

Changes in Hypothalamic Gene Expression Associated with the Arrest of Pulsatile Gonadotropin-Releasing Hormone Release during Infancy in the Agonadal Male Rhesus Monkey (*Macaca mulatta*)*

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ABSTRACT

This study examined whether changes in the levels of the messenger RNAs (mRNAs) encoding the γ -aminobutyric acid (GABA) synthesizing enzymes, glutamate decarboxylase (GAD)₆₅ and GAD₆₇ and transforming growth factor- α (TGF α) in the hypothalamus are correlated with the arrest of pulsatile GnRH release during infancy in the agonadal male monkey. This experiment also provided the opportunity to examine changes in hypothalamic GnRH gene expression during this critical phase of primate development. Male rhesus monkeys were castrated at 1 week of age: four were killed 4–7 weeks after orchidectomy while pulsatile GnRH release was robust as reflected by high circulating LH levels, and four were killed at 12–15 months of age after establishing that pulsatile GnRH release had been arrested. GAD₆₅, GAD₆₇, TGF α , and GnRH mRNA levels were estimated using RNase protection assays employing homologous

probes and the results were expressed relative to cyclophilin mRNA levels. GnRH peptide was measured by RIA. GAD₆₅ and GAD₆₇ mRNA levels in the hypothalamus of juveniles were significantly greater than those in neonatal monkeys. On the other hand, hypothalamic TGF α and GnRH mRNA (and peptide) levels in agonadal neonate and juvenile monkeys were indistinguishable. These results indicate that the molecular concomitants associated with bringing the hypothalamic GnRH pulse generator into check in agonadal neonatal males are not a mirror image of those previously reported at the time this neuronal network is reactivated at puberty when TGF α and GnRH gene expression increase and GAD₆₅ and GAD₆₇ mRNA levels remain unchanged. Thus, the neurobiological mechanism that reactivates pulsatile GnRH release at puberty is likely to involve more than a simple reversal of that underlying inhibition of the same network in late infancy. (*Endocrinology* 141: 3273–3277, 2000)

THE FINDINGS that embryonic GnRH neurons from the rhesus monkey secrete their peptide in a pulsatile mode *in vitro* (1) and that, in this species, LH secretion by the fetal pituitary occurs intermittently (2), suggest that the neuronal network that governs pulsatile GnRH release in primates becomes functional during early fetal development. Certainly, the capacity of this neuroendocrine system for generating a robust hypophysiotropic drive during neonatal development is demonstrated by the striking LH discharges that are readily detected in infantile male and female monkeys after gonadectomy (3, 4).

Hypothalamic GnRH stimulation of the pituitary gonadotrophs of the infantile primate, however, is not sustained and gonadotropin secretion later declines guaranteeing a delay in the onset of puberty, which in these species is characteristically protracted (5). Thus, the mechanism that brings pulsatile GnRH release into check during the neonatal-juvenile transition must be viewed as a fundamental component of the control system that times the onset of primate puberty. While the neurobiology of the reactivation of pulsatile GnRH

release at the end of the juvenile phase of development has been examined extensively (6–9), that associated with restraining pulsatile GnRH release during the neonatal-juvenile transition has received little attention.

The idea that the mechanism responsible for imposing the brake on pulsatile GnRH release in infancy is thrown into reverse at the termination of the juvenile phase of development to trigger the onset of puberty is the most parsimonious explanation to account for the time course of GnRH release from birth until puberty in primates. With this in mind, hypothalamic levels of the messenger RNAs (mRNAs) encoding transforming growth factor- α (TGF α) and the γ -aminobutyric acid (GABA) synthesizing enzymes, glutamate decarboxylase (GAD)₆₅, and GAD₆₇, were compared in agonadal monkeys during the neonatal (robust GnRH pulse generator activity) and juvenile (arrested GnRH pulse generator activity) phase of development. TGF α and GABA have been implicated in the postnatal regulation of GnRH in primates (7–9) and, during the juvenile-pubertal transition, hypothalamic levels of the mRNA encoding TGF α have been shown to increase (7, 10), whereas those encoding the GADs have been reported not to change during this developmental stage (10, 11). Additionally, this experiment provided an opportunity to examine changes in hypothalamic GnRH gene expression that occur as the GnRH pulse generator is brought into check in late infancy. Male rhesus monkeys were used because the prepubertal hiatus in pulsatile GnRH release is most marked in the male (5, 12), and it was reasoned

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that hypothalamic concomitants would be correspondingly exaggerated and therefore easier to identify for the first time in the male. The agonadal model was employed to eliminate any secondary molecular changes in the hypothalamus, which in intact animals may be anticipated to occur in response to the declining levels of testicular steroids during the neonatal-juvenile transition.

It should be noted that the hypothalamic mRNA and protein extracts from the animals employed in this study were used to examine changes in neuropeptide Y (NPY) expression during the neonatal-juvenile transition. The NPY results comprise a component of a comprehensive study of the role of this neuropeptide in regulating pulsatile GnRH release throughout postnatal development in the male monkey, and have been previously reported (10).

Materials and Methods

Animals and experimental design

The animals and the experimental design employed in the present study have been described (10). In brief, rhesus monkeys born at the Center for Research in Reproductive Physiology were maintained in accordance with the NIH Guide for the Care and Use of Laboratory animals, and experimental protocols were approved by the institutional animal care and use committee. Eight monkeys were bilaterally orchidectomized when 7–10 days old, using a sterile technique, after anesthesia with ketamine hydrochloride (Vetalar, Parke-Davis, Morris Plains, NJ; approximately 40 mg/kg body wt im). The testes were removed from the inguinal canals via a midline incision. The infantile animals were housed with their mothers in individual cages under a controlled photoperiod (lights on, 0700–1900 h) until they were killed, either 4–7 weeks after castration (neonate group; $n = 4$) while GnRH pulse generator activity was robust, or at 12–15 months of age (juvenile group; $n = 4$) after establishing that pulsatile GnRH release had declined to low prepubertal levels, as indicated by undetectable circulating LH levels (Fig. 1). To monitor circulating LH concentrations, blood samples (1–3 ml) were collected on the day of castration and thereafter every other week by femoral venipuncture, while the monkeys were sedated with ketamine hydrochloride (approximately 20 mg/kg BW). During neonatal development, the infants were temporarily taken from their mothers while the latter were sedated with ketamine hydrochloride. The four monkeys studied as juveniles were separated from their mothers

when 6–9 months of age and housed in pairs in individual cages located in the same building as their mothers. These animals were fed once a day with Purina Monkey Chow (Ralston Purina Co., St. Louis, MO) at approximately 1100 h, and fruit was invariably provided in the afternoon. Water was provided *ad libitum*.

Tissue collection

As described previously, the animals were killed with a lethal dose of pentobarbital sodium (10). Brains were removed immediately and blocks containing the combined preoptic area (POA) and medial basal hypothalamus (MBH) were isolated by placing two coronal cuts, one immediately rostral to the anterior commissure and one directly in front of the mammillary bodies, and two parasagittal cuts approximately 5 mm from each side of midline. The POA and MBH-containing blocks were then separated by a coronal cut at the caudal boundary of the optic chiasm. Hypothalamic tissue, together with samples of frontal and/or parietal cerebral cortex (taken from the vicinity of the central sulcus) were snap-frozen in liquid nitrogen and kept at -80°C until RNA and protein extraction.

RNA and protein preparation

Total RNA was isolated using RNazol (RNA STAT-60) followed sequentially by precipitation with isopropanol, washing with ethanol and solubilization in DEPC-treated water according to the manufacturer's instructions (Tel-Test, Friendswood, TX). RNA integrity was checked by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA bands after migration on agarose gel, and RNA concentration was determined by measuring absorbance at 260 nm.

Proteins were precipitated sequentially with ethanol and isopropanol from the organic phase after isolation of RNA, followed by washing with 0.3 M guanidine hydrochloride-95% ethanol, and solubilization in 1% SDS according to the manufacturer's instructions (Tel-Test, Friendswood, TX). Protein concentration was measured by the Bradford assay (Bio-Rad Laboratories, Inc. Hercules, CA).

Complementary DNAs (cDNAs) and riboprobe synthesis

A 204-bp fragment of monkey GnRH and a 364-bp fragment of monkey TGF α cDNAs (7) were kindly provided by Dr. S. R. Ojeda (Oregon Regional Primate Research Center, Beaverton, OR). A 382-bp fragment of monkey GAD $_{65}$ and a 210-bp fragment of monkey GAD $_{67}$ cDNAs (13) were kindly provided by Drs. T. G. Golos and E. Terasawa (Wisconsin Regional Primate Research Center, Madison, WI). A 117-bp fragment of rat cyclophilin cDNA (14) was kindly provided by Dr. J. L. Roberts (Mount Sinai School of Medicine, New York, NY).

The cDNAs were linearized with *EcoRI* (GnRH, TGF α , cyclophilin) or *BamHI* (GAD $_{65}$, GAD $_{67}$), then *in vitro* transcribed with SP6 (GnRH, TGF α) or T7 (GAD $_{65}$, GAD $_{67}$, cyclophilin) RNA polymerase using a transcription kit (Ambion, Inc., Austin, TX), to synthesize ^{32}P UTP-labeled RNA antisense probes. Sense probes were generated from the same cDNA fragments that were used to synthesize the respective antisense probes. The cDNAs were linearized with *HindIII* (GnRH, TGF α) or *BamHI* (cyclophilin), then *in vitro* transcribed with T7 (GnRH, TGF α) or T3 (cyclophilin) RNA polymerase.

RNase protection assay

RNase protection assay was performed as described previously (15, 16). Briefly, 30 μg of RNA were allowed to hybridize with the ^{32}P -labeled antisense RNA probes in solution at 45°C overnight, followed by combined RNase A and T1 digestion of nonhybridized RNA. TGF α and GnRH probes were used simultaneously in the same assay, as were the probes for GAD $_{65}$ and GAD $_{67}$. Stable hybrids were extracted, ethanol-precipitated, and then denatured and separated on 6% polyacrylamide-8 M urea gels. The dried gel was exposed in a Bio-Rad Laboratories, Inc. (Hercules, CA) CS Molecular Imaging Screen for 16–22 h, and an image of each gel was acquired by a Molecular Imager (Model GS-525; Bio-Rad Laboratories, Inc.). Antisense probes with 30 μg transfer RNA were run as controls and were completely digested by RNase A and T1. In addition, increasing amounts (0, 5, 10, 20, and 40 pg) of *in vitro* transcribed sense GnRH, TGF α and cyclophilin mRNAs were hybridized with the

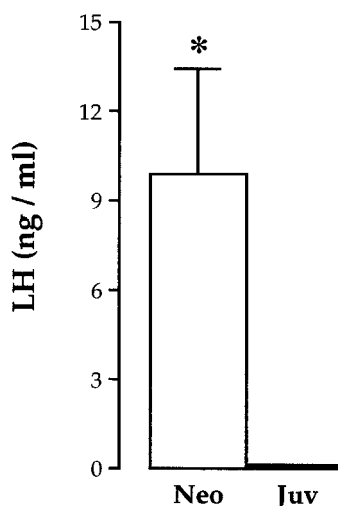


FIG. 1. Circulating LH concentrations (mean \pm SD) in 4 neonate (Neo; open bar) and 4 juvenile (Juv; closed bar) agonadal male rhesus monkeys on the day they were killed. *, $P < 0.05$, Mann-Whitney *U* test. Values calculated from previous publication (10).

corresponding ^{32}P -labeled RNA antisense probes. In each case, a linear relationship was observed between mRNA mass and integrated optical density, quantified by Molecular Analyst Software (Bio-Rad Laboratories, Inc.) after subtraction of background. In the case of GAD_{65} and GAD_{67} , for technical convenience, standard curves were not generated. Increasing amounts (up to 80 μg) of hypothalamic mRNA yielded dose-dependent increases in integrated optical density for all transcripts studied.

The integrated optical density of the $\text{TGF}\alpha$, GnRH , GAD_{65} , and GAD_{67} hybrids were normalized to that of cyclophilin and expressed as relative optical density. The use of homologous antisense probes ($\text{TGF}\alpha$, GnRH , GAD_{65} , and GAD_{67}) yielded full-length hybrids, although a weak secondary signal was also detected in the GAD_{65} assay. In contrast, the use of a heterologous antisense probe to assay cyclophilin produced two hybrids of approximately 65 and 45 bp, respectively. This was presumably the result of sequences mismatches between the monkey mRNA and the rat antisense probe. As in an earlier study (16), all relative optical densities were determined using the 45 bp cyclophilin hybrid.

RIAs

Circulating LH concentrations were estimated by a previously described RIA (10). GnRH content was measured by RIA using the antiserum R1245 from Dr. T. Nett (17) at a final dilution of 1:90,000. Synthetic GnRH from Dr. G. Bialy (NICHHD) was used for both the radiolabeled antigen and reference standard. The antigen-antibody complex was precipitated with a goat antirabbit γ globulin (Antibodies Inc., Davis, CA) at a final dilution of 1:120. The ED_{50} of the assay was 15 ng/tube and the minimal detectable concentration of GnRH was 0.03 ng/tube. The average intraassay coefficient of variation was 15%. GnRH levels were expressed as ng/mg protein.

Numerical analysis

Values were expressed as mean \pm SD. Statistical comparisons between experimental groups were made using the nonparametric Mann-Whitney U test, and differences were considered to be significant if $P < 0.05$.

Results

The mean (\pm SD) optical densities of the cyclophilin hybrids in hypothalamic extracts from neonate and juvenile monkeys were not significantly different ($18,430 \pm 1,947$ and $18,160 \pm 1,552$ for MBH; $18,940 \pm 1,817$ and $20,520 \pm 1,983$ for POA; in neonate and juvenile animals, respectively).

In both the MBH and POA, the mean relative optical density of GAD_{65} and GAD_{67} mRNA signals in the juvenile group were approximately 30% greater than those in the neonatal group (Fig. 2). In contrast, $\text{TGF}\alpha$ mRNA levels in both MBH and POA were indistinguishable in neonate and juvenile animals (Fig. 2). These three transcripts were detected in the cortex, but developmental differences in GAD_{65} , GAD_{67} and $\text{TGF}\alpha$ levels were not observed in this brain region (Table 1).

The mean GnRH mRNA level in the MBH and POA of neonatal monkeys were not different from those observed in juvenile monkeys (Fig. 3). Developmental changes in GnRH peptide content in these brain regions were also unremarkable (Fig. 3). The GnRH mRNA signal in cortex was weak and was not quantitated.

Discussion

As described in the *Introduction*, the postnatal ontogeny of GnRH release in primates is characterized by phases of robust pulse generator activity during neonatal and pubertal development that are separated by a prolonged period of prepubertal development when GnRH release is held in check. Because this developmental pattern in GnRH release

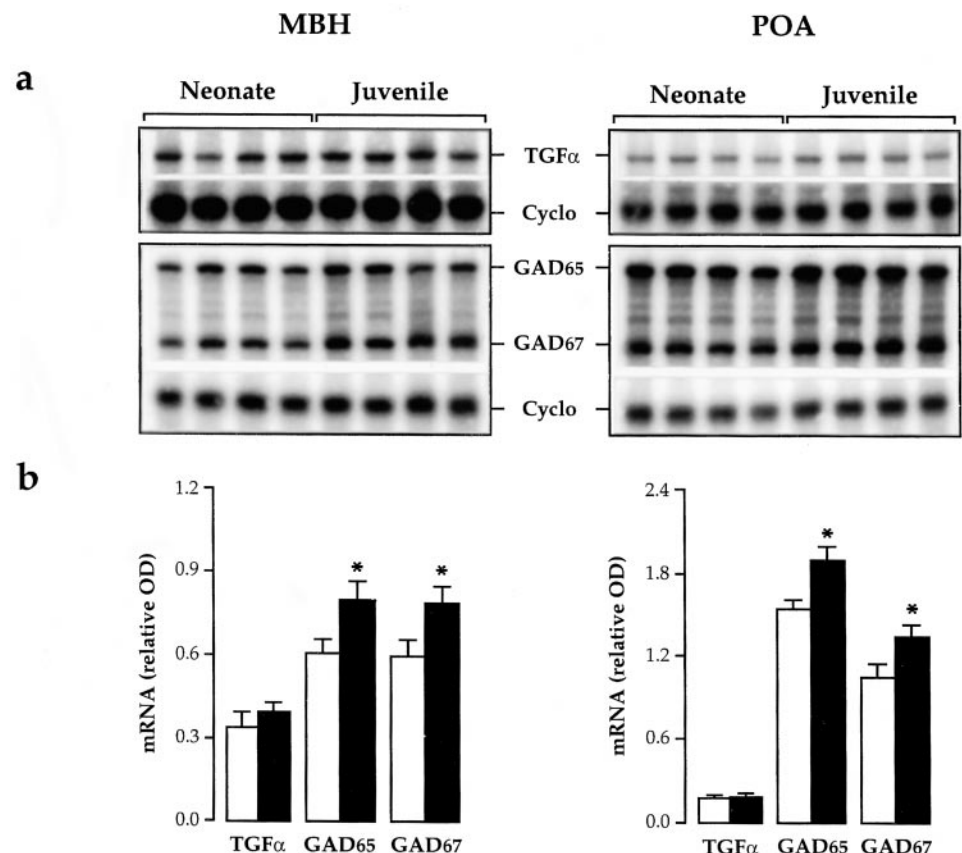


FIG. 2. $\text{TGF}\alpha$, GAD_{65} , GAD_{67} and cyclophilin (Cyclo) mRNA levels in the MBH (left hand) and POA (right hand) of neonate and juvenile agonal male monkeys. The upper panels show, for 4 neonate and 4 juvenile monkeys, autoradiograms of the mRNAs hybridized to ^{32}P -labeled antisense probes. The lower panels depict the corresponding relative optical densities (mean \pm SD) of the $\text{TGF}\alpha$, GAD_{65} , and GAD_{67} mRNA levels in neonate (open bar) and juvenile (closed bar) monkeys. *, $P < 0.05$, Mann-Whitney U test.

appears unique to higher primates, studies of the monkey are particularly relevant to the human situation, and the present findings are unlikely ever to be replicated in man.

We had anticipated that hypothalamic levels of the mRNA encoding GnRH would decrease during the neonatal-juvenile transition, *i.e.* in a manner to mirror the previously reported increased expression of this gene during the juvenile-pubertal transition in agonadal males (10). On the other hand, hypothalamic GnRH peptide content was not expected to change in accordance with the results of an earlier study (18). Although the latter prediction was confirmed, surprisingly GnRH mRNA levels in the MBH of neonatal and juvenile animals were indistinguishable; a result similar to that previously reported for the intact female monkey (7). Presumably a decrease in GnRH secretion in the neonatal-juvenile transition is achieved in the absence of a change in GnRH gene expression.

The present study also failed to provide evidence for the hypothesis that the mechanisms responsible for imposing the brake on pulsatile GnRH release in infancy are simply

TABLE 1. Relative optical density (mean \pm SD) of the GAD₆₅, GAD₆₇ and TGF α hybrids in cortex from four neonate and four juvenile agonadal monkeys

mRNA	Neonate	Juvenile
GAD ₆₅	0.88 \pm 0.02	0.88 \pm 0.04
GAD ₆₇	0.98 \pm 0.04	1.03 \pm 0.02
TGF α	0.25 \pm 0.01	0.26 \pm 0.01

thrown into reverse at the termination of the juvenile phase of development to trigger the onset of puberty. Levels of the mRNAs encoding the GABA synthesizing enzymes, GAD₆₅ and GAD₆₇ in both MBH and POA of juvenile monkeys, in which GnRH pulse generator activity had been arrested, were greater than those in neonatal animals exhibiting robust pulsatile GnRH release. This is in contrast to the absence of a change in these mRNA levels previously established using identical methodology during the juvenile-pubertal transition in the agonadal model (10). Changes in the expression of these genes from the early juvenile stage to adulthood have also been shown to be unremarkable in the intact male monkey (11).

In the rat, GABA levels in the MBH and POA increase during the first 15–20 days of postnatal life (19, 20). Moreover, during this phase of development the electrophysiological action of GABA in regions of the rodent CNS including the hypothalamus changes from one that is stimulatory to one that is inhibitory (21, 22), which has been reported to occur as a result of an increase in the expression of the neuronal Cl[−] extruding K⁺/Cl[−] cotransporter, KCC2 (23). While it is likely that this developmental “maturation” in GABA action is recapitulated in the primate brain, the timing of this event in the monkey is unknown and it is therefore not possible to place into comparative perspective the present finding of an increase in hypothalamic GAD expression during the neonatal-juvenile transition. It should be noted, however, that in rat the increase in hypothalamic GAD levels

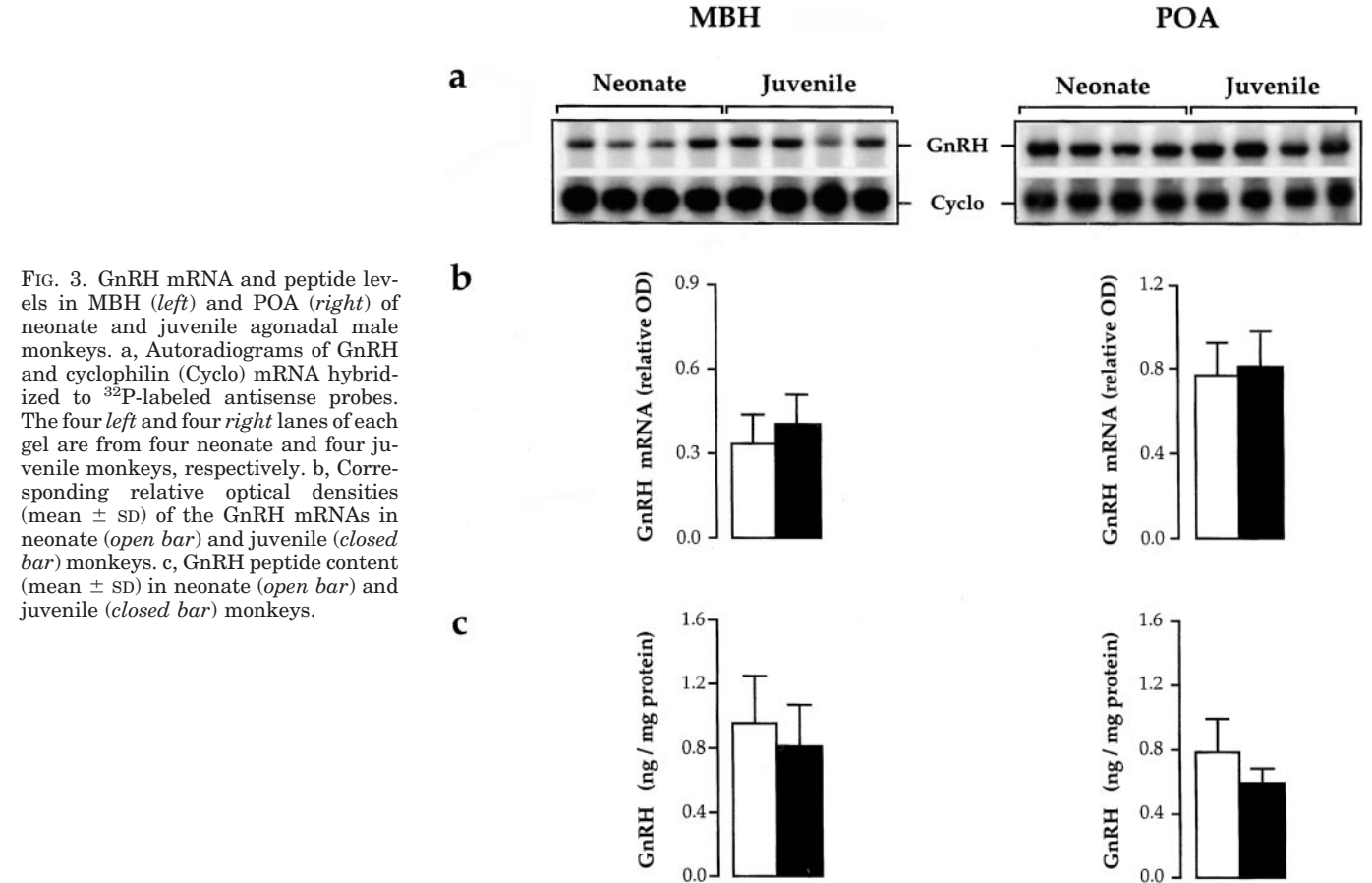


FIG. 3. GnRH mRNA and peptide levels in MBH (left) and POA (right) of neonate and juvenile agonadal male monkeys. **a**, Autoradiograms of GnRH and cyclophilin (Cyclo) mRNA hybridized to ³²P-labeled antisense probes. The four left and four right lanes of each gel are from four neonate and four juvenile monkeys, respectively. **b**, Corresponding relative optical densities (mean \pm SD) of the GnRH mRNAs in neonate (open bar) and juvenile (closed bar) monkeys. **c**, GnRH peptide content (mean \pm SD) in neonate (open bar) and juvenile (closed bar) monkeys.

during neonatal development was paralleled by a similar change in the cortex (20). In the present study of the monkey, however, there was no evidence for changes in GAD expression in the cortex during the neonatal-juvenile transition.

In the adult monkey, orchidectomy results within 42 days in a decrease in the levels of the mRNAs encoding both GAD₆₅ and GAD₆₇ in the MBH (16), and therefore it is unlikely that the increased levels of GAD mRNAs in the MBH observed between 2 months and 15 months of age in the agonadal male monkey was due to an insidious effect on GAD expression triggered by removing the testes at 1 week age.

Studies by Terasawa and her colleagues (8) have provided compelling evidence for the view that GABA plays an important role in restraining GnRH release in the juvenile female monkey before menarche. GABA content in perfusates from the MBH of prepubertal female monkeys is greater than that from pubertal females, and inhibition of GABA synthesis or action results in precocious GnRH release and premature menarche and ovulation (8, 9, 13). We have previously argued that this inhibitory GABA tone may not be as robust in the juvenile male (10), and if this is the case it might be predicted that the increase in the hypothalamic levels of the mRNAs encoding the GADs during the neonatal-juvenile transition would be more striking in the female.

As with changes in GAD gene expression during the neonatal-juvenile transition, those in TGF α at this developmental stage also failed to mirror those that were previously described during peripubertal development in the agonadal male (10). In the latter situation, TGF α mRNA levels in the MBH in pubertal monkeys are higher than those in juvenile animals (10). A similar observation has also been made in the intact female monkey, although in the latter case the pubertal increase in TGF α gene expression was observed in the both hypothalamic regions (7). In the case of the female, however, hypothalamic TGF α mRNA levels were also elevated during the neonatal period (7). Because estradiol treatment of ovariectomized adults castrated prepubertally does not appear to influence TGF α gene expression (15), it seems reasonable to conclude that the developmental pattern in hypothalamic TGF α expression in the female monkey is a gonadal independent event. The reasons for the apparent sex difference in the expression of TGF α during the neonatal juvenile transition remains to be established.

In summary, while GnRH and TGF α mRNA levels in the hypothalamus of the agonadal male monkey do not change at the time when the GnRH pulse generator is being brought into check during the neonatal-juvenile transition, expression of the genes encoding GAD₆₅ and GAD₆₇ increase at this critical developmental stage. These results are to be contrasted with those demonstrating that during removal of the prepubertal brake upon the GnRH pulse generator at the time of the juvenile-pubertal transition, GnRH and TGF α mRNA levels increase while those encoding the GADs do not change (10). Taken together, these findings indicate that the molecular concomitants associated with bringing the GnRH pulse generator into check during infancy are not mirror images of those seen at the time this neuronal network is reactivated at the end of the juvenile phase of development. Thus, the neurobiological mechanism that activates the GnRH neuronal network at the onset of puberty is likely to involve more than a simple reversal of those underlying inhibition of the same network in late infancy.

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