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Testis of Prepubertal Rhesus Monkeys Receives a Dual Catecholaminergic Input Provided by the Extrinsic Innervation and an Intragonadal Source of Catecholamines[1]

Artur Mayerhofer,[2,3,4] Michael Danilchik,[6] K.-Y. Francis Pau,[5] Hernan E. Lara,[7] Lonnie D. Russell,[8] and Sergio R. Ojeda[4]

Molecular Anatomy,[3] Anatomical Institute, Technical University, Munich, Germany
Divisions of Neuroscience,[4] and Reproductive Sciences,[5] Oregon Regional Primate Research Center, Beaverton, Oregon 97006
Department of Biological Structure and Function,[6] School of Dentistry, Oregon Health Sciences University, Portland, Oregon 97201
Department Bioquimica y Biologica Molecular,[7] Universidad de Chile, Santiago, Chile
Department of Physiology,[8] Southern Illinois University, Carbondale, Illinois 62901

ABSTRACT

The mammalian testis is innervated by extrinsic catecholaminergic nerves and responds to catecholamines with steroid secretion. Although the primate testis has also been shown to be innervated, potential differences in the density of this innervation between immature and sexually developed individuals have not been described. A recent study demonstrated that the primate ovary contains a network of neuron-like cells and that some of these cells are catecholaminergic. It is thus possible that the male gonad is also endowed with a similar intragonadal source of catecholamines. The present study addresses these two issues. Catecholaminergic nerves were identified as such by their content of immunoreactive tyrosine hydroxylase (TH; the rate-limiting step in catecholamine biosynthesis), and in some cases by glyoxylic acid histochemistry. Fibers containing TH were abundant in testes from juvenile animals (1–2 yr of postnatal life), but the density of this innervation was not maintained in adult animals, whose testis showed only a few TH-positive fibers scattered in the interstitial tissue. Testicular norepinephrine (NE) concentration was much lower in adult than in juvenile animals, suggesting that the marked increase in testicular weight that occurs with the attainment of sexual maturity is not accompanied by corresponding changes in NE content. At the ultrastructural level, testicular nerve fibers contained pleiomorphic, dense-core and clear vesicles, suggesting the presence of catecholamines and other neurotransmitters. In addition to this extrinsic catecholaminergic innervation, prepubertal testes, but not adult gonads, contained an intrinsic population of TH-immunopositive neuron-like elements, identified as cells by confocal scanning laser microscopy. To determine whether the prepubertal monkey testis indeed expresses the TH gene, testicular RNA was subjected to reverse transcriptase polymerase chain reaction to amplify the 5′ end of TH mRNA, which encodes the regulatory domain of the enzyme. The cDNA that was obtained predicts an amino acid sequence similar, but not identical, to that encoded by the alternatively spliced type 1 TH mRNA form present in the adrenal gland. These results indicate 1) that the primate testis receives a dual catecholaminergic input, one provided by the extrinsic innervation and the other by neuron-like cells located within the gonad itself, and 2) that the influence exerted by both sources on testicular function may be more prominent during the prepubertal period than in adulthood. The presence in the testis of a TH mRNA variant encoding amino acid substitutions in its 5′ end suggests that regulation of testicular TH enzyme activity may include a gonad-specific component.

INTRODUCTION

Longitudinal studies in peripubertal human males have shown that serum testosterone levels rise sharply during the early phases of puberty, a time during which the concentration of serum LH is also rapidly increasing [1]. Serum testosterone levels, however, begin to increase in juvenile individuals even before the changes in LH become apparent [1], suggesting that changes in testicular responsiveness to LH may contribute to the pubertal change in testosterone output. Studies in rodents have provided experimental evidence for this concept [2]. That these changes in target organ responsiveness may contribute to both the initial and the LH-dependent increase in peripubertal serum testosterone levels is suggested by the fact that, during the phase of rapid increase in serum LH levels at puberty, serum testosterone levels increase more than 10-fold despite a mere 3-fold increase in circulating LH levels [1].

Several gonadal factors may facilitate the testicular response to LH, including, for example, the recently described metalloproteinase-1/procathepsin L complex produced by Sertoli cells [3], and neurotransmitters arriving at the testis via the extrinsic innervation [4]. It is well established that the bulk of testicular innervation is sympathetic [4, 5], and that the main neurotransmitter associated with the postganglionic sympathetic fibers innervating the testis is norepinephrine (NE). Although the testes of all mammalian species thus far examined are innervated [4–7], the density of this innervation appears to be greater in the human testis [8, 9].

There exists a large body of evidence showing that catecholamines are able to influence Sertoli cell and especially Leydig cell function via specific adrenergic receptors [10–24]. The distinctiveness of these actions has led to the concept that catecholamines may act as a backup mechanism for steroid production by Leydig cells during stress [25] and may act to potentiate the effect of gonadotropins during

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[2]Correspondence: Dr. Artur Mayerhofer, Molecular Anatomy, Anatomical Institute, Technical University-Munich, Biedersteiner Str. 29, 80802 Munich, Germany. FAX: 49 89 397035.

phases of development when circulating gonadotropins are low [19, 21, 22]. There is also evidence suggesting that catecholamines may be able to influence testicular and Leydig cell development and differentiation [23, 26–28].

It is generally accepted that the only source of catecholamines found in mammalian testis is its extrinsic innervation. This, however, may not be the case, as it has recently been found that the primate ovary contains an intrinsic network of neuron-like, tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine biosynthesis)-immunoreactive cells, endowed with low-affinity nerve growth factor (NGF) receptors [29]. Since these cells have not been found in rodents, their presence in rhesus monkeys suggests that the primate gonad in particular may be subjected to the influence of an intragonadal neuronal regulatory system.

The present study provides morphological evidence for the concept that the catecholaminergic input to the primate testis is greater before puberty than during adulthood, owing to a higher density of the extrinsic innervation and to the presence of an intrinsic source of catecholamines provided by a population of TH-immunoreactive neuron-like cells located in the interstitial tissue of the gland. Evidence is also provided that the developing primate testis expresses the TH gene, but that the encoded mRNA, though similar, is not identical to that produced in the adrenal gland. A partial report of these studies has appeared previously [30].

MATERIALS AND METHODS

Tissue Collection

The testes used in this study were obtained from the Tissue Distribution Program at the Oregon Regional Primate Research Center (ORPRC). Testes from a total of 15 rhesus monkeys (*Macaca mulatta*) ranging from 1 to 25 yr of postnatal age were examined. Upon collection of the testes, they were divided into smaller pieces that were used for various procedures. Thus, for measurement of NE, a total of 6 fragments from the testes of six monkeys were used; for polymerase chain reaction (PCR) cloning, we used pieces from two animals. Fragments from three monkeys were used for electron microscopy; from four animals, for glyoxylic acid histochemistry; and from eight animals, for immunohistochemistry. The ages of the monkeys used are listed below. In addition, the adrenals from two adult monkeys were collected. Upon dissection from surrounding tissue, the organs were rapidly frozen on dry ice for RNA extraction or were immersed in fixative for immunohistochemistry or electron microscopy examination (see below).

Measurement of NE

Testicular fragments (40–230 mg) from six monkeys of different ages (prepubertal: 1 yr, 1 yr and 8 mo, and 2 yr and 1 mo of age; adult: 11, 13, and 16 yr of age) were homogenized in 0.7–1 ml of 0.2 M perchloric acid (PCA) and centrifuged at 15 000 × *g* for 10 min; the supernatants were frozen until NE measurement. Norepinephrine content was determined by either the radioenzymatic method of Saller and Zigmond [31], as previously reported [32], or by HPLC using a Waters 717 Plus automated HPLC system with a Waters 460 electrochemical detector [33]. The mobile phase (Waters Chromatography, Milford, MA) was recirculated through the analytical column (Waters Resolve C18; 5-μm silica, 15 cm in length) at a rate of 1 ml/min. The sensitivity of this unit for catecholamine determination was routinely maintained at 1–2 pg/μl.

Immunohistochemistry

The cellular distribution of TH in the testes was determined by two immunohistochemical methods (avidin-biotin-peroxidase [ABC] and fluorescence), using a well-characterized polyclonal antibody (rabbit anti-TH; Eugene Tech, Ridgefield Park, NJ) at a 1:500–1:1000 dilution. For both methods, freshly collected testes from five prepubertal (4 mo, 11 mo, 1 yr, 1 yr and 7 mo, and 1 yr and 11 mo of age) and three adult monkeys (13, 16, and 25 yr of age) were cut into small fragments and immersion-fixed in Zamboni's fixative for 18–20 h at 4°C, rinsed for 24 h in PBS (pH 7.4), and transferred to PBS containing 20% sucrose. The next day, the tissues were embedded in O.C.T. compound (Miles Inc., Elkhart, IN) and frozen on dry ice before cryostat sectioning. Ten 12-μm-thick sections were incubated overnight at 4°C with the TH antibody.

Immunohistochemistry using the ABC method was performed as described previously [34, 35]. For the fluorescent method, the reaction was developed with a fluorescein isothiocyanate (FITC)-labeled affinity-purified goat anti-rabbit gamma globulin (1:200; Jackson Immunoresearch Laboratories, West Grove, PA) as previously described [29, 36]. In most cases, the cell nuclei were counterstained with propidium iodide (Sigma Chemical Co., St. Louis, MO) at 2 μg/ml PBS for 2 min as previously reported [29]. Sections incubated without the primary antibody and with normal rabbit serum substituting for the primary antibody served as controls. The immunohistochemical reaction was viewed under fluorescence illumination with a Zeiss Axiovert (Carl Zeiss, Thornwood, NY) microscope equipped with appropriate filters for FITC and rhodamine fluorescence [36]. Specificity of the reaction was verified by incubating sections in the absence of the TH antibody and by examination of adrenal gland sections. Cells of the adrenal medulla were strongly immunoreactive, whereas the endocrine cells of the cortex were immunonegative.

Confocal Laser Scanning Microscopy

Double-labeled sections, combining the nuclear DNA dye propidium iodide and the immunohistochemical stain for TH, were also analyzed with a confocal scanning laser microscope (Bio-Rad MRC 500 [Richmond, CA] system coupled to a Zeiss Axiovert 100 inverted microscope). The emissions of FITC and propidium iodide were detected with A1/A2 two-channel filter sets, which were optimized for fluorescein and Texas Red fluorescence, respectively (for details see [29, 37]). Gray-scale images were collected separately and fused. They were rescaled to normalize contrast and then merged via Adobe Photoshop 2.5 (Adobe Systems, Inc., Mountain View, CA) to produce CMYK mode images, which were printed with a Tektronix Phaser II SDX (Tektronix Inc., Beaverton, OR).

Glyoxylic Acid Histochemistry for Catecholamines (NE)

For identification of catecholamine-containing elements, cryostat sections (10–12 μm) of freshly frozen unfixed testicular fragments were utilized. The testes used were obtained from three prepubertal (1 yr and 9 mo, 1 yr and 11 mo, 2 yr and 1 mo of age) and one adult (13 yr old) monkey. Immediately after sectioning, the tissues were treated for 1–3 sec with glyoxylic acid (Sigma) dissolved at 1

g/100 ml in 0.2 M potassium phosphate buffer (pH 7.4) containing sucrose (6.8 g/100 ml). Sections were dried under a stream of cool air, covered with mineral oil, and placed onto metal blocks in an oven at 100°C for 2–3 min, as described by de la Torre [38]. Catecholamine histofluorescence was then examined with a Zeiss Axiovert microscope equipped with appropriate filters. Specificity of the reaction was assessed by examination of sections of adrenals containing both positive (medulla) and negative (cortex) cells.

Transmission Electron Microscopy (TEM)

The testes from two prepubertal monkeys (1 yr and 9 mo, and 2 yr and 1 mo of age) and one adult monkey (11 yr and 2 mo of age) were examined by TEM. Small fragments of testes were fixed in 5% glutaraldehyde in 0.05 M cacodylic acid, pH 7.4. They were postfixed in a mixture of OsO_4 containing potassium ferrocyanide (1:1.5% final concentration [39]) and embedded in araldite plastic. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with a Hitachi H500H (Tokyo, Japan) transmission electron microscope.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Cloning

Total testicular RNA from two prepubertal monkeys (1 yr, and 1 yr and 7 mo of age) was prepared by the acid phenol extraction method [40] as previously described [41, 42]. Aliquots of 200–500 ng of total testicular or adrenal RNA were subjected to reverse transcription utilizing an 18-mer polydeoxythymidine primer containing *Xho* I and *Eco* RI restriction sites and Superscript II reverse transcriptase (Gibco BRL, Grand Island, NY). The procedure employed has been described in detail elsewhere [43].

The gene-specific oligodeoxynucleotide primers used to amplify testicular TH were a 5′-sense primer (5′-TCTGAGCTGGACGCCAAGCAG-3′) complementary to a sequence conserved in humans and monkeys [44, 45], which spans nt 74–94 in exon 1 of TH mRNA, and a 3′-antisense primer (5′-TGGAGGCTGTGGCCTTTGAGGAG-3′) complementary to another conserved region in exon 3 of TH mRNA (nt 222–244). Another 5′-sense primer was used to amplify a fragment of adrenal TH mRNA; this primer was similar to that used for amplification of testicular TH, except for an additional 5 nucleotides (CCGTG) added to the 5′ terminus. Synthesis of the second DNA strand following reverse transcription was performed using one cycle of denaturing (94°C, 2 min), annealing (55°C, 3 min), and extension (72°C, 10 min). The subsequent amplification reaction consisted of 35 cycles of denaturing (94°C, 1 min), annealing (55°C, 2 min), and extension (72°C, 3 min), followed by a final extension at 72°C for 7 min. The PCR products were loaded on 2% agarose gels and visualized by staining with ethidium bromide. Identity of the amplified TH cDNAs was initially assessed by Southern blot analysis after the PCR products were transferred to nylon membranes. The hybridizing probe used for this procedure was a 28-mer ^{32}P-5′-labeled oligodeoxynucleotide complementary to nt 133–161 in exon 3 of the human TH sequence [44]. To confirm the identity of the TH cDNAs, they were subcloned into the pGEM-T vector (Promega, Madison, WI) and sequenced by the dideoxynucleotide termination method of Sanger et al. [46] using Sequenase T7 polymerase and a kit (Sequenase version 2.0) purchased from U.S. Biochemicals (Cleveland, OH). Most cDNAs were also sequenced in an ABI model 373A automatic DNA sequencer (Perkin Elmer, Foster City, CA) using a fluorescein dye terminator reaction (Prism Ready Reaction Dye terminator Cycle Sequencing kit) and Amplitaq DNA polymerase.

To determine whether TH mRNA is represented in a rhesus monkey testicular cDNA library (constructed by S. Nagalla and A. Mayerhofer, ORPRC), aliquots of the library were also subjected to PCR amplification using the same primers that amplified a fragment of TH mRNA from total testicular RNA. The library (Uni-ZAP XR vector; Stratagene, La Jolla, CA), constructed from 5 μg of testicular polyadenylated RNA (derived from one immature and one adult testis), contains approximately 600 000 independent recombinants per milliliter. Positive clones were identified by Southern blot analysis, subcloned, and sequenced as described above.

RESULTS

Catecholaminergic Nerves Are More Abundant in the Testes of Prepubertal than Adult Monkeys

Catecholaminergic nerves, identified by their content of immunoreactive TH, were readily detected in the prepubertal gonad (Fig. 1A). The fibers not only had a perivascular localization, but were also found associated with clusters of interstitial cells (box in Fig. 1A; for morphological identification see Fig. 1B). A similar density and distribution of the fibers was observed when catecholamine-containing fibers were identified by glyoxylic acid histochemistry (Fig. 1C). No appreciable differences in the density of this innervation were observed when the testes of four juvenile animals ranging from 1 to 2 yr of age were compared.

In contrast, adult monkey testes (13, 16, and 25 yr of age) appeared to contain only a few catecholaminergic nerve fibers, identified by either glyoxylic acid histochemistry (not shown) or by their TH immunoreactivity (Fig. 1D). Most of these nerves had a perivascular localization, usually near arterioles in the interstitial space. Only rarely were these nerves seen associated with Leydig cells.

At the ultrastructural level (Fig. 2A), the nerve fibers innervating the testis were unmyelinated and contained pleiomorphic, dense-core (arrow inside box) and clear vesicles (arrowhead inside box), suggesting that they contain catecholamines as well as other neurotransmitters.

Immature Primate Testis Contains TH-Immunoreactive Neuron-Like Cells in Addition to Its Extrinsic Innervation

The testes from prepubertal animals, but not those from adult monkeys, were found to contain elongated/stellate-shaped elements positive for TH immunoreactivity (Fig. 2B) and catecholamine-specific glyoxylic acid histochemistry (not shown). These elements had a neuronal appearance identical to that of a network of neuron-like cells recently described in the monkey ovary [29]. As in the case of the ovary, these immunopositive elements were positively identified as cell bodies by confocal scanning laser microscopy analysis (Fig. 3, A and B). Through counterstaining of the tissue sections with propidium iodide, which binds to double-stranded DNA, the nucleus of these cells was unambiguously visualized; thus the TH-immunoreactive structures were identified as cells. It is noteworthy that the majority of the TH-positive neuron-like cells detected in the testis had a bipolar configuration. The cells were found scattered throughout the interstitial compartment of the gland.

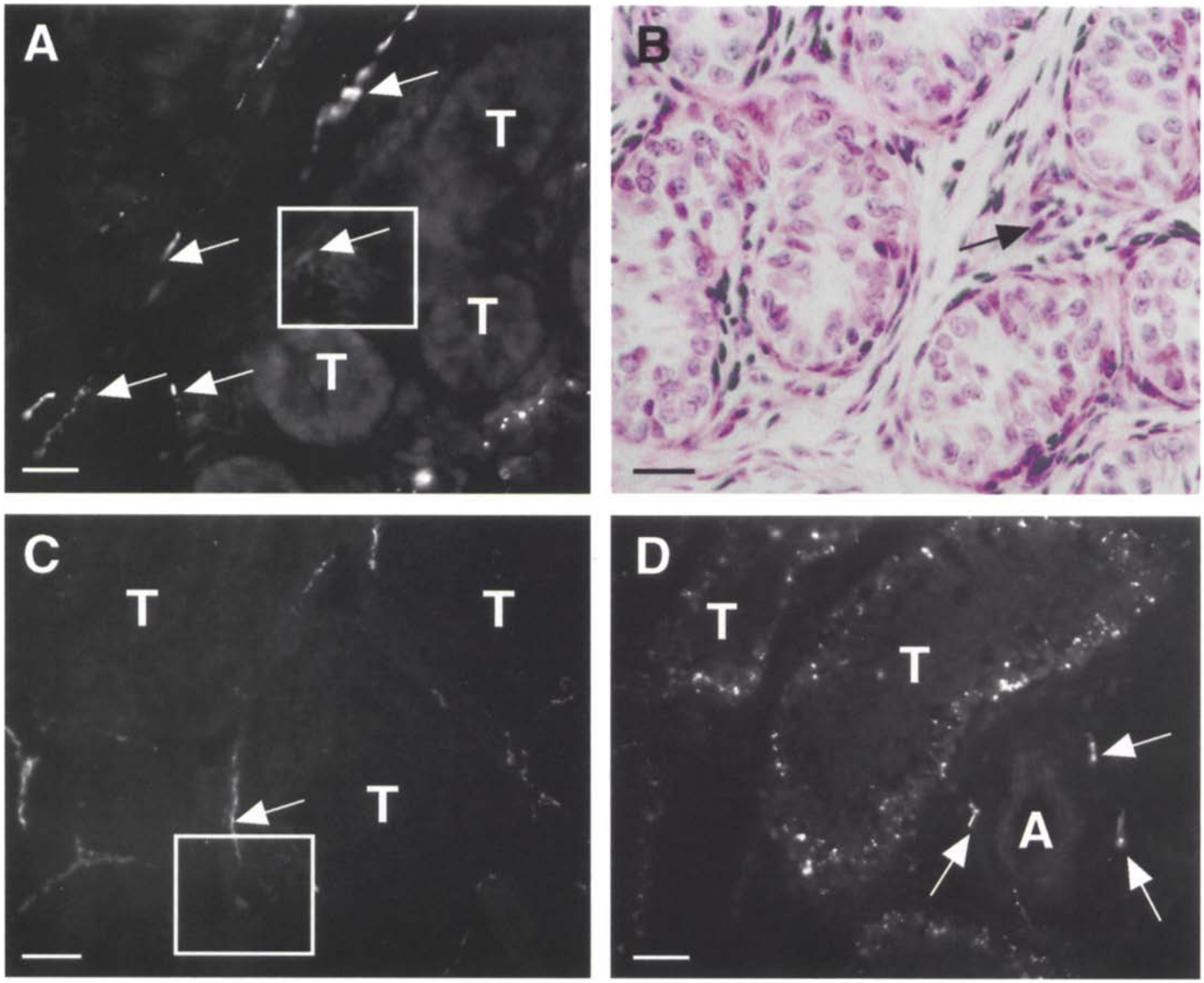


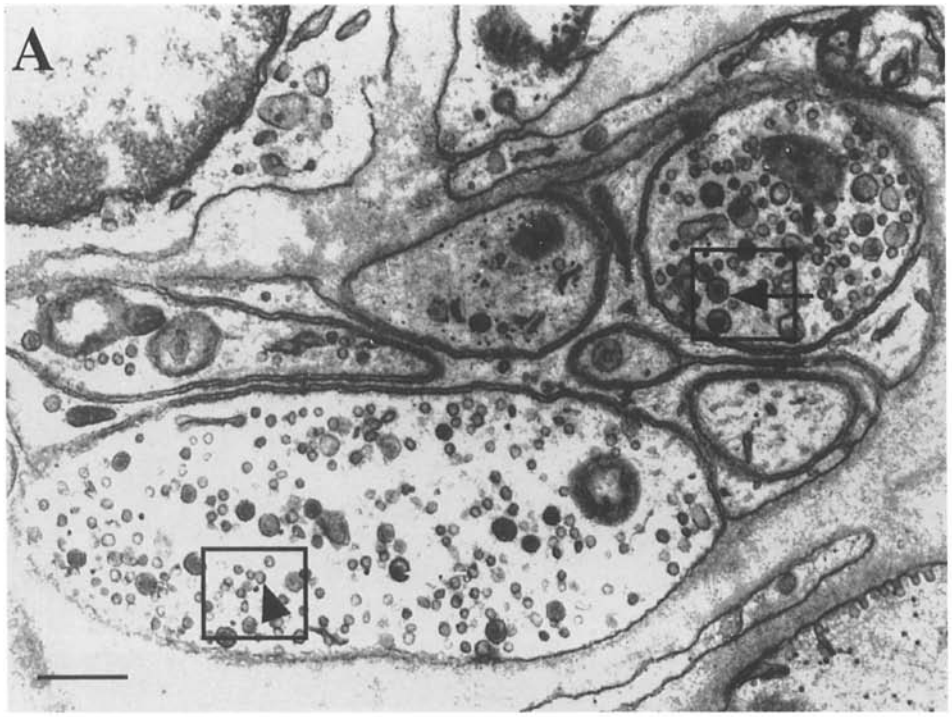
FIG. 1. TH-immunoreactive nerve fibers are more abundant in prepubertal than in adult testes. **A**) In the prepubertal gonad, the fibers transverse the interstitial space (arrows) between the seminiferous tubules (T) and appear to approach clusters of interstitial cells (box). The section shown is from an 11-mo-old monkey. Bar = 18 μm. **B**) Hematoxylin/eosin-stained section of the testis shown in **A**. Note a cluster of Leydig cells/Leydig cell precursors in the interstitial space (arrow). Bar = 30 μm. **C**) A similar profile is observed when catecholaminergic fibers are identified by glyoxylic acid-induced fluorescence. The section depicted is from the testis of an animal 2 yr and 1 mo of age. Bar = 20 μm. **D**) In the adult testis, TH-immunoreactive fibers were sparse (arrows) and were confined to the immediate neighborhood of blood vessels, such as arterioles (A). The section shown is from the testis of a 13-yr-old animal. Bar = 12 μm. Note the nonspecific fluorescence of inclusion bodies in the basal portion of the seminiferous epithelium in the adult testis (yellow color in the original preparation). The microphotographs depicted are representative of immunohistochemical reactions performed on testes from five prepubertal and three adult monkeys (TH) and on testes from three prepubertal monkeys and one adult (glyoxylic acid). For additional details about ages of the animals used, see *Tissue Collection* and *Immunohistochemistry* sections.

Testicular NE Concentration Is Lower in Adult Than in Prepubertal Monkeys

The testicular concentration of NE was highest in the prepubertal gonad (NE in ng/g wet testis weight: 1 yr, 945; 1 yr and 8 mo, 488; 2 yr and 1 mo, 586) than in adult testes (11 yr/nonbreeding season, 112; 13 yr/breeding season, 202; 16 yr/breeding season, 67). Combined results for prepubertal and adult animals are presented in Figure 4.

TH Gene Is Expressed in the Monkey Testis

To determine whether the monkey testis has some of the basic molecular components required for catecholamine biosynthesis, experiments were performed to isolate testicular mRNA forms that may encode TH, the rate-limiting enzyme in catecholamine biosynthesis. Since 5′ heterogeneity of the human TH mRNA gives rise to at least four alternatively spliced mRNA forms [34, 44, 45], we selected oligodeoxynucleotide primers complementary to conserved sequences in exon 1 and 3 of the human sequence to amplify potential testicular TH-encoding mRNA forms by RT-PCR. Two identical cDNA clones were isolated and found to have a nucleotide sequence highly homologous (85%) to the TH mRNA type 1 isoform expressed in *Macaca fuscata* [34] (Fig. 5). The testicular sequence was also very similar (85% similarity) to that of a TH cDNA isolated with the same primers from the adrenal gland of *Macaca mulatta* (Fig. 5). Most of the nucleotide substitutions in the



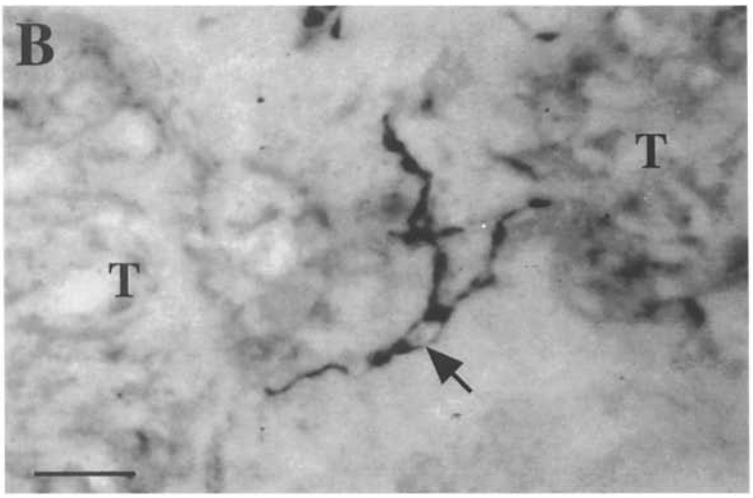


FIG. 2. **A**) Ultrastructural aspect of a cross section through a bundle of unmyelinated nerve fibers in the testis of a rhesus monkey 2 yr and 1 mo of age. The nerve fibers contain pleiomorphic, dense-core (arrow inside box) and clear (arrowhead inside box) vesicles. Bar = 0.3 μm. A similar profile was seen in fibers found in the testes of two other monkeys (one juvenile and one adult). **B**) Immunohistochemical detection (using ABC immunohistochemistry and diaminobenzidine as chromogen) of TH-immunoreactive neuron-like cells bearing processes in the juvenile monkey testis (1 yr and 7 mo of age). The unstained area in the middle of the soma (arrow) corresponds to the nucleus of the cell. Bar = 10 μm.

testicular TH cDNA sequence were conservative, as only 8 of 56 of the encoded amino acids were different from those of rhesus monkey or *Macaca fuscata* adrenal TH (Fig. 5). That the cDNA fragments isolated from testicular total RNA do indeed correspond to a TH mRNA sequence expressed in the testis was indicated by the PCR isolation of exactly the same sequence (two independent clones) from a rhesus monkey testicular cDNA library.

DISCUSSION

The results of this study indicate that the testis of the prepubertal rhesus monkey receives a dual catecholaminergic input: one provided by the extrinsic sympathetic innervation and the other by neuron-like cells located in the interstitial compartment of the gland. Both sources appear to be much more abundant during the prepubertal period than in adulthood. The existence of this particular developmental profile, however, requires quantitative verification, as the dramatic increase in testicular size that accompanies the acquisition of reproductive competence may have prevented us from detecting nerve fibers and neurons in the limited number of sections analyzed.

The prominence of these two regulatory components in the prepubertal testis suggests that the catecholaminergic control of testicular function may be maximally operative during prepubertal development of the primate gonad. Studies in human subjects have revealed that at the end of childhood, serum testosterone levels begin to increase in the face of still unchanged LH levels [1] and that the subsequent increase in LH secretion is accompanied by a much more pronounced increment in testosterone production [1]. Our results in juvenile monkeys, and previous findings demonstrating the ability of catecholamines to potentiate the stimulatory effect of gonadotropins on testicular androgen

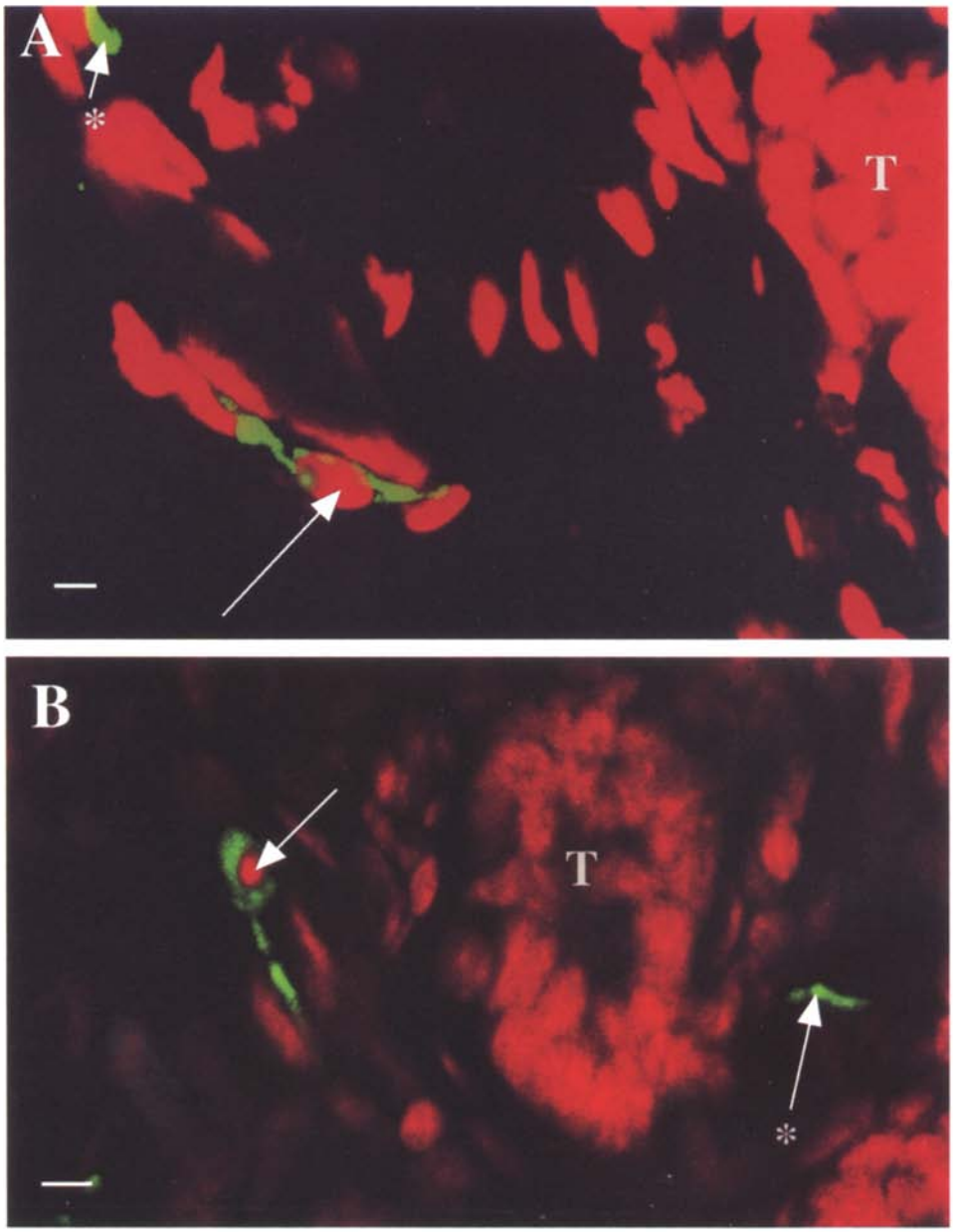


FIG. 3. Identification by confocal microscopy of TH-immunopositive cell bodies bearing processes in the monkey testis. **A**) TH-immunoreactive bipolar cell in the testis of an 11-mo-old monkey. Note the (green) cytoplasmic staining (representing TH immunoreactivity) in both the soma and processes of the cell. The nucleus (arrow) is clearly identifiable. A TH-positive nerve fiber (arrow-asterisk) present in the plane of section is also shown; T: seminiferous tubule. Bar = 4 μm. **B**) The plane of this section through a TH-positive cell in the testis of the same animal is nearly perpendicular to the one shown in **A**. Note also a TH-positive nerve fiber traveling in and out of the plane of focus (arrow-asterisk); T: seminiferous tubule; bar = 5 μm.

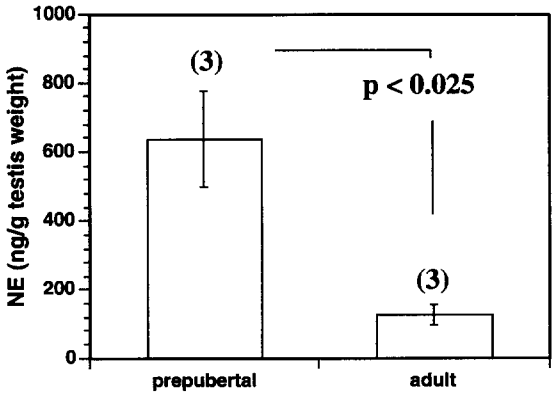


FIG. 4. Testicular concentrations of NE are much higher in prepubertal than in adult rhesus monkeys. Each bar represents the mean ± SEM of the three animals. Individual values for each of these animals are provided in the text.

release in rodents [19, 21, 22], raise the possibility that an enhanced catecholaminergic input to androgen-producing cells may play a role in amplifying the steroidogenic response of the developing gonad to gonadotropins at the initiation of puberty.

Most target cells of the peripheral nervous system are indirectly innervated [47]; that is, rather than reaching the target cell via direct contact or synaptic specializations, neurotransmitters and neuromodulators are released from axonal varicosities (boutons en passant) and diffuse towards the target cells, where they bind to specific receptor molecules. Normally, these varicosities are located more than 150 nm away from the target cell. Ultrastructural evidence [48] indicates that this basic mechanism also operates in the testis, where Leydig cells appear to be a major target cell for catecholamine action. The production of steroids, the LH receptor content, and possibly the development of Leydig cells are affected by catecholamines [19, 20, 26]. In addition to being indirectly innervated, it has been recently shown [48] that Leydig cells in the human testis receive

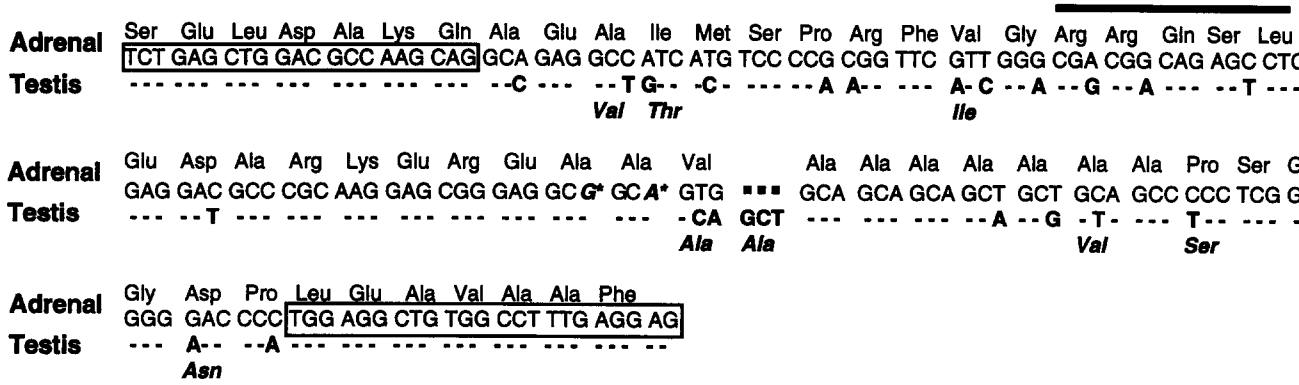


FIG. 5. Nucleotide and amino acid sequences of a cDNA encoding the 5′ end of a testicular form of TH mRNA type 1, isolated by RT-PCR from immature monkey testes. Four independent clones having the same sequence were isolated: two from total RNA and two from a testicular cDNA library. The sequence is compared with that of TH mRNA type 1 isolated from the adrenal gland of *Macaca mulatta*. Nucleotides or amino acids in the testicular sequence that are identical to those in the adrenal sequence are shown as dashes. Letters in bold denote nucleotide or amino acid substitutions in the testicular sequence relative to the adrenal sequence. A 3-nt addition that results in an extra amino acid (Ala) in the testicular sequence is shown by a dotted line. Boxed regions correspond to the deoxyoligonucleotide sequences used for PCR amplification. The sequence of adrenal TH mRNA is identical to that reported for *Macaca fuscata* [34] with the exception of two conservative nucleotide substitutions (denoted by asterisks on the adrenal sequence: G instead of A, and A instead of G). The consensus phosphorylation sequence surrounding Ser-40 is indicated by a heavy overline.

direct, selective innervation by noradrenergic nerve terminals/varicosities, which were found to be only 20 nm apart from the cell membrane. This direct innervation is consistent with previous findings made in other species [8, 9, 49] (see reviews by Setchell et al. [5] and Nistal et al. [50]).

While much is known about the distribution and ultrastructural characteristics of the autonomic innervation of the testis, less attention has been given to the possibility that the degree and pattern of the innervation may be developmentally regulated. One study [50] reported an apparent increase of numbers of axonal varicosities adjacent to myoid cells (which may be another target cell for catecholamines in the testis [51]) in the testes of infants and hypogonadal men as compared to adult, normal men. It has also been reported that testicular catecholamine concentrations may be higher in prepubertal than in adult rats [52] and that immature golden hamster testes have higher intratesticular NE concentrations than adult testes [20, 21]. In harmony with these results, Prince [48] recently described a marked increase in the density of the innervation to the human testis between the neonatal and the prepubertal stage.

In the present study, TH-immunopositive or glyoxylic acid-reactive fibers were always more readily detectable in prepubertal than adult gonads. Although the apparent "loss" of testicular innervation at adulthood may be more related to the large increase in seminiferous tubule size that occurs at puberty in the face of an unchanging density of innervation, the net outcome would still be the same, i.e., a decreased catecholaminergic influence on testicular function. The lower concentration of NE observed in adult compared to prepubertal testes also suggests a decrease in total NE input to the gland. While testicular weight increases 20- to 40-fold between the juvenile period and adulthood, NE concentration decreased 6-fold, indicating that the availability of NE within the gland does not keep up with the massive changes in testicular mass that occur with the advent of sexual maturation. Importantly, there was also an age-related qualitative difference in the distribution pattern of testicular nerves. In immature testes, nerve fibers were always seen associated with interstitial cells. In contrast, nerve fibers in the adult testes were almost always seen in the proximity of blood vessels. Thus, catecholaminergic fibers may more likely influence Leydig cell function in prepubertal animals than in adults. It is possible that after the pubertal activation of testicular function is ended and a new steady-state level is attained, the influence of catecholamines on steroidogenesis decreases but their involvement in modulating testicular blood flow remains [4, 5]. The ultrastructural detection of clear vesicles in nerve fibers of the immature testis indicates that testicular nerves also contain neurotransmitters other than catecholamines. The precise nature of these neurotransmitters and the potential significance of their contribution to testicular homeostasis remain to be elucidated.

An intriguing observation, made during the course of studying the developmental profile of the testicular innervation, was the detection of elongated, mainly bipolar neuron-like elements exhibiting immunoreactivity for TH. That these elements indeed correspond to cell bodies was proven by confocal scanning laser microscopy. A double-labeling technique, which combines staining with the nuclear dye propidium iodide and TH immunohistochemistry, allowed us to unambiguously identify a population of TH-positive cells. These neuron-like cells also appear to contain catecholamines, as judged by their content of glyoxylic acid-derived catecholamine histofluorescence. Interestingly, they were detected only in the immature, prepubertal testes. They may, however, also be present, albeit in lower numbers, in adult testes. The present experiments did not permit us to clearly establish a morphological distinction between nerve fibers derived from the extrinsic innervation and processes extended by intrinsic neuron-like cells. Presumably, most of the nerve fibers seen in the interstitium represent axonal fibers from extrinsic neurons projecting to the testis, whereas the neuron-like cells are single cells with attenuated cytoplasmic processes. Further experiments are required to determine the relative contribution of the extrinsic nerves and the TH-positive neurons to the overall testicular innervation. Our results do not confirm earlier reports showing TH-immunoreactive material in Leydig cells [53, 54]. The reasons for this discrepancy are not apparent, but they may be related to the different antibodies used. In our study, we found TH-like material in a subset of interstitial cells, but these cells were also positive in controls incu-

bated without the TH antiserum or with rabbit serum and did not have a neuronal appearance.

We recently described the presence of a group of TH-expressing neuron-like cells in the rhesus monkey ovary [29]. These cells appear to be more numerous in the ovary and, in contrast to those in the testis, remain detectable throughout adulthood. The density of the ovarian catecholaminergic innervation also increases postnatally and reaches a maximum peripubertally [55], but in contrast to what occurs in the testis, it does not decrease in adulthood. The reasons for these fundamental differences are unclear, but it is possible that catecholaminergic regulatory influences are important for growth and differentiation processes that take place recurrently in the adult ovary but that may occur only during prepubertal development of the testis and predominantly affect Leydig cell function. Inferential support for this concept comes from observations that catecholamines may act as "trophic" factors on testicular cells [56] and that they are able to promote Leydig cell hyperplasia [26]. Clearly, the precise reasons for these developmental sex differences in the catecholaminergic input to the primate gonad remain to be elucidated.

The mechanisms underlying the inability of the dual catecholaminergic innervation to the primate testis to keep up with the massive increase in testicular mass that occurs during sexual maturation are, likewise, unknown. Since the testis is a source of neurotrophins such as nerve growth factor [57, 58] and neurotrophin-4 [59], it is possible that the testicular catecholaminergic system fails to grow at puberty along with the somatic and germinal components of the testis because of a reduction in neurotrophin support.

While the presence of both catecholamine fluorescence and TH-immunoreactive material in testicular neuron-like cells indicates that they are indeed able to synthesize catecholamines, it is still possible that the detection of TH-immunoreactive material is confounded by the presence of another immunologically related protein. Phenylalanine hydroxylase and TH are mixed-function oxydases evolved from a common ancestor gene that share extensive nucleic acid and amino acid homology throughout their central and carboxy terminal regions [60]. This homology, however, is lost at the amino terminus, which encodes the regulatory domain of the enzymes. Cloning of the amino terminus-encoding, 5′ region of TH mRNA by RT-PCR unambiguously demonstrated that the monkey testis does express the TH gene. The authenticity of the sequence was verified by the isolation of four identical cDNAs from either total testicular RNA or a testis cDNA library.

Both humans and monkey TH mRNA exhibit 5′ heterogeneity [34, 61], which in humans has been shown to result in the expression of four different mRNAs [61, 62]. While all four types share the first exon, type 2 has a 12-bp insertion at the end of exon 1; type 3 lacks this insertion, but has an alternatively spliced 81-bp insertion encoded by exon 2 between exons 1 and 3. Type 4 contains both the 12- and the 81-bp insertions. All four variants result from alternative splicing of a single primary transcript [62]. While types 1 and 2 are the predominant forms expressed in brain, the adrenal gland also contains the other two types [61], albeit at lower levels. Most nonhuman primates, including the rhesus monkey, on the other hand, produce only the TH mRNA forms 1 and 2 [45]. Our results indicate that the monkey testis may express only a variant of type 1 TH mRNA; the type 2 isoform may be absent or at levels too low for detection. Unexpectedly, the sequence of the type 1 testicular form differed in 8 amino acids from that isolated from the adrenal gland [63]. This is in contrast to the absolute amino acid sequence homology found between adrenal TH mRNA type 1 from *Macaca mulatta* (Fig. 4) and that of *Macaca fuscata* [34].

Since in both humans and monkeys the 5′ heterogeneity of TH mRNA results from alternative splicing of a single gene [61, 62], it is difficult to explain the substitutions of the testicular sequence as due to expression of a second gene. The isolated nature of the substitutions makes it equally unlikely that the 5′ heterogeneity of testicular TH mRNA is due to additional alternative splicing events. It is conceivable, however, that the substitutions are attributable to RNA editing [64], a process by which the sequence of the mRNA transcript is posttranscriptionally modified by either insertion or deletion of nucleotides [65]. Further studies are necessary to resolve this issue.

It does not appear that the amino acid substitutions observed in the 5′ end of testicular TH mRNA would compromise the short-term regulation of the enzyme's activity. It is known that this short-term regulation is mediated by direct phosphorylation [66] of four sites (Ser-8, Ser-19, Ser-40, and Ser-153) in the N-terminal region of the enzyme [67]. Of these phosphorylation sites, Ser-40 is contained within the sequence encoded by the isolated DNA fragment. The amino acids surrounding this sequence (Arg-Arg-Gln-Ser-Leu) correspond to the consensus sequence (Arg-Arg-X-Ser-Y), known to be a substrate for cAMP-dependent kinase, protein kinase C, and calmodulin-dependent kinase [67]. Thus, at least theoretically, testicular TH is endowed with the same regulatory motifs as brain or adrenal TH.

In summary, the present results indicate that, like the ovary, the primate testis contains extrinsic catecholaminergic nerves and intrinsic nerve-like cells. Both potentially regulatory components, however, are more prevalent—and thus may operate most predominantly—during prepubertal development of the gonad, as few nerves and no neuron-like cells are detected in adulthood. This is in contrast to the situation in ovary, in which the extrinsic nerves and the intragonadal cells are readily detectable throughout adulthood. The fact that ovarian target cells undergo recurrent cycles of growth and differentiation throughout the reproductive life span, whereas testicular interstitial cells do so more prominently only during prepubertal development, suggests that a predominant role of the catecholaminergic input to the primate testis may be to facilitate the functional development of interstitial, androgen-producing cells at puberty.

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REFERENCES

1. Faiman C, Winter JSD. Gonadotropins and sex hormone patterns in puberty: Clinical data. In: Grumbach MM, Grave GD, Mayer FE (eds.), Control of the Onset of Puberty. New York: John Wiley & Sons; 1974: 32–55.
2. Odell WD, Swerdloff RS. Etiologies of sexual maturation: a model system based on the sexually maturing rat. Recent Prog Horm Res 1976; 32:245–288.
3. Boujrad N, Ogwuegbu SO, Garnier M, Lee C-H, Martin BM, Papadopoulos V. Identification of a stimulator of steroid hormone synthesis isolated from testis. Science 1995; 268:1609–1612.
4. Setchell BP. Nerves of the testis and scrotum. In: Finn CA (ed.), The

Mammalian Testis. Reproductive Biology Handbooks. Ithaca, NY: Cornell University Press; 1978: 77–89.
5. Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction, 2nd Edition. New York: Raven Press; 1994: 1063–1175.
6. Rauchenwald M, Steers WD, Desjardins C. Efferent innervation of the rat testis. Biol Reprod 1995; 52:1136–1143.
7. Zhu BC, Chiocchio SR, Suburo AM, Tramezzani JH. Monoaminergic and peptidergic contributions of the superior and the inferior spermatic nerves to the innervation of the testis in the rat. J Androl 1995; 16: 248–258.
8. Baumgarten HG, Holstein AF. Catecholaminhaltige Nervenfasern im Hoden des Menschen. Z Zellforsch Mikroskop Anat 1967; 79:389–395.
9. Okkels H, Sand K. Morphological relationship between testicular nerves and Leydig cells in man. J Endocrinol 1940; 2:38–46.
10. Heindel JJ, Steinberger A, Strada SJ. Identification and characterization of a beta-1-adrenergic receptor in the rat Sertoli cell. Mol Cell Endocrinol 1981; 22:349–358.
11. Anakwe OO, Moger WH. Ontogeny of rodent testicular androgen production in response to isoproterenol and luteinizing hormone in vitro. Biol Reprod 1984; 30:1142–1152.
12. Anakwe OO, Moger WH. Catecholamine stimulation of androgen production by rat Leydig cells: interaction with luteinizing hormone and luteinizing hormone-releasing hormone. Biol Reprod 1986; 35:806–814.
13. Anakwe OO, Murphy PR, Moger WH. Characterization of beta-adrenergic binding sites on rodent Leydig cells. Biol Reprod 1985; 33: 815–826.
14. Poyet P, Labrie F. Characterization of beta-adrenergic receptors in dispersed rat testicular interstitial cells. J Androl 1987; 8:7–13.
15. Cooke BA, Golding M, Dix CJ, Hunter MG. Catecholamine stimulation of testosterone production via cyclic AMP in mouse Leydig cells in monolayer culture. Mol Cell Endocrinol 1982; 27:221–231.
16. Levin J, Lloyd CW, Lobotsky J, Friedrich EH. The effect of epinephrine on testosterone production. Acta Endocrinol (Copenh) 1967; 55: 184–192.
17. Eik-Nes KB. An effect of isoproterenol on rates of synthesis and secretion of testosterone. Am J Physiol 1969; 217:1764–1770.
18. Damber J-E, Janson PO. The effects of LH, adrenaline and noradrenaline on testicular blood flow and plasma testosterone concentrations in anesthetized rats. Acta Endocrinol (Copenh) 1978; 88:390–396.
19. Mayerhofer A, Bartke A, Steger RS. Catecholamine effects on testicular testosterone production in the gonadally active and the gonadally regressed adult golden hamster. Biol Reprod 1989; 40:752–761.
20. Mayerhofer A, Amador AG, Steger RS, Bartke A. Testicular function after local injection of 6-hydroxydopamine or norepinephrine in the golden hamster (*Mesocricetus auratus*). J Androl 1990; 11:301–311.
21. Mayerhofer A, Bartke A, Steger RS, Gow G. Catecholamines stimulate testicular testosterone release in the immature golden hamster via interaction with testicular alpha- and beta-adrenergic receptors. Acta Endocrinol (Copenh) 1992; 127:526–530.
22. Mayerhofer A, Began T, Bartke A. Catecholamines stimulate testicular steroidogenesis in vitro in the Siberian hamster, *Phodopus sungorus*. Biol Reprod 1993; 48:883–888.
23. Mayerhofer A, Amador AG, Steger RS, Shire JGM, Bartke A. Catecholamines and testicular function: a study in BALB/cJ mice, a substrain with high levels of adrenomedullary catecholamine synthesizing enzymes. Neuroendocrinol Lett 1993; 15:417–424.
24. Favaretto ALV, Valenca MM, Hattori M, Wakabayashi K, Antunes-Rodriges J. Characterization of adrenergic control of the Leydig cell steroidogenesis: identification of both stimulatory and inhibitory components. Braz J Med Biol Res 1988; 21:539–543.
25. Frankel AI, Ryan EL. Testicular innervation is necessary for the response of plasma testosterone levels to acute stress. Biol Reprod 1981; 24:491–495.
26. Bergh A, Blom H, Damber JE, Henriksson R. The effect of long-term variation in sympathetic activity on testicular morphology in immature rats. Andrologia 1987; 19:448–451.
27. Gerandai I, Nemeskeri A, Csernus V. Depending on the dose 6-OHDA stimulates or inhibits the testis of immature rats. Exp Clin Endocrinol 1984; 84:27–36.
28. Nagai K, Murano S, Minokoshi Y, Okuda H, Kinutani M. Effects of denervation and local 6-hydroxydopamine injection on testicular growth in rats. Experientia 1982; 38:592–594.
29. Dees WL, Hiney JK, Schultea TD, Mayerhofer A, Danilchik M, Dissen GA, Ojeda SR. The primate ovary contains a population of catecholaminergic neuron-like cells expressing nerve growth factor receptors. Endocrinology 1995; 136:5760–5768.
30. Mayerhofer A, Russell LD, Ojeda SR. The primate testis contains an intratesticular source of catecholamines complementary to its extrinsic innervation. Proc XIIIth Testis Workshop: Cell Mol Regulation of Testicular Cells 1995; 54.
31. Saller CF, Zigmond MJ. A radioenzymatic assay for catecholamines and dihydroxphenylacetic acid. Life Sci 1978; 23:1117–1130.
32. Lara HE, Ferruz JL, Luza S, Bustamante DA, Borges Y, Ojeda SR. Activation of ovarian sympathetic nerves in polycystic ovary syndrome. Endocrinology 1993; 133:2690–2695.
33. Hirst JJ, Haluska GJ, Cook MJ, Hess DL, Novy MJ. Comparison of plasma oxytocin and catecholamine concentrations with uterine activity in pregnant rhesus monkeys. J Clin Endocrinol & Metab 1991; 73: 804–810.
34. Ichikawa S, Ichinose H, Nagatsu T. Multiple mRNAs of monkey tyrosine hydroxylase. Biochem Biophys Res Commun 1990; 173:1331–1336.
35. Mayerhofer A, Russell LD, Grothe C, Rudolf M, Gratzl M. Presence and localization of a basic fibroblast growth factor-like protein in rodent testes. Endocrinology 1991; 129:921–924.
36. Dissen GA, Hill DF, Costa ME, Dees WL, Lara HE, Ojeda SR. A role for *trk*A nerve growth factor receptors in mammalian ovulation. Endocrinology 1996; 137:198–209.
37. Savage RM, Danilchik MV. Dynamics of germ plasm localization and its inhibition by ultraviolet irradiation in early cleavage *Xenopus* embryos. Dev Biol 1993; 157:371–382.
38. de la Torre JC. Standardization of the sucrose-potassium phosphate-glyoxylic acid histofluorescence method for tissue monoamines. Neurosci Lett 1980; 17:339–340.
39. Russell L, Burguet S. Ultrastructure of Leydig cells as revealed by secondary tissue treatment with a ferrocyanide-osmium mixture. Tissue & Cell 1977; 9:751–766.
40. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–159.
41. Lara HE, Hill DF, Katz KH, Ojeda SR. The gene encoding nerve growth factor is expressed in the immature rat ovary: effect of denervation and hormonal treatment. Endocrinology 1990; 126:357–363.
42. Ojeda SR, Hill DF, Katz KH. The genes encoding nerve growth factor and its receptor are expressed in the developing female rat hypothalamus. Mol Brain Res 1991; 9:47–55.
43. Ma YJ, Costa ME, Ojeda SR. Developmental expression of the genes encoding transforming growth factor alpha (TGFα) and its receptor in the hypothalamus of female rhesus macaques. Neuroendocrinology 1994; 60:346–359.
44. O'Malley KL, Anhalt MJ, Martin BM, Kelsoe JR, Winfield SL, Ginns EI. Isolation and characterization of the human tyrosine hydroxylase gene: identification of 5′ alternative splice sites responsible for multiple mRNAs. Biochemistry 1987; 26:6910–6914.
45. Ichinose H, Ohye T, Fujita K, Yoshida M, Ueda S, Nagatsu T. Increased heterogeneity of tyrosine hydroxylase in humans. Biochem Biophys Res Commun 1993; 195:158–165.
46. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463–5467.
47. Burnstock G. Autonomic neuromuscular junctions: current developments and future directions. J Anat 1986; 146:1–30.
48. Prince FP. Ultrastructural evidence of indirect and direct autonomic innervation of human Leydig cells: comparison of neonatal, childhood and pubertal ages. Cell Tissue Res 1992; 269:383–390.
49. van Campenhout E. Les relation nerveuses de la glande interstitielle des glandes genitales des mammiferes. Rev Can Biol 1949; 8:374–429.
50. Nistal M, Paniagua R, Asuncion-Abaurrea M. Varicose axons bearing "synaptic" vesicles on the basal lamina of the human seminiferous tubules. Cell Tissue Res 1982; 226:75–82.
51. Miyake K, Yamamoto M, Mitsuya H. Pharmacological and histological evidence for adrenergic innervation of the myoid cells in the rat seminiferous tubule. Tohoku J Exp Med 1986; 149:79–87.
52. Zieher LM, Debeljuk L, Iturriza F, Mancini RE. Biogenic amine concentration in testes of rats at different ages. Endocrinology 1971; 88: 351–354.
53. Davidoff MS, Schulze W, Middendorf R, Holstein AF. The Leydig cell of the human testis—a new member of the diffuse neuroendocrine system. Cell Tissue Res 1993; 271:429–439.
54. Middendorf R, Davidoff M, Holstein AF. Neuroendocrine marker sub-

stances in human Leydig cells—changes by disturbances of testicular function. Andrologia 1993; 25:257–262.
55. Schultea TD, Dees WL, Ojeda SR. Postnatal development of sympathetic and sensory innervation of the rhesus monkey ovary. Biol Reprod 1992; 47:760–767.
56. Lauder JM. Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci 1993; 16:233–240.
57. Ayer-LeLievre C, Olson L, Ebendal T, Hallböök F, Persson H. Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. Proc Natl Acad Sci USA 1988; 85:2628–2632.
58. Seidl K, Holstein AF. Evidence for the presence of nerve growth factor (NGF) and NGF receptors in human testis. Cell Tissue Res 1990; 261: 549–554.
59. Ip NY, Ibañez CF, Nye SH, McClain J, Jones PF, Gies DR, Belluscio L, Le Beau MM, Espinosa III R, Squinto SP, Persson H, Yancopoulos GD. Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc Natl Acad Sci USA 1992; 89:3060–3064.
60. Ledley FD, DiLella AG, Kwok SCM, Woo SLC. Homology between phenylalanine and tyrosine hydroxylases reveals common structural and functional domains. Biochemistry 1985; 24:3389–3394.
61. Grima B, Lamouroux A, Boni C, Julien J-F, Javoy-Agid F, Mallet J. A single human gene encoding multiple tyrosine hydroxylases with different predicted functional characteristics. Nature 1987; 326:707–711.
62. Nagatsu T. The human tyrosine hydroxylase gene. Cell Mol Neurobiol 1989; 9:313–321.
63. Mayerhofer A, Dissen GA, Pau KYF, Ojeda SR. Novel tyrosine hydroxylase mRNA forms in rhesus monkey testis and ovary. Soc Neurosci Abstr 1994; 20:11.
64. Cattaneo R. Different types of messenger RNA editing. Annu Rev Genet 1991; 25:71–88.
65. Adler BK, Hajduk SL. Mechanisms and origins of RNA editing. Curr Opin Genet Dev 1994; 4:316–322.
66. Pigeon D, Ferrara P, Gros F, Thibault J. Rat pheochromocytoma tyrosine hydroxylase is phosphorylated on serine 40 by an associated protein kinase. J Biol Chem 1987; 262:6155–6158.
67. Campbell DG, Hardie DG, Vulliet PR. Identification of four phosphorylation sites in the N-terminal region of tyrosine hydroxylase. J Biol Chem 1986; 261:10489–10492.