Immunohistochemical characterization of efferent neurons innervating the oviduct in the pig located in the sympathetic chain ganglia

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ABSTRACT: The present study was aimed at disclosing the pattern(s) of putative co-incidence of tyrosine hydroxylase (TH), dopamine β-hydroxylase (DβH), neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP) and nitric oxide synthase (NOS) within the porcine "oviductal" efferent neurons using combined retrograde tracing and double-labelling immunohistochemistry. The fluorescent retrograde tracer Fast Blue (FB) was injected into the wall of the ampullar ($n = 5$) and isthmal ($n = 5$) part of the organ in ten sexually immature female pigs. After a survival period of three weeks sympathetic chain ganglia (SCG) were collected. 10 μm-thick cryostat sections of the ganglia were examined for the presence of FB-positive (FB^+) nerve cells under the fluorescent microscope. Tracered neurons were processed for double-labelling immunofluorescence according to the method of Wessendorf and Elde. Retrograde labelling revealed a population of "oviductal" efferent neurons located in the thoracic (T) and lumbar (L) SCG at the level of T_{14} to L_5. Double-labelling immunofluorescence allowed several subpopulations of the studied perikarya to be distinguished. The largest one consisted of $TH^+/D\beta H^+$ (immunopositive) nerve cells. The moderate number of FB^+ nerve cells expressed TH/NPY- immunoreactivity (IR). The tracered neurons did not show SP, CGRP and NOS immunoreactivity. Because identically coded nerve fibres have been observed within the wall of the porcine oviduct it can be assumed that $TH^+/D\beta H^+$ or TH^+/NPY^+ neurons are involved in the control the oviductal tonus and ovum transport.

Keywords: sympathetic chain ganglia; oviduct; pig; immunohistochemistry; tracing

There are relatively many papers dealing with the distribution and chemical nature of efferent neurons innervating genital organs in animals (Brundin *et al.*, 1969; Lakomy *et al.*, 1983; Kannisto *et al.*, 1986; Majewski *et al.*, 1995; Czaja *et al.*, 1996; Houdeau *et al.*, 1998). However, the sources of origin of nerve fibres supplying reproductive organs in breeding animals have not been studied thoroughly (Welento *et al.*, 1984, 1987; Flieger *et al.*, 1984, 1988; Boratynski *et al.*, 1988). The above mentioned investigations performed in breeding animals involved extirpations of fragments of the reproductive organs what allowed a detection of nerve centers including those localized in thoraco-lumbar SCG contributing to the innervation of the removed segments of the organs. Only application of the retrograde tracing method was considered to be one of the most advanced and precise approaches in localizing specific neuronal populations supplying any particular organ under study (Kobbert *et al.*, 2000). Retrograde tracing has been used in combination with histo- and/or immunohistochemistry to disclose neurochemical characteristics of retrogradely labelled neurons supplying genital organs (Nance *et al.*, 1988; Kaleczyc *et al.*, 1994, 1995; Pidsudko *et al.*, 2001; Czaja *et al.*, 2001b). Although the previous studies have revealed that some nerve fibres supplying the porcine oviduct may be of SCG origin (Welento *et al.*, 1984, 1987; Czaja *et al.*, 2001a) the neurochemical nature of their perikarya is still obscure.

Therefore, by means of combined retrograde tracing and double-labelling immunofluorescence (Wessendorf *et al.*, 1987), the present study was aimed at disclosing the pattern(s) of putative co-incidence of tyrosine hydroxylase (TH), dopamine β-hydroxylase (DβH), neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP) and nitric oxide synthase (NOS) within the efferent neurons supplying the oviduct in the pig.

MATERIAL AND METHODS

The experiment was performed on ten sexually immature female pigs of the Great Polish breed, about 15 kg of body weight (b.w.), obtained from a commercial fattening farm. The animals were kept under standard laboratory conditions. Thirty min. before the main anaesthetic was given, all the animals were pre-treated with atropine (Polfa, Poland; 0.04 mg/kg b.w., *s.c.*) and propionyl-promasine (Combelen, Bayer, Germany; 0.4 mg/kg b.w., *i.m.*). The main anaesthetic, sodium pentobarbital (Vetbutal, Biovet, Poland; 30 mg/kg b.w.) was given intravenously. During laparotomy the right oviduct was gently removed from surrounding tissues and the fluorescent retrograde tracer Fast Blue (FB; Dr. K. Illing GmbH, Groβ-Umstadt, Germany) was injected into the wall of the ampullar (n = 5) and the isthmal (n = 5) part of the organ. A total volume of 10 μl of FB was injected into each part of the oviduct using a Hamilton syringe. After a survival period of three weeks the animals were deeply reanaesthetised (following the same procedure as applied before the laparotomies) and perfused transcardially with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4). Both, oviducts and SCG were collected. All the tissue specimens were overnight postfixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) and then stored at 4°C in 0.1M phosphate buffer containing 18% sucrose and 0.01% NaN_3 until sectioning. 10 μm-thick cryostat sections containing FB^+ neurons were processed for double-labelling immunofluorescence (Wessendorf *et al.*, 1987). Primary and secondary antisera used are listed in Table 1. Following combinations of the primary antisera were applied: TH/DβH, TH/NPY, TH/SP, TH/CGRP, and TH/NOS. The specificity of the primary antisera was tested by the preabsorption control. 1 μM concentration of the respective peptide completely abolished the fluorescence. There was also no immunostaining when primary antisera were omitted or replaced by a normal rabbit, rat or mouse serum. Labelled neurons were counted in every third section. This strategy eliminated the likelihood of counting the same neuron twice. The labelled sections were studied and photographed with a Nikon Microphot FXA microscope, equipped with epi-illumination fluorescence and an appropriate filter set for Texas Red and fluoresceine izothiocyanate (FITC).

In the experiments, the principles of laboratory care as well as the specific national laws on the protection of animals were followed.

RESULTS AND DISCUSSION

Retrogradely labeled efferent neurons supplying both the isthmus and ampulla of the porcine oviduct were found in the ipsilateral thoraco-lumbar SCG at the level of T_{14}-L_5 (Figures 1a, 2a). The distribution of neurons projecting to the studied parts of the oviduct was very similar to that, found in the previous studies (Welento *et al.*, 1984, 1987; Czaja *et al.*, 2001a). In both

Table 1. Primary antibodies and secondary reagents used in the study

Antigen	Species	Code	Dilution	Supplier
Primary antisera				
TH	mouse	2/40/15	1 : 60	Boehringer
DβH	rabbit	TE103	1 : 400	ETI
NPY	rabbit	RPN1702	1 : 500	Amersham
NOS	rabbit		1 : 1 000	B. Mayer
SP	rat	NC1	1 : 300	Serva
CGRP	rabbit	000114	1 : 1 800	Penisula
Secondary reagents				
FITC-conjugated mouse anti-rabbit IgG			1 : 400	Cappel
FITC-conjugated rabbit anti-mouse IgG			1 : 400	Cappel
FITC-conjugated rabbit anti-rat IgG			1 : 400	Cappel
Biotinylated mouse anti-rabbit IgG			1 : 100	Cappel
Biotinylated rabbit anti-mouse IgG			1 : 100	Cappel
Biotinylated rabbit anti-rat IgG			1 : 100	Cappel
Texas Red-conjugated streptavidin			1 : 100	Amersham

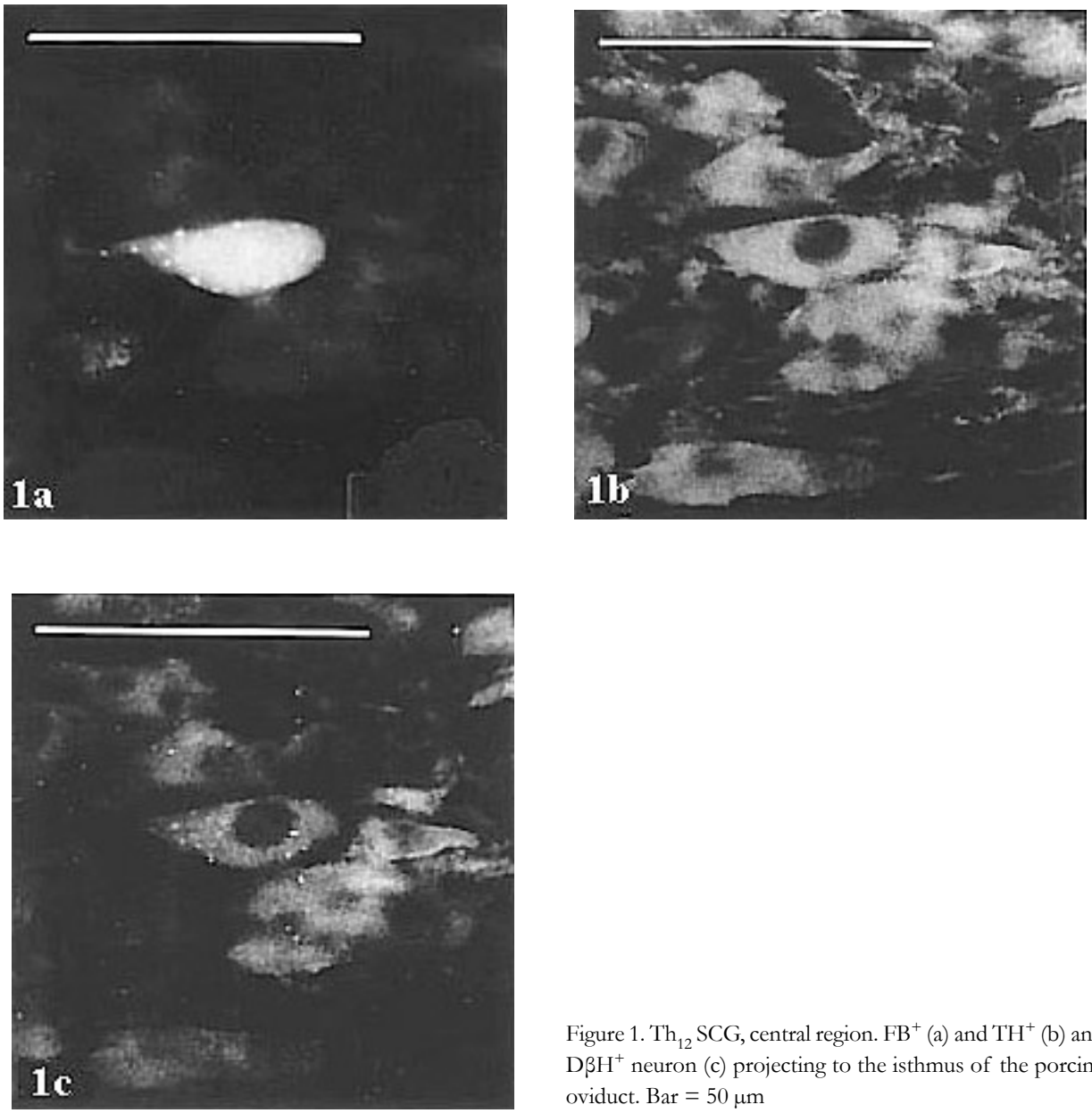
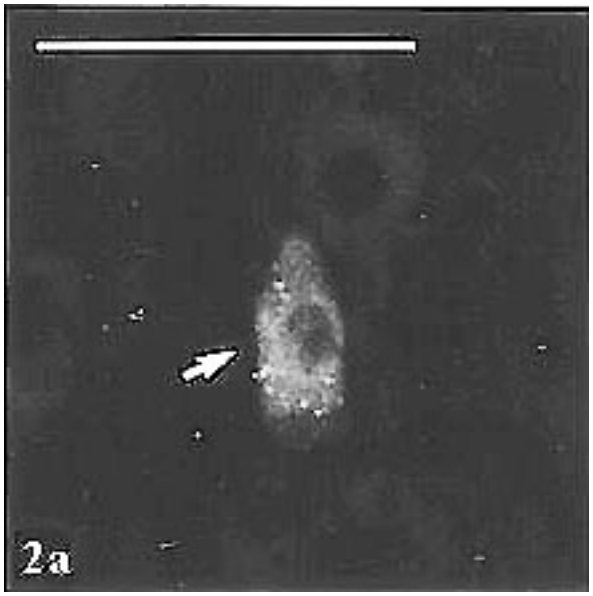
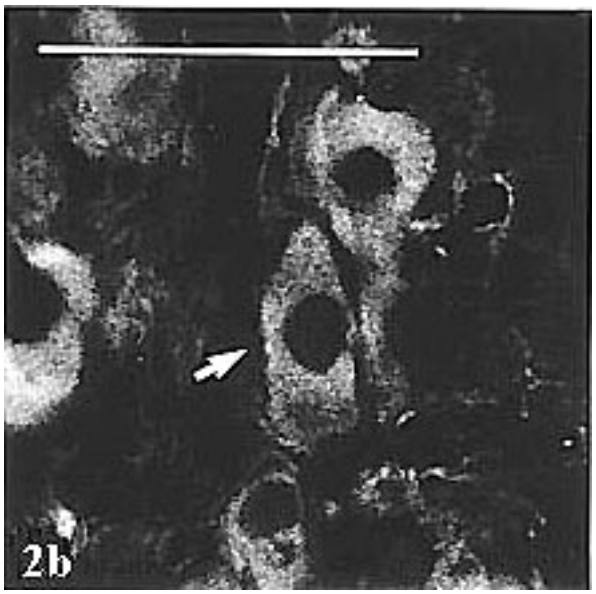


Figure 1. Th_{12} SCG, central region. FB^+ (a) and TH^+ (b) and $D\beta H^+$ neuron (c) projecting to the isthmus of the porcine oviduct. Bar = 50 μm

subpopulations, the average number of labelled perikarya varied from 50 to 300 per ganglion. The majority of labelled neurons localized in all the ganglia studied were of 20–35 μm in diameter and lenticular or oval in shape. Immunohistochemical examination of the ganglia revealed that FB^+ neurons, irrespective of the neuromere and the portion of the oviduct studied, were TH-, DβH- or NPY-IR. No SP^+, $CGRP^+$ and/or NOS^+ oviduct-projecting neurons were found in the ganglia studied. Furthermore, the SCG neurons supplying the porcine oviduct were found to contain various combinations of the antigens studied. The population of "oviductal" neurons localized in thoraco-lumbar SCG, based on their immunohistochemical properties, can be divided into several groups: $FB^+/TH^+/DbH^+$ (Figures 1a, b, c), $FB^+/TH^+/NPY^+$ (Figures 2a, b, c), $FB^+/TH^+/NPY^-$, $FB^+/TH^+/SP^-$, $FB^+/TH^+/CGRP^-$ and $FB^+/TH^+/NOS^-$. It was found that 100% of double-labelled FB^+ perikarya were $TH^+/D\beta H^+$ while 41% of them displayed immunoreactivity to TH/NPY. The chemical coding of SCG neurons in many animal species was the point of wide interest. It was found that not only the great neuronal subpopulation containing TH/DβH and/or NPY is present, but also perikarya of these ganglia contain various combinations of VIP, CGRP, SP (Landis and Fredieu, 1986; Kummer and Heym, 1991; Schotzinger *et al.*, 1994 or NOS (Anderson *et al.*, 1993;





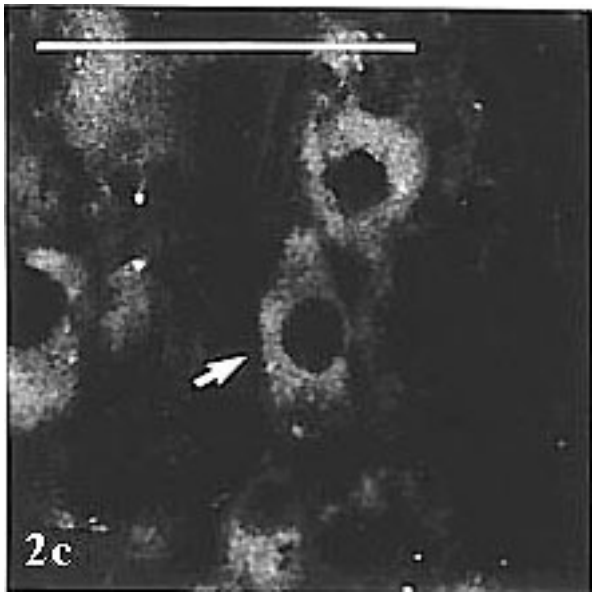


Figure 2. L_1 SCG, lateral part of the peripheral region. FB^+ (a) and TH^+ (b) and NPY^+ neuron (c) innervating the ampulla of the porcine oviduct (arrow). Bar = 50 μm

Modin, 1994; Majewski *et al.*, 1995). Results of the present study show that all the neurons innervating the porcine oviduct were those containing TH and DβH i.e. commonly accepted markers for noradrenergic nerves. Double labelling immunofluorescence has shown a total colocalization of TH and DβH. This finding confirms the results of many histochemical studies reporting the relatively rich noradrenergic innervation of the oviduct in mammalian species studied (Brundin *et al.*, 1969; Owman and Sjoberg, 1972; Paton, 1976; Chernaeva and Milenov, 1978; Kannisto *et al.*, 1986; Wrobel and Kujat, 1993; Czaja *et al.*, 1993, 1996, 2001b; Anderson *et al.*, 1993; Modin, 1994; Majewski *et al.*, 1995). In mammalian species, the majority of adrenergic nerve terminals in the isthmal region are non-vascular, but located mainly in muscular membrane of the organ, whereas within the ampulla, these nerve fibres were predominantly related to blood vessels (Paton *et al.*, 1977; Moawad *et al.*, 1977; Wrobel and Kujat, 1993; Czaja *et al.*, 1993, 1996). Physiologically, catecholamines have been proved to affect the oviductal musculature by inducing and/or enhancing organ contractions (Ohkawa, 1982; Sorger *et al.*, 1983), so the noradrenergic neurons seem to play a similar role in the motor function of the porcine organ. TH/ DβH-IR nerves accompaying blood vessels may exert significiant microvessel regulation and they can also be involved in mechanisms of neurogenic control of blood flow (Appenzeller *et al.*, 1984; Zochodne and Low, 1990). This study has revealed moderate number of

TH/NPY-IR neurons supplying both the isthmus and ampulla of the oviduct. NPY-IR nerve fibres are richly distributed in the oviduct of several species (Blank *et al.*, 1986; Owman *et al.*, 1986; Reinecke *et al.*, 1989; Czaja *et al.*, 1996) including human. The smooth musculature of the isthmic region has been found to be the most abundantly innervated by NPY-IR neurons, whereas in the ampulla and infundibulum, nerve terminals are particularly richly distributed around blood vessels. Subepithelial localization of TH/NPY-IR nerve terminals was also observed. Several regulatory functions may be attributed to NPY, as revealed by previous studies. Experimental investigations performed in rabits have shown that NPY induces a dose-related stimulatory effect on non-vascular uterine smooth muscle (Tenmoku *et al.*, 1988. Moreover, in the rat uterine cervix, NPY inhibits the release of acetylcholine, probably through a prejunctional action on cholinergic nerve endings (Stjernquist *et al.*, 1983). Subepithelially located fibres in the oviductal mucosa may be involved in secretory functions (Samuelson *et al.*, 1985). This peptide has been shown to inhibit the release of noradrenaline from sympathetic nerve endings by presynaptic mechanism, but it can also directly affect postsynaptic receptors enhancing contractile effects of noradrenaline (Lundberg *et al.*, 1991). The presence of either NPY-IR alone, or TH/NPY neurons projecting to the oviduct, as observed in our study, suggests that both the mentioned mechanisms can be involved in the contraction of the oviductal smooth myocytes by NPY. Thus, our findings provide further evidence for the coexistence and a possibile cooperation of NPY with noradrenaline in sympathetic neurons. The conclusion of the presented results is that efferent neurons innervating porcine oviduct localized in SCG, send only noradrenergic axons to the oviduct. The above mentioned data indicate that the noradrenergic neurons supplying the ampulla, take part in the transport of egg cell to the place of fertilization whereas FB^+ neurons sending their fibers to the oviduct isthmus may control the sperm passage towards the egg cell. Both groups of described neurons: these projecting to the isthmus and those projecting to the ampulla may indirectly participate in the fertilization.

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