’ ratio thereby ensures better
survival of the offspring (Dardente, 2012). The onset of breeding season has specific physiological underpinnings and is associated with activation of the HPG axis in males (Aleandri, 1996, Greives et al., 2008).

Factors regulating seasonal reproduction

The synchronization of the HPG axis to specific environmental cues is of prime importance in male reproduction in many species (Chemineau et al., 2008). The control of seasonal reproduction is an intricate interplay of both exogenous and endogenous factors (Bronson, 1989; Bronson, 2009). The exogenous factors involved in the control of reproduction are environmental factors such as photoperiod (day-length), temperature, humidity, rainfall, food availability, social cues, predation pressure, etc. The environmental variables affecting seasonal reproduction can be broadly categorized into following factors:

Ultimate factors

Ultimate factors are those variables which in the course of evolution exert selection pressure to restrict breeding activity to a particular time of the year when it is most likely to be successful. Reproductive timing mechanisms are under high selection pressure because temporal errors can result in wasted effort and can jeopardize survival (Baker, 1938; Reiter, 1975). The main ultimate factors include dietary and climatic factors particularly food availability, rainfall and temperature. Predator pressure and inter-specific competition are also regarded as ultimate factors.

Proximate factors

The immediate environmental cues that control the annual rhythm by regulating physiological processes are known as proximate factors. The proximate factors include photoperiod, temperature, humidity, rainfall etc. For a proximate factor to be reliable and accurate predictor for seasonal events it must be consistent and stable year after year (Hoffmann, 1973; Steger et al., 1985). Among the proximate factors the most accurate and reliable proximate factor is the proportion between the environmental light-dark hours i.e. photoperiod (Baker and Ranson, 1932).
Photoperiod: The core proximate factor

Due to the inclination of the Earth’s rotational axis and translation along its orbit, the duration of day-light changes throughout the year at a particular geographical location. This variation in photoperiod is regarded as the most accurate and reliable natural measure for determining the season thus, acts as the priming factor for reproduction (Goldman, 2001; Zucker, 2001; Gwinner, 2003). Therefore, photoperiod has been accepted as the most ‘noise free’ annual predictor of seasons in mammals (Turek and Campbell, 1979). Since, seasonal changes in climate and food availability can be predicted on the basis of changing photoperiod, it is considered as the most reliable predictor of upcoming environmental conditions, (Bradshaw and Holzapfel, 2007). Therefore, among the different exteroceptive factors, the photoperiod is the most extensively studied one in temperate as well as in tropical countries (Gwinner, 1981). Photoperiodism is the ability of organisms to assess and use the day-length information as an anticipatory cue to time seasonal events in their life history (Goldman, 2001). Photoperiodism is especially important in initiating physiological and developmental processes that are typically irrevocable and that culminate at a future time (Bradshaw and Holzapfel, 2007).

The first demonstration of the role of photoperiod as a predictive cue and in the control of reproductive function in mammals was found in a colony of field voles where reproduction was continuous when exposed to 15 hours of light; however, exposure to 9 hours of light hampered the reproductive processes (Baker and Ranson, 1932). Since then, photoperiodism has been shown to influence reproduction in almost all mammalian orders. The effects of photoperiod on reproduction have been classically investigated in sheep (Thwaites, 1965), Syrian hamsters (Reiter, 1975; Steger et al., 1985) and Siberian hamsters (Hoffmann, 1973). In Syrian and Siberian hamsters, exposure to short days results in marked decline in the concentration of peripheral luteinizing hormone (LH), follicle-stimulating hormone (FSH), and testosterone, accompanied by testicular regression and cessation of spermatogenic activity within 8-12 weeks, whereas maintenance of hamsters under long day condition testicular involution
(Hoffmann, 1973; Steger et al., 1985). However, in sheep, exposure to short days induces HPG axis and activates reproduction whereas exposure to long days leads to reproductive inhibition (Chemineau et al., 2010).

Both the Syrian and Siberian hamsters have been used extensively as mammalian models for investigating the phenomenon of photoperiodism, as their neuroendocrine axis is highly sensitive to the changing photoperiod and can be maintained easily in the laboratory. Later on, a number of wild rodents such as the vole (*Microtus agrestis*; Spears and Clark, 1986), the four-striped grass mouse (*Rhabdomys pumilio*; Jackson and Bernard, 1999), the white-footed mouse (*Peromyscus leucopus*; Young et al. (2000), Mongolian Gerbils (Karkas et al., 2005), Tete veld rats (*Aethomys ineptus*), Namaqua rock mice (*Aethomys namaquensis*; Muteka et al., 2006), Indian palm squirrel (*Funambulus pennant; Ahmad and Haldar, 2010a) have also been investigated to elucidate the effect of photoperiod on seasonal reproduction.

**Critical photoperiod: The duration matters**

The minimum duration of light exposure that supports reproduction in a seasonal animal is called the ‘critical photoperiod’ (Elliot, 1976). Critical photoperiod is not a fixed variable, but differs among populations of animals living at different altitudes and latitudes (Bradshaw and Holzapfel, 2001). Adult male Syrian hamsters undergo gonadal regression when exposed to < 12.5 h of light/day (Elliot, 1981) however, Siberian hamsters, which live at higher latitudes than Syrian hamsters, display a critical photoperiod of 13 h of light/day (Hoffmann, 1982). In addition to latitudinal variation, manipulation of photoperiodic history can also influence the critical photoperiod. In contrary to the Syrian hamster, where the critical photoperiod is independent of the previous light regime, the critical photoperiod in the Siberian hamster appears to be dependent on the photoperiodic history (Pevet, 1988).

**Quality and quantity of light affects reproduction**

Photoperiodism is the result of perception of light information (duration) and its interpretation *via* the photoperiodic response system. Apart from the
duration, other important characteristics of daily light are its quantity (light intensity) and quality (colour and spectral composition). The intensity refers to the quanta of photons emitted from the light source and the colour refers to the sensation experienced as a result of activation of specific photoreceptors by specific wavelength of visible light spectrum (380-760 nm). Therefore, the gradual shifts in the intensity and wavelength of light can modulate the entrainment and induction of the photoperiodic clock system and thus, provide reliable information regarding the time-of-day and time-of-year. The wavelength and intensity of light to which photoreceptors are maximally sensitive can induce a maximal response. Substantial research demonstrates the effect of light intensity on various reproductive (Asil et al., 2013) and immune responses (Lahiri and Haldar, 2009).

Black-headed bunting maintained under stimulatory (13L: 11D) photoperiod in white, green (528 nm) and red (654 nm) light with an intensity of 100 lux, showed a significant increase in testes weight under red light exposure (Kumar and Rani, 1999).

**Photo-refractoriness: A disobedience to photoperiod**

The term photo-refractoriness refers to the loss of responsiveness to photoperiod. In seasonal animals, prolonged exposure to constant photoperiod induces photo-refractoriness, causing spontaneous reversion in physiology and behaviour to a state associated with the opposite photoperiod (Lincoln et al., 2005). This is particularly evident at the spring and autumn equinoxes when seasonal mammals have completely different physiology, despite the photoperiod and the associated melatonin signal, being the same at the two times of the year (Freeman and Zucker, 2001). In Syrian hamsters, prolonged exposure to short photoperiod (10-12 week) causes gonadal regression and the development of a winter phenotype that is followed by a progressive and regulated recovery back to a summer physiology within a further period of 12–20 week, with no change in the prevailing photoperiod (Pevet, 1988). Thus, this SD refractory response permits animals in the natural winter environment to reactivate physiology in anticipation
of spring, without the requirement for the stimulus of long photoperiod (Reiter, 1972). Photorefractoriness in hamsters can be terminated by exposure to long photoperiod for 10-15 weeks, following such treatment the animals can again respond to short photoperiod (Pevet, 1988).

**Regulation of seasonal reproduction: From photonic energy to perpetuation of species**

Reproduction is a highly complex and energy consuming physiological function orchestrated by the hypothalamic-pituitary-gonadal (HPG) axis. Substantial research demonstrates that photoperiod regulate the seasonal reproductive activity through modulation of pineal function that ultimately alters GnRH release by the hypothalamus (Roy and Belsham, 2002) and gonadotropin (FSH/LH) secretion from the pituitary (Filippa et al., 2005). Maintenance of reproductive function in mammals is dependent upon activation of the hypothalamic–pituitary–gonadal (HPG) axis. Pulsatile gonadotropin releasing hormone (GnRH) secretion from the hypothalamus into the hypophysial portal system stimulates releases of gonadotropins (luteinizing hormone–LH, follicle-stimulating hormone–FSH) from the anterior pituitary, which in turn act on the gonads to stimulate the production of gonadal steroids and promote spermatogenesis and therefore supports development and maintenance of the mature gonad (Levine, 2003; Bliss et al., 2010). Gonadal steroids control secondary sexual characteristics and also feedback to the brain, where they improvise sexual behaviour and modulate GnRH and gonadotropin secretion. Throughout the lifetime, the HPG axis experiences episodes of activation/deactivation. The HPG axis can be modified at multiple levels to regulate reproductive function, some of which are influenced by photoperiod (Arendt, 1998) and the photoperiodic message appears to be conveyed by the chronobiotic neurohormone melatonin.
Melatonin: The regulator of seasonal endocrine orchestra

Melatonin (N-acetyl-5-methoxytryptamine) was first isolated and identified in the nineteen-fifties, as the hormone of the pineal gland (Lerner et al., 1958). Its name is indicative of melatonin’s first identified function, its skin-lightening properties in fish and amphibia. However, alteration in skin colouration is not applicable to mammals, whose melanocytes are devoid of physiologically controlled, mobile melanosomes. The hormone received considerable attention when it was found to regulate and reset circadian rhythms (Redman et al., 1983; Armstrong et al., 1986). In photoperiodic responders it is also involved in the measurement of day length (photoperiodic time measurement, PTM), an environmental variable used for seasonal timing of reproduction, metabolism and behaviour (Tamarkin et al., 1985; Arendt, 1986; Reiter, 1991). The occurrence of melatonin is not restricted to vertebrates, but is almost ubiquitously present in numerous taxa including, e.g., bacteria, unicellular eukaryotes and plants (Hardeland and Fuhrberg, 1996; Hardeland and Poeggeler, 2003; Hardeland et al., 2007; Paredes et al., 2009) indicating that this molecule has gained many additional functions during the course of evolution (Hardeland et al., 1995; Tan et al., 2010). Melatonin displays an exceptional multiplicity in its actions. These can
only be understood on the basis of an integrative, orchestrating role by which melatonin is distinguished from many other important signalling molecules.

**Melatonin: Biosynthesis and its photic regulation**

The daily changes in environmental light-dark conditions have profound impact on all aspects of life. As a response, wide panoply of physiological and behavioural processes in all kinds of living organisms is rhythmic (Hastings et al., 2003) and being anticipatory are strictly controlled by an internal clock (Benarroch, 2008). In vertebrates, the master clock is located in the anterior hypothalamus above the optic chiasma, in the suprachiasmatic nucleus (SCN) and generates an endogenous “circadian” rhythm with a period coinciding the 24 hours (Reppert and Weaver, 2002). In mammals, lesions in the SCN abolish the rhythm of melatonin synthesis in the pineal gland, establishing the fact that the circadian pattern of melatonin secretion is regulated by the biological clock resides in the SCN (Klein and Moore, 1979). The SCN is synchronized to the environmental light–dark cycle via light perceived through the retina, acting mainly on a subgroup of retinal ganglion cells (RGCs) that contain the photopigment melanopsin (Berson et al., 2002). These RGCs connect to the SCN via the retinohypothalamic tract (RHT). Function of the pineal gland is regulated through a complex multi-synaptic network involving the paraventricular nucleus (Larsen et al., 1998; Moore and Speh, 2004). Postganglionic sympathetic fibers from the superior cervical ganglia reach the pineal gland and regulate melatonin biosynthesis through the presynaptic release of norepinephrine (NE). NE release occurs during the “night” portion of the circadian pacemaker cycle.

The chronobiotic neurohormone melatonin is secreted during the dark phase of the light-dark cycle by the vertebrate pineal gland (Ganguly et al., 2002; Klein, 2007; Acuna-Castroviejo et al., 2014). Biosynthesis of melatonin is a four step phenomenon. First, Tryptophan, the precursor for melatonin biosynthesis, is taken up from the circulation into the pinealocyte and converted to 5-hydroxy tryptophan by tryptophan 5-monoxygenase/hydroxylase and further decarboxylated by L-aromatic amino acid decarboxylase to form 5-
hydroxytryptamine (5-HT) or serotonin. Serotonin is acetylated (N-acetylation), to form N-acetyl serotonin using arylalkylamine N-acetyl transferase (AA-NAT) formerly known as arylalkylamine N-acetyl transferase (AA-NAT). Finally, N-acetylserotonin is methylated by N-acetylserotonin-α-methyltransferase (ASMT) formerly known as hydroxyindole-α-methyltransferase (HIOMT) to form melatonin (Axelrod and Wurtman, 1968). AA-NAT is the rate-limiting enzyme in melatonin synthesis and thus playing an important regulatory role in this process and known as ‘timezyme/ melatonin rhythm enzyme’ (Klein, 2007).

Fig. 2: Melatonin biosynthetic pathway and dynamic expression pattern of ‘melatonin rhythm enzyme’ Arylalkylamine N-acetyltransferase (Adapted from Ganguly et al., 2002).

The molecular basis of rhythmic melatonin synthesis

In mammals, AA-NAT and melatonin rhythms are derived from nocturnal activation of the noradrenergic innervations of the pineal gland (Stehle et al., 2001; Klein, 2007). Cyclic AMP (cAMP) plays a highly conserved role in regulating rhythmic synthesis of AA-NAT (Ganguly et al., 2002). The marked increase in the cAMP concentration is controlled through a combinatorial
mechanism involving the activation of both α1- and β1-adrenergic receptors (Vanecek et al., 1985; Sugden et al., 1985). NE-dependent activation of β1-adrenergic receptors elevates the intracellular concentration of cAMP by increasing adenylate cyclase activity. Moreover, NE activation of α1-adrenergic receptors potentiates the β1-adrenergic effects on intracellular cAMP levels, AA-NAT activity, and melatonin synthesis through increasing [Ca^{2+}] ion concentration and activation of protein kinase C (PKC). In rodents, the cAMP/PKA pathway also allows AA-NAT stabilization by binding to 14-3-3 proteins (Schomerus and Korf, 2005; Ganguly et al., 2005) and thereby controls transcriptional mechanisms to regulate melatonin synthesis (Klein et al., 1997; Korf et al., 1998). β1-Adrenergic stimulation of the cAMP/PKA pathway leads to cyclic AMP response element (CRE)–binding protein (CREB) phosphorylation in the nuclei of pinealocytes (Roseboom and Klein, 1995; Tamotsu et al., 1995). Phosphorylated (p) CREB enhances transcription of genes (notably Aanat) bearing a CRE in their upstream promoter regions (Baler et al., 1997). Enhanced transcription of the Aanat gene elicits a dramatic increase in Aanat mRNA followed by an increase in the AA-NAT protein levels and melatonin production in the middle of the night (Borjigin et al., 1995; Roseboom et al., 1996). Other targets of pCREB in the pineal gland are the genes for the β1-adrenergic receptor (Pfeffer et al., 1999) and for the transcription factor ICER (inducible cAMP early repressor) (Stehle et al., 2011). ICER is a potent inhibitor of cAMP-inducible genes that is involved in the inhibition of Aanat mRNA accumulation at the end of the night (Maronde et al., 1999).

Switch-off mechanism of melatonin synthesis

The rhythmic expression of AA-NAT in pinealocyte is tightly regulated through both transcriptional and posttranslational mechanisms that control its proteolytic degradation in a cAMP-dependent manner (Gastel et al., 1998). This mechanism allows for a more rapid ‘switch-off’ of AA-NAT protein and melatonin production upon unexpected light at night. Under these conditions, cAMP levels immediately drop and terminate the protection of AA-NAT from
proteolytic degradation whereas AA-NAT mRNA is still elevated (Schomerus and Korf, 2005; Ganguly et al., 2005, Gupta et al., 2005). Down-regulation of melatonin production involves precise crosstalk between several regulatory processes. First, the SCN-regulated release of NE from sympathetic nerve fibres is attenuated as the night progresses (Drijfhout et al., 1996). Second, dephosphorylation of pCREB is one of the crucial mechanism for the terminate melatonin production is the pineal gland. Experimental evidences indicate that withdrawal of NE through activation of protein serine/threonine phosphatase (PSP1) causes rapid pCREB dephosphorylation followed by a rapid decrease in levels of Aanat mRNA and protein (Koch et al., 2003). Finally, the pCREB-dependent activation of ICER binds directly to CRE element in the AA-NAT promoter and represses transcription of aa-nat gene resulting in the inhibition of melatonin synthesis (Maronde et al., 1999; von Gall et al., 2000; Gupta et al., 2005).

**Fig. 3:** Melatonin biosynthesis in the pineal gland and its regulation (Modified from Arendt, 1998; Schomerus and Korf, 2005)
Neuroendocrine regulation of seasonal reproduction

In seasonal animals, photoperiod tightly regulates the reproductive activity to a particular time of the year ensuring the birth of the offspring at the most favourable time of year and thereby assures survival (Reiter, 1972; Goldman, 2001; Reiter et al., 2009). In mammals, a distinct photo-neuroendocrine circuit controls seasonal breeding via modulation of pineal hormone melatonin (Dardente, 2012). Melatonin is responsible for the seasonal regulation of reproduction, but the anatomical substrate and the cellular mechanism through which melatonin modulates sexual activity is far from complete understanding. However, recent discoveries indicate that the synthesis and release of hypothalamic GnRH require the stimulatory action from hypothalamic areas upstream to the HPG axis (Simonneaux et al., 2012). One of the major breakthroughs of the decade in the field of reproductive biology pointed out the involvement of kisspeptin (Kp) and its receptor GPR54 in the control of seasonal reproductive activity (Revel et al., 2008).

Pars tuberalis (PT): From circadian clock to seasonal output

The crucial role of melatonin in mammalian photoperiodism has been established in different seasonal breeders (Reiter, 1972; Bittman ans Karsch, 1984). Within the pineal, melatonin is produced and released during the night and therefore constitutes an internal neurochemical representation of photoperiod (Goldman, 2001). The ability of photoperiod to dictate seasonal reproductive activity via the circadian timing system was first suggested by Bunning and popularly known as “the external coincidence model” (Goldman, 1999; Goldman, 2001). SCN, the master circadian clock contains the clock genes that are known to regulate circadian rhythmicity in physiology and behaviour depending on pineal melatonin rhythm (Pevet and Challet, 2011). However, the genetic and molecular bases and organization of circadian clocks have recently been identified (Dibner et al., 2010; Mohawk et al., 2012). Recent reports indicate that besides SCN, clock genes are also present in almost every tissue and cell to regulate “local” physiology (Dardente and Cermakian, 2007). PT being not an exception also
expresses a full set of clock genes and displays persistent circadian rhythmicity (Dupre et al., 2008; Guilding et al., 2009). Melatonin is the fundamental regulator of both the rhythmic expression and relative phasing of clock genes present in PT (Carr et al., 2003; Lincoln et al., 2002; von Gall et al., 2005) that displays highest density of melatonin binding sites (Roca et al., 1996). Therefore, PT can be regarded as a melatonin dependent circadian oscillator (Dardente, 2007; Dardente, 2012). Collectively, these findings suggest that melatonin receptors act as ‘seasonal time-keepers’ that decode the neuroendocrine signal for seasonal time-keeping in the PT through activation of melatonin receptor and thus help to maintain circadian rhythms in peripheral tissues (Morgan and Hazlerrig, 2008). In this context, the regulation and dynamic expression of Per1 and Cry1, two crucial circadian clock genes, could be important mediator in the regulation of circadian rhythmicity (Pevet and Challet, 2011).

Melatonin is known to inhibit the forskolin induced cAMP accumulation in PT through activation of Gi (Hazlerigg et al., 1991) leading to the inhibition of CREB phosphorylation (von Gall et al., 2000) and subsequently suppression of Per1 expression (von Gall et al. 2002a). Further, the temporal variation in Per1 expression modulates the secretion of prolactin (PRL) (Morgan, 2000) that regulates circadian rhythmicity in animals (von Gall et al., 2002b). Further, being a key repressor of the circadian clock, acute induction of Cry1 expression via melatonin is essential to reset the PT clock (Hazlerigg et al., 2004; Maywood et al., 2013). The expression of Cry1 is tightly regulated to the onset of melatonin secretion via the expression of transcriptional co-activator EYA3 (eyes absent family of protein) (Lincoln et al., 2002). The clock-controlled expression of Eya3 is suppressed by melatonin in short photoperiod exposed animals (Dardente et al., 2014). The increased level of EYA3 is known to be responsible for the photoperiodic control of Tshβ expression in PT and therefore seasonal and circadian rhythmicity in animals (Tsujino et al., 2013).

Kiss-1/GPR54 system: The central gatekeeper of photo-neuroendocrine axis

Recent evidences indicate that kisspeptins, encoded by Kiss-1 gene and its specific receptor GPR54 represent potent regulators of the reproductive axis
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(Kisspeptins (10-54 amino acids, with common C-terminus), are regarded as the missing link between melatonin and seasonal regulation of reproduction (Simonneaux et al., 2009; Ansel et al., 2011; Simonneaux et al., 2012; Clarke and Caraty, 2013). In Syrian hamster Kiss-1 gene is expressed in the arcuate nucleus of the hypothalamus at significantly higher levels in hamsters kept in long photoperiod as compared to short photoperiod (Mikkelsen and Simonneaux, 2009; Ansel et al., 2010). The down-regulation of Kiss-1 expression in the arcuate nucleus under short photoperiod is mediated via melatonin and not by the secondary changes in gonadal steroids (Revel et al., 2006a; Ansel et al., 2010). Moreover, the chronic administration of kisspeptin restores testicular activity in hamsters exposed to short photoperiod, despite persisting photo-inhibitory conditions (Ansel et al., 2011). Therefore, a central role of Kiss-1/ GPR54 system in the photoperiodic modulation in the secretion of GnRH from hypothalamus and gonadotrophins (FSH/LH) from pituitary has been established (Revel et al., 2006a; Simonneaux et al., 2013). However, in situ hybridization and RT-PCR analysis confirmed that the kiss-1 expressing arcuate nucleus is devoid of melatonin receptor (Li et al., 2011). Therefore, the mechanisms underlying the regulation of Kiss-1 expression in the arcuate nucleus via melatonin remained unknown (Revel et al., 2007). However, recent research in this area established two more candidate genes which are regarded to be essential mediators in regulating kiss-1 expression and therefore seasonal variation in reproduction.

RFRP neurons: A missing link between melatonin and Kiss-1

The RFamide-related peptide (rfrp) gene, the mammalian ortholog of avian gonadotropin inhibitory hormone (GnIH), encodes a precursor that produces two peptides, RFRP-1 and RFRP-3 (Ukena and Tsutsui, 2005; Kriegsfeld et al., 2006; Clarke et al., 2008). RFRP neurons are located in the dorsomedial hypothalamus (DMH) and ventromedial hypothalamus (VMH) (Ukena and Tsutsui, 2001; Johnson et al., 2007; Mason et al., 2010). Substantial research indicate that both central and peripheral administration of RFRP-3 inhibits LH secretion in various mammalian species (Anderson et al., 2009; Clarke et al., 2008; Kadokawa et al.,
Therefore, an antagonistic relationship between kisspeptin and GnIH/RFRP has been emerged as a modern concept in regulation of the gonadotrophic axis (Bentley et al., 2010; Smith and Clarke, 2010; Clarke, 2011). However, recent studies established that the expression of \( rfrp \) mRNA and the number of RFRP-immunoreactive cell bodies down-regulated in the DMH/VMH of sexually quiescent Syrian hamster \( \text{via} \) melatonin under short photoperiod (Revel et al., 2008). Therefore, decreased \( rfrp \) levels in sexually inactive hamsters under short photoperiod challenged the hypothesis that RFRP-3 is a negative regulator of the gonadotrophic axis (Revel et al., 2008). Further, reduction in the Rfrp expression under short photoperiod is independent from secondary changes on gonadal steroid synthesis, whereas its regulation is strictly melatonin-dependent as evident from experiments involving pinealectomy and exogenous melatonin administration (Revel et al., 2008). Central administration of RFRP-3 in sexually active/quiescent male Syrian hamsters produced a significant increase in plasma concentrations of both LH and testosterone (Ancel et al., 2012). Interestingly, the administration of RFRP-3 has been proved to increase the Kiss1 mRNA levels in the ARC (Ancel et al., 2012). Altogether, these reports indicate that melatonin acting thorough its receptor present on the DMH/VMH and thereby modulates the expression of Rfrp which further stimulates the reproductive activity might be \( \text{via} \) the Kiss1 neurons of the ARC.

**TSH driven tanycyte deiodinase (Dio2/Dio3) system**

The crucial role played by thyroid hormone in the seasonal regulation of reproduction has long been recognized however, understanding of the underlying mechanisms and its relationship with photoperiod and thus melatonin started quite recently (Karsch et al., 1995; Shinomiya et al., 2014). Deiodinase 2 (\( \text{Dio} \, 2 \)) and deiodinase 3 (\( \text{Dio} \, 3 \)) are enzymes involved in the metabolism of thyroid hormone and thereby regulate its local availability. \( \text{Dio} \, 2 \) converts the relatively inactive T4 into the active T3 while \( \text{Dio} \, 3 \) inactivates T4 by converting it into rT3, and also degrades T3 into T2 (Dardente et al., 2014). The central expression of \( \text{Dio} \, 2 \) and \( \text{Dio} \, 3 \) are expressed specifically in pineal gland (Dardente et al., 2014), tanycytes
and astrocytes (Tu et al., 1997), lining the third ventricle and median eminence, in a photoperiod dependent manner in different seasonal breeders (Yoshimura et al., 2003; Revel et al., 2006b; Hanon et al., 2008; Hanon et al., 2010). Tanycytes are a heterogeneous population of ependymal cells, which constitute a gateway between the CSF and the MBH and median eminence (Guerra et al., 2010). The expression of Dio2 and Dio3 displays reciprocal regulation by photoperiod and thus melatonin, independently of sex steroids (Revel et al., 2006b; Helfer et al., 2013). Dio2 and Dio3 are highly expressed under long and short photoperiod respectively and thereby regulate T3 content within the MBH (Yoshimura et al., 2003; Revel et al., 2006b). Therefore, it can be suggested that the local T3 levels in the MBH could increase under long photoperiod (Dardente et al., 2014). Recent studies indicate that mammalian PT is capable of synthesizing TSH in a photoperiod dependent manner with lower levels under short photoperiod (Dardente et al., 2003; Dardente et al., 2010). TSH secreted from the PT stimulates Dio2 activity via TSH receptors located on the tanycytes of the MBH (Hanon et al., 2008; Nakao et al., 2008). Therefore PT can be regarded as an ‘indirect-T3-generator’ in the MBH (Dardente et al., 2014). Further, chronic central infusion of T3 reactivates photoinhibited reproductive activity in the Siberian hamster (Barrett et al., 2007). Altogether, these recent findings point to the PT-TSH/tanycyte-T3 model as a pivotal system for the photoperiodic control of reproduction (Simonneaux et al., 2012). Interestingly, recently findings suggest that melatonin induces a profound remodeling of the tanycyte in the median eminence to regulate GnRH secretion (Bolborea et al., 2013).

**T3/RFRP-3/ kisspeptin: A unifying photo-neuroendocrine regulation**

The individual participation of kisspeptin, RF-amide related peptides and local availability of T3 in regulation of seasonal reproductive activity has already been proposed however, the underlying dialogue between these regulators is not fully understood. Interestingly, the MBH hosts two cell populations expressing RF-amide related peptides; the neurons of the ARC, which express Kiss1 and neurons of the VMH/DMH, which express Rfrp precursor (Dardente et al.,
Both *Kiss1* and *Rfrp* expression display marked photoperiod dependent variation in mammals (Revel et al., 2008; Simonneaux et al., 2012) however, the photoperiodic control of *Kiss1* and *Rfrp* is likely to be indirect (Dardente, 2012; Beltramo et al., 2014). In this context, a possible role of PT-derived TSH could be suggested (Dardente et al., 2012). Intra cerebroventricular infusion of TSH induces *Dio2* expression within ependymal cells and restores the expression of *Kiss1* and *Rfrp* in MBH similar to long photoperiod (Klosen et al., 2013). This mechanism might underlie the reactivation of the gonadal axis in Siberian and Syrian hamsters following T3 administration (Henson et al., 2013). Therefore, the most parsimonious model could be that the photoperiodic regulation of TSH within the PT modulates the local availability of T3 in tanycytes, which further stimulate the kiss-1 and rfrp-3 and thereby participate in the seasonal control of GnRH secretion and reproductive activity.

**Fig. 4:** Schematic representation of a putative hypothalamic T3/RFRP/Kp network in regulation of seasonal reproduction in mammals (Adapted from Simonneaux et al., 2012)
Melatonin Receptors

The study of melatonin receptors can be traced back to the demonstration of skin blenching activity of bovine pineal extract in *Rana pipsiens* tadpole by McCord and Allen in 1917 leading to the elucidation of its chemical structure (Lerner et al., 1959). However, the first evidence that suggested the existence of melatonin receptors culminated from the experiments carried out to measure the efficacy of melatonin and melatonin analogues to induce aggregation of melanosomes of amphibian dermal melanophores that postulated the presence of melatonin receptors and established structure-activity relationships of melatonin analogues (Heward and Hadley, 1975). This bioassay was further utilized to demonstrate that melatonin induced pigment aggregation could be blocked by the use of pertussis toxin (PTx) suggesting that melatonin receptors are coupled to a pertussis toxin-sensitive G-protein (White et al., 1987).

The first attempts to identify brain melatonin receptors employed $[^3H]$-melatonin as a radioligand to label binding sites in membranes from bovine hypothalamus, cerebral cortex, and cerebellum (Cardinali et al., 1980). However, the development of 2-$[^{125}I]$-idomelatonin analog (Vakkuri et al., 1984) was a major breakthrough and can be regarded as a silver bullet in the field of melatonin receptor research. The first prominent use of 2-$[^{125}I]$-idomelatonin to identify melatonin receptor binding was accomplished by Dubocovich and Takahashi (1987); they identified high affinity melatonin binding sites in chicken retina. Dubocovich, Nile and their colleagues identified a 2-$[^{125}I]$-idomelatonin binding site in hamster brain with a fundamentally different pharmacological profile, in particular a relatively higher affinity for N-acetyl serotonin and prazosin (Duncan et al., 1986; Niles et al., 1987; Duncan et al., 1988; Pickering and Niles, 1990). Furthermore, by using iodinated radioactive probe, melatonin binding was also detected in several areas of brain e.g. choroid plexus and in some brain arteries (Stankov et al., 1993; Capsoni et al., 1994), as well as in peripheral organs like the Harderian glands, lymphoid organs, the adrenals, heart, lungs, the gastrointestinal tract, the mammary glands, the kidney and the male reproductive organs (Poon
Characterization of 2-[\(^{125}\)I]-idomelatonin binding sites

Based on the pharmacological and kinetic differences in 2-[\(^{125}\)I]-idomelatonin binding affinities, the melatonin binding sites have been characterized into two putative melatonin receptors and termed as ML\(_1\) and ML\(_2\) receptors (Dubocovich, 1988; 1995). The ML\(_1\) pharmacological profile (2-iodomelatonin > melatonin >> \(N\)-acetylserotonin) was exhibited by both 2-[\(^{125}\)I]-iodomelatonin binding in mammalian retina and pars tuberalis (PT) and the functional presynaptic receptor characterized in rabbit retina (Dubocovich, 1988, 1995; Hagan and Oakley, 1995). In contrast, 2-[\(^{125}\)I]-iodomelatonin binding to the ML\(_2\) site (later termed MT3) in hamster brain was distinguished by another endogenous ligand, \(N\)-acetylserotonin, that showed equal affinity with melatonin (ML\(_2\): 2-iodomelatonin > melatonin = \(N\)-acetylserotonin) (Dubocovich, 1988, 1995; Molinari et al., 1996). The pharmacological binding specification for ML\(_1\) and ML\(_2\) sites (Dubocovich, 1995; Dubocovich et al., 2010) is summarized in the table:

Table: 1 Characteristic features of 2-[\(^{125}\)I]-idomelatonin binding sites

<table>
<thead>
<tr>
<th>Attributes</th>
<th>ML(_1)</th>
<th>ML(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common name</td>
<td>High affinity (pM)</td>
<td>Low affinity (nM)</td>
</tr>
<tr>
<td>Affinity states</td>
<td>High (10-300 pM)</td>
<td>High (0.9-10 nM)</td>
</tr>
<tr>
<td></td>
<td>Low (0.3-5 nM)</td>
<td></td>
</tr>
<tr>
<td>Kinetics:</td>
<td>Slow (t_{1/2} = 9-60) min</td>
<td>Fast (t_{1/2} = 1-2) min</td>
</tr>
<tr>
<td>Association</td>
<td>Slow (t_{1/2} = &lt; 40) min</td>
<td></td>
</tr>
<tr>
<td>Dissociation</td>
<td></td>
<td>Fast (t_{1/2} = 1-2) min</td>
</tr>
<tr>
<td>Regulation</td>
<td>GTP: Yes</td>
<td>Na(^+): Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td><strong>Ca&lt;sup&gt;2+&lt;/sup&gt;</strong></td>
<td><strong>Mg&lt;sup&gt;2+&lt;/sup&gt;</strong></td>
<td><strong>Temperature</strong></td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>Yes (affinity increases)</td>
</tr>
<tr>
<td>No</td>
<td>No</td>
<td>Yes (affinity decreases)</td>
</tr>
</tbody>
</table>

6-OHMEL, 6-hydroxymelatonin; 2-IMEL, 2-[125I]-idomelatonin; MEL, melatonin; 6-CLMEL, 6-chloromelatonin; 5-HT, 5-hydroxytryptamine; NA 5-HT, N-acetyl 5-HT; AP, area postrema; CA, caudal artery; CW, circle of Willis; PT, pars tuberalis; PVNT, paraventricular thalamic nucleus; SC, super colliculus; SCN, suprachiasmatic nucleus.

**Cloning of the melatonin receptors**

Till date two high affinity melatonin receptor subtypes have been cloned and characterized in mammals and were initially termed as MT<sub>1</sub> and MT<sub>2</sub> (formerly known as Mel<sub>1a</sub> and Mel<sub>1b</sub>) (Reppert et al., 1995a; Roca et al., 1996; Browning et al., 2000; Dubcovich et al., 2010). A third subtype of high affinity receptor was cloned from chicken brain with 80% sequence similarity to *Xenopus* receptor and was termed as Mel<sub>1c</sub> (Reppert et al., 1995b). More recently two other isoforms of melatonin receptors have been identified from *Xenopus laevis* skin. These receptor cDNA appears to be alleles of a single locus and were designated as Mel<sub>1c (α)</sub> and Mel<sub>1c (β)</sub> (Jockers et al., 1997). So far no mammalian homolog of Mel<sub>1c</sub> receptor has been reported and no full length sequence is available for non-mammalian Mel<sub>1b</sub>. Thus, of the three subtypes two are present in mammals and one in lower vertebrates like amphibians and birds however, characteristics of high affinity ML<sub>1</sub> binding existed in each of the three subtypes reported.
Nomenclature of melatonin receptor

The International Union of Pharmacology (NC-IUPHAR) has renamed the existing mammalian melatonin receptor clones in 1998 (Dubocovich et al., 1998). The official nomenclature designated melatonin receptors with letters MT (Melatonin) based on its endogenous ligand, each particular receptor type was denoted by a numerical subscript (e.g., MT₁, MT₂). MT₁ (referred to as MT1 hereafter) and MT₂ (referred to as MT2 hereafter) belongs to the ML₁ class of the receptor based on their ¹²⁵I-mel binding pharmacokinetics while, MT₃ (referred to as MT3 hereafter) belongs to ML₂ type receptor class. The IUPHAR classifies the two cloned mammalian melatonin receptors into two types MT1 and MT2. These two high-affinity melatonin receptor types, MT1 and MT2, have been cloned in mammals: humans, sheep, Siberian hamsters, mice, and rats (Reppert et al., 1994, 1995a,b; Roca et al., 1996; Reppert et al., 1996; Browning et al., 2000; Jin et al., 2003; Audinot et al., 2008; Coge et al. 2009) till date. Unfortunately this nomenclature does not take into account the receptors found in the non-mammalian species (i.e., Mel₁c; Dubocovich et al., 2010).

Melatonin binding sites: Beyond membrane receptors

Melatonin is also known to be a ligand for a retinoid related orphan nuclear hormone receptor (RZR/ROR) (Becker-Andre et al., 1994). These nuclear receptors belong to the RZR/ROR orphan receptor subfamily, which includes three subtypes (α, β, γ) and four splicing variants (Becker-Andre et al., 1993). RORα₁ and RORα₂ seem to be involved in some aspects of immune modulation, and RZR-β is expressed in the central nervous system, including the pineal gland (Wiesenber et al., 1998; Carlberg, 2000). Moreover, ROR-α was assumed to mediate up regulations of antioxidant enzymes (Wiesenber et al., 1998). Still, the full spectrum and physiological meaning of these receptors remains to be clarified. In addition, melatonin interacts with intracellular proteins such as calmodulin (Benitez-King and Anton-Tay, 1993), calreticulin (Macias et al., 2003) or tubulin (Cardinali and Freire, 1975) and antagonizes the binding of Ca²⁺ to calmodulin (Benitez-King, 2006). These interactions are most likely related to some of the
physiological effects of melatonin but critical data regarding this point have yet to be obtained.

**Distribution of membrane melatonin receptors**

The distribution of membrane melatonin receptors (MT1 and MT2) have been investigated through functional, immunohistochemical, and genetic knock-out studies. The presence of MT1 receptors have been reported in various regions of the central nervous system (SCN) including the pars tuberalis (PT) (von Gall et al., 2002b; 2005) and the SCN (Liu et al., 1997; Dubocovich et al., 2005; Dubocovich, 2007), hippocampus (Savaskan et al., 2002), central dopaminergic pathways (i.e., substantia nigra, ventral tegmental area, nucleus accumbens, caudate-putamen) (Uz et al., 2005).

Among the peripheral targets MT1 enjoys a wide distribution in retina (Scher et al., 2002; 2003), ovary (Clemens et al., 2001), testis (Valenti et al., 1997; Izzo et al., 2010; Frungieri et al., 2005), mammary gland (Ram et al., 2002), coronary blood vessels and aorta (Ekmekcioglu et al., 2001), liver and kidney (Naji et al., 2004), adrenal (Torres-Farfan et al., 2003; Richter et al., 2008), gallbladder (Aust et al., 2004), exocrine pancreas (Aust et al., 2008), β cells of endocrine pancreas (Mühlbauer and Peschke, 2007), skin (Slominski et al., 2005) and the immune system (Carrillo-Vico et al., 2003; Pozo et al., 2004, Ahmad and Haldar, 2010a,)

MT2 melatonin receptors are more restrictively expressed in the brain and its mRNA has been found in human retina, hippocampus, and whole brain (Reppert et al., 1994, 1995a; Weaver and Reppert, 1996). Among the peripheral organs, the membrane receptors MT2 were demonstrated in duodenal enterocytes (Sjöblom et al., 2001, 2003), β cells of endocrine pancreas (Mühlbauer and Peschke, 2007; Mulder et al., 2009), skin (Slominski et al., 2005), myometrium (Schlabritz-Loutsevitch et al., 2003; Sharkey et al., 2010), testes (Izzo et al., 2010), placenta (Lanoix et al., 2008), granulosa and luteal cells (Woo et al., 2001; Tamura et al., 2009), cardiac ventricular wall (Ekmekcioglu et al., 2003), aorta, coronary and cerebral arteries and other parts of peripheral vasculature (Ekmekcioglu, 2006; Cui et al., 2008), brown and white adipose tissues (Brydon et
al., 2001) and various immune cells (Pozo et al., 2004; Dubocovich and Markowska, 2005; Sánchez-Hidalgo et al., 2009a, b; Ahmad and Haldar, 2010a). Interestingly, Siberian hamsters Phodopus sungorus, could not express a functional MT2 receptor and is popularly called as “nature’s knock-out for MT2”.

**Multiplicity of target organs**

Melatonin receptors have been detected in numerous tissues. In preliminary investigations, this was revealed by $[^{3}H]$-melatonin (Cardinali et al., 1979; Niles et al., 1979) and subsequently, $^{125}$I-2-iodomelatonin binding (Vanecek et al., 1987). Later, after characterization of the membrane-bound receptors MT1 and MT2 (Reppert et al., 1994, 1995a, b), corresponding data were obtained in expression studies at mRNA or protein levels. In many mammalian species, the membrane receptors have been identified in various sites of the central nervous system and in peripheral organs, such as gastrointestinal tract, liver, lung, skin, Harderian gland, adrenal gland, gonads and male accessory organs, mammary tissue, kidney, heart, blood vessels, adipose tissue, neutrophils, lymphocytes and lymphoid tissues (Dubocovich and Markowska, 2005; Sallinen et al., 2005; Pandi-Perumal et al., 2008; Ishii et al., 2009). However, considerable species differences exist in the distribution melatonin receptors. A comprehensive survey of the literature suggests a wide distribution of peripheral (non-neural) melatonin receptors while a few tissues are obviously devoid of those (Slominski et al., 2012). Moreover, the reason underlying the absence of melatonin receptors in some tissues may be merely because no one investigated their presence at these sites (Reiter et al., 2013).

**Functional pleiotropism of melatonin**

Melatonin is a pleiotropic molecule and acts as a conductor of the large endocrine orchestra (Hardeland and Poeggeler, 2008; Hardeland and Coto-Montes, 2010; Hardeland et al., 2011). The pleiotropy of melatonin has been suggested at different levels, from the sites of synthesis and local dynamics, distribution of receptors and other binding sites in target organs, cell specific differences in signalling as related to the presence of G-protein variants, intracellular effects and further signal molecules (Radogna et al., 2010; Luchetti et al., 2010). Melatonin
participates in diverse physiological functions and has great functional versatility related to the regulation of circadian rhythms and seasonal behaviour in seasonal breeders (Reiter, 1991; Frungieri et al., 2005), the pre-mammillary hypothalamus and the PT (Dardente, 2012, Dardente et al., 2014) and peripheral reproductive organs (Reiter et al., 2009; Reiter et al., 2013), sexual development (Roy and Belsham, 2002), retinal physiology (Lundmark et al., 2006), osteogenesis (Sanchez-Hidalgo et al., 2007), tumour inhibition, antioxidant (Rodriguez et al., 2004; Galano et al., 2011, Reiter, 2014), anti-aging (Anisimov et al., 2006; Vishwas et al., 2013a; Singh and Haldar, 2014) and immunoenhancer properties (Guerrero and Reiter, 2002; Gupta et al., 2015), energy metabolism (Lardone et al., 2014; Rocha et al., 2014) and also influences mitochondrial electron flux, and mitochondrial biogenesis (Pandi-Perumal et al., 2006).

**Fig. 5:** Overview of the functional pleiotropism of melatonin (Adapted from Hardeland et al., 2011)
Signalling of membrane melatonin receptors

The signal-transduction pathways triggered by MT1 and MT2 receptors were characterized in various primary cell cultures and tissues and in different mammalian cell lines expressing the recombinant receptors through activation of both pertussis toxin-sensitive and -insensitive G proteins (Masana and Dubocovich, 2001; von Gall et al., 2002b; Witt-Enderby et al., 2003). However, the signalling pathways for melatonin receptors appear to vary among different tissues and cell types (von Gall et al., 2002b; Witt-Enderby et al., 2003). The classical effect of both the receptors is transmitted by pertussis toxin-sensitive Gi proteins which cause a decrease in cAMP, followed by decline in PKA activity and CREB phosphorylation (Godson and Reppert, 1997; Jin et al., 2003; Dubocovich et al., 2003; Dubocovich and Markowska, 2005; Dubocovich et al., 2010). This pathway is classically involved in the acute effects of melatonin on a small cluster of genes in the pars tuberalis region of the anterior pituitary, which are associated with the neuroendocrine functions of the indole and include the same MT1 receptor gene (Fustin et al., 2009). In addition, the MEK1/2-ERK1/2 pathway is stimulated by MT1 receptors in non-neuronal cells (Witt-Enderby et al., 2000; New et al., 2003; Radio et al., 2006). Melatonin similarly attenuates cGMP accumulation in cell lines expressing recombinant *Xenopus* Mel1c or Mel1cß receptors (Jockers et al., 1997). The induction of cGMP levels by melatonin has also been reported in cells such as lymphocytes (Lopez-Gonzalez et al., 1992). Activation of MT1 receptor also induces a transient elevation of cytosolic Ca²⁺ and inositol phosphate accumulation (Brydon et al., 1999b; Roka et al., 1999). MT1 receptor mediates vasoconstriction by decreases in cAMP-mediated phosphorylation of calcium activated potassium channels (BKCa) through G/Go protein coupled to MT1 melatonin receptors present in the smooth muscle (Nelson and Quayle, 1995; Geary et al., 1998; Masana et al., 2002).

Similarly, activation of the MT2 receptor inhibits forskolin stimulated cAMP production (Reppert et al., 1995a; MacKenzie et al., 2002) and stimulates JNK (Chan et al., 2002) and phosphoinositide turnover (MacKenzie et al., 2002). Collectively, the repertoire of G-protein-dependent signaling pathways activated...
by MT1 and MT2 receptors is very similar. Subtype-specific differences have only been reported in some cases. For instance, the MT2 receptor inhibits cGMP formation through the soluble guanylyl cyclase pathway, but MT1 does not in HEK293 cells (Brydon et al., 1999a; Petit et al., 1999). Furthermore, activation of PKC in the SCN occurs only through MT2 receptors and not MT1 despite the expression of both subtypes (Hunt et al., 2001). Although the multiple signaling pathways of melatonin, along with their cell type-specific consequences appear highly complex, it should be noted that the pleiotropy of melatonin’s actions is not yet fully described. The basic pathway of action of melatonin signal transduction is explained in the following figure.

**Fig 6:** Melatonin receptor MT1 (A) and MT2 (B) signalling pathways (Adapted from Dubocovich et al., 2010).
**Testes: The primary male reproductive organ**

The testes (sing: testis) are twin ovoid glandular organs that are central to the function of the male reproductive system. It is the unique site for the production of spermatozoa (spermatogenesis) and the primary organ for the synthesis of male sexual hormone (steroidogenesis). The term spermatogenesis describes and includes all the processes involved in the production of male gametes, whereas steroidogenesis refers to the enzymatic cascade leading to the production of male steroid hormones. Spermatogenesis and steroidogenesis take place in two morphologically and functionally distinguishable compartments due to the presence of blood-testes-barrier. These are the tubular compartment, consisting of the seminiferous tubules (tubuli seminiferi) and the interstitial compartment (interstitium) in between the tubules. Although anatomically separate, both compartments are closely connected with each other. For quantitatively and qualitatively optimal production of sperm, the integrity of both compartments is necessary. The function of the testis is primarily governed by endocrine regulation *via* the hypothalamus and the pituitary gland. These endocrine effects are mediated and modulated at the testicular level by paracrine and autocrine control mechanisms (Weinbauer et al., 2010).

**Neuroendocrine regulation of testicular functions**

The primary functions of the testis are the production of spermatozoa and biosynthesis of androgen. The endocrine regulation of spermatogenesis and steroidogenesis is primarily governed by the hypothalamus and hypophysis *via* GnRH and gonadotropins. The gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are produced and secreted by the gonadotrops of the anterior pituitary and regulate steroidogenesis and spermatogenesis in the testis. Pituitary gonadotropes in turn, are regulated by the hypothalamic gonadotropin-releasing hormone (GnRH). Since GnRH secretion is pulsatile, gonadotropin release also occurs in discrete peaks, more evident in the case of LH, due to its shorter half-life in circulation compared to FSH. In turn, GnRH secretion
depends on the activation of the GPR54 receptor and stimulated by kisspeptin (Simonneaux et al., 2012). Due to their strict anatomical and functional connection, hypothalamus and pituitary gland are considered to be a unique functional unit for the maintenance of testicular functions (Weinbauer et al., 2010). Importantly, the hypothalamo-hypophyseal circuit is subject to negative feedback regulation mediated by testicular factors. Testosterone inhibits the secretion of LH and FSH. For FSH, the protein hormone inhibin B also plays an important regulatory role (Boepple et al. 2008).

Fig. 7: Hormonal regulation of the testicular functions  
(Adapted and modified from www.austincc.edu)
Neuroendocrine regulation of spermatogenesis

Spermatogenesis is the process through which the spermatogonia undergo sequential mitotic and meiotic cell divisions and differentiation to produce mature spermatozoa. Spermatogenesis is an extremely active and finely orchestrated physiological process and is exclusively dependent on testicular steroid biosynthesis (Holdcraft and Braun, 2004). For the better understanding of the hormonal regulation of spermatogenesis, the following terminologies are essential:

a) **Initiation of spermatogenesis:** First complete cycle of spermatogenesis during puberty

b) **Maintenance of spermatogenesis:** Hormonal requirements of intact spermatogenesis in the adult

c) **Reinitiation of spermatogenesis:** Hormonal requirements for the restimulation of gametogenesis after transitory interruption

d) **Qualitatively normal spermatogenesis:** All germ cells are present although in subnormal numbers

e) **Quantitatively normal spermatogenesis:** All germ cells are present in normal numbers

Considerable efforts have been made to understand the relative importance of LH/testosterone and FSH for qualitative and quantitative initiation, maintenance and reinitiation of spermatogenesis. It is generally assumed that either testosterone or FSH alone is able to initiate, maintain and reinitiate spermatogenesis but only to a qualitative extent (Weinbauer et al. 2004). In order to achieve quantitative effects on germ cell production and sperm numbers, at least under physiological conditions, both LH and FSH activities are necessary. Complete spermatogenesis is seen in the vicinity of testosterone-producing Leydig cell tumors and in patients with activating mutations of the LH receptor, suggesting that pharmacologically high local testosterone concentrations induce sperm formation (Walker, 2011). This is normally pursued clinically via the administration of hCG, which contains high LH activity, together with FSH. Further, defective FSH β subunit
presents with azoospermia (Lindstedt et al. 1998; Phillip et al. 1998) suggesting the need of FSH for complete initiation of spermatogenesis. Conversely, in Pasqualini syndrome, a disorder with selective LH deficiency complete spermatogenesis is achieved, indicating the ability of FSH to initiate the entire male germ cell development cascade. Exogenous administration of supranormal doses of testosterone suppresses gonadotropin secretion through the negative feedback mechanism and leads to a drastic decrease in the number of spermatozoa. In gonadotropin-suppressed condition, either FSH or LH maintained spermatogenesis (Matthiesson et al. 2006). The importance of FSH is also evident from a hypophysectomized patient in whom an activating mutation of the FSH receptor coexisted with normal spermatogenesis in the absence of LH (Gromoll et al. 1996). Conversely, inactivating mutations of FSH action do not necessarily lead to a complete block of spermatogenesis (Huhtaniemi, 1996). However, either hormone on its own has the potential to elicit the entire spermatogenic process. In certain animal species, e.g., Djungarian hamsters, FSH is the only hormone responsible for spermatogenesis, while LH and testosterone stimulate the development of androgen-dependent organs and sexual behaviour. Conversely, in primates, both gonadotrophins are necessary for spermatogenesis. The biological meaning of this dual regulation system is not clear yet (Weinbauer et al. 2004).

A. Neuroendocrine regulation of testicular steroidogenesis

Androgen biosynthesis in testes leads to the conversion of cholesterol to testosterone. This transformation is achieved through five different enzymatic steps in which the side chain of cholesterol is shortened through oxidation from 27C to 19C. Biosynthesis of testosterone starts with the shortening of the cholesterol side chain through C 22 and C 20 hydroxylases, followed by cleavage of the bond between C 20 and C 22, leading to production of pregnenolone. The formation of pregnenolone from cholesterol occurs in the mitochondria whereas the steps following pregnenolone formation occur in the smooth endoplasmic reticulum (Zirkin et al., 2000). Testosterone synthesis occurs through the intermediates progesterone, 17α-OH-progesterone, androstenedione and finally testosterone. Cholesterol has to be transported within the cell to the mitochondria.
where it is imported into the cristae of the mitochondria. The discovery of the steroidogenic acute regulatory protein (StAR) and related proteins containing StAR-related lipid transfer domains have helped much to understand this limiting step of testosterone synthesis. StAR mRNA expression is triggered by endocrine stimuli and is rapidly and widely distributed in steroidogenic tissues. StAR moves cholesterol from the outer to the inner mitochondrial membrane, but acts exclusively on the outer membrane (Stocco, 2001). The precise mechanism by which StAR’s action stimulates the influx of cholesterol remains unclear, but when StAR connects to cholesterol it performs a conformational change that opens a cholesterol-binding pocket (Miller, 2007). After a phosphorylation StAR interacts with voltage-dependent anion channel 1 (VDAC1) on the outer membrane, which processes the phospho-StAR to a smaller intermediate. If VDAC1 is lacking, phospho-StAR is degraded by cysteine proteases preventing the mitochondrial membrane transport (Bose et al. 2008). It is well known that StAR transcripts are rapidly synthesized in response to luteinizing hormone and cAMP (King et al., 2002). At the inner mitochondrial membrane site cytochrome P450ssc (ssc = side chain cleavage) catalyzes the conversion of cholesterol into pregnenolone (Payne and Hales, 2004). The enzyme cytochrome P450ssc is responsible for the different enzymatic reactions leading to the production of pregnenolone. Like other steroid synthetic enzymes, it belongs to the group of monooxygenases, containing a prosthetic hemogroup and is localized on the internal membrane of mitochondria. This reaction consists of three consecutive monooxygenations requiring two electrons to activate molecular oxygen; a 22-hydroxylation, 20-hydroxylation and the cleavage of the C20-C22 bond, yielding pregnenolone and isocaproic aldehyde. Pregnenolone diffuses across the mitochondrial membranes and is transformed into testosterone through sequential actions of 3β-HSD, 17α-hydroxylase, C17-20 lyase and 17-KSR (17-ketosteroid reductases, also known as 17β-HSD). Testosterone is the main secretory product of the testis, along with 5α-dihydrotestosterone (DHT), androsterone, androstenedione, 17-hydroxyprogesterone, progesterone and pregnenolone.
B. Local regulation of testicular functions

The regulation of testicular function is primarily controlled by central structures. The complexity of the testicular cell types and architecture also mandates a variety of local control and regulatory mechanisms. The categories of local interactions and communication can be classified as paracrine, autocrine and intracrine. The term “paracrinology” has inadvertently been used earlier to characterize all types of testicular cell interactions which seem better described by “local interaction” (Weinbauer and Wessels 1999). In addition, the interplay between the different testicular compartments is also subsumed under local interactions. It is evident that the endocrine mechanisms play the central role in the regulation of testicular function and factors produced locally are important for the modulation of hormone activity and local factors could thus be seen as mediators of hormone action and intra-/intercellular communication. From this point of view both gametogenesis and endocrine function of the testis are under local control. An example for this might be the earlier report of stage-specific expression of androgen receptor in the human testis (Suarez-Quian et al. 1999). While Sertoli
cells were viewed as coordinators and regulators of germ cell development and maturation for a long time, these cells are now believed to be influenced by germ cell products that can influence the secretory activity of Sertoli cells. Hence, Sertoli cells are under the local control of germ cells having varying requirements for metabolic substances depending on the spermatogenic cycle phase (Franca et al. 1998). A plethora of factors with potentially local testicular activity has accumulated, e.g., growth factors, stem cell factors, immunological factors, opioids, oxytocin and vasopressin, peritubular cell modifying substance, renin and angiotensin, growth hormone-releasing hormone (GHRH), corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH), gonadotropin-releasing hormone (GnRH), calmodulin, ceruloplasmin, transport proteins, glycoproteins, plasminogen activator, metalloproteases, dynorphin, pituitary adenylate cyclase-activating peptide (PACAP), etc. Moreover, it can be reasonably assumed that other, still unidentified factors mediate the communication between interstitial and tubular compartments, between Sertoli cells and germ cells and between germ cells (Roser, 2001).

**Fig. 9:** Paracrine/autocrine regulation of testicular function (Adapted from Roser, 2001).
Melatonin and melatonin receptor in regulation testicular steroidogenesis

Endogenously released melatonin resulting from changes in day length modulates reproduction in seasonal breeders in part through activation of melatonin receptors at multiple levels of the hypothalamic-pituitary-gonadal (HPG) axis (Reiter, 1980; Tamarkin et al., 1985; Vanecek, 1998; Malpaux et al., 2001; Roy et al., 2001; Soares et al., 2003; Frungieri et al., 2005). In immortalized GnRH-releasing cells, activation of endogenous MT1 and MT2 receptors decreased the expression of GnRH mRNA in a 24-h cyclical manner, which can be blocked by luzindole (Roy et al., 2001). In the neonatal rat pituitary gland, melatonin inhibits GnRH-induced LH release, cAMP and cGMP accumulation, and increases in intracellular Ca\(^{2+}\) through activation of a pertussis toxin-sensitive GPCR (Martin et al., 1980; Vanecek and Vollrath, 1990; Vanecek and Klein, 1995). The mechanism(s) by which melatonin modulates pituitary gonadotropin secretion involves activation of MT1 melatonin receptors (Johnston et al., 2003); however, participation of MT2 receptors (Balik et al., 2004) cannot be excluded. Regulation of ovarian and testicular function by melatonin also involves activation of both MT1 and MT2 receptors along the hypothalamic-pituitary-gonadal axis (Frungieri et al., 2005). Melatonin may also exert direct effects on ovarian function, because it is found in ovarian follicular fluid (Brzezinski et al., 1987; Ronnberg et al., 1990). Specific 2-[\(^{125}\)I]-iodomelatonin binding, as well as MT1 and MT2 melatonin receptor mRNAs and MT1 melatonin receptor protein, was identified in various ovarian structures (Niles et al., 1999; Clemens et al., 2001; Woo et al., 2001; Soares et al., 2003). Endogenous estrogens regulate the functional activity of melatonin receptors (Soares et al., 2003), whereas melatonin stimulates progesterone secretion from granulosa cells in several species including humans (Schaeffer and Sirotkin, 1995; Woo et al., 2001). In human granulosa-luteal cells, melatonin increases LH and decreases GnRH receptor density (Woo et al., 2001). In hamster testicular Leydig cells, melatonin inhibits basal and chorionic gonadotropin-stimulated cAMP and androgen production through activation of MT1 receptors which can be blocked by melatonin receptor antagonist, luzindole (Frungieri et al., 2005). In several mammalian species,
changes in photoperiod regulate reproduction via the duration of the melatonin signal known to encode the length of the night (Bartness et al., 1993). Reproduction in hamsters is regulated by melatonin-mediated photoperiodic signals; however, this species does not express functional MT2 melatonin receptors (Weaver et al., 1996b). Moreover, the presence of two nonsense mutations in the coding frame of the MT2R subtype renders it inactive in the transduction of photoperiodic stimuli in hamster (Weaver et al., 1996b; Jin et al., 2003) to an extent that transduction of photoperiodic stimuli occurs principally via the MT1R (Yasuo et al., 2009). Therefore, the reproductive responses are assumed to be mediated by activation of MT1 melatonin receptors. Yasuo et al. (2009) reported differential regulation of photoperiod-induced changes in Diodinase 2 and Diodinase 3 expression in C3H mice with genetic disruption of the melatonin membrane receptors. These studies provided evidence for distinct functions for the MT1 and MT2 melatonin receptors in the regulation of photoperiodic responses in pars tuberalis and confirmed that melatonin through activation of the MT1 melatonin receptor transmit photoperiod information to the hypothalamus-hypophysial axis to regulate reproductive function (Yasuo et al., 2009).

**Opiatergic control of testicular steroidogenesis**

Opioid peptides are well known for their role in the inhibition of pain transmission or analgesia (Stein, 1999). Moreover, the participation of opioid peptides in the regulation of reproductive physiology at multiple sites has been established (Fabbri et al., 1989). Endogenous opioid peptides are neurotransmitters involved in cell communication (Subirán et al., 2011). They are ubiquitously distributed in different organs and tissues of the male and female reproductive tract, suggesting that they may regulate some of the processes involved in reproductive function (Subirán et al., 2011). Endogenous opioid peptides (EOPs) are synthesized from the processing of their precursors, which are encoded by three different genes: pro-enkephalin (PENK), pro-opiomelanocortin (POMC) and pro-dynorphin (PDYN) (Cesselin, 1995). The POMC precursor, which in part contains the β-endorphin peptide, is a precursor of adrenocorticotropic hormone (ACTH), α- and β-melanotropin (MSH) (Turner, 1986). EOPs exert their action
through opioid receptors. Opioid receptors are G-protein–coupled receptors that are monomeric and exhibit seven transmembrane domains. There are three principal types of opioid receptors: the delta-opioid receptor (DOR), the mu-opioid receptor (MOR) and the kappa-opioid receptor (KOR) (Jordan and Devi, 1998; Gaveriaux-Ruff and Kieffer, 1999). EOPs exhibit different affinities for these opioid receptors. β-Endorphins bind to DOR and MOR with similar affinity, whereas dynorphins preferentially bind to KOR (Stein, 1999). Opioid receptors are known to couple to G0 and Gi proteins, since signaling by these receptors is effectively blocked by pertussis toxin. Opioid signaling pathways involve the inhibition of the adenylate cyclase enzyme (Sharma et al., 1977) which might underlie the inhibitory effect of opioids on testicular function. In fact, opioid abuse primarily leads to hypogonadism and includes decreased libido and erectile dysfunction in men and infertility (Vuong et al., 2010). Administration of opioid antagonists such as naltrexone can improve symptoms of hypogonadism and improved erectile function, although the antagonist did not increase testosterone or LH levels, suggesting regulation at the central rather than the peripheral level (Fabbri et al., 1989). Numerous studies have demonstrated the presence of EOPs in different testicular cell types (Tsong et al., 1982a; Soverchia et al., 2006). In addition, binding studies have revealed the presence of all three types of opioid receptors (DOR, MOR and KOR) in rat testes (Pintar et al., 1984; Kilpatrick and Millette, 1986). Identification and the expression of β-EP in rat Leydig cells (Bardin et al., 1984) suggests a role of endorphinergic system in testicular function. Substantial research demonstrates that β-EP modulates gonadal function primarily by acting on opioid receptors in the hypothalamus (Vuong et al., 2010), inducing the decreased release or disruption of the normal pulsatility of gonadotropin releasing hormone (GnRH) secretion. This results in a reduction of the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland and of testosterone from the testes (Meites et al., 1979; Bicknell et al., 1986). Further, local synthesis of β-EP in the testis and in different compartments of the male genital tract (Pintar et al., 1984; Kilpatrick and Millette, 1986) suggests a paracrine action of β-EP in regulation of male reproduction (Fabbri et al., 1988; Adams et al., 1993; Aloisi et al., 2010). These effects of β-EP
might culminate into the impaired male sexual behaviour and loss of libido as evident from treatment with opiate drugs (Parr, 1976) and administration of β-EP to male rats (Meyerson and Ferenius, 1977). Moreover, disruption of the testicular endocrine function has also been reported in rats following administration of β-EP (Chandrashekar and Bartke, 1992). Reduction in testicular steroid biosynthesis upon gonadotropin stimulation by β-EP has been shown in testicular cells via in vitro studies (Knotts and Glass, 1988). In contrast, intratesticular treatment with naloxone, an opioid receptor antagonist, has been reported to decrease serum testosterone levels in pituitary-intact, hemicastrated rats (Gerendai et al., 1984). Therefore, a dilemma exists regarding the effect of β-EP on testicular steroid biosynthesis. Reports suggest an increase in plasma β-EP as a function of photoperiod and increased chronological age (Chen et al., 1984). However, the cross-talk between the melatonergic system and endophinergic system in regulation of seasonal variation of testicular steroidogenesis has not been investigated.

![Diagram](image.png)

**Fig. 10:** Opiatergic regulation of male reproduction at multiple levels of the HPG axis (Adapted from Subirán et al., 2011)

**Cytoprotective attributes of melatonin**

Cytoprotective properties (antioxidant and anti-apoptotic) of melatonin have become an important field of melatonin physiology. The cytoprotective attributes of melatonin have been established in various organs through
experimental manipulations such as exposure to oxidotoxins, ischemia/reperfusion, trauma, ionizing radiation, proapoptotic and proinflammatory signals (Karbownik and Reiter, 2000; Hardeland et al., 2003; Jaworek et al., 2005; Tengattini et al., 2008; Reiter et al., 2014). Further, the neuroprotective actions of melatonin has become an emerging field (Srinivasan et al., 2005; Korkmaz et al., 2009;), which exceed the experimental challenges mentioned and include counteractions against neurodegenerative disorders, processes of normal aging and interventions to promote mitochondrial biogenesis and, where possible, neurogenesis (Hardeland, 2009; Hardeland and Coto-Montes, 2010).

**Melatonin: As an Antioxidant**

Melatonin is a potent antioxidant and being an amphiphilic molecule it can easily cross almost all physiological barrier including the blood-brain barrier and blood-testis-barrier (Hardeland, 2005, Tan et al. 2007; Hardeland et al., 2011). Melatonin exerts its antioxidant activity through different pathways: (i) Direct free radical scavenging; (ii) Indirect stimulation of antioxidant enzyme activity; (iii) Protection of antioxidant enzymes from oxidative damage; (iv) Increasing the efficiency of the mitochondrial transport chain and (v) Reduction in the generation of free radicals (Tan et al., 2010; Rosales-Corral et al., 2010). It does not undergo redox cycling unlike other antioxidants such as vitamin C which act as pro oxidants counter intuitively promoting free radical formation. The free radical scavenging capacity of melatonin extends to its secondary, tertiary and quaternary metabolites (Hardeland, 2009). Once oxidized, melatonin cannot be reduced to its former state since it forms several stable end-products on reacting with free radicals (Hardeland, 2009). Melatonin as well as its metabolites (such as AFMK, AMK) form “free radical scavenging cascade” therefore act as good antioxidants. A single AFMK molecule can neutralize up to ten ROS/RNS (Galano et al., 2013). Due to its free radical scavenging cascade reactions, one melatonin molecule has the potential to scavenge up to four or more reactive species, hence several times more potent than vitamin C and E in protecting tissues from oxidative injury (Tan et al., 2007; Reiter et al., 2014).
Melatonin: As a direct free radical scavenger

Substantial reports have directly or indirectly established that melatonin is a potent free radical scavenger (Tan et al. 1993). Due to its high resonance stability and very low activation energy barrier towards the free radical reactions, the indole moiety of the melatonin molecule acts as the reactive centre of interaction with oxidants. The methoxy and amide side chains also contribute significantly to melatonin’s antioxidant capacity. The N-C=O structure in the C3 amide side chain is the functional group (Tan et al., 2007). Melatonin scavenges a variety of reactive oxygen and nitrogen species (ROS/RNS) including hydroxyl radical (•OH), hydrogen peroxide (H₂O₂), singlet oxygen (O₂), nitric oxide (NO) and peroxynitrite anion (ONOO⁻) (Reiter et al., 2014). It interacts with reactive species to form the melatoninyl cation radical by donating an electron or through radical addition (Tan et al., 1993; 2007).

Fig. 11: Melatonin as a potent antioxidant
(Modified from Tan et al., 1993, Tomas-Zapico and Montes, 2005).
Melatonin: An indirect stimulator of antioxidant molecules

Although direct radical scavenging has been effective under numerous experimental conditions, at clearly supraphysiological concentrations, its relevance at physiological levels has been questioned already for reasons of stoichiometry. However, this reservation is not generally valid, especially not for organisms producing by orders of magnitude higher levels of melatonin than vertebrates, such as various plants and dinoflagellates (Antolín et al., 1997; Hardeland et al., 2007). Even though a single melatonin molecule may generate products in a scavenger cascade which may collectively eliminate up to ten free radicals (Rosen et al., 2006), such findings from chemical systems may not be fully applicable to physiological conditions. From a stoichiometric point of view, it may only contribute to antioxidative protection in some melatonin-producing vertebrate organs, such as the rodent Harderian gland, but, in most mammalian organs, the levels do presumably not suffice for a substantial contribution to radical detoxification. Despite this conclusion, melatonin was shown to protect from oxidotoxicity already at physiological concentrations (Tan et al., 1994). Thereafter, various studies demonstrated the upregulation of several antioxidant enzymes by melatonin, such as glutathione peroxidase, glutathione reductase, $\gamma$-glutamylcysteine synthase, glucose-6-phosphate dehydrogenase, hemoperoxidase/catalase, Cu, Zn- and Mn-superoxide dismutases (Reiter et al., 2003a; Pandi-Perumal et al., 2006; Hardeland and Poeggeler, 2008). However, the relevance of these findings may be easily overestimated, perhaps, with the exception of glutathione peroxidase, which has repeatedly, and widely consistently, been shown to be increased by melatonin (Hardeland, 2005) and which has gained new relevance in the context of mitochondrial function (Acuña-Castroviejo et al., 2007; Hardeland and Coto-Montes, 2010). Glutathione reductase may mainly respond to changes in the redox equilibrium. The other enzymes, especially catalase and the superoxide dismutases, exhibited highly variable responses depending on sources and conditions. Sometimes, the increases were only in the lower percent range, often only demonstrated at the mRNA level (Jiménez-Ortega et al., 2009), and, in several cases, no effects (Okatani et al., 2001; Ohta et al., 2004; Mauriz et al.,
2007) or even decreases (Gürdöl et al., 2001) were observed. Even glutathione peroxidase, which was mostly upregulated, was not stimulated, e.g., at protein level in the liver of young– but in aged–rats (Mauriz et al., 2007) or, in the murine cerebral cortex, at the mRNA level (Olcese et al., 2009). Notably, protection by melatonin was achieved in all these studies in which antioxidant enzymes were not upregulated. Therefore, explanations different from radical scavenging and enzyme induction have to be sought for the protective potential of melatonin, especially at physiological or low pharmacological concentrations.

**Contributions of melatonin metabolites**

When high pharmacological levels of melatonin are used to combat oxidotoxicity, and also under conditions of inflammation, the metabolic route of pyrrole-ring cleavage leads to methoxylated kynuramine metabolites AFMK and AMK (Hardeland, 2009b; Galano et al., 2013). The relevance of this pathway under basal conditions is, however, uncertain in vertebrates. The anti-inflammatory properties of AFMK and AMK (Kelly et al., 1984; Mayo et al., 2005), potent inhibition of nNOS (Entrena et al., 2005; León et al., 2006) and downregulation of iNOS expression by AMK (Tapias et al., 2009) indicate that these kynuramines contribute to mitochondrial protection, at least in pharmacological experiments. Additionally, AMK was shown to be a potent scavenger of all NO congeners (Guenther et al., 2005; Hardeland et al., 2007), of CO3•- and •OH (Ressmeyer et al., 2003; Guenther et al., 2005). In contrast to melatonin, the cyclic AMK-NO adduct does not re-donate NO. In fact, AMK was shown to protect mitochondria and to enhance complex I activity (Acuña-Castroviejo et al., 2003; Tapias et al., 2009).

**Melatonin: The regulator of programmed cell death**

For every cell, there is a time to live and time to die. Balance maintained between cell division and cell death is vital for normal development, maintenance of tissue homeostasis. Numerous reports have confirmed the essential role played by melatonin in regulation of inflammation and apoptotic cell death (Casado-Zapico et al., 2010; Luchetti et al., 2010; Radogna et al., 2010). Recent evidences
suggest that the so-called intrinsic pathway might represent the main target of melatonin to antagonize apoptosis (Acuna-Castroviejo et al., 2007; Radogna et al., 2008). The increasing evidence for melatonin–mitochondria relationship includes the anti-apoptotic properties of the indoleamine through its interaction with mitochondrial permeability transition (MPT; Andrabi et al., 2004; Martinis et al., 2012). Emerging evidences suggest that lowered production of ROS in cells exposed to pharmacological concentrations of melatonin could prevent apoptotic signalling in mitochondria via specific targeting of Bcl2/Bax levels. In leukocytes melatonin mainly exerts an anti-apoptotic role (Radogna et al., 2007) by influencing regulation of the Bcl-2 family proteins (Weinreb et al., 2003) to support cell viability. Melatonin by interacting with its MT1/MT2 receptors can retard the dimerization/activation of the pro-apoptotic Bax to antagonize the damage induced by apoptogenic stimuli (Radogna et al., 2008). Melatonin is also able to reduce caspase-3 and caspase-9 activation and thereby rescue cells undergoing apoptosis (Espino et al., 2010).

**Melatonin: Role in energy homeostasis**

Individuals of most species are faced with marked seasonal fluctuations in environmental conditions (e.g., temperature, rainfall, food availability) and must adapt to the challenge of obtaining energetic resources (Bronson and Heideman 1994; Bronson, 2009). The energy available to an animal under most natural conditions is finite and will depend on the quality and abundance of these energetic resources in the environment. Once an animal obtains energy, it is then faced with the challenge of balancing energy allocation among different physiological processes (Ricklefs and Wikelski, 2002; Sheldon and Verhulst, 1996). The balancing of allocation towards diverse biological processes often results in energetic trade-offs among physiological systems. The energetic trade-offs between the reproductive and immune systems are essentially necessary to maintain these two mega-physiological process (Demas et al., 2012; Fedorka, 2014), where increased investment into one system results in minimal investment to the other one. The central nervous system (CNS) is composed of the most energetically demanding cells in the body (Ames, 1992). Minimizing CNS
metabolic demands during times of restricted energy availability (viz., short days), without impairing CNS function to the point of impacting long term reproductive fitness and survival, presumably provides adaptive advantages (Jacobs, 1996). However, the direct contribution of varying seasonal demands in energy homeostasis to the evolution of these adaptations remains unspecified. Many non-tropical mammalian and avian species use photoperiodic information to orchestrate energetically expensive activities (e.g., reproduction and immune function) to coincide with times of adequate resources (Nelson et al., 1990). The use of photoperiod to time life history events energetically prepares organisms for the upcoming season and is advantageous when factors are highly predictable from year to year (Goldman and Nelson, 1993; Bronson and Heideman, 1994). Studies of seasonal changes in mammalian physiology have generally focused on reproductive function due to its high energetic costs and implications for organismal fitness (Bronson, 2009). Metabolic fuels such as glucose affect reproductive function in seasonally breeding rodents and reduction in glucose availability inhibits testicular steroidogenesis (Amrolia et al., 1988). In theory, seasonal fluctuations in reproductive status have evolved as adaptive mechanisms to cope with seasonal fluctuations in energy availability (Zysling et al., 2009). However, the impact of energy availability in regulation of testicular steroidogenesis and antioxidant status has never been investigated for any seasonal breeder in general and especially for golden hamster.

A. Long days: Positive energy balance  B. Short days: Negative energy balance

Fig. 12: Hypothetical illustration of photoperiodic differences in allocation of among competing processes (Adapted from Walton et al., 2011)
Extrapineal melatonin: Influence on local physiology

After the pioneer discovery of melatonin (N-acetyl-5-methoxytryptamine) by Lerner in 1958 (Lerner et al., 1958), melatonin was considered for five decades to be exclusively produced in the pineal gland (Sánchez-Hidalgo et al., 2009a; Acuna-Castroviejo et al., 2014). With the discovery of the enzymatic machinery (AA-NAT and HIOMT) necessary for melatonin biosynthesis in pinealocytes, the presence of AA-NAT and HIOMT was subsequently uncovered in the retina, lens and cerebellum (Cardinali and Rosner, 1971; Itoh et al., 2007; Bubenik et al., 1974). This was followed by the identification of melatonin synthesis in a large number of extrapineal sites retina, hardarian gland, brain, gut, ovary, testes, inner ear and immune system including bone marrow, lymphocytes, macrophages, thymus, skin and, more recently in thyroid cells (Kvetnoy, 1999; Bubenik, 2002; Carrillo-Vico et al., 2004; Slominski et al., 2005; García-Marín et al., 2012) Although the potential physiological actions of extrapineal melatonin is not fully understood, it has been suggested that extrapineal melatonin may play a key role as an intra-, auto- and paracrine signal molecule in those tissues where it is synthesized (Carrillo-Vico et al., 2004; Sánchez-Hidalgo et al., 2009a; Markus et al., 2007). The demonstration of the presence of key melatonin synthetic enzymes established local synthesis of melatonin in testis (Borjigin et al., 1995; Tijmes et al., 1996, Stefulj et al., 2001) and ovary (Itoh et al., 1997, 1999). The synthesis and presence of melatonin in multiple sites of the ovary and testes reflect its potential intra-, auto-, and paracrine regulation of reproductive physiology, which guarantees the quality of the egg and sperm (Acuna-Castroviejo et al., 2014). Recent evidences suggest the involvement of local melatonin in regulation of testicular steroidogenesis (Frungieri et al., 2005) and oxidant-antioxidant profile in testes (Rossi et al., 2014). However, the exact mechanism underlying the regulation testicular function via local melatonergic is not clearly understood.

Glucocorticoid, stress and reproduction

In addition to the well-characterized role of the sex steroid receptors in regulation of fertility and reproduction, reproductive events are also mediated by the hypothalamic-pituitary-adrenal axis in response to an individual's environment.
Glucocorticoid secretion in response to stress contributes to the well-characterized suppression of the hypothalamic-pituitary-gonadal axis through central actions in the hypothalamus and pituitary (Whirledge and Cidlowski, 2013). However, in the absence of stress, it appears that homeostatic glucocorticoid signaling plays a significant role in reproduction and fertility in all tissues comprising the hypothalamic-pituitary-gonadal axis (Sayers, 1950; Schwartz and McCormack, 1972; Michael and Papageorghiou, 2008; Shi et al., 2011). Therefore, when circulating levels of glucocorticoids surpass levels shown to promote fertility, survival occurs at the expense of reproduction. Glucocorticoids are multitasking molecules (Julia, 2006) influencing almost all physiological functions including reproduction (Sapolsky et al., 2000). The adverse effects of stress on reproduction, mediated primarily by high levels of GCs, have been recognized and reported in a number of species ranging from rodents to ruminants and primates including humans (Maeda and Tsukamura, 2006). While excessive exposure to glucocorticoids on the pituitary–gonadal axes of both genders may be detrimental, physiological levels of GC are generally beneficial and important for normal function of the reproductive system at its various levels or stages of the development (Brann and Mahesh, 1991). The inhibitory effects of glucocorticoid are in part due to direct action on testicular Leydig cells (Hales and Payne, 1989; Sapolsky, 1992; Orr et al., 1994). Increased glucocorticoid leads to decreased testosterone through suppressed androgen synthesis and reduction in the number of Leydig cells as a result of apoptosis (Yazawa et al., 1999; Gao et al., 2003; Yazawa et al., 2000). Increased glucocorticoid levels have been reported to disrupt and suppress endocrine signalling in the male reproductive axis (Baldwin et al., 1990; Suter and Schwartz, 1985).

The molecular actions of glucocorticoids are mediated by their circulating levels, local metabolism, and intracellular signaling through the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of ligand-dependent transcription factors (Evans, 1988; Ramamoorthy and Cidlowski, 2013). The male gonads are the direct targets of glucocorticoid action, mediating stress-induced inhibition of testicular steroidogenesis (Orr and Mann, 1992; Hardy et al., 2005).
The ability of glucocorticoids to control testosterone biosynthesis in Leydig cells through binding intracellular GR has already been established (Evain et al., 1976). Expression of GR has been described in multiple cell types of the testis (Schultz et al., 1993; Herrera-Luna et al., 2012). Glucocorticoids also inhibit the synthesis of the cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and the steroidogenic acute regulatory protein (StAR), key regulators of steroidogenesis (Stocco, 2001).

Glucocorticoid-induced apoptosis resulting from exogenous exposure has also been described for other cell types in the testis. Spermatogonia, spermatocytes, and spermatids are susceptible to apoptosis by exogenous glucocorticoids in murine models (Orazizadeh et al., 2010). Furthermore, following 7 days of dexamethasone, the seminiferous tubules display widespread alterations to morphology, including vacuolization, disorganization of the germ cell layer, loss of elongated spermatids, and atrophy. The changes to testicular morphology indicate exogenous glucocorticoids have both cytotoxic and apoptotic effects in the testis. Apoptosis in Leydig and germ cells is thought to be mediated by GR, although the exact mechanisms involved in cytotoxicity and apoptosis remain unclear. Deprivation of gonadotropins and testosterone also results in the stage-specific germ cell apoptosis (Sinha Hikim and swerdloff, 1999). However, glucocorticoid-mediated apoptosis occurs in androgen-dependent and -independent stages, suggesting increased apoptosis induced by exogenous glucocorticoids may not be entirely related to changes in testosterone biosynthesis. Glucocorticoids up-regulate the expression of Bax and Fas ligand (FasL), two important proapoptotic proteins, in testicular germ cells in vivo (Mahmoud et al., 2009; Khorsandi et al., 2008). However, it is possible that up-regulated Bax and FasL are secondary to androgen withdrawal induced by glucocorticoids, and further studies are needed to clarify the direct vs. indirect contributions of glucocorticoids to germ cell apoptosis (Kondo et al., 2002).

**Melatonin deficiency: A hallmark of physiological aging**

With regard to the orchestrating role of the pineal hormone, a plethora of effects can be expected to result from melatonin deficiency. Countless
publications have dealt with experimental melatonin deficiency by pinealectomy in animals. Studies using aged pinealectomized rats, oxidative damage to membrane lipids, protein and DNA were enhanced in various organs, compared to controls of the same age (Reiter et al., 1999), that completely consistent with the amply documented antioxidant properties of melatonin. Moreover, an increase of homocysteine because of pinealectomy, might indicate a higher risk of cardiovascular disease, results that were in line with the homocysteine-reducing action of melatonin (Baydas et al., 2002a). In models of neurodegeneration, based on focal brain ischemia or glutamate toxicity, the damaged areas were larger in pinealectomized rats than in control animals (Manev et al., 1996). An age-associated decline in the secretion of a pleiotropically acting melatonin has to have profound consequences for the functioning of the organism. Continuous administration of melatonin, e.g., via drinking water has generally not been found to extend the lifespan (Poeggeler, 2005). In several mouse strains, especially those which are melatonin-deficient, animals die from cancer, and melatonin administration may result in a kind of chemoprevention. Instead of prolongation of the lifespan, melatonin-treated rodents frequently show the so-called “Methuselah syndrome”, i.e., they remain in apparently healthy condition concerning mobility, glossy fur, absence of skin inflammations and low osteoporosis (Poeggeler, 2005). Melatonin may contribute in many fold ways to healthy aging, by various actions. This should include phasing of the circadian system (Korf and von Gall, 2006), having important consequences for sleep (Saper et al., 2005; Kocher et al., 2006), support of the immune system (Vishwas et al., 2013a; Singh and Haldar, 2014), antioxidant and anti-inflammatory actions (Carrillo-Vico et al., 2005; Reiter et al., 2014), perhaps oncostatic effects (Chubb, 1999; Cucina et al., 2009), prevention of neuronal overexcitation (Tapias et al., 2009; Hardeland and Coto-Montes, 2010) and, not least, safeguarding of mitochondrial electron flux and minimizing electron leakage (Hardeland, 2009a). Though, the antioxidant potential of melatonin is well documented, its role in the maintenance of testicular antioxidant status following reproductive senescence is not known.
In the light of the reports cited above, it can be summarized that melatonin via receptor dependent and independent pathways regulate a wide array of physiological functions including the regulation of seasonal reproduction. Based on the lacunae observed in literature we proposed our objectives of the present dissertation to understand the regulatory mechanisms involved in seasonal variation in testicular steroidogenesis.

It is well known that testicular steroidogenesis is regulated through extremely complex mechanisms. However, in the present dissertation we have chosen the key regulators as markers of testicular steroidogenesis to elucidate the effect of photoperiod and melatonin. It is believed that the rate-limiting step in the steroidogenesis is the movement of cholesterol across the mitochondrial membrane so that it can be acted upon by the cytochrome P450 side chain cleavage enzyme (P450\textsubscript{SCC}; Payne and Hales, 2004). The movement has been shown to be mediated by the steroidogenic acute regulatory protein (StAR; Stocco, 2001). Further, LH-R signalling in testes is known to up-regulate the expression of StAR and P450\textsubscript{SCC} in a cAMP mediated pathway and thereby stimulate the synthesis of testosterone (Payne and Hales, 2004; Ascoli et al., 2002). Further, 3β-HSD and 17β-HSD were chosen as markers that catalyzes the conversion of pregnenolone to dihydroepiandrosterone (DHEA) and androstenedione to testosterone, the two important steps in testicular steroid biosynthesis. Moreover, spermatogenesis and male fertility are exclusively dependent on testosterone concentration and androgen receptor expression in the testes (Walker, 2011). Therefore, we analyzed the receptor expression for AR, StAR, P450scc, LH-R along with the enzyme activity for 3β-HSD and 17β-HSD and the level testosterone. Melatonin is known to inhibit the basal and chronic gonadotropin-stimulated cAMP and androgen production through activation of MT1R which can be blocked by melatonin receptor antagonist, luzindole (Reiter et al., 1977; Frungieri et al., 2005). With the aforementioned reports, it is imperative that photoperiodic stimulus is an important modulator of the testicular steroidogenic pathway. Since, melatonin is the principal transducer of such stimuli it can be presumed that the local melatonergic system may have substantial cross-talk with
the testicular steroidogenic pathway, via the MT1R. Based on the above hypothesis, we attempted to explicate the photoperiodic regulation of the expression of MT1R and its interaction with testicular steroidogenic pathways.

Moreover, spermatogenesis is a highly active and replicative process and is exclusively dependent on testicular steroid biosynthesis. Physiological metabolism incessantly generates reactive oxygen species (ROS) and the testis, the primary reproductive organ in males, is not an exception in this regard. Elevated oxidative stress results in increased levels of circulating glucocorticoid that give rise to multiple complex physiological effects. The complexity of the glucocorticoid actions depend on the wide range of target tissues and the duration of exposure. Recent studies have revealed that rapid glucocorticoid actions provide an integrative signal linking stress with the regulation of energy homeostasis. Testicular oxidative insult increases under metabolic stress, with increasing chronological age and glucocorticoid-induced stress conditions. Oxidative stress is known to induce a progressive functional impairment of the HPG axis and has been suggested as a possible explanation for the age-dependent suppression of testicular function and reproductive senescence. Melatonin is a pleiotropic molecule and acts as a direct antioxidant as well as an indirect stimulator of antioxidant enzymes and is thereby considered as an endogenous protective agent. The co-operative response of melatonin in regulation of glucose metabolism, homeostasis and the reciprocal interaction between melatonin and glucocorticoid might be beneficial in testicular protection against elevated oxidative injury. Therefore, it would be interesting to elucidate the crucial role played by melatonin in protecting the testes against oxidative insults arising from a plethora of physiological and pathophysiological conditions.

I do hope that the present dissertation would provide some relevant ecological perspective to expand our understanding regarding the involvement of melatonin and its receptor in seasonal regulation of male reproductive status. Our results would suggest a fundamental basis for the direct effect of melatonin on testicular steroid biosynthesis and spermatogenesis, which is collectively expressed by its anti-gonadotropic, antioxidant and anti-apoptotic effects. This small effort would definitely add some extra knowledge to our present understanding about the role of melatonin and its receptors in regulation of seasonal testicular physiology.