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Role of GABA in the Mechanism of the Onset of Puberty in Non-Human Primates

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Abstract

Evidence indicates that GABA is an inhibitory neurotransmitter responsible for restricting luteinizing hormone-releasing hormone (LHRH) release before the onset of puberty. LHRH neurons in the hypothalamus of female rhesus monkeys are already active during the neonatal period, but subsequently enter a dormant state in the juvenile/prepubertal period because of an elevated level of GABA in the stalk-median eminence (S-ME). The developmental reduction in tonic GABA inhibition results in an increase in LHRH release in the S-ME, triggering puberty. The reduction in GABA also appears to allow an increase in glutamate release in the S-ME and this glutamate seems to further contribute to the pubertal increase in LHRH release. These observations conducted in non-human primates, as a model for humans, provide some insights into future studies of the importance of GABAergic mechanisms in the relation between onset of puberty and neurodevelopmental disorders including autism.

Introduction

There are several neurological and psychiatric diseases associated with puberty and changes in GABAergic function may, at least in part, be responsible for underlying etiology. First, recent discoveries by several laboratories indicate that an abnormality of the GABAergic neuronal system may be a cause of autism or responsible for some symptoms of autism (Prosser et al., 1997; Hussman, 2001; Menold et al., 2001; Cohen et al., 2002; ; Nurmi et al., 2003; Muhle et al., 2004). Moreover, in some patients, a worsening of autistic behavior is observed in association with puberty (Gillberg, 1984; Mouridsen et al., 1999). Second, it has been well documented that the onset of schizophrenia occurs between late puberty and early young adulthood (Lewis, 1997, Lewis et al., 2005). A significant decrease in the GABA transporter, GAT-1, immuno-reactivity on axonal terminals of a subset of GABA neurons that innervate pyramidal cells in the frontal cortex is observed in patients with schizophrenia when compared to normal human subjects (Woo et al., 1998). Third, the new onset of epileptic seizures tends to occur early in life and during the adolescent period (Robertson et al., 1990; Appleton and Gibbs, 1998). Precocious puberty is also often associated with epilepsy in children (Lennox and Lennox, 1960; Elian, 1970; Mouridsen et al., 1999; Shenoy and Raja, 2004). Furthermore, treatment with sodium valproic acid, a GABA agonist, delays the timing of puberty in children with seizure disorders (Lundberg et al., 1986; Cook et al, 1992) and in genetically epilepsy-prone mice (Snyder and Badura, 1995). It is possible that the pubertal increase in gonadal steroids may sensitize neurocircuits involved in epileptic seizures, but it is also possible that there is a common mechanism of developmental deficiency, i.e., weakened GABA inhibition in the LHRH neuronal system resulting in precocious puberty and weakened GABAergic inhibition in the brain at the pubertal age resulting in epilepsy (Olsen and Avoli, 1997).

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Bourguignon and colleagues treated an 11-month old child who exhibited severe epileptic seizures and precocious puberty with loreclezole and vigabatrin, GABA agonists. At an earlier stage traditional treatment for epilepsy with phenobarbital was not effective in this patient. However, treatment with loreclezole followed by vigabatrin not only regressed all signs of precocious puberty, but also settled seizure attacks (Bourguignon et al., 1997).

This laboratory has been studying the mechanism of the onset of puberty in the rhesus monkey, as a model for humans. Specifically, results from a series of experiments suggest that the GABAergic neuronal system is, in part, responsible for the timing of puberty in primates (Terasawa, 1995; Terasawa, 2000). Puberty is an important developmental stage during which not only reproductive function is attained (Terasawa and Fernandez, 2001), but also the maturation of the prefrontal cortex, responsible for adolescent behaviors, occurs (Gogtay et al., 2004). Recently, a concept has been proposed that the maturation of the hypothalamus, responsible for puberty, may occur independently from the maturation of the cortices, but there may be common mechanisms governing the maturation of reproductive function and behaviors (Sisk and Foster, 2004). Therefore, a series of observations from this laboratory conducted in non-human primates provide some insights into better understanding the role of GABA function in the possible relation between onset of puberty and clinical changes in autism and other neuropsychiatric disorders.

Developmental changes in luteinizing hormone-releasing hormone release

The decapeptide, luteinizing hormone-releasing hormone (LHRH, also called gonadotropin-releasing hormone or GnRH), is synthesized in the preoptic area and hypothalamus and is released into the pituitary portal circulation in a pulsatile manner at approximately 60 min intervals in mature primates including humans (Hotchkiss and Knobil, 1994). In juveniles the pulse interval of LHRH release is much longer, 90–120 min (Plant, 1994). Acceleration of the pulse frequency accompanied by an increase in the pulse amplitude, hence an increase in total output of LHRH release, triggers the onset of puberty (Watanabe and Terasawa, 1989). It has also been shown that pulsatile administration of LHRH into juvenile monkeys results in precocious puberty (Wildt et al., 1980), and pulsatile administration of LHRH agonist and antagonist analogs has been used for the treatment of precocious and delayed puberty in humans (Crowley et al., 1985).

Although LHRH neurons in the preoptic area and hypothalamus are reasonably mature at birth (Terasawa and Fernandez, 2001) and release the decapeptide in a pulsatile manner shortly after birth, the adult type of secretory pattern with a higher pulse frequency interval (~60 min) is not established until puberty. This is probably due to the immaturity of transsynaptic input regulating LHRH neurons before puberty, either by 1) insufficient excitatory neuronal input to LHRH neurons, or 2) by inhibitory neuronal input to LHRH neurons suppressing activity of LHRH neurons. Electrical stimulation of the medial basal hypothalamus in prepubertal monkeys results in LHRH release of the same magnitude observed in pubertal monkeys (Claypool *et al.*, 1990). However, the immaturity of inhibitory input in control of LHRH release is the predominant mechanism over the immaturity of excitatory input before the onset of puberty, i.e., activity of LHRH neurons during the neonatal period is elevated for the first few months after birth in rhesus monkeys and several months after birth in humans, but is then suppressed by an unknown source of inhibition until shortly before puberty (Plant, 1994), and this inhibition is central in origin, independent from suppression by the ovarian steroid hormone estrogen (Terasawa et al., 1983; Chongthammakun et al., 1993). Thus, we investigated inhibitory neurotransmitters for LHRH release before puberty.

At an early stage we examined the role of beta-endorphin, an inhibitory neuropeptide. However, we excluded it as a candidate for prepubertal inhibition of LHRH release: The release of beta-

endorphin increased concomitant with the pubertal increase in LHRH release (Terasawa and Fernandez, 2001). Subsequently, we examined the role of GABA, a dominant inhibitory neurotransmitter in the hypothalamus, (Decavel and van den Pol, 1990) and have found that GABA is an important neurotransmitter responsible for the timing of puberty.

Developmental changes in GABA release

LHRH neurons release the decapeptide into portal circulation located in the median eminence (ME) and pituitary stalk (S), and appear to be controlled by presynaptic input from GABA neurons. Modulation of LHRH neurons by GABA neurons appears to occur at the cell body and dendrites as well as at the neuroterminals. As the first step to assess the role of GABA in puberty, we measured the simultaneous release of LHRH and GABA in the S-ME. The collection of samples from the S-ME located in the base of the hypothalamus in unanesthetized monkeys is a challenging task, but we have been successful in collecting hypothalamic perfusates for the detection of neurochemical substances using a push-pull perfusion method. As described previously (Terasawa, 1994), a double lumen cannula is inserted into the S-ME with aid of x-ray ventriculograms, and artificial CSF is slowly infused to the area, approximately 1 mm³, through the push cannula while perfusates are continuously collected through the pull cannula using two peristaltic pumps calibrated at identical speeds. Using this method, we are able to measure LHRH, GABA, glutamates, beta-endorphin, neuropeptide Y, prostaglandin E₂, and catecholamines and their metabolites in various physiological conditions (Terasawa, 1994). This method is also useful in examining the effects of neurotransmitter agonists and antagonists on neurotransmitter/neuromodulator release, such as LHRH and/or GABA, by direct application through the push cannula.

Developmental changes in GABA and LHRH levels in the same samples were assessed using this method. Perfusate samples from the S-ME are collected from prepubertal monkeys at 13–21 months of age (before any sign of puberty is apparent), early pubertal monkeys at 22–28 months of age (after some signs of puberty, before menarche) and midpubertal monkeys at 34–46 months of age (after menarche, but before first ovulation). LHRH and GABA levels were measured by radioimmunoassay and HPLC with electrochemical detection, respectively. In prepubertal monkeys LHRH levels are low, whereas GABA levels measured in the same samples are high. LHRH levels significantly increase in early pubertal and midpubertal monkeys, whereas GABA levels are significantly low in both early and midpubertal monkeys (Fig. 1, Terasawa et al., 1999). These observations are similar to those reported previously (Mitsushima et al., 1994). Although we were not able to obtain data from monkeys between the neonatal period and 12 months of age because they were not weaned, a scatter plot of GABA levels during the ages of 13 to 46 months (Fig. 2) indicates that there is a clear developmental GABA decrease. Interestingly, this profile in female rhesus monkeys resembles to that described for circulating GABA levels in normal children (Dhossche et al., 2002).

Evidence for GABA as an inhibitory neurotransmitter before puberty

A question arises as to whether high levels GABA release in the S-ME before puberty have any physiological significance. To answer this question, two experiments were conducted. First, we examined the effect of the GABA_A receptor antagonist, bicuculline, on LHRH release (Mitsushima et al., 1994). Results suggest that bicuculline stimulates LHRH release in prepubertal monkeys by removing endogenous GABA inhibition, whereas exogenous GABA is not effective in suppressing LHRH release until after the onset of puberty, when endogenous GABAergic tone is reduced. The GABA_B receptor blocker, saclofen, was not effective in prepubertal monkeys. Second, we examined whether lowering GABA levels in the S-ME by chronic infusion of bicuculline triggers puberty (Keen et al., 1999). The average ages of menarche and first ovulation in female rhesus monkeys in our colony are approximately 30

and 45 months, respectively (Terasawa et al., 1983). Pulsatile infusion of bicuculline into the third ventricle of prepubertal monkeys results in precocious menarche, which occurs 6–8 weeks after the initiation of bicuculline infusion, and in precocious first ovulation, which occurs by 30 months, the age of menarche in control females (Fig. 3, Keen et al., 1999; Richter and Terasawa, 2001). However, since the interval between menarche and first ovulation is not shortened by bicuculline infusion, additional mechanisms, such as the establishment of the stimulatory neuronal system for pulsatile LHRH release, are necessary for the pubertal transition in female primates. The mean ages of menarche and first ovulation in control monkeys receiving saline infusions are not different from the data in colony controls. The results of these two experiments indicate that tonic GABAergic inhibition in the S-ME is, at least in part, responsible for restraining the activity of LHRH neurons before the onset of puberty and reduction in GABAergic inhibition triggers the pubertal increase in LHRH release resulting in the onset of puberty.

Role of glutamic acid decarboxylase in puberty

In presynaptic neurons GABA is synthesized from glutamate by decarboxylation in the presence of glutamic acid decarboxylase (GAD), stored in vesicles, and released by exocytosis upon depolarization in the presence of extracellular Ca^{2+} (Rando et al., 1981). There are two different proteins, GAD67 and GAD65, derived from respective genes (Martin and Rimvall, 1993; Erlander and Tobin, 1991). To assess the possible involvement of GADs in puberty, we examined whether interference in GAD67 and GAD65 synthesis in prepubertal monkeys modifies the LHRH release pattern (Mitsushima et al., 1996). Infusion of antisense oligodeoxynucleotides for GAD67 and GAD65 mRNAs into the S-ME of prepubertal monkeys results in a dramatic increase in LHRH release (Mitsushima et al., 1996; Kasuya et al., 1999), presumably due to the reduction in GABA synthesis and subsequent GABA release (Mitsushima et al., 1996; Terasawa et al., 1999). Scrambled oligodeoxynucleotides for GAD67 and GAD65 mRNAs as controls did not induce any significant effect. Observations from this experiment indicate that interference in GAD67 and GAD65 synthesis is effective in reducing tonic GABAergic inhibition, resulting in an increase in LHRH release.

Developmental changes in GABA and GAD in primates are not well studied, and there are conflicting data from the postnatal period. GAD activity in the human neocortex sharply increases at birth and continues to increase until 1 year of age, after which it declines gradually until pubertal age and then slightly increases at adulthood (Diebler et al., 1979; Johnston and Coyle, 1981). Urbanski *et al.* 1998 reported that the distribution pattern and concentration of GAD67 and GAD65 mRNAs in hypothalamic nuclei assessed by *in situ* hybridization in gonadally intact juvenile (~0.6 years of age) male rhesus monkeys were not different from those in adult (~10 years of age) male monkeys, and a report by Plant and his colleagues () indicates that GAD mRNA levels in the basal hypothalamus of juvenile castrated male rhesus monkeys did not differ from those in adult castrated male monkeys. Although these data do not appear to support the hypothesis that GAD plays an important role in puberty, more precise developmental studies with the exact regional distribution pattern of GABA neurons in non-human primates, including in females, are needed before conclusions can be drawn.

GAD67 and GAD65 exist in the enzymatically active holo form and inactive apo form, and conversion of holo-GAD67 or holo-GAD65 to and from apo-GAD67 or apo-GAD65 is determined by the presence of the cofactor, pyridoxal-5'-phosphate, which is influenced by physiological states as well as by experimental conditions (Erlander and Tobin, 1991; Kaufman et al., 1991). We do not have any data showing how the ratio of the active and inactive form changes during development.

Other possible factors associated with developmental changes in GABAergic function

GABA transporters

Elevated local concentrations of GABA in the synaptic cleft are actively removed by GABA transporters (GAT), located on presynaptic terminals and surrounding glial cells, where the neurotransmitters are recycled. Four GABA transporters (GAT-1, GAT-2, GAT-3 and BGT-1), classified as the Na⁺ and Cl⁻-coupled transporter family, have been described (Guastella et al., 1990; Nelson et al., 1990). GAT-1 is the predominant GABA transporter in the mammalian brain (Borden, 1996) and the GAT-1 transcript contains an estrogen responsive element (Herbison et al., 1995). Thus, it is possible that the reduction of GABA concentration in the S-ME at the onset of puberty might be due to a developmentally regulated increase in GABA transporter activity. At this time there is no information on the role of GAT activity in puberty.

GABA_A receptors and their subunit composition

The developmental pattern of each GABA_A receptor subunit is complex and differs from region to region or neuron to neuron, which insures the functional heterogeneity of GABA input. Nonetheless, it has been consistently reported that, in general, α_2 subunit expression is very high before birth to shortly after birth, and decreases gradually toward adult levels, whereas α_1 expression is minimal in prenatates and then gradually increases after birth until adulthood (Brooks-Kayal and Pritchett, 1993; Hendrickson et al., 1994; Fritschy et al., 1994; Fritschy and Mohler, 1995; Laurie et al., 1992). In fact, it appears that GABA_A receptors containing α_1 subunits gradually replace GABA_A receptors containing the α_2 subunit during postnatal maturation in the rat, monkey and human brain, and that the increase in the α_1 subunit is an indication of brain maturation, i.e., the onset of synaptic GABA inhibition, whereas β subunits do not generally undergo developmental changes (Brooks-Kayal and Pritchett, 1993; Hendrickson et al., 1994; Laurie et al., 1992). An example of developmental changes in subunit composition in association with function has been shown: Changes in GABA_A receptor subunit composition in hippocampal neurons preceded the onset of epilepsy by weeks in epileptic rats (Brooks-Kayal et al., 1998).

It is, therefore, possible that developmental changes in the GABA_A subunit composition in neuronal cells may occur prior to the onset of puberty. Analysis of the literature provides support for the hypothesis that GABA disinhibition of LHRH neurons through GABA_A receptors at the onset of puberty in female monkeys (Mitsushima et al., 1994) is due to changes in GABA_A receptor subunit composition. For example, a report using single cell RT-PCR (Sim et al., 2000) suggests that the pattern of LHRH neurons expressing GABA subunits in the POA and medial septum of sexually immature mice at neonatal and juvenile ages is more heterogeneous than that in adults, and it becomes homogeneous when the mice mature. Interestingly, the same authors have reported that sensitivity to GABA in LHRH neurons of juvenile mice is lower than that in adult mice and that the pattern of the dose response curve to GABA in prepubertal LHRH neurons is more heterogeneous than that in adult LHRH neurons (Sim et al., 2000). At this time, we have little knowledge of developmental changes in the GABA_A subunit composition of LHRH neurons in non-human primates.

The pubertal reduction in GABAergic inhibition is followed by an increase in glutamatergic tone

Glutamate is profoundly involved in pulsatile LHRH release *in vivo* and *in vitro* through NMDA and kainate receptors. NMDA stimulates release of LH and LHRH in adult rats and monkeys *in vivo* (Price et al., 1978, Olney et al., 1976; ; Brann and Mahesh, 1997; van den Pol

et al., 1994; Bourguignon et al., 1995), and glutamate, NMDA, and kainate all stimulate LHRH/LH release in sexually immature monkeys (Gay and Plant, 1987; Medhamurthy et al., 1990), rats (Bourguignon et al., 1989; Brann and Mahesh, 1992; Cicero et al., 1988), sheep (T'Anson et al., 1993), and fetal sheep (Bettendorf et al., 1999) *in vitro* and *in vivo*. Moreover, stimulation of NMDA receptors results in precocious puberty in rats and monkeys (Plant et al., 1989; Urbanski and Ojeda, 1990), whereas administration of the NMDA receptor blockers, MK-801 or 2-amino-5-phosphonovaleric acid (AP-5), delays the timing of puberty in rats (Meiji-Roelofs et al., 1991; Urbanski and Ojeda, 1990; Wu et al., 1990; MacDonald and Wilkinson, 1990). In contrast, the non-NMDA receptor antagonist, 6,7-dinitroquinoxaline-2,3-dione (DNQX), fails to change the timing of puberty (Brann and Mahesh, 1994). The excitatory action of glutamate on LHRH release may occur not only through NMDA receptors, but also through metabolic receptors. Therefore, the developmental changes in NMDA and kainate receptors are integrated parts of the mechanism of the onset of puberty.

We have measured glutamate release in the S-ME using the push-pull perfusion method (Terasawa et al., 1999). Glutamate levels during the prepubertal period are very low, but increase strikingly during the early pubertal period, and remain high during the midpubertal period, although midpubertal levels decline slightly from early pubertal levels (Fig. 1). However, this observation from monkeys at different ages (cross-sectional study) does not provide the exact timing of glutamate increase during puberty. Fortunately, there is an indication that the pubertal elevation in glutamate release may occur promptly following GABA reduction. The results of the antisense GAD67 infusion experiment in prepubertal monkeys suggest that the reduction in GABA release induced by the antisense GAD67 treatment is followed by an increase in glutamate release for several hours (Terasawa et al., 1999).

Sensitivity to glutamatergic stimulation increases after the onset of puberty. For example, 1) infusion of NMDA into the S-ME at 10 μ M-100 μ M stimulates LHRH release in pubertal monkeys, whereas only 100 μ M NMDA results in LHRH release in prepubertal monkeys (Claypool et al., 2000), and 2) *i.v.* injection of NMDA at 10 mg/kg results in LHRH responses with a longer duration in pubertal monkeys than in prepubertal monkeys (Claypool et al., 2000). Although this increase in the responsiveness of LHRH neurons to NMDA after puberty may, in part, be due to an increase in circulating estrogen, glutamatergic tone is more elevated after the onset of puberty.

Conclusions

The mechanism of the onset of puberty is complex. As we discussed above, LHRH neurons are reasonably mature at birth and are already active during the neonatal period. However, in primates "central inhibition" suppresses pulsatile LHRH release during the juvenile period. Studies from this laboratory suggest that the GABAergic neuronal system appears to be a substrate for "central inhibition" in primates. When approaching puberty, this GABA inhibition is removed or diminished, and an increase in LHRH release occurs. Subsequently, increases in stimulatory input from glutamatergic neurons as well as new stimulatory input from norepinephrine and NPY neurons (which we did not discuss here, see Terasawa and Fernandez, 2001) and inhibitory input from β -endorphin neurons to the LHRH neuronal system become active to establish the adult type of regulatory mechanism for pulsatile LHRH release. This pubertal increase in LHRH release results in a cascade of events during puberty, such as increases in synthesis and release of gonadotropins, and increases in steroidogenesis and gametogenesis, followed by the appearance of secondary sexual characteristics.

The most important question still remains: What determines the timing to remove "GABA inhibition"? Because many genes in the brain are turned on or turned off to establish a complex

series of events occurring during puberty, the timing of spontaneous puberty must be regulated by a master gene or genes, as part of a series of developmental events. We expect that future studies will include a search for genes determining events to remove "GABA inhibition" and genes which ultimately trigger the onset of puberty in primates.

Understanding the mechanism of the onset of puberty in detail is very important, as puberty is associated with onset of or changes in many neurodevelopmental disorders, including autism, schizophrenia, and affective disorder. At this time it is unclear whether the series of events in the hypothalamus with puberty also occur in the higher brain regions where cognitive function is controlled. Future studies to assess whether similar events in the hypothalamus also occur in the higher brain regions that are involved in autism and other disorders will be useful for treatment strategies.

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Figure 1.

Developmental changes in luteinizing hormone-releasing hormone (LHRH, top), GABA (middle), and glutamate (bottom) levels in the stalk-median eminence of the hypothalamus in female monkeys. Samples were obtained using the push-pull perfusion method. Note that GABA release in the stalk-median eminence decreases, whereas glutamate release increases, when the pubertal increase in LHRH release occurs. The ages of prepubertal, early pubertal, and midpubertal monkeys are 13–20 months (before any sign of puberty), 21–30 months (some signs of puberty, but before menarche), and 34–46 months (after menarche, but before first ovulation), respectively. Ages of menarche and first ovulation in our colony females are ~30 months and ~45 months, respectively. *P<0.05 vs. prepubertal; **p<0.01 vs.

prepubertal; ^aP<0.05 vs. early pubertal monkeys. Modified from Terasawa et al. (1999) with permission.

Figure 2.
A scatter plot of GABA release in the stalk-median eminence of female monkeys at ages of 13–46 months. A gradual decline of GABA levels with age is seen.

Figure 3.

Reducing GABA neurotransmission with the GABA_A receptor blocker, bicuculline, advances puberty in female rhesus monkeys. (a) The age of menarche and first ovulation in female rhesus monkeys treated by chronic infusion of bicuculline occur at a significantly earlier age than that in controls. Bicuculline treated group, filled bar; saline-treated control group, open bars. **P<0.01.

(b) Representative examples showing LH concentration in a bicuculline treated (filled circles) monkey and a saline-treated control (open circles) monkey. The dramatically earlier onset of puberty is shown by menarche (M) and first and second ovulations (O). Modified from Keen et al., (1999) and Richter and Terasawa (2001) with permission.