Role of Glutamic Acid Decarboxylase in the Prepubertal Inhibition of the Luteinizing Hormone Releasing Hormone Release in Female Rhesus Monkeys

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To investigate further the role of GABA in the onset of puberty, this study examines whether glutamic acid decarboxylase (GAD), the catalytic enzyme for GABA synthesis, is involved in the suppression of luteinizing hormone releasing hormone (LHRH) before puberty in rhesus monkeys. First, both GAD67 and GAD65 mRNAs were detectable by reverse transcription-PCR analysis in the preoptic area, medio-basal hypothalamus, posterior hypothalamic area, and hippocampus of the monkey brain. Second, effects of antisense oligodeoxynucleotides (D-oligos) for GAD67 and GAD65 mRNAs on LHRH release were examined in conscious female rhesus monkeys at the prepubertal stage using a push-pull perfusion method. The GAD67 or GAD65 antisense D-oligos or scrambled D-oligos were infused directly into the stalk-median eminence. Both the GAD67 and the GAD65 antisense D-oligos induced a large and prompt

increase in LHRH release, whereas the scrambled D-oligos did not induce any significant effect. The results suggest that the removal of GABA inhibition by interfering with GAD synthesis is effective in increasing LHRH release in prepubertal monkeys. Third, the specificity of the antisense D-oligos on GAD levels was examined by incubating basal hypothalami with D-oligos *in vitro* and subsequent Western blot analysis. The antisense D-oligos consistently decreased the proteins GAD67 and GAD65 compared with respective control D-oligos. We conclude that the decrease of tonic GABAergic inhibition and maturational changes in GAD synthesis may be critical factors for the onset of puberty in nonhuman primates.

Key words: antisense oligodeoxynucleotide; GAD67; GAD65; GABA; LHRH; puberty

An increase in pulsatile release of luteinizing hormone releasing hormone (LHRH) is a critical factor for the onset of puberty in primates (Watanabe and Terasawa, 1989; Terasawa et al., 1990). However, the underlying mechanism of the pubertal increase in LHRH release is unclear. It has been speculated that in primates there is a "central inhibition" of LH release and presumably LHRH release, starting a few months after the neonatal period and ending before the onset of puberty (Terasawa et al., 1983; Plant, 1988; Grumbach and Kaplan, 1990). LH and LHRH levels are low in juvenile monkeys, in which the negative feedback influence of gonadal steroids was removed during the neonatal period (Plant, 1983; Terasawa et al., 1983; Chongthammakun et al., 1993). Because LHRH neurons themselves appear to be mature well before the onset of puberty (Ronnekleiv and Resko, 1980; Silverman et al., 1982; Goldsmith and Song, 1987; Claypool et al., 1990; Vician et al., 1991), immaturity of the regulatory mechanisms

for the LHRH neurosecretory system must be responsible for the low levels of LHRH release before the onset of puberty (Terasawa et al., 1990; Terasawa, 1995).

GABA is known as a dominant inhibitory neurotransmitter in the brain. Recently, we have proposed a hypothesis that GABA is an inhibitory neurotransmitter restricting LHRH release before the onset of puberty and that the removal of this inhibition is important for the pubertal increase in LHRH release (Mitsushima et al., 1994). This hypothesis was based on the following facts. (1) Direct infusion of the GABA_A receptor blocker bicuculline into the stalk-median eminence (S-ME) dramatically stimulated LHRH release in prepubertal monkeys, whereas bicuculline stimulated LHRH release very little in pubertal monkeys. (2) Infusion of GABA into the S-ME of pubertal monkeys suppressed LHRH release, whereas it failed to cause any change in prepubertal monkeys. (3) GABA release in the S-ME in prepubertal monkeys was much higher than that in pubertal monkeys (Mitsushima et al., 1994).

Glutamic acid decarboxylase (GAD) is the enzyme catalyzing the synthesis of GABA from glutamate. Two subclasses of GAD have been described in the mammalian brain (Michelsen et al., 1991; Bu et al., 1992). The cDNAs encoding for human GAD67 and GAD65 have been cloned (Karlsen et al., 1991; Bu et al., 1992), and RNA blot hybridization with cDNA probes for human GADs has revealed that the brain of the rhesus monkey expresses both GAD mRNAs (Karlsen et al., 1991). It is plausible, therefore, that if synthesis of GADs is decreased by treatment with GAD-specific antisense oligodeoxynucleotides (D-oligos), it may

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alter the synthesis of GABA and, subsequently, LHRH release. In the present study, to investigate whether GADs are involved in the prepubertal suppression of LHRH release and to determine further the physiological role of GABA in the control of the onset of puberty, we have examined the effect of antisense *d*-oligos for GAD67 and GAD65 mRNAs on LHRH release in prepubertal monkeys.

MATERIALS AND METHODS

Animals

Ten prepubertal female rhesus monkeys (*Macaca mulatta*) ranging from 13 to 20 months of age were used in push-pull perfusion studies. The monkeys were weaned at 10–11 months of age and housed in rooms with controlled lighting of 12 hr light (lights on 6:00 A.M.) and 12 hr darkness and a controlled temperature (22°C). Monkeys were fed a standard diet of Purina monkey chow daily, supplemented with fresh fruits several times per week. Tap water was available *ad libitum*.

Implantation of cranial pedestal

Monkeys were implanted with a cranial pedestal under halothane anesthesia as described previously (Gearing and Terasawa, 1988; Terasawa, 1994). During the surgery, a radio-opaque dye was injected into the foramen of Monro to visualize the third ventricle, and x-ray pictures were taken to allow accurate positioning of the cannula in the S-ME in later experiments. Animals were allowed to recover at least 1 month before the initiation of experiments. During this period, monkeys were well adapted to the primate chair, the experimental environment, and investigator before the initiation of the experiments.

Push-pull perfusion

Two to three days before a push-pull perfusion experiment, a monkey under ketamine/xylazine anesthesia was placed in a stereotaxic apparatus for cannula implantation. An outer cannula with stylet was inserted into the S-ME using a microdrive unit (MO95-B, Narishige, Tokyo, Japan), and cannula placement was compared to ventriculograms taken during pedestal implantation as described previously (Gearing and Terasawa, 1988; Terasawa, 1994). The monkeys were placed in a primate chair. On the day of the experiment, the stylet was replaced with an inner cannula (29 gauge) through which a modified Krebs–Ringer phosphate buffer solution was infused at 23.5 μ l/min via a peristaltic pump (Minipulse3, Gilson Medical Electric, Middleton, WI). Perfusates were collected continuously in 10 min fractions on ice from the outer cannula using an identically calibrated pump. Volumes of 150 and $\sim$ 85 μ l were aliquoted for radioimmunoassay (RIA) of LHRH and later HPLC analysis of GABA, respectively. Samples were snap-frozen and stored at -70°C until assay.

Experimental design

Experiment 1. To determine whether the rhesus monkey brain contains GAD67 and GAD65 mRNAs, RNA from the preoptic area, medio-basal hypothalamus with median eminence, posterior hypothalamic area, and hippocampus was isolated from two adult rhesus monkey brains. The brain tissues of the rhesus monkeys were obtained through the Tissue Distribution Program of the Wisconsin Regional Primate Research Center. For negative controls, RNA from the liver of a monkey fetus and the placenta of a female monkey at day 73 of gestation was also obtained. RNA samples were analyzed for GAD67 and GAD65 mRNAs by reverse transcription (RT)-PCR. For reverse transcriptases, 10 μ g of total RNA was denatured in water at 70°C for 10 min, added to a 20 μ l (final volume) reaction containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 1 U of Inhibit-ace ribonuclease inhibitor (5 Prime-3 Prime, Boulder, CO), 100 ng of an oligo(dT) primer (GACTCGAGTCGACATCGAT₁₇), and 0.125 mM each deoxynucleotide triphosphate and 100 U of Superscript reverse transcriptase (Gibco, Gaithersburg, MD), and incubated at 37°C for 1 hr. One to twenty microliters of reverse transcriptase of the reaction was then subjected to PCR. The PCR primers for GAD67 and GAD65 mRNAs were based on sequences common to human and rat mRNAs and amplified a 211 bp cDNA and a 382 bp cDNA for GAD67 and GAD65, respectively. The primers for glyceraldehyde 3-phosphate dehydrogenase (G3PDH) amplified a 980 bp cDNA as described previously (Golos et al., 1993). The PCR primers were synthesized by the University

of Wisconsin Biotechnology Center. The aliquot of the RT reaction was added to 12.5 pmol each of GAD or G3PDH primer, 0.25 mM *d*-NTPs (Pharmacia, Piscataway, NJ), and 1 $\times$ AmpliTaq buffer in which final reaction concentrations were 10 mM Tris, pH-adjusted to 8.3 with 50 mM KCl, 1.5 mM MgCl₂, 0.001% (w/v) gelatin, 15% glycerol (10% for G3PDH), and 1.25 U of AmpliTaq (Perkin-Elmer, Oak Brook, IL) in a

Figure 1. RT-PCR analysis of rhesus monkey brain mRNAs for GAD67 (A) and GAD65 (B). Two micrograms of total RNA from each brain sample shown, as well as fetal liver and midpregnancy placenta (negative controls for GAD mRNAs), were reverse-transcribed, and 4.5 μ l of RT reactions was subjected to PCR amplification with the indicated primers. Amplification products (25 μ l for GAD67, 40 μ l for GAD65) were fractionated through 3.0% agarose gels. DNA size markers are indicated at the left, and tissue samples are labeled across the bottom. 1, Medio-basal hypothalamus; 2, 3, posterior hypothalamus; 4, 5, preoptic area; 6–9, hippocampus; 10, fetal liver; 11, placenta. The same alphabet letter indicates that tissues were obtained from the same monkey. Amplification for G3PDH (C) is shown to verify RNA integrity. Because the amount of PCR product in the initial GAD65 amplification was quite low, B presents amplification product from a second round of PCR, using 0.1 μ l of the first-round reaction as template.

total volume of 50 μ l. The first cycle consisted of 1 min at 94°C, 30 sec at 55°C, and 1 min at 72°C. In subsequent cycles, the 94°C step was held for 30 sec. A total of 35 cycles was run. Samples were held at 72°C for 7 min after completion of the final amplification cycle. After amplification, 25 μ l aliquots of the PCR reactions were fractionated in 3% Nusieve (FMC Bioproducts, Rockland, ME) agarose gels (1 $\times$ TBE running buffer), and DNA bands were visualized by ethidium bromide staining. Because the amount of PCR product for GAD65 was consistently lower than that of GAD67 in the first PCR reaction, a second round of 35 PCR cycles was carried out for GAD65 with 0.1 μ l of the first PCR reaction as template, and 40 μ l of the second reaction was loaded for visualization.

Experiments 2 and 3. To examine whether GADs are involved in the suppression of LHRH release before the onset of puberty, the effects of antisense D-oligos for GAD67 mRNA or for GAD65 mRNA on LHRH release were examined in conscious prepubertal female monkeys using a push-pull perfusion method. For antisense D-oligos, 20-mer oligodeoxynucleotides that corresponded to the GAD enzyme N terminus immediately downstream from the initiation codon were used. For controls, oligonucleotides with scrambled sequences of the respective antisense D-oligos were used.

The sequences of the oligonucleotides were as follows:

GAD67 antisense D-oligo: 5'-GAAGATGGGGTCTGAAGACGC-3'
 GAD67 scrambled D-oligo: 5'-TAGGAGCAGACTGAGAGGCG-3'
 GAD65 antisense D-oligo: 5'-AAGCCAGAGCCCGGAGATGC-3'
 GAD65 scrambled D-oligo: 5'-CGGCACGTCAACGGAAGAGC-3'

After a 3 hr control perfusion, an antisense or scrambled D-oligo for either GAD67 mRNA or GAD65 mRNA at 10^{-6} M in a modified Krebs–Ringer phosphate buffer was continuously infused for 6 hr. Perfusion was continued for an additional 5–6 hr. Of 10 monkeys, 4 were exclusively used for experiment 2 (GAD67), 3 were exclusively for experiment 3 (GAD65), and the remaining 3 were for both studies. Between perfusion experiments, the monkey was rested in her home cage for at least 1 month.

Experiment 4. Specificity of the antisense D-oligos for GAD67 and GAD65 mRNAs was examined by exposing brain tissues to the D-oligos for either GAD67 mRNA or GAD65 mRNA *in vitro* and subsequent Western blot analysis for respective enzyme proteins. The cerebellum and the hypothalamus of the rhesus monkeys were obtained through the Tissue Distribution Program of the Wisconsin Regional Primate Research Center. The basal hypothalamus was dissected out on ice caudal to the optic chiasm anteriorly, posterior to the mamillary body caudally, at the infundibular sulcus laterally, and 3 mm above the base of the brain dorsally, and was bisected further at the midline. The cerebellar cortex from the superior hemisphere of both sides was dissected out and cut into four small pieces of similar size on ice. Each tissue was then placed in a small perfusion chamber and perfused with medium consisting of 50% artificial CSF (Gearing and Terasawa, 1988) and 50% M199 (Gibco) under 95% O₂/5% CO₂ at 37°C at a rate of 6 μ l/min using a perfusion system (Endotronics, Minneapolis, MN).

After 1 hr of control perfusion, hypothalamic tissues were continuously exposed to either the antisense D-oligos or the scrambled D-oligos at a concentration of 10^{-6} M for a 6 hr period. Similarly, three cerebellar tissues were each exposed to antisense D-oligos, scrambled D-oligos, or sense D-oligos. The remaining cerebellar tissue was not exposed to any D-oligos as a control. After completion of the perfusion, the tissues were removed from the chambers, quickly weighed, and homogenized in buffer at a concentration of 10 mg/ μ l tissue weight. Homogenates were then centrifuged (15,000 $\times$ g) at 0°C and supernatants were obtained, aliquoted, and stored at –70°C until Western blot analysis.

Samples adjusted to contain the same concentration of protein were denatured at 95°C in a loading buffer containing SDS (final concentration 2%) and β -mercaptoethanol (final concentration 2%) and subjected to SDS-PAGE (10%). Fractionated proteins were then electroblotted (Hoefer Scientific Instruments) onto polyvinylidene fluoride membrane (Millipore, Bedford, MA).

A monoclonal antibody for GAD65 immunodetection was obtained from the Hybridoma Bank (Iowa City, IA), and a polyclonal antibody for

RHESUS GAD67 cDNA

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578  GCAACCTCCTCGAACGCGGGAGCGGACCCCAATACCACTAACCCTGCGCCCCACAACGTACGATACCTGGTGC GCGCTGGCCCATGGATGCACCAGA
    A T S S N A G A D P N T T N L R P T T Y D T W C G V A H G C T R

674  AAAGTGGGGCTCAAGATCTGCGGCTTCTTGACAGAGGACCAACAGCCTGGAAGAGAAGAGTCGCCTTGTGAGTGCCTTCAAGGAGAGGCAGTCTCTCC
    K L G L K I C G F L Q R T N S L E E K S R L V S A F K E R Q S S
                                A                                G A human
                                                                (R)

770  AAGAACCCTGCTTTCCTGTGA
    K N L L S C
  
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RHESUS GAD65 cDNA

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146  CATCTCCGGGCTCTGGCTTTTGGTCTTCGGGTGCGAAATGGCTCCGGGGATTCCGACAAATCCCGGCACAGCGCGAGCCTGGTGCCAAGTGGCTC
    S P G S G F W S F G S E N G S G D S D N P G T A R A W C Q V A Q
                                T                                T                                G human
                                                                (D) (E)

242  AGAAGTTCACGGGCGGCATCGGAACAACTGTGCGCCCTGCTCTACGGAGACGCCGAGAAGCCGGCGGAGAGCGGGAGCCAAACCCCGCGGG
    K F T G G I G N K L C A L L Y G D A E K P A E S G G S Q P P R V
                                                                (A)

338  TCGCCGCCCGGAAGGCCGCTGCGCCTGCGACCAGAAGCCCTGCAGCTGTCCCAAGCGGATGTCAACTACGGGTTTCTCCATGCAACAGACCTGC
    A A R K A A C A C D Q K P C S C P K A D V N Y G F L H A T D L L human
    C                                CT T C                                (S) (V) (A)

434  TGCCGCGGTGTGATGGAGAAAGGCCCACTTTGGCGTTTCTGCAAGATGTTATGAACATTTTACTTCAGTATGTGGTGAAGTTTCGATAGATC
    P A C D G E R P T L A F L Q D V M N I L L Q Y V V K S F D R
  
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Figure 2. Partial nucleotide and predicted protein sequences of GAD67 and GAD65 in rhesus monkeys. The 211 bp cDNA from GAD67 and 382 and 232 bp cDNAs from GAD65 PCR reactions were isolated from agarose gels, subcloned, and sequenced. The sequences of the cDNAs appear *above* the predicted amino acid sequences, and are numbered according to the full-length human mRNAs. The nucleotide sequences and deduced amino acid sequences of the cloned rhesus monkey fragments appear in full. Nucleotides of the human mRNAs that differ from those of the rhesus monkey are shown *below* the rhesus protein sequences, and amino acids that differ from the rhesus protein are shown in *parentheses*. The boxed region in the GAD65 clone represents sequences not present in the 232 bp PCR amplification product, which results in an in-frame deletion of 50 amino acids.

GAD67 immunodetection was obtained from Biogenesis (Sandown, NH). Antibodies were used at dilutions of 1:160 and 1:8000, respectively. For the second antibodies, goat anti-mouse and goat anti-rabbit, respectively, conjugated with alkaline phosphatase were used. Finally, the substrate 5-bromo-4-chloro-3-indolyl phosphate and chromagen *p*-nitroblue tetrazolium chloride were applied to the membrane.

Assays

LHRH in perfusates was measured by RIA using antiserum R1245, kindly provided by Dr. T. Nett (Rasmussen et al., 1988), as described previously (Gearing and Terasawa, 1988; Watanabe and Terasawa, 1989). Synthetic LHRH (Richelieu Laboratory, Montréal, Québec, Canada) was used for the radiolabeled antigen and reference standard. Sensitivity of the assay was 0.1 pg/tube. In the present report, the data on the release of GABA are not included because the assay has not been completed.

Statistics

To determine the effects of infusion of D-oligos on LHRH release, mean LHRH levels during each hour before the D-oligos infusion were compared with mean LHRH levels during each hour after the infusion using two-way ANOVA with repeated measures in which variables were D-oligos and time. To determine the specific time of the effects of D-oligo on LHRH release, data were analyzed further by Student-Newman-

Keuls multiple-range test. Significance was attained at $p < 0.05$. For graphic expression, normalized data are used: in each animal, the mean LHRH level in the 3 hr before the D-oligo infusion was designated as 100%, and the mean LHRH levels in each hour before and after the initiation of D-oligos were calculated relative to the preinfusion mean.

All experiments presented in this manuscript were performed following the standards established by the Animal Welfare Act and Principles for Use of Animals and Guide for the Care and Use of Laboratory Animals. The protocol for this study was reviewed and approved by the Research Animal Resource Center, University of Wisconsin.

RESULTS

GAD mRNAs in the monkey brain (experiment 1)

Samples of total RNA isolated from various brain regions, liver, and placenta were subjected to RT-PCR analysis for GAD67 and GAD65 mRNAs. Figure 1*A* illustrates that a single band of 211 bp for GAD67 was present in all brain samples, but not in the liver or placenta. Figure 1*B* demonstrates specific amplification from the same RT reactions of the expected cDNA of 382 bp for GAD65, and an additional band of ~232 bp. All bands were subcloned and sequenced, and the 211 and

Figure 3. Effects of an antisense oligodeoxynucleotide for the GAD67 mRNA (*GAD67AS*) on LHRH release in the S-ME of two conscious prepubertal female rhesus monkeys. Note that the LHRH release started to increase 3–4 hr after the initiation of *GAD67AS* infusion, and the increase returned to control levels 1–2 hr after the termination of infusion.

382 bp bands represented the expected cDNAs for GAD67 and GAD65, respectively. The relative amounts of GAD65 amplification product were consistently lower than those of GAD67 in all brain samples (data not shown). Figure 1C shows abundant amplification product from all samples for the control mRNA G3PDH; no bands were obtained in the absence of reverse transcriptase or input RNA (data not shown).

Two bands were seen in PCR amplifications with primers specific for the GAD65 mRNA (Fig. 1B). When both fragments were subcloned and sequenced, they were found to represent authentic GAD65 cDNAs, but the smaller fragment of 232 bp had a deletion of 150 bp (Fig. 2), which would result in a predicted protein lacking amino acids 46–95 of the human protein.

Effects of an antisense D-oligo for GAD67 mRNA on LHRH release (experiment 2)

Before the D-oligo infusion, LHRH levels in prepubertal monkeys were very low, as were typical at this stage (Figs. 3, 4). Infusion of antisense D-oligo to GAD67 (10^{-6} M) into the S-ME resulted in a

drastic increase in LHRH release starting 3–4 hr after the initiation of the infusion. Commonly, this increase lasted for ~1–2 hr after the cessation of the antisense infusion (Fig. 3A), but in some cases a residual effect was observed several hours after the cessation of the infusion (Fig. 3B). In contrast, scrambled D-oligo to GAD67 infusion did not cause any significant effects on LHRH release (Fig. 4). Statistical analysis indicated that antisense D-oligo to GAD67 (10^{-6} M) induced a significant effect on LHRH release ($p < 0.001$). Post hoc analysis further revealed that a significant increase in LHRH release occurred from the third hour of initiation of antisense D-oligo to GAD67 infusion until 1 hr after the cessation of the infusion compared both with preinfusion levels and with levels of the corresponding periods after infusion of scrambled D-oligo to GAD67 (Fig. 5).

Effects of an antisense D-oligo for GAD65 mRNA on LHRH release (experiment 3)

Infusion of antisense D-oligo to GAD65 (10^{-6} M) also induced an increase in LHRH release (Fig. 6), whereas infusion of scrambled

Figure 4. Effects of a scrambled oligodeoxynucleotide for GAD67 mRNA (GAD67SC) on LHRH release in the S-ME of two conscious prepubertal female rhesus monkeys. Case *B* is the control from the same monkey shown in Figure 3. Note that no changes were observed throughout the experiment in these as well as other cases not shown.

Figure 5. Effects of GAD67AS (*A*) or GAD67SC (*B*) on mean LHRH release in prepubertal monkeys. Mean LHRH release was calculated for each 60 min period. Data were analyzed using ANOVA with repeated measures followed by Student–Newman–Keuls multiple-range test (mean $\pm$ SEM, $n = 6$ in GAD67AS, $n = 4$ in GAD67SC; ** $p < 0.01$; * $p < 0.05$). Overall significance between two treatments was $p < 0.001$.

D-oligo to GAD65 did not cause any significant effects (Fig. 7). ANOVA indicated that the effect of antisense D-oligo to GAD65 was significant from that of scrambled D-oligo ($p < 0.01$). Post hoc analysis revealed that the increase in LHRH release from hours 2, 3, and 5 of the antisense D-oligo to GAD65 infusion was significantly greater than preinfusion levels or levels of the corresponding periods after infusion of scrambled D-oligo to GAD65 (Fig. 8), and a significant increase was also observed from the 1st to 6th hour after the cessation of the GAD65 antisense infusion compared with preinfusion levels.

Interestingly, the maximum increase during the exposure to antisense GAD67 D-oligo (Fig. 3) was larger than that during antisense GAD65 D-oligo infusion (Fig. 6).

Effects of antisense D-oligos on behaviors

Because GABA is involved in various physiological functions, we have made careful observations on behavioral changes during and after infusion of D-oligos. Although infusion of antisense D-oligo to GAD67 appeared to increase food intake very slightly, a quantitative analysis was not conducted. No other typical changes in behavior were observed with antisense D-oligo to GAD67. The infusion of antisense D-oligo to GAD65 induced epileptic seizure in one of the four monkeys, and another one had some symptoms of epilepsy several hours after the termination of sample collection, when the animals were already returned to their home cages. The remaining two monkeys did not exhibit any significant behavioral changes.

Specificity of the antisense D-oligos for GAD67 and GAD65 mRNAs (experiment 4)

The expression of GAD67 in the hypothalamic sample exposed to the antisense D-oligo for GAD67 mRNA was modestly but consistently lower than that in the sample exposed to the scrambled D-oligo (Fig. 9). Similarly, the expression of GAD67 in the cerebellar sample exposed to the antisense D-oligo was lower than that in the samples exposed to the scrambled D-oligo for GAD67 mRNA, the sense D-oligo for GAD67 mRNA or the control (Fig. 9). Very similar results were seen for GAD65 expression in both tissues, i.e., expression of GAD65 in tissues exposed to the antisense D-oligo for GAD65 mRNA was less than that in tissues exposed to the scrambled or sense D-oligos for GAD65 mRNA (Fig. 9).

DISCUSSION

The findings of the present study are summarized as follows. First, we have found that both GAD67 and GAD65 mRNAs and their proteins are present in the rhesus monkey brain. Second, direct infusion of the GAD67 antisense into the S-ME dramatically stimulated the release of LHRH, whereas the GAD67 scrambled did not. Third, the GAD65 antisense also enhanced the release of LHRH, whereas the GAD65 scrambled did not. Finally, the antisense D-oligos specifically decreased expression of the proteins GAD67 and GAD65.

Human cDNAs for both forms of GADs have been isolated and sequenced from the brain (Karlsen et al., 1991; Bu et al., 1992).

Figure 6. Effects of an antisense oligodeoxynucleotide for GAD65 mRNA (*GAD65AS*) on LHRH release in the S-ME of two conscious prepubertal female rhesus monkeys. Note that LHRH started to increase 2–3 hr after the initiation of *GAD65AS* infusion, and the increase lasted for several hours after the termination of infusion. However, the increased levels of LHRH were not as large as those observed with *GAD67AS*.

The presence of both forms of GAD mRNAs in the monkey brain was also reported using Northern blot analysis (Karlsen et al., 1991) and *in situ* hybridization (Benson et al., 1991). Our RT-PCR results are consistent with those findings. Moreover, the fact that the *GAD65* mRNA in the hypothalamus and hippocampus is less abundant than *GAD67* mRNA suggests that *GAD67* may be a dominant enzyme for GABA synthesis in these areas of the monkey brain.

In the present study, although the 382 bp fragment represented the previously cloned *GAD65* cDNA, the 232 bp fragment had a novel deletion of 150 bp, which would result in a predicted protein lacking amino acids 46–95 (see Fig. 2). To our knowledge, the gene coding for the *GAD65* mRNA has not been sequenced in any species; thus, it is unclear whether the bands represent alternatively spliced mRNAs or products of separate genes. However, the fact that all brain tissues obtained from two monkeys expressed both fragments suggests that this may be characteristic of the *GAD65* mRNA popula-

tion in the brain. The functional significance of these results remains to be determined.

It has been speculated that both unmodified (β -) and phosphorothioate-substituted oligonucleotides enter the cells via a receptor-mediated endocytosis with mechanisms yet to be identified, or by passive diffusion (Loke et al., 1989; Kregenow et al., 1995). Moreover, the uptake of β -oligo is relatively quicker than that of phosphorothioate-substituted oligo (Woolf et al., 1992; Legrand et al., 1993). Antisense β -oligos can interfere with protein synthesis by mechanisms such as arresting mRNA hybridization mechanically (Helene and Toulme, 1990; Kregenow et al., 1995) or the cleavage of mRNA with ribonuclease H (Walder and Walder, 1988; Woolf et al., 1992). The observation that the LHRH increase occurs consistently 2–3 hr after the initiation of the antisense infusion suggests that the uptake of the antisense oligonucleotide and inhibition of GAD protein synthesis occurs in a relatively short time course. However, it is unclear whether the prolonged effects of the antisense *GAD65* on LHRH release are

Figure 7. Effects of scrambled oligodeoxynucleotide for GAD65 mRNA (*GAD65SC*) on LHRH release in the S-ME of two conscious prepubertal female rhesus monkeys. Both cases are controls for experiments shown in Figure 6. Note that no apparent changes were observed throughout the experiment.

related to the longer half-life (20–30 hr) of GAD65 than that of GAD67 (<4 hr) (Christgau et al., 1991), to a difference in subcellular distribution of the two enzymes (Esclapez et al., 1994), or to a difference in GAD65 and GAD67 syntheses (Martin and Rimvall, 1993). It is possible that intracellular GAD67 levels may recover quickly after the cessation of antisense GAD67 infusion, whereas GAD65 levels may stay low after the antisense GAD65 because, unlike GAD65 protein levels, GAD67 protein levels are sensitive to small increases in intracellular GABA levels (Rimvall and Martin, 1994).

Incubation of hypothalamic tissue and cerebellar cortex with antisense D-oligo decreased expression of both GAD67 and GAD65 (Fig. 9) compared with those incubated with scrambled or sense D-oligos. These findings, together with preliminary data showing a reduction in *in vivo* release of GABA in the S-ME by antisense D-oligos (E. Terasawa, unpublished data), suggest that the antisense D-oligos for GAD mRNAs infused into the S-ME were specific in their interference with GAD synthesis. The inhi-

bition of protein synthesis by antisense oligonucleotides has been reported for LH receptors (West and Cooke, 1991), neuropeptide Y (NPY) receptors (Wahlstedt et al., 1993), estrogen and progesterone receptors (McCarthy et al., 1993; Ogawa et al., 1994), GADs (McCarthy et al., 1994), and potassium channels (Chung et al., 1995).

The hypothesis for a central inhibition to LHRH release before the onset of puberty has been proposed for some time. Gonadotropin levels of children with agonadal syndrome are very low, and gonadotropins do not increase until the age of puberty (Winter and Faiman, 1972; Conte et al., 1975). The release of LHRH and gonadotropins is not altered by gonadectomy until the onset of puberty in monkeys (Plant, 1983; Terasawa et al., 1983; Wilson et al., 1986; Chongthammakun et al., 1993). Precocious puberty has been observed in children with brain tumors and brain injury (Bauer, 1959; Byrnes et al., 1974) as well as in female rhesus monkeys with lesions in the posterior portion of the hypothalamus (Terasawa et al., 1984;

Figure 8. Effects of GAD65AS (A) or GAD65SC (B) on mean LHRH release in prepubertal monkeys. Mean LHRH release was calculated for each 60 min period. Data were analyzed using ANOVA with repeated measures followed by Student–Newman–Keuls multiple-range test (mean $\pm$ SEM, $n = 4$ in GAD65AS, $n = 6$ in GAD65SC; * $p < 0.05$). Overall significance between the treatments was $p < 0.01$.

Schultz and Terasawa, 1988). Nevertheless, little evidence is available for the neural substrate responsible for the central inhibition before the onset of puberty.

Previously, we reported that a decrease in GABA release and a concomitant increase in LHRH release occurred in the S-ME of prepubertal monkeys (Mitsushima et al., 1994), and the infusion of bicuculline into the S-ME in prepubertal monkeys stimulated LHRH release drastically, whereas bicuculline infusion in pubertal monkeys stimulated LHRH release very little (Mitsushima et al., 1994). Based on these observations, we proposed a hypothesis that GABA is a neurotransmitter restricting LHRH release before the onset of puberty. The present study further examines this hypothesis by interfering

with GAD synthesis using the antisense D-oligos for GAD mRNAs. The treatment with both antisense D-oligos for GAD67 and GAD65 mRNAs resulted in an increase in LHRH release in prepubertal monkeys, although the response to the antisense GAD65 was smaller than that seen for the antisense GAD67. Because the antisense D-oligos decrease GAD expression examined by Western blot analysis, and because GABA release decreases after exposure to the antisense D-oligos (E. Terasawa, unpublished data), we can speculate that infusion of the antisense GADs may cause the reduction of GABA, which in turn results in the increase in LHRH release. These results are consistent with our hypothesis.

In this study, whereas infusion of antisense D-oligos resulted

Figure 9. Specificity of the antisense D-oligos for GAD mRNAs. Expressions of GAD65 (left) and GAD67 (right) examined by the Western blotting analysis are shown. Lanes are labeled as follows: hypothalamus exposed to scrambled (1) and to antisense (2) D-oligos; cerebellum exposed to scrambled (3), to antisense (4), and to sense (5) D-oligos; and cerebellum not exposed to any D-oligo (6). Arrows indicate the migration of 66 kDa molecular weight marker (bovine serum albumin).

in a large response of LHRH release (Figs. 3, 5, 6, 8), incubation of hypothalamic and cerebellar tissues with antisense D-oligos only decreased GAD expression by a small amount (Fig. 9). Furthermore, the effects of GAD antisense infusion into the S-ME on LHRH release in this study were much greater than those of GABA_A receptor antagonist infusion examined with the same method (Mitsushima et al., 1994). These observations raise two questions. (1) Is GAD reduction linear to GABA reduction? (2) Can the increase in LHRH release by antisense D-oligos be solely attributed to GABA reduction? There are at least three possible explanations for these questions. First, because excessive amounts of GADs are present in GABA neurons (Martin and Rimvall, 1993), only a small decrease in GADs may be sufficient for the reduction of GABA release, resulting in an increase in LHRH release. Second, the effects seen with GAD antisense infusion may be partly attributable to an extracellular increase in glutamate, which has been shown to be a stimulatory neurotransmitter for LHRH release in prepubertal animals (Claypool and Terasawa, 1989; Plant et al., 1989; Urbanski et al., 1990; Bourguignon et al., 1992; Nyberg et al., 1995). The reduction of GADs by the antisense D-oligos could result in the intracellular accumulation of glutamate, which might be transported out to the extracellular space by glutamate transporters where it can act as an excitatory neurotransmitter. It has been shown that glutamate transporters can play a role in transporting the amino acid in both directions (Kanai et al., 1993), and there is the possibility of metabolic glutamate release induced by reversed uptake, described as nonvesicular release (Szatkowski et al., 1990; Attwell et al., 1993). This speculation is not totally unfounded, because a recent report using immunocytochemistry indicated that some GAD-positive neurons in the medio-basal hypothalamus were also glutamate-positive in monkeys (Thind and Goldsmith, 1993). Moreover, our preliminary data measuring glutamate in samples from an *in vivo* push-pull perfusion study suggest that glutamate increases during exposure to the antisense GAD67. Third, because inhibition of GAD synthesis may facilitate other less dominant synthetic pathways for GABA, such as formation from putrescine or γ -hydroxybutyrate, the GAD reduction may not be linear to the GABA reduction. Further studies are necessary to pursue these issues.

There appears to be a two-step process for the mechanism of puberty. First, disinhibition of LHRH neurons from GABA may trigger the onset of puberty. A concurrent excitatory input from glutamate may also be very important at this time, as discussed earlier. Second, the contribution from norepinephrine and NPY neurons is important for the further pubertal increase in LHRH release and, hence, the additional progress of puberty, as we reported previously (Gore and Terasawa, 1991; Gore et al., 1993).

In conclusion, interference of GAD synthesis with antisense D-oligos for GAD mRNAs induced a prompt and large increase in LHRH release in prepubertal monkeys. The results not only support our hypothesis that GABA is an inhibitory neurotransmitter restricting LHRH release before the onset of puberty, but also lead to the concept that maturational changes in GAD synthesis may be important for the onset of puberty in primates.

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