Timing of puberty – Which factors trigger pulsatile GnRH release and the onset of puberty?

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Abstract

Puberty is the process of physical and psychological development towards adulthood, ultimately marked by the ability to reproduce. This development requires activation at all levels of the hypothalamus-pituitary-gonadal axis (HPG-axis): the hypothalamus for pulsatile secretion of gonadotropin-releasing hormone (GnRH), the pituitary for the pulsatile release of gonadotrophic factors and the gonads for generating gametes and gonadal steroids in response to these pulses. Pulsatile GnRH release from the hypothalamus is the primary drive to the HPG-axis. It seems that this is the limiting factor for the initiation of puberty. This system has the potential the function at birth, but is being held in check. Interestingly, several direct and indirect upstream signalling pathways regulate the GnRH-secreting neurons: such as kisspeptin, leptin and gonadal steroids. Changes in these upstream factors might explain the initiation of pulsatile GnRH secretion and pubertal development. However, despite extensive research, it remains a mystery as to what exactly triggers the sudden onset of puberty. Furthermore, a clear sex difference exists in the presentation of puberty, occurring 1-2 years earlier in girls. This suggests that underlying mechanisms controlling GnRH secretion are differentially regulated in both sexes. The current review aims to give a critical overview of recent findings on factors that contribute to the initiation of puberty. Mechanisms that could underlie the sex difference in onset of puberty will be highlighted. New lines of research and remaining questions in the field will be discussed.

Contents

Introduction
Hallmarks of puberty
Puberty onset

Puberty – lifting the neurobiological brake
Changes in GnRH morphology
Kisspeptin – Gatekeeper to puberty onset
Organization of the kisspeptin system
Kisspeptin signaling - kisspeptin as a GnRH modulator
Development of the kisspeptin system
Epigenetic changes
Linking metabolism and reproduction: Leptin – a metabolic sensor

Conclusions
References

Introduction

Puberty is the process of physical and psychological development towards adulthood, due to changes in the activity of the hypothalamus-pituitary-gonadal axis (HPG-axis). These changes or developments ultimately lead to the ability to reproduce. It requires activation at all major levels of the HPG-axis: the hypothalamus for episodic secretion of its releasing factor, the pituitary for the pulsatile release of the gonadotrophic factors and the gonads for generating gametes and gonadal steroids in response to these pulses. The rise in gonadal steroids, testosterone for boys and estradiol for girls, is accompanied by the appearance of secondary sexual features (Bianco, 2012).

Hallmarks of puberty

Throughout postnatal development of the human reproductive system, a few major processes can be discerned. The most important one is the *gonadarche*. It indicates the true start of central puberty, through the reactivation of the HPG-axis, which has been dormant since early infancy (figure 1)(see Box 2: Before and towards puberty). The resulting increased levels of gonadotrophic factors from the pituitary, i.e. gonadotropins, stimulate gonadal growth and development of sexual features. The first clinical sign of the *gonadarche* in girls is the start of ovarian growth and breast development, named *thelarche*. In boys, it is marked by testicular enlargement (Delemarre - van de Waal, 2002; Dorn, Dahl, Woodward, & Biro, 2010; Neill, 2006). Testicular volume is a very precise measure for pubertal onset. Breast tissue, on the other hand, is indiscernible from adipose tissue, which may cause overestimations of reproductive maturation in overweight girls. Areolar development can therefore be used as a more precise measure for pubertal maturation (Dorn et al., 2010).

The *gonadarche* is preceded by the *adrenarche*, characterized by an increase in adrenal androgen production. This develops relatively independent from changes in the HPG-axis. Namely, increasing circulating levels of dehydroepiandosteron (DHEA), which is a precursor hormone for testosterone and estradiol, cause this increased steroidogenesis. Growth of the innermost layer of the adrenal cortex also contributes to the increase in secretion of adrenal androgens. The adrenarche is accompanied by the development of pubic hair, axillary hair and adult sweat odor (Burt Solorzano & McCartney, 2010; Neill, 2006).

Within two years after the onset of breast development, well into the pubertal process, *menarche* occurs in most girls (Dorn et al., 2010). At first, the menstrual cycles are generally irregular and anovulatory, but usually develop gradually into a regular pattern of ovulatory cycles within 5 years after onset (Delemarre - van de Waal, 2002).

A species-specific sex difference exists in the onset of puberty (see Box 1). In humans, girls are more likely to enter puberty 1-2 years before boys do. Moreover, a pathological condition of precocious puberty is more common in girls whereas hypogonadotropic hypogonadism occurs more

frequently in boys (Bianco, 2012; Palmert & Boepple, 2001; Sykiotis et al., 2010). The mechanisms underlying these sex differences are fairly unknown and require further investigation. One possible cause for changes in puberty onset might be environmental neuroendocrine disruptors or chemicals (ECDs). It is beyond the scope of this review to evaluate these effects. A thorough discussion of the role of ECDs in pubertal onset can be found elsewhere (Bourguignon et al., 2013).

Puberty onset

The major marker for puberty is thus the increased pulsatile secretion of gonadotropins: the protein hormones luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. These gonadotropins target the gonads, i.e. the ovaries and testes. Secretion of FSH and LH is essential for gonadal steroid synthesis and thus the secretion of estradiol and testosterone from the ovaries and testes, respectively.

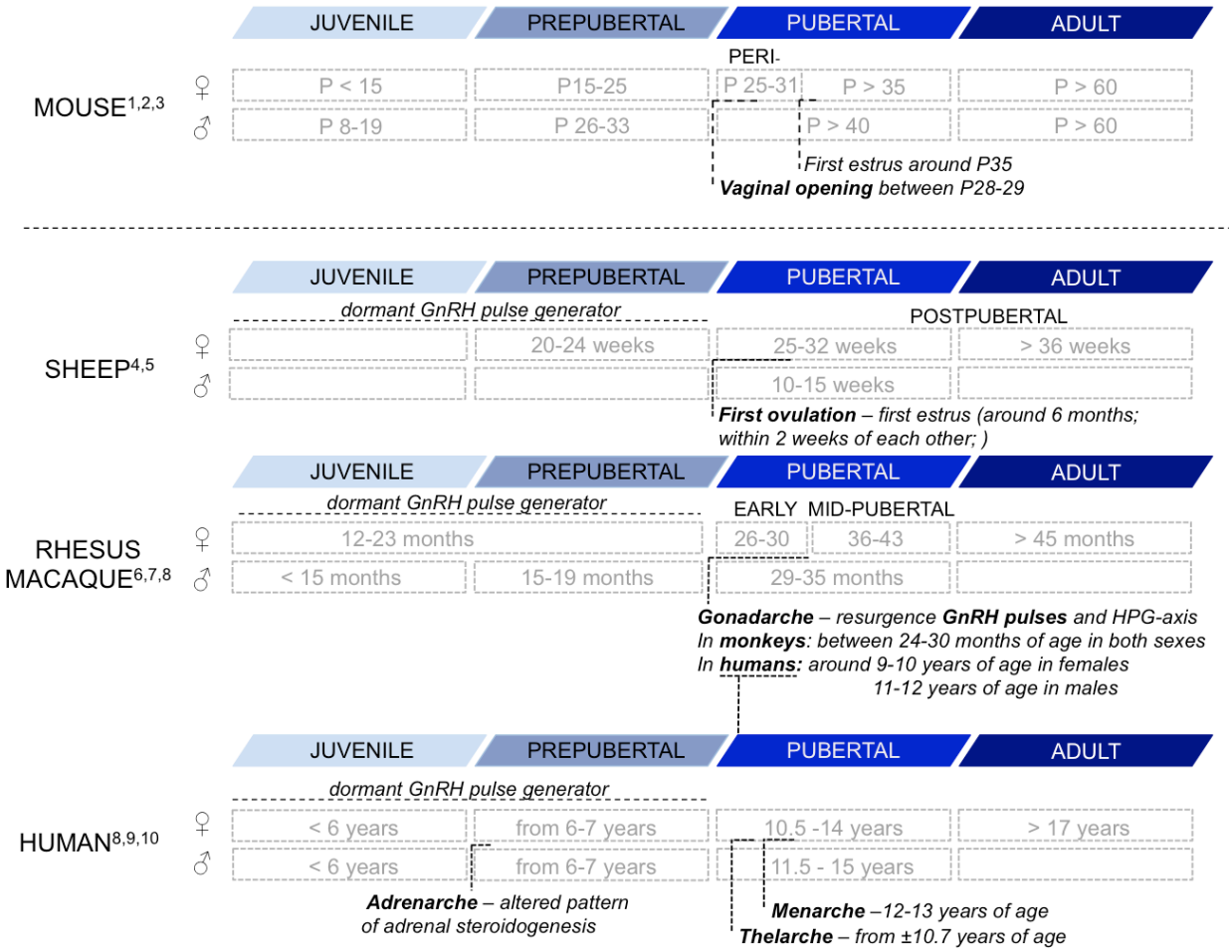
The increase in pulsatile LH and FSH occurs before most physical signs of puberty (Terasawa & Fernandez, 2001). In early puberty, pulsatile secretion is limited to the inactive phase of the day. As puberty progresses, gonadotropin release develops into the established adult pulsatile pattern throughout the day (Burt Solorzano & McCartney, 2010). Importantly, secretion of LH and FSH depends on the release of gonadotropin-releasing hormone (GnRH) from GnRH neurons in the hypothalamus. The importance of these neurons for normal reproductive development has been stressed in patients suffering from Kallmann's syndrome, in which the GnRH neurons fail to migrate from the olfactory placode to the hypothalamus due to specific gene mutations (see Box 2: Before and towards puberty). It is marked by a failure to start puberty and renders these patients infertile.

Through coordinated, episodic firing of GnRH neurons, distinct pulses of GnRH-secretion are being released from axon terminals (figure 2a)(see Box 2: Architecture)(Carmel, Araki, & Ferin, 1976). The ability to generate these GnRH pulses is intrinsic to these neurons and serves as the primary drive to the HPG-axis (Plant & Barker-Gibb, 2004; Plant, 2006). This pulse generator is fully intact and has the potential to function at birth. Rather than requiring progressive maturation throughout postnatal and juvenile phases, it thus seems that this network is being held in check. These findings, combined with the fact that the pituitary and gonads are able to respond to a prepubertal hypophyseal and gonadotropic stimulus, point towards the hypothalamus as the limiting factor of the HPG-axis for the initiation of puberty (Plant, 2006).

The rapid reawakening of the dormant pulse generator results in changes in pulsatile secretion of GnRH from the hypothalamus (Hemond, O'Boyle, Hemond, Gay, & Suter, 2013; Nestor et al., 2012; Plant, 2008; Terasawa & Fernandez, 2001). When talking about processes that trigger puberty, one is therefore inevitably talking about processes that trigger GnRH pulsatility. Interestingly, GnRH neurons are controlled by several upstream hormonal and neural pathways that convey different types of regulatory information (metabolic, etc.)(see Box 2: Architecture). This governs the timing of

puberty, to adjust it to optimal internal and external conditions of the body, and demands exquisite coordination and functional integration, i.e. concerted action of all of the upstream signaling pathways (Kauffman, 2010; Ojeda & Lomniczi, 2013; Sanchez-Garrido & Tena-Sempere, 2013).

Despite the immense amount of research, what underlies the lifting of the restraint on GnRH neurons during the juvenile stages, has not been resolved yet. Whether the physiological changes in GnRH output occur in response to changes at the level of neuronal inputs, i.e. extrinsic, or at the level of sensitivity changes or structural remodeling, i.e. intrinsic, remains to be determined. The current review aims to give a critical overview of recent findings in the field. Which factors contribute to the initiation of GnRH pulsatility and puberty? It is beyond the scope of this review to deal with all of the possible contributing factors in detail. This review will therefore focus firstly on possible morphological changes in the GnRH neurons as an explanation for initiation of puberty. Second, the kisspeptin system, as an essential factor for puberty timing, will be discussed. Finally, it will describe an example of metabolic regulation of puberty onset. Throughout the review, sexually different signaling pathways that underlie and can predict sex differences in puberty onset are highlighted.



Box 1. Reproductive maturation in different species

Research findings regarding reproductive maturation are based on different species, such as rodents, sheep and primates. Although such findings help to better understand mechanisms underlying the human development, some important differences between those species should be noted. First, in most species puberty results from an increase in the frequency of GnRH and gonadotrophic pulses, but what underlies this increase can be very different for these species. In sheep, primates and humans, GnRH pulses exist shortly after birth and in early infancy, followed by a *silent phase* or *juvenile pause*, in which the GnRH pulse generator is dormant (see Box 2: GnRH neurons – Before and towards puberty). Puberty is initiated through reawakening of the GnRH pulse generator. Such a *silent phase* is absent in rodents (Foster & Jackson, 2006; Ojeda & Skinner, 2006; Plant, 2006). This complicates the translation of rodent findings directly to the human development and hampers its use as an animal model for reproductive maturation.

Another notable difference between species is their speed of development. Sheep are relatively developed at birth, as compared to rodents and primates. Part of the maturation that is achieved postnatally in these species was therefore attained prenatally in sheep (Foster & Jackson, 2006). Also, sheep and rodents mature much quicker, when compared to primates and humans, which show prolonged development from birth to adulthood. In humans specifically, two additional phases can be discerned before the juvenile phase: the infantile and childhood phase. These are marked by a decreasing dependence on the parents for survival and nutrition (Plant & Witchel, 2006). For clarity, these are left out of the figure. The differences in speed of development demand some caution in translating the development of one species to another.

Although different terms of postnatal phases, such as *juvenile, pre-, peri- and pubertal* are often used in the literature, these are not always specified to the age of the species. Furthermore, different studies have used different nomenclature for animals of the same age. In rodents especially, where reproductive maturation spans only 60 days, such differences in terminology can have detrimental effects when elucidating on the differences between phases in for example cell numbers or mRNA expression. Based on studies used in this review, the developmental postnatal phases of different animal models have been specified here.

Sex differences in developmental maturation are also indicated in the figure. In most species, puberty occurs earlier in females than males, except for sheep, where rams show increases in GnRH and gonadotrophic pulses a couple of weeks before ewes (Nestor et al., 2012). The fact that sheep are furthermore seasonal breeders, in contrast to rodents, primates and humans, should also be taken into account.

Finally, the onset of puberty is usually defined by species-specific hallmarks of this development. In female rodents, an external index used to determine the onset of puberty is the vaginal opening, which occurs around postnatal day (P) 28-29 in mice. This index is however not immediately followed by the first estrus, which is the phase in which the animals are receptive to sexual behavior. Whether vaginal opening is a good parameter for the onset of puberty in these animals remains a matter of debate. In female sheep, puberty onset is alternatingly defined as the initiation of ovulation or the first estrus, that occur at least six months after birth and usually within two weeks of each other. In primates including humans, the *gonadarche* denotes puberty onset. These hallmarks have been indicated in the figure.

1. Mayer et al., 2010 2. Clarkson & Herbison, 2006; 3. Han et al., 2005; 4. Nestor et al., 2012; 5. Foster & Jackson, 2006; 6. Majoubi et al., 2000; 7. Shahab et al., 2005; 8. Plant & Witchel, 2006; 9. Delamarre - van de Waal, 2002; 10. Dorn et al. 2006

Box 2. GnRH neurons

Before and towards puberty

The anatomical development of the GnRH secretory system in humans occurs early in life. The capacity to synthesize and secrete GnRH is likely well present before puberty (Ebling, 2005). Namely, during the embryonic phase, GnRH neurons arise in the olfactory placode. These neurons migrate towards the hypothalamus where they are located in the tuberal zone, including the periventricular region and the infundibular nucleus (Inf)(Baroncini et al., 2007). During the first half of gestation, the portal capillary network is completed. This allows communication between the hypothalamus and the pituitary, resulting in an initial sudden rise in gonadotropin levels. The development of a negative feedback system to gonadal steroids and inhibiting factors onto GnRH neurons in the second half of gestation cause a decrease in those gonadotropin levels (figure 1)(Delemarre - van de Waal, 2002; Grumbach, 2002). Immediately after birth, the rapid withdrawal of placental sex steroids disturbs the negative-feedback balance between sex steroids and GnRH release, causing a transient increase in gonadotropin levels. This process has been termed *mini-puberty*. The GnRH neurons exhibit spontaneous auto-rhythmicity, functioning as an intrinsic mechanism to secrete GnRH in a pulsatile manner (Delemarre - van de Waal, 2002; Grumbach, 2002). From here on, there is a gradual dampening from the oscillating GnRH-neurons in infancy, towards a state of quiescence in childhood and juvenile years. This juvenile pause has been shown in other species, such as sheep and rhesus macaques, but is absent in rodents (see Box 1)(Foster & Jackson, 2006; Ojeda & Skinner, 2006; Plant, 2006). With puberty approaching, a progressive resurgence of the GnRH-neuron activity occurs (Delemarre - van de Waal, 2002; Grumbach, 2002; Plant, 2008).

Architecture

GnRH neurons receive both inhibitory as well as excitatory afferent signals from distinct neurotransmitters (see figure 2A). Glutamate, acting via AMPA and NMDA receptors, provides important excitatory input to GnRH neurons. Furthermore, these are very strongly modulated by kisspeptin neurons through direct projections. Kisspeptin exerts a potent depolarizing effect on the excitability of nearly all adult GnRH neurons, but the responsiveness of GnRH to kisspeptin likely develops postnatally (Han et al., 2005). GABA can also depolarize GnRH neurons. Whether GABAs role in GnRH modulation is purely excitatory remains a matter of debate: it seems dependent on the state of maturation and its binding to different GABA receptors (Hemond et al., 2013; Herbison & Moenter, 2011; Lomniczi et al., 2013; Terasawa & Fernandez, 2001). Neuropeptide Y (NPY), involved in food intake and metabolism, exerts positive or negative control over GnRH neurons, depending on the maturational stage (Castellano, Bentsen, Mikkelsen, & Tena-Sempere, 2010; Terasawa & Fernandez, 2001). GnRH neurons furthermore receive inhibitory input from opioidergic systems. The gonadal steroids, estrogen and testosterone, also inhibit GnRH release through negative feedback onto the hypothalamus. In females, estradiol can also provide positive feedback effects, to evoke the preovulatory GnRH/LH surge. Interestingly, GnRH neurons are devoid of estrogen and androgen receptors (ERα and AR). The regulating effects of gonadal steroids on these neurons must thus be mediated via other pathways, likely via kisspeptin (Mayer et al., 2010).

GnRH neurons send their axon terminals into the portal blood system, where GnRH is released into the median eminence, serving as a connection site between the hypothalamus and the pituitary (Low, 2011). Following secretion, GnRH binds to a G protein-coupled membrane receptor, GnRHR, on endocrine cells in the anterior pituitary, called gonadotrophs (figure 2A). Through this binding, GnRH functions as a releasing factor, stimulating both the synthesis as well as episodic secretion of LH and FSH. In addition to gonadotroph control, GnRH secretion seems to be involved in other reproductive functions, such as *lordosis* in female mice, mounting in males and regulation of olfactory sensitivity to socially relevant pheromones. Namely, GnRH might act by targeting downstream neurons expressing GnRHR in parts of the central nervous system, including different nuclei within the amygdala, thalamus and hypothalamus that are related to these functions. Interestingly, in contrast to GnRHR expression on gonadotrophs in the anterior pituitary of mice, arising during early stages of gestation, GnRHR expression in the brain was not detected until the juvenile phase (Wen et al., 2011).

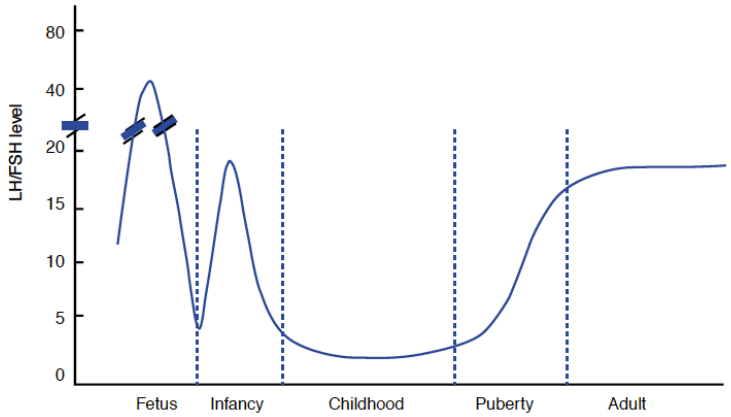


Figure 1. Pattern of gonadotropin levels throughout development. During gestation, communication between de pituitary and hypothalamus and development of a negative feedback system causes the rise and drop in LH/FSH levels. Removal of negative feedback from the placenta at birth causes *mini-puberty*. During childhood, LH/FSH secretion is restrained. GnRH pulsatility at the onset of puberty causes a sudden rise in LH/FSH levels to ultimately reach an adult pattern of secretion. Reprinted from "Regulation of puberty" by H.A. Delamarre – van de Waal 2002, *Best practice & Research clinical Endocrinology and Metabolism, 16, p. 1-12*. Copyright 2002 by Elsevier Science

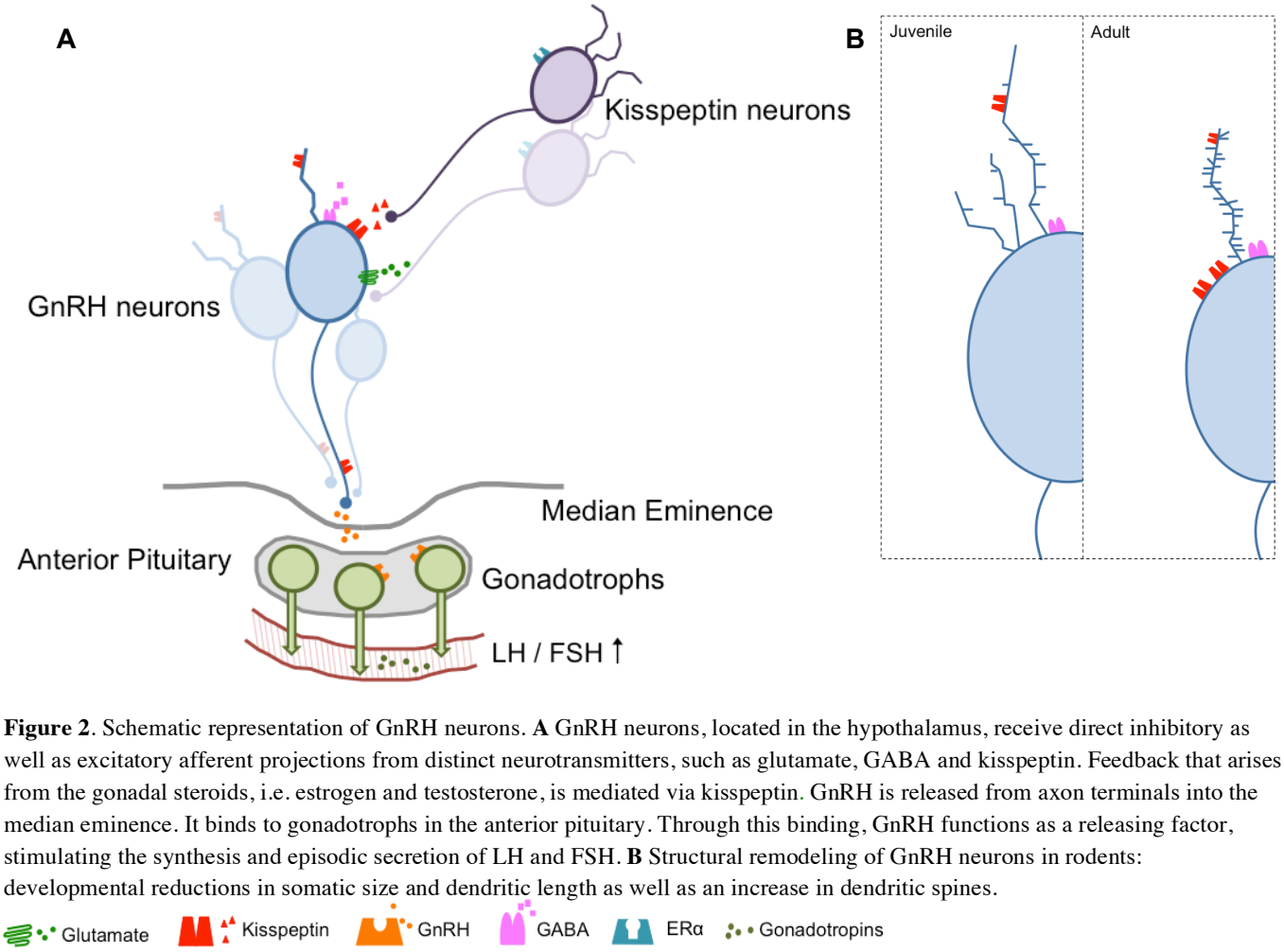


Figure 2. Schematic representation of GnRH neurons. **A** GnRH neurons, located in the hypothalamus, receive direct inhibitory as well as excitatory afferent projections from distinct neurotransmitters, such as glutamate, GABA and kisspeptin. Feedback that arises from the gonadal steroids, i.e. estrogen and testosterone, is mediated via kisspeptin. GnRH is released from axon terminals into the median eminence. It binds to gonadotrophs in the anterior pituitary. Through this binding, GnRH functions as a releasing factor, stimulating the synthesis and episodic secretion of LH and FSH. **B** Structural remodeling of GnRH neurons in rodents: developmental reductions in somatic size and dendritic length as well as an increase in dendritic spines.

Puberty: lifting the neurobiological brake

Several hypotheses have been put forward in an attempt to explain the sudden onset of puberty. According to the *Gonadostat hypothesis*, the gonadal steroids provide the main negative feedback onto the gonadotrophin regulating system. Puberty onset was in this regard thought to mark a sharp decrease in the sensitivity of this system to negative feedback of the gonadal steroids, thereby lifting the brake that is held onto GnRH neurons throughout infancy. This lifting would then result in an increase in the release of gonadotropins and ultimately gonadal steroids. However, development of patients suffering from gonadal dysgenesis and castrated primates contradicted this hypothesis, since a similar biphasic development of gonadotrophic secretion was still seen in the absence of gonadal steroids. It therefore seemed that aside from the negative feedback of gonadal steroids, GnRH release is restrained by a central inhibitory system, which is lifted at puberty onset (Delemarre - van de Waal, 2002; Terasawa, Guerriero, & Plant, 2013).

The current view states that the onset of puberty occurs due to gonad-independent processes (Chongthammakun & Terasawa, 1993; Terasawa et al., 2013). Rather, changes in afferent synaptic input to GnRH neurons, consisting of a removal of inhibitory input or an increase in stimulatory input, cause the initiation of puberty. Lifting of inhibitory signals onto the GnRH neurons would by itself not be sufficient to set puberty in motion. It seems instead that puberty onset requires an additional gain in

stimulatory, excitatory signals. Such a gain in combination with a lift of inhibitory signals culminates in an acceleration of puberty onset.

Changes in GnRH morphology

As mentioned before, GnRH neurons receive afferent input from many different transmitters and peptides, projecting directly or indirectly onto the dendritic spines (figure 2A). One possible level at which changes occur that initiate GnRH pulsatility, is a structural remodeling of the dendrites or GnRH somata (figure 2B)(Hemond et al., 2013; Plant, 2006). In male rats, the architecture of dendrites changes from one that is relatively complex during infancy, to a compact and uncomplicated one in pubertal and adult animals. The larger, more complicated GnRH neurons during infancy and prepuberty limit their responsiveness to synaptic input. The prepubertal transformation involves reductions in dendritic length and halving of the somatic areas. Moreover, throughout the maturation, the infantile GnRH neuron, showing many primary dendrites, changes into a bipolar adult neuron, with only a single dendrite and axon. Human studies have also reported predominantly bipolar adult GnRH neurons (Baroncini et al., 2007). Altogether, this results in a simplified configuration of the mature GnRH neuron and dendrite, sensitizing their responsiveness to afferent synaptic input (Hemond et al., 2013; Ybarra, Hemond, O'Boyle, & Suter, 2011). In addition, the somata and proximal parts of the dendrites show a prepubertal increase in the area of specialized excitatory synaptic contacts, i.e. dendritic spines. A similar plasticity in dendritic and soma morphology was found in male mice, with changes in dendritic spine density and prepubertal pruning of dendritic branches and in dendritic trees (Cottrell, Campbell, Han, & Herbison, 2006). Interestingly, no differences in GABA input were found between infantile and adult animals, suggesting an increase in excitatory inputs to these areas only. These morphological changes were not dependent on gonadal steroids, since these were roughly similar for castrated, testosterone-treated and intact male rats. This leaves the possibility of intrinsic hypothalamic factors initiating this maturation.

Whether these findings can be translated into a human model of reproductive maturation, remains an important question. Since rodents do not show the juvenile pause that is typical for the GnRH pulse generator in sheep, primates and humans, structural remodeling of these neurons before puberty might specifically occur in species that show a progressive maturation, rather than a biphasic pattern of development.

Kisspeptin – gatekeeper to puberty onset

Kisspeptins are the protein products of the Kiss1 gene, expressed in many peripheral tissues, such as the placenta, testes, ovaries and adipose tissue, as well as brain areas such as the hypothalamus (reviewed in Kauffman, 2010). These peptides bind with high affinity to a G-protein-coupled receptor encoded by the gene Gpr54. Interestingly, nearly all GnRH neurons express Gpr54 and this homogeneous expression pattern is found in different species, such as fish, rats and monkeys (Han et al., 2005). The involvement of kisspeptin in reproductive maturation and puberty onset was revealed by inactivating mutations in the human Kiss1 and Gpr54 gene, resulting in absence of puberty. Lack of spermatogenesis and menstrual cyclicity, low levels of gonadal steroids and hypogonadotropic hypogonadism were other consequences of mutations in the Gpr54 or Kiss1 gene (reviewed in Kauffman, Clifton, & Steiner, 2007). Similarly, ablation of these genes in mice rendered them infertile and hypogonadotropic (Mayer et al., 2010). Kisspeptin signaling through Gpr54 thus seems to be essential for normal reproductive maturation.

Organization of the kisspeptin system

Kisspeptin neurons can be roughly divided between two hypothalamic populations. The first is comprised of kisspeptin neurons located in the rodent arcuate nucleus (Arc). A similar population has been reported for rhesus macaques and sheep, containing most of the kisspeptin neurons (Lehman, Merkley, Coolen, & Goodman, 2010; Ramaswamy, Guerriero, Gibbs, & Plant, 2008; Shahab et al., 2005). In humans, this area is most similar to the Inf, where most kisspeptin cell bodies are identified in adults (figure 3A)(Hrabovszky et al., 2010; Hrabovszky, 2013). Interestingly, the Arc kisspeptin population coexpresses other neuropeptides as well, that are likely involved in reproductive maturation

Box 3. KNDy neurons – Kisspeptin, Neurokinin B and dynorphin

The neuropeptide neurokine B (NKB) is also thought to be involved in pubertal timing, since it is coexpressed with kisspeptin in the same Arc (Inf) neurons in rodents, sheep, primates and humans (figure 3A)(Goodman et al., 2007; Hrabovszky et al., 2010; Ramaswamy et al., 2010). NKB expression is, however, not limited to this area only. A clear sex difference has been found in the NKB system in the human Inf, with a denser innervation and more intense staining in females than males (Taziaux, Swaab, & Bakker, 2012). Most of the kisspeptin neurons that cosynthesize NKB, also express ERα. Its precise role in reproduction and pubertal development long remained unclear, but inactivating mutations in the gene encoding NKB or its functional receptor (NK3R) caused a state of hypogonadotropic hypogonadism, clearly pointing towards its involvement in reproductive maturation.

Recent extensive research has since shown that NKB is implicated in the control of GnRH secretion. A central or peripheral injection of the NK3R agonist senktide stimulated LH release in prepubertal ewes, female prepubertal female rats and prepubertal castrated male rhesus monkeys (Navarro et al., 2012; Nestor et al., 2012; Ramaswamy et al., 2010). In contrast, treatment with an NKB antagonist was able to moderately delay vaginal opening and thus pubertal onset in female rats (Navarro et al., 2012). In rhesus monkeys, use of a GnRH receptor antagonist abolished all LH responses in response to an NKB or senktide treatment. NKB, like kisspeptin, therefore seems to exert a potent stimulatory role on LH secretion in a GnRH-dependent manner (Ramaswamy et al., 2010). The fact that injection of an NK3R antagonist abolished the increased LH secretion after treatment with senktide, though not with a kisspeptin agonist, would even suggest that NKB signaling is upstream from kisspeptin (Ramaswamy et al., 2010). NKB might thus influence pubertal onset through kisspeptin output emerging onto GnRH neurons.

In contrast to kisspeptin and NKB, stimulating episodic GnRH secretion, an inhibiting neuropeptide that is found in these neurons is dynorphin, which holds GnRH and gonadotrophic pulses in check. Altogether, the specific set of these kisspeptin-neurokinin B and dynorphin (KNDy) Arc neurons thus serves a critical role in synchronizing GnRH pulsatility.

(see Box 3). This review will, however, focus on the role of kisspeptin mainly. In sheep, a sex difference has been reported: kisspeptin cell numbers in the Arc of ovariectomized ewes outnumbered those of castrated rams, suggesting an organizational role of gonadal steroids in establishing this effect (see Box 4)(reviewed in Lehman et al., 2010). A similar sex difference was found in the human Inf (Hrabovszky et al., 2010). The latter study did not control for possible sex and age differences in circulating hormone levels, however. Although no sex differences in kisspeptin populations were found in adult mice, early postnatal sex differences have been reported (see: *Development of the kisspeptin system*)(Brock & Bakker, 2013; Han et al., 2005).

In rodents, numerous kisspeptin somata are furthermore found in the anteroventral periventricular nucleus and the preoptic periventricular nucleus (AVPV/PeN). Although less numerous, kisspeptin neurons are also found in the preoptic region of sheep (Franceschini et al., 2006). An anatomically corresponding human population consists of neurons located in the preoptic area of female hypothalami only, although these are more scattered (figure 3A)(Hrabovszky et al., 2010). However, previous studies in primates state that kisspeptin-producing neurons outside of the Arc or Inf are scarce (Ramaswamy et al., 2008; Shahab et al., 2005). Distinct methodologies or a lack in sensitivity of the antisera used for immunocytochemical studies may explain these different results. Whether kisspeptin neurons in the preoptic area of humans are homologue to the AVPV/PeN in rodents, remains thus a matter of debate. More research on kisspeptin expression in the human brain is clearly needed. Interestingly, the rodent AVPV/PeN shows a similar sex difference, with much higher numbers of neurons in females. This difference likely arises from organizational effects of gonadal steroids. In males, perinatal testosterone exposure likely actively masculinizes, i.e. diminishes the AVPV/PeN nucleus. In females, estradiol developmentally feminizes the AVPV/PeN kisspeptin population (see Box 4)(Clarkson & Herbison, 2006; Hrabovszky, 2013; Kauffman, 2010; Mayer et al., 2010; Takumi, Iijima, & Ozawa, 2011).

Interestingly, neurons in both kisspeptin populations express the estrogen receptor ERα, posing a possible role for these receptors and gonadal steroids to modulate puberty progression. It has been proposed that the AVPV/PeN population is responsible for the positive feedback of sex steroids mediated via kisspeptin neurons, to evoke a preovulatory GnRH/LH surge in female rodents, and accordingly, this population shows sex differences with higher numbers of kisspeptin neurons in females than males. This positive-feedback mechanism does not function during the juvenile phase, matures just before puberty is initiated and drives the generation of the preovulatory GnRH/LH surge (Clarkson, Han, Liu, Lee, & Herbison, 2010; Takase et al., 2009). By contrast, in sheep, it appears that the critical region for the preovulatory GnRH/LH surge is the Arc, rather than the preoptic region. Likewise, in humans, the Inf seems to be responsible for generating the preovulatory GnRH/LH surge in females (reviewed in Lehman et al., 2010). Both in sheep and humans, females showed higher numbers of kisspeptin cells in the Arc/Inf than males. Contrarily to its effect in the AVPV/PeN, estradiol exerts potent inhibitory effects on the Arc neurons in female rats, suppressing kisspeptin

Box 4. Organizational and activational effects of gonadal steroids

When talking about gonadal steroid effects on the central nervous system, two different types of effects can be discerned. On the one hand, organizational effects are those that influence brain and sex organ development. They are permanent, occur during a critical period and change the responses of target tissue to future exposure of the hormone. According to the organizational hypothesis, most neural sex differences arise due to such organizational effects. More specifically, the brain initially has the potential to develop both male-like as well as female-like. In this regard, masculinization or feminization of the brain occurs in response to fetal or prepubertal active exposure to high-level gonadal steroids: testosterone (or its metabolite dihydrotestosterone) and estradiol, respectively (Brock, Baum, & Bakker, 2011; Kauffman, 2010; Simerly, 2002). In the hypothalamus, changes in regional anatomy and total cell number in the distinct nuclei, survival or facilitation of loss of neurons, but also altered connectivity between neurons are examples of such organizational gonadal steroid effects (Simerly, 2002). One consequence of this sex-defined differential exposure to testosterone or its metabolites during perinatal or prenatal life is the ability of females to generate a GnRH/LH surge. This ability is averted in males due to testosterone exposure (Kauffman et al., 2007).

On the other hand, activating effects occur later in life and are acute or transient, dissipating after the hormonal signal is relieved. In females, for example, the GnRH/LH surge is known to be estrogen-*induced*. These latter effects can thus be viewed as hormonal effects *activating* tissues that were prenatally *organized* or wired. In other words: early (fetal) hormonal, organizational actions lay a neural substrate in specific periods, on which acute hormonal actions can act in later developmental stages (Arnold, 2009; Brock & Bakker, 2013; Poling & Kauffman, 2013)

expression before puberty onset (Takase et al., 2009). Absence of ERα in kisspeptin neurons precociously activated GnRH pulsatility and LH secretion, advancing pubertal onset in female mice. This restraint effect on GnRH pulsatility via ERα is likely mediated via the kisspeptin neurons located in the Arc (Mayer et al., 2010).

Axonal fiber projections from kisspeptin neurons are found in both the Arc as well as preoptic region, similar to the location of most cell bodies. Interestingly, very dense preoptic kisspeptin fibers seem to project in part onto the kisspeptin neurons of the Arc (Clarkson et al., 2010). In addition, in both primates and rodents, fiber projections extend into the median eminence, albeit fewer in density and number (Clarkson & Herbison, 2006; Lehman et al., 2010; Ramaswamy et al., 2008). Since GnRH neurons send their axon terminals into the portal system to release GnRH into the median eminence, axon-axon contacts between kisspeptin and GnRH neurons in this region could provide a mechanism through which this release can be regulated outside of the hypothalamus. Close associations were indeed found at the level of the median eminence between kisspeptin and GnRH fibers in male rhesus monkeys (figure 3A)(Ramaswamy et al., 2008). Axosomatic and in some cases axodendritic contacts between kisspeptin and GnRH neurons have been described for different species (Clarkson & Herbison, 2006; Hrabovszky, 2013). Besides their efferent projections, Arc kisspeptin neurons seem to establish frequent contacts onto one another as well (Hrabovszky, 2013; Lehman et al., 2010). This intranuclear network connectivity adds to the complexity of the kisspeptin signaling system.

Kisspeptin signaling – kisspeptin as a GnRH modulator

As mentioned before, kisspeptin serves as a potent GnRH modulator. Mutations in the Gpr54 receptor resulted in a deficient release of LH and FSH in rodents, due to a diminished GnRH secretion (Kauffman et al., 2007). Furthermore, in response to a brief kisspeptin stimulus *in vitro*, GnRH neurons in the adult mouse showed a clear depolarization. This was found even after low

concentrations of kisspeptin and persisted until after the administration period. Depolarization was smaller in female as compared to male mice (Han et al., 2005). Kisspeptin also showed to be a strong activator of the LH pulse in male adult mice *in vivo*, regardless of the kisspeptin concentration that was applied (Han et al., 2005). Similarly, in female rhesus monkeys, pulses of kisspeptin and pulsatile GnRH release into the median eminence were for the most part paralleled (Keen, Wegner, Bloom, Ghatei, & Terasawa, 2008). Furthermore, GnRH release in female rhesus monkeys was increased or decreased through stimulation of a kisspeptin agonist or antagonist *in vivo,* respectively (Guerriero, Keen, Millar, & Terasawa, 2012). All these findings, combined with the fact that almost all GnRH neurons express the kisspeptin receptor, Gpr54, indicate a direct innervation of GnRH neurons by kisspeptin.

Development of the kisspeptin system

The kisspeptin system undergoes an extensive and complex development, which might be essential for the proper timing of puberty. Throughout development, the responsiveness of GnRH neurons to kisspeptin signaling changes, gradually acquiring adult sensitivity levels. A developmental increase in the expression of GPR54 at GnRH neurons as a mechanism for this increased sensitivity is still debatable. No clear differences in mRNA levels of GPR54 between juvenile and adult male mice were found (Han et al., 2005). Alternatively, Shahab and colleagues (2005) showed developmental changes in the Gpr54 expression pattern in juvenile to prepubertal female rhesus macaques only. The possibility of a development in the expression of Gpr54 occurring at an earlier stage, i.e. before the juvenile phase, remains. Indeed, in rats, differences in GPR54 mRNA expression levels were increasingly found from the neonatal and juvenile phase onwards (Navarro et al., 2004). All in all, a large population of GnRH neurons seems to express Gpr54 at birth or the neonatal stage and hence, is able to respond to kisspeptin (Kumar et al. 2014). The amount of GnRH neurons expressing Gpr54 reaching adult-like levels then increases in advance of puberty (Clarkson et al., 2010).

Besides, the depolarizing effect of kisspeptin on the excitability of GnRH neurons is regulated developmentally. Increasing percentages of the GnRH neuronal population in mice show depolarization upon a kisspeptin stimulus, from juvenile to adulthood onwards (Han et al., 2005). This maturation of the electrical response of GnRH neurons to kisspeptin is an important step in the initiation of puberty (Clarkson et al., 2010). However, whether a similar development is seen in species in which a juvenile pause exists remains to be elucidated.

In mice, pre- and postnatal development of the kisspeptin neuron populations occurs. This development is likely part of the kisspeptin-GnRH activation that triggers puberty. A clear developmental timing difference exists between the Arc and AVPV/PeN. The kisspeptin population in the Arc of female mice shows kisspeptin expression well before the AVPV/PeN. Namely, Arc kisspeptin neurons first appear around mid-gestation, rapidly developing until half of the population is already in place near the end of embryonic development (Kumar et al., 2014). A clear sex difference

has been shown in the development of the Arc kisspeptin population: kisspeptin expression in females gradually increased from the early postnatal phase, whereas in male mice expression increased only from the prepubertal phase onwards (Brock & Bakker, 2013). No differences were found between juvenile and adult levels of positive-stained kisspeptin neurons in male mice (Han et al., 2005). However, the aforementioned dense fibers surrounding the kisspeptin cell bodies in the Arc hamper the ability to discern individual cell bodies from fibers in mice, which needs to be implemented when studying cell numbers. Alternatively, a developmental increase in the amount of kisspeptin neurons and kisspeptin expression in the Arc has been reported in ewes, male and female rats and in the medial basal hypothalamus, presumably the Arc, of rhesus monkeys (figure 3B)(Nestor et al., 2012; Shahab et al., 2005; Takumi et al., 2011).

As mentioned before, NKB is cosynthesized in the same Arc neuronal population as kisspeptin. In female rats, the expression of NKB develops postnatally, reaching a maximum around puberty onset. Hypothalamic expression of its functional receptor showed maximal levels even before puberty onset (Navarro et al., 2012). In the Arc specifically, no differences in NKB expression level were found in intact ewes or female rats from the prepubertal to pubertal transition (Navarro et al., 2012; Nestor et al., 2012). These results could also indicate that NKB expression develops before these stages in the Arc, which would correspond to findings in the kisspeptin system.

In the AVPV/PeN of mice, kisspeptin-containing cells were detected from the juvenile phase, gradually increasing towards adulthood, with females expressing kisspeptin neurons earlier than males (Brock & Bakker, 2013; Clarkson & Herbison, 2006; Han et al., 2005; Mayer et al., 2010). In female rats, the increase in kisspeptin mRNA expression just before puberty depends on organizational effects of estradiol, and might thus underlie the establishment of a positive-feedback mechanism of increasing estradiol levels (Bakker, Pierman, & González-Martínez, 2010; Takase et al., 2009). A model has been proposed in which GABA and glutamate initially excite GnRH neurons, stimulating LH and FSH release which results in rising gonadal steroid levels. The increase in estradiol then acts to increase kisspeptin expression in the AVPV/PeN, resulting in an amplification of GnRH neuron activity in a positive feedback manner (Clarkson et al., 2010; Mayer et al., 2010).

As mentioned before, in rodents, estradiol exerts inhibitory control over Arc kisspeptin neurons. A change in sensitivity to this suppression that has been previously been associated with pubertal onset might occur in the Arc. In female prepubertal but not adult rats, estradiol suppressed Arc kisspeptin expression (Takase et al., 2009). Combined with the increased expression of AVPV/PeN kisspeptin shortly before puberty, this reset in hypothalamic sensitivity serves as an important part of the pubertal accelerator in rodents. It should be stressed, however, that the restraint on GnRH pulsatility during the juvenile phase in primates is predominantly steroid-independent (Chongthammakun & Terasawa, 1993; Plant & Witchel, 2006; Mayer et al., 2010). A role for ERα mediated via Arc neurons in pubertal timing in humans might therefore not be as important. This issue clearly requires further research.

Aside from increasing kisspeptin cell numbers, another level at which the activation of GnRH develops is the coupling between kisspeptin inputs and GnRH neurons. Kisspeptin-GnRH appositions in male and female mice were only gradually detected from the prepubertal phase onwards, although these were probably underestimated at an earlier age due to technical limitations (Clarkson & Herbison, 2006). Alternatively, Kumar and colleagues (2014) found communication between GnRH and kisspeptin neurons in female mice already during embryonic development. In post-pubertal ewes, the amount of close contacts between GnRH neurons and kisspeptin neurons was greater than before puberty. This increase in synaptic input correlated with the growing amount of kisspeptin neurons throughout maturation (Nestor et al., 2012).

It might seem paradoxical that the supposed gatekeeper for puberty timing is in some cases already present long before puberty onset (Brock & Bakker, 2013; Han et al., 2005; Kumar et al., 2014). Moreover, part of the GnRH neurons is already able to respond to kisspeptin and close appositions between GnRH and kisspeptin neurons in female mice were found just shortly after GnRH migration into the preoptic area (Kumar et al., 2014). If that is the case, what holds puberty onset in check? Besides kisspeptin afferents, some of which will be discussed in more detail below, the above implies that the kisspeptin system does in fact require developments at several different levels, before puberty is initiated.

Epigenetic changes

An exciting new line of evidence provides another mechanism at the transcriptional level that likely modulates pubertal timing. According to this view, target genes involved in the stimulatory control over reproductive maturity, i.e. GnRH secretion, are being repressed. Through silencing of upstream genes that activate puberty, such as Kiss1, puberty is kept in check (Ojeda & Lomniczi, 2013). These genes are repressed by a polycomb-group (PcG) protein complex, which is able to epigenetically silence genes and block DNA transcription. Around puberty, DNA methylation of the promoter regions of these repressor genes simultaneously increased, while the overall expression of these specific genes in the medial basal hypothalamus and the associations between their protein products and the promoter region of Kiss1 decreased (Ojeda & Lomniczi, 2013). The net result is thus a methylation-induced suppression of the repressing PcG complex, thereby lifting an inhibitory mechanism (figure 3C)(Lomniczi et al., 2013). This transcriptional repression, or gene silencing, is a promising and relatively new mechanism involved in the timing of puberty. In the same regard, expression of the gene MKRN3 in the Arc of mice gradually declined throughout postnatal development and mutations in the same gene caused precocious puberty in humans (Abreu et al., 2013). This potentially poses a similar role for this gene as the PcG in transcriptionally repressing stimulatory factors of the initiation of puberty (Ojeda & Lomniczi, 2013).

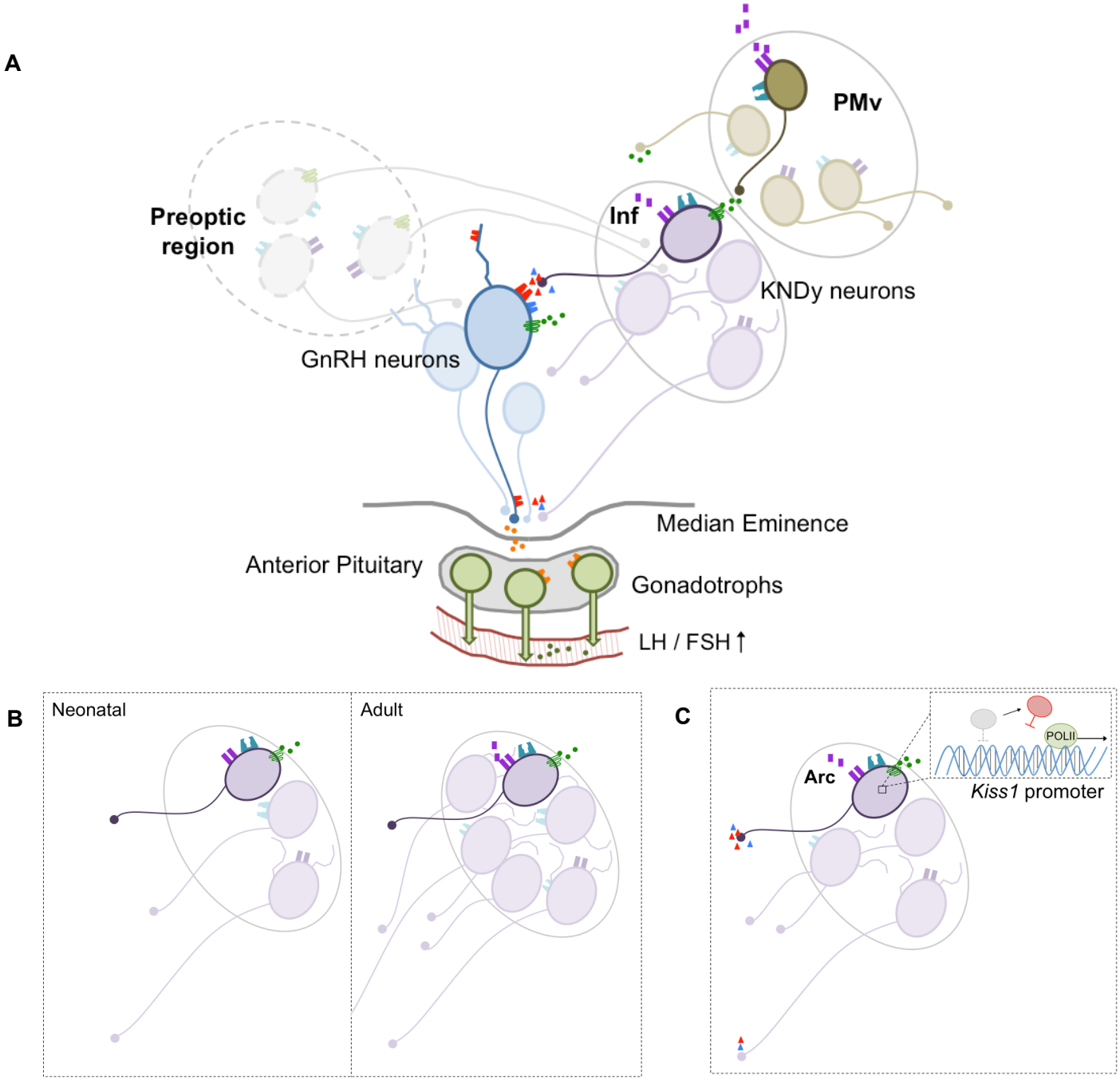


Figure 3. Puberty onset is caused by developmental changes in afferent input of GnRH neurons. **A** Schematic overview of different upstream pathways of GnRH neurons. Most kisspeptin-neurokinin B-dynorphin (KNDy) neurons are located in the Infundibular nucleus (Inf). Possibly, other kisspeptin neurons are located in the preoptic region. KNDy neurons synthesize both kisspeptin and neurokinin B (NKB) that directly innervate GnRH neurons. Receptors for kisspeptin and NKB are highly expressed on most GnRH somata. Close axon-axon appositions between kisspeptin- and GnRH neurons also exist. GnRH neurons are devoid of leptin and estrogen receptors (ERα). These signals are mediated through kisspeptin neurons that express leptin receptors, ERα and glutamatergic receptors. Kisspeptin neurons integrate these different upstream signals and convey that information to the GnRH neurons. Leptin also binds to glutamatergic neurons in the premammillary nucleus (PMv) that directly innervate GnRH neurons as well as kisspeptin neurons. **B** Increasing numbers of kisspeptin neurons in the Arcuate nucleus (Arc) of rodents, sheep and rhesus macaques are found throughout development. This increase might also be part of the kisspeptin-GnRH activation that triggers puberty in humans. **C** In mice, transcription of positive genetic factors upstream from GnRH, such as kisspeptin (*Kiss1* gene), is prevented before puberty by repressing genes. Around puberty, methylation of these repressing genes is increased and their overall expression decreases, thereby removing the brake that was held on transcription of *Kiss1*. Lifting of this inhibited transcription might be part of the trigger for puberty onset. POLII: RNA polymerase II. Figure 3C adapted from "Unraveling the mystery of puberty" by S.A. Ojeda & R. Lomniczi 2014 *Nat. Rev. Endocrinol., 10, p 67-69*. Copyright 2014 Macmillan Publishers.

Linking metabolism and reproduction: Leptin – a metabolic sensor

Pubertal development is highly dependent on the physiological condition of an individual. Nutritional state is one such key factor. Since the process of reproduction is very energy demanding, especially pregnancy and lactation in females, the neural network controlling fertility is sensitive to metabolic status. The metabolic state of an organism is thus transmitted to GnRH neurons (Sanchez-Garrido & Tena-Sempere, 2013).

Leptin is a peptide primarily produced by white adipose tissue that conveys the energy state information of peripheral adipose tissue to the brain (Delemarre - van de Waal, 2002). Since GnRH neurons are devoid of receptors for leptin, its effects in metabolically controlling puberty are likely conducted via leptin-sensitive neurons that converge onto GnRH neurons, such as the Kiss1 system (figure 3A)(Donato Jr et al., 2011; Elias, 2012; Sanchez-Garrido & Tena-Sempere, 2013). In extreme cases of fasting and energy insufficiency, hypothalamic Kiss1 expression was suppressed in various species (Elias, 2012; Navarro et al., 2012). Similarly, the NKB system is also subjected to metabolic regulation during puberty. Food deprivation decreased expression of the NKB receptor in the Arc of female rats (Navarro et al., 2012). In this way, metabolic cues are used to prevent fertility in times of energy insufficiency. Adequate nutritional status, i.e. an adequate leptin level, is thus a limiting condition for the initiation of puberty: when it reaches a certain level, it permits the activation of the GnRH pulse generator (Burt Solorzano & McCartney, 2010). However, leptin is rather an essential permissive factor in the initiation of puberty than the definite trigger of its onset (Bianco, 2012; Elias, 2012). Although its effects are less well characterized, persistent energy excess increased the number of kisspeptin fibers in the AVPV/PeN in rodents before puberty onset, thereby causing precocious occurrence of puberty (see Box 5)(Castellano et al., 2011).

The expression of functional leptin receptors in the Arc of various species, furthermore underscores the way in which leptin, acting via kisspeptin, can modulate the timing of puberty. It seems that the signals from extracellular metabolic sensors such as leptin, are passed on to specific intracellular pathways. These intracellular pathways can respond to binding hormones and modulate cellular physiology through functions such as cell proliferation, growth, cytoskeleton organization and protein translation (Roa & Tena-Sempere, 2010). It is beyond the scope of this review to thoroughly describe all of the intracellular mechanisms that underlie leptin signaling, but the mammalian target of rapamycin (mTOR) is one such pathway that mediates peripheral energy homeostasis cues conveyed by leptin. Blocking mTOR signaling through rapamycin in female rats prevented the permissive effects of leptin on pubertal timing. Interestingly, rapamycin also suppressed kisspeptin mRNA levels in the Arc of female rats, although whether this is a direct or rather indirect control of kisspeptin remains to be seen. All in all, a leptin-mTOR-kisspeptin pathway for the metabolic regulation of puberty is suggested (Roa & Tena-Sempere, 2010; Roa et al., 2009). These findings await validation in other animal models of reproductive maturation.

Box 5. Obesity and altered pubertal timing

Since childhood obesity prevalence is rising and puberty onset is metabolically regulated, the mechanisms underlying this regulation are of growing interest. Since the 19th century, the age at which girls reach thelarche and gonadarche has dropped, due in part to nutritional improvements (Biro & Wien, 2010; Biro et al., 2010). Excess childhood adiposity in girls seems to enhance pubertal onset even further. A direct relation between increasing adiposity and pubertal timing through premature activation of the GnRH pulse generator has therefore often been assumed. This issue has often been put forward in recent literature (Bianco, 2012; Donato Jr et al., 2011; Elias, 2012; Roa & Tena-Sempere, 2010; Sanchez-Garrido & Tena-Sempere, 2013), but the underlying mechanism remains controversial and unsolved. Furthermore adding to the complexity is the fact that in contrast to girls, obesity in boys delays pubertal timing (Bianco, 2012; Burt Solorzano & McCartney, 2010).

Precocious or a delay in pubertal onset can have serious psychological as well as medical consequences (Burt Solorzano & McCartney, 2010; Dorn et al., 2010). The impact of obesity on pubertal development should therefore be carefully studied. Future research should critically evaluate whether these effects are caused by a central problem, or rather by a peripheral effect. Increased aromatization of androgens to estrogens by fat tissue, a decrease in estrogen metabolism and increased bioavailability of sex steroids in the prepubertal phase as a result of excess fat tissue, all promote breast tissue development, i.e. thelarche. Such possible peripheral effects thus do not necessarily represent neuroendocrine HPG-axis maturation (Burt Solorzano & McCartney, 2010; Elias, 2012).

Discussion of all the possible factors contributing to these changes in pubertal timing is beyond the scope of this review. It remains to be seen, whether the delay or rather enhancement of central puberty initiation is a direct result of excess adiposity. In other words: do changes in pubertal onset genuinely reflect changes in HPG axis activation? This issue requires further studying.

Interestingly, leptin also targets the ventral premammillary nucleus (PMv) that seems to mediate the effects of leptin directly towards the hypothalamic area (Sanchez-Garrido & Tena-Sempere, 2013). Due to its expression of functional receptors for metabolic hormones, such as ghrelin, insulin and leptin, as well as androgen and estrogen receptors, this nucleus likely integrates environmental cues and reproductive and energy store signals. In rodents, direct glutamatergic projections to the GnRH neurons and neurons in the AVPV/PeN and Arc exist, serving as a possible mechanism to convey this integrated information towards the reproductive endocrine axis (figure 3A)(Donato & Elias, 2011). PMV neurons are thus stimulated by increasing levels of leptin, activating their targets via the release of excitatory neurotransmitters (Elias, 2012).

Conclusions

This review has described major factors implicated in the onset of puberty. Besides, the current literature has been critically evaluated, pointing out issues and remaining questions for the field. It can be concluded that puberty onset requires the combined activation of different signaling pathways, rather than an isolated pathway being responsible for the neuroendocrine control of puberty. Maturation of these signaling pathways changes their output to GnRH neurons. In this view, it seems that GnRH neurons serve as a central output pathway.

Kisspeptin and NKB are essential factors in reproductive maturation. Since absence or malfunctioning of these neuropeptides results in an absence of puberty, these factors have been termed gatekeepers of puberty. Integration of upstream genetic and environmental cues takes place at the level of these so-called KNDy neurons. Discovery of the involvement of these neuropeptides in bringing about puberty has greatly improved our knowledge. However, the question as to what exactly triggers these gatekeepers still remains. Recent work by Lomnicnizi et al., (2013) is pointing towards epigenetic changes in these upstream positive factors shortly before puberty, thereby relieving a repressing brake. Although these results are very promising, the question then remains: what brings about these sudden epigenetic changes of these repressing genes? This issue stresses the complexity of the system.

It was beyond the scope of this review to include all of the different factors that contribute pubertal onset. Besides recent work that focuses on epigenetic effects in the control of GnRH pulsatility, the role of microRNAs in pubertal timing is currently being studied (Sangiao-Alvarellos et al., 2013). MicroRNAs fine-tune expression of many of the protein-encoding genes in mammals. It is expected that research will focus on these exciting areas in the forthcoming years.

The review has also highlighted modulating or permissive factors, such as leptin, that tune pubertal timing to the most optimal conditions of the body. These effects are not directly exerted onto GnRH neurons, but via upstream pathways, including the kisspeptin system and the PMv. Metabolic regulation of pubertal timing is often an issue of interest because of the rising incidence of childhood obesity and correspondingly precocious puberty, at least in females. However, peripheral effects of excess adiposity might just as well explain the earlier onset of breast development, as seen in some obese girls. This issue also highlights the discrepancies that can exist between external cues that are used to assess puberty onset, such as breast development, and the actual stage of HPG-axis maturation. The precise role of excess adiposity on influencing central puberty initiation remains to be elucidated.

Other important modulators of GnRH activity are the gonadal steroids. Again, these effects are mediated onto GnRH neurons via upstream pathways, including kisspeptin. The gonadal steroids generally provide negative feedback to the hypothalamus. In females, however, a positive feedback mechanism also exists that causes the preovulatory GnRH/LH surge. Different kisspeptin nuclei are thought to mediate the positive or negative feedback in rodents, but this issue requires validation in

humans. Clear differences between species furthermore exist in the role of these steroids. Whereas rodents show a sex steroid-dependent mechanism in the onset of puberty, this process develops relatively independent of the steroidal effects in humans and primates. Changes in gonadal feedback systems in primates and probably humans do not account for the increased pulsatile GnRH release that marks puberty onset.

The use of different animal models for reproductive maturation is very valuable. However, this review has also stressed the need for caution in translating the development of one species to another. Besides the aforementioned steroid-dependent or rather independent trigger for puberty onset, the fact that the rodent is the only animal model that does not show a juvenile pulse of the GnRH pulsatility is another very important difference. Comparing a gradual, progressive maturation to a system that has full potential to function at birth, but is restrained until puberty onset, are very different things. Whether prepubertal changes in GnRH neuron morphology, increases in sensitivity to kisspeptin stimulation or receptor expression are part of the trigger machinery for human pubertal timing awaits validation in other animal models, preferably primates. Despite its great value, these differences hamper the use of the rodent as a model for human pubertal timing.

In the same regard, research focusing on the expression patterns of kisspeptin neuronal populations in humans is absolutely necessary. Although rodents clearly show another kisspeptin population outside of the Arc, this was less evident for primates and humans. Also, some of the characteristics and developments of the system that controls GnRH pulsatility possibly explain sex differences in pubertal onset, which occurs earlier in females in most species. These could include kisspeptin cell numbers, which were higher in the Inf of adult women as compared to men. However, most of the literature to date, both reviews and studies, has cited a single study by Hrabovszky et al. (2010). This study included a total of twelve adult participants, their age ranging from 26 to 74 years of age. Developmental changes at the level of cell numbers, fiber growth and appositions in males and females at a younger age, especially neonatal and around puberty, clearly requires further investigation. Such findings, combined with new advances in the field, will further help to understand the complex system that controls the development towards reproductive maturation.

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