

NERVE FIBERS IMMUNOREACTIVE TO SENSORY NEURONAL FACTORS IN THE PORCINE ADRENAL GLANDS

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Abstract. The distribution pattern of nerves immunoreactive to neuronal factors typical for sensory neurons, such as substance P (SP), calcitonin gene – related peptide (CGRP), somatostatin (SOM), galanin (GAL) and nitric oxide synthase (NOS), used here as the marker of nitrergic neurons was studied by single immunofluorescence technique in various parts of the porcine adrenal gland i.e. subcapsular region, zona glomerulosa, zona fasciculata and zona reticularis of the cortex, as well as in the medulla of the gland. Evaluation of the density of nerves was performed by the semi quantitative method, where (-) means the absence of fibers (+) – single fibers, (++) – rare nervous processes, (+++) – depicts a dense and (++++) - very dense meshwork of fibers. All substances studied were observed in the porcine adrenal gland and the density of nerve fibers immunoreactive to particular factors depends on the adrenal gland region. The largest density of nerve fibers immunoreactive to neuronal factors studied was observed in subcapsular region and adrenal medulla. The study shows that nerves within the porcine adrenal gland exhibit significant differentiation in terms of expression of neuronal factors typical for sensory neurons.

Keywords: adrenal gland, immunohistochemistry, neuronal factors, sensory nerves, pig.

Introduction

It is generally accepted that adrenal glands are innervated by different types of neurons. It is also known that autonomic nervous system plays a pivotal role in adrenal functions (Kesse et al., 1988; Coupland et al., 1989). Adrenal cortex is mainly innervated by postganglionic sympathetic fibres, which derive from paravertebral trunk and prevertebral ganglia, such as coeliac or suprarenal ganglia (Kesse et al., 1988), as well as by parasympathetic processes within the vagus nerve (Coupland et al., 1989). On the other hand, the innervation of adrenal medulla derives mainly from cholinergic (sympathetic) preganglionic neurons, which are located in thoracic and lumbar spinal cord (Kesse et al., 1988). Other types of neuronal cells that take part in the innervation of adrenal medulla are afferent neurons situated in vagal sensory ganglia as well as within thoracic and lumbar dorsal root ganglia (DRG) (Kesse et al., 1988; Coupland et al., 1989; Gonkowski 2002).

Various localizations of neurons supplying the adrenal gland cause that broad spectrum of neuronal factors has been described in nerve fibres within this organ. Till now, it has been reported that nerve fibres in the adrenal gland can contain a variety of substances such as, for example, substance P (SP) (Heym et al., 1995a, 1995b; Marabayashi et al., 2009), calcitonin gene related peptide (CGRP) (Heym et al., 1995a; Tornoe et al., 2000), pituitary adenylate cyclase activating polypeptide (PACAP) (Tornoe et al., 2000), nitric oxide synthase (NOS) (Heym et al., 1995a) or leu-enkephalin (LENK) (Kong et al., 1989). But it should be pointed out that the knowledge concerning sensory innervation of adrenal glands in the pig, which becomes in Europe a very popular laboratory animal (Verma et al., 2011), is rather

fragmentary (Kong et al., 1989; Tornoe et al., 2000; Gonkowski 2002). So, the aim of this study was the identification of sensory neuronal factors, which have been studied within DRG neurons supplying adrenal glands in other species (Heym et al., 1995a) or within porcine DRG (Bossowska et al., 2009), in nerve fibres innervating the porcine adrenal glands.

Materials and methods

The present study was performed on five juvenile female pigs of the Large White Polish breed (12–18 kg body weight, approximately 8 weeks old) obtained from a commercial fattening farm in Niedzwiedz (Poland). Pigs were kept in standard laboratory conditions with admission to species-specific fodder and water *ad libitum*. All experimental procedures were performed in compliance with the regulations of the Local Ethical Committee, with special attention paid to the minimising of stress reaction. The animals were euthanized with an overdose of sodium thiopental (Thiopental, Sandoz, Kundl-Rakúsko, Austria; 20 mg/kg of body weight given intravenously) and then perfused transcardially with 4% buffered paraformaldehyde (pH 7.4) prepared *ex tempore*. Both adrenal glands were collected from each animal, post-fixed by immersion in the same fixative for several hours and, finally, stored in 18% sucrose until sectioning. Ten- μ m-thick cryostat sections of adrenal glands were processed for standard single-labeling immunofluorescence (Gonkowski et al., 2009), using antisera raised in different species and directed towards substance P (SP; rat monoclonal, Biogenesis Ltd, England; in working dilution 1:300), calcitonin gene – related peptide (CGRP; rabbit polyclonal; Amersham Pharmacia Biotech., England; 1:1800), somatostatin

(SOM; rat monoclonal; Chemicon Int. Inc., USA; 1:50), galanin (GAL; rabbit polyclonal; Peninsula, England; 1:2400) and nitric oxide synthase (NOS – marker of nitrergic nerves; mouse monoclonal; Sigma, Germany; 1:1000). Primary antisera were visualized by species-specific secondary antisera conjugated to FITC or biotin (all from Jackson Immunochemicals, USA, in working dilution 1:800). The latter antibodies were then visualized using a streptavidin-CY3 complex (Jackson, 1:8000). Labeled nerve fibers were evaluated using an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets. Nerve profiles were estimated in 10 sections of each adrenal gland *per* animal, in 5 fields *per* section per each part of adrenal gland i.e. in subcapsular region, zona glomerulosa, zona fasciculata and zona reticularis of the cortex as well as in the medulla. To prevent double evaluation of the same nerve fibers, the sections were located at least 250 μm apart from each other. For the semi-quantitative evaluation of the density of nerves immunoreactive to each substance studied, an arbitrary scale was used, where (-) means the absence of fibers (+) – single fibers, (++) – rare nervous processes, (+++) – depicts a dense and (+++++) – very dense meshwork of fibers. Standard controls, i.e. pre-absorption of the neuropeptide antisera with appropriate antigen, omission and replacement of primary antisera by non-immune sera were performed to test the antibodies and specificity of the method.

Results

The presence of nerve fibers immunoreactive to all neuronal factors studied was observed within the porcine

adrenal glands and clear differences in density of nerve processes positive for particular neuronal factors were seen depending on the adrenal gland region (Table, Figs 1, 2, and 3). On the other hand, visible differences in density of fibers immunoreactive to neuronal factors studied both between particular animals and between right and left adrenal glands were not observed in the present study.

In the subcapsular region, rare nerve fibers immunoreactive to SP (Figure 1A), CGRP and/or NOS (Figure 1B) were observed, whereas GAL- and/or SOM-LI nerve processes occurred only occasionally as thick nervous bundles, which innervate deeper located parts of adrenal glands (Fig. 1C).

Within the cortex of adrenal medulla (Fig. 2), nerve fibers immunoreactive to majority of neuronal factors studied were thin, delicate and occurred as single processes except for zona fasciculata, where CGRP- and/or NOS- like immunoreactive nerves were somewhat more numerous (Figure 2 IIA, IIB). Moreover, in the all zones of the cortex, like in the subcapsular region thick nervous bundles probably supplying the adrenal medulla were occasionally observed.

In the adrenal medulla meshwork of nerve fibers immunoreactive to majority of substances studied was denser and more visible compared to the cortex (Fig. 3). Nerve processes immunoreactive to NOS (Fig. 3A) were thick, clearly visible and built the very dense meshwork. SOM- and/or GAL – like immunoreactive processes (Fig. 3 B, D) were observed quite rarer and nerves positive for SP and/or CGRP (Fig. 3 C, E) were single, thin and delicate.

Table: **Arbitrary evaluation of density of nerve fibers in the porcine adrenal gland positive for particular substances, presented in arbitrary units** (- absence, + single fibers, ++ rare nerve fibers, +++ dense meshwork of fibers, +++++ very dense meshwork of fibers)

Part of adrenal gland	SP	CGRP	SOM	GAL	NOS
Subcapsular region	++	++	+	+	++
Zona glomerulosa	+	+	+	+	+
Zona fasciculata	+	++	+	+	++
Zona reticularis	+	+	+	+	+
Adrenal medulla	+	+	++	++	++++

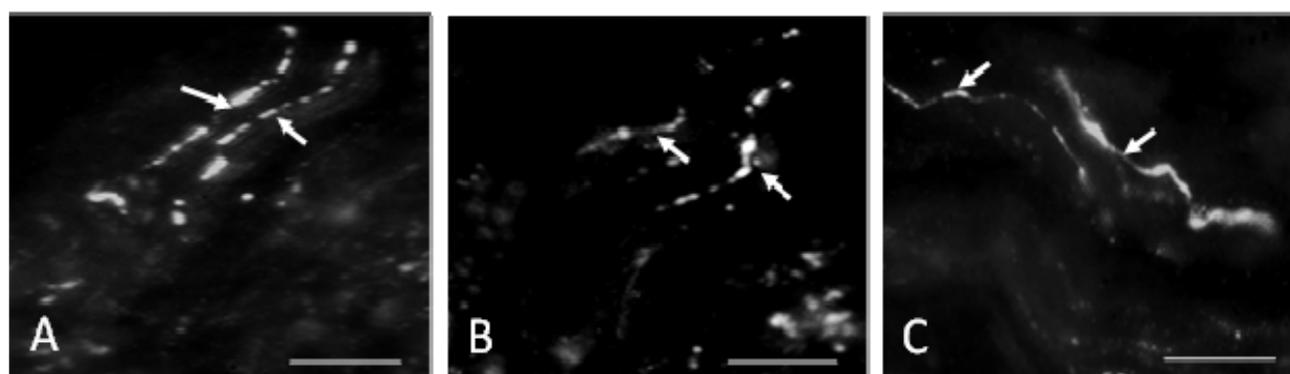


Fig. 1. Subcapsular region of the porcine adrenal gland. Nerve fibers (arrows) immunoreactive to SP (A), NOS (B) and GAL (C). Scale bar 30 μm .

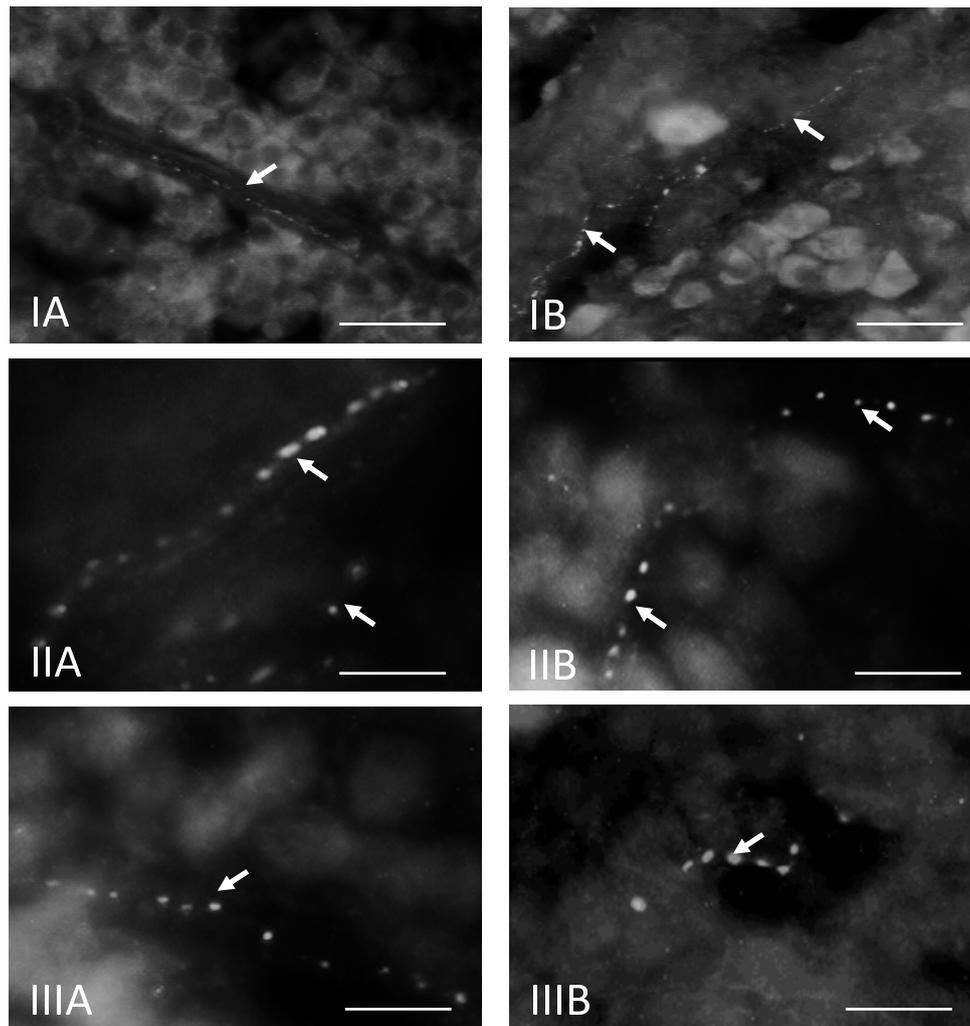


Fig. 2. Cortex of the porcine adrenal gland: I - zona glomerulosa with nerves (arrows) immunoreactive to SP (A) and SOM (B); II - zona fasciculata with nerves (arrows) immunoreactive to CGRP (A) and NOS (B); III - zona reticularis with nerves (arrows) immunoreactive to GAL (A) and SOM (B). Scale bar 30 μ m.

Discussion

During the present investigation, all neuronal factors studied were observed in the nerve fibers within the porcine adrenal glands, what is in accordance with previous studies in humans and other mammal species (Heym et al., 1995a, 1995b; Tornoe et al., 2000; Marabyashi et al., 2009). It should be pointed out that till now the knowledge concerning neurochemical characterization of porcine intra-adrenal nerve fibers is rather poor and limited to two studies (Kong et al., 1989; Tornoe et al., 2000), where LENK-, CGRP-, SP- and PACAP - like immunoreactive nerve processes have been described. The results concerning SP- and CGRP - immunoreactivity obtained in the present study somewhat differ from the previous, mentioned above observations, which noted the denser meshwork of such fibers. This fact can suggest that intra-adrenal nerves in pigs may undergo structural, functional and/or chemical changes as a result of various stimuli such as development, aging, environmental stressors or pathological stimuli, what has been confirmed in other species (Whithworth et al., 2003). All substances studied during the present

investigation have been previously noted in primary sensory neurons supplying the adrenal glands in other species (Heym et al., 1995a) or within dorsal root ganglia in pigs (Bossowska et al., 2009), what can suggest that nerve fibers observed in this study play roles in sensory stimuli conducting. On the other hand, it is well known that studied neuronal factors are broadly spread in various parts of nervous system and involved in different regulatory processes. So, part of observed nerve fibers can play other, not sensory, functions within the adrenal glands, what has been confirmed by previous investigations. Even CGRP or SP, which are regarded to be markers of sensory neurons (Bossowska et al., 2009), may also play other roles in adrenal glands. Namely, CGRP has been reported to be involved in catecholamine release (Tortorella et al., 2001) and stimulation of steroidogenesis and/or increase adrenal blood flow (Thakor and Giussani, 2005), what is confirmed by an increase in steroid output from the adrenal glands during exogenous treatment with CGRP (Hinson and Vinson, 1990).

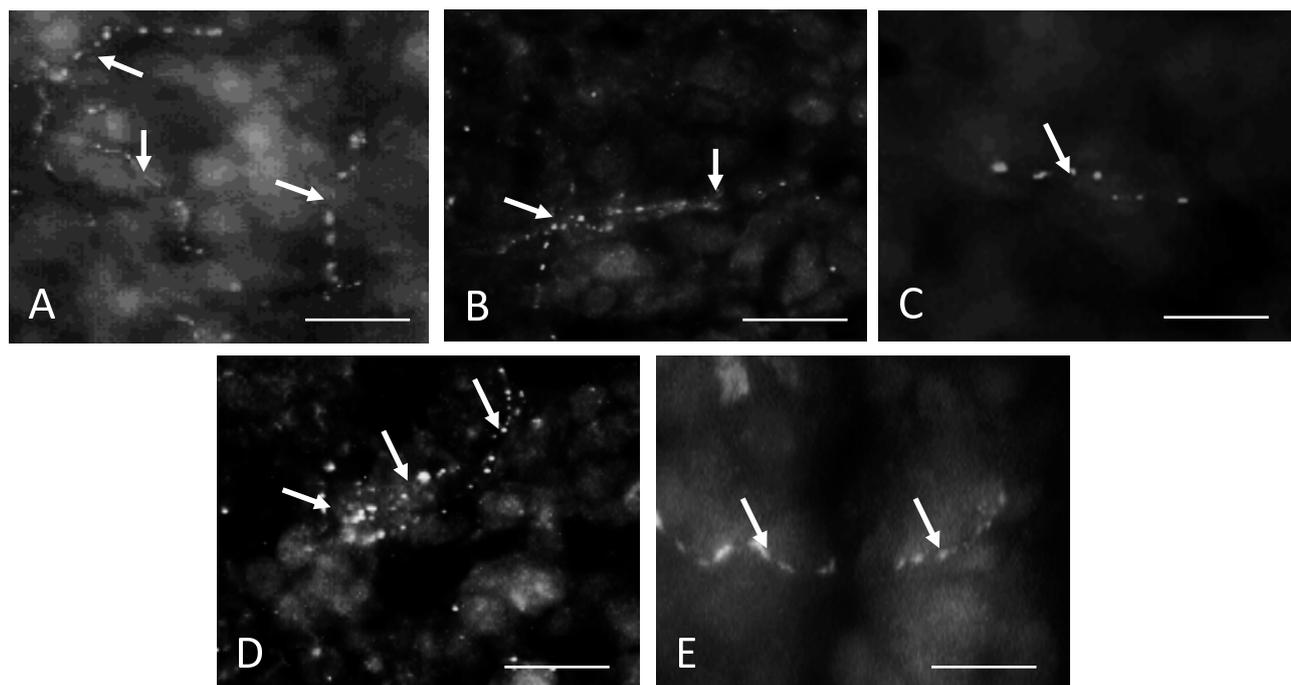


Fig. 3. Porcine adrenal medulla. Nerve fibers (arrows) immunoreactive to NOS (A), SOM (B), SP (C) GAL (D) and CGRP (E). Scale bar 30 μ m.

Interestingly, during the present investigations the biggest density of CGRP positive fibers were observed in subcapsular region and zona fasciculata of adrenal cortex. It is partly in agreement with previous studies in rats when the dense plexus of CGRP-positive nerve fibers have been studied in adrenal capsule (Kuramoto et al., 1987) and it may be connected with functions of this neuropeptide in glucocorticoids release (Thakor and Giussani, 2005). On the other hand, only rare CGRP-LI nerves were observed in adrenal medulla unlike to previous studies on rats (Murabayashi et al., 2007) and knowledge about participation of CGRP in catecholamine release (Tortorella et al., 2001). This fact suggests significant differences in innervation of adrenal glands between particular species of mammals.

SP is also involved in secretory actions of adrenal glands (Zhou and Livett, 1991), what can be an effect of vasodilatory action of this substance (Marabayashi et al., 2007). In turn, NOS is a marker of nitrergic neurons which, although its direct influence on catecholamine secretion has not been observed (Marley et al., 1995), can take part in the regulation of adrenal activity by participation in genes expression, ions channels regulation or vasodilatory effects (Faraci et al., 1989). It also is known that, apart from sensory roles, GAL is a factor that mainly interacts with adrenal cortex affecting mineralo- and glucocorticoids release and this action is different in immature and mature organisms (Hochól et al., 2000).

To sum up, the obtained results show the presence of nerve fibers immunoreactive to sensory neuronal factors in the porcine adrenal glands, but some of these nerves can also play other functions and determination how many of them in fact conduct sensory stimuli, requires further investigations.

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