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Segment-Specific Decrease of Both Catecholamine Concentration and Acetylcholinesterase Activity Are Accompanied by Nerve Refinement in the Rat Cauda Epididymis During Sexual Maturation

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ABSTRACT: In the present work, histochemical and biochemical studies were conducted to analyze changes in the pattern of autonomic innervation during sexual maturation, using the rat epididymis as a model. Glyoxylic acid histochemistry and immunohistochemical studies against dopamine β-hydroxylase (DβH) and acetylcholinesterase (AChE) indicated a reduction in the amount of catecholaminergic and AChE-positive neurons, fibers, and *puncta* detected in the cauda epididymis of adult rats (120 days old), when compared to immature (40 days) and young adult (60 days) animals. No obvious age-related variations were detected in the few catecholaminergic and AChE-positive fibers and *puncta* present in the caput region. AChE-positive fibers were found sorting out among epithelial cells and ending free upon the epithelial surface or into the tubular lumen of the cauda region of adult rats. Furthermore, a positive staining for AChE in epithelial cells was also detected in the caput and cauda epididymis in all ages studied. Biochemical analysis confirmed a significant decrease in noradrenaline concentration as well as AChE activity in the cauda epididymis with sexual maturation. Immunohistochemical studies against microtubule-associated protein 1B (MAP 1B), a neuronal cytoskeletal marker, further substantiated the quantitative changes observed in catecholaminergic and AChE-positive neuronal elements in the cauda epididymis. Thus, our results documented segment-specific variations in noradrenaline concentration and AChE activity during epididymal sexual maturation and suggest that such variations result, at least in part, from the refinement of the autonomic innervation pattern with age.

Key words: Autonomic innervation, male reproductive tract, development, neurons, age.

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The epididymis contains a plexus of autonomic nerves that arise from the inferior mesenteric, major pelvic (Hodson, 1970), and pelvic accessory ganglions (Ricker et al, 1996, 1997). These nerves are in close association with smooth muscle fibers, epithelial cells, and vasculature (Baumgarten et al, 1968; Nouhouayi and Négulesco, 1985). Such anatomical relationships, together with pharmacological and physiological data (Hib, 1976; Laitinen and Talo, 1981; Pholpramool and Triphrom, 1984), suggest that the major role of the adrenergic and cholinergic innervation in adult animals is to control excurrent duct system contraction, sperm transport through it, and blood flow (Baumgarten et al, 1968; Kuwahara and Frick, 1974; Damber et al, 1982; Billups et al, 1990; Santamaria et al, 1995). Epithelial exo/endocytotic events and ionic exchange between cellular and luminal compartments are other processes that are influenced by autonomic innervation in adult animals (Mayerhofer et al, 1992; Chan et al, 1994; Kempinas et al, 1995; Lamano-Carvalho et al, 1996; Zhu et al, 1998).

Studies using both surgical and guanethidine-induced denervation have shown that the decreased contractility, observed in the rat epididymis with the loss of adrenergic innervation, induces a delay in cauda luminal transit, with a significant increase in the number of spermatozoa present in the cauda epididymis (Billups et al, 1990; Ricker

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et al, 1996; Kempinas et al, 1998a,b). The consequences of the loss of innervation to the quality of sperm are, however, contradictory. Billups et al (1990) reported changes in sperm motion parameters after removal of the rat inferior mesenteric ganglion. Ricker et al (1996) also found significant decreases in the fertility of cauda epididymis sperm 1 and 4 weeks after surgical denervation. Kempinas et al (1998a,b), on the other hand, observed that either surgical or chemical sympathectomy, in this case induced by a low level of guanethidine exposure, resulted in a prolonged transit time of the sperm within the epididymis, with no effects on the quality and fertility of the sperm collected from the distal cauda epididymis.

Pharmacological and surgical denervation experiments have also documented the importance of autonomic, and especially adrenergic, innervation for sustaining both the normal growth pattern and the rate of development of testes and other male reproductive organs (Nagai et al, 1982; Gerendai et al, 1984, 1989; Bergh et al, 1987; Zhu et al, 1998; Chow et al, 2000). In fact, autonomic innervation is necessary to maintain mature ovary (Burden and Lawrence, 1977; Gerendai et al, 1978; Burden et al, 1981, 1983) and testicular functional and structural integrity (Hodson, 1965; Nagai et al, 1982; Bergh et al, 1987; Lamano-Carvalho et al, 1996; Zhu et al, 1998; Chow et al, 2000). It has also been suggested that autonomic innervation is even required for the process of gonadal sex determination and/or differentiation in some vertebrate species (Gutiérrez-Ospina et al, 1999). Taken together, these observations suggest that the autonomic innervation might control cell differentiation and development throughout the male reproductive tract.

In the present work, we combined histochemical and biochemical techniques to illustrate how the epididymal innervation pattern might change during the rat sexual maturation. We focused our study at this developmental period because of our interest in understanding the possible role of neuron–target-cell reciprocal trophic interactions on male reproduction and fertility.

Materials and Methods

Animals

Male Wistar rats of 40 (immature), 60 (young adults), and 120 days (adult) of age were used. Animals were housed in the Animal Facility at Instituto Nacional de Farmacologia (INFAR), Universidade Federal de São Paulo-Escola Paulista de Medicina (UNIFESP-EPM), and kept on a 12-hour light:12-hour dark lighting schedule, at 20°C, with food and water ad libitum. These ages were chosen on the basis of the testosterone plasma levels of each animal: 0.42 plus or minus 0.07 (40 days), 1.61 plus or minus 0.19 (60 days), and 1.92 plus or minus 0.27 ng/mL (120 days) (Queiróz et al, 2001). Animal procedures were performed using the guidelines for the care and use of laboratory animals, approved by the Research Committee from UNIFESP-EPM. All animals were either euthanized or perfused at the same hour to avoid circadian variation in catecholamine content (Reuss et al, 1999). Rat body weight was determined. The epididymides were removed; dissected on an ice-chilled plate, freed of fat; and sectioned into 3 segments: the caput, corpus, and cauda. The caput and cauda were used in our experiments.

Glyoxylic Acid Histochemistry

Anesthetized rats were placed on an ice tray and perfused through the left ventricle with ice-cold saline, followed by an ice-cold phosphate-buffered (61 mM, pH 7.4) fixative solution containing paraformaldehyde (0.5%) and glyoxylic acid (2%). The caput and cauda epididymis were each isolated, dissected, and frozen each in dry-ice prechilled 2-methyl butane. The tissue samples were then cryostat cut (20 μm), mounted onto silane-coated slides, and incubated (60 seconds) in an ice-cold phosphate-buffered solution containing glyoxylic acid (2%) for 1 minute. Slides were air dried and placed in an oven at 100°C for 10 minutes. The slides were coverslipped with glycerol and visualized using a Zeiss epifluorescence microscope (Carl Zeiss, Jena, Germany) with a 395- to 440-nm excitation filter.

Immunohistochemistry

The caput and cauda epididymides from each rat were dissected, embedded in Jung tissue freezing medium (Leica Instruments, Nussloch, Germany), rapidly frozen in dry-ice–prechilled 2-methyl butane, and stored at −75°C until use. Cryostat caput and cauda epididymis transverse sections (8 μm) were fixed in formalin (4%) in phosphate buffer (0.1 M, pH 7.4) for 30 minutes. The sections were then incubated with blocking solution (albumin 3% and Triton X-100 0.3% in phosphate buffer 0.1 M, pH 7.4) for 1 hour at room temperature. After several washes with phosphate buffer, sections were then incubated with primary goat polyclonal antibodies raised against rat dopamine β-hydroxylase (DβH), acetylcholinesterase (AChE) (1:25 each, Santa Cruz Biotechnologies, Santa Cruz, Calif), and microtubule-associated protein 1B (MAP 1B 1:500, Santa Cruz Biotechnologies) diluted in blocking solution, overnight at 4°C. Following three 10-minute washes in blocking solution, sections were incubated for 90 minutes at room temperature with rabbit anti-goat secondary antibody conjugated to biotin (1:200) diluted in blocking solution. An avidin-biotin complex (ABC) staining system (Santa Cruz Biotechnologies) was used to localize the biotinylated antibody according to manufacturer's instructions. Peroxidase activity was revealed by using a phosphate buffer containing 3,3-diaminobenzidine (0.05%) and hydrogen peroxide (0.01%) for 3 minutes at room temperature. The enzyme reaction was stopped by washing several times in phosphate buffer. Air-dried slides were then coverslipped with entellan. Controls for DβH and AChE immunohistochemistry included preadsorption of the primary antibody for 2 hours with a fivefold excess of the corresponding blocking peptide (Santa Cruz Biotechnologies). Thus, the immunostaining obtained with the preadsorbed antibody was always compared to the nonpreadsorbed primary antibody in serial sections, in order to analyze specific staining. Negative controls, in the absence of the primary antibody, were also processed. Regional differences in the inten-

sity of staining in epithelial cells, being nonexistent in the efferent ducts, intermediate in the initial segment and caput region, and highest in the cauda epididymis at all ages analyzed, were observed in experiments done in the absence of primary and secondary antibodies. The incubation of an excess of unlabeled avidin (1 μg) for 90 minutes before detection of peroxidase activity in these experiments prevented the epithelial staining, indicating a nonspecific epithelial staining associated with the ABC. The sections were visualized with a Nikon E800 microscope (Nikon, Melville, NY). Images were processed using Image-Pro Express Software Program (Media Cybernetics, Silver Spring, Md).

High-Performance Liquid Chromatography for Monoamine Determination

High-performance liquid chromatography (HPLC) for detection of monoamines (noradrenaline and adrenaline) was carried out according to the protocol described by Cavalheiro et al (1994). Briefly, the caput and cauda epididymis from rats of different ages were dissected on an ice-chilled plate, snap frozen in liquid nitrogen, weighted, and stored at −75°C until use. The tissue samples were ultrasonically homogenized (15 μL/mg tissue) in a solution containing $HClO_4$ (0.1 M), $Na_2S_2O_5$ (0.02%), and dihydroxybenzylamine (0.7 μM), the latter used as a monoamine internal standard. Samples were then centrifuged at 11 000 × *g* at 4°C for 40 minutes, and the supernatant was filtered and injected into the HPLC system. An isocratic system consisting of an LKB pump, a clamper, and a column oven fitted with a rheodyme loop injector (20 μL) was used. Electrochemical detection of monoamines was performed with an LKB detector with an electrode potential of −0.5 V. An LKB 2-channel recorder was used, and the chromatographic peak height was measured. An OD-224 Spheri-5 RP-18 (220 by 4.6 mm) column (Brownlee Precision Co, San Jose, Calif) with a flow rate of 0.8 mL/min was used. The phosphate/citrate buffer (0.02 M, pH 2.64) mobile phase contained methanol (90/10 [vol/vol]), disodium EDTA (0.12 mM), and heptanesulfonic acid (0.06%). Standard monoamine mixtures were injected at the beginning and end of each set of experiments to control the performance of the system. Monoamine recoveries and epididymal concentration calculations obtained after acid treatment were made as described by Mazzacoratti et al (1990). Chromatograms were computer recorded, and the peak height was measured. Results were expressed in picograms of monoamines per total tissue weight or per milligram of tissue.

Extraction of Total AChE and Enzyme Activity Assay

The caput and cauda epididymis were dissected, snap frozen in liquid nitrogen, weighted, pooled in a tube, and stored at −75°C until use. The tissue samples were homogenized in 1 mL of borate extraction buffer (20 mM, pH 9.0) containing NaCl (1 M), EDTA (5 mM), Triton X-100 (0.5%), *n*-ethylmaleimide (5 mM), benzamidine (2 mM), and bacitracin (0.7 mM), with an Ultra-Turrax homogenizer (T-25, Ika Labortechnik, Stanfeni, Germany). Each homogenate was centrifuged for 30 minutes (20 000 × *g*, 4°C), and total AChE activity from the supernatant was assayed by a radiometric procedure (Johnson and Russell, 1975), as described by Rotundo and Fambrough (1979), using 0.1 μCi [^{3}H]-acetylcholine iodide (2.0 GBq/mmol, 55.2 μCi/mmol, New England Nuclear, Boston, Mass) as substrate. The enzyme activity was assayed in the presence of 10 μM butyrylcholinesterase inhibitor tetraisopropyl pyrophosphoramide (Iso-OMPA; Sigma Chemical Co, St Louis, Mo), and the total AChE activity (dpm/min) was calculated as arbitrary units (AU). Results were expressed as AU per total tissue weight or per milligram of tissue.

Statistics

Data were expressed as mean plus or minus standard error of the mean. Statistical analysis was determined by analysis of variance, followed by Bonferroni multiple range analysis, using the Instat program (GraphPad Software, San Diego, Calif). *P* values less than .05 were accepted as significant.

Results

Glyoxylic Acid Histochemistry and DβH, AChE, and MAP 1B Immunohistochemical Studies

In the caput region, DβH immunohistochemistry (Figure 1a and b) and glyoxylic acid histochemistry (data not shown) indicated very few catecholaminergic nerve fibers and *puncta*, mostly associated with blood vessels and smooth muscle fibers. Qualitative observations demonstrated no obvious variations in the number of these elements in the caput epididymis with increasing age (Figure 1a and b). Although DβH immunohistochemistry revealed no major differences in the number of catecholaminergic fibers and *puncta* between 40- and 60-day-old rat cauda epididymis (data not shown), the abundance of these elements decreased significantly in the cauda epididymis of adult animals when compared to immature rats (Figure 1c and d). Glyoxylic acid histochemistry followed a similar profile (Figure 1e and f). Catecholaminergic innervation was more abundant in the cauda (Figure 1c through f) than in the caput epididymis (Figure 1a and b) in all ages analyzed. The DβH-positive staining observed in the caput and cauda epididymis was blocked when experiments were performed in the presence of the respective blocking peptide (Figure 1, inserts).

AChE-positive neurons, nerve fibers, and *puncta* were identified in the caput epididymis interstitial space (Figure 2a and b). Qualitative observations demonstrated no obvious variations in the few numbers of AChE-positive staining in the caput, as animals matured (Figure 2a and b). Although no major differences were observed in the number of neurons, fibers, and *puncta* positively labeled for AChE when 40- and 60-day-old rat cauda epididymides were compared (data not shown), a significant reduction in the amount and intensity of these AChE-positive neuronal elements occurred in the adult cauda epididymis when compared to immature rats (Figure 2c and d). The number and density of staining of AChE-positive

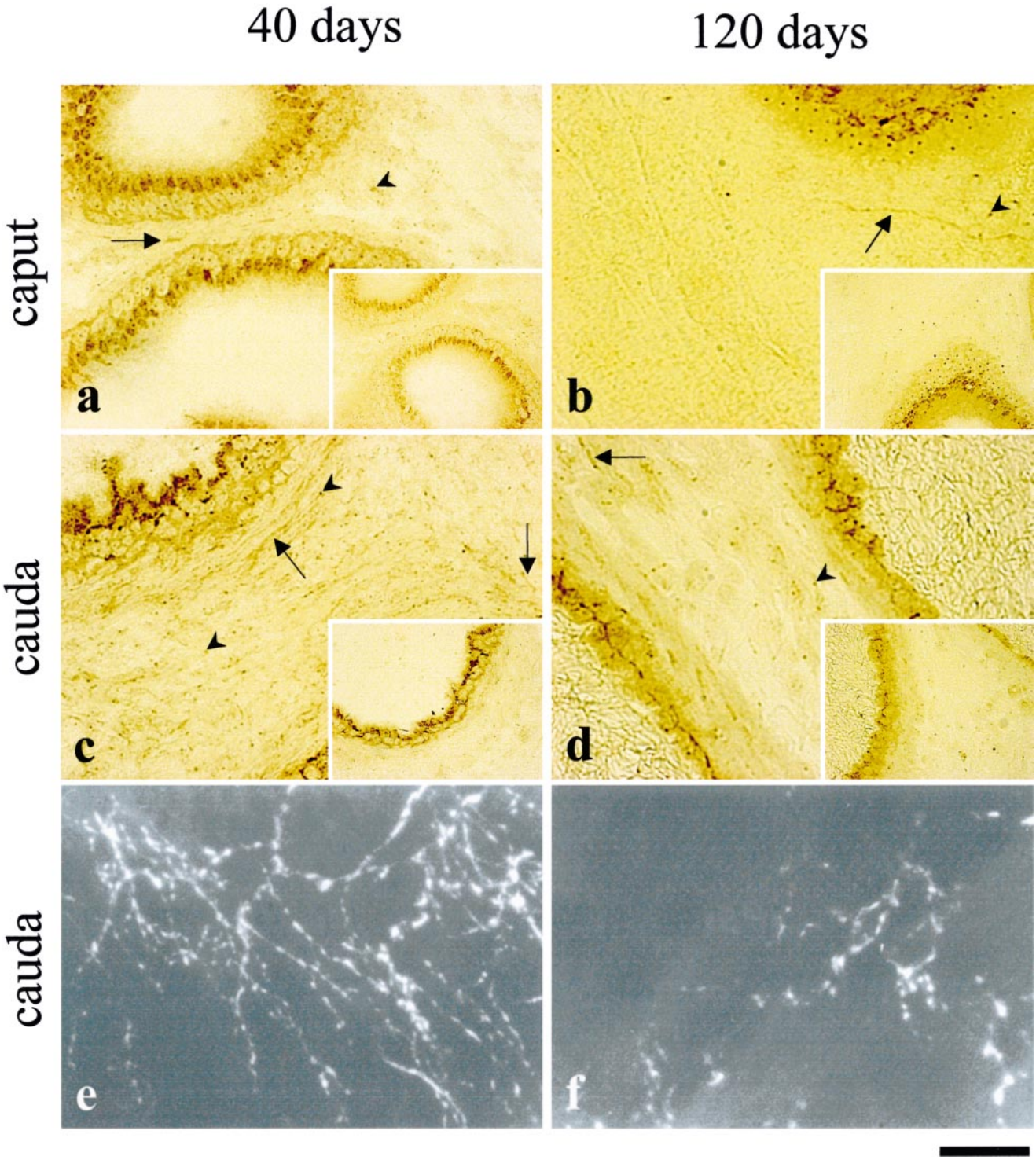
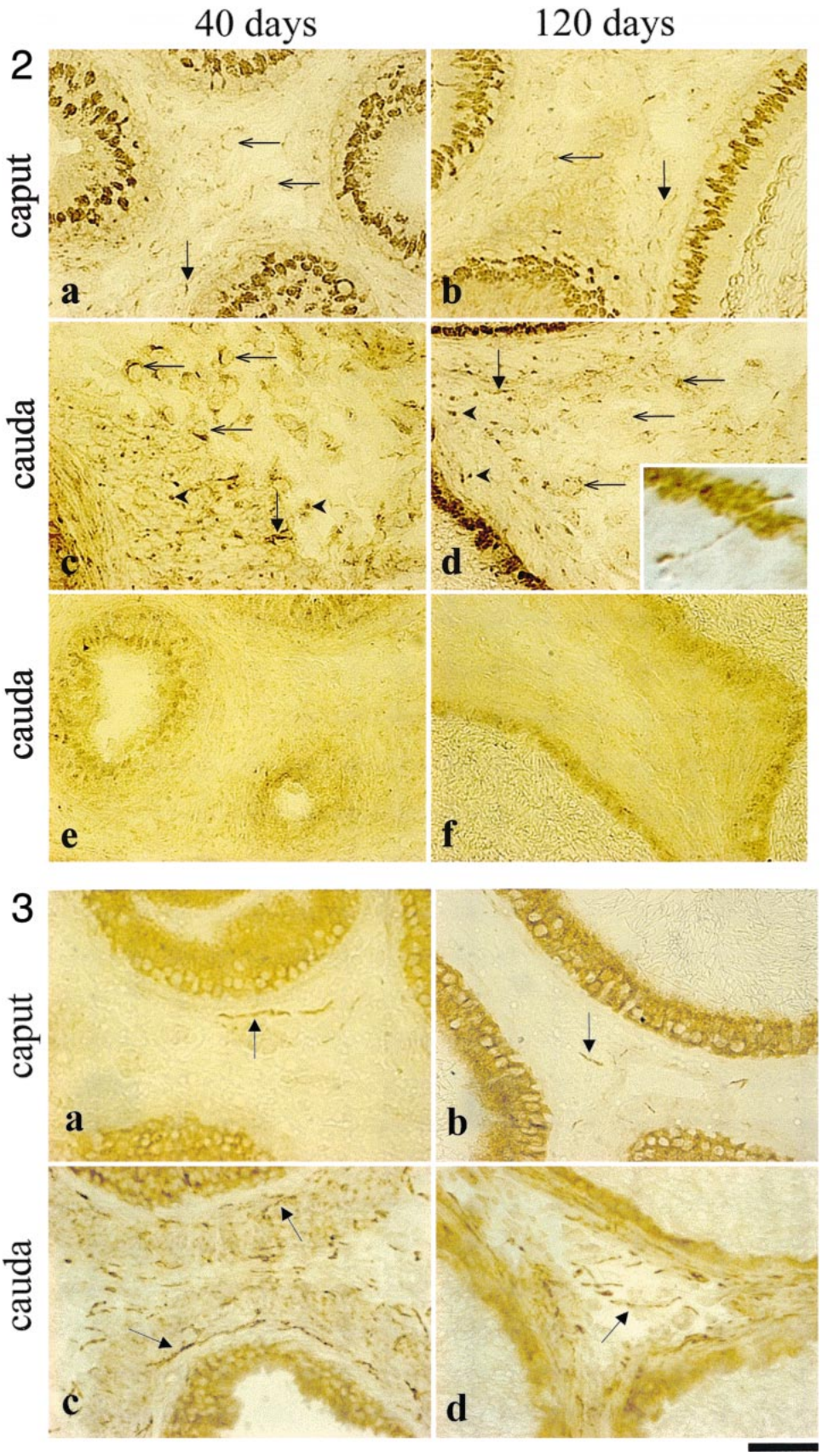


Figure 1. Representative immunohistochemistry localization of dopamine β-hydroxylase (DβH) and glyoxylic acid histochemistry in the rat epididymis. Immunohistochemical localization of DβH is shown in the caput **(a)** and cauda **(c)** epididymis from 40-day-old rats and in the caput **(b)** and cauda **(d)** from 120-day-old rats. Controls, in the presence of blocking peptide, were performed for the immunohistochemical detection of DβH (inserts). Glyoxylic acid histochemistry is shown in the cauda epididymis from 40- **(e)** and 120-day-old rats **(f)**. Arrows and arrowheads indicate labeling of nerve fibers and *puncta,* respectively. Scale bar = 50 μm.


40 days
120 days
2
caput
cauda
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a
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c
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3
caput
cauda
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neuronal elements were greater in the cauda (Figure 2c and d) than in the caput epididymis (Figure 2a and b) in all ages analyzed.

Numerous AChE-positive nerve fibers were found sorting out among epithelial cells and ending free upon the epithelial surface or into the tubular lumen in the cauda region of adult animals (Figure 2d, insert). AChE-positive staining of epididymal epithelial cells was currently observed in both the caput and cauda epididymis of 40-, 60-, and 120-day-old rats (Figure 2). All the AChE-positive staining observed in the rat epididymis was blocked when experiments were performed in the presence of the respective blocking peptide (Figure 2e and f).

Immunohistochemical studies against MAP 1B, a neuronal cytoskeletal marker, were used to identify neuronal processes in the caput and cauda epididymis of maturing animals (Figure 3). The amount of fibers labeled in the caput (Figure 3a and b) was lower than that in the cauda epididymis (Figure 3c and d) regardless of animal age. MAP 1B immunoreactive fibers in the caput epididymis did not change with progression of sexual maturation. However, qualitative observations indicated that the amount of MAP 1B-positive elements in the cauda epididymis did not change from 40- to 60-day-old rats (data not shown) but decreased in the adult rats when compared to younger animals (Figure 3c and d).

Rat Body and Tissue Weight

The effect of sexual maturation on rat body weight and on caput and cauda epididymis wet weight is shown in Table 1. Rat body weight significantly increased with age. Caput and cauda epididymis wet weight also increased significantly with progression of sexual maturation.

Monoamine Determination in the Caput and Cauda Epididymis of Sexually Maturing Rats

The caput and cauda epididymis presented a gradual increase in noradrenaline content, expressed per total tissue weight, with sexual maturation (Table 2). Noradrenaline concentration, expressed per milligram of tissue, did not change in the caput epididymis with increasing age. Although there was no difference in noradrenaline concentration between 40- and 60-day-old rats, a marked decrease in noradrenaline concentration occurred in the cauda region of adult rats (Table 2). Noradrenaline, either expressed per total tissue weight or per milligram of tissue, was higher in the cauda than in the caput epididymis in all ages studied (Table 2).

Table 1. *Effects of sexual maturation in the rat body weight and in the wet weight of the caput and cauda epididymis from 40-, 60-, and 120-day-old rats**

Age, d	Rat Body Weight, g	Tissue Wet Weight, mg	
		Caput	Cauda
40	106.0 ± 1.1 a†	31.1 ± 1.4 a	20.5 ± 2.1 a
60	206.0 ± 2.9 b	120.8 ± 8.9 b	68.9 ± 6.3 b
120	306.4 ± 15.3 c	279.6 ± 13.9 c	286.6 ± 20.8 c

* Data are expressed as mean plus or minus standard error of the mean from 5 experiments.

† Different letters in the same column indicate significant differences with age ($P < .05$).

Adrenaline was detected in the caput and cauda epididymis of 40-, 60-, and 120-day-old rats within the low range of 12.67–58.33 pg/mg tissue, indicating that noradrenaline is the main catecholamine in both regions of the rat epididymis.

AChE Activity in the Caput and Cauda Epididymis of Sexually Maturing Rats

The caput and cauda epididymis presented an increase in AChE activity per total tissue weight with sexual maturation (Table 2). When results were expressed per milligram of tissue, AChE activity showed a biphasic profile in the caput epididymis since the activity increased from 40- to 60-day-old rats and then dropped from 60- to 120-day-old rats to similar values observed in the immature animals. In the cauda epididymis, on the other hand, there was a significant progressive decline of AChE activity with increasing age. AChE activity was higher in the cauda than in the caput epididymis, regardless of animal age (Table 2).

Discussion

Previous studies have shown that peripheral innervation exerts trophic effects on the development, maturation, structural maintenance, and function of peripheral targets (reviewed in Purves, 1988). Furthermore, various neurotransmitters display a variety of trophic effects, when available during developmental stages, in various organs including the genital tract (Buznikov et al, 1999). Ac-

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Figure 2. Representative immunohistochemical detection of acetylcholinesterase (AChE) in the caput **(a)** and cauda **(c)** epididymis from 40-day-old rats and in the caput **(b)** and cauda **(d)** from 120-day-old rats. Controls, in the presence of blocking peptide, were performed for the immunohistochemical detection of AChE in the cauda from 40- **(e)** and 120-day-old rats **(f)**. AChE-positive fibers ending free upon the tubular lumen are shown in the cauda region **(d)** (see insert). Closed arrows, opened arrows, and arrowheads indicate labeling of nerve fibers, neuronal *somata,* and *puncta,* respectively. Scale bar = 50 μm.

Figure 3. Representative immunohistochemical detection of microtubule-associated protein 1B (MAP 1B) in the caput **(a)** and cauda **(c)** epididymis from 40-day-old rats and in the caput **(b)** and cauda **(d)** from 120-day-old rats. Arrows indicate labeling of nerve fibers. Scale bar = 50 μm.

Table 2. *Determination of noradrenaline and acetylcholinesterase activity in the caput and cauda epididymis from 40-, 60-, and 120-day-old rats*†*

Tissue	Age, d	Noradrenaline		Acetylcholinesterase Activity	
		pg/Total Tissue	pg/mg Tissue	AU/Total Tissue	AU/mg Tissue
Caput	40	5.3 ± 0.1 [a‡]	189.7 ± 4.9	18.1 ± 1.8 [a]	0.59 ± 0.05 [a]
	60	15.3 ± 1.9 [b]	172.7 ± 21.1	123.1 ± 4.5 [b]	1.04 ± 0.08 [b]
	120	29.1 ± 3.3 [c]	135.3 ± 14.4	158.6 ± 11.0 [c]	0.57 ± 0.03 [a]
Cauda	40	151.9 ± 6.5 [a]	5107.0 ± 354.0 [a]	132.9 ± 7.6 [a]	6.7 ± 0.7 [a]
	60	238.8 ± 34.4 [a]	3852.3 ± 505.3 [a]	218.8 ± 20.2 [b]	3.2 ± 0.2 [b]
	120	658.6 ± 56.6 [b]	3231.0 ± 309.9 [b]	426.0 ± 27.7 [c]	1.5 ± 0.1 [c]

* Data are expressed as mean plus or minus standard error of the mean from 3–5 experiments.
† AU indicates arbitrary units of enzymatic activity.
‡ Different letters indicate significant differences among ages in the same tissue ($P < .05$).

cordingly, published evidence suggests that autonomic innervation might be required for cell differentiation, development, and structural integrity of various male reproductive tract organs (Hodson, 1965; Nagai et al, 1982; Gerendai et al, 1984, 1989; Bergh et al, 1987; Zhu et al, 1998; Gutiérrez-Ospina et al, 1999; Chow et al, 2000). To evaluate this possibility further, it is necessary to know how the adult innervation pattern arises, as well as to document possible changes in the availability of nerve-derived trophic molecules (eg, catecholamines), during critical times of sexual maturation. Using the rat epididymis as a model, changes in the pattern of the autonomic innervation during the rat sexual maturation were analyzed in the present study.

Glyoxylic acid histochemistry and immunohistochemical studies against DβH and AChE indicated that catecholaminergic and AChE-positive neuronal elements were more abundant in the cauda than in the caput epididymal region, as previously described (El-Badawi and Schenk, 1967; Baumgarten et al, 1968). Furthermore, qualitative observations demonstrated a sensible reduction in catecholaminergic and AChE-positive neurons, fibers, and *puncta* detected in the cauda epididymis of 120-day-old rats when compared to immature (40 days) and young adult (60 days) animals. No obvious variations in the few catecholaminergic and AChE-positive fibers and *puncta* were observed in the caput region with age.

In accordance with the results obtained with glyoxylic acid histochemistry and immunohistochemical studies, a decrease in the cauda epididymis noradrenaline concentration, as well as in the AChE activity as rats mature sexually, was observed. No obvious shifts in these parameters were observed in the caput epididymis with age, suggesting that noradrenaline and AChE maturational variations are segment-specific. These results support the hypothesis that autonomic nerve remodeling through trophic factor occurs in the cauda epididymis with sexual maturation.

A reduction with age in the number of innervating adrenergic fibers has been reported in other organs of the male reproductive tract of rats (Zieher et al, 1971), macaques (Mayerhofer et al, 1996), and humans (Baumgarten et al, 1968). The results of the present work add, however, that such maturational changes also affect the cholinergic system. It is important to emphasize that, although most AChE-positive staining observed in the present work is generally associated with cholinergic innervation, AChE-positive fibers can also be related to noncholinergic nerves (Papka et al, 1981, 1985), such as neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI), calcitonin gene-related peptide (CGRP), and substance P-immunoreactive, reported to be present in the cauda epididymis (Lamano-Carvalho et al, 1986). It is worth mentioning that the immunohistochemical studies performed in the present work with the antibody against MAP 1B, a cytoskeletal protein expressed in nerve cells only while remodeling (Schoenfeld et al, 1989; Viereck et al, 1989), also indicated a reduction in the number of MAP 1B-positive nerves in the cauda epididymis. Thus, the results suggest that nerve refinement might also occur in other types of fibers reaching the epididymis or in fibers rising from intrinsic neurons.

The development of the epididymis can be divided into 3 distinct postnatal phases: a proliferative phase, in which undifferentiated cells undergo mitotic activity (days 0–15); a period of differentiation when the blood epididymal barrier is formed, and the columnar cells differentiate into principal cells (days 16–44); and a phase of expansion in which spermatozoa enter the epididymis and are stored in the lumen of the cauda epididymis (days 44–91) (Sun and Flickinger, 1979, 1982). Histological and functional differentiation of the caput during postnatal development precedes that of the cauda epididymis (Rajalakshmi, 1985; Limanowski et al, 2001), mainly due to varying dependence on testicular fluid and the age-dependent and segment-specific role of testosterone during epididymal development (Sun and Flickinger, 1982; Viger and Robaire, 1994). Our results suggest that the age-related reduction in the cauda noradrenaline concentration and

AChE activity might be associated in part with a decrease in the number of cauda autonomic neuronal elements. Possible, however, is the involvement of a nerve fiber dilution effect associated with the process of growth of the epididymal structure on these observations. Interestingly, the cauda epididymal weight increased 14-fold between immature and adult animals, while noradrenaline concentration and AChE activity decreased 1.5- and 4.5-fold (or increased 4.3- and 3.2-fold per total tissue weight), respectively. Thus, these results indicate that the availability of noradrenaline and AChE within the tissue does not keep up with the changes in epididymal mass that occur with progression of sexual maturation. Although the biochemical data appear to support the existence of segment-specific remodeling of autonomic innervation through afferent elimination, changes in catecholamine and acetylcholine synthesis and/or degradation as animals mature sexually can not be ruled out. Both processes are not mutually exclusive.

Androgen concentration in epididymal tissue extracts is high relative to plasma, especially in the caput epididymis (Vreeburg, 1975; Pujol et al, 1976; Turner et al, 1989). The circulating levels of testosterone modify in different ways the monoaminergic activity in the central and peripheral nervous systems (Battaner et al, 1987; Siddiqui and Shah, 1997; Kritzer, 2000). It is then plausible to raise the possibility that increased testosterone availability induces epididymal autonomic, especially adrenergic, nerve refinement. Against this possibility, however, is the fact that testosterone serves as a potent trophic signal for the somas of pelvic ganglion neurons supplying adrenergic innervation to vas deferens, urinary bladder, and rectum and for cholinergic neurons supplying innervation to the penis, vas deferens, and prostate gland (Keast and Saunders, 1998). In our study, no major differences were observed in the number of catecholaminergic fibers and the concentration of monoamines when 40- and 60-day-old rat cauda epididymides were compared, although the testosterone levels in the plasma of these animals significantly increased during this period (Queiróz et al, 2001).

In 120-day-old rats, numerous AChE-positive fibers were found sorting out among epithelial cells and ending free upon the epithelial surface or into the tubular lumen of the cauda region. These findings closely resemble those previously reported for histochemically stained AChE in the adult dog and the rat epididymis (El-Badawi and Schenk, 1967). Although the role of these nerves is currently unknown, they have been thought to serve sensory functions (El-Badawi and Schenk, 1967). They might also be the source of minute amounts of acetylcholine that could in part explain the presence of cholinergic receptors (Florman and Storey, 1982; Ward et al, 1994; Baccetti et al, 1995), as well as AChE activity in spermatozoa (Egbunike, 1980). Immunohistochemical studies also revealed the localization of AChE in the epithelial cells of the caput and cauda epididymis in all ages studied. There is speculation in the literature that acetylcholine might be metabolized by an epithelium-dependent AChE activity in guinea pig airways (Small et al, 1990; Koga et al, 1992; Folkerts et al, 2001). Further experiments will be necessary to confirm if the presence of AChE in the epididymal epithelium compartment is correlated with enzyme activity.

Why reduce autonomic innervation during cauda epididymis sexual maturation? We have no definitive answer to this fundamental question. Increased availability of catecholamines at least in the adult testis has deleterious effects on the germinal epithelium growth and differentiation (Chow et al, 2000). In fact, a possible relation between increased number of catecholaminergic neural elements and testicular pathologies has been suggested (Mayerhofer et al, 1999). Also, El-Badawi and Schenk (1967) discussed the inverse relation between innervation density and epithelial cell secretory function. Autonomic innervation growth-promoting actions might then be restricted to certain points along epididymal development. Also, decreased innervation may improve secretory processes in the maturing cauda epididymis, while the lack of innervation may keep ongoing secretion high in the caput epididymis.

It is known that neurotrophic factor-dependent peripheral nerve elimination is a common process during maturation (for a review, see Purves, 1988). In this regard, it would be instructive to evaluate whether the expression of target-derived neurotrophic signals by cells within the reproductive organs (eg, neurotrophins; Russo et al, 1999) covary with the amount of innervation they receive at different times during sexual maturation. Also, it is known that neurotrophic signals may have deleterious effects on the development of neuronal processes depending on the type of the neuron and the time that developing cells and cell factors interact with one another (McAllister et al, 1999). Epididymal autonomic nerve retraction might thus also result from ‘‘negative’’ interactions with neurotrophic signals.

Finally, the present work and previous data (Lamano-Carvalho et al, 1986; Lakomy et al, 1997) show that catecholaminergic, cholinergic, and peptidergic innervation is mainly concentrated in the cauda epididymis. This condition appears permanent throughout development since the present work and others (Properzi et al, 1992) have failed to demonstrate the appearance of significant innervation in the caput epididymis with age. Although the reason for these segmental differences in the epididymal autonomic innervation pattern is unknown, it might reflect the expression of segment-specific morphogenes (or the lack of them; for reviews, see Viger and Robaire,

1995; Serre and Robaire, 1998; Kirchhoff, 1999), whose translation products allow or prevent nerves from growing into the cauda or caput, respectively. Protein families such as netrins, ephrins, semaphorins/collapsins, and slit, known to be involved in nervous system axon guidance (Mark et al, 1997), are just a few potential candidates among others to be considered in the search for epididymal chemoattractant or chemorepellant molecules.

Thus, in this work, we present data showing a segment-specific nerve refinement during rat sexual maturation in the cauda epididymis.

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