

Neuroendocrine mechanisms controlling female puberty: new approaches, new concepts

Sergio R. Ojeda, Christian Roth,¹ Alison Mungenast, Sabine Heger,² Claudio Mastronardi, Anne-Simone Parent, Alejandro Lomniczi and Heike Jung³

Division of Neuroscience, Oregon National Primate Research Center/Oregon, Health & Science University, Beaverton, OR, USA

Summary

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Correspondence:

Sergio R. Ojeda, Division of Neuroscience, Oregon National Primate Research Center/Oregon Health & Science University, 505 N.W. 185th Avenue, Beaverton, OR 97006, USA. E-mail: ojedas@ohsu.edu

¹Present address: Department of Pediatrics, University of Bonn, Adenauerallee 119, 53113 Bonn, Germany.

²Present address: Hospital for Children and Adolescents, University of Leipzig, Oststr.21–25, 04317 Leipzig, Germany.

³Present address: Lilly Deutschland GmbH, Niederlassung Bad Homburg, Saalburgstrabe, 611350 Bad Homburg, Germany.

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Sexual development and mature reproductive function are controlled by a handful of neurones that, located in the basal forebrain, produce the decapeptide luteinizing hormone releasing hormone (LHRH). LHRH is released into the portal system that connects the hypothalamus to the pituitary gland and act on the latter to stimulate the synthesis and release of gonadotrophin hormones. The pubertal activation of LHRH release requires coordinated changes in excitatory and inhibitory inputs to LHRH-secreting neurones. These inputs are provided by both transsynaptic and glia-to-neurone communication pathways. Using cellular and molecular approaches, in combination with transgenic animal models and high-throughput procedures for gene discovery, we are gaining new insight into the basic mechanisms underlying this dual control of LHRH secretion and, hence, the initiation of mammalian puberty. Our results suggest that the initiation of puberty requires reciprocal neurone-glia communication involving excitatory amino acids and growth factors, and the coordinated actions of a group of transcriptional regulators that appear to represent a higher level of control governing the pubertal process.

Introduction

During the last few years significant progress has been made towards elucidating the basic cellular and molecular mechanisms underlying the neuroendocrine control of mammalian puberty. The search for the neuronal networks most critically involved in controlling luteinizing hormone releasing hormone (LHRH) release during sexual development has been narrowed down to those systems that utilize excitatory/inhibitory amino acids (reviewed in Ojeda & Terasawa, 2002; Ojeda & Skinner, 2006), and the recently identified neuropeptide metastin/kisspeptin (de Roux *et al.*, 2003; Seminara *et al.*, 2003), for neurotransmission. Another major conclusion derived

from these investigative efforts is that the pubertal activation of LHRH secretion can no longer be considered as an event exclusively driven by purely transsynaptic influences (Ojeda *et al.*, 2000a; Ojeda & Terasawa, 2002). Cell–cell signalling molecules – which are produced in astroglial cells and are able to facilitate LHRH secretion – have been identified, and genetic approaches employed to define the physiological contribution of these molecules to the pubertal process (reviewed in Ojeda *et al.*, 2003). In this article we will mostly discuss studies performed in our laboratory. The interested reader can find much more comprehensive reviews of the subject elsewhere (Terasawa & Fernandez, 2001; Ojeda & Terasawa, 2002; Plant, 2002).

The transsynaptic control of LHRH secretion

It is now clear that the major transsynaptic events involved in the initiation of puberty are an increase in glutamatergic and metastatin stimulation of LHRH neurones (Bourguignon *et al.*, 2000; Ojeda & Terasawa, 2002; de Roux *et al.*, 2003; Seminara *et al.*, 2003; Shahab *et al.*, 2005), and a concomitant decrease in gamma aminobutyric acid (GABA)ergic inhibition (Terasawa, 1999).

Activation of glutamatergic neurotransmission – the primary mode of excitatory transsynaptic communication in the hypothalamus (van den Pol & Trombley, 1993) – increases LHRH secretion (Donoso *et al.*, 1990; Claypool *et al.*, 2000) and accelerates sexual maturation in both rats and monkeys (Plant *et al.*, 1989; Urbanski & Ojeda, 1990). Glutamate stimulates LHRH secretion by acting directly on LHRH neurones and on neurones synaptically connected to the LHRH network (Gore *et al.*, 1996; Eyigor & Jennes, 1997). It is still uncertain if the *primus movens* of puberty is the loss of a ‘central restraint’ (Terasawa & Fernandez, 2001) or the activation of stimulatory inputs to LHRH neurones (Ojeda & Terasawa, 2002). The recent finding that mutations of GPR54, a seven transmembrane G-protein coupled receptor that recognizes the KiSS1-derived peptide metastatin (Kotani *et al.*, 2001; Muir *et al.*, 2001; Ohtaki *et al.*, 2001), causes hypothalamic hypogonadism (de Roux *et al.*, 2003; Seminara *et al.*, 2003) supports the latter view as GPR54 signalling is coupled to stimulation of LHRH release rather than to inhibition (Navarro *et al.*, 2004; Shahab *et al.*, 2005).

GABAergic neurones acting via GABA_A receptors provide the most important inhibitory transsynaptic influence controlling LHRH secretion during prepubertal development (Mitsushima *et al.*, 1994; Mitsushima & Kimura, 1997). This inhibitory mechanism, most elegantly demonstrated in rhesus monkeys (Mitsushima *et al.*, 1994, 1996), appears to play a major role in restraining the initiation of primate puberty, as evidenced by the dramatic advancement of female sexual development induced by the intrahypothalamic blockade of GABA_A receptors (Keen *et al.*, 1999). As in the case of glutamate, GABA modulates LHRH secretion by acting directly on LHRH neurones (DeFazio *et al.*, 2002; Han *et al.*, 2004) and on neurones synaptically connected to the LHRH neuronal network (reviewed in Ojeda & Terasawa, 2002; Ojeda & Skinner, 2006).

The glial control of LHRH neurones

A remarkable feature of hypothalamic morphology is the strong association that exists between LHRH neurones and glial cells (Silverman *et al.*, 1994). Glial processes appose most of the LHRH cell membrane, especially

along the secretory axons terminating in the median eminence (Witkin *et al.*, 1991; King & Letourneau, 1994; Silverman *et al.*, 1994). In this region, LHRH nerve terminals are in intimate contact with both astroglial and modified ependymogial cells (Kozłowski & Coates, 1985; King & Letourneau, 1994).

During the past 5 years, it has become clear that glial cells and LHRH neurones share a relationship much more dynamic than a simple structural togetherness. Receptor tyrosine kinases of the erbB family expressed in glial cells have emerged as key components of communication between these two cell types (reviewed in Ojeda *et al.*, 2000b; Ojeda *et al.*, 2003). Transforming growth factor alpha (TGF α) and neuregulins (NRGs) bind to erbB receptors (erbB-1 and erbB-4 respectively) located on astrocytes eliciting the release of chemical messengers, such as prostaglandin E₂ (PGE₂), that act directly on LHRH neurones to stimulate LHRH secretion (Ma *et al.*, 1997, 1999).

The TGF α –erbB-1 signalling complex

The importance of astrocytic erbB-1 receptors in the control of female sexual development has been demonstrated using various approaches. Either the pharmacological blockade of erbB-1 receptors in the median eminence (Ma *et al.*, 1992), or a point mutation of the erbB-1 gene (Apostolakis *et al.*, 2000) results in delayed puberty. Conversely, puberty is advanced in transgenic mice expressing the human TGF α gene under the control of an inducible promoter (Ma *et al.*, 1994), and rats carrying intrahypothalamic grafts of cells genetically engineered to secrete TGF α (Rage *et al.*, 1997). Because some hypothalamic hamartomas (HH) associated with human sexual precocity have a rich network of astroglial cells containing TGF α and its erbB-1 receptor (Jung *et al.*, 1999), erbB-1 signalling has also been implicated in the aetiology of this form of precocious puberty. It is now clear that erbB-1 receptors not only mediate astrocyte-to-LHRH neurone communication, but also control a critical aspect of tanyctic function (Prevot *et al.*, 2003a). Tanyocytes contain erbB-1 receptors and their ligand-dependent activation results in plastic changes that, involving PGE₂ and transforming growth factor- β ₁ as downstream effectors, mimic the morphological plasticity displayed by tanyocytes during the hours encompassing the preovulatory surge of LHRH (Prevot *et al.*, 2003a).

The NRG–erbB-4 signalling complex

In addition to TGF α , hypothalamic astrocytes produce NRGs, which upon binding to erbB-4 receptors elicit the formation of erbB-4/erbB-2 receptor heterodimers

(Ma *et al.*, 1999). As in the case of erbB-1, the importance of erbB-2 and erbB-4 receptors in the control of female puberty has been defined by using molecular and genetic approaches. For instance, *in vivo* disruption of hypothalamic erbB-2 receptor synthesis via intraventricular infusion of an antisense oligodeoxynucleotide directed against erbB-2 mRNA resulted in a striking delay of female puberty (Ma *et al.*, 1999). Transgenic mice overexpressing, in an astrocyte-specific fashion, a truncated erbB-4 protein (DNerbB-4) had reduced plasma gonadotropin levels and delayed puberty, due to a disrupted ability of hypothalamic astrocytes to produce LHRH secretagogues in response to NRGs. The signalling capability of the intact erbB4 receptor is reduced because the truncated receptor, lacking the intracellular domain, acts in a dominant negative fashion to suppress erbB-4 signalling (Prevot *et al.*, 2003b).

The coordination of neuronal and glial facilitatory inputs to LHRH neurones

It is now clear that the activity of glial cells is influenced by neuronal inputs throughout the nervous system. An example of such neurone-to-glia communication in the neuroendocrine brain can be found in the finding that stimulation of astrocytic glutamatergic receptors activates the astrocytic erbB signalling system (Dziedzic *et al.*, 2003). Hypothalamic astrocytes express metabotropic receptors (mGluRs) and ionotropic alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Combined metabotropic/AMPA receptor activation resulted in phosphorylation of erbB receptors, indicating that glutamate stimulation of astrocytes facilitates the interaction of erbB receptors with their TGF α /NRG ligands. This ligand-dependent activation requires a metalloproteinase activity that promotes the cleavage of both TGF α and NRG mature forms from their respective precursors, making them available for interaction with their receptors (Dziedzic *et al.*, 2003). In the case of TGF α the enzyme involved is a metalloproteinase termed tumour necrosis factor alpha converting enzyme (TACE) or a disintegrin and metalloproteinase-17 (ADAM-17) (Peschon *et al.*, 1998). Concomitant activation of AMPA and metabotropic receptors enhances TACE activity in hypothalamic astrocytes, and TACE activity increases in the median eminence at the time of the first preovulatory surge of gonadotropins (Lomniczi & Ojeda, 2003), suggesting that an enhanced TGF α ectodomain shedding from cells near LHRH nerve terminals is a required component of the neurone–glia signalling process controlling LHRH release. Consistent with this view, blockade of TACE activity

targeted to the median eminence resulted in a striking delay of puberty in female rats (Lomniczi & Ojeda, 2003).

A global view of the changes in protein expression that occur in the hypothalamus of animals with delayed puberty

The finding that a single gene defect results in delayed puberty raises the question as to the proteins that may be affected by the deficiency. Recently, high-throughput approaches have been devised to assess differences in protein expression (Tao & Aebersold, 2003). To identify proteins that might be affected by the disruption of astrocytic erbB-4 signalling, we compared protein expression profiles in the hypothalamus of wild-type mice and mutant DN-erbB4 animals, using the method of isotope-coded affinity tag microcapillary liquid chromatography tandem mass-spectrometry (Gygi *et al.*, 1999). The results of this study show that the content of five proteins was decreased in the hypothalamus of DN-erbB4 mice. Among these five proteins, the levels of SynCAM, an immunoglobulin-like adhesion molecule recently described to play a critical role in homophilic adhesion and synapse formation and function (Biederer *et al.*, 2002), were the most consistently decreased (Mungenast *et al.*, 2003). SynCAM protein expression in the rat brain increases over the first three postnatal weeks (Biederer *et al.*, 2002), the major period of synaptogenesis. The decrease in SynCAM expression that occurs following the loss of astrocytic erbB4 receptor function is exciting because it raises the intriguing possibility that a single astrocytic signalling system involved in glia-to-neurone communication may play a fundamental role in controlling synaptic assembly and synaptic communication in the neuroendocrine brain. If a functional erbB4 receptor is necessary for the expression and/or function of SynCAM, this could provide a link between erbB4 signalling and neuronal excitability during the onset of puberty.

This possibility notwithstanding, additional studies revealed that SynCAM is present not only in LHRH neurones (GT1-7 cell line) but also in hypothalamic astrocytes (Mungenast *et al.*, 2003). SynCAM mRNA expression was reduced in the DN-erbB4 mouse astrocytes compared with wild-type controls. Sequencing of SynCAM-containing polymerase chain reaction (PCR) products revealed that of the six possible alternate splice forms of the SynCAM mRNA, the same isoform is present in both GT1-7 cells and hypothalamic astrocytes. Because SynCAM is a homophilic adhesion molecule (Biederer *et al.*, 2002), the occurrence of the same isoform in these two cells types suggests that SynCAM-

mediated adhesion is an intrinsic component of LHRH neurone–astrocyte communication.

A global view of the hypothalamic changes in gene expression during female puberty in nonhuman primates

Although a number of genes have been implicated in the central process that controls sexual development in higher primates (for review see Ojeda & Terasawa, 2002; Plant, 2002), progress towards identifying the gene networks that, operating within the hypothalamus, are ultimately responsible for setting in motion the pubertal process has been hampered by the lack of global, high-throughput approaches. We have begun to use cDNA arrays to not only identify some of these gene networks, but also to define the existence of region-specific changes in gene expression that occur in the hypothalamus at critical windows of primate sexual development. Puberty in primates is accompanied by profound changes in cortical synaptic connectivity and neuronal morphology (e.g. the adolescent ‘pruning’ of cortical neurones) (Huttenlocher, 1984). Similar plastic changes also occur in the hypothalamus (Perera & Plant, 1997). By comparing gene expression profiles between these two brain regions, we are identifying those changes that are specific to the neuroendocrine brain, those that are common to the hypothalamus and cerebral cortex, and those that are specific to the cerebral cortex, and that consequently, may be related to the establishment of adult cognitive functions, and not to the neuroendocrine control of reproductive development.

Using these two brain regions, we interrogated 8500 genes by comparing the gene expression profiles of juvenile female monkeys to that of animals initiating puberty and monkeys more advanced in the process, but still far from the first ovulation. An important conclusion derived from these experiments is that distinct changes in gene transcription do accompany the initiation of the pubertal process. The existence of such changes suggests that different transcriptional regulators, acting within different neuronal and glial contexts, might establish the conditions required for the productive interaction of neurones and glial cells at puberty.

The identification of transcriptional regulators of neurone–glia communication

Two candidates for such a role were identified by the DNA arrays. One of them is thyroid transcription factor 1 (TTF-1), a homeobox gene; the other is a gene provisionally termed chromosome 14 open reading frame 4

(C14ORF4) (Rampazzo *et al.*, 2000), which we have now termed Enhanced at Puberty (EAP-1) (Heger *et al.*, 2003). Both genes map to regions of chromosome 14 (14q13 and 14q24.3 respectively) implicated by linkage analysis in the control of puberty (Tomkins *et al.*, 1996; Martin *et al.*, 1999; Sutton & Shaffer, 2000).

Thyroid transcription factor-1

After birth, TTF-1 remains expressed in discrete cell populations of the hypothalamus, including LHRH neurones, enkephalinergic neurones of the lateral-ventromedial nucleus and tanycytes of the median eminence (Lee *et al.*, 2001). Additional results showed that TTF-1 controls the transcription of the LHRH, erbB-2 and pro-enkephalin genes by binding to specific recognition motifs in their promoter regions (Lee *et al.*, 2001). In recent studies we used the Cre-loxP system to conditionally delete the TTF-1 gene from those neuronal subsets of the hypothalamus where it is normally expressed. The mutant mice have delayed puberty (evidenced by a delayed age at first ovulation), disruption of initial oestrous cyclicity, and decreased reproductive capacity (assessed by a lower number of pups per litter and premature termination of litter production) (Mastronardi *et al.*, 2004). These results support the hypothesis that TTF-1 is a hypothalamic transcriptional regulator of neuronal networks required for the initiation of female reproductive capacity.

Enhanced at Puberty

Enhanced at Puberty-1 is an intronless gene encoding a protein endowed with a RING finger motif at the C-terminus (Rampazzo *et al.*, 2000). Using quantitative PCR studies we observed that hypothalamic EAP-1 mRNA levels increase at puberty in both monkeys and rats. We also know that the EAP-1 protein localizes to neuronal nuclei and that EAP-1 is able to transregulate the promoter activity of genes involved in the transsynaptic control of LHRH secretion (Heger *et al.*, 2006). These and other observations suggest that EAP-1 is a second upper-echelon gene controlling the expression of subordinate genes that, differentially expressed in neurones, are necessary for the neurone-to-neurone regulation of LHRH secretion at puberty.

Are there hypothalamic gene networks able to provide an integrative level of control for the initiation of mammalian puberty?

To date, attempts to answer this question would have been premature not only because of the scarcity of the

information provided by our current reductionist approaches but, most of all, because of the unavailability of appropriate technology.

To identify such gene networks, we used both DNA microarrays and quantitative proteomics (Gygi *et al.*, 1999). The arrays interrogated two related sources of candidate gene transcripts: (i) the hypothalamus of female rhesus monkey at different phases of pubertal development, and (ii) HH from human subjects. These rare, non-neoplastic congenital malformations of the basal hypothalamus are usually associated with precocious puberty (Grumbach & Styne, 2003). The quantitative proteomics approach searched for hypothalamic proteins that might be downregulated or upregulated in DNerbB-4 mice with delayed puberty (Prevot *et al.*, 2003b). Analysis of the monkey arrays results showed that expression of four genes previously described as being involved in 'tumour suppression', but that otherwise play a role in maintaining normal cell differentiation processes, increases in the hypothalamus at the time of puberty (Roth *et al.*, 2004). This change was specific to the hypothalamus, as it did not occur in the cerebral cortex, a region of the brain not directly involved in neuroendocrine function. The hamartoma arrays identified four additional candidates with purported roles in tumour pathology (Parent *et al.*, 2005). Consistent with these findings, the quantitative proteomics analysis of the hypothalamus from mice with delayed puberty revealed that, as indicated earlier, the content of SynCAM was strikingly decreased in the mutant animals (Mungenast *et al.*, 2003). Before the synaptic function of SynCAM was discovered, SynCAM was studied as 'tumour-suppressor in lung cancer-1' (TSLC1) (Kuramochi *et al.*, 2001). Finally, in a seemingly unrelated development, two groups of investigators independently reported 2 years ago that the initiation of human puberty is critically dependent upon the functional integrity of an entirely new system composed of a 53-amino acid peptide, product of the KiSS1 gene, and its GPR54 receptor (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Before this seminal discovery, the only known function of the KiSS1 gene was as a suppressor of tumour metastases (Ohtaki *et al.*, 2001; Steeg *et al.*, 2003).

Using real-time PCR we validated the array results, and showed that – as predicted by the hypothesis of a neuroendocrine tumour suppressor gene (TSG) network – SynCAM, KiSS1 and GPR54 mRNA expression also increases in the hypothalamus at the time of puberty in primates (Roth *et al.*, 2004; Shahab *et al.*, 2005). Additional experiments resulted in the unexpected, but internally consistent, finding that the changes in TSG mRNA prevalence are accompanied by increased expression of p53, the most important upper-echelon TSG affected in human cancer

(Vousden & Lu, 2002). Further experiments showed that the abundance of DNA methyltransferase 1 (DNMT-1) mRNA declines at the time when TSG gene expression is increasing. Such a change is also internally consistent, because DNMT-1 is the maintenance enzyme for DNA cytosine methylation most critically involved in modulating normal TSG expression and aberrantly silencing it in cancer cells (Robert *et al.*, 2003).

Altogether, these observations raise the exciting hypothesis that SynCAM and KiSS-1 are simply the first two recognized members of a network of genes that, instead of functioning in the neuroendocrine brain as bona fide TSGs, integrate hypothalamic function to bring about the initiation of puberty.

Conclusions

Our results suggest that activation of LHRH secretion at puberty requires the coordinated activation of transsynaptic and astroglial regulatory systems. Key neuronal systems of this process are those that utilize glutamate, metastin and GABA as neurotransmitters. Astroglial cells facilitate LHRH secretion via pathways initiated by growth factors that act directly and indirectly on LHRH neurones to stimulate neurosecretion. One of these pathways is provided by the epidermal growth factor family of growth factors and their erbB receptors. Integrity of this signalling system is not only important for the normalcy of glia–neuronal communication, but it also appears to be required for maintaining adequate levels of expression of at least one protein (SynCAM) involved in synaptic organization. Glutamatergic neurones coordinate the facilitatory transsynaptic and astroglial inputs to LHRH neurones by activating erbB signalling in glial cells. Finally, the overall process of neurone–glia communication that leads to the initiation of puberty appears to be controlled by a hierarchy of genes, some of which were previously described as having tumour suppressor activity. Two of these genes, TTF-1 and EAP-1, appear to be components of an upstream transcriptional regulatory hierarchy controlling the changes in neuronal–glial bidirectional communication underlying the initiation of puberty.

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Discussion

Dr J Högberg (Stockholm, Sweden)

Is the p53 gene active at the onset of puberty?

Dr SR Ojeda (Beaverton, OR, USA)

We have not tested the activity of p53 as a regulator of the onset of puberty but other workers have found that p53 is preferentially expressed in hypothalamic nuclei such as the paraventricular nucleus where p53 appears to participate in stress-mediated responses. We are currently testing siRNAs against p53 to knock down p53 expression in discrete hypothalamic regions in order to determine if the loss of expression affects the time of puberty. The siRNAs can be delivered into specific hypothalamic nuclei using lentiviruses.