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Changes in the number and volume of NPY and VIP neurons from periprostatic accessory vegetative ganglia in pre- and peripubertal rats. A stereological study

J.M. Pozuelo [a], R. Rodríguez [a], R. Arriazu [a], I. Ingelmo [b], R. Martín [c], L. Santamaría [d,*]

[a] Department of Physiology, Morphology, and Nutritional Sciences, San Pablo University CEU, Madrid, Spain
[b] Department of Anesthesiology, Hospital Ramón y Cajal, Madrid, Spain
[c] Service of Pathology, Hospital N. Sra de Sonsoles, Ávila, Spain
[d] Department of Anatomy, Histology, and Neuroscience, School of Medicine, Autonomous University of Madrid, C/Arzobispo, Morcillo 2, 28029 Madrid, Spain

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ABSTRACT

The amount of neurons of periprostatic accessory ganglia in pre- and peripubertal rats was studied to ascertain whether the development of these autonomic ganglia is androgen-dependent. Stereological estimates of the volumes and number of neurons immunoreactive to protein gene product 9.5 (PGP 9.5), neuropeptide Y (NPY), and vasoactive intestinal polypeptide (VIP) were carried out. Immunostaining of androgen receptors (AR) in the ganglia was also performed. The ganglionic neurons from the two groups studied were immunoreactive to PGP 9.5, NPY, and VIP. Almost all the neurons were immunostained for AR. The ganglionic volume showed a significant increase in peripubertal prostate in comparison with the prepubertal gland. No significant changes were observed with respect to the absolute number of neurons immunoreactive to all the antigens. The neuronal volume was significantly increased in peripubertal rats in comparison with prepubertal animals. These findings led us to the following conclusions: There is no evidence of neurogenesis during pubertal development in the periprostatic accessory ganglia of the rat. The increase of ganglionic volume in puberty is due to the growth in neuronal volume. There were no differences between the sizes of NPY and VIP neurons in pubertal periprostatic accessory ganglia. The development of periprostatic vegetative neurons is androgen-dependent.

1. Introduction

The importance of the autonomous nervous system in the function, maintenance, and growth of rat prostate has been ascertained by a number of authors (Wang et al., 1991; Martinez-Pineiro et al., 1993; Lujan et al., 1998). The role of prostatic innervation has traditionally been ascribed to the classic autonomic neurotransmitters, noradrenaline and acetylcholine. However, in the last fifteen years, evidence suggests that neuropeptides contained in autonomic nerves play a role in the regulation of prostatic function (Gkonos et al., 1995).

Vasoactive intestinal polypeptide (VIP) is a well-studied neuropeptide, highly abundant in rat prostate nerves. The presence of receptors for VIP in epithelial prostatic cells, together with the occurrence of a remarkable periglandular VIP innervation, suggest that this peptide plays a role in the physiological regulation of prostatic epithelium (Polak and Bloom, 1984; Vega et al., 1990; Properzi et al., 1992; Kepper and Keast, 1995; Rodriguez et al., 2005). Neuropeptide Y (NPY) is also widely distributed in the prostatic autonomic nerves of the rat (Properzi et al., 1992; Kepper and Keast, 1995; Rodriguez et al., 2005). Several studies indicate that NPY could act in the prostate, modulating the effects of VIP or other neurotransmitters on epithelial cells (Harada et al., 1992; Torres et al., 1992; Zhu et al., 1992; Solano et al., 1994).

An interesting question arises concerning the origin of peptidergic fibers innervating the prostate. In this respect, the composition of major pelvic ganglia (MPG) is well known. Nervous innervation of the prostate is believed to be mainly sympathetic. These nerves descend through the hypogastric nerves and synapse on post-ganglionic neurons in MPG, from which the prostate receives direct innervation. The sympathetic nervous innervation is split into two portions: post-ganglionic adrenergic nerves, which synapse on blood vessels and the smooth muscle that surrounds the alveoli, and sympathetic cholinergic nerves, which innervate the glandular epithelium. Thus, control of prostatic secretion is mediated by a sympathetic cholinergic pathway. Evidence for parasympathetic innervation of the prostate is present, but its function is largely unknown. Speculation suggests some form of control of the volume or composition of the secretion (Nadelhaft et al., 2002).

The pelvic ganglia of the rat contain both para- and ortosympathetic neurons in approximately equal proportions, with the presence of NPY and VIP neuropeptides (Keast, 1995; Wanigasekara et al., 2003). Abundant projecting neurons from MPG reach the prostate, and post-ganglionic nerve fibers distribute them through-

* Corresponding author. Tel.: +34 914975367.
E-mail address: luis.santamaria@uam.es (L. Santamaría).

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out the prostatic stroma (Rodriguez et al., 2005; Santamaria et al., 2007; Rodriguez et al., 2007). There is also evidence of the presence of periprostatic and capsular ganglia, respectively, inside and outside the prostatic capsule, but not much is known about their pharmacology or function (Nadelhaft et al., 2002).

Although there are abundant studies related to the influence of sexual hormones on the plasticity of the neurons from the major pelvic ganglia (Keast, 1995; Keast and Saunders, 1998; Keast, 2006), there is scarce information about the relationship between androgenic stimulus (i.e., at the onset of puberty) and the development of periprostatic accessory ganglia, as was ascertained with respect to intra-prostatic innervation (Rodriguez et al., 2005; Rodriguez et al., 2007). These authors observed an increase of NPY- and VIP-immunoreactive fibers related to pubertal development. Other studies show a significant decrease in the length of VIP fibers in rats with a pharmacologic blockade of androgen receptors (Santamaria et al., 2007), and the androgenic enhancement of the receptor–effector VIP system in the prostate of the rat (Juarranz et al., 1994). However, the structure and quantification of these accessory vegetative ganglia have not been thoroughly analyzed to date.

In order to ascertain if the development of periprostatic accessory autonomic ganglia undergoes androgenic influence, this study has investigated the analysis of the distribution of immunoreactive PGP 9.5, NPY, and VIP neurons of periprostatic accessory ganglia in pre- and peripubertal rats. To achieve this, we carried out stereological estimates of the mean volumes of the neurons and the numerical density and absolute number of neurons immunoreactive to the different markers employed. To confirm the androgenic action on the periprostatic accessory ganglia, immunohistochemical visualization of androgen receptors in the ganglia was also performed.

2. Materials and methods

2.1. Animals

The study was carried out on 40 male Wistar rats. The animals were placed in 2 groups (20 rats per group), according to post-natal development: prepubertal, 15–30-day-old animals from the first to the second peaks of prostate epithelial proliferation (i.e., included in the resting phase prior to the beginning of pubertal growth) (Vilamaior et al., 2006), and peripubertal, 30–70-day-old rats from the second peak of epithelial cell proliferation to the acquisition of the adult prostate profile (Gomez et al., 2009; Vilamaior et al., 2006). Animal protocols agreed with the guidelines for the care and use of research animals adopted by the Society for the Study of Reproduction. During this experiment, all animal studies were conducted in accordance with the European Community Council ruling of 24 November 1986 (86/609/EEC) (Van Coppenolle et al., 2001) and Spanish and local directives ruled by the "Real Decreto 1201/2005". All rats were killed by exsanguination after CO_2 narcosis. The prostate complex was dissected from the abdominal cavity of each animal, its fresh volume was estimated by water displacement, and it was then exhaustively cut into 2-mm-wide slices. The section plane was perpendicular to the sagital axis of the gland. All specimens were fixed by immersion in 10% paraformaldehyde in phosphate-buffered saline (PBS) pH 7.4 during 24 h and then embedded in paraffin.

2.2. Sampling procedure

For each prostate, the paraffin blocks were serially sectioned. Five μm-thick sections (for immunohistochemistry and routine hematoxylin–eosine techniques), alternating with 10 μm-thick sections (for stereological methods), were performed on each block. Hematoxylin–eosine sections with periprostatic accessory vegetative ganglia were visualized and employed to select the corresponding paraffin blocks for immunohistochemical and stereological studies. These blocks were exhaustively cut and twenty sections were selected by random systematic sampling from each block obtained from each animal (Gundersen and Osterby, 1981; Gundersen, 1986).

2.3. Immunohistochemistry

Ganglionic neurons immunoreactive to PGP 9.5, NPY, and VIP were studied in all rat prostates from both groups. At least ten selected slides per animal (per prostate and per antigen) were immunostained. Deparaffinized and rehydrated tissue sections were treated for 30 min with hydrogen peroxide 0.3% in phosphate-buffered saline (PBS) pH 7.4 to block endogenous peroxidase. To detect PGP 9.5 immunoreactivity, sections were incubated with a monoclonal anti-PGP 9.5 antibody (Biomeda, Foster City, CA, USA) at a dilution of 1:25. To detect NPY and VIP immunoreactivities, sections were incubated respectively with a polyclonal anti-NPY antibody (Hammersmith Hospital London, UK) at a dilution of 1:1000 and with a polyclonal anti-VIP antibody (Biomeda) at a dilution of 1:50. Androgen receptor (AR) immunoreactivity in the periprostatic accessory ganglia was detected by incubating sections with a polyclonal anti-AR antibody (Calbiochem, Cambridge, MA, USA) diluted at 1:60. Pretreatment of sections by heat in citrate buffer pH 6.0, using a pressure cooker, (Martin et al., 2001) was performed to enhance AR immunostaining. All primary antisera were diluted in PBS, pH 7.4, containing 1% bovine serum albumin (BSA) plus 0.1% sodium azide. All incubations with primary antisera were kept overnight at 4 °C. The second antibody used for primary monoclonal antibodies was a biotin–caproyl–anti-mouse immunoglobulin (Biomeda), while the second antibody used for primary polyclonal antibodies was a biotin–caproyl–anti-rabbit immunoglobulin (Biomeda). Both were diluted at 1:400 in PBS containing 1% BSA without sodium azide. The tissues were incubated for 30 min at room temperature. Thereafter, sections were incubated with a streptavidin–biotin–peroxidase complex (Biomeda). The immunostaining reaction product was developed using 0.1 g diaminobenzidine (DAB) (3,3′,4,4′-Tetraminobiphenyl, Sigma, St Louis, USA) in PBS (200 mL), plus 40 μL of hydrogen peroxide.

After immunoreactions, sections were counterstained with methyl green or Harris hematoxylin. All slides were dehydrated in ethanol and mounted in a synthetic resin (Depex, Serva, Heidelberg, Germany). The specificity of the immunohistochemical procedures was checked by incubation of sections with non-immune serum instead of the primary antibody.

2.4. Stereological methods

2.4.1. Ganglionic volume

The volume of the periprostatic accessory ganglia (G_V) was estimated in all the rats studied. G_V was measured by multiplying the total fresh volume of the prostate by the volume fraction (V_V G) occupied by the ganglionic structures. The estimates of V_V G were performed on three sections, randomly selected from all the hematoxylin–eosin stained sections per each specimen, by counting the points hitting either the prostate or the ganglionic tissues from all the regions present in each section, using the CAST-GRID software package (Interactivision, Silkeborg, Denmark).

The V_V G was thus equal to:

$$\frac{\Sigma \text{ points on the ganglionic tissue}}{\Sigma \text{ points on all the prostate tissues}}.$$