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Original article

The expression of androgen receptor in neurons of the anterior pelvic ganglion and celiac-superior mesenteric ganglion in the male pig

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Abstract

The present study investigated the expression of androgen receptor (AR) in neurons of the anterior pelvic ganglion (APG) and celiac-superior mesenteric ganglion (CSMG; ganglion not involved in the innervation of reproductive organs) in the male pig with quantitative real-time PCR (qPCR) and immunohistochemistry. qPCR investigations revealed that the level of *AR* gene expression in the APG tissue was approximately 2.5 times higher in the adult (180-day-old) than in the juvenile (7-day-old) boars. Furthermore, in both the adult and juvenile animals it was significantly higher in the APG than in CSMG tissue (42 and 85 times higher, respectively). Immunofluorescence results fully confirmed those obtained with qPCR. In the adult boars, nearly all adrenergic (DβH-positive) and the majority of non-adrenergic neurons in APG stained for AR. In the juvenile animals, about half of the adrenergic and non-adrenergic neurons were AR-positive. In both the adult and juvenile animals, only solitary CSMG neurons stained for AR. The present results suggest that in the male pig, pelvic neurons should be considered as an element of highly testosterone-dependent autonomic circuits involved in the regulation of urogenital function, and that their sensitization to androgens is a dynamic process, increasing during the prepubertal period.

Key words: androgen receptor, neurons, anterior pelvic ganglion, celiac-superior mesenteric ganglion, quantitative real-time PCR, immunohistochemistry, male pig

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Table1. List of primary antisera and secondary reagents used in this study.

Primary antibodies					
Antigen	**Clonality**	**Host**	**Dilution**	**Company**	**Catalog No.**
Androgen receptor	polyclonal	rabbit	1:1000	Chemicon	AB561
Dopamine beta hydroxylase	monoclonal	mouse	1:500	Millipore	MAB308
Secondary antibodies					
Antigen	**Fluorophore**	**Host**	**Dilution**	**Company**	**Catalog No.**
Mouse IgG	Alexa 488	goat	1:1000	Invitrogen	A-11001
Rabbit IgG	Alexa 555	goat	1:1000	Invitrogen	A-21428

Introduction

It has been found that major components of pelvic autonomic pathways, including preganglionic neurons, autonomic ganglion cells and primary afferent neurons, are influenced by testosterone (for review, see Keast 2000). Investigations performed mainly in rats suggest that testosterone affects the development and maturation of pelvic autonomic neurons, and it is necessary to maintain their proper function. Testosterone deprivation (achieved by orchidectomy) causes changes in morphological (Melvin et al. 1988, 1989, Keast and Saunders 1998), biochemical (Melvin and Hamill 1987, Melvin et al. 1988, Melvin et al. 1989, Melvin and Hamill 1989a,b, Squillacioti et al. 2008, Huang et al. 2011) and electrophysiological (Kanjhan et al. 2003, Huang et al. 2011) properties of the nerve cells. These testosterone actions are partly mediated by androgen receptors (AR) found in pelvic neurons (Schirar et al. 1997, Keast and Saunders 1998).

It should be mentioned that the literature in the field contains many contributions dealing with effects of gonadal steroids on neuronal circuits in the mammalian central nervous system (for review, see Kawata 1995, Panzica et al. 2012). However, the information concerning steroid (particularly androgens) action on peripheral neurons in male animals is much more limited and derives almost entirely from investigations performed in the rat major pelvic ganglion (MPG).

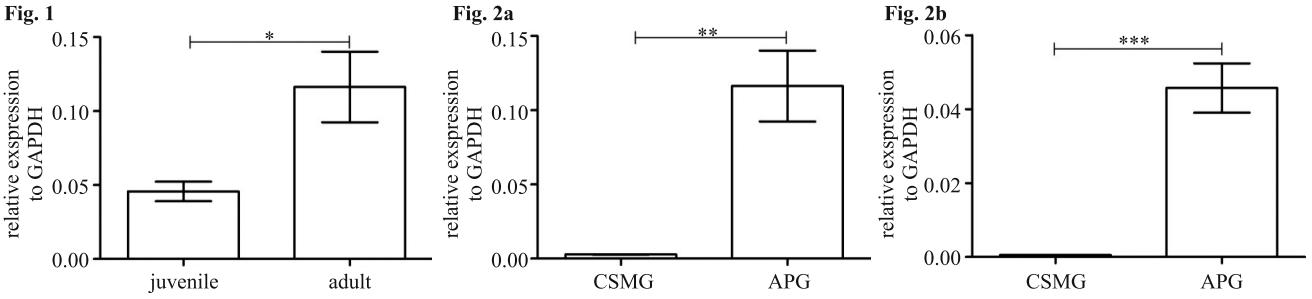
The pelvic plexus in the male pig contains one larger ganglion, the anterior pelvic ganglion (APG), found between the urethral end of the vas deferens and the caudal part of the seminal vesicle, and numerous smaller ganglia distributed on and along lateral sides of the pelvic part of the urogenital duct (Kaleczyc 1998). The anterior pelvic ganglion is a “mixed” autonomic ganglion consisting of sympathetic adrenergic and parasympathetic cholinergic neurons. Retrograde tracing investigations have revealed that the APG supplies the testis (Sienkiewicz 2010), vas deferens (Kaleczyc 1998), accessory genital glands (prostate, vesicular gland; Kaleczyc, unpublished data) and the urinary bladder (Pidsudko 2014).

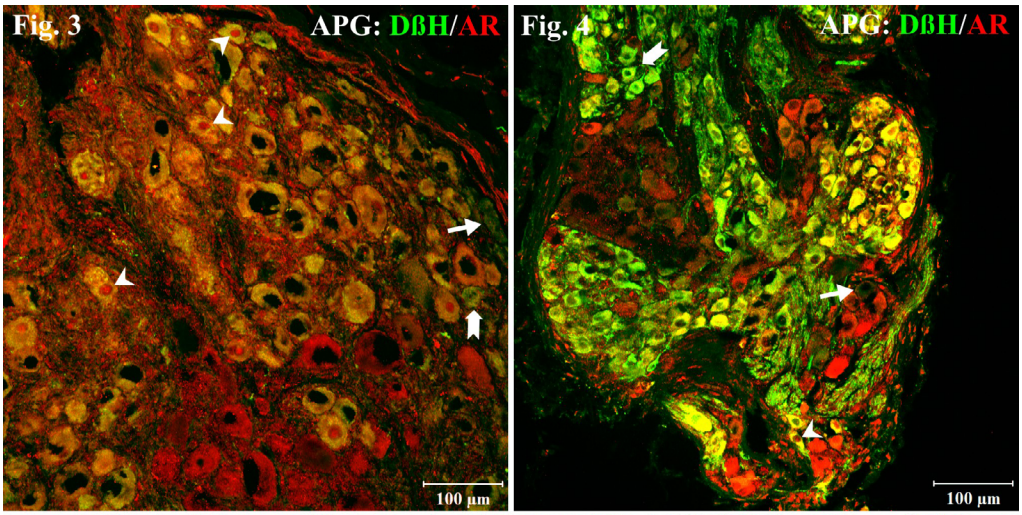
To verify whether the neuroendocrine relationships concerning rat pelvic neurons may occur in larger mammalian species, the present study investigated the expression of androgen receptor (AR) in neurons of APG and celiac-superior mesenteric ganglion (CSMG; ganglion not involved in the innervation of reproductive organs) in the male pig using double-labelling immunofluorescence, and quantitative real-time PCR (qPCR) methods.

Materials and Methods

The study was performed in 5 juvenile (7-day-old) and 5 adult (180-day-old) male pigs. All the animals were housed and treated in accordance with the rules approved by the local Ethics Comission. Prior to tissue collection, all the pigs were deeply anaesthetized with ketamine (10 mg/kg BW, i.m.), xylazine (2 mg/kg BW, i.m.) and sodium pentobarbital (20 mg/kg BW, i.v.). The right APGs and right parts of CSMGs assigned for qPCR investigations were placed into RNAlater. The left APGs and left parts of CSMGs assigned for immunohistochemistry were fixed by immersion in 4% buffered paraformaldehyde (pH 7.4).

Total RNA was extracted using Total RNA Mini isolation kit (AA Biotechnology) and the cDNA samples were synthesized from respective high quality matrix samples using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Quantitative real-time PCR was performed according to the method described by Kasica-Jarosz et al. (2018) using SYBR Green (SYBR Select Master Mix, Applied Biosystems) on 7500 Fast Real-Time PCR System instrument (Applied Biosystems). The primers were as follows: *GAPDH* F: 5’TTCCACCCACGGCAAGTT3’ and R: 5’GGCCTTTCCATTGATGACAAG3’; *AR* F: 5’TGCAGCCTATTGCACGAGAA3’ and R: 5’TCTGGAAAGTCCACGCTCAC3’. The qPCR data





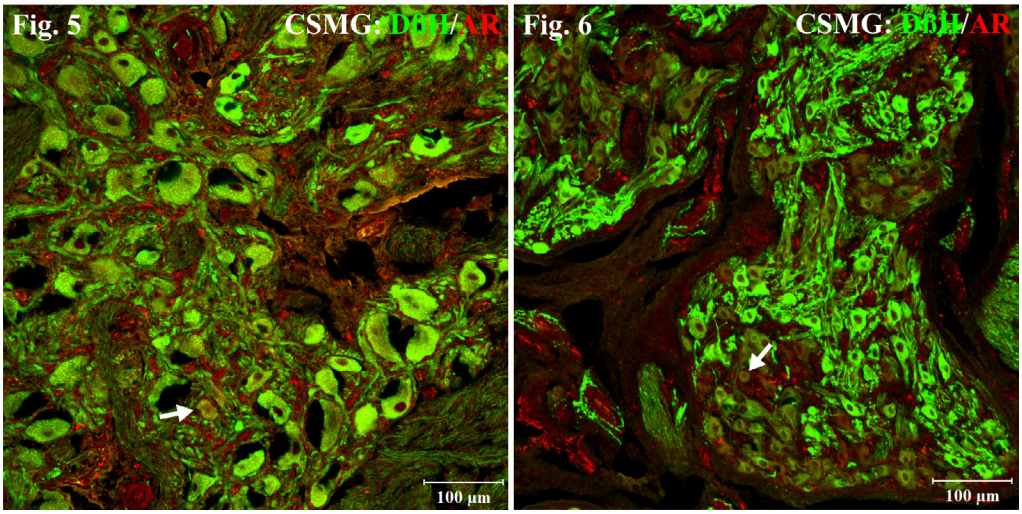


Fig. 1. Gene expression of androgen receptor (AR) in anterior pelvic ganglion (APG) tissue in 7-day-old (juvenile) and 180-day-old (adult) male pigs measured by quantitative real-time PCR (qPCR). Bars represent means and error bars correspond to SEM. * difference significant at $p \leq 0.05$

Figs. 2a and 2b. Gene expression of AR in APG and celiac-superior mesenteric ganglion (CSMG) tissue in the adult (Fig. 2a) and juvenile (Fig. 2b) male pigs measured by qPCR. Bars represent means and error bars correspond to SEM. ** and *** differences significant at $p \leq 0.01$ and $p \leq 0.001$, respectively

Figs. 3 and 4. Sections from APG in the adult (Fig. 3) and juvenile (Fig. 4) male pigs stained for AR (red; Alexa 555 visualization) and dopamine-β-hydroxylase (DβH; green; Alexa 488 visualization). Red and green channels were superimposed, double-labeled elements are yellow to orange. In the adult pigs (Fig. 3), nearly all DβH-positive and many DβH-negative neurons exhibited cytoplasmic and nuclear AR-immunoreactivity; arrowheads show some AR-positive neuronal nuclei. In the juvenile animals (Fig. 4), the number of AR-positive neurons was smaller. Thin and thick arrows show DβH-negative/AR-negative and DβH-positive/AR-negative neurons, respectively.

Figs. 5 and 6. Sections from CSMG in the adult (Fig. 5) and juvenile (Fig. 6) male pigs stained for AR (red; Alexa 555 visualization) and DβH (green; Alexa 488 visualization). Green and red channels were superimposed, double-labeled elements are yellow to orange. In both the adult and juvenile animals only solitary neurons (arrows) stained for AR.

were analyzed by the Student t-test (using GraphPad Prism 5.0 software). Each bar represents mean ± SEM. The differences were considered significant at $p<0.05$.

Double-labelling immunofluorescence using antibodies (Table 1) against AR and dopamine-β-hydroxylase (DβH; adrenergic marker) was performed according to the method described by Sienkiewicz et al. (2010).

The labelled sections were investigated and images were recorded with Zeiss LSM 700 confocal laser microscope. The relative frequency of the labelled neurons was assessed and the results were appointed by two independent investigators. The assessment was done in APG and CSMG sections from different, representative regions of the ganglia (from their upper, middle and lower one-third).

Negative controls for immunostaining included omission or replacement of primary antisera with the corresponding non-immune sera.

Results

Quantitative real-time PCR investigations revealed that the level of *AR* gene expression in the APG tissue was approximately 2.5 times higher in the adult than in the juvenile boars (Fig. 1). Furthermore, the *AR* gene expression level in both the adult (Fig. 2a) and juvenile (Fig. 2b) animals was significantly higher in the APG than in CSMG tissue (42 and 85 times higher, respectively).

The results of immunofluorescence investigations fully confirmed those obtained with qPCR.

In the adult boars, nearly all DβH-positive (adrenergic; about 95%) and the majority of non-adrenergic (about 70%; virtually all non-adrenergic neurons have previously been found to be cholinergic in nature; Kaleczyc et al. 2003) neurons in APG stained for AR (Fig. 3). In the juvenile animals, even though the number of APG AR-positive adrenergic and non-adrenergic neurons was lower than that found in the adult boars, they were still numerous and made up about half of all ganglionic nerve cells (Fig. 4). The AR-positive perikarya exhibited weak to strong cytoplasmic staining, however, many, mostly adrenergic, neurons in the adult boars and some in the juvenile animals also displayed intensely stained nuclei (Figs. 3, 4). In both the adult and juvenile boars, only solitary adrenergic or non-adrenergic CSMG neurons mostly weakly stained for AR (Figs. 5, 6).

Discussion

The present study has revealed that the expression of androgen receptor in pelvic neurons is significantly higher in adult than in juvenile male pigs. Moreover, it has been found to be immensely higher in pelvic neurons than in neurons potentially not contributing to the innervation of reproductive organs, in both groups of the animals. The expression was measured, to our knowledge for the first time in such tissues, with qPCR, and assessed by immunohistochemistry. It should be emphasized, that the results obtained by the two methods were highly consistent and complementary. Immunohistochemistry has been used to precisely locate AR in ganglionic structures and revealed that many both adrenergic (in the adult pigs, nearly all) and putative cholinergic APG neurons express immunoreactivity to this component. Accordingly, a very low level of *AR* gene expression in CSMG tissue (affirmed by immunohistochemistry) further confirms the reliability of the findings dealing with APG and suggests, that presumably only neurons supplying reproductive organs are highly susceptible to androgens. It should also be noted that uniform (especially in the adult animals) as regards the expression of AR APG adrenergic neurons most likely project to smooth muscles of the reproductive organs; it has been found that nearly 90% of APG neurons projecting to the vas deferens belong to this neuronal set (Kaleczyc 1998), and in turn, the population of adrenergic nerve fibres distributed in the smooth muscle layer of the porcine vas deferens significantly outnumbers the other intramural axonal populations (Kaleczyc 1998).

In contrast to our findings, immunohistochemical studies in the rat major pelvic ganglion (MPG) have failed to identify AR expression in pelvic adrenergic neurons, thus the mechanism of the androgen action (which, parenthetically, is evident) in this species is not obvious (Keast and Saunders 1998, Keast 2000). Population of rat MPG cholinergic neurons can be divided into two main, virtually separate, groups distinguished by the presence of vasoactive intestinal peptide (VIP) or neuropeptide Y (NPY; Keast 1991). Interestingly, unlike pelvic adrenergic neurons which are homogeneously androgen sensitive (but did not express AR), AR is expressed only by pelvic cholinergic VIP-positive neurons which innervate reproductive organs (Schirar et al. 1997, Keast and Saunders 1998). The cholinergic NPY-positive neurons, which project mainly to the urinary bladder and rectum (Keast and De Groat 1989), did not contain AR. It is doubtful that similar associations are attributed to APG cholinergic neurons, because earlier investigations have revealed that VIP and NPY are not expressed by separate populations of these nerve cells, respectively, but on the contrary, the majority of cholinergic neurons co-express both peptides (and also nitric oxide synthase and somatostatin; Kaleczyc et al. 2003). Therefore, whether AR-positive pelvic cholinergic neurons in the male pig project

to reproductive organs only remains to be elucidated with tracing experiments.

It should be mentioned that in the rat, preganglionic autonomic neurons involved in pelvic reflexes are potential targets for circulating terstosterone, because numerous spinal neurons retrogradely labelled from the MPG express AR immunoreactivity (Watkins and Keast 1999). If this holds true for the male pig, it can be assumed that also in this animal pelvic (APG) neurons constitute a significant element of highly testosterone-dependent autonomic circuits involved in the regulation of reproductive function. The present results also suggest that sensitization of the pelvic neurons to androgens is a dynamic process, increasing during the prepubertal period, because both the number of APG AR-positive neurons, and the *AR* gene expression level measured by qPCR were distinctly lower in the juvenile than in the adult pigs.

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