Editorial Article

The interrelationship of sleep, biologic clocks, neurotransmitters, gonadotropins and pubertal development

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Summary

Purpose: To evaluate the influence of sleep on early pre-pubertal and pubertal development and to explore the importance of circadian rhythms and gonadotropin secretion. *Methods:* Mechanisms of suppression and turning on of the hypothalamic gonadotropin releasing hormone (GnRH) pulse generator at different times in development are evaluated. Furthermore, the influence of neurotransmitters in controlling pubertal development is also considered. *Results:* By the end of the first year, certain genes are activated that cause marked sensitivity of the GnRH pulse generator to negative feedback of circulating sex steroid. Furthermore, a central nervous system mechanism contributes greatly to the juvenile pause. Biologic clocks help to turn on the gonadostat and loss of negative feedback to sex steroid. This occurs during the sleeping hours. Equally important is to neutralize the neurotransmitter gamma amino butyric acid (GABA) which is the main central nervous system inhibitor. *Conclusions:* Pubertal development is a complex process requiring the activation of certain genes which activate biologic clocks. This results in the increased secretion of certain neurotransmitters, for example, leptin and kisspeptin, which are very important in awakening the GnRH pulse generator. Suppression of the inhibitory neurotransmitter GABA is equally important.

Key words: Biologic clocks; Sleep; Neurotransmitters; Gene activation; Gonadotropin releasing hormone; Pulse generator.

Gonadotropins and sex steroids in the fetus neonates and infants

The various hypothalamic factors responsible for the production and the release of various pituitary hormones are not fully developed at birth [1]. Minutes after birth in the male, neonate serum luteinizing hormone (LH) significantly increases in the serum which causes an increase in serum testosterone within three hours that persists for 12 hours or more [2]. This very early LH secretion is not found in the female neonate [2].

Within the first few days of life, differences in follicle stimulating hormone (FSH) and LH are found between male and female infants with both sexes displaying pulsatile patterns, but the amplitude of FSH pulses is much greater in the female infant [1]. The difference between sexes may be related to the effect of the early testosterone secretion of the male from 11 - 24 weeks related to hypothalamic development [1].

Toward birth, a drop in serum FSH and LH occurs in relation to the development of estrogen, testosterone, and progesterone receptors in the hypothalamus, thus allowing negative feedback from these sex steroids [3, 4]. Inhibin can also exhibit a negative feedback in late gestation [5]. Actually the change from high levels of sera FSH and LH begins at mid-gestation with increasing sensitivity to the negative effect of sex steroids on hypothalamic GnRH secretion with the formation of sex steroid receptors. The placenta is the source of estrogen and progesterone which exert the negative feedback effect and testosterone from the fetal testes from mid-gestation and testicular inhibin in late gestation [5, 6].

Differences in the pulsatility and concentration of FSH and LH in males vs female neonates

The aforementioned rise in LH shortly after birth not only leads to a rise in serum testosterone, but high LH leads to a proliferation of sertoli cells and spermatogonia [2, 7]. Furthermore, more Leydig cells are produced leading to an increase in serum testosterone during the first few months of life [8].

The sensitivity of hypothalamic sex steroid receptors to negative feedback reaches a maximum by six months of age in the male. Thus the gonadotropins are maximally suppressed by six months and stay suppressed until puberty [8].

The gonadotropin patterns are somewhat different in the female neonate and infant. FSH pulse amplitude is much greater in the female infant. This leads to a rise in estradiol in the first 1.5 years of life [9]. The peak negative sensitivity of sex steroids on gonadotropins does not occur until two to three years of age in the female when the gonadotropins drop to very low levels which remain low until puberty [10]. Thus the inhibition of the GnRH pulse generator and thus the suppression of GnRH from the hypothalamus does not occur until late infancy or early childhood in females and even earlier in males.

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Pubertal development – influence of genetics

Genetic factors (especially polygenic) are estimated to be 50 - 80% responsible for determining the timing of puberty when the GnRH pulse generator loses its sensitivity to the negative feedback effects of sex steroids [11, 12]. These genes control the development of the GnRH pulse generator through their effect of increasing stimulatory factors, for example, glutamate and kisspeptin, and decreasing inhibitory factors such as GABA and opiloids [12]. The mechanism of delivering these stimulatory and inhibitory factors is through transsynaptic and glial-neuronal communications [12]. An example of a pathological state related to polygenic control of onset of puberty is constitutional delayed pubescence; however the exact gene loci for this polygeneic influence on onset of puberty has not been completely identified.

Pubertal development – influence of leptin

Leptin is a protein resembling cytokines which is predominantly made by adipose tissue [13, 14]. It resembles cytokines and, in fact, its receptor is a member of the qp family of cytokines receptors [15]. Leptin acts on the hypothalamus to cause appetite satiety and thus plays a key role in metabolism and weight control [16, 17].

A mutant leptin gene in a group of very obese mice known as ob / ob mice was found to be etiologic for their obesity [18]. These mice have also been found to have hypogonadotropic hypogonadism [19, 20].

Some have considered leptin as the possible main peripheral trigger to the central nervous system "awakening" the hypothalamic pulse generator [21, 22]. Indeed leptin is secreted in a pulsatile manner [23]. Similar to gonadotropins at puberty leptin has a diurnal pattern with the highest levels at night and the lowest in the morning [16, 24]. Most of the studies favor a permissive role for leptin rather than leptin being the trigger for puberty [25-27]. It is believed that its main role is to signal the GnRH pulse generator that a critical energy store has been achieved [21]. The data actually favor that rather than leptin stimulating GnRH, it is in fact, the increase in GnRH pulsatility at puberty that causes rise in serum leptin [28].

Thus previous observations have found an earlier onset of menarche in moderately obese girls [29]. Similarly low weight states, such as anorexia nervosa or a decrease in body fat without low weight as in strenuous exercise has been associated with a delay in menarche [30-34]. With the discovery of the correlation of serum leptin levels with adipose mass and percentage of body fat [26, 27], at first it seemed reasonable to consider leptin as the initiating agent for puberty [26, 27]. However, as stated above, its secretion plays a role in the hypothalamic secretion of GnRH, but it may be more involved in enabling the hypothalamus and suprahypothalamus factors involved in the manner of GnRH secretion knowing that there is sufficient energy available to continue the pattern of pulsatile GnRH secretion that was originally initiated by other factors [28].

The permissive role of leptin cannot be overlooked although it maybe not the trigger; the ob / ob mice have increased body fat but because of mutated leptin receptors have hypogonadotropic hypogonadism [19, 35]. Actual analogous mutations have been found in either leptin or in the leptin receptor leading to marked obesity, and hypogonadotropic hypogonadism in humans [36, 37]. Evaluation of various pathological states, such as constitutional delayed pubescence and several genetic disorders leading to severe leptin deficiency have led to the conclusion that a critical level of leptin and a leptin signal are required to attain puberty (permissive role), but a rise in leptin is not needed to trigger the hypothalamic GnRH pattern needed to initiate puberty [27, 38].

Inhibitory effect on pubertal development of the central nervous system (CNS) after infancy

The intrinsic CNS inhibitory effect lasts for approximately a decade of life. As mentioned, there is an increased amount of LH and FSH in infants. However, shortly after infancy, there is a suppression of GnRH activity leading to diminished FSH and LH secretion. Certainly an important part of the low gonadotropin secretion is related to exquisite sensitivity to negative feedback of sex steroids.

Gonadal dysgenesis in Turner's syndrome is a classic example of the influence of negative feedback effect of sex steroids, but also the presence of a central gonadotropin inhibitory mechanism and their ontogeny [39, 40]. Related to the agonadal state (and thus even less sex steroids made by the ovaries than the normal infant), baseline sera FSH and LH levels are higher in infant females with Turner's syndrome than normal gonadal infant females [39, 40]. Furthermore, infant females with Turner's syndrome show an exaggerated response to exogenous GnRH compared to normal infant females [39, 40].

However, the influence of the CNS inhibitory effect is evident even in females with gonadal dysgenesis because the mean gonadotropin levels drop to similar values, as their age peers with normal ovaries between the ages of 4 - 10 years [39, 40]. Since the inhibitory mechanism of the GnRH pulse generator occurs in agonadal children who do not make sex steroids, it is clear that this CNS inhibitory mechanism is independent of the sex steroid mechanism of negative feedback.

As puberty approaches the CNS inhibitory mechanism which has been dominant since three to four years of age gradually loses its initial influence during nighttime sleep [1], but also the GnRH pulse generator becomes less sensitive to

the negative effects of sex steroids [1]. Nevertheless in the post-pubertal individual, whereas the CNS inhibitory mechanism seems to be completely eradicated, there still remains some sensitivity to the negative effects of sex steroids, and only the set point has been raised. The end of the so-called juvenile pause is initially shown by an increase in LH pulse amplitude during the early hours of sleep [41, 42].

Neurotransmitter and other potential influencing factors on the intrinsic CNS inhibitory mechanism: GABA

One of the most important inhibitory neurotransmitters in the brain is GABA. Most studies support GABA, which is generated by interneurons, as the intrinsic CNS inhibitor of the GnRH pulse generator during the juvenile phase before puberty [41-45].

GABA may actually have an excitatory effect on synaptic transmission in the post-natal period contributing to the higher basal gonadotropin levels in the post-natal period and increase intracellular calcium concentration [46, 47]. A developmental switch from GABA ergic excitatory to inhibitory states is most likely responsible for the diminished GnRH pulse generator activity in childhood [47].

The onset of puberty in the rhesus monkey (and probably in humans) is characterized by the decrease in GABA ergic and neuropeptide Y inhibitor of the hypothalamic GnRH pulse generator [48]. It is also characterized by the increased release of glutamate, which is considered to be the major excitatory amino acid neurotransmitter in the hypothalamous [49]. Of the two mechanisms, inhibition of the GABA ergic inhibitor of the GnRH pulse generator, is considered the more important event in the initiation of puberty [48].

Another excitatory neuropeptide may be N-methyl-d-aspartate (NMDA) which has been found to stimulate LH release in prepubertal and adult rhesus monkeys [50, 51]. N-methyl-d-aspartate has been found to induce the release of GnRH from the hypothalamus and in fact, one can cause prepubertal rhesus monkeys to enter puberty earlier by repeated infusions of NMDA [48].

The importance of kisspeptins

The KISS-1 gene is a human metastasis suppressor gene and KISS-1 mRNA is found in several tissues in the body, but in the brain it is mainly found in the hypothalamus and basal ganglion [52]. The secreted product of the KISS-1 gene is a 54-amino acid peptide called Kisspeptin (metastin is another name). The receptor for kisspeptin is a G protein known as GPR54 and is similarly found predominantly in the hypothalamus and basal ganglia. There are data suggesting that KISS-1 signaling through the GPR54 receptor of the primate and human hypothalamus may be activated at the end of the juvenile pause. This gene activation thus may play an important role in the turning on of the GnRH pulse activator at the initiation of puberty [53].

Ablating the pulsatility of GnRH by continuous infusion of kisspeptin suppresses GnRH release and subsequent secretion from the pituitary of LH and FSH. Similarly, continuous infusion of kisspeptin decreases the response of the gonadotropin cells to boluses of kisspeptin by down-regulation of the GRP54 receptors [54]. Kisspeptin-GPR54 signaling seems to play a critical part in the initiation and maintenance of puberty [55-57].

Summary of the relationship of neurotransmitters and initiation of puberty

Thus to summarize these events, the GABA ergic neuronal network with its neurotransmitter GABA is the main inhibiting transmitter in the hypothalamus and is responsible for the juvenile pause during the pre-pubertal years. The waning of GABA inhibitor of the GnRH pulse generator allows its reactivation. Kisspeptin excitant amino acids help to reactivate the GnRH pulse generator. The activation of the kisspeptin amino acids is enhanced by excitatory neuropeptides, such as: glutamate and N-methyl-d-asperate (NMDA) and by nitric oxide, noradrenergic pathways, and growth peptides. Furthermore the increase in sex steroids exerts a positive effect on some of the kisspeptin excitatory amino acids and other neuroexitatory factors.

However, independent of sex steroids, there is an increase in KISS-1 mRNA expression in kisspeptinergic neurons in the medical basal hypothalamus which allows the secretion of kisspeptins. As a consequence of kisspeptins binding to the GPR54 receptor on the surface of the GnRH neurons, this sequence increases the amplitude of GnRH pulses and also, but to a lesser degree, increases the frequency of GnRH pulses. The increase amplitude and frequency of GnRH pulses then increases the amplitude and frequency of LH and FSH pulses and this causes an increase is sex steroid production by the ovary or testes leading to pubertal changes. The mechanism is not yet completely understood. Though it is clear that puberty results from the removal of GABA inhibiter and reactivation of the GnRH pulse generator, it is unclear what event triggers this transition.

Biological clocks and sleep

Critical changes in the secretion of various endocrine hormones frequently have cyclical or periodic changes. These periodic or cyclical changes are independent of the environment. The biologic clock that drives these rhythms are mainly under the control of the nervous system.

Though these biologic clocks are independent of the environment, they are coordinated by external signals. One of these signals is light - dark changes and another somewhat-related signal is the ratio between the length of day and night [58-60]. These light - dark signals are known as Zeitgeber or "time givers". Light-dark signals influence the most diurnal rhythms (approximately a day). The 28-day menstrual cycle is referred to as infradian (longer than a day) rhythm.

Most endocrine rhythms are circadian. The secretion of growth hormone and prolactin (PRL) in humans is maximal shortly after the onset of sleep. For adreno corticotropic hormone (ACTH), the secretion begins about 4:00 a.m. and peaks at 7:00 a.m. As previously mentioned, gonadotropin secretion in adolescents is increased at night and characterized by rapid high amplitude pulsations. This phenomenon stops, however, when full maturity is reached.

The suprachiasmatic nuclei, as the name implies, lies above the optic chiasm as paired nuclei in the hypothalamus and is considered to be the area responsible for most of the circadian rhythms. Isolated cells of the suprachiasmatic nucleus have intrinsic capacity to oscillate in a circadian pattern [61]. The suprachiasmatic nucleus is rich in neuropeptides including neurotensin, neuropeptide Y, somatostatin, and vasoactive intestinal peptide.

The majority of the suprachiasmatic neuromal activity terminates in the dorsal medial nucleus of the hypothalamus. Among other functions through this pathway, the suprachiasmatic nucleus produces circadian rhythms involved in sleep and arousal [60].

The suprachiasmatic nucleus has been found to possess MT1 and MT2 melatonin receptors [62]. Thus the suprachiasmatic nucleus can respond to the secretion of melatonin from the pineal gland. The suprachiasmatic nucleus projects to the pineal gland through the autonomic nervous system and the paraventricular nucleus. Noradrenergic sympathetic nerve terminals are critical regulators of melatonin production and release of melatonin from the pineal gland. The retina directly innervates the suprachiasmatic nucleus through the retino-hypothalamic tract. In the absence of light, the pineal gland rhythms persist but are not entrained to the external light-dark cycle

The main substance secreted by the pineal gland is melatonin. Melatonin is very important in the regulation of many circadian rhythms. One of the most important signals is darkness. In many mammalian species, melatonin levels are highest during darkness and suddenly plummet in the presence of light.

In certain animals, melatonin plays a critical role in the initiation of puberty and in the control of gonadotropin secretion. Removal of the pineal gland in some species leads to precocious puberty. Male rats that are kept in constant darkness or are made to become blind will develop testicular atrophy. However, this can be prevented by removing the pineal gland, thus suggesting the mechanism of gonadotropin suppression is through melatonin. This mechanism can play a role in breeding of some species at certain times to allow a favorable time for delivery.

The pineal gland probably does not have a significant effect in the development of puberty or gonadotropin secretion in humans [63]. As mentioned in rats, creation of blindness by enucluation show testicular atrophy and reduced testosterone secretion. Yet in humans, early onset of menarche has been described in studies of blind women [64]. It has to be realized that certain species depend more on melatonin controlling and entraining circadian rhythms than the human species, and some species are more sensitive to the effects of light - dark on melatonin synthesis. It takes much more light in humans to produce an equivalent nocturnal suppression of melatonin compared to rodents [65].

Regardless of the species, the mechanism of melatonin control seems to be similar. Melatonin mediates its effects by interacting predominantly with an MT1 receptor (which is from the family of G proteins – couples receptors) and to a lesser degree with MT2 receptors (from the same family). Its main action is to inhibit the activity of neurons in the suprachiasmatic nucleus and thus acts to inhibit the master mammalian circadian pacemaker in the brain [66-68]. It is not completely clear, but it is known that melatonin plays some role (and it may vary according to species) in the effect of light to induce certain circadian phase shifts.

Actually, melatonin therapy has been used in humans to treat certain circadian-based sleep disorders by resetting circadian rhythms and causing some phase shifting [69]. It has been used with limited efficacy as a sleep aid in humans who do not have a disturbance in their circadian rhythms.

Peripheral extra-SCN oscillators: biologic clocks and gonadotropin secretion

As mentioned, in the early 1970's, the SCN based on extensive experimentation was considered the master circadian clock in mammals [70, 71]. However, with the discovery of clock genes, it became clear that there are multiple peripheral oscillators throughout the body [72-74].

Nakao *et al*. recently discovered that clock genes "per 2" and "per 3", as well as "clock" and "bam/1" are expressed in preovulatory follicles [75]. Nakao *et al*. found that "per 2" and "per 3" expression is only found in the largest follicle that is first in line to ovulate [75]. They were not found in the smaller follicles.

It has been well known that the dominant follicle is in charge of its own destiny, i.e., effects the control of the complex relationship of positive or negative feedback control of LH and FSH release from the pituitary, especially with certain timed cyclic events the sex steroids, as estrogen, inhibit LH release then change to now exert a positive effect on LH release. The study by Nakao strongly suggests that clock genes, and thus peripheral oscillations in the developing follicle itself, must play a role in circadian mechanisms involving gonadotropins and sex steroids [75].

Nakao *et al*. found a gene related to increased synthesis of progesterone called steroidogenic acute regulatory protein (STAR). The gene was found to demonstrate a 24-hour cycle in the largest follicle which coincided with the expression of "per 2". Thus this study suggests that LH induction of progesterone synthesis is gated by a circadian rhythm in clock gene expression in the largest follicle that is ready to ovulate [75]. Nakao *et al*. demonstrated that the fifth flanking region of the STAR gene contains E-box enhancers which can bind to CLOCK / BMALI heterodimers to activate gene transcription (clock genes through a process of coordinated feedback between transcription and translation are able to produce an oscillation that controls many different circadian rhythms).

Thus the data from Nakao *et al*. strongly suggest that the brain is not solely in control of regulating the ovary and timing of the secretion of sex steroids. Instead, the dominant ovarian follicle and the brain seem to equally share as circadian clocks gating the timing of the LH surge and thus ovulation.

The interrelationship of sleep, biologic clocks, gonadotropin secretion, and pubertal development

Sleep and circadian rhythmicity (intrinsic effects of time of day, irrespective of the sleeping or waking state) interact to produce the overall temporal pattern of the majority of hormones [76]. Growth hormone, PRL, LH, and FSH are secreted in large amounts during sleep, whereas thyroid-stimulating hormone (TSH) and ACTH secretion are reduced during the first half of the sleep period [76].

Even before any physical changes associated with pubertal development in five to six year-old children, a diurnal rhythm of serum LH and FSH and testosterone is able to be detected using a very sensitive radioimmunoassay [42, 77, 78]. During the early and mid-pubertal stages, LH pulses show greater amplitude and frequency during sleep [78, 79]. LH pulses are not detected during the day time at this stage of puberty. In late puberty, there are LH surges detected even during the daytime, but the amplitude and frequency is still higher during sleep. Once adulthood is reached, there is no longer a difference between awakness and sleep. In males, the higher LH during sleep leads to an increase of testosterone. This increased LH secretion during sleep may be in some part caused by the infradian (referring to longer than a day) awakening on turning on of a suppressed gene in the CNS. Furthermore, the increased pulsatility is in part related to diminished negative feedback effect on the hypothalamus during the night.

There are data suggesting that regulation of fetal circadian rhythms may be mediated by maternal circulatory melatonin [65]. In fact, the timing of the human circadian pacemaker can be altered by the administration of melatonin [65]. It is tempting in view of the well-known association of darkness and melatonin to consider that melatonin plays some role in the initial activation of the CNS aspect of the nocturnal awakening of the hypothalamic GnRH pulse generator in pre-pubertal children [65]. However, there are no data at present to substantiate this hypothesis.

It is clear however that once GnRH activity is initiated, the GnRH itself has a self-priming effect on the gonadotropins increasing their sensitivity, and thus enhanced secretion of sex steroids to GnRH. This is substantiated by the demonstration that pubertal children secrete more LH and FSH in response to exogenous GnRH compared to pre-pubertal children. It is the increased LH response to synthetic GnRH that is one of the earliest hormonal markers of the onset of puberty.

The development of the positive feedback effect of estrogen, which is needed to allow ovulation, is a relatively late event probably commencing in mid-puberty. Thus the removal of the exquisite sensitivity of the negative feedback effects coupled with positive stimulatory effects from the CNS to the GnRH pulse generator allows sufficient follicular development to permit sufficient antral progression to make sufficient estrogen (though the follicles are probably androgen dominant at this stage) to allow menarche and other changes associated with increased estrogen production.

However, there is insufficient secretion of estrogen level attained to induce a positive feedback effect on LH, leading to a surge that is able to advance meiosis of an oocyte to a metaphase two stage and cause sufficient receptor changes to change the follicle from androgen dominance to estrogen dominance. During this time of estrogen production from antral follicles, they do not reach the dominant follicle stage. Nevertheless the amount of estrogen produced allows endometrial proliferation and eventually breakthrough bleeding occurs heralding the menarche.

For the first two years of menarche, the large majority of menstrual periods which occur irregularly (and especially with widespread intervals shortly after menarche), are anovulatory and are related to breakthrough bleeding [80].

Over time, first commencing during sleep and then extending to the awakening hours, the GnRH amplitude and pulse frequency progressively increase. The increased GnRH stimulation sensitizes the gonadotropins of the pituitary to increase FSH outright from a given GnRH bolus. Eventually the level of FSH becomes sufficient to allow one of the antral follicles to become the dominant follicle. According to the most recent theory, the Zeitgeber or "time giver" or "gate keeper" that synthesizes infradian (refers to a rhythm longer then the circadian day, e.g., one month as seen with the LH surge) monthly LH surge are the clock genes (e.g., "clock" and "BAM/1" and "per 2" and "3"), which are activated at a certain stage of folliculogenesis in the follicle destined to ovulate. This follicle is now able to synchronize the complicated events of turning estradiol from an inhibitor of LH to a stimulator in a given ovulatory cycle.

Thus the timing of puberty involves a "developmental clock", a singular timer, or a series of timers [81]. Using the clock analogy, the alarm but not the clock itself is species specific [81]. Expression of the alarm but not the clock itself is determined by the integration of the alarm with multiple permissive clues. As an example, one would consider the

clock itself that determines the year that a person will activate the GnRH pulse generator, it is the permissive cues that ultimately determines the month in which one or more genes that control the regulation of GnRH secretion are activated [81].

Though sleep and darkness have significant effects on the secretion of several pituitary hormones and melatonin from the pineal gland, there does not seem to be much influence of the suprachiasmatic nuclei on gonadotropin secretion. Similarly in humans there does not appear to be much influence of melatonin on gonadotropin secretion. Teleologically there must be some reason why the initiating events preceding menarche involve the removal of inhibitory factor for the GnRH pulse generator during the sleeping hours, but it is not yet clear as to whether there is some benefit to allow gonadotropins to be produced at the same time as other pituitary trophic hormones. Finally, it is unclear as to how the sleeping hours help to remove the CNS inhibitory neurotransmitters and the sensitivity to negative feedback effect of sex steroids to allow the development of GnRH and thus LH and FSH pulsatility and increased secretion during sleeping hours.

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