

Minireview: The Neuroendocrine Regulation of Puberty: Is the Time Ripe for a Systems Biology Approach?

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The initiation of mammalian puberty requires an increase in pulsatile release of GnRH from the hypothalamus. This increase is brought about by coordinated changes in transsynaptic and glial-neuronal communication. As the neuronal and glial excitatory inputs to the GnRH neuronal network increase, the transsynaptic inhibitory tone decreases, leading to the pubertal activation of GnRH secretion. The excitatory neuronal systems most prevalently involved in this process use glutamate and the peptide kisspeptin for neurotransmission/neuromodulation, whereas the most important inhibitory inputs are provided by γ -aminobutyric acid (GABA)ergic and opiate neurons. Glial cells, on the other hand, facilitate GnRH secretion via growth factor-dependent cell-cell signaling. Coordination of this regulatory neuronal-glia network may require a hierarchical arrangement. One level of coordination appears to be provided by a host of unrelated genes encoding proteins required for cell-cell communication. A second, but overlapping, level might be provided by a second

tier of genes engaged in specific cell functions required for productive cell-cell interaction. A third and higher level of control involves the transcriptional regulation of these subordinate genes by a handful of upper echelon genes that, operating within the different neuronal and glial subsets required for the initiation of the pubertal process, sustain the functional integration of the network. The existence of functionally connected genes controlling the pubertal process is consistent with the concept that puberty is under genetic control and that the genetic underpinnings of both normal and deranged puberty are polygenic rather than specified by a single gene. The availability of improved high-throughput techniques and computational methods for global analysis of mRNAs and proteins will allow us to not only initiate the systematic identification of the different components of this neuroendocrine network but also to define their functional interactions. (*Endocrinology* 147: 1166–1174, 2006)

IN MAMMALS, INCLUDING humans, developmental changes in gonadotropin secretion are controlled by changes in pulsatile release of GnRH. At puberty, pulsatile gonadotropin secretion increases in a diurnal fashion, initially characterized in humans (1), but that also occurs in other species, including the rat (2). This change, necessary for normal gonadal development and function, is determined by activation of a hypothalamic GnRH pulse generator (for review, see Refs. 2 and 3). The pubertal increase in GnRH secretion is, in turn, prompted by changes in transsynaptic and glial inputs to the GnRH neuronal network. Studies conducted by different groups have identified these inputs as being both facilitatory and inhibitory. The former use excitatory amino acids (for review, see Refs. 2 and 4) and the recently identified neuropeptide metastin/kisspeptin (5, 6) for neurotransmission/neuromodulation. γ -Aminobutyric acid (GABA) and opioid peptides provide the inhibitory inputs (7, 8). It is also clear that the pubertal activation of GnRH secretion can no longer be considered as an event driven solely by transsynaptic inputs (2, 9). Glial cells pro-

duce cell-cell signaling molecules that stimulate GnRH release and that have been shown to be critical for the correct timing of the pubertal process (for review, see Ref. 10).

This article will briefly review our current understanding of the cell-cell mechanisms underlying the neuroendocrine control of puberty. We will also begin to develop the broad (but obviously imperfect) concept that the pubertal activation of GnRH secretion is controlled by a network of genes that, having diverse functions, operate within different cell contexts to coordinate the secretory activity of the GnRH neuronal network at puberty. We will propose the concept that coordination of GnRH release requires the participation of two sets of genes, those that are subordinate and those that govern the pubertal process at a higher hierarchical, transcriptional level of control.

Using a systems biology approach, *i.e.* the coordinated study of a biological system (11), for the understanding of these neuroendocrine regulatory networks, requires by definition (12): 1) identifying the genes, proteins, and other small molecules constituting the pathway of interest; 2) perturbing each pathway component through genetic manipulations and detecting the global cellular response to each perturbation with the help of high-throughput and whole-genomics techniques; 3) integrating the observed mRNA and protein responses with existing models of protein-protein, protein-DNA, and other interactions, using appropriate computational methods; and 4) formulating new hypotheses to ex-

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Abbreviations: GABA, γ -Aminobutyric acid; HH, hypothalamic hamartoma; ME, median eminence; NRG, neuregulin; PG, prostaglandin; TSG, tumor suppressor gene; TTF-1, thyroid transcription factor-1.

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plain observations not predicted by the model. As the reader will readily appreciate from this article, the application of these principles to the understanding of the neuroendocrine control of puberty is, at best, in an early embryonic stage.

Subordinate Genes Required for Cell-Cell Communication

This category include all downstream genes that participate in the excitatory and inhibitory control of GnRH neurons, whether this control is exerted transsynaptically or via glia-to-neuron communication (Fig. 1). Subordinate genes execute specific cellular functions required for cell-cell signaling, but their expression is hypothetically regulated by a

higher order of system network control. For operational purposes they can be considered as the last to be activated.

It was earlier established that the major excitatory transsynaptic event prompting the initiation of puberty is an increase in glutamatergic neurotransmission (2, 13), the primary mode of excitatory transsynaptic communication in the hypothalamus (14). Activation of glutamatergic inputs increases GnRH secretion (15, 16) and accelerates sexual maturation in both rats and monkeys (17, 18). Glutamate acts both directly (19–22) and via regulatory neuronal subsets (23) to stimulate GnRH secretion.

Glutamatergic neurotransmission is a complex process controlled by a plethora of genes required for synthesis,

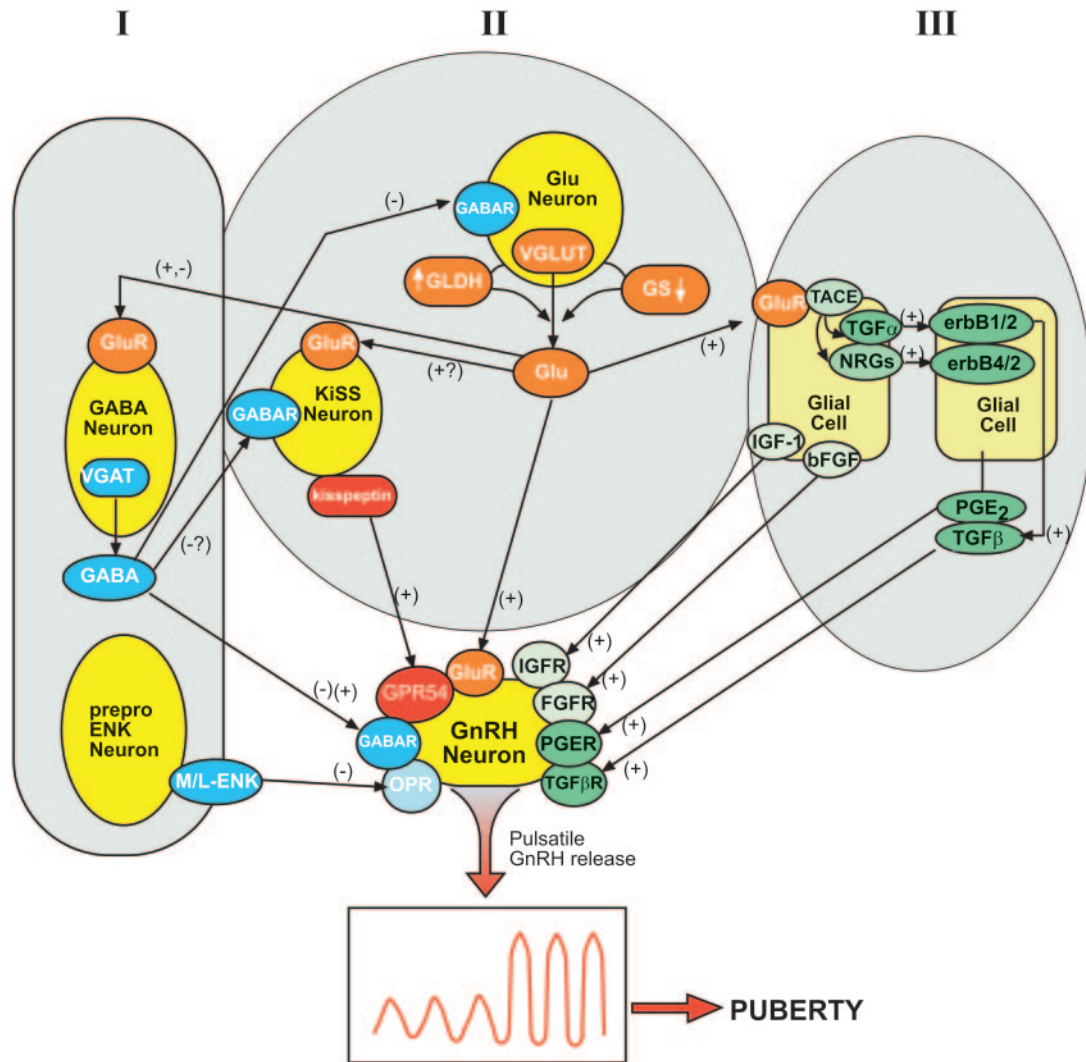


FIG. 1. Some of the subordinate genes involved in the transsynaptic and glial control of GnRH neurons at the time of female puberty. These genes are postulated to function within a large cellular network organized into three interacting domains. Domain I contains the transsynaptic inhibitory components of the system, *i.e.* GABAergic and opiate neurons (represented here by preproenkephalineric neurons); domain II contains the excitatory neuronal subsets (represented by glutamatergic and kisspeptin-producing neurons); and domain III is composed of astroglial and ependymoglial cells. Not all the potential cell-cell communication pathways are shown. Also notice that the direct GABA_A receptor-mediated effects of GABA on GnRH neurons can be excitatory. VGLUT, Vesicular glutamate transporters 1 and 2; VGAT, vesicular GABA transporter 1; GLDH, glutamate dehydrogenase; GS, glutamine synthase; Glu, glutamate; GluR, ionotropic and/or metabotropic glutamate receptor; GABA_A, GABA receptor (A or B); M/L-ENK, Met- or Leu-enkephalin; OPR, opioid receptor; TACE, tumor necrosis factor- α -converting enzyme; erbB1, 2, and 4, receptors for TGF α (erbB1/2) and NRGs (erbB4/2); TGF β R, TGF β receptors (I and III); bFGF, basic fibroblast growth factor; IGFR, IGF-I receptor; FGFR, FGF receptor; PGER, PG receptor; (+), stimulation; (-), inhibition; ?, not known.

transport, and release of the amino acid, as well as for the expression of the various receptors that mediate glutamate actions. Puberty-related changes in glutamate receptor expression might be restricted to specific hypothalamic cellular subsets. For instance, the binding capacity of NMDA and kainate receptors (which presumably reflect changes in gene expression) does not change in cell membranes derived from whole hypothalami (24). On the other hand, kainate receptor expression measured by *in situ* hybridization increases in GnRH neurons during sexual development (21). The upstream genes controlling this change are not known. Even less is known about the transcriptional control of genes encoding enzymes involved in the synthesis, metabolism, and transport of glutamate. The importance of these homeostatic systems has been made evident by recent studies in which we used a quantitative proteomics approach (25) to identify proteins whose expression is increased in the hypothalamus at the time of rat puberty (26). We observed that the abundance of glutamate dehydrogenase, one of the enzymes that catalyzes the synthesis of glutamate (27), increases in the hypothalamus of female rats undergoing puberty. In contrast, the abundance of glutamine synthase, which catalyzes the metabolism of glutamate into glutamine (27), decreases at this time (Fig. 1). These changes were accompanied by an increased capacity of the hypothalamus to release glutamate after blockade of glutamate transport, suggesting that more glutamate is available for both synaptic transmission and glia-to neuron signaling at the time of puberty. Because both enzymes are predominantly expressed in glial cells, the results also indicate that an increased glutamate output of glial origin plays a major role in the control of GnRH release at puberty. The upstream genes controlling the transcriptional activity of the glutamate dehydrogenase and glutamine synthase genes remain to be identified. Transcriptional regulation of vesicular glutamate transporter expression might represent an even more important control point because vesicular glutamate transporters (28, 29) are critical for the homeostatic minute-to-minute control of glutamate release (30).

Much discussion has been centered on the question of the *primus movens* of puberty: is it the loss of a central restraint (7) or the activation of stimulatory inputs to GnRH neurons (2)? The recent finding that mutations of GPR54, the receptor for the KiSS1-derived peptide metastin (31–33), causes hypothalamic hypogonadism (5, 6) suggests that the latter view is correct because GPR54 signaling is coupled to stimulation of GnRH release, instead of inhibition (34, 35). The KiSS1-GPR54 signaling complex is a novel, and unsuspected, system involved in the control of GnRH secretion. Metastin/kisspeptin is a 53-amino acid peptide encoded by the KiSS1 gene (5, 6); proteolytic cleavage of the primary KiSS1 protein product originates the decapeptide kisspeptin-10, which is extraordinarily potent in eliciting LH release (36–38). The comparable effectiveness of intracerebral and systemic administration (36, 39) suggests either a dual hypothalamic-pituitary site of action or a main effect at the median eminence (ME) of the hypothalamus, a region of the brain located outside the blood-brain barrier. Although KiSS1-containing neurons are located in discrete neuronal subsets of the preoptic area (37) and the arcuate nucleus (36, 37), GPR54-con-

taining cells are more diffusely distributed (36, 40), including GnRH neurons (40, 41) and the adenohypophysis (31, 32). This distribution suggests that KiSS1 neurons may not only facilitate GnRH secretion by acting on GnRH neuronal perikarya and GnRH nerve terminals at the ME but also stimulate gonadotropin secretion directly (39) by releasing metastin into the portal system. KiSS1 and GPR54 mRNA abundance increases in the nonhuman primate hypothalamus at the time of puberty, indicating that increased GPR54-mediated signaling contributes to the pubertal activation of GnRH secretion (36) (Fig. 1). The ability of centrally administered kisspeptin to advance puberty in juvenile female rats (38, 39) supports this concept.

GABAergic neurons acting via GABA_A receptors provide a major inhibitory transsynaptic influence controlling GnRH secretion during prepubertal development (42, 43). Although this restraining influence has been unambiguously demonstrated in primates (42, 44, 45), an inhibitory role of GABAergic neurotransmission in rodent puberty is much less clear because both inhibitory and stimulatory effects have been reported (for review, see Ref. 2). Like glutamate, GABA regulates GnRH secretion by binding to receptors located both on GnRH neurons (46, 47) and on their synaptically connected neuronal partners (for review, see Refs. 2 and 4). It appears that the main effect of GABA acting via GABA_A receptors on GnRH neurons is excitation (47), but inhibitory effects have also been reported (46). Like glutamate, GABA production requires the participation of different proteins involved in the synthesis, metabolism, transport, and release of the amino acid. Because no changes in hypothalamic expression of the mRNAs encoding GAD-65 and GAD-67 (the enzymes responsible for GABA synthesis) have been detected during primate sexual development (48), it does not appear that regulation of their gene expression is an event related to the onset of puberty. By analogy to the glutamatergic system, however, it might be inferred that important control points reside at the level of GABA vesicular transport (30, 49) and/or GABA receptors (Fig. 1). Again, the upstream genes involved in the transcriptional control of these components at the time of puberty remain to be identified.

The other major inhibitory transsynaptic input to the GnRH neuronal network is provided by opiateergic neuronal systems (such as preproenkephalin-containing neurons). 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. However, opioid peptides may provide additional homeostatic counterbalance to the cascade of excitatory events leading to the pubertal increase in GnRH output. In rodents, the strength of the prepubertal opioid peptide inhibitory tone (50) diminishes at the time of puberty (51). Opioid peptides as a group do not appear to restrain the initiation of puberty (for review, see Refs. 2 and 52), but it is possible that this inhibitory tone is exerted by a neuronal subset selectively using β -endorphin, dynorphin, or Met/Leu-enkephalin for neurotransmission (Fig. 1).

GnRH neurons and glial cells share an intimate morphological association (53). In the ME, both astroglia (53–55) and modified ependymoglia cells known as tanycytes (55, 56) appose GnRH terminals. Tanycytic end-feet contacting the portal vessels intervene between GnRH nerve endings and

endothelial cells of the portal vessels (55, 56) but retract at the time of the preovulatory surge of gonadotropins allowing the terminals to directly contact the endothelial cells (57). It is now clear that glial cells and GnRH neurons also share a functional relationship. This relationship depends upon growth factors acting via serine threonine kinase receptors, such as TGF β 1, and growth factors signaling through receptors with tyrosine kinase activity, like IGF-I, basic fibroblast growth factor, and the members of the epidermal growth factor family, TGF α , and neuregulins (NRGs) (Fig. 1). We will discuss here only the latter because their role in the control of puberty has been more extensively characterized than the others. A more comprehensive discussion of the roles of IGF-1 and basic fibroblast growth factor in the control of GnRH neurons can be found in (2, 58, 59).

TGF α binds to erbB1 receptors located on astrocytes and tanyocytes, whereas NRGs are recognized by erbB4 receptors expressed only in astrocytes. Both receptors recruit the co-receptor erbB2 for signaling, and in both cases, a major outcome is the release of chemical messengers, such as prostaglandin E₂ (PGE₂), that act directly on GnRH neurons to stimulate GnRH secretion (60–62) (Fig. 1). Pharmacological and genetic approaches have been used to define the involvement of glial erbB1 receptors in the control of female sexual development. Although blockade of erbB1 receptors in the ME (63) or a point mutation of the erbB1 gene (64) result in delayed puberty, sexual development is advanced in transgenic mice conditionally overexpressing the TGF α gene (65) and rats carrying intrahypothalamic grafts of cells genetically engineered to secrete TGF α (66). Ligand-dependent activation of erbB1 receptors in tanyocytes results in plastic changes that, involving PGE₂ and TGF β 1 as downstream effectors, mimic the morphological plasticity displayed by tanyocytes during the hours encompassing the preovulatory surge of GnRH (62). ErbB1 signaling also has been implicated in the etiology of precocious puberty induced by hypothalamic hamartomas (HHs) in humans (67).

In vivo disruption of hypothalamic erbB2 receptor synthesis using antisense oligodeoxynucleotides resulted in delayed puberty (61). Such a delay was also observed in transgenic mice overexpressing, in an astrocyte-specific fashion, a truncated erbB4 protein (DNerbB4) that, lacking the intracellular domain, acts as a dominant negative receptor to block the signaling capability of the intact receptor (68). A combined deficiency achieved by generating DNerbB4 mice carrying a point mutation of the erbB1 receptors accentuated the effect of the single deficiencies (69), indicating that both systems work in a coordinated fashion to facilitate the onset of female puberty.

Are there mechanisms in place able to coordinate the trans-synaptic and glial influences on GnRH neurons? One of these mechanisms, initiated by excitatory amino acids, has been shown to target the astrocytic erbB signaling system for regulation (70). Hypothalamic astrocytes express metabotropic and ionotropic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. Upon concomitant stimulation of both receptor subtypes, astrocytes respond with mobilization of erbB receptors to the cell surface and TGF α /NRG-dependent phosphorylation of these receptors, indicating that glutamate stimulation of astrocytes facilitates the interaction of TGF α /NRG ligands

with their receptors (70). Studies in other cell systems have shown that a surface protein with adhesion and protease activity termed tumor necrosis factor- α -converting enzyme or a disintegrin and metalloproteinase-17 cleaves TGF α and NRGs from their transmembrane precursors, allowing the growth factors to bind their erbB receptors (71, 72). This is precisely what activation of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and metabotropic glutamate receptors does in astrocytes, *i.e.* it enhances tumor necrosis factor- α -converting enzyme-like activity, which in turn elicits TGF α release (73) (Fig. 1).

Second Tier Genes Controlling Cell-Cell Interactions

The late, but still present, initiation of puberty displayed by animals in which expression of certain candidate genes has been reduced (*e.g.* TGF α , glutamate receptors, *etc.*) and the very low incidence in the human population of hypothalamic hypogonadism attributed to a single gene defect (for instance, GPR54) suggest that no isolated pathway or cellular subset is solely responsible for the neuroendocrine control of puberty. Instead, this control is more likely exerted by functionally interconnected regulatory networks. As indicated in the introductory section, a first step in the implementation of a system biology approach is to identify all the genes, proteins, and other molecules constituting the pathway of interest (12). The conventional single gene/single protein approaches referred to in the previous section do obviously contribute to accomplish this first step. However, a thorough investigation of the diverse constituents of the various cellular networks implicated in the process requires the use of global, high-throughput approaches. Using a combination of DNA microarrays, proteomics, guilt by association, and retrospective approaches, we have singled out a group of genes that may represent a novel genetic network involved in the neuroendocrine control of female puberty (74). These genes have diverse cellular functions but share the common feature of having been earlier identified as involved in tumor suppression.

With the help of DNA microarrays, we queried the hypothalamus of female rhesus monkey at different phases of pubertal development and HHs from human subjects in search of candidate gene transcripts. We investigated HHs because these rare, nonneoplastic congenital malformations of the basal hypothalamus are usually associated with sexual precocity (75). To complement this genomic approach, we used quantitative proteomics (25) to identify hypothalamic proteins that might be down- or up-regulated in DNerbB4 mice, which as indicated above have delayed puberty (68). Analysis of the monkey arrays results showed that expression of four tumor-related genes that otherwise participate in normal cell differentiation processes increases selectively in the hypothalamus at the time of monkey puberty (74). The hamartoma arrays identified four additional candidates also implicated in tumor pathology (76). In keeping with these observations, quantitative proteomics using isotope-coded affinity tag labeling revealed that the content of SynCAM, an immunoglobulin-like adhesion molecule required for synapse formation (77), was decreased in the DNerbB4 mice (78). Before its synaptic function was discovered, SynCAM was

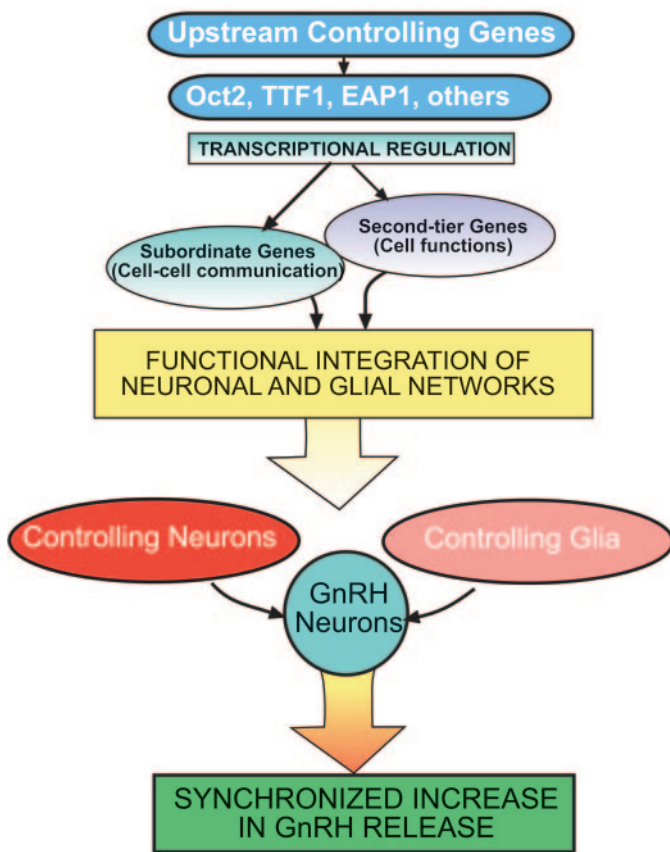


FIG. 3. The transcriptional control of the GnRH neuronal network at puberty by upper echelon genes. Changes in the secretory activity of GnRH neurons are specified by transsynaptic and glial inputs. Physiological modification of these inputs requires a host of subordinate genes (examples shown in Fig. 1) that, differentially expressed in neurons and glia, are necessary for the integration of neuron-to-neuron, glia-neuron, and glia-to-glia communication. In turn, upper echelon genes control expression of these subordinate genes at the transcriptional level. Oct-2, TTF-1, and EAP-1 have been tentatively identified as three of these upstream genes. It is envisioned that this hierarchical arrangement is required to initiate and maintain an enhanced level of pulsatile GnRH secretion at puberty.

amus, such as GnRH and preproenkephalergic neurons and tanycytes of the ME (89). TTF-1 acts on each of these cell types to promote cell-specific functions. For instance, it enhances GnRH and *erbB2* gene transcription but inhibits preproenkephalin promoter activity (89). DNA arrays and quantitative PCR analysis of the female rhesus monkey hypothalamus revealed a pubertal increase in TTF-1 expression. Employing the Cre-loxP system to conditionally delete the TTF-1 gene from those neuronal subsets of the hypothalamus where it is normally expressed, we found that TTF-1 null mutants have delayed puberty, a disruption of initial estrous cyclicity, and decreased reproductive capacity (90). These deficiencies were accompanied by increased preproenkephalin gene expression (90) and by suppressed hypothalamic GnRH and *Kiss1* mRNA levels (Mastronardi, C. A., G. Smiley, T. Kusakabe, A. Kawagushi, S. Heger, R. Cabrera, A. E. Mungenast, S. Kimura, and S. R. Ojeda, unpublished data). Thus, TTF1 enhances the transcriptional activity of genes required for the facilitatory control of puberty

(GnRH, *erbB2*, *Kiss1*) while repressing the transcription of a gene involved in the inhibition of GnRH secretion.

The third candidate was also discovered using cDNA arrays to interrogate the primate hypothalamus at the time of puberty (91). It is a gene earlier known as C14ORF4 (92), but that we have now termed EAP-1 (91). Like TTF-1, EAP-1 maps to human chromosome 14. Hypothalamic EAP-1 mRNA levels increase in both monkeys and rats during female puberty (93), suggesting an involvement in the control of the pubertal process. EAP-1 encodes a nuclear protein, which is expressed in neuronal subsets involved in the stimulatory and inhibitory control of GnRH secretion, such as glutamatergic, GABAergic, proenkephalergic, and *Kiss1* neurons, in addition to GnRH neurons themselves (93). Like TTF-1, EAP-1 transactivates the promoter of genes involved in facilitating the advent of puberty (e.g. GnRH) while suppressing the expression of genes inhibitory to the pubertal process (such as the preproenkephalin gene). Knocking down hypothalamic EAP-1 expression via siRNA technology delayed puberty and disrupted estrous cyclicity, confirming the importance of EAP-1 as an upper echelon gene necessary for the neuron-to neuron regulation of GnRH secretion at puberty (93) (Fig. 3).

Conclusion

These observations suggest that the neuroendocrine control of puberty is provided by a gene network of hierarchical nature similar in principle to those postulated to exist in less complex cellular systems (see Refs. 12, 82, and 83 and references therein). Essential features of such networks are the dominance of a few highly connected upper echelon gene hubs, the partial overlap of second tier gene subnetworks, the large number of less-connected subordinate gene nodes, and the remarkable redundancy of the system (83). Although the overall validity of this concept (summarized in Figs. 2 and 3) remains to be experimentally tested, the existence of a hypothalamic gene network composed of genes situated at different, but interactive, hierarchical levels is consistent with the idea that the onset of puberty is genetically determined and depends on the contribution of more than one gene (94–96). Further supporting this idea is the recent identification of key quantitative trait loci regulating the abundance of thousands of transcripts in the nervous system in a region-specific manner (97). Recent reports have also made clear that, contradicting the current dogma that human central precocious puberty is sporadic in nature, a significant number of cases of this disorder is caused by genetic factors (98). Future studies should make clear whether those genes implicated in the control of puberty in animal models are, in fact, required for the normalcy of human puberty.

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