

Innervation of the pineal gland in the Arctic fox (*Vulpes lagopus*) by nerve fibres immunoreactive to substance P and calcitonin gene-related peptide

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Background: The study demonstrates, for the first time, the presence of substance P (SP) and calcitonin gene-related peptide (CGRP) in the nerve fibres supplying the pineal gland in the Arctic fox.

Materials and methods: The expression and distribution pattern of the studied substances were examined by double-labelling immunofluorescence technique.

Results: The SP-positive fibres enter into the pineal gland through the capsule as the nervi conarii. The fibres formed thick bundles in the capsule and connective tissue septa, from where they penetrated into the pineal parenchyma. Inside the parenchyma, the nerve fibres created basket-like structures surrounding clusters of pinealocytes. The density of intrapineal SP positive fibres was slightly higher in the distal and middle parts of the gland than in the proximal one. Double immunostaining with antibodies against SP and CGRP revealed that the vast majority of SP positive fibres were also CGRP positive. The fibres showing a positive reaction to SP and negative to CGRP were scattered within the whole gland. The fibres immunopositive to CGRP and immunonegative to SP were not observed. In the habenular and posterior commissural areas adjoining to the pineal gland the immunoreactive nerve fibres were not found. Moreover, no immunopositive cell bodies were observed in both the pineal gland and the commissural areas.

Conclusions: These results reveal that SP and CGRP are involved in the innervation of pineal gland in carnivores. In turn we suggest that these peptides can regulate/modulate melatonin secretion. (*Folia Morphol* 2019; 78, 4: 695–702)

Key words: pineal gland, nerve fibres, substance P, calcitonin gene-related peptide, immunohistochemistry, Arctic fox

INTRODUCTION

The pineal gland is an endocrine organ involved, due to the rhythmic secretion of melatonin, in regulation of many phenomena occurring in daily and seasonal cycles [38]. In mammals, this rhythmical secretory activity is generated by the suprachiasmatic nucleus of the hypothalamus, which receives informa-

tion about the environmental light conditions from the retina. The neuronal signals from suprachiasmatic nucleus are transmitted via the multisynaptic pathway to the pineal gland. The last element of this pathway comprises the sympathetic nerve fibres, which perikarya are located in the superior cervical ganglia [21, 40]. Noradrenaline is rhythmically released from

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these fibres and induces the nocturnal increase in melatonin synthesis and secretion [38]. In addition to the sympathetic innervation, the mammalian pineal organ contains a large number of nerve fibres expressing numerous biologically active peptides that originate from the brain and various peripheral ganglia [13, 20, 24, 25, 28–31, 37]. Among them, there are nerve fibres containing substance P (SP) and calcitonin gene-related peptide (CGRP).

Substance P is an undecapeptide and belongs to the tachykinin/neurokinin family. This family comprises, in addition to SP, neurokinin A (NKA) and neurokinin B (NKB) [6]. The tachykinins are encoded by two different genes. Thus, SP and NKA are encoded by the preprotachykinin I gene, while NKB is encoded by the preprotachykinin II gene [8]. SP is considered to be the neurotransmitter for primary sensory afferent nerve fibres, where is involved in nociception. Another functions performed by SP include involvement in the regulation of pituitary hormone release, cardiovascular control and baroreceptor reflex [7, 26]. The immunohistochemical studies performed on few species demonstrated considerable differences between mammals in the density and distribution of SP-positive fibres in the pineal gland [10, 12, 18, 25, 28, 33, 37]. The putative sources of the intrapineal SP-positive fibres are the medial habenular nucleus and the trigeminal ganglion [32]. The data on function of SP in the mammalian pineal gland are enigmatic [22].

Calcitonin gene related peptide is a member of the calcitonin family of peptides [35]. CGRP is one of the most abundant peptides produced in the peripheral and central nervous systems [2, 5, 9]. This peptide is synthesized in the cell bodies of motor neurons in the anterior horn of the spinal cord and may contribute to the regeneration of nervous tissue after injury. Together with SP, CGRP may be linked to the transmission of pain in the posterior horn of the spinal cord [2, 4]. In the pineal gland, expression of CGRP was exclusively described in rodents, the tree shrew and the pig [10, 12, 25, 31, 37]. In the latter case, a significant degree of co-localisation between CGRP and SP was reported [25]. A role of these peptides in the pineal gland is completely unknown.

Except the histology and ultrastructure, which have been studied in the dog, cat, fox and mink [1, 3, 4, 41] the knowledge about the carnivore pineal gland is fragmentary and largely limited. As concerning innervation, the available data describe only the

sympathetic [15] and vasopressinergic inputs [14] in the dog pineal gland as well as the neuropeptide Y-immunoreactive fibres in the cat and mink pineal glands [17, 19]. The experimental studies showed that melatonin plays an important role in the control of the breeding season and the mounting cycle in foxes [16, 23, 39]. Melatonin has also positive effect on mink coat [34]. In view of the data concerning significance of melatonin in the carnivore physiology, further studies on the pineal gland in this group of mammals are highly desirable.

The aim of present study was to investigate by double immunohistochemistry the presence and distribution of structures containing SP and CGRP in the pineal gland of the Arctic fox. This species was chosen as a study object because of two reasons:

- the Arctic fox should be considered as a model for research on the pineal gland physiology in accompanying carnivores, as the studies on dogs and cats are significantly limited due to ethical reasons and legal regulations;
- the pineal gland and melatonin play an essential role in the seasonal changes in fox physiology and the knowledge about these phenomena is important due to practical reasons.

MATERIALS AND METHODS

Animals and material collection

Adult Arctic foxes ($n = 8$), aged 3–4 years, housed in natural light conditions in outdoor cages in a farm near Olsztyn (Poland) were used. They were fed in accordance to the nutritional regime for foxes and had free access to water. The animals were killed in Winter, according to the approved procedure for fur animal farms. The pineal glands were removed immediately after skinning and prepared for immunohistochemical examinations.

Immunohistochemical studies

The pineal glands were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS), pH = 7.4 for 60 min, rinsed several times with PBS, transferred into 30% sucrose solution and stored at 4°C until sectioning. The tissue blocks were cut in the frontal or sagittal planes using Microm HM 560 cryostat (Carl Zeiss, Germany) at a thickness of 12 μm and mounted on gelatinised glass slides. The sections were processed for a double-immunofluorescence staining followed by DAPI nuclear staining. Shortly, after drying at 32°C for 45 min, the sections were rinsed

Table 1. List of primary and secondary antisera used in the study

Antigen	Species of origin	Code	Dilution	Supplier/Country
Primary antisera				
Substance P	Rat	8450-0505	1:100	AbD Serorec/USA
Calcitonin gene-related peptide	Rabbit	AB5920	1:2000	Chemicon/USA
Secondary antisera				
Alexa Fluor 488	Donkey anti-rat	A11081	1:1000	Molecular Probes/USA
Alexa Fluor 546	Donkey anti-rabbit	A10040	1:1000	Molecular Probes/USA

in PBS (3 × 10 min) and incubated in 10% horse serum in PBS with 0.3% Triton X-100 and 1% bovine serum albumin (BSA) for 20 min. Then, the sections were incubated overnight at 4°C with primary antibodies (listed in Table 1) diluted in PBS containing 0.3% Triton X-100 and 1% BSA. On the following day, the sections were rinsed in PBS (3 × 5 min) and incubated with secondary antibodies (Table 1) at a concentration 7.5 µg/mL (in PBS containing 0.25% BSA and 0.1% Triton X-100) for 4 h. Then, the sections were again rinsed in PBS (3 × 5 min), incubated for 10 min in a 500 nM solution of DAPI (Invitrogen, USA), rinsed in PBS (3 × 5 min) and cover slipped with polyethylene glycol/glycerin solution. The prepared specimens were viewed and photographed using an Axioimager microscope Z1 equipped with an Apotome and an AxioCam HRm digital camera (Carl Zeiss, Germany). We analysed all sections per animals, whereas to prevent photographed the same nerve fibres the pictures were made from every fifth sections. Negative controls used in the immunofluorescence procedure included pre-absorption of antisera with appropriate neuropeptides (100 µg of appropriate antigen per 1 mL of corresponding antibody at working dilution), omission of the primary antisera and replacement of the primary antisera with non-immune sera.

RESULTS

The SP-immunoreactive (SP-IR) nerve fibres entered into the pineal gland as the nervi conarii (Fig. 1A). They formed thick bundles in the capsule and just beneath it (Fig. 1B). The nerve fibres penetrated from the capsule to the connective tissue septa (Fig. 1C, D) and later to the parenchyma, where they created a dense network (Fig. 1E). Inside the parenchyma, the fibres formed basket — like structures surrounding clusters of pinealocytes (Fig. 1F). The density of SP-IR

fibres was slightly higher in the distal and middle parts of the pineal gland than in the proximal one (Fig. 2).

Double immunostaining (Fig. 3A–F) against SP and CGRP demonstrated that the vast majority of SP-positive fibres were also CGRP-immunoreactive (CGRP-IR). The fibres showing a positive reaction to SP and negative to CGRP were present within the whole gland. The fibres immunopositive to CGRP and immunonegative to SP were not found.

In the habenular and posterior commissural areas adjoining to the pineal gland, the fibres under interest were not present (Fig. 2). Moreover, no immunopositive cell bodies were observed in both the pineal gland and the commissural areas.

DISCUSSION

To the best of our knowledge, this paper describes for the first time the presence and distribution of SP- and CGRP-immunopositive nerve fibres in the carnivore pineal gland. Up till now, SP has been detected in the nerve terminals supplying the pineal gland in rodents, the rat [13], cotton rat [12], gerbil [37], Chinese hamster [13], tupaiidae, the tree shrew [10], ungulates, the bovine [18], pig [25, 29] and primates, the macaque [33] as well as in the human [27]. A list of species, where the presence and distribution of CGRP-IR structures have been investigated in the pineal gland is much shorter and includes the gerbil [37], rat [13], cotton rat [12], tree shrew [10] and pig [29].

Interestingly, our data showed that the fox pineal gland possesses an exceptionally rich innervation by SP-containing fibres comparing to many other investigated species. The density of positive nerve fibres was apparently higher in the fox than in rodents and ungulates [13, 25, 28] and similar to that in the macaque [33].

The obtained results demonstrated some region-dependent differences in the distribution of

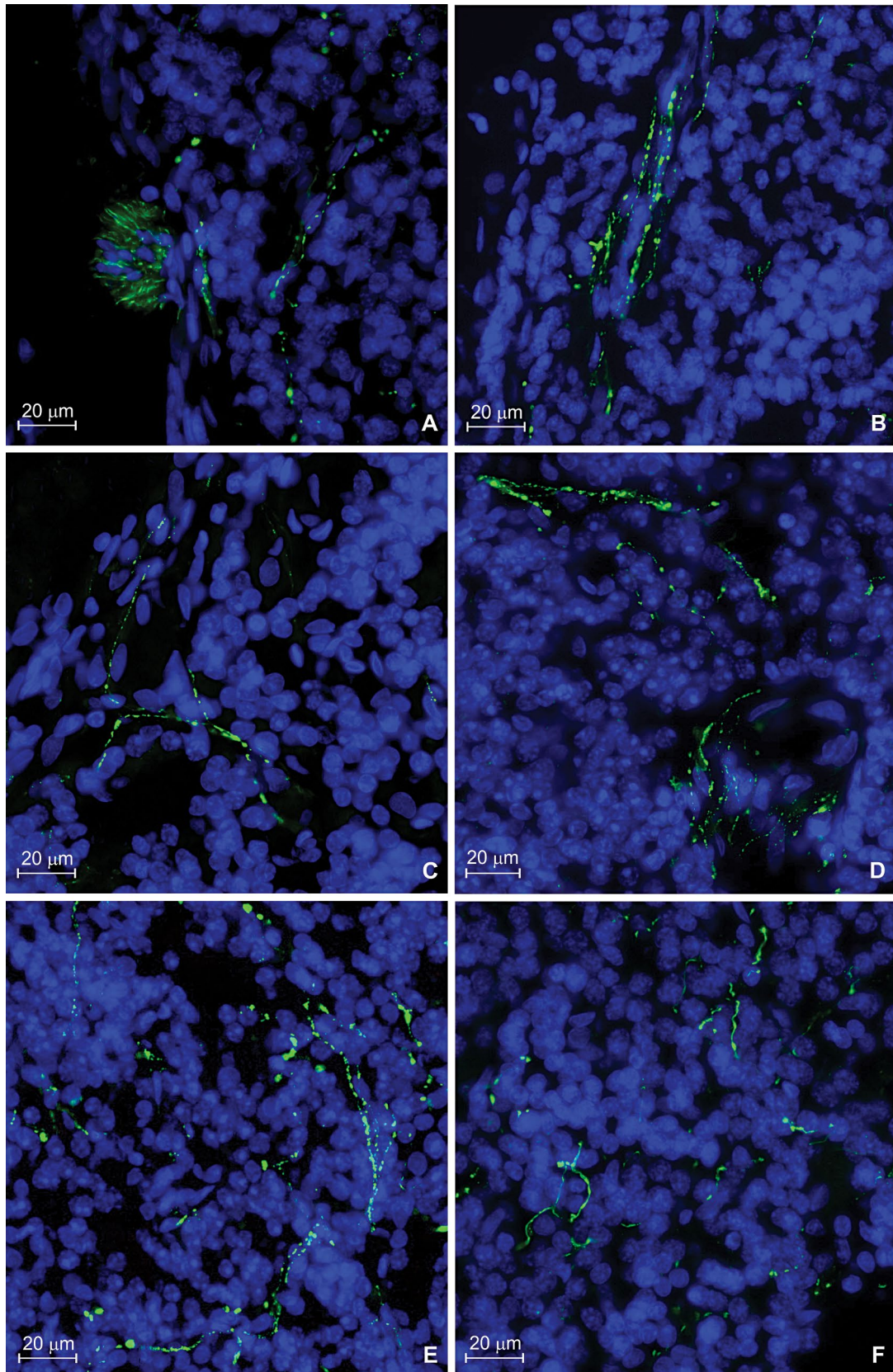


Figure 1. Localisation of substance P-immunoreactive (SP-IR) nerve fibres in the fox pineal gland; **A.** Immunopositive nerve fibres entering into the pineal gland as nervi conarii; **B.** Thick bundles of positive fibres beneath the capsule; **C.** Immunopositive fibres penetrating from the capsule to the connective tissue septa; **D.** Positive nerve fibres in the connective tissue septa and parenchyma; **E.** A dense network of positive nerve fibres inside parenchyma; **F.** Positive nerve fibres between pinealocytes.

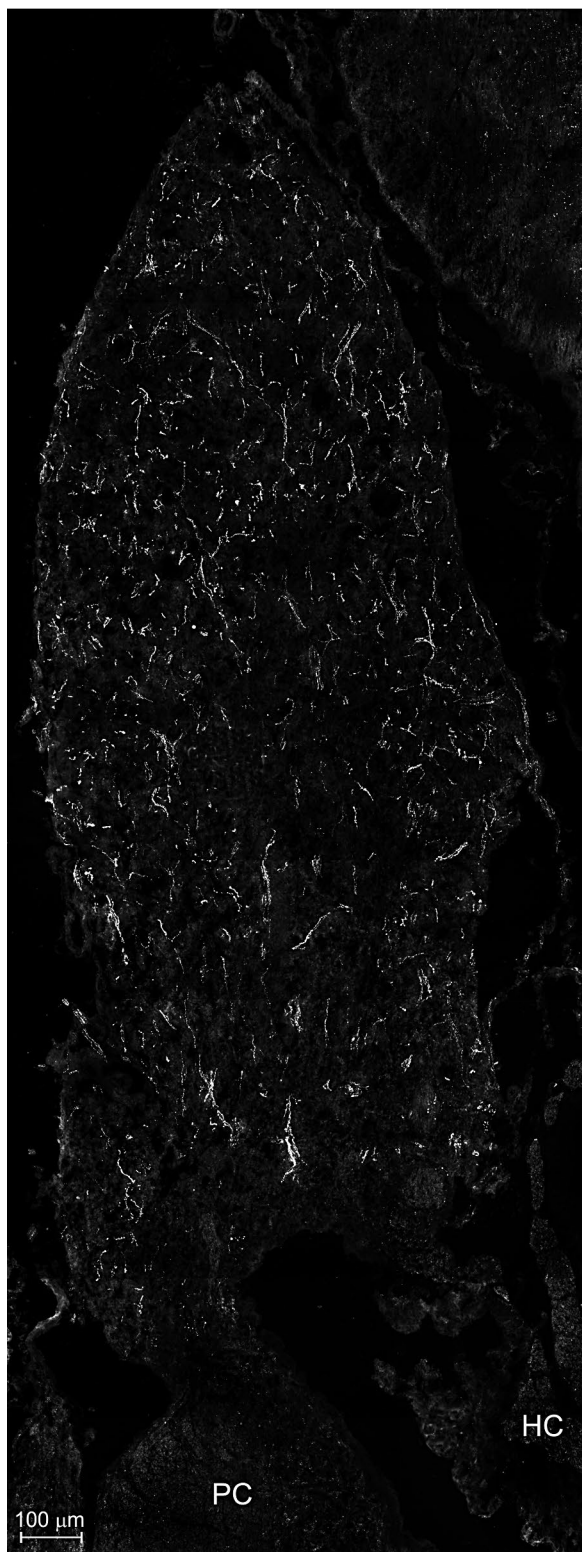


Figure 2. Distribution of substance P-immunoreactive (SP-IR) in the fox pineal gland; PC — the posterior commissure; HC — the habenular commissure.

SP-positive nerve fibres. Namely, the SP-IR fibres were slightly more numerous in the distal and middle parts of the fox pineal gland than in the proximal one. The prominent regional differences in the density of SP nerve fibres have been described in rodents [13]. In the rat and the cotton rat, SP nerve fibres were mainly located in the superficial pineal gland and in the pineal stalk, while only single nerve terminals were found in the deep pineal gland [13]. Similarly, the nerve fibres were present almost exclusively in the superficial part of the pineal gland in the hamster [11]. In contrast, the uniform distribution of SP-positive fibres was reported in the bovine [13], pig [25, 29] and macaque [33].

The interspecies differences in distribution of SP-positive fibres are probably related to variable sources of these fibres. In rodents, the vast majority of SP-IR fibres comes from the peripheral ganglia and enters into the pineal gland via the nervi conarii [13]. The use of combined technique of neuronal tracing and immunohistochemistry revealed that SP-IR fibres in the rat pineal gland originate, at least in part, from the trigeminal ganglion [32]. SP-IR fibres remained unchanged following bilateral removal of the superior cervical ganglia in the rat pineal gland [42]. In contrast, the superior cervical ganglionectomy performed in the cotton rat resulted in considerable decrease in the number of intrapineal SP-positive nerve fibres [13]. It is worth to note that this procedure completely eliminated the fibres immunopositive to tyrosine hydroxylase and neuropeptide Y, but no SP-IR fibres from the nervi conarii. Thus, it could be concluded that both sympathetic and non-sympathetic nerve fibres form the nervi conarii and enter into the pineal gland via this route [13]. The sparse fibres present in the deep pineal gland of rodents may have their source in the central nervous system. In the rat pineal gland, it was demonstrated that SP-positive cells located in the medial habenular nucleus give their processes to the deep pineal gland [13]. In turn, the results obtained in the bovine suggest mainly the central origin of SP-IR fibres [18]. In this species numerous SP-IR cells occur in the habenular nucleus, from where the positive fibres penetrate towards the pineal gland. The positive fibres are also present in the posterior commissure. Similarly, in the pig, numerous SP-IR nerve penetrate from the habenular and posterior commissures to the

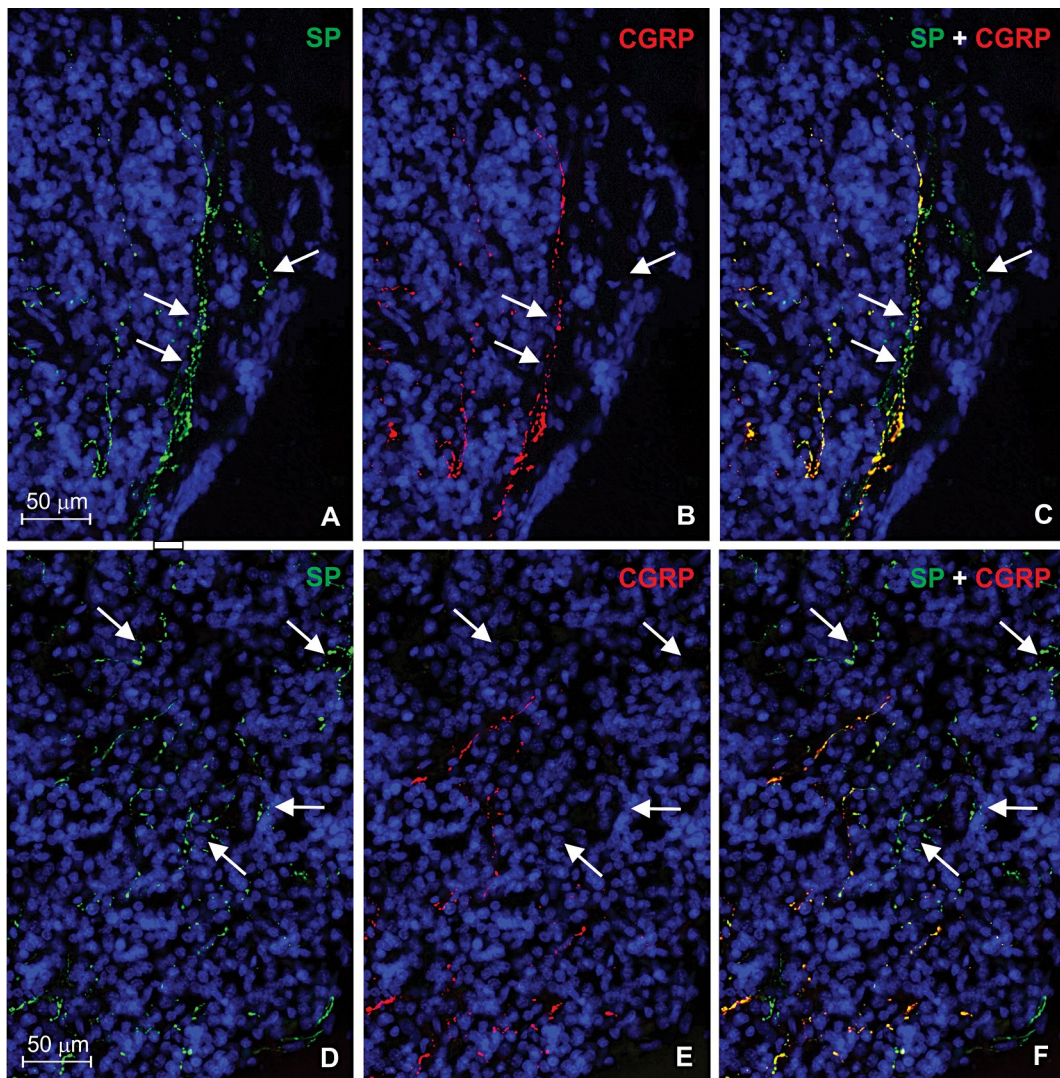


Figure 3. Double immunostaining with antibodies against substance P (SP) and calcitonin gene-related peptide (CGRP); **A, B, C.** Bundles of nerve fibres in the capsule and in the peripheral part of parenchyma; **D, E, F.** Network of nerve fibres in the parenchyma. Note that the vast majority of SP-positive fibres are also CGRP-positive. Some fibres show positive reaction to SP and negative to CGRP (arrows).

pineal gland, and form thick bundles in the proximal part of the gland [28]. Double immunocytochemistry with antibodies against SP and CGRP demonstrated two populations of SP-IR fibres in the pig pineal gland [11]: (1) SP-positive/CGRP-negative fibres present in the proximal part of the organ and the commissural areas, originating, at least in part, from the neurons located in the habenular nucleus, (2) SP-positive/CGRP-positive fibres present in the distal and middle parts of the organ, having probably a peripheral origin. The intrapineal neuronal-like cells should be considered as another source of SP-IP fibres. Such cells have been found in the cotton rat and the human; however, they are rather sparse [13, 27].

In our study, the careful examination of serial sections demonstrated that the SP-IR fibres penetrate inside the gland parenchyma through the pineal capsule as the *nervi conarii*. Furthermore taking into consideration the intrapineal distribution of fibres we can deduce the SP-positive innervation of the fox pineal gland has a peripheral origin. This assumption is strongly confirmed by the absence of SP-IR cells and fibres in the posterior and habenular commissural areas adjoining to the pineal gland well as the lack of SP-IP cell bodies inside the gland.

Another peptide examined in our study was CGRP. The CGRP-IR fibres were less abundant than SP-IR fibres in the fox pineal gland, however the distribution

pattern of CGRP was essentially the same as that of SP. Like, SP-IP fibres, CGRP-IR fibres were slightly more numerous in the distal and middle parts of the gland than in the proximal one. The habenular and commissural areas were devoid of any CGRP positive elements.

The double immunofluorescence staining has revealed that all fibres containing CGRP are also SP positive; however, there are some nerve fibres with the positive reaction for SP and negative for CGRP. Our results demonstrate the presence of two populations of SP-IR nerve fibres in the fox pineal gland: these simultaneously expressing CGRP and those without CGRP. Both populations enter the pineal gland via the nervi conarii.

The distribution patterns of CGRP-IR nerve fibres are similar in the pineal glands of all studied species up till now. In rodents, the density of CGRP-positive fibres is moderate in the superficial pineal gland and the stalk while in the deep pineal only single fibres are presented [17]. The fibres are absent in the habenular and posterior commissures. In the pig, the nerve fibres containing CGRP occur exclusively in the distal and middle parts of the pineal gland.

The question arises, what are the physiological roles of SP and CGRP in the pineal gland? Until recently, it has been considered that SP has no influence on the pineal secretory activity. The research performed on the rat pinealocytes demonstrated that the application of SP did not influence the cAMP formation as well as the basal or norepinephrine-stimulated serotonin N-acetyltransferase activity and melatonin secretion [38]. Similar data have been obtained for melatonin secretion from bovine pinealocytes [36]. The recent studies performed on the rat pineal organ cultures, shed new light for the role SP [22]. At the presynaptic site, SP potentiates the release of norepinephrine from the sympathetic preganglionic fibres endings. This effect is mediated through neurokinin 1 receptor. Activation of this receptor by SP augments the intracellular Ca^{2+} concentration what stimulates the release of norepinephrine. At the postsynaptic site, SP inhibits the norepinephrine-induced increase N-acetyltransferase activity and melatonin synthesis [22]. These data clearly demonstrate that SP plays a modulatory role in the adrenergic regulation of melatonin synthesis, acting both at the presynaptic and postsynaptic levels. The role of CGRP in the pineal gland has been not investigated. This peptide may participate in the control of blood flow.

CONCLUSIONS

Taking all these together, it is reasonable to conclude that the fox pineal gland is richly supplied by SP- and CGRP-positive nerve fibres. The fibres enter into the pineal gland via the nervi conarii and create numerous bundles in the capsule, from where they penetrate to the connective tissue septa and later to the parenchyma. There are two population of SP-positive fibres, differing by the presence or lack of CGRP. The obtained results seem to be interesting because SP/CGRP innervation of the fox pineal gland, especially their density and origin, differs from hitherto examined mammals. In our opinion, the presence of nerve structures immunoreactive to SP and CGRP indicates participation of these peptides in the regulation of synthesis and secretion of melatonin. Further research, taking into account the presence of receptors of SP and CGRP as well as effects of the peptides on melatonin secretion *in vitro*, are necessary to fully understand the role of SP and CGRP in the pineal physiology in carnivores.

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