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Gene Networks and the Neuroendocrine Regulation of Puberty

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Abstract

A sustained increase in pulsatile release of gonadotrophin releasing hormone (GnRH) from the hypothalamus is an essential, final event that defines the initiation of mammalian puberty. This increase depends on coordinated changes in transsynaptic and glial-neuronal communication, consisting of activating neuronal and glial excitatory inputs to the GnRH neuronal network and the loss of transsynaptic inhibitory tone. It is now clear that the prevalent excitatory systems stimulating GnRH secretion involve a neuronal component consisting of excitatory amino acids (glutamate) and at least one peptide (kisspeptin), and a glial component that uses growth factors and small molecules for cell-cell signaling. GABAergic and opiateergic neurons provide transsynaptic inhibitory control to the system, but GABA neurons also exert direct excitatory effects on GnRH neurons. The molecular mechanisms that provide encompassing coordination to this cellular network are not known, but they appear to involve a host of functionally related genes hierarchically arranged. We envision that, as observed in other gene networks, the highest level of control in this network is provided by transcriptional regulators that, by directing expression of key subordinate genes, impose an integrative level of coordination to the neuronal and glial subsets involved in initiating of the pubertal process. The use of high-throughput and gene manipulation approaches coupled to systems biology strategies should provide not only the experimental bases supporting this concept, but also unveil the existence of crucial components of network control not yet identified.

Keywords

hypothalamus; GnRH neurons; glial-neuronal communication; transcriptional regulation; timing of puberty

General Concepts

A wealth of evidence has accumulated in recent years demonstrating the contribution of different, yet partially overlapping, sub-systems to the neuroendocrine mechanism controlling the pubertal process in mammals. The final, *sine qua non*, event required for puberty to occur is a sustained increase in pulsatile gonadotrophin-releasing hormone (GnRH) release from GnRH neurons. In primates, these neurosecretory cells are mostly located in the medial basal hypothalamus; in rodents, they reside in the preoptic area (Ojeda and Skinner 2006; Plant and Witchel 2006). The pubertal changes in GnRH output are, in

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turn, determined by modifications in transsynaptic (Kordon et al., 1994; Ojeda and Terasawa 2002) and glial (Ojeda et al., 2003; Ojeda and Terasawa 2002) inputs to the GnRH neuronal network. Studies in monkeys and rodents have shown that while the transsynaptic changes involve an increase in excitatory inputs and a reduction in inhibitory influences (Ojeda and Terasawa 2002; Plant and Witchel 2006; Terasawa and Fernandez 2001), the glial component of the system is predominantly facilitatory, and consists of growth factors and small diffusible molecules that directly or indirectly stimulate GnRH secretion (Ojeda et al., 2003; Ojeda and Skinner 2006).

Individual components of each of these regulatory systems continue to be identified. For instance, we now know that in addition to glutamatergic neurons (Brann 1995; Ojeda and Skinner 2006; Plant and Witchel 2006), which are widely distributed throughout the basal forebrain, the hypothalamus contains neurons that use the peptide kisspeptin for neurotransmission and to stimulate GnRH release (Kauffman et al., 2007; Oakley et al., 2009). These neurons also produce neurokinin B (Navarro et al., 2009), a peptide encoded by a gene recently implicated in the control of human puberty (Topaloglu et al., 2008), and dynorphin, an opioid peptide that inhibits GnRH secretion (Kinoshita et al., 1982; Navarro et al., 2009; Schulz et al., 1981). In rodents, one group of kisspeptin neurons is located in the periventricular region of the anteroventral periventricular nucleus (AVPV), and the other in the periventricular region of the arcuate nucleus (ARC) (Clarkson et al., 2009b). Thus, the excitatory transsynaptic regulation of GnRH secretion is provided by neurons that use glutamate, kisspeptin (Dungan et al., 2006; Ojeda and Skinner 2006), and apparently, neurokinin B (Topaloglu et al., 2008), as excitatory neurotransmitters/neuromodulators.

In general terms, it can be stated that the inhibitory counterpart of this circuitry is provided by both GABAergic neurons and opiateergic neurons [reviewed in (Terasawa and Fernandez 2001)]. In addition to these two systems, recent evidence suggest that a product of the RFamide-related peptide gene (*RFRP*), which is the mammalian ortholog of the peptide gonadotrophin-inhibiting hormone (GnIH) in birds (Ebling and Luckman 2008), is able to inhibit GnRH neuronal activity in mammals (Ducret et al., 2009; Gibson et al., 2008). It is thus possible that RFRP-containing neurons provide an additional set of inhibitory inputs to GnRH neurons during reproductive maturation. Of these three inhibitory systems, it would appear that RFamide-dependent inhibition is the simplest, because it is exerted by one or two peptides (RFRP1 and RFRP3) acting on a single receptor (Hinuma et al., 2000), which appears to be expressed in GnRH neurons (Ducret et al., 2009). The actions of GABA are more complex, because GABA both inhibits GnRH secretion by acting on subsets of neurons connected to the GnRH neuronal network (Ojeda and Skinner 2006; Terasawa and Fernandez 2001), and stimulate GnRH neurons directly via activation of GABA_A receptors (DeFazio et al., 2002; Moenter and DeFazio 2005). Opioid-producing neurons, on the other hand, employ different peptides and a variety of different receptors to inhibit GnRH release (Kordon et al., 1994); this inhibition may be exerted directly (Dudas and Merchenthaler 2006) or indirectly on neurons involved in the stimulatory control of the GnRH neuronal network, such as kisspeptin neurons (Navarro et al., 2009).

It is now clear that the pubertal activation of GnRH secretion is not only driven by transsynaptic inputs, but also by glial cells (Ojeda et al., 2000; Ojeda and Terasawa 2002), which produce cell-cell signalling molecules that stimulate GnRH release, and that have been shown to be critical for the correct timing of the pubertal process [reviewed in (Lomniczi and Ojeda 2009)]. Glial cells contribute to the pubertal activation of GnRH secretion via two related mechanisms. One of these mechanisms involves growth factors. Some growth factors, such as transforming growth factor-beta (TGF β), are recognized by cell-membrane receptors endowed with serine-threonine kinase activity. Others are recognized by receptors with tyrosine kinase activity. They include the epidermal growth

factor (EGF) family, basic fibroblast growth factor (bFGF), and insulin-like growth factor 1 (IGF-I). A neuron-to-glia regulatory pathway is, in turn, provided by glutamatergic neurons which facilitate astrocytic signaling mediated by erbB receptors. These belong to the EGF receptor family and mediate the actions of the EGF family of trophic factors [reviewed in (Holbro and Hynes 2004)]. Genetic disruption of erbB receptors delays female sexual development due, at least in part, to impaired erbB ligand-induced glial prostaglandin E₂ (PGE₂) release (Lomniczi and Ojeda 2009).

The second mechanism of glia-to-GnRH neuron communication involves plastic rearrangement of cell-cell adhesive behavior. The adhesiveness of glial cells to GnRH neurons appears to require at least two different cell-cell communications systems, one provided by Synaptic Cell Adhesion Molecule 1 (SynCAM1) (Ojeda et al., 2008), and the other resulting from the interaction of neuronal contactin with glial Receptor-like Protein Tyrosine Phosphatase- β (RPTP β) (Parent et al., 2007). Because both systems are endowed with signaling capabilities, the interaction of glial cells with GnRH neurons may not only involve secreted bioactive molecules, but also the activation of cell-cell signaling mechanisms set in motion by adhesive molecules.

The concept of gene networks involved in the neuroendocrine control of puberty

Can the initiation of puberty be attributed to the activation of a single gene? On the surface, the answer to this question may be affirmative if one considers that rare single gene mutations, such as those affecting *GNRHR* (Bedecarrats and Kaiser 2007), *GPR54* (de Roux et al., 2003; Seminara et al., 2003), *KISS1* (Lapatto et al., 2007), *TAC3* and *TACR3* (Topaloglu et al., 2008), result in pubertal failure. However, none of these genes appears to be in a commanding position to synchronize neuronal and glial networks involved in the initiation of puberty. Illustrating the general validity of this concept is the emerging view that – contrary to earlier expectations – *KISS1* is not a gene that sets in motion the pubertal process. Instead, *KISS1*-expressing neurons seem to be activated in response to developmental changes in estradiol production (Clarkson et al., 2009a). These observations and the recent demonstration that common genetic variation in a set of genes thought to play an essential role in puberty does not affect the timing of this process in humans (Gajdos et al., 2008), have made clear that no isolated pathway or cellular subset is responsible for the neuroendocrine control of puberty (Eaves et al., 2004; Krewson et al., 2004; Ojeda et al., 2006; Seminara and Crowley, Jr. 2001). This notion is further supported by recent genome-wide association studies demonstrating an association of a sequence variation in *LIN28B* (a gene encoding an RNA-binding protein) with early menarche (Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009). These studies also found a significant association of sequence polymorphisms in at least 9 other genes with the age at menarche (Perry et al., 2009; Sulem et al., 2009). A plausible explanation for these findings can be provided by the hypothesis, put forward earlier, that puberty is controlled by regulatory gene networks composed of multiple functional modules (Ojeda et al., 2006) and that operate with overlaps of partially redundant pathways as opposed to being in a strict hierarchy.

This article will discuss and attempt to refine this concept in the light of new findings that have become recently available to the field.

General Structure of a Genetic Network

In the broadest sense, genetic networks consist of central “hubs”, which direct the flow of information throughout the entire network, and subordinate “nodes” that, located at various hierarchical positions (and at different cellular levels), execute commands flowing from the

central hubs. Genetic networks are also scale-free and hierarchical. They are scale-free because they show a strong interconnectivity if they are composed of a few nodes, and this connectivity does not increase (i.e. it is not “scaled up”) as the complexity of the network increases (Basso et al., 2005). They are hierarchical, because highly connected nodes exhibit preferential interactions among themselves. Thus, networks show an “elite” of major nodes that are highly interconnected, and a multitude of peripheral nodes that become increasingly less connected as they move away from the major hubs (Fig. 1). This loss of connectivity is to be expected considering that the complexity of a network can increase strikingly as it expands from its central hubs. For instance, the c-Myc network has 56 first neighbors to c-Myc and near 2,000 second neighbors! (Basso et al., 2005). In addition to these characteristics, gene or protein networks are robust, i.e., they exhibit a great degree of redundancy in their pathways. It is not surprising, therefore, that when a given pathway within a network is disabled, there is rapid activation of a redundant, or more accurately described, “back-up” pathway.

A Gene Network Involved in the Neuroendocrine Control of Puberty

There are genes required for the acquisition of reproductive function, such as *GPR54*, *Kiss1* and *TAC*, and genes that, expressed in different cellular subsets of the hypothalamus contribute to defining the correct timing of the pubertal process. Some of these genes play major roles in controlling astroglial function, such as those encoding erbB receptors (Lomniczi and Ojeda 2009); others encode novel molecules required for glutamate release (Choi et al., 2008; Ha et al., 2008), homeostatic maintenance of GnRH neuron excitability (Garcia-Rudaz et al., 2008), unidirectional glia-to-GnRH neuron signaling (Lomniczi et al., 2006), and glia-GnRH neuron adhesive communication (Ojeda et al., 2008; Parent et al., 2007). Still, other genes appear to be involved in the transcriptional regulation of puberty; they include the POU-domain gene *OCT2* (Ojeda et al., 1999), the homeodomain gene *TTF1/NKX2.1* (Mastronardi et al., 2006), and a novel gene provisionally termed chromosome 14 open reading frame 4 (C14ORF4) (Rampazzo et al., 2000), which we have termed *EAP1* (Enhanced At Puberty1) (Heger et al., 2007). Based on a variety of experimental approaches, including the use of antisense oligodeoxynucleotides (Ojeda et al., 1999), Cre-loxP-mediated, neuron-specific conditional gene deletion (Mastronardi et al., 2006) and siRNA-mediated region-specific knock-down of gene expression (Heger et al., 2007) it has been proposed that *OCT2*, *TTF1* and *EAP1* are upper-echelon (upstream) genes controlling the expression of subordinate genes that, differentially expressed in neurons and glial cells, are necessary for the neuron-to neuron and glia-to neuron regulation of GnRH secretion at puberty.

While advancing our knowledge, these findings have also made apparent the need for a global approach that can place these diverse components within the framework of a regulatory network (or set of networks) controlling puberty (Ojeda et al., 2006). Until now, studies addressing this issue have not been forthcoming, essentially due to the lack of appropriate technologies, and the relative paucity of genetic and biochemical details that can be assimilated into a testable biological model. The emergence of high-throughput approaches and computational methods to create, organize, display and analyze the plethora of results derived from such approaches is rapidly changing this landscape, and giving us for the first time the opportunity of identifying and demonstrating functional genetic modules involved in the hierarchical control of puberty.

To begin exploring the notion of gene networks controlling the pubertal process, studies were performed interrogating three complementary sets of samples: the juvenile-early pubertal female monkey hypothalamus using human cDNA arrays, the juvenile-peripubertal female rat hypothalamus using Affymetrix rat arrays, and a group of human hypothalamic

hamartomas (HHs) associated with precocious puberty, also interrogated using Affymetrix arrays (Parent et al., 2008; Roth et al., 2007). Additional genes were identified using quantitative proteomics (Ojeda et al., 2008), “guilt by association” (Lockhart and Winzler 2000; Oliver 2000), and retrospective approaches. HH were investigated because these rare, non-neoplastic congenital malformations of the basal hypothalamus are usually associated with sexual precocity (Grumbach and Styne 2003). To complement this genomic approach, quantitative proteomics (Gygi et al., 1999) was employed to identify hypothalamic proteins that might be down- or up-regulated in transgenic (DNerBB4) mice exhibiting delayed puberty due to an astrocytic-specific defect in erbB receptor signalling (Prevot et al., 2003). Analysis of the monkey arrays results showed that expression of four genes implicated in tumor formation, but that otherwise participate in normal cell differentiation processes, increases selectively in the hypothalamus at the time of female monkey puberty (Roth et al., 2007). The hamartoma arrays identified four additional candidates also implicated in tumor formation (Parent et al., 2008). Consistent with these observations, quantitative proteomics revealed that the content of SynCAM1, an immunoglobulin-like adhesion molecule, earlier known as “tumor-suppressor in lung cancer-1” (TSLC1) (Kuramochi et al., 2001) and more recently shown to be required for synapse formation (Biederer et al., 2002), was decreased in the DNerBB4 mice (Ojeda et al., 2008). Quantitative PCR studies verified the array results (Parent et al., 2008; Roth et al., 2007) and indicated that that *KiSS1* and *GPR54* are also part of this neuroendocrine gene network, because their mRNA expression increases in the hypothalamus at the time of primate puberty (Shahab et al., 2005). Before the role of kisspeptin in the control of puberty was discovered, the *KiSS1* gene was known as a suppressor of tumor metastases (Ohtaki et al., 2001; Steeg et al., 2003).

With this information in hand, computational and systems biology methods were employed to determine if these genes are functionally organized, and the conclusion was reached that they form part of a gene network. Although the genes composing this network have diverse cellular functions, they share the common feature of being earlier identified as involved in tumor suppression/tumor formation (Roth et al., 2007). Because of this, the aforementioned network was termed TSG (for Tumor Suppressor Gene) network.

The model predicts that *KiSS1* and *SynCAM1* are subordinate genes. While *KiSS1* is essential for the occurrence of puberty in mice and humans, *SynCAM1* was recently shown to play a pivotal role in the developmental control of glia-GnRH neuron adhesive communication (Ojeda et al., 2008). *Cis*-regulatory analysis of shared transcription factor binding sites predicted the existence of five major hubs (*CDP/CUTL1*, *MAF*, *p53*, *YY1*, and *USF2*) controlling the network at the transcriptional level (Roth et al., 2007). These hubs are not only connected to genes encoding proteins required for intracellular signaling, and cell-cell communication, but also with the other upper-echelon genes (*OCT2*, *TTF1*, and *EAP1*) postulated to contribute to the transcriptional regulation of the pubertal process. Cellular localization studies using a combination of immunohistochemistry and *in situ* hybridization demonstrated that these three genes, and other subordinate nodes of the network are expressed in neuronal and/or glial subsets involved in the control of GnRH secretion, including GnRH neurons themselves (Heger et al., 2007; Mastronardi et al., 2006; Ojeda et al., 2008; Roth et al., 2007). Remaining to be defined is not only the identity of the neuronal and glial subsets expressing each putative central hub, but also the possibility that individual central hubs have different, cell-specific, puberty-related developmental patterns of expression or epigenetic control.

Are There Additional Gene Networks Controlling Puberty?

It would not appear that the TSG network is the only network involved in the control of puberty. *OCT2*, *TTF1*, and *EAP1* appear to form part of another network(s) yet to be

characterized, because they do not behave as TSGs. In fact, examination of *EAP1* expression in a panel of human tumors, revealed no discernible pattern of underexpression (S. Heger, T. Neff and S.R. Ojeda, unpublished), reinforcing the idea that *EAP1* may not be a TSG. Although it would not be surprising to find that transcriptional regulators are shared by different neuronal and glial subsets, specialized networks must exist that include subordinate genes specifically expressed in particular neuronal subsets. In the case of glutamatergic neurons, genes encoding receptors, and enzymes involved in the synthesis and metabolism of glutamate should be considered, in addition to those encoding vesicular transporters, and transporters required for glial glutamate uptake. For instance, the abundance of glutamate dehydrogenase, [one of the enzymes catalyzing the synthesis of glutamate (Erecinska and Silver 1990)], increases in the hypothalamus of female rats undergoing puberty. In contrast, the abundance of glutamine synthase [which catalyzes the metabolism of glutamate into glutamine (Erecinska and Silver 1990)] decreases at this time (Roth et al., 2006). These changes were accompanied by an increased capacity of the hypothalamus to release glutamate following blockade of glial glutamate uptake, suggesting that more glutamate is released into the synaptic cleft at the time of puberty.

Like glutamate, GABA synthesis, metabolism, transport and release requires the participation of different proteins. Because no changes in hypothalamic levels of the mRNAs encoding GAD-65 and GAD-67 (the enzymes responsible for GABA synthesis) have been detected during female sexual development (Urbanski et al., 1998), it does not appear that changes in expression of these genes have a role in the onset of puberty. It is, however, plausible that the critical steps subjected to network control, are those involving GABA vesicular transporters (De Gois et al., 2005; McIntire et al., 1997) and GABA receptors, because they provide a more dynamic means of regulation.

A reduction in opioid input to the GnRH neuronal network at the time of puberty may not be as critical as the loss of GABAergic inhibitory control, but there is little doubt that opioid peptides are inhibitory to GnRH release. In rodents, the strength of the prepubertal opioid peptide inhibitory tone (Blank et al., 1979) diminishes at the time of puberty (Wilkinson and Bhanot 1982). Although opioid peptides as a group do not appear to restrain the initiation of puberty [reviewed in (Ojeda and Terasawa 2002)], dynorphin, an individual component of this system has been shown to be a potent inhibitor of GnRH secretion (Navarro et al., 2009; Schulz et al., 1981). Because dynorphin is also involved in the control of kisspeptin neuron activity (Navarro et al., 2009), it seems important to identify the network components that regulate dynorphin expression. Of additional importance is the identification of the factors involved in regulating the transcriptional activity of the *RFRP* gene, which may turn out to play a significant role in the inhibitory control of GnRH secretion.

A possibility that has never been considered is that a regulatory step affecting the function of peptidergic neurons controlling the GnRH neural network is the transcriptional control of genes encoding for enzymes involved in processing the prepropeptide of interest, such as kisspeptin, dynorphin, RFRP1/3, etc. Finally, little has been done to identify regulatory networks that, operating in hypothalamic astrocytes, control glial production of growth factors (e.g. TGF α , neuregulins), and small molecules (e.g., prostaglandin E₂, glutamate, ATP) involved in stimulating GnRH release. Of all of these factors, only TGF α has been found to be transcriptionally regulated by a gene (*Oct2*) considered to be involved in the upstream regulation of the pubertal process.

Perturbation of Network Function

Genetic networks can be perturbed for experimental verification of computational predictions using either gain-of-function or loss-of function approaches (Ideker et al., 2001;

Vidal 2001). The latter can be expeditiously implemented in both *in vitro* and *in vivo* settings, using RNAi technology. Because lentiviruses (Tiscornia et al., 2003) can efficiently deliver siRNAs to tissues or cells of interest (Garcia-Rudaz et al., 2007; Heger et al., 2007), experiments were conducted in female rats to determine if decreasing the production of EAP1, a purported transcriptional regulator of the pubertal process, would alter the onset of female puberty and adult reproductive cyclicity (Heger et al., 2007). *Eap1* siRNA-producing lentiviral particles were injected bilaterally into the preoptic area (POA) of juvenile 23-day-old rats. Control animals were injected with enhanced green fluorescent protein (eGFP)-producing viruses. Identification of eGFP-expressing cells two months after the injections showed that well-placed injections infected neurons located in or near the AVPV, the region of the POA required for the preovulatory discharge of gonadotropins (Simerly 2002). Image analysis showed that the EAP1 content was significantly lower in *Eap1* siRNA-expressing cells than in non-infected cells (Heger et al., 2007). The onset of puberty was delayed in *Eap1* knock-down rats, which also failed to cycle normally, exhibiting prolonged episodes in estrus, reduced plasma LH, FSH and estradiol levels, and delayed growth of ovarian follicles.

To determine if EAP1 is also important for the hypothalamic control of menstrual cyclicity in nonhuman primates, a modified delivery vector was used (Dissen et al., 2009). This vector consists of three hairpin (hp)RNAs targeting *EAP1* mRNA, embedded into the body of a miRNA (miR130), and incorporated into a third generation lentiviral vector (Dull et al., 1998; Stegmeier et al., 2005). In contrast to siRNAs, shRNAs delivered as integral part of a miRNA can be processed by the cellular machinery as if they were endogenous miRNAs, thus avoiding cellular toxicity caused by oversaturation of the pathway that processes miRNAs into functional siRNAs (Boudreau et al., 2009; Grimm et al., 2006). These viral particles were injected into the hypothalamus of cycling adult female rhesus monkeys, utilizing stereotaxic techniques and MRI guidance. Each monkey received three injections per side targeting the antero-caudal dimensions of the arcuate nucleus, which controls cyclic GnRH release in humans and monkeys. Menstrual cycles preceding and following the intrahypothalamic injections were assessed by measuring serum estradiol and progesterone levels. The results showed that knocking down *EAP1* expression in this brain region abolished menstrual cyclicity, indicating that *EAP1* is crucial for the ARC to maintain menstrual cyclicity in higher primates (Dissen G, Lomniczi, A, Heger, S, and Ojeda S.R., et al., in preparation).

The Transcriptional Repression of Puberty: A speculation or an emerging concept?

Much discussion has been given to the question as to the *primum movens* of puberty: is it the loss of a “central restraint” (Terasawa and Fernandez 2001) or the activation of stimulatory inputs to GnRH neurons (Ojeda and Terasawa 2002)? Perhaps a more fundamental question resides at another, hitherto unsuspected level of control: Would it be possible that the pubertal process is held in check during juvenile days by a developmental program involving transcriptional/posttranscriptional repression of genes that are stimulatory to the pubertal process? Although this possibility has never been formally considered, the time to do so may have arrived. Recent reports have shown that a single nucleotide polymorphism near the *LIN28B* gene in chromosome 6(q21) is associated with earlier puberty and shorter stature in girls (Ong et al., 2009; Perry et al., 2009; Sulem et al., 2009). *LIN28B* and its homolog *LIN28* (located on a different chromosome) encode cytoplasmic proteins that control gene expression via posttranscriptional regulation (Moss et al., 1997). A structural hallmark of these proteins is the presence of a cold shock domain and retroviral-type zinc finger motifs, both of which are found in RNA-binding proteins. Despite the similarity of these two homologs, only sequence variation in *LIN28B* is associated with

early menarche (Ong et al., 2009). A role for *LIN28B* in controlling specific aspects of mammalian development can be inferred from the demonstration that mutations of its ortholog *lin28* in *C. elegans* result in precocious larval development, whereas increasing *Lin28* expression leads to developmental delay (Moss et al., 1997). *Lin28* exerts this effect by preventing the early expression of genes, which should normally be activated at a subsequent phase of development (Ambros and Horvitz 1984; Moss et al., 1997). Mammalian *LIN28* and *LIN28B* control cellular function via a posttranscriptional regulatory mechanism that consists of blocking the maturation of *let-7* miRNA precursors into mature miRNAs. This blockade then results in overall repression of this miRNA family and derepression of *let-7* miRNA target genes (Viswanathan et al., 2009). Because some of these targets are oncogenic (for instance *K-Ras* and *c-Myc*), *let-7* miRNAs are considered to be TSGs (Viswanathan et al., 2009). It then follows that if *LIN28B* is involved in the control of mammalian puberty, *let-7* miRNAs might be components of the TSG network controlling the onset of puberty. Within this scenario, *LIN28B* expression would be expected to decline in the hypothalamus at the time of puberty, resulting in derepression of *let-7* miRNA maturation. Preliminary results showing that *LIN28B* mRNA abundance decreases in the agonadal monkey hypothalamus at the expected time of puberty provide initial support for this notion (V. Matagne, S. R. Ojeda and T. Plant, unpublished). Whether mature *let-7*-miRNAs are TSGs required for the pubertal activation of GnRH secretion is not known, but their contribution would be expected to involve suppressing the expression of genes inhibitory to the pubertal process.

The composition of a potential transcriptional/posttranscriptional repressive mechanism controlling the timing of puberty is not yet known; however, it may have an unsuspected degree of complexity. For instance, a recent high-throughput interrogation of the agonadal male monkeys transcriptome revealed that expression of a highly conserved family of genes encoding zinc-finger (ZNF)-containing proteins changes selectively in the hypothalamus with the advent of puberty, with expression profiles inversely correlated to the prepubertal changes in LH output, i.e., increased expression during the late juvenile period when GnRH secretion is turned off, followed by decreased expression as the GnRH neuronal network becomes activated at the initiation of puberty (Matagne et al., 2009). ZNF genes encode proteins that function as transcriptional repressors (Urrutia 2003; Vogel et al., 2006). In ongoing studies, using Illumina DNA arrays to interrogate the female rat hypothalamus during pre and peripubertal development, we identified two families of transcriptional repressors, and made the puzzling observation that expression of genes belonging to one of these families decreases at puberty, while that of members from the other family increases. Family 1 is composed of TRG genes of the POZ-ZF (poxvirus and Zinc finger) family of transcriptional regulators, also known as BTB (broad complex, Tramtrack, bric à brac) (Kelly and Daniel 2006). POZ-ZF genes are transcriptional repressors implicated in the control of a variety of developmental processes, with alterations of their function resulting in tumorigenesis and developmental disorders (Kelly and Daniel 2006). Our results showed that expression of a subset of the 12 known POZ-ZF genes, known as the POK (POZ and Krüppel) subfamily, *increases* in the hypothalamus of female rats at puberty, suggesting that these genes may be repressing downstream repressors of the pubertal process. The second family of transcriptional repressors identified by our studies consists of genes of the Polycomb group (PcG). The PcG silencing complex imposes gene silencing in a developmentally regulated fashion (Kohler and Villar 2008; Schwartz and Pirrotta 2007; Simon and Kingston 2009). We observed that expression of key members of this complex *decreases* in the hypothalamus at puberty, suggesting that – as it occurs in other developmental processes – these genes might prevent the untimely, earlier initiation of puberty by inhibiting the expression of downstream genes whose activation is ultimately required for puberty to occur. Both POK genes and the Polycomb silencing complex can be considered as TRGs, because both have tumor suppressor activity (Classen et al., 2009;

Kelly and Daniel 2006; Martinez et al., 2009). These observations, though preliminary, give initial support to the idea that transcriptional repression may be an integral component of the developmental program controlling mammalian puberty.

Conclusions and Perspectives

The above reviewed observations are consistent with the notion that the neuroendocrine control of puberty is provided by overlapping and interacting gene networks endowed with elements of hierarchical and scale-free features. As such, they are expected to be dominated by a few highly connected central hubs, and use second-tier gene sub-networks and a large number of less-connected subordinate nodes for integrative performance. Overall, these networks should be extremely robust, because they possess a high degree of redundancy. Though such an arrangement requires detailed experimental verification, its very existence is consistent with the notion that the onset of puberty is genetically determined and depends on the contribution of more than one gene (Eaves et al., 2004; Krewson et al., 2004; Seminara and Crowley, Jr. 2001). It is, indeed, only recently that the complexity of the developmental program underlying the neuroendocrine control of puberty has begun to be appreciated. There are many genes and many pathways that contribute to the process, but in resemblance to developmental process operating during embryonic life, the initiation of the pubertal process may also be controlled at the transcriptional/post-transcriptional level by discrete groups of genes. One group of “activators” might be able to move the process along by sequentially eliciting key developmental events; another group might repress the untimely activation of these genes (Fig. 2). Needless to say, the overall concept that puberty is determined by the functional interaction of more than one gene network operating within the basal forebrain is still in its infancy. Even younger, and thus much less developed, is the notion of “in-tandem” repressive mechanisms that act during juvenile development to prevent the premature activation of puberty-advancing genes, and during puberty to diminish the influence of puberty-delaying genes (Fig. 2). The latter exert their effects by supporting transsynaptic inhibitory mechanisms controlling GnRH secretion. At the risk of being overly speculative, it may be suggested that both inhibitory and stimulatory pathways of transcriptional/post-transcriptional regulation operate within neuronal and glial circuitries involved in controlling the onset of puberty, and that a higher level of network control is provided by at least two layers of gene repression (Fig. 3). One of these layers, formed by transcriptional/posttranscriptional “repressors” (e.g. PcG genes, LIN28B, etc) may function to prevent the premature activation of puberty-inducing genes (such as TTF1, EAP1, Oct2, etc); the other layer, located more centrally in the network (e.g., POK, ZNF genes, others), may modulate in a temporal dynamic manner the expression of repressors (Fig. 3). As such, these more centrally located genes can be considered as “repressors of repressors”. Both layers may be also directly control key subordinate genes of the network (such as those operating within kisspeptin, GABAergic, glutamatergic, opioid, and RFRP neurons, in addition to genes expressed in glial cells). In addition, it appears that an encompassing level of coordination and transcriptional plasticity to these gene networks might be provided, not by variations in DNA sequence, but by heritable changes in gene expression that occur without changing the primary nucleotide sequence of a gene, i.e. epigenetics (Ojeda and Lomniczi 2010). Much research is obviously needed to verify or challenge the validity of these concepts.

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Figure 1.

General structure of a gene/protein network. One central hub (red) is shown surrounded by first neighbor nodes (blue circles labeled 1st N). These nodes are connected to secondary nodes (green circles), which in turn are connected to additional, more peripheral nodes (orange circles). The interconnectivity is greatest among the central hub and first neighbors, diminishing progressively among nodes located more distally from the central hub (green and orange circles). The area delineated by a triangular outline shows a segment of the network displaying all contributing nodes.

Figure 2.

The hypothetical transcriptional control of mammalian puberty by overlapping and interactive gene networks operating in the neuroendocrine brain. It is envisioned that during early juvenile days there are sets of genes strongly repressing downstream genes involved in advancing the pubertal process (such as the transcriptional regulators *Ttf1*, *Eap1* and *Oct2*, in addition to key subordinate genes such as *Kiss1*). In contrast, transcriptional repression of genes inhibitory to puberty (e.g., dynorphin, RFRP, and/or genes encoding prepropeptide processing enzymes) would be negligible. At this time of development, trans-activation of puberty-advancing genes would be minimal, whereas trans-activation of puberty-delaying genes might be enhanced (indicated by question mark). According to this model, the transcriptional inhibition of puberty-advancing genes would be lifted at puberty, and replaced by increased trans-activation of gene expression. Simultaneously, the repression of puberty-delaying genes would be enhanced. ← = stimulation; ⊥ = inhibition; ? = possible.

Figure 3.

A speculative view of the general structure of a gene network operating neuronal and glial networks involved in the neuroendocrine control of mammalian puberty. It is envisioned that the highest level of control is exerted by *repressors of repressors*, and that the influence of these genes on the inhibitory neuronal component of puberty is low during juvenile development (Juv), increasing at puberty (Pub). A similar temporal change in repressive influence would occur in the control of a second, less “central” level of control formed by “repressors” that may act during juvenile development to prevent the premature activation of “puberty-inducing” genes. As a result of these changes in repressive tone, expression of subordinate genes sub-serving the inhibitory transsynaptic control of GnRH secretion would decrease at puberty, whereas expression of key genes involved in the transsynaptic and glial stimulation of GnRH secretion would be enhanced. EAP1 is shown as both repressor and activator, because it has this dual function.