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NEUROENDOCRINE CONTROL OF THE ONSET OF PUBERTY

Tony M. Plant

Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh
School of Medicine and Magee-Womens Research Institute

Abstract

This chapter is based on the Geoffrey Harris Memorial Lecture presented at the 8th International Congress of Neuroendocrinology, which was held in Sydney, August 2014. It provides the development of our understanding of the neuroendocrine control of puberty since Harris proposed in his 1955 monograph [2] that “a major factor responsible for puberty is an increased rate of release of pituitary gonadotrophin” and posited “that a neural (hypothalamic) stimulus, via the hypophyseal portal vessels, may be involved.” Emphasis is placed on the neurobiological mechanisms governing puberty in highly evolved primates, although an attempt is made to reverse translate a model for the timing of puberty in man and monkey to non-primate species.

Keywords

human; rhesus monkey; rat; sheep; GnRH pulse generation; puberty; kisspeptin; GnRH surge generation

Introduction

Puberty - the period of becoming first capable of reproducing sexually, marked by maturation of the genital organs, development of secondary sex characteristics, and, in the human and other highly evolved primates¹, by the first occurrence of menstruation in the female [1] - has not previously provided the central theme of the Geoffrey Harris Memorial Lecture. In the context of this important developmental event, however, it is interesting to note that Harris in his renowned 1955 monograph proposed that “a major factor responsible for puberty is an increased rate of release of pituitary gonadotrophin” and posited “that a neural (hypothalamic) stimulus, via the hypophyseal portal vessels, may be involved.” [2]. Harris was correct on both accounts. In 1971, the hypothalamic factor that he argued was transmitted via the hypophyseal portal system was finally isolated from bovine and ovine

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Correspondence to: Dr. Tony M. Plant, Magee-Womens Research Institute, 204 Craft Avenue; Room B311, Pittsburgh, PA 15213, USA, Tel: 1-412-641-7663, planttm@mwri.magee.edu.

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¹Highly evolved primates or Catarrhini include the Old World monkeys, such as the macaques and baboons, apes and humans, and may be divided into two super families: Cercopithecidae (Old World monkeys and Hominoidea (apes and humans) [12]. For simplicity, the term primate will also be frequently used throughout this review to describe these two families.

hypothalamus independently by the laboratories of Schally and Guilleman, respectively [3] [4]. It was termed luteinizing hormone releasing hormone (LHRH) or luteinizing hormone releasing factor (LRF). This releasing hormone or factor was a decapeptide, which is now generally referred to as gonadotropin releasing hormone (GnRH). Two years before the isolation and characterization of GnRH, Knobil's laboratory had demonstrated an episodic pattern of gonadotropin release in the ovariectomized monkey and had proposed that this mode of secretion may be due to intermittent signals from the central nervous system that are relayed to the pituitary by a luteinizing hormone releasing factor [5]. Knobil's laboratory went on to establish in 1978 the critical importance of this pulsatile mode of hypothalamic GnRH release in sustaining gonadotropin secretion [6], but empirical confirmation of the episodic nature of GnRH levels in hypophyseal portal blood was not realized until 1982 after Clarke and Cummins had pioneered the development of a technique to sample hypophyseal portal blood in the unrestrained and unsedated ewe [7]. These observations seeded the idea that intermittent GnRH release was driven by a hypothalamic control system known as the hypothalamic GnRH pulse generator. This concept was championed during the early 80s by Knobil and Karsch working independently with the monkey and sheep, respectively [8] [9]. The acceptance of Knobil and Karsch's "black box" hypothalamic GnRH pulse generator was greatly reinforced by the subsequent finding that brief increases in multiunit electrophysiological activity (MUA) in the medial basal hypothalamus tightly coincided with episodes (pulses) of LH secretion [10]. This electrophysiological correlate of pulsatile GnRH release may be monitored and so provide a precise measure of GnRH pulse generator activity [11].

It is now generally accepted that the hypothalamic GnRH pulse generator drives "basal" or "tonic" gonadotropin secretion that is responsible for folliculogenesis, maintenance of the corpus luteum and the synthesis of ovarian estradiol and progesterone in the female and for maintaining spermatogenesis and testicular testosterone secretion in the male [13]. An additional mode of gonadotropin release, the pre-ovulatory LH surge observed at the end of the follicular phase of the ovarian cycle, is required for ovulation and therefore for puberty in the female [13]. In all species, the GnRH pulse generator plays an important role in producing the surge mode of gonadotropin release because it drives the rise in estradiol secretion late in the follicular phase that in turn serves as the ovarian component of the trigger for the pre-ovulatory LH surge.

From a pedagogic perspective, the core components of the physiological control system that governs the onset of puberty are most readily identifiable in our own species and in other highly evolved primates. There are two primary reasons for this. First, puberty in these species occurs after a protracted period of relatively stable gonadal quiescence during infancy and juvenile development² [13]. Therefore in primates the onset of puberty is clearly demarcated from earlier embryonic and perinatal periods of pre-pubertal development. For example, in the male monkey, the appearance of differentiating spermatogonia, which is an early event of male puberty marking the initiation of spermatogenesis, is not typically observed until 36 months of age [13]. In the mouse on the

²Childhood is observed only in hominids [15], and for the purpose of the present review the term juvenile will be used to describe the phase of development between infancy and puberty in all highly evolved primates including human.

other hand differentiating spermatogonia appear in the testis as early as postnatal day 3 [14]. Thus, in the primates temporal changes in endocrine, neuroendocrine and somatic parameters that are temporally correlated with the onset of puberty, and which may be used as markers of the onset of this developmental stage, unfold in an identifiable manner. Likewise, it is also in these species that changes in parameters associated with perinatal development (see below) are most easily compartmentalized and separated from pubertal changes that occur several years later.

Second, the control system that governs the preovulatory gonadotropin surge in primates is less complex than that in rodents. In fact, in the former species the minimal hypothalamic input required to support ovarian cycles and ovulation, and therefore puberty in both males and females, is intermittent GnRH stimulation of the pituitary gonadotropes [16]. In rodents, on the other hand, an additional hypothalamic component, namely, a specific neural signal that originates in an anterior region of the hypothalamus (the preoptic area) and one that is tightly coupled to the light-dark cycle is needed, together with the GnRH pulse generator, for initiation of the preovulatory LH surge [17]. The circadian neural signal is relayed to the pituitary by a large discharge of GnRH, which is therefore conceptualized in models of the rodent ovarian cycle to be produced by a GnRH “surge” generator. Thus, models for the control of puberty in rodents must include a hypothalamic GnRH surge generator to fully account for this developmental phase in the female. This is not the case for primates where puberty in both males and females may be achieved with pulsatile GnRH stimulation, alone; a situation again favoring the development of fundamental models of puberty.

Thus, when taken together, the protracted delay to the onset of puberty and the relative emancipation of ovulation from control by the preoptic area of the hypothalamus in primates facilitates the development of fundamental models to account for the neuroendocrine control of puberty. For the foregoing reason, this review will initially focus primarily on the control of puberty in primates and then briefly examine the extent to which models for the control system governing primate puberty may be applied to other species, using the rodent and sheep as examples.

Neither gonad, pituitary, nor GnRH neuron of the juvenile is limiting to the onset of puberty

It was known at the time of Harris that the quiescence of the ovary and testis in juvenile animals, and therefore the pre-pubertal condition of this stage of development cannot be accounted for by an intrinsic immaturity of these glands [2]. In primates, spermatogenesis or ovulation may be induced during juvenile development as a result of premature gonadotropic stimulation, which in man occurs on occasion spontaneously, and which may be imposed experimentally in laboratory primates, such as the rhesus monkey [18] [13]. Conversely, in boys and girls of “pubertal” age but with low circulating gonadotropin concentrations the onset of puberty is delayed or absent [18]

That the anterior pituitary is not a limiting component to the onset of puberty has also been recognized for many decades as a result of the early finding by Harris and Jacobsohn [19] that, in the rat, transplantation of the pituitary from prepubertal animals to the empty sella

turcica of hypophysectomized adult females led to a resumption of ovarian cyclicity in the adults before vaginal opening was observed in the litter mates of prepubertal donors. Similarly, in primates exposure of the pituitary to pulsatile GnRH stimulation prior to puberty, that in man occurs spontaneously in cases of GnRH dependent precocious puberty, results in a premature pubertal pattern of gonadotropin secretion, which if sustained will lead in turn lead to ovarian cyclicity and spermatogenesis [13]. Experimentally, administration of a chronic intermittent iv infusion of GnRH to pre-menarcheal monkeys results in the initiation of premature ovarian cyclicity with ovulation [20] (Figure 1). Following withdrawal of the exogenous GnRH stimulation, the pituitary-ovarian axis reverts back to a prepubertal condition.

During early embryonic development, GnRH neurons, which are born outside the brain in the olfactory placode, migrate through the forebrain to the hypothalamus [21] [22]. In primates, the fetal hypothalamus at mid-gestation is endowed with an adult number of GnRH neurons, distributed diffusely in both the preoptic area and medial basal hypothalamus with extensive projections to the median eminence [13] [23]. Interestingly, at the juvenile stage of development in the gonadal (surgically castrated) male monkey the hypothalamic contents of GnRH and the mRNA encoding this releasing factor are indistinguishable from those observed in gonadal adult animals exhibiting robust GnRH pulsatility and elevated levels of LH secretion [13]. Consistent with the later observations, is the finding that the distribution of GnRH perikarya and their projections to the median eminence, as revealed by immunohistochemistry, are similar at these two stages of development [13]. In contrast to the GnRH neuronal network of the juvenile hypothalamus, which may be viewed as being held in a state of suspended animation, the gonadotrophs of the anterior pituitary show little evidence of biosynthetic activity. LH and FSH contents of the juvenile pituitary and corresponding levels of the mRNAs that encode the β -subunits of the intact gonadotropin molecules are low and in line with the low levels of circulating gonadotropin at this stage of development [13]. Although the juvenile pituitary is relatively unresponsive to acute stimulation with GnRH, the gonadotrophs may be easily up-regulated by administration of a chronic intermittent iv infusion of the synthetic decapeptide [13].

Because of the upregulated biosynthetic state of the GnRH neuron in the hypothalamus of the prepubertal monkey, it is perhaps not surprising that this neuroendocrine neural network in the juvenile monkey may be readily provoked into producing a sustained pulsatile pattern of GnRH release by intermittent neurochemical stimulation with *N*-methyl-D-aspartate (NMDA), an amino acid analog that mimics the excitatory action of the neurotransmitter, glutamate [13] or by repetitive electrical stimulation [24]. In the testis (and presumably ovarian) intact situation, stimulation of the juvenile with NMDA leads to an adult pattern of gonadotropin and gonadal steroid secretion [25] [26] (Figure 2). The foregoing findings indicate the GnRH neurons of juvenile primate are endowed with the molecular and cellular machinery required for generating a functional hypophysiotropic drive to the pituitary gonadotrophs: all that is required for the initiation of puberty is the imposition of an appropriate afferent input to the GnRH neuronal network. In other words, the entire GnRH neuron-pituitary-gonadal axis is non-limiting to the onset of puberty.

The Hypothalamic GnRH Pulse Generator

There are two schools of thought regarding the neurobiological bases of the hypothalamic GnRH pulse generator [23]. The first proposes that pulsatility in the GnRH neuronal network is intrinsic to the GnRH neuron itself and that synchrony between the GnRH cells is achieved by extensive inter-cellular communication. This hypothesis, however, is difficult to reconcile with the finding that retrochiasmatic hypothalamic explants from the rat, which contain few if any GnRH cell bodies, continue to exhibit pulsatile GnRH release in culture [27]. The second hypothesis, which was generated by early findings that discrete lesions of the arcuate nucleus in the mediobasal hypothalamus of the female monkey abolished gonadotropin secretion without compromising the blood supply to the anterior pituitary [28], while surgical interruption of all neural inputs to this hypothalamic region did not block pulsatile LH secretion [29], posits that neurons in the arcuate nucleus are responsible for pulse generation. The later notion has recently gained considerable credence following the recognition of the importance of hypothalamic kisspeptin in regulating the GnRH-pituitary-gonadal axis. In 2003, the signal observation was made that loss of function mutations of the kisspeptin receptor (KISS1R, aka GPR54) were associated with hypogonadotropism and delayed or absent puberty in both man and mice [30] [31], and it soon became apparent that the arcuate nucleus is one of two major hypothalamic sites where *KISS1*, the gene encoding kisspeptin, is expressed and immunoreactive kisspeptin perikarya are found in abundance [32] [33]. Moreover, kisspeptin is an exceptionally potent GnRH secretagogue, GnRH neurons express KISS1R, and kisspeptin fibers project to GnRH cell bodies and GnRH fibers [23]. Of particular interest are those GnRH fibers that target the median eminence. Because these fibers exhibit characteristics of both axons and dendrites, they have been recently termed dendrons by Herbison and colleagues [34].

Many neurons in the arcuate nucleus also express, in an apparently species dependent manner, two other peptides, namely, neurokinin B, a tachykinin, and dynorphin, an endogenous opioid peptide [35] [36], and this pattern of coexpression led Lehman, Goodman and colleagues to suggest the acronym, KNDy, as a name for these cells [37]. Interestingly, loss of function mutations in man in either neurokinin B or its receptor (TAC3R) are associated with a very similar phenotype to that described earlier for inactivating mutations of KISS1R [38] i.e. hypogonadotropic hypogonadism and delayed or absent puberty. In monkeys, neurokinin B is stimulatory to GnRH release; an action that appears to be mediated indirectly via kisspeptin [39] [40]. Doses of neurokinin B that stimulate GnRH secretion in the monkey have not been administered to humans [41]. Dynorphin is generally recognized as inhibiting the release of the GnRH [35]. These three peptides have emerged as major components of the arcuate nucleus model of GnRH pulse generation. A critical evaluation of this model is beyond the scope of this review but the evidence upon which the KNDy model of pulse generation is based has been recently documented in several excellent papers [35] [42] [43] [44].

In essence, the model proposes that pulse generation is initiated in the KNDy neuronal network by a reciprocating interplay of stimulatory neurokinin B signals and inhibitory dynorphin inputs. The output of the pulse generator, on the other hand, is relayed from the midline arcuate nucleus to the more lateral and basal network of GnRH neurons by release

of kisspeptin from axonal terminals originating from KNDy neurons. It should be noted that the model does not exclude the possibility that other neurons in the arcuate nucleus, such as glutamate interneurons are an important component of pulse generation ([45], [46]). Moreover, for the model to be comprehensive it will also need to incorporate earlier findings indicating the importance of noreadrenergic and neuropeptide Y signaling to GnRH pulse generation ([47] [48] [49]). Notwithstanding, if one accepts the fundamental features of this model, kisspeptin expressed by KNDy neurons, albeit critical for the onset of puberty and, parenthetically, for the maintenance of fertility in adulthood, should be viewed not as a puberty activating neuropeptide but rather as a GnRH pulse generating peptide [50]. Further, according to this conceptualization kisspeptin neurons in the arcuate nucleus play no “regulatory” role in controlling the timing of puberty; instead as a component of the neural network responsible for GnRH pulse generation, they are slave to upstream regulatory mechanisms that are responsible for the timing of puberty (see later).

GnRH pulse generator activity at the onset of puberty

Consistent with the KNDy neuron model for GnRH pulse generation, Terasawa’s laboratory using microdialysis to sample kisspeptin release in the median eminence of the female monkey has recently shown that during juvenile development release of this KNDy neuron peptide occurs in low amplitude pulses, whereas in pubertal animals high amplitude and high frequency release was observed [51].

More precise temporal characteristics of the increase in GnRH pulse generator activity during the peripubertal period have been inferred largely from assessment of the frequency and amplitude of pulsatile LH secretion [13]. This approach, while sufficiently sensitive to reveal that enhanced pulse generator activity is first apparent at night, is limited because, as described above, the responsiveness to GnRH of the pituitary gonadotrophs that readout GnRH pulse generator activity is poor immediately before the initiation of puberty, and therefore discharges of GnRH, particularly those of small amplitude, may not be registered by this indirect assay during the early stages of pubertal development. In order to eliminate the early hyporesponsivity of the juvenile pituitary to GnRH, Suter et al [52] first “primed” the pituitary of gonadal male monkeys with a prolonged intermittent iv infusion of GnRH (1 pulse of GnRH every hr) before tracking the pubertal increase in GnRH pulse generator activity. In such animals, nocturnal GnRH pulse frequency accelerated immediately and explosively at the termination of the juvenile phase of development from <1 pulse/7h to approximately 4 pulses/7h over a period of less than 6 weeks. This finding indicates that the potential for adult GnRH pulse generator activity at the termination of the juvenile phase of development is rapidly achieved early in the pubertal process in the male monkey.

GnRH pulse generator activity prior to puberty

Interestingly, in primates, circulating levels of both LH and FSH are markedly elevated before the generally recognized hypogonadotropic state of the prepubertal condition is achieved [13]. Moreover, gonadotropin secretion during infancy is dependent upon GnRH, as reflected by the finding that, in the monkey, LH and FSH levels are suppressed by postnatal treatment with a GnRH receptor antagonist [53]. These observations suggest that

GnRH pulse generator activity may be robust many years before the onset of puberty. This proposal is supported by the finding of hypogonadotropism in an infant boy in association with a loss of function mutation in KISS1R [54]. In addition, LH secretion during infancy is pulsatile and in the monkey this mode of secretion is amplified following either ovariectomy or orchidectomy. Although the frequency of pulsatile LH release, and therefore that of the GnRH pulse generator, in the agonadal infant female monkey is less than that observed in the adult [13], the conclusion that infancy is a phase of robust GnRH pulse generator activity is inescapable. Moreover, the finding that the duration of the period of elevated gonadotropin secretion of preterm infants is greater than that of term infants suggests the expression of GnRH pulse generator activity postnatally is determined by developmental events in the brain rather than by escape from the suppressive hormonal milieu of pregnancy [55]. Although this phase of primate development has been termed the “mini-puberty” of infancy, this descriptor is misleading because at the gonadal level of the axis neither ovulation, nor spermatogenesis, is initiated in spite of an adult-like endocrine milieu to which the ovary and testis are exposed [56] [57; 58]. Here, it is interesting to note that by the time the post natal gonads do acquire the full ability to respond to stimulation by gonadotropin, the GnRH pulse generator has been brought into check and the hypogonadotropic state of the human child or juvenile monkey has been attained thus guaranteeing the continued relative quiescence of the gonad that is associated with the prepubertal situation.

Since intermittent neurochemical NMDA stimulation of the hypothalamus of the juvenile male monkey is able to drive the pituitary-gonadal axis into a pubertal mode of operation in a GnRH dependent manner (see above), it is not surprising that a similar mode of exogenous kisspeptin administration provides the dormant GnRH neuronal network of the juvenile hypothalamus with an “exogenous” pulse generating signal that induces a sustained train of GnRH discharges as reflected by the pattern of LH secretion in the agonadal GnRH primed juvenile monkey [59] (Figure, 3).

Although the neurobiological development of the GnRH pulse generator during embryonic and fetal development is only beginning to emerge from contemporary studies of genetically manipulated mice [60], studies of the time course of fetal gonadotropin secretion during human gestation and of the operation of the pituitary-gonadal axis in fetal sheep and monkeys indicate that the hypothalamic GnRH pulse generator is capable of generating a functional hypophysiotropic drive to the fetal pituitary by the second trimester of pregnancy [13].

The question therefore becomes is the subsequent hypogonadotropic state of the juvenile due to suppression of hypothalamic GnRH pulse generator activity from infancy until the termination of the juvenile phase of development or to its uncoupling from the GnRH neuronal network during this time. While this issue has not been systematically addressed, the finding that low amplitude pulsatile LH release is observed in boys and girls prior to the onset of puberty [13] indicates that activity of this neuroendocrine system is diminished rather than uncoupled from the GnRH neuronal network during juvenile development. This view is supported by the report in the juvenile female monkey that release into the median eminence of kisspeptin, the posited output of the GnRH pulse generator, occurs at a greatly

reduced amplitude when compared to the adult [51]. Also consistent with this view is the finding from this laboratory that in the male rhesus monkey the number of neurons in the arcuate nucleus expressing kisspeptin is reduced in association with the transition from the infantile to juvenile stage of development [61]. Similarly, expression of *KISS1*, the gene that encodes for kisspeptin increases in the arcuate nucleus of male and female monkeys in association with the transition from the juvenile to pubertal stage of development [62].

The hiatus in robust pulsatile GnRH release during juvenile development may be conceptualized to result from a prepubertal “brake” or restraint that during this developmental stage is imposed upon the GnRH pulse generator in the arcuate nucleus [63]. Additionally, puberty may be further conceptualized to be timed by two postnatal switches: the first applies the “brake” or “restraint” that holds the GnRH pulse generator in check during the infantile-juvenile transition and the second removes the brake at the termination of juvenile development leading to a re-activation of GnRH pulse generation and the onset of puberty. The brake of course is conceptual and may be mediated by either the application of inhibitory signals to the pulse generator or the loss of stimulatory inputs or a combination of both.

In the absence of negative feedback from gonadal hormones, circulating levels of LH and FSH reflect more robustly the activity of the GnRH pulse generator. In this regard, the time course of FSH secretion in neonatally castrated male and female monkeys from birth until the time at which puberty would have been anticipated had the animals remained intact is particularly informative [13] [64] [65] (Figure 4). This is because a clear sex difference in the duration and degree of suppression of this gonadotropin, and presumably therefore that of the hypothalamic GnRH pulse generator, is apparent, with the brake being imposed with greater intensity and for a longer duration in the male. This sex difference also indicates that in the gonadally intact situation negative feedback signals from the ovary play a greater role in amplifying the consequence of the brake on FSH and LH secretion during juvenile development than do those from the testes [65]. Comparable, albeit less complete data are available for agonaladal human subjects during analogous stages of post natal development [13]. This sex difference in postnatal development of GnRH pulse generator activity, likely underlies the earlier onset of female puberty in primates, and the findings that constitutional delay of puberty in children is most frequently reported in boys, while GnRH dependent precocious puberty is relatively more common in girls [18].

The study of agonaladal subjects also reveals that the suppression of the GnRH pulse generator during juvenile development occurs in the absence of either the ovary or testis. This characteristic of the postnatal pattern of hypothalamicpituitary activity in primates was first demonstrated in 1975 by Grumbach and his colleagues [66] who studied girls with Turner syndrome and other forms of gonadal dysgenesis. This signal observation made 4 decades ago remains the cornerstone of the current view that puberty in primates is delayed at the level of the brain by what Grumbach and Kaplan termed an “intrinsic central nervous system inhibitory mechanism” [67], and what is now conceptualized as being imposed on the hypothalamic GnRH pulse generator.

The impact of robust GnRH pulse generator activity during infancy and at the initiation of puberty on gonadotropin secretion is modulated by the negative feedback actions of the gonadal steroids that are produced in response to the elevated hypophysiotropic drive to the gonadotroph at these stages of development. These steroid dependent actions, probably exerted at both hypothalamic and pituitary sites, determine the precise duration of the hypogonadotropic state of the juvenile and the tempo at which puberty progresses once the neurobiological brake on the GnRH pulse generator begins to wane [13]. In the female, for example, there is generally a significant delay between menarche and first ovulation, and it has been posited that this is because the rise in tonic gonadotropin secretion which is being driven by a progressive reactivation of the GnRH pulse generator is initially damped by the relatively small amounts of estradiol produced by the ovary at this stage of development [68].

The dramatic changing steroid milieu to which the brain is exposed during the “turn off” of the GnRH pulse generator at the time of the infant-juvenile transition and its subsequent “turn on” during the juvenile-pubertal transition generates a major caveat to studies aimed at interrogating the neurobiological mechanisms underlying the two critical switches regulating the pattern of pulsatile GnRH release from birth to puberty. This is because gonadal steroids have profound effects on most regions of the brain, including those that are not generally recognized to be involved with the postnatal regulation of the GnRH pulse generator. These steroid actions may effect the molecular, cellular and structural biology of the brain. Thus, if an association is made between a change, for example, in the expression of a gene, or in the release of a neuropeptide, or in a structural parameter such as synaptic density, on the one hand, and the onset of puberty on the other, it is impossible to decipher whether the change being observed is associated with the primary neurobiological event (ie the removal of the brake) or is simple a manifestation of a secondary or tertiary consequence of increased gonadal steroid secretion driven by the fundamental hypothalamic event. To eliminate this caveat, my laboratory has made extensive use of the gonadal monkey, castrated either at birth to examine the turn off of the GnRH pulse generator (Switch 1), or during the mid juvenile stage of development to examine the mechanisms responsible for the pubertal activation of the GnRH pulse generator (Switch 2). In these models, the impact of gonadal steroids on the brain is eliminated and any change in a neuronal or glial parameter(s) in association with either the turn off or turn on of the GnRH pulse generator is more likely to reflect a component of the fundamental neurobiology dictating(s) the postnatal ontogeny of the GnRH pulse generator [69].

Neurobiology of the prepubertal brake delaying reactivation of GnRH pulsatility

In general, two experimental strategies have been taken to probe the nature of the neurobiological mechanisms that are associated with suppression of the GnRH pulse generator during juvenile development, and, are in turn responsible for the hypogonadotropic state that characterizes this stage of prepubertal development. The first of these may be viewed as the “classical” approach, and ranges from lesioning/ablation techniques to neuropharmacological interventions to morphological analysis employing

immunohistochemistry, in situ hybridization and electron microscopy. The second approach may be termed the “omics” approach, which is based on high throughput sequencing of DNA, RNA transcripts and protein combined with computational biology. The latter systems biology approach seeks to extract from global gene expression data (to date derived from hypothalamus) insight into the genes that underlie the onset of puberty [70].

The classical approach has been to test a particular hypothesis based on the prevailing understanding of the action of a particular neurochemical or on the established function of a particular area of the brain. For example, in the case of the former, melatonin is considered to have anti-gonadotropic properties and therefore the impact of pinealectomy during juvenile development on the pubertal reactivation of pulsatile GnRH release has been investigated: incidentally with a negative outcome [13]. Another example, and one that is worthy of more extensive comment is γ -aminobutyric acid (GABA), the major inhibitory neurotransmitter utilized by the mammalian brain, and a rational candidate for a component of a prepubertal brake suppressing the GnRH pulse generator prior to the onset of puberty. In this regard, Terasawa's group has performed an extensive series of experiments that provide compelling evidence for the view that this amino acid plays an important role in the timing of the onset of puberty in the female rhesus monkey [71]. Notably, the pubertal increase in GnRH release in the region of the stalk-median eminence of the female monkey is correlated with a concomitant decrease in GABA content in this region of the brain. Chronic repetitive administration of the GABA_A receptor antagonist, bicuculline, into the base of the third ventricle led to early menarche and precocious ovulation. Moreover, acute inactivation of the GABA_A receptor or reduction of GABA tone in this region of the hypothalamus of the prepubertal female with bicuculline or antisense oligodeoxynucleotide for the mRNA encoding the GABA synthesizing enzyme, glutamic acid decarboxylase 67 (GAD67), respectively, elicited an immediate discharge of GnRH. On the other hand, injection of GABA into the region of the stalkmedian eminence inhibited GnRH release in pubertal monkeys but not in prepubertal animals [72]. The action of GABA_B receptor antagonism with saclofen on GnRH release was inconsistent and did not reach significance [72].

The location of the GABAergic neurons responsible for the peripubertal changes in GABA content of the stalk–median eminence of the female monkey, and whether this hypothalamic area is the major site at which GABA inhibits GnRH release during juvenile development are unknown. Moreover, studies of the neuronal targets and electrophysiology underlying the action of GABA to regulate GnRH release are incompletely understood, even in rodent models that have been studied extensively [23].

As described above, the intensity of the neurobiological brake on the GnRH pulse generator during juvenile development in the female is less than that in the male, and its effect on gonadotropin secretion is amplified by the negative feedback action of ovarian steroids. Therefore, since the foregoing studies of the female monkey were conducted with ovarian intact animals it is unclear whether the stimulation of GnRH secretion that followed interference of GABA signaling was the result of interrupting negative feedback by the ovary or removing the non-gonadal restraint on the GnRH pulse generator. The later possibility is supported by the finding that application of similar strategies to reduce GABA

tone in the pubertal monkey elicited GnRH responses of lesser magnitude than those observed in juvenile animals, presumably because GABA tone in the pubertal situation was significantly reduced due to loss of the component mediating the non-gonadal brake.

Unfortunately, analogous studies examining the role of GABA have not been conducted in the male monkey, although the pubertal re-activation of GnRH pulse generator activity in the male hypothalamus does not appear to be associated with a change in expression of either *GAD 65* or *GAD67* [73] [74]. In the infantile-juvenile transition, expression of these mRNAs actually increases [75].

Interestingly, the reciprocal decrease in GABA release and increase in GnRH levels in the median eminence of the female monkey at puberty is temporally correlated with an increase in the content of kisspeptin [76], the posited output of the GnRH pulse generator, and the excitatory amino acid, glutamate [71]. As mentioned above, intermittent activation of the NMDA receptor (one of the receptor subtypes which transduce glutamate signals) has been shown to mimic the action of intermittent kisspeptin stimulation in inducing a precocious pubertal pattern of GnRH release from the hypothalamus of the agonadal juvenile male monkey [77]. The functional significance of the pubertal increase in glutamate is unclear. This amino acid transmitter, like the neuropeptide, kisspeptin, might be a component of the GnRH pulse generator and its increase at the onset of puberty simply reflecting the reactivation of GnRH pulse generation. Alternatively, glutamate may represent a component of the brake.

In the case of the male, my laboratory invested significant effort into pursuing the hypothesis that neuropeptide Y (NPY) was an important component of the brake exerted upon the GnRH pulse generator during juvenile development. Although the pattern of NPY expression from birth to puberty in the agonadal male was inversely related to that of pulsatile GnRH release, as reflected by circulating LH concentrations [74], we were never able to demonstrate using pharmacological approaches that inhibition of NPY signaling in the hypothalamus of the juvenile resulted in a lifting of the brake as evidenced by precocious GnRH release [78].

The omics approach to the mystery of puberty has been pioneered by Ojeda and his colleagues at the Oregon National Primate Research Center [79]. Using human cDNA microarrays, the Oregon group has identified that expression of many hypothalamic genes, which encode for a diverse variety of proteins, changes during the transition into puberty in the female rhesus monkey: levels of the mRNA transcripts of more than a hundred of these genes was several fold greater in pubertal animals than those in juveniles [80]. Importantly, a pubertal increase in the expression of these genes was not observed in the cortex, and in the case of genes of particular interest the initial array results were confirmed by real-time PCR [80]. Coupling the foregoing expression data with contemporary bioinformatic tools, enabled Ojeda and his colleagues to first highlight a subset of these developmentally regulated genes that encoded transcription factors with tumor suppressing or oncogenetic properties. Next, the promiscuousness of each of the transcription factors encoded by a developmentally regulated gene was predicted from their relative potential to interact with cis regulatory sequences upstream from the promoter of the other genes in the

developmentally regulated cohort. This analysis led to a model whereby a few of the tumor related genes (termed “hub genes”), were posited to govern a plethora of the other genes (termed “subordinate genes”). Based on the algorithm, subordinate genes, on the other hand, were themselves capable of regulating only a limited number of genes in the network [80].

A non-tumor related gene, thyroid transcription factor 1 (*TFT1*), the expression of which is also elevated in the mediobasal hypothalamus of pubertal female monkeys [81] was also indicated to be a hub gene by this systems biology approach [80]. A second non-tumor related gene expressed in the mediobasal hypothalamus of the pubertal female monkey was identified as a predicted gene of unknown function [82]. Additional work demonstrated that this gene encoded a 15 transcription factor, containing a Zinc finger domain of the C3HC4 subclass, and that, in the female monkey, was expressed in hypothalamic regions controlling the reproductive axis, such as the arcuate nucleus. The gene was termed enhanced at puberty 1 (*EAP1*), and was later considered as a hub gene [79]. Interestingly, *EAP1* is expressed in kisspeptin neurons in the arcuate nucleus of the rat [83]. Moreover, knockdown of *EAP1* using a lentivirus approach interrupts menstrual cyclicity in the monkey and a SNP upstream of the *EAP1* gene has been associated with irregular menses in this primate [84] [85]. Interrogation of global gene expression in the agonadal male rhesus monkey, however, using a monkey cDNA array failed to reveal an increase expression of *EAP1* in the mediobasal hypothalamus during the juvenilepubertal transition when GnRH pulse generation is reactivated (Ojeda and Plant, unpublished observations). The latter study, on the other hand, did find that several genes encoding other Zinc finger transcriptional repressors did decrease during the juvenile-pubertal transition. These included a sub-class of Zinc finger proteins endowed with a Kruppel-associated box (KRAB) domain, and GATA zinc finger domain-containing protein 1; the later interacts with histones in chromatin to regulate transcription epigenetically [86].

As a result of the foregoing data, and other results obtained since 2007 from studies of non primates, the original proposal of Ojeda and his colleagues of a central group of hub genes governing sub-ordinate gene networks posited to underlie the initiation of puberty has grown in complexity with additional layers of genes added to the network in a hierarchical fashion [79].

At the time the array data from the agonadal male monkeys were being analysed, the groups of Kaiser and Latronico together published the interesting finding that loss of function mutations in another Zinc finger protein, makorin RING finger protein 3 (*MKRN3*), were associated with GnRH dependent precocious puberty in both boys and girls [87]. Precocity was noticed between the ages of 5.0 to 6.5 years in girls and 5.9 to 8.5 years in boys. Consistent with this clinical observation, qPCR analysis of the agonadal male monkey experiment revealed that expression of *MKRN3* in the MBH of agonadal juvenile monkeys was higher than that in “pubertal” animals, in which GnRH pulse generator activity had been re-activated as reflected by elevated LH secretion (Ojeda and Plant, unpublished observations). In contrast to the KRAB Zinc finger transcriptional repressors, but like *EAP1*, *MKRN3* contains a C3HC4 RING zinc finger motif, which is a motif considered to bestow ubiquitin-ligase activity on the protein [88]. This has led to the proposal that the elevated expression of *MKRN3* during juvenile development leads to ubiquitination, and therefore

degradation, of proteins in a signaling pathway driving the secretion of GnRH that in turn contributes to the diminished pulsatile GnRH release characteristic of this phase of development [89]. Where *MKRN3* will be placed in the Ojeda model for the genomic basis of puberty has yet to be determined.

With regard to the arrest of robust pulsatile GnRH release during infancy, it might be predicted that the changes in hypothalamic gene expression that underpin this developmental event are the reverse of those that are associated with the pubertal reawakening of the GnRH pulse generator. Although data on this issue are limited, results to date suggest that this expectation is not going to be the case. The expression of the KRAB Zn fingers genes and *MKRN3* that all decrease during the juvenile-pubertal transition in agonadal male monkeys do not change during the infantile-juvenile transition in this primate model (Ojeda and Plant, unpublished). In addition, a similar dichotomy is seen with *LIN28B*, a gene implicated in timing the age of menarche in girls (see below). In the female rhesus monkey, the levels of *LIN28B* mRNA in the MBH have been reported to fall by more than 50% in the infantile-juvenile transition but to remain unchanged during the transition into puberty [90].

Regardless, the computational analysis of gene function at puberty in the systems biology approach is currently conducted largely without regard to the location of the neuronal systems (or glia) in which the empirically determined developmental changes in gene expression are occurring. Since the operation of the posited gene interactions is based on transcriptional regulation, the *in silico* network hypothesized to control puberty must be contained within at least a subset of communicating cells in a particularly significant neuronal system, for example KNDy neurons, which by utilizing classical signaling pathways to effect transcellular actions can, in turn, govern downstream events leading to pulsatile GnRH release. To date this has not been established. Thus, the immediate challenge is to integrate models generated by classical and omics strategies and thereby obtain unified paradigms. Once this is achieved, the overarching question of whether the developmental changes in transcriptional, cellular and inter-cellular machinery within the pubertal hypothalamus are intrinsic to the postnatal development of this region of the brain, or are set in motion by an extra-hypothalamic pubertal trigger will remain (see below for discussion of the timing of puberty).

Timing of Puberty

Fundamental Control Systems

The fundamental control system governing pubertal timing may be viewed as comprising two switches, the first turns off the active GnRH pulse generator of the infant to guarantee the hypogonadotropic state of the juvenile, and the second reactivates this hypothalamic mechanism at the termination of juvenile development and thus triggers puberty. As noted above, the key issue relating to the control of these two switches is the question of whether they are regulated by extra-hypothalamic signals or, on the other hand, governed by an intrinsic timing mechanism that unfolds according to a specific developmental program within the postnatal hypothalamus?

With regard to the second - pubertal switch - the notion of a central growth tracking system that enables the re-activation of the GnRH pulse generator and therefore puberty to be synchronized with the attainment of adult somatic size has particular appeal. Here, the brain is proposed to monitor a circulating endocrine or metabolic cue that reflects somatic development, and that is transduced on line into a signal that gates the reemergence of robust GnRH pulsatility with the attainment of an appropriate stage of somatic development. The central neural component that tracks the somatic cue has been conceptualized as a “somatometer” [91]. Conceivably, the neural network that underlies GnRH pulse generation could also serve as the putative somatometer, but distinct locations of the putative somatometer and pulse generator are also possible. The idea of a somatometer dates back to the work and ideas of Frisch in the 1940s: she proposed that in girls a critical fat mass or fat/lean ratio, rather than a critical age, had to be attained for menarche to occur [92] [93]. With the discovery of the adipose tissue hormone, leptin, in 1994 [94] interest in the ideas of Frisch were rekindled. However, while the action of leptin on the hypothalamus is essential for sustained activity of the hypothalamic-pituitary-gonadal axis both during puberty and adulthood, the hormone does not function as a somatic trigger that times the onset of puberty by reactivating robust GnRH pulsatility. This view is best supported by the finding that in young children with leptin deficiency initiation of treatment with the recombinant hormone does not induce puberty immediately but rather this occurs only following prolonged exposure to the hormone and after a typical pubertal bone age has been achieved [95] [96] [97]. That bone age and the onset of puberty are more tightly associated with chronological age and sexual maturation has also been recognized for many years [98], and the idea that maturation of the skeleton might govern the timing of puberty is intuitively reasonable. Moreover, bone is now an established endocrine organ [99], although direct empirical evidence that this organ may contribute to the putative somatic signal that is posited to time puberty has yet to be obtained. Conceptualization as to how a posited somatometer would control the timing of the first (off) switch of infancy that is responsible for curtailing the activity of the GnRH pulse generator during subsequent prepubertal development is not as straightforward as that underlying the pubertal switch.

The alternative model for the control of the timing of puberty, namely that the initiation of this developmental event is governed by a “clock” intrinsic to the brain that unfolds postnatally according to a specific developmental program that enables time to be tracked has yet to be pursued empirically. It is nevertheless interesting to note that peripubertal changes in several structural parameters of human brain maturation such as total cerebral volume and grey matter volume occur earlier by several years in girls than in boys [100]. Moreover, such peripubertal changes in the human brain are associated with marked changes in sleep architecture, and in particular in slow wave sleep [101]. Whether these changes in brain activity, like those in GnRH pulse generator activity, are independent of the dramatic pubertal increases in gonadal steroids, which also unfold with a similar temporal sex difference is unclear, but it is tempting to speculate that some aspect of brain development, such as maturation of sleep architecture, might be causally related to the re-initiation of GnRH pulse generator activity. In this regard, it has been recognized since the 1970s that the pubertal reactivation of GnRH pulsatility, as reflected by LH secretion is first observed during sleep [102]. If changes in sleep architecture represent a component of the output of a

putative pubertal clock, the relationship to GnRH pulse generation would appear to be non-linear, because although the majority of LH pulses during sleep in pubertal boys and girls are preceded by slow wave sleep [103] [104], this sleep stage declines markedly during pubertal development [101], and in the follicular phase of the menstrual cycle GnRH pulse frequency decelerates at night [105] [106].

Inheritable pubertal traits

The second switch that reactivates robust GnRH pulsatility at the termination of juvenile development sets in motion the pubertal process and many traits of this developmental phase such as a tendency to early or late menarche in girls have been well recognized to be inheritable [13]. The genetics underpinning such associations, however, have only recently begun to emerge. In 2009, four papers were published describing for the first time the application of genome wide association studies (GWAS) to identify genetic variants associated with the timing of menarche [107] [108] [109] [110]. A single nucleotide polymorphisms (SNPs) that was consistently and strongly associated with an earlier age of menarche was found with a high frequency in a locus on chromosome 6 (6q21) in the region of *LIN28B*: a gene that encodes for a micro-RNA binding protein. Subsequently, additional GWAS studies of large cohorts of women have now identified over 100 loci on the human genome that are associated with age at menarche [111]. Interestingly, another locus of genetic variation with an effect on the timing of menarche as strong as that of *LIN28B* was found in the region (15q11-13) of the paternally expressed gene, *MKRN3*. As described above loss of function mutations of the protein encoded by *MKRN3* have been associated with GnRH dependent precocious puberty in both boys and girls. It should be noted that the age of menarche in the population as a whole may extend from 10 to 16 years [18], and even loci with strong associations to this developmental event, such as that for *LIN28B*, appear to account for only a small fraction (1–2 months) of the normal variation. In addition, many of the loci that have been tied to the age at menarche are also associated with variation in growth and body mass and may therefore influence pubertal timing as secondary modulators of this developmental event. Thus, the exact relationship between the neurobiological changes within the hypothalamus that dictate the developmental pattern of GnRH, on the one hand, and the genetic bases of the timing of various heritable pubertal traits, on the other, remain to be elucidated.

Other factors

Pubertal traits may also be related to epigenetic mechanisms brought into play by environmental factors such as those associated with sub-optimal nutrition or exposure to certain chemicals, particularly at the time of fetal and early postnatal development. In this regard, the finding of Barker in the 1990's that birth weight in man was correlated with the onset of certain diseases in adulthood, led him to propose that susceptibility to disease in adulthood can be programmed during early development [112]. In keeping with the Barker hypothesis of the developmental origin of health and disease (DOHAD), it has more recently emerged that intrauterine growth restriction, and the degree of catch up growth during the early postnatal period, interact in a manner that may result in earlier puberty as reflected primarily by age of menarche [113]. Again, as with the genetic basis of pubertal timing, the question of whether environmentally induced epigenetic changes impact the fundamental

neurobiology governing the pubertal re-surgence of GnRH release or influence processes controlling growth and metabolism, which in turn indirectly modulate the pubertal process is unclear.

To conclude discussion on the timing of puberty, three other factors that impact the age at which this developmental event is manifest need to be recognized. First, as discussed above, puberty in primates is initiated earlier in the female than in the male [18], and this sex difference is reflected in the developmental pattern of gonadotropin secretion in agonadal male and female rhesus monkeys [13] (Figure 4). In the former, the duration of the hiatus in LH and FSH secretion from infancy until the termination of the juvenile phase of development is greater than that in their female counterparts, and circulating gonadotropin levels, particularly those of FSH, are much lower in the male during the juvenile phase of development. These observations indicate that the restraint imposed upon the GnRH pulse generator during juvenile development is most intense and applied for a longer duration in the male. This sex difference in the postnatal time course of GnRH pulse generator activity, which likely is responsible for the later onset of male puberty, is presumably dictated by a programming action of testicular testosterone on the fetal hypothalamus. This view is consistent with the finding that in humans with a male karyotype loss of function mutations in the androgen receptor, which result in a syndrome known as “androgen insensitivity”, is associated with peak growth velocity at puberty occurring at an age earlier than that observed in normal boys [114]. Additionally, androgenization of the female monkey in utero is associated with delayed menarche [115], while administration of the antiandrogen, Flutamide, at this stage of development accelerates the onset of puberty in the male offspring [116].

Second, the dramatic increase in circulating gonadal steroids that follows the initiation of puberty exert negative feedback actions on the LH and FSH secretion that is occasioned by the reawakening GnRH pulse generator. These feedback actions may be exerted at both hypothalamic and pituitary sites and serve to dampen the pubertal reactivation of the hypothalamic-pituitary unit that occurs rapidly in the agonadal situation. Thus, the timing and tempo of gonadal maturation and puberty will be regulated by ovarian and testicular feedback to the hypothalamus and pituitary [13].

Finally, the timing and tempo of puberty may also be delayed or suppressed by conditions prevailing in late juvenile development and continuing thereafter, including under nutrition and the presence of social factors such as stress. As previously discussed [13], however, since these conditions also suppress the hypothalamic-pituitary-gonadal axis in adulthood it is likely that they influence the timing of puberty simply by masking the manifestation of the pubertal activity of the GnRH pulse generator, as they do post-pubertally.

GnRH Surge Generator and Puberty

As indicated above, male puberty and the transition into adulthood in this sex is achieved by a single mode (tonic) of gonadotropin release, which is driven by the re-activation of the hypothalamic GnRH pulse generator. In females, however, ovulation and therefore completion of puberty and fertility in adulthood requires not only tonic LH and FSH release

but also a second mode of gonadotropin secretion known as surge secretion. The neurobiological basis of the pre-ovulatory LH surge in primates is poorly understood, and the extent to which models developed from both classic and contemporary studies of non-primate species may be translated to the human has remained a topic of debate ever since 1980 when Knobil's laboratory posited that the role of the primate hypothalamus in the control of the menstrual cycle was permissive [117], ie pituitary gonadotropin secretion is driven by pulsatile GnRH release from the hypothalamus but the patterns of LH and FSH secretion throughout the menstrual cycle including the midcycle pre-ovulatory surge are dictated by the negative and positive feedback actions of ovarian estradiol at the level of the pituitary. The cardinal question in the context of puberty is whether the triggering of the pre-ovulatory LH surge in female primates involves estrogen dependent activation of a hypothalamic GnRH surge generator, as it does in non-primate species. If this were the case, a complete understanding of the neurobiology underlying puberty in female primates would require a full appreciation of the development and operation of the hypothalamic GnRH surge generator.

Studies aimed to address this question in the human female, conducted primarily by Hall and colleagues, have of course been indirect in nature, but as reviewed recently by the author of this article, indicate that ovulation in women may occur in the absence of a GnRH surge [17], thus providing support for the hypothesis of Knobil. In the monkey, on the other hand, compelling evidence is at hand indicating that an unambiguous surge of GnRH is triggered at the time of the spontaneous midcycle LH surge [118]. Moreover, in the monkey the activity of the hypothalamic GnRH pulse generator, as reflected by MUA in the mediobasal hypothalamus, is arrested during the preovulatory gonadotropin surge [119]. Until the hypothalamic components of the GnRH surge generator in the monkey have been delineated, it will not be possible to study the postnatal development of this system and therefore our overall understanding of the neurobiology of puberty in this species will be incomplete. In the case of the human, where it seems to be reasonable to conclude that during the course of evolution ovulation has become emancipated from control by the GnRH surge generator [17], puberty may well be accounted for solely by the mechanisms that reawaken the GnRH pulse generator at the termination of the juvenile phase of development.

Reverse translation of the primate model

As articulated above, the neurobiology underlying the pre-ovulatory gonadotropin surge in primates has received little attention since the emergence over the last decade of the profound importance of kisspeptin in regulating ovulation in rodents [23]. For this reason, discussion here will be limited to the extent to which the model positing that male puberty in monkeys and boys is triggered by a reawakening of the GnRH pulse generator, which for the greater part of pre-pubertal development has been held in check by mechanisms that are independent of the gonads, may be applied to mammalian species other than primates. Sheep and rodents will be used as examples because most of what is known regarding the control of puberty in non-primate species is based upon studies in these animals [120] [121].

In male primates, the reawakening of the GnRH pulse generator at the termination of the juvenile phase of development leads, in a hierarchical and temporally ordered sequence, to

re-initiation of gonadotropin release, reactivation of testicular testosterone secretion and the initiation of spermatogenesis driven by the combined intratesticular action of testosterone and FSH and resulting in an upswing in testicular growth. Because these pubertal events are robustly separated from any perinatal changes in the activity of the hypothalamic-pituitary-testicular axis, and follow a protracted period of comparative quiescence in this neuroendocrine axis, the onset of puberty in primates is relatively readily identified.

In the case of rodents, where sexual maturation follows closely on the heels of fetal and neonatal development, a distinct point in development when pubertal activity in the hypothalamic-pituitary axis is initiated is more difficult to pin down. This problem is further exacerbated because comprehensive longitudinal descriptions of the precise time courses of circulating gonadotropin and testosterone concentrations from birth until the completion of puberty are scant and those studies that have been conducted report highly variable results, particularly in the case of LH [122] [123] [124] [125] [126] [127] [128]. In male primates, the pubertal reactivation of gonadotropin secretion results in the initiation of spermatogenesis, as first reflected by the appearance of differentiating pre-meiotic spermatogonia [56]. In mice and rats, such differentiating spermatogonia are observed as early as postnatal day 3 [14], and it is doubtful that this critical developmental event in the testis can be used as a marker of the pubertal activation of the hypothalamic-pituitary component of the neuroendocrine axis in these species, as it may in the monkey, because spermatogonial differentiation in rodents occurs independently of gonadotropin signaling [129]. Meiosis, on the other hand, is a testosterone, and therefore LH, dependent event and in the mouse is completed for the first time approximately 2 weeks after the appearance of differentiating spermatogonia, ie around 3 weeks of age [130]. Thus, it seems reasonable to posit that, in rodents, the pubertal rise in LH and FSH secretion is likely initiated between 1 and 3 weeks of age. This view is consistent with findings in the mouse that 1) GnRH mRNA levels are low during the first week of life but increase significantly by 15–25 days of age [128], 2) a distinct mode of episodic LH release is apparent at 28 days of age [131], and 3) kisspeptin projections to GnRH neurons markedly increase between day 25–31 [132].

Regardless of the precise timing of the pubertal rise in gonadotropin secretion, the question becomes is this endocrine event triggered by a **reactivation** of GnRH pulsatility, as it is in primates, or is it simply a reflection of the earliest manifestation of GnRH pulse generator activity. In this regard, an increase in testosterone secretion during the first few postnatal days in male rodents, predicted many years ago from classical studies of the role of the neonatal testis in programming brain structures responsible for sexually differentiated function in the adult [133], has been empirically verified [134] [135] [136] [137]. However, the obvious difficulty of obtaining serial blood samples from newborn pups has prevented characterization of any moment to moment changes in plasma LH concentrations there may be at this critical stage of development, and the evidence to support the notion that the postnatal surge in testicular testosterone in these species is driven by GnRH dependent LH secretion is contradictory [135] [136] [137]. Kisspeptin (KNDy) neurons are in synaptic communication with a subset of GnRH neurons by late embryonic development in the mouse [60], and transgenic male mice with GnRH neuron specific deletion of the kisspeptin receptor exhibit a blunted testosterone surge on postnatal day 1 [137]. On the other hand, the

recent application of fast scan cyclic voltametry to detect GnRH in brain slices of male mice indicate that release of the decapeptide in the median eminence on the day of birth and for the first week of postnatal life exhibits a unique ultra-high frequency, low amplitude pattern that is independent of kisspeptin signaling [138]. Moreover, the hypophysial portal circulation at birth in the rat and mouse is relatively immature and invasion of the median eminence by GnRH terminals at this stage of development is incomplete [139] [140] [141] [142] [143] [144].

Taking the foregoing considerations together, it seems reasonable to propose that the completion of puberty in male rodents requires an increase in gonadotropin secretion that is driven by GnRH pulse generator activity, but that, in contrast to primates, this pubertal hypothalamic activity is probably the reflection of the final maturation of the neuronal network generating pulsatility, and not the reactivation of a fully differentiated system that was established prenatally as in primates. As Harris pointed out in 1955 [2], the pituitary is not limiting to the onset of puberty, and therefore the delay from birth until the pubertal rise in gonadotropin in rodents must be determined by mechanisms within the developing hypothalamus. One component of such hypothalamic development involves an age dependent reduction in the efficacy of testicular steroids to suppress gonadotropin secretion; as reflected by the finding that castration before the predicted age of the pubertal rise in gonadotropin secretion leads to an immediate hypersecretion of LH in rat and guinea pig [145] [146] [126] [147] (Figure 5). Surprisingly, however, this has not been found for the mouse [148] [149].

Sheep, in contrast to mice and rats, are precocial and male fertility is typically achieved between 16 to 18 weeks of age, which in spring born lambs occurs a month or two before the fall breeding season when first estrus and first ovulation occur in female siblings [121]. Since the spermatogenic process in the ram requires a duration of approximately 7 weeks [150], the initiation of puberty in male lambs, regardless of the precise marker used to identify this stage of development, is separated from birth by a distinct window of prepubertal development [121]. Moreover, as in primates, robust GnRH pulse generator activity is initiated during fetal development as may be deduced from the classic studies of gonadotropin secretion in the catheterized ovine fetus by Grumbach and his colleagues during the 1980s [151]. They demonstrated that circulating levels of LH and FSH in male and female fetuses during mid gestation were similar to those observed in castrated adult sheep. Moreover, sequential sampling of the fetal circulation identified distinct episodes of GnRH dependent LH secretion at this stage of fetal development presumable reflecting corresponding discharges of GnRH from the fetal hypothalamus [152] [153]. The latter view is supported by the contemporaneous finding that administration of an iv bolus of the GnRH secretagogue, NMDA (see above) elicited LH discharges from the fetal pituitary [154]. Again, as in primates, the elevated levels of gonadotropin secretion observed in the ovine fetus at midgestation decline as term approaches at day 145 due, as is also presumed in primates, to the ability of elevated levels of fetoplacental steroids to exert negative feedback actions at this early stage of development [151]. Following birth, pulsatile GnRH release in ram lambs as reflected by circulating LH concentrations remain low until 4–8 weeks of age when a dramatic rise in this gonadotropin is observed in association with increasing frequency of the GnRH pulse generator [121]. Thus, in sheep, as in primates, puberty results

from the re-emergence of a robust pattern of GnRH pulse generation that was first established during fetal development. Whether the brief hiatus in GnRH pulse generator activity observed postnatally in the male sheep is determined by the same non-gonadal mechanisms that hold this neuroendocrine system in check for 2 years in the monkey and 10 years in boys has received scant attention. In this regard, orchidectomy during the first week of life resulted in a hypersecretion of gonadotropin after a delay of 1 week [155], whereas castration at 4 weeks of age resulted in an immediate increase in LH secretion [156]. The possibility that this suggestion of a non-gonadal restraint of GnRH pulse generation in the neonatal male lamb may be the result of a delay in recovery from the suppressive effects of the hormonal milieu to which the fetus is exposed in late gestation, however, cannot be excluded.

Summary

Puberty and the control of the timing of this critical phase in development is most easily conceptualized for highly evolved primates such as the human and the rhesus monkey, an Old-World monkey. In males of such species, puberty is triggered by the re-awakening of the hypothalamic GnRH pulse generator after a prolonged period of relative hypoactivity from late infancy until the termination of the juvenile stage of development. Although robust GnRH pulse generator activity during early infancy in boys and male monkeys drives tonic LH and FSH secretion in an adult manner, which in turn leads to secretion of testicular testosterone, spermatogenesis is not initiated because of limited androgen and FSH signaling by the Sertoli cell at this stage of development [157]. Interestingly, by the time the testis acquires the capacity to respond to androgen and FSH stimulation during subsequent pre-pubertal development, a hypogonadotropic state is in place as a result of the turn off of GnRH pulse generator activity, thereby guaranteeing continued gonadal quiescence. A similar postnatal pattern of GnRH pulse generator activity also unfolds in female primates, but the question of whether puberty in these species also involves the expression of a GnRH surge generator is not entirely clear, and may well differ between species. The restraint of the GnRH pulse generator from infancy until puberty is largely independent of the gonads, and is conceptualized as being imposed by a neurobiological brake that is switched on in late infancy and switched off at the termination of the juvenile stage of development (Figure 6). Although several classical neurotransmitters and neuropeptides, and a rapidly increasing number of hypothalamic genes have been implicated as components of the brake, a compelling unifying hypothesis for the neurobiological control of GnRH pulse generator activity during primate post natal development has not been forthcoming. Similarly, the mechanisms underlying how inheritable traits of pubertal timing are integrated with the developmental control of the GnRH pulse generator remain obscure. Also, whether the fundamental operation of the prepubertal brake and the timing of the “off” and “on” switch is governed by cues related to somatic development or by an intrinsic clock mechanism with which the brain is endowed has not been empirically addressed. From a comparative perspective, although puberty in sheep is triggered by a reactivation of hypothalamic GnRH pulse generation, the mechanism underlying the prepubertal suppression of pulsatile GnRH release has received scant attention, and in rodents GnRH pulse generator activity appears to develop for the first time during puberty rather than being reactivated as it is in sheep and

primates. From an evolutionary perspective it seems reasonable to argue that in primates with extensive development of the neocortex, the evolution of a mechanism to effect a prolonged delay in the onset of puberty (the neurobiological brake on GnRH pulse generator prepubertally) would be adaptive, while in a species such as rodents, which must breed before they fall prey to others there would be little advantage to such a mechanism. Finally, although it is recognized that factors such as nutrition, season, stress and other psychosocial considerations may profoundly modulate the timing and tempo of puberty these determinants have not been discussed in this review.

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1. *KISS1* is a GnRH pulse generating gene not a puberty gene!
2. New ideas on the molecular bases for the control of the delay and onset of puberty.
3. Models for the developmental control of the hypothalamic GnRH pulse generator.

Figure 1.

Ovulatory ovarian cycles in two premenarcheal rhesus monkeys induced by a chronic intermittent intravenous infusion of GnRH (1 pulse/hr) initiated on day 0. Note that the pituitary-ovarian axis reverted to a prepubertal state following termination of GnRH treatment on days 92 and 111, respectively, and subsequent administration of estradiol (indicated by the open bar labeled E2) failed to induce a gonadotropin surge. The occurrence of menstruation is indicated by M. (Reprinted with permission from AAAS from Ref. 20).

Figure 2.

Premature activation of the hypothalamic GnRH-pituitary-Leydig cell axis of a prepubertal male rhesus monkeys by repetitive neurochemical stimulation with NMDA administered iv once every 3 h for 8 weeks. NMDA stimulation was initiated at week 0 when the animal was between 15 – 16 months of age: 1.5 – 2 years before the expected age of puberty. Although intermittent stimulation with NMDA was maintained without interruption, circulating LH and testosterone concentrations were only monitored during a 6 h window at weekly or biweekly intervals. The right hand panel shows pulsatile profiles of plasma LH and testosterone levels in a male monkey during spontaneous puberty. Reprinted from Ref. 26. Testicular sperm and motile epididymal sperm are typically observed in juvenile monkeys after 16–26 weeks of NMDA stimulation [25].

Figure 3.

LH responses in agonadal GnRH primed juvenile male rhesus monkeys (N=4) during the last two priming infusions of GnRH (administered on Day 1 at 0900 and 1000 h, open arrows) and during brief hourly intravenous infusions of either kisspeptin or vehicle (black arrows) initiated on Day 1 at 1100 h and maintained for 48 h. The LH response to kisspeptin is shown by black data points. Note that although the kisspeptin and vehicle injections were administered without interruption for 48 h, only those injections to which the LH response was monitored are indicated. The LH response to the first 2 re-priming pulses of GnRH are shown for the kisspeptin experiment (administered on Day 3 at 1100 and 1200 h, open arrow). The GnRH priming infusions before and after kisspeptin administration produced a pulsatile discharge of LH comparable to that observed spontaneously in pubertal animals. The response to repetitive kisspeptin administration was abolished by concomitant treatment with a GnRH receptor antagonist (data not shown), indicating the intermittent kisspeptin infusion provides the GnRH neuronal network of the juvenile hypothalamus with a stimulus similar to that produced endogenously by the GnRH pulse generator in pubertal animals. Vertical lines above data points indicate SEM. Reprinted with approval from Ref. 58.

Figure 4.

Time courses of circulating LH (top panel) and FSH (bottom panel) concentrations (means $\pm$ SE) determined in blood samples collected in the morning from birth until 142–166 weeks of age in rhesus monkeys ovariectomized ($\bullet$, $N = 6$) and orchidectomized (*stippled area*, $N = 4$) at 1 week of age. Note that the prepubertal hiatus in the secretion of FSH, and LH to a lesser extent, in agonadal females is truncated in comparison to that in castrated males. This difference between agonadal males and females, which presumably underlies the earlier onset of female puberty, is further exaggerated when nighttime concentrations of LH and FSH are examined (not shown). (The data for males are redrawn with approval, from Ref. 64).

Figure 5.

Time courses of circulating LH concentrations (mean $\pm$ SE) in male guinea pigs bilaterally orchidectomized at 2 days of age (closed data points) and in intact controls (open data points). LH secretion increased dramatically immediately after castration to plateau in the adult range by 44 days of age. Also, note the unremarkable changes in circulating LH concentrations in intact males during the period of study (birth to 98 days of age). Reprinted with approval from Ref. 147.

Figure 6.

A model for the control of GnRH pulse generator activity and the resulting drive to the pituitary-gonadal-axis in primates. Kisspeptin (KP, green) signaling is posited to be a critical component of the neural machinery essential for generation of pulsatile GnRH (red) release in the hypothalamus. In this model, the GnRH pulse generating mechanism resides in the arcuate nucleus (ARC) and the output of this signaling is relayed to GnRH terminals in the median eminence (ME) by KP projections arising from perikarya in the ARC. During infancy (left panel), ARC GnRH pulse generating activity is robust leading to intermittent release of KP in the ME, resulting in a corresponding pattern of GnRH release into the portal circulation. This, in turn, drives pulsatile gonadotropin (LH and FSH) secretion. In the transition from infancy to the juvenile phase of development (middle panel), a neurobiological brake holds the ARC GnRH pulse generating mechanism in check and pulsatile release of KP in the ME is markedly suppressed. This leads to reduced GnRH release and to a hypogonadotropic state in the juvenile period. The onset of puberty is initiated when the brake is removed and GnRH pulse generation with robust intermittent release of KP in the ME is reactivated (right panel). According to this model, the mystery of primate puberty lies in the molecular basis of the neurobiological brake, and the mechanism that times the application of the brake during infancy and its release at the end of the juvenile phase of development. Two possible timing models are proposed. The first is based on the idea of a pubertal clock resident in the primate brain (represented by the clock face in the hypothalamus at the stages of development). The second, posits that a growth tracking device in the brain, termed a somatometer (SM, indicated by the grey boxes) is able to monitor a circulating signal of somatic development (perhaps skeletal, as shown in this model) and thereby co-ordinates the reactivation of the GnRH pulse generator with the impending attainment of adult somatic size. The thickness of the blue (T, testosterone) and gold (E, estradiol) arrows indicating negative feedback by the testis and ovary, respectively, reflect the degree of gonadal steroid inhibition exerted on LH secretion at these three stages of primate development. It should be noted that the ability of the post natal gonad to respond fully to gonadotropin stimulation is not acquired until the juvenile stage of development by which time the hypothalamic GnRH pulse generator has been brought into check and a hypogonadotropic state prevails. AC, anterior commissure; AP, anterior pituitary gland,

ARC, arcuate nucleus; OC, optic chiasm; ME, median eminence; MMB, mammillary body.
Modified from Ref. 50.

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