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# Neuronal Elements in the Testis of the Rhesus Monkey: Ontogeny, Characterization and Relationship to Testicular Cells

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**Key Words**
Catecholamines · Gonads · Mast cells · Primates · Peripheral neuroendocrinology · Testis

**Abstract**
Intrinsic neuron-like cells expressing the catecholamine-biosynthetic enzyme tyrosine hydroxylase (TH) were recently identified in the testis of the prepubertal rhesus monkey. In this study, we characterized the neuron-like nature of these cells and examined distribution and frequency of neuronal elements in the testes of monkeys during postnatal development, puberty and adulthood. Using immunohistochemical methods, we detected both nerve fibers and cell bodies, immunoreactive for the neuronal markers neurofilament 200 (NF-200) and synaptosomal associated protein of 25 kDa (SNAP-25), TH and neuropeptide Y (NPY) in perivascular locations, intermingled with interstitial cells and close to the wall of seminiferous tubules. Marked age-related differences in the numbers of these neuronal elements became apparent, when we quantified NF-200-immunoreactive neuronal elements. Thus, intrinsic neuron-like cell bodies were found only in the testes from immature animals (i.e., until about 3 years of age). Conversely, nerve fibers, presumably representing mainly the extrinsic innervation, were observed at all ages although they became more prominent after the pubertal increase in LH and testosterone levels. Interestingly, another testicular cell type known to contain potent regulatory substances, mast cells, was found to be in close anatomical proximity to nerve fibers. The number of these cells, positively identified with an antibody to tryptase, increased significantly after puberty following the same pattern as nerve fibers. These results confirm that the testicular nervous system of the monkey is composed of two components, intrinsic nerve cells and extrinsic fibers, both of which are catecholaminergic and peptidergic in nature. Furthermore, both components show a marked degree of plasticity during development, especially around the time of puberty. The intratesticular locations of neuron-like cells and fibers suggest that catecholamines and neuropeptides are likely to have multiple sites of actions, and may affect Leydig cells, cells of the tubular wall and vascular cells directly and/or indirectly via intermediation of mast cells.



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## Introduction

In addition to the crucial role of gonadotropins for the regulation of testicular function, there is growing evidence that other factors participate in the control of gonadal functions. These factors include growth factors and neurotransmitters, in particular the catecholamines norepinephrine (NE) and epinephrine (EPI) [see summary and additional references in 1, 2]. NE and EPI are able to activate catecholaminergic receptors present on Leydig cells, Sertoli cells and smooth muscle cells in the testis [3–7]. The consequences are, as shown in vivo and in vitro, changes in steroid production and LH receptors of Leydig cells [cf. 6, 8–12], increased cyclic adenosine monophosphate (cAMP) and lactate formation of Sertoli cells [cf. 5, 13] or altered smooth muscle/vascular tonus [cf. 3]. Whether other somatic cells of the testis, e.g. mast cells, are among the targets for catecholamines is not known, but studies in other organs have indicated such a possibility [14–16]. Since the mast cell products histamine and serotonin affect testicular steroidogenesis, this aspect appears worth being investigated [17–20]. Despite these well-established actions of catecholamines, the significance of testicular catecholamines for the in vivo regulation of testicular function is not clear. To have physiological meaning, it would be required that these substances can be delivered in sufficiently high levels close to their receptor-bearing testicular targets. Several routes are possible: Adrenal catecholamines can travel from the adrenal medulla via the bloodstream to their targets in the testis and/or catecholamines can be released from primarily catecholaminergic nerve fibers present in the testis [3, 4, 21–24]. Compared to the bloodstream, the innervation may allow a more precise way of delivery neurotransmitters to target cell in the testis. Indeed testicular nerve fibers form 'synapses en passant' with Leydig cells and peritubular cells in the human testis [25–27], implicating release of neurotransmitters into the interstitial space in the close neighborhood of the targets. Moreover, testicular innervation, appears also to be important for a functional direct link between testes and brain [cf. 28].

Another, as yet little examined potential source of catecholamines exists in the interstitial spaces of the rhesus monkey testis, which contains a population of phenotypically elongated neuron-like cells, immunoreactive for tyrosine hydroxylase (TH), the rate-limiting enzyme for catecholamine biosynthesis [29]. Moreover, the gene for TH is expressed in the monkey testis and similar cells were found in the ovary of the monkey [30, 31], lending further support for the existence of these cells at least in primates. This cell type may act alone and/or in concert with the extrinsic sympathetic innervation of the testis. In contrast to the ovary, the testicular TH cells were described to exist mainly in prepubertal monkey testes and were not found in adult testes in a previous study [29]. Whether the cell expressing TH are indeed of neuronal nature is not clear. To address this point, we therefore examined if they possess other neuronal proteins. We also examined the distribution and frequency of the neuronal elements (cell bodies and fibers) in the testes of monkeys during postnatal development, puberty and adulthood using immunohistochemical techniques. Special attention was paid to the anatomical relationship between neuronal elements and testicular cells including mast cells.

## Materials and Methods

*Tissue Collection*

All tissues were obtained from animals involved in other unrelated studies. For our study, the testes were obtained from rhesus monkeys *(Macaca mulatta)* aged 100–300 days (infantile group, n = 6), 1–2 years (juvenile group, n = 7), 3–4 years (peripubertal group, n = 6), 6–8 years (adult group, n = 5). The animals were cared for by the Oregon Regional Primate Research Center in accordance with the NIH Guide for the Care and Use of Laboratory Animals. They were housed in a 12L:12D photoperiod (i.e., 12 h of light per day) and fed twice daily with Purina monkey chow; fresh fruit was also provided daily and drinking water was made available ad libitum. They were painlessly killed using an overdose of ketamine and pentobarbital, according to procedure established by the Panel on Euthanasia of the American Veterinary Society. For the purpose of this study, the testes were fixed for at least 48 h in Bouin's fluid, followed by 70% ethanol, and then embedded in paraffin wax. Sections (5 μm) were prepared for immunohistochemistry. Serum was aliquoted and stored at –20°C until assayed for LH and testosterone.

*Hormone Assays*

Bioactive LH concentrations were measured in serum samples using a previously reported mouse Leydig cell bioassay [32], which could detect as little as 3 ng LH/ml using the cynomologus LH RP-1 as the reference preparation. Testosterone concentrations in the serum were measured by radioimmunoassay (RIA) as previously described [33]. The antiserum used shows 67% cross-reactivity with dihydrotestosterone, but less than 4% with other steroids.

*Immunohistochemistry*

*Avidin-Biotin-Peroxidase (ABC) Method.* The testicular distribution of NF-200, TH, NPY, synaptosomal associated protein of 25 kDa (SNAP-25) and mast cells tryptase was examined in monkey testes using an ABC-immunohistochemical method as described previously [31, 34, 35]. Specific antibodies were employed (mouse monoclonal antibody anti-NF-200: Boehringer Mannheim Inc., Indianapolis, Ind., USA; dilution 1:50; rabbit polyclonal antibody anti-TH: Chemicon International Inc., Temecula, Calif., USA, dilution 1:400; rabbit polyclonal antibody anti-NPY: Peninsula Laboratories Inc., Belmont, Calif., USA, dilution 1:70,000; mouse monoclonal

antibody directed against a synaptosomal associated protein of 25 kDa, SNAP-25: Sternberger Monoclonals Inc., Baltimore, Md., USA, dilution 1:500; mouse monoclonal antibody antitryptase: Dako, Hamburg, Germany, dilution 1:50). Sections incubated in buffer (without primary antibody) or sections incubated with buffer containing mouse or rabbit normal serum, respectively, served as controls for all samples.

*Double Staining: A Combination of ABC and Immunogold-Silver Methods.* Co-location of TH-immunoreactive fibers and tryptase-positive mast cells was assayed by a double staining immunohistochemical method described originally by van der Loos and Becker [36]. Commercially available antibodies (rabbit polyclonal antibody anti-TH: Chemicon International Inc.; mouse monoclonal antibody anti-tryptase: Dako, Denmark) were employed. In brief, sections were first deparaffinized, endogenous peroxidase reactivity was quenched by a 10-min pretreatment with 10% methanol, 0.3% $H_2O_2$ in 0.01 *M* phosphate-buffered saline (PBS, pH 7.4), and residual aldehyde groups present after aldehyde fixation were inactivated by pretreatment with 0.05 *M* glycine and 0.1% $NaBH_4$ in PBS for 15 min. The cells were permeabilized by a 5-min incubation with 0.5% saponin in PBS and nonspecific proteins were blocked by subsequent incubation with protein block buffer (5% bovine serum albumin, 0.1% fetal calf serum and 5% goat normal serum in PBS) for 30 min. After several wash steps the incubation with a TH antisera (1:100) and tryptase antibody (1:50) diluted in incubation buffer (0.2% BSA-acetylated: Biotrend, Cologne, Germany; 20 m*M* $NaN_3$ in PBS, pH 7.4) was carried out overnight in a humidified chamber at 4°C. The second day, testicular sections were washed and incubated with biotinylated secondary antisera (goat anti-rabbit IgG preabsorbed against human proteins; 1:500 diluted: Camon, Wiesbaden, Germany) and a gold conjugate reagent (goat anti-mouse, gold particle diameter 0.8 nm: Immuno Gold Aurion, Wageningen, The Netherlands, 1:100 diluted) for 2 h at room temperature. The specimens were postfixed in 2% glutaraldehyde in PBS and enhanced by silver stained (R-GENT, Biotrend) followed by a commercial ABC kit (Vectastain, Camon, Burlingame, Calif., USA). TH immunoreaction was visualized with 0.01% $H_2O_2$ and 0.05% 3,3-diaminobenzidine (DAB) solution (in 0.05 *M* Tris-HCl, pH 7.6). For control purposes the first antiserum was omitted. Sections were examined with a Zeiss Axiovert microscope (Oberkochen, Germany) with a 400× magnification using a combination of epipolarization for immunogold-silver and transmitted light for ABC staining that allow to visualize both reaction products simultaneously.

*Quantification of NF-200-Immunoreactive Elements and Tryptase-Positive Mast Cells*

Because the monoclonal antibodies against the neuronal marker NF-200 and the tryptase mast cells produced a strong staining without significant background staining, we used these antibodies to evaluate the frequency of neuronal elements (single nerve fibers, nerve fiber bundles and cell bodies) and mast cells in monkey testes during the postnatal development and sexual maturation. The number of NF-200-immunoreactive structures and tryptase-immunopositive cells were quantified with a Leica microscope with a 250× magnification and a gridded eyepiece. In each testicular section, 4–5 fields were evaluated for the presence of immunoreactive neuronal elements or mast cells and for the seminiferous tubules sectioned. The results were expressed as immunoreactive nerve fibers/tubule, immunoreactive neuron-like cells/tubule and immunoreactive mast cells/tubule as described previously [see 37]. Results obtained were statistically analyzed using ANOVA and Fisher PLSD test. Data were expressed as mean ± SEM. $p < 0.05$ was considered significant.

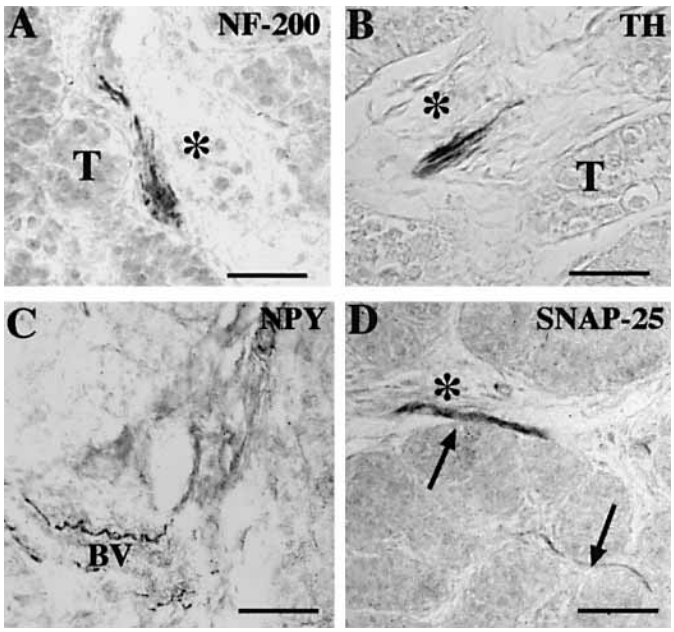


**Fig. 1.** Nerve fibers in the monkey testis. **A** NF-200-immunoreactive nerve fiber bundle in the interstitial space of a monkey testis. Note that interstitial cells (asterisks) and seminiferous tubules (T) are in close proximity (age 30 months, bar ca. 40 μm). **B** Similar area as in **A**: TH-immunoreactive nerve fiber bundle is seen (age 38 months, bar ca. 40 μm). **C** Example of perivascular (BV, blood vessel) and interstitial nerve fibers immunoreactive for NPY (age 38 months, bar ca. 60 μm). **D** Two SNAP-25-immunoreactive nerve fibers of different sizes are seen in the testis of a 16-month-old monkey (bar ca. 50 μm).

## Results

*Testicular Catecholaminergic/Peptidergic Nerve Fibers in the Testis*

Nerve fibers immunoreactive for NF-200, TH and NPY were identified in the interstitial spaces and within the neighborhood of the seminiferous tubules and blood vessels at all ages studied. This staining was robust and observed in all sections (fig. 1A–C). In contrast, the SNAP-25 antibody was less robust (i.e. not detected in every section) but specific staining was seen in the same locations in some of the sections (fig. 1D). Mast cells immunoreactive for tryptase were seen in the same areas as nerve fibers (not shown). Double immunocytochemical staining methods allowed to clearly identify close anatom-

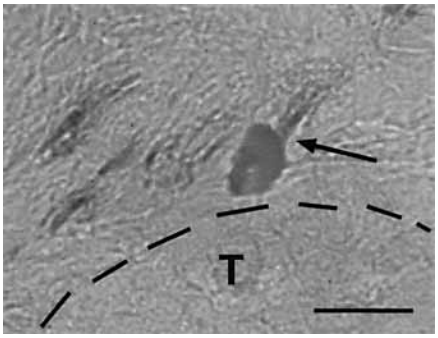


**Fig. 2.** Anatomical proximity between nerve fibers and mast cells. Immunohistochemical detection of a mast cell (antitryptase; epipolarization) in close contact with TH-immunoreactive nerve fibers (arrow) (16 months, bar ca. 30 μm); T, seminiferous tubule. Note that the original color of the mast cell was blue, the color of the TH fibers brown.

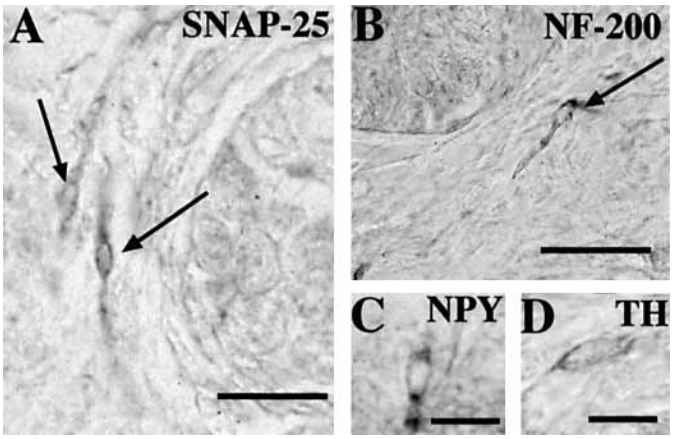


**Fig. 3.** Identification of neuron-like cells in monkey testes. **A** The arrows point to the unstained nuclei of elongated bipolar cells showing cytoplasmatic SNAP-25 immunoreactivity. The cells are located in the interstitial space of a testicular section (age 16 months, bar ca. 30 μm). **B** A small NF-200-immunoreactive neuron-like cell (arrow) is seen in the interstitial space in close association with interstitial cells of a testicular section (age 16 months, bar ca. 30 μm). **C** Example of a cell expressing NPY (interstitial space of 38-month-old monkeys, bar ca. 15 μm). **D** Immunoreactive cell body expressing TH (interstitial space of 46-month-old monkeys, bar ca. 15 μm).

ical proximity between mast cells (tryptase-positive) and nerve fibers (TH-positive) (fig. 2).

*Catecholaminergic/Peptidergic Neuron-Like Cells*

In the interstitium of seven testes of immature monkeys (infantile and juvenile animals, from birth to about 3 years old, and in one case of a 46-month-old monkey), but not those from adult monkeys, we found elongated cells with a mainly bipolar and occasionally multipolar phenotype, which stained with specific antibodies against SNAP-25, NF-200, NPY and TH (fig. 3A–D). These neuron-like cells were similar in phenotype to the TH-positive cells previously described and identified by confocal scanning laser microscopy analysis in the prepubertal monkey testis by Mayerhofer et al. [29].

*Developmental Pattern of Nerve Fibers, Neuron-Like Cells and Mast Cells – Correlation with Hormone Levels*

The changes in serum LH and testosterone levels are indicated in figure 4A. Thus a marked increase of LH levels was found at 3–4 years of age. Levels remained constant during the adult period. Circulating testosterone levels were significantly elevated in the adults. The number of nerve fibers/tubule and neuron-like cells/tubule, was studied by immunohistochemistry using a monoclonal antibody against NF-200 in one set of slides from all animals (fig. 4B). The number of nerve fibers/tubule was unchanged from birth to 3 years of age (infantile and juvenile groups), then exhibited a significant rise (peripubertal group), and a subsequent gradual increase during adulthood. After birth until about 2 years of age the number of neuron-like cells/tubule remained nearly constant, however after about 3–4 years of age, these neuronal cells were not detected in the monkey testis. The number of mast cells/tubule remained constant in the infantile and juvenile groups, but exhibited a significant increase at peripubertal time (3–4 years of age), and adulthood (fig. 4C).

## Discussion

In the rat testis, NPY and NPY receptors have been detected in the tunica albuginea and around the intracapsular blood vessels [24, 38–40] and NPY has profound effects on testicular blood flow [41]. Furthermore, it has been demonstrated that monoamines and neuropeptides can coexist in nerve fibers of the rat testis [24, 42, 43]. To our knowledge, however, in monkey testes neither innervation nor neuron-like cells have been well characterized, although TH-positive fibers and cells have previously been described [29] and were also found in this study. The present study indicates moreover that both the extrinsic nerve fibers and the intrinsic neuron-like cells contain SNAP-25, a protein present in synaptic vesicles, chromaf-

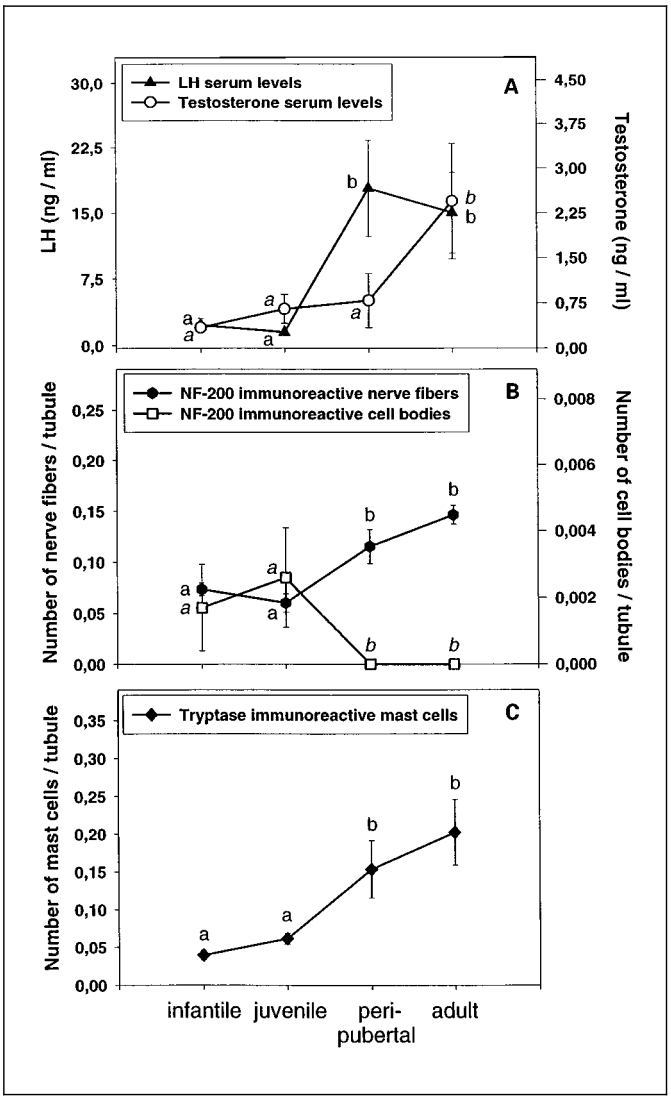


**Fig. 4.** Summary of hormone levels, frequency of testicular neuronal elements and testicular mast cells during ontogeny. Graphic representation of the hormone values (**A**) and frequency of NF-200-positive neuronal elements (**B**), as well as mast cells (**C**) in corresponding testicular sections. Immunohistochemical results were obtained from one set of randomly selected slides from 24 monkeys (infantile group: age 100–300 days, n = 6; juvenile group: age 1–2 years, n = 7; peripubertal group: age 3–4 years, n = 6; adult group: age 6–8 years, n = 5) immunostained for NF-200 and tryptase. Results shown represent means ± SEM. Different letters denote statistically significant differences between groups ($p < 0.05$).

fin vesicles and in neuronal plasmalemma [44–46], and also NF-200, an intermediate filament specific for neurons. Moreover, at least some interstitial and perivascular nerve fibers and neuron-like cells also contain NPY. The fact that electron-dense vesicles coexist with clear vesicles in varicosities of nerve fibers in the monkey testis [29] indicates that catecholamines and peptides, as well as other yet unidentified neurotransmitters, are present in monkey testicular nerve fibers and presumably also in testicular neuron-like cells.

To determine whether testicular neuronal elements change during development, a point raised by our previous finding [29], the presence of NF-200-immunopositive nerve fibers (single fibers and fiber bundles) and neuron-like cells was evaluated in one randomly selected set of testicular sections. Immunoreactive structures and seminiferous tubules in the same viewing area were counted and results expressed per seminiferous tubule, as described previously [37]. The evaluation of larger sections or whole testes was not possible, but since the number (in contrast to the diameter) of tubules in the testes presumably is the same in young versus adult testes, this approach provided an appropriate semiquantitative way of evaluating neuronal elements. The data indicate that the number of testicular nerve fibers significantly increases throughout life. On the other hand, neuron-like cells were detectable only in immature gonads, but not in the adult testes, suggesting an unexpected neuronal plasticity during sexual maturation (i.e., despite the apparent loss of neuron-like cells, an overall enrichment of the testis by neuronal elements with age was apparent). Although previously the ontogeny of neuronal elements in the testis was not studied in detail, there are some reports which hint at changes in nerve fiber density and changes in the levels of testicular catecholamines. Namely, a marked increase in the number of testicular nerve fibers was observed during the neonatal and prepubertal period in the human testis by Prince [27]. Also, alterations in the testicular content of catecholamines during sexual development of humans and rodents were noted by Zieher et al. [47] and by Mayerhofer et al. [11]. It is plausible that these previous results reflect changes in the composition and function of the gonadal nervous system, suggesting that testicular neuronal plasticity may also occur in nonprimate species.

The plasticity observed in our study occurred both at the level of neuron-like cells, which disappeared, and at the level of nerve fibers, which increased with age. However, it is important to bear in mind that the fibers observed could be processes of intrinsic neuron-like cells or branches of extrinsic fibers, an issue which can presently not be resolved. Our data also do not allow us to draw conclusions to the reason(s) and the mechanism(s) underlying the observed plasticity. High levels of androgens present only after puberty in the testis might have

caused neuronal cell death, as described in certain brain areas [48]. Alternatively, the state of activation of intratesticular neuron-like cells and/or neurons residing outside the testis (presumably in the para-aortic ganglia and possibly pelvic and accessory ganglia [see 24]) may change around puberty. Consequently, secretory products like neurotransmitters, neuropeptides and components of the secretory machinery may not be visible any more close to the nucleus, but rather in the processes of neuron-like cells. It is plausible that we therefore did not detect immunoreactive cell bodies of testicular neuron-like cells using immunohistochemical staining methods. Neurotrophic factors (nerve growth factor, NGF [cf. 49–52]) or other growth factors [50] may be involved in such an activation process. Especially in neuron-like cells in the monkey ovary [30] and in sympathetic nerve fibers of human testis [49] receptors for NGF were described. The striking correlation between testicular neuronal changes and the onset of puberty in the present study may be indicative of a hormone dependency, possibly involving LH. In neurons of the fetal and adult rat brain, which express LH receptors [53], LH or hCG acted as a neurotrophic factor in vitro and promoted neurite outgrowth [53]. Assuming that neuron-like cells of the testis may contain LH receptors, the peripubertal rise in LH might have activated testicular neuron-like cells. Additional studies are required to clarify this point.

The role of neuronal elements and the one of neurotransmitters released from these structures in the testis is far from being understood. The pure presence of the testicular neuronal elements in immature monkey testes, i.e. during a time when the pituitary-gonadal axis is not yet functional, implies however involvement in as yet unknown testicular functions or in testicular development. In other systems, growth-promoting effects of neurotransmitters are established [cf. 54, 55] and for example in the ovary, catecholamines and the neuropeptide vasoactive intestinal peptide (VIP) induce functional maturation of small follicles and expression of receptors for FSH [56]. The increase of nerve fibers after puberty leads to an enrichment of neuronal elements in the testicular interstitium in the adult testis and suggests a different role of neuronal elements now. That substances, such as catecholamines and neuropeptides in general can affect testicular cells in the absence and in the presence of pituitary hormones, is supported by a number of studies [9–13, 29, 57–59]. Documented close anatomical proximity between neuronal elements and testicular cells (Leydig cells, cells of the tubular wall and vascular cells [cf. 25–27]) in vivo, make testicular cells direct targets of catecholamines and neuropeptides and provide further evidence for a functional link. It is possible that while all Leydig cells in the adult may be subjected to regulation by LH, some, the ones contacted by neuronal element in particular, may be subjected to dual regulation, one by pituitary LH and one by catecholamines and/or neurotransmitters.

While little is known about testicular neuronal elements, even less is known about testicular mast cells and their function. It is thought that progenitor cells migrate to peripheral tissues including the testis and undergo proliferation and differentiation to typical mast cells apparently under the influence of local factors [60]. Importantly in this context is that neurotransmitters, neuropeptides and NGF are able to induce proliferation, but also degranulation of mast cells as well as induction of expression of cytokines [61–64]. On the other side, mast cells have been shown to be a source of NGF and produce neuropeptide-degrading proteases [65, 66]. These results indicate a mutual interaction between neuronal elements and mast cells.

We found that not only the testicular distribution of neuronal elements and mast cells were strikingly similar, but also demonstrated close anatomical proximity between mast cells and catecholaminergic nerve fibers in monkey testes, similar to a relation in skin and gut [14–16]. In addition, both the number of mast cells and the number of nerve fibers in monkey testes increased after puberty and are thus inversely related to the number of neuron-like cells. Previous reports described age-dependent increases in the number of testicular mast cells occurring around puberty in hamster, rat and human testes [20, 25, 67], and mast cells and Leydig cells, which developed simultaneously after chemical destruction of Leydig cells in the rat [67]. Mast cells produce biologically highly active substances, including proteases, cytokines, histamine and serotonin, which can be released in response to different stimuli [19, 67]. For some of these substances a possible role in the testis was shown. Thus, histamine and serotonin can affect Leydig cells and regulate testosterone production [17–20]. The present paper clearly indicates that at least the anatomical prerequisites for interactive mutual paracrine influences to occur between testicular neuronal elements on one side, mast cells and Leydig cells on the other side exist in the monkey testis.

In summary, our study presents evidence that the testis of the rhesus monkey contains catecholaminergic/peptidergic extrinsic nerves and intrinsic neuron-like cells, as well as mast cells, all of which undergo marked age-related changes during postnatal development and sexual maturation. Although the mechanisms governing these changes

are currently unexplored, these results in conjunction with a host of data from in vitro and in vivo studies, imply that neuronal signals may act directly and/or indirectly, e.g. via mast cells, to regulate testicular function and development.


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