

Innervation of the canine mammary gland: an immunohistochemical study

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Summary. The distribution of peptidergic nerves in canine mammary tissues was studied by immunohistochemical techniques. In addition, the general and the noradrenergic innervations were demonstrated using protein gene product 9.5 and tyrosine hydroxylase immunoreactivities as markers, respectively. Tissue specimens from the caudal mammary glands were obtained from adult, non-lactating, female dogs. The overall innervation of the mammary gland tissue was sparse and primarily associated with the arterial vasculature. Nerve fibres positive for protein gene product 9.5 were rarely found in the secretory parenchyma. The nipple was not richly innervated, although it displayed a greater amount of nerve fibres than the mammary parenchyma. Nerve fibres supplying nonvascular structures of the nipple expressed immunoreactivity for the sensory neuropeptides calcitonin gene-related peptide, substance P and neuropeptide K, but not for vasoactive intestinal peptide, peptide histidine isoleucine and C-flanking peptide of neuropeptide Y. Somatostatin immunoreactivity was not detected in mammary gland tissue. Our results indicate that the innervation of the canine mammary gland is mainly affiliated with the vasculature and comprises peptidergic nerves which may be involved in the regulation of local blood flow. The presence of sensory neuropeptides in nerves supplying the mammary nipple suggest that these peptides may play a role in the afferent pathway of the milk ejection reflex.

Key words: Mammary gland, Innervation, Dog, Neuropeptides, Immunohistochemistry

Introduction

Most of the studies concerning the innervation of the mammary gland have focused on the neuronal basis of the milk ejection reflex and, in particular, on the sympathetic and sensory mechanisms involved in that process (Moos and Richard, 1975; Haller, 1985; Clapp et al., 1985; Tasker et al., 1986; Tasker et al., 1988; Poulain and Wakerley, 1986; Rousselot et al., 1994; Mena et al., 1995). In contrast, only a few studies on the occurrence and functional significance of nerves containing neuropeptides in the mammary gland have so far been reported. The first description of a neuropeptide in mammary tissue dates back to the mid-1980s with the demonstration of substance P immunoreactivity in nerves supplying the rat mammary nipple and secretory parenchyma (Traurig et al., 1984). Subsequently a few studies have revealed the presence of other peptides within nerves innervating the mammary gland in woman (Eriksson et al., 1996a), rat (Thulesen et al., 1994; Eriksson et al., 1996a; Skakkebæk et al., 1999) and pig (Franke-Radowiecka et al., 2002; Franke-Radowiecka, 2003). These observations have been expanded in a number of studies documenting the expression of neuropeptide receptors, such as vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (PACAP) receptors, in neoplastic and non-neoplastic mammary tissues (Reubi, 1995; Reubi et al., 2000, 2002; Dagar et al., 2001; Moody et al., 2004; Schulz et al., 2004; García-Fernández et al., 2005), as well as in normal mammary epithelial cells and breast cancer cell lines (Gespach et al., 1988; Berthon et al., 1992; Waschek et al., 1995). In the last years, it has become increasingly evident that neuropeptides may have a role on the growth, differentiation, and function in normal and neoplastic breast. One of the examples is that of VIP, a peptide suspected to have a physiological role in regulating milk secretion and ejection from the mammary glands (Werner et al., 1985; Uvnas-Moberg et al., 1984; Rolandi et al., 1987; Eriksson et al., 1987) and to be an additional breast cancer growth factor (Zia et

al., 1996; Csernus et al., 1999; Moody et al., 2004). More recently, the involvement of neuropeptides of the tachykinin family and their receptors in breast cancer development has also been an area of research interest (Singh et al., 2000; Patel et al., 2005; Bigioni et al., 2005).

Despite their potential roles in the mammary gland biology, the occurrence of neuropeptide-containing nerves in canine mammary tissues has not so far been reported. The fact that the mammary gland is one of the most common sites of tumour development in dogs (Misdorp, 2002), further strengthens the need for studies addressing the role of neuropeptides in canine mammary tissues. Accordingly, we describe here the distribution of nerve fibres containing peptides in the nipple and mammary parenchyma of the female dog by immunohistochemical techniques. Particular classes of nerves were studied using antisera for the following neuropeptides: C-flanking peptide of neuropeptide Y (CPON), calcitonin gene-related peptide (CGRP), substance P (SP), neuropeptide K (NPK), vasoactive intestinal peptide (VIP), peptide histidine isoleucine (PHI) and somatostatin (SOM). In addition, protein gene product 9.5 (PGP 9.5), which is considered a highly sensitive marker of neuronal elements including peripheral axonal projections (Thompson et al., 1983; Wilson et al., 1988), was employed as a pan-peripheral nerve marker, and tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, was evaluated to visualize the distribution of noradrenergic nerves.

Materials and methods

Animals

Healthy sexually mature, non-lactating, female mixed-breeds dogs (n=5), housed at the Oeiras County Animal Shelter (Portugal), were used in this study. The animals were abandoned dogs that were to be sacrificed. Tissue specimens from the last two sets of mammary

glands (the 4th and 5th glands) were collected immediately after euthanasia with an intravenous injection of sodium pentobarbital.

Tissue processing

Mammary tissues were fixed by immersion in Zamboni's fixative (Stefanini et al., 1967) for 16-24h at 4°C. Following thorough rinsing in phosphate-buffered saline (PBS; pH 7.2) containing 15% (w/v) sucrose and 0.1% (w/v) sodium azide, tissues were embedded in Tissue-Tek O.C.T.compound (Sakura Finetech Europe BV, Zoeterwoude, The Netherlands) and snap-frozen in nitrogen-cooled isopentane. Sections, 12 µm thick, were cut at -20°C in a cryostat (Leica CM3050S). All sections were collected on Superfrost Plus slides (Menzel-Gläser, Braunschweig, Germany) and air-dried for 1 hr at room temperature (RT).

Immunohistochemistry

Sections were treated with 0.2% Triton X-100 in PBS (30 min; RT) and stained with 0.05% Chicago Sky Blue (Sigma, St. Louis, MO, USA) (30min; RT). The sections were then rinsed in PBS (3x10 min), incubated in primary antisera (Table 1) overnight at 4°C, rinsed again in PBS (3x10 min), and incubated with biotinylated goat anti-rabbit immunoglobulin G (1:200 dilution; Vector Laboratories, Burlingame, CA, USA) for 1 h at RT. After rinsing in PBS (3x10 min), the sections were further incubated for 1 h in fluorescein isothiocyanate (FITC)-conjugated streptavidin (1:100 dilution; Vector Laboratories, Burlingame, CA, USA) at RT. After final washing in three changes of PBS, the sections were coverslipped in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and examined using an Olympus BX-50 microscope equipped for fluorescence epi-illumination. Images were captured with an Olympus DP-10 digital camera, imported into Corel[®] Paint Shop Pro[®] X, contrast and brightness adjusted if necessary, labelled and then

Table 1. Source and characterization of the primary antisera.

Antigen	Donor Species	Code	Working dilution	Source
PGP 9.5	Rabbit	Ra95103	1:9600	Ultraclone, UK
TH	Rabbit	Te101	1:1200	Eugene Tech, USA
CPON	Rabbit	1411	1:1200	Hammersmith Hosp.,UK
CGRP	Rabbit	1208	1:1600	Hammersmith Hosp.,UK
SP	Rabbit	910	1:1600	Hammersmith Hosp., UK
VIP	Rabbit	652	1:4000	Hammersmith Hosp., UK
NPK	Rabbit	15-36R2	1:3000	Dr. Valentino, USA
PHI	Rabbit	938	1:1600	Hammersmith Hosp., UK
SOM-28 (Tyr ⁴⁻¹⁴)	Rabbit	1082	1:1600	Immuno Nuclear, USA

PGP 9.5: protein gene product 9.5; TH: tyrosine hydroxylase; CPON: C-flanking peptide of neuropeptide Y; CGRP: calcitonin gene-related peptide; SP: substance P; VIP: vasoactive intestinal peptide; NPK: neuropeptide K; PHI: peptide histidine isoleucine. SOM-28: somatostatin. References for characterization of the antisera: Gulbenkian et al., 1985, Gulbenkian et al., 1987, Gulbenkian et al., 1990, Wharton et al., 1988.

Innervation of canine mammary gland

printed. Control conditions included the omission of the primary antiserum, replacing the primary antiserum with preimmune serum, and preabsorbing the antisera with the corresponding antigens (10^{-5} - 10^{-6} M). No immunostaining was observed after any of these control procedures.

Results

The use of an antiserum raised against the general

neuronal marker PGP 9.5 showed that the innervation of the canine mammary gland was sparse and mostly affiliated with the arterial vasculature. In general, arteries and arterioles, as distinguished by a thick muscular wall and the presence of an elastica interna, were ensheathed in a moderately dense network of innervation (Fig. 1A). Veins and venules, as identified by a thin wall and a relatively large lumen, were devoid of demonstrable nerves (Fig. 1B). PGP 9.5-positive nerve fibres were rarely found among the alveoli in the

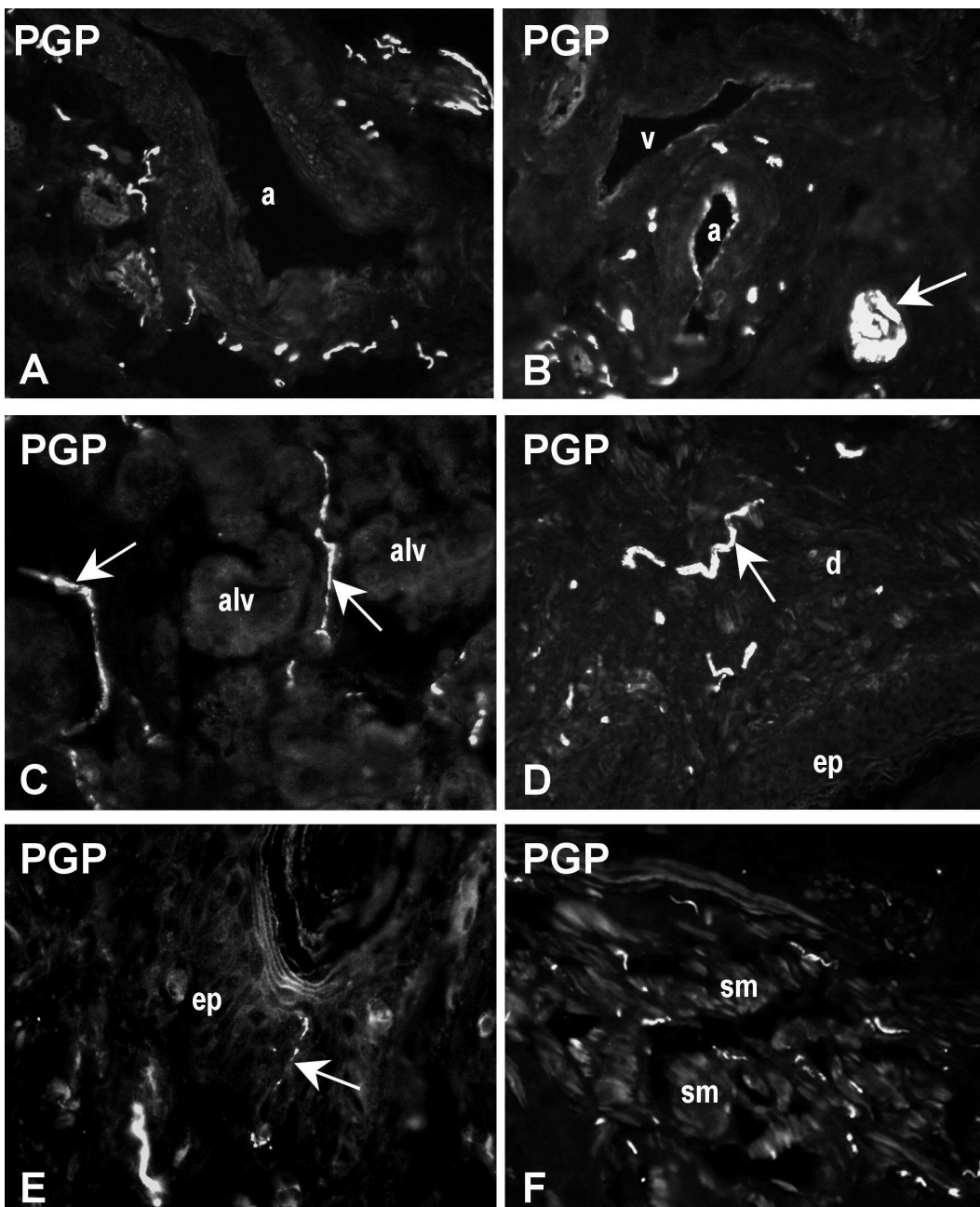


Fig. 1. Cryostat sections of canine mammary gland tissue immunostained for the general neuronal marker PGP 9.5 (PGP). The overall innervation of the dog mammary tissues is primarily associated with arteries and arterioles (a) (A, B). Veins (v) are not innervated. A nerve fascicle (arrow) is observed in relation to an arterial vessel (a) (B). The nerve supply of the mammary parenchyma is scarce. A few delicate varicose fibres (arrows) can be detected between alveoli (alv) (C). Small nerve fascicles (arrow) and single fibres are found scattered in the dermis (d) of the nipple (D). Occasional fine calibre axons (arrow) are observed within the nipple epidermis (ep) (E). Nerve fibres with PGP 9.5 immunoreactivity are also associated with smooth muscle fascicles (sm) of the nipple (F). A, B, D, x 200; C, E, x 400

secretory parenchyma. Only sporadically, a single fine-caliber axon could be detected between alveoli and, in these areas, mostly associated with an intralobular small-sized blood vessel (Fig. 1C). The interlobular stroma housed small nerve bundles which usually accompanied the interlobular arteries (Fig. 1B).

The results showed also that the nipple was not richly innervated, although it displayed a greater amount of PGP 9.5-containing nerve fibres than the mammary parenchyma. In the dermis of the nipple, nerve fibres were seen as components of thin nerve bundles or as freely coursing axons (Fig. 1D). Some of these dermal nerve fibres were found in association with blood vessels and skin appendages; others were seen immediately beneath the epidermis covering the nipple and sometimes even within it (Fig. 1E). Single nerve fibres with PGP 9.5 immunoreactivity were also observed in association with smooth muscle fascicles of the nipple (Fig. 1F). In contrast, no contacts were found between PGP 9.5-positive nerve fibres and the epithelium of the lactiferous ducts.

Immunoreactivity for TH was predominantly vascular (Fig. 2A). However, TH-containing fibres represented just a fraction of all periarterial fibres stained by PGP 9.5 antiserum. TH-containing axons were also observed as components of nerve bundles running in the interlobular stroma (Fig. 2B) and, occasionally, in and around the smooth muscle fascicles in the nipple. Only very sporadically, a solitary nerve fibre displaying TH immunoreactivity could be detected within the mammary lobules, apparently associated with an intralobular small arteriole.

As regards the peptidergic innervation, CGRP-immunoreactive fibres and CPON-immunoreactive fibres appeared to represent the main peptide-containing subpopulations in the mammary gland tissue of the dog. Both types of nerve fibres were mainly encountered in large interlobular arteries, forming incomplete cuffs around the vessel wall (Fig. 3A,B). Few nerve fibres displaying immunoreactivity for either SP, NPK, VIP or PHI were identified in the wall of arterial vessels (Fig. 3C-F). In this particular, both the VIP- and PHI-immunoreactive innervations were exceptionally sparse being apparently restricted to the wall of the larger arteries where they occurred as solitary thin-caliber profiles located in the media-adventitia border of the vessel (Fig. 3E,F).

Individual peptide-containing nerve fibres were also found in interlobular nerve trunks but almost never within the mammary lobules among alveoli. In addition, scattered nerve fibres displaying immunoreactivity for either CGRP, SP or NPK but not for CPON, VIP or PHI were detected at low density in the dermis of the nipple with no apparent relation to blood vessels. Some of these nonvascular peptidergic fibres were seen in close association with the basal lamina of the epidermis (Fig. 4A-C). However, fibres penetrating the basal lamina were not found. Occasional axons with immunoreactivity for CGRP, NPK or SP were also observed adjacent to and within the smooth muscle fascicles of the nipple (Fig. 4D). Of the few peptidergic nerve fibres visible in the dermis of the nipple, the most numerous were those displaying CGRP-immunoreactivity.

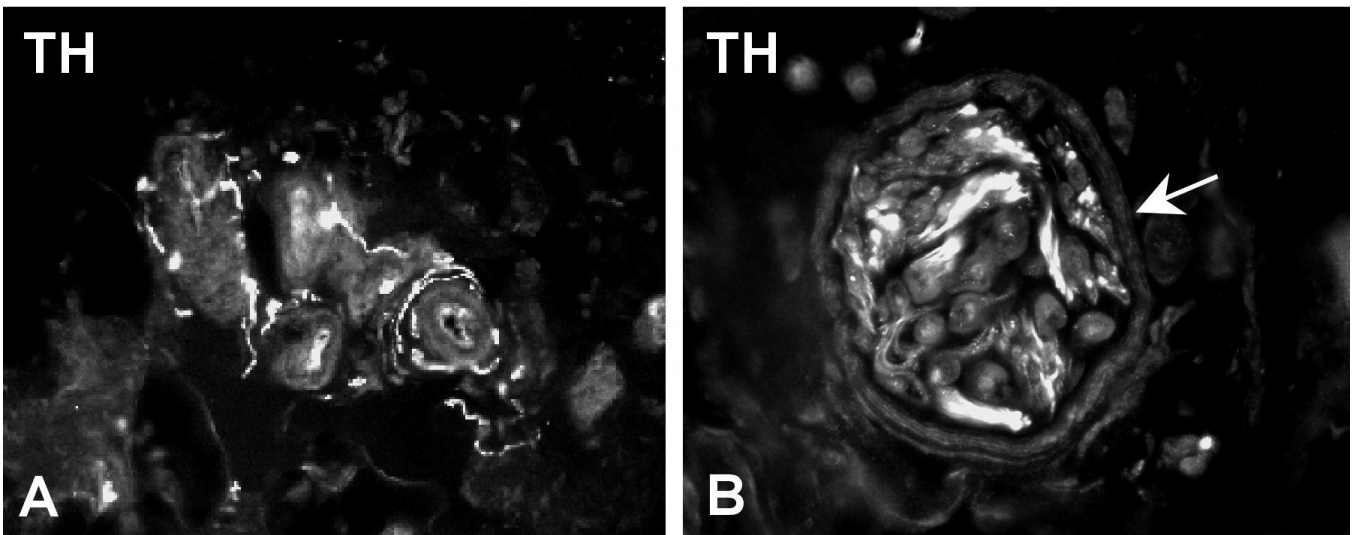


Fig. 2. Cryostat sections of canine mammary gland tissue immunostained for tyrosine hydroxylase (TH). TH-containing nerve fibres are found predominantly in relationship to arteries and arterioles (A). A nerve fascicle (arrow) with TH-immunoreactivity is shown in cross section in the interlobular stroma of the gland (B). A, x 200; B, x 400

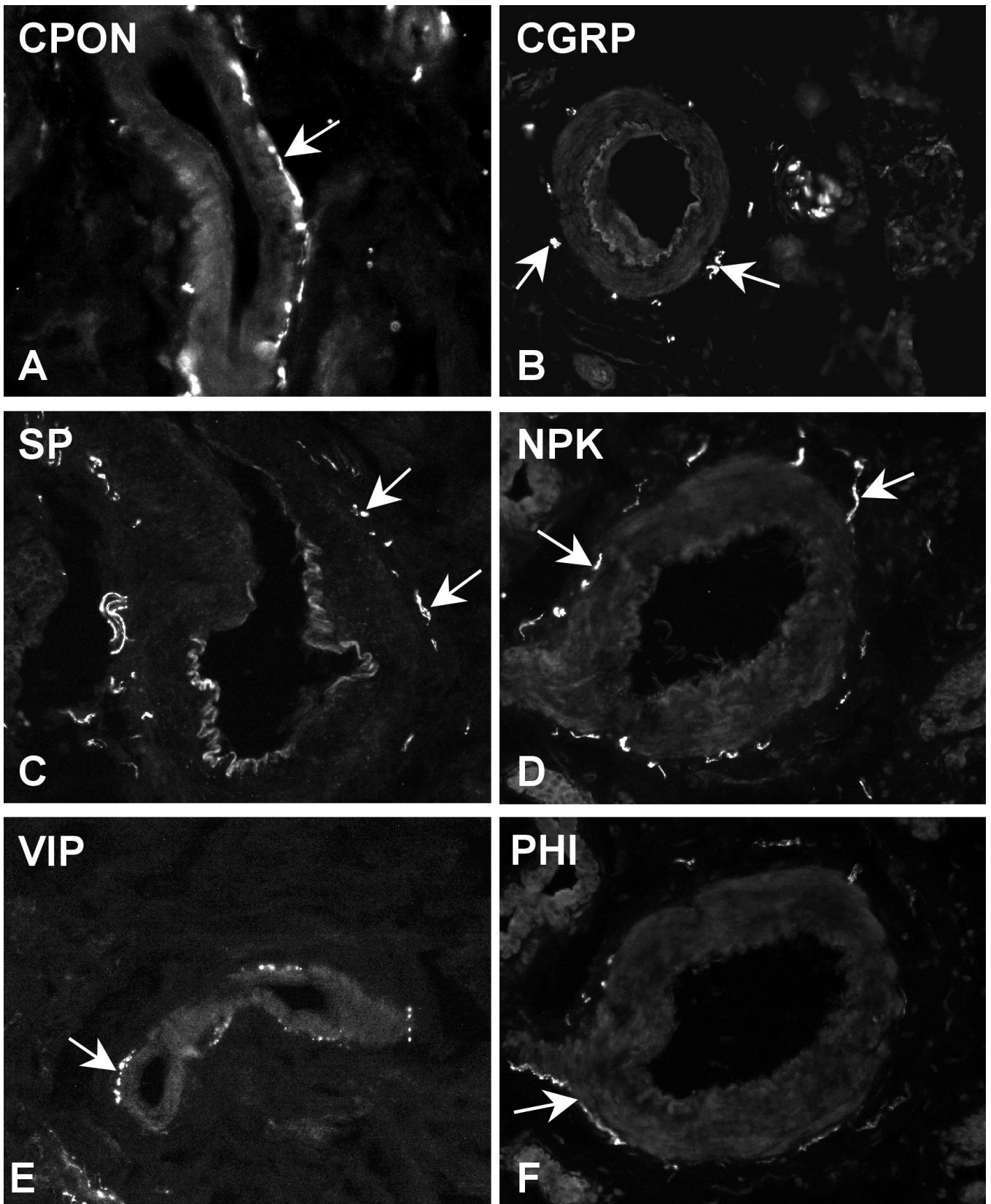


Fig. 3. Cryostat sections of canine mammary gland tissue immunostained for CPON (A), CGRP (B), SP (C), NPK (D), VIP (E) and PHI (F). Immunoreactivities for CGRP, SP, NPK, VIP and PHI are mainly recognized in nerve fibres (arrows) distributed around arteries and arterioles. B-D, x 200; A, E, F, x 400

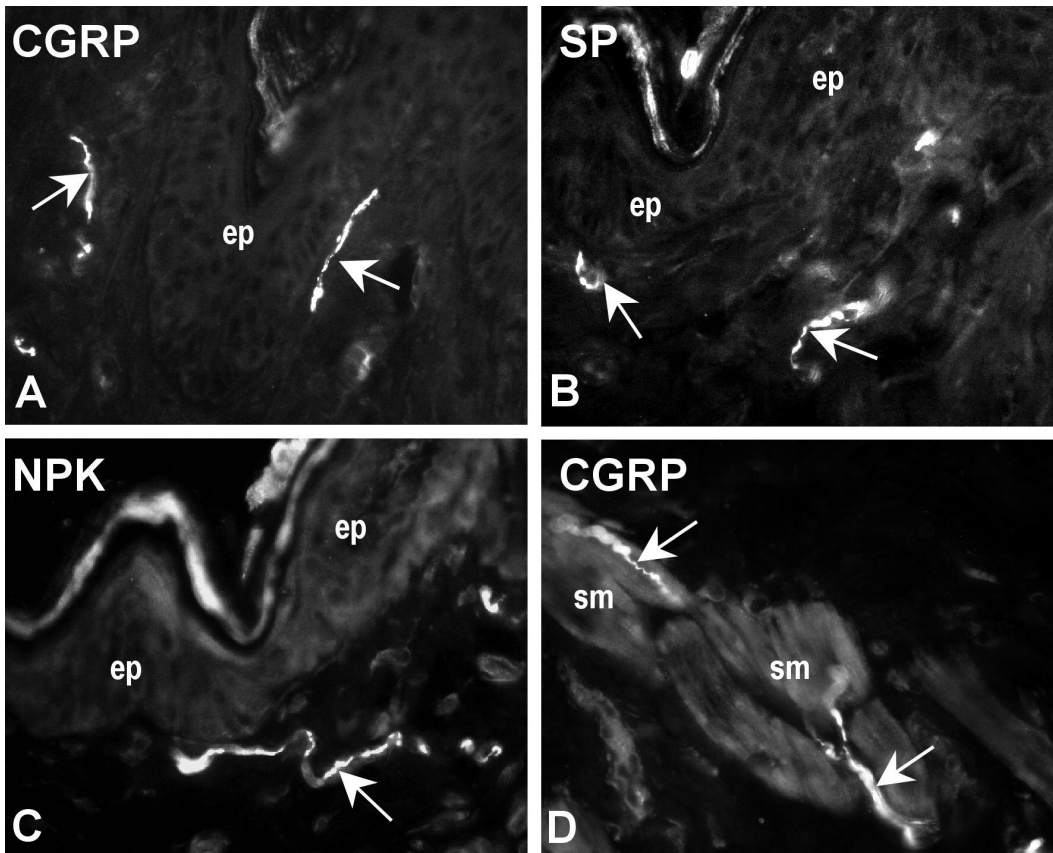


Fig. 4. Cryostat sections of canine mammary nipple immunostained for CGRP (A, D), SP (B), and NPK (C). CGRP-, SP- and NPK-immunoreactivities are detected in a few scarcely scattered nerve fibres (arrows) within the connective tissue underneath the epidermis (ep) of the nipple (A, B, C). Nerve fibres with CGRP-immunoreactivity (arrows) are also found between smooth muscle fascicles (sm) in the nipple (D). A, x 200; B-D, x 400

Somatostatin immunoreactivity was not detected in the mammary gland tissue of any of the examined dogs.

Discussion

The present study shows that in the female dog the overall innervation of the mammary tissues, as visualized by the general neuronal marker PGP 9.5, is primarily associated with arteries and arterioles. Similarly, the vast majority of peptidergic nerve fibres, as well as of nerves containing the catecholamine synthesizing enzyme TH, are found in close proximity to arterial blood vessels. Except for this perivascular location, peptide- and TH-containing nerve fibres are poorly represented in canine mammary tissues. When encountered, noradrenergic and peptidergic fibres are localized in nerve trunks or sparsely distributed in the dermal connective tissue of the nipple, mainly in association with smooth muscle fascicles. It is noteworthy that large parts of the secretory parenchyma can be found without any innervation. In particular, peptidergic nerve fibres seem to be lacking around alveoli or ducts. Comparing these results with those obtained in the human (Eriksson et al., 1996a), rat (Traurig et al., 1984; Thulesen et al., 1994; Eriksson et al., 1996a) and pig (Franke-Radowiecka et al., 2002;

Franke-Radowiecka, 2003) mammary gland, it appears that there are distinct interspecies differences in the occurrence and distribution of peptide-containing nerve fibres in mammary tissues. Peptidergic nerves have been described in high density in the nipple in all of these species, but to a lesser extent in the mammary parenchyma. Nerve fibres expressing CGRP immunoreactivity are particularly abundant in the nipple of both rat and human, with fewer fibres associated with the secretory parenchyma (Thulesen et al., 1994; Eriksson et al., 1996a). This is in marked contrast to the dog, in which there is a sparse supply of CGRP-immunoreactive nerves in the nipple and an almost complete lack of this innervation in the mammary parenchyma.

Although relatively few nerve fibres display CGRP immunoreactivity, this neuropeptide, along with CPON, seems to be the most abundant one in nerves associated with arterial vessels. A recent report by Blacklock and Smith (2004) also noted a predominant association of CGRP-fibres with arterioles in the mammary gland of ovariectomized rats. Although fewer than the CGRP-containing nerve fibres, tachykinins (SP or NPK)-immunoreactive nerve fibres were also found in the arterial supply of the mammary gland tissue of the dog. Co-localization of tachykinins and CGRP has been

Innervation of canine mammary gland

documented in peripheral endings of primary afferent neurons in different organs of mammalian species, and these neuropeptides have been shown to act as potent vasodilators (Maggi, 1995; Lundberg, 1996). A possible relevance of CGRP and tachykinins as modulators of mammary blood flow is further suggested by the vasodilatory actions exerted by CGRP and SP in the precontracted human internal mammary artery (Luu et al., 1997; Raddino et al., 1997; Wiley and Davenport, 2002) and in the isolated bovine intra-mammary artery (Trakranungsie and Will, 1997), respectively. CGRP has further been reported to cause an increase in cutaneous blood flow when injected in the skin overlying the rat mammary gland (Eriksson et al., 1996b). Thus, it can be postulated that in canine mammary tissues, perivascular CGRP- and tachykinins-containing nerves exert vasorelaxant activities, raising blood flow and therefore increasing substrate supply for milk synthesis.

In addition to an efferent function regulating blood flow, CGRP and SP have been suggested to be involved in the transmission of the suckling stimuli centrally. The high density of CGRP-immunoreactive nerve fibres and to a lesser extent of SP-positive fibres in structures of both the human and rat nipple (Eriksson et al., 1996a), as well as the presence of CGRP-immunoreactivity in neurons of the dorsal root ganglia projecting to the mammary gland (Tasker et al., 1988) supports this notion. However, as already mentioned, our study revealed that, with the exception of the vasculature, the nipple of the dog is poorly innervated (PGP 9.5-immunoreactivity), receiving only a sparse supply of nerve fibres containing CGRP or tachykinins. It is worthwhile to note that McGrouther and Ahmad (1998) also reported a relatively sparse innervation of the human breast skin by CGRP-containing nerve fibres. These discrepancies may be explained either by species differences or by methodological factors. In the present study, some of the nerve fibres expressing immunoreactivity for CGRP, SP or NPK were found in close proximity to the epidermis covering the nipple. However, contrary to what has been reported in the human and rat nipple (Thulesen et al., 1994; Eriksson et al., 1996a; McGrouther and Ahmad, 1998; Blacklock and Smith, 2004), we were not able to demonstrate intraepidermal free nerve fibres endings expressing CGRP- or tachykinins-immunoreactivity in the dog nipple. In spite of this, and in spite of their low density, CGRP and tachykinins-containing nerve fibres may play a role in the afferent pathway of the milk ejection reflex in the dog as suggested for the other mammalian species. Presumably, the fine caliber epidermal innervation evidenced in the dog by means of the anti-PGP 9.5 antibody is mostly nonpeptidergic since it does not label with antibodies against other neuropeptides.

The present study also reports the localization of CPON-immunoreactive nerve fibres in canine mammary tissues. CPON is a peptide produced by posttranslational processing of a molecular precursor which also yields neuropeptide Y (NPY). As expected, CPON has an

identical distribution pattern to NPY (Gulbenkian et al., 1985), but no role has yet been proposed for CPON as an effector of biological function. Since NPY and CPON occur together in the same precursor molecule and show full colocalization, immunostained fibres detected using antisera raised against CPON can be regarded also as NPY-ergic. Our results show that in canine mammary tissues, nerve fibres displaying immunoreactivity to CPON are sparse and restricted almost exclusively to the wall of arteries. These findings differ somewhat from previous immunohistochemical demonstrations of NPY-containing nerve fibres in mammary tissues of rats (Thulesen et al., 1994; Eriksson et al., 1996a), pigs (Franke-Radowiecka et al., 2002) and humans (Eriksson et al., 1996a). In all of the three species, NPY-positive vascular nerves seem to be more numerous than CPON-containing vascular nerves in the dog. Moreover, in both rat and human tissue, in addition to arterial vessels, NPY-containing nerve fibres are seen around veins and also found in association with non-vascular smooth musculature and lactiferous ducts of the nipple. On the contrary, virtually none of the CPON-positive nerve fibres observed in canine mammary tissue are affiliated with veins, ductal structures or non-vascular smooth musculature. Despite these species-related differences, it is obvious that the vasculature is one of the main targets for the CPON-immunoreactive innervation in mammary tissue. In vascular beds, both CPON and NPY are usually colocalized with noradrenaline in sympathetic nerves and NPY has been regarded as a modulator of peripheral autonomic vasoconstriction (Edvinsson et al., 1984). Thus, the present demonstration of CPON immunoreactivity in relation to arterial vessels might lead to the suggestion that NPY/CPON-containing nerves also in canine mammary tissues participate in the local control of the vascular tone. On the other hand, the possibility that NPY may be involved in the contraction of the smooth muscle fibres which leads to nipple erection and the emptying of the lactiferous ducts, as suggested for the woman and rat (Eriksson et al., 1996a), is not corroborated by our morphological results showing no association of CPON-immunoreactive fibres with structures other than blood vessels.

We did not examine whether immunoreactivities for CPON and TH are colocalized in dog mammary tissues as was observed for NPY and TH in the woman, rat and pig (Eriksson et al., 1996a, Franke-Radowiecka et al., 2002). However, in canine mammary tissues, the CPON- and TH-immunoreactivity differ in their localization in that TH is occasionally found in association with the nonvascular smooth muscle of the nipple and CPON is not. As already mentioned, mammary tissue of dogs receives only a sparse supply of TH-containing nerve fibres. Immunostaining for TH indicates that arteries and arterioles are the main targets for noradrenergic fibres. These findings correlate with the observations of Blacklock and Smith (2004) who reported a low density of TH-immunoreactive fibres in mammary tissues of rats and a predominance of these fibres around arterial

vessels.

Our results also show that the mammary gland tissue of the dog receives a very sparse supply of VIP-positive nerves, which are limited to the wall of larger arteries. In particular we could not find these peptide-containing nerves within the secretory parenchyma or in association with the lactiferous ducts and smooth muscle cells of the nipple. This is in contrast to the woman, pig and lactating rat in which a more extensive VIP-immunoreactive innervation has been reported in the mammary gland tissue (Eriksson et al., 1996a; Franke-Radowiecka, 2003). However, another study in the rat found, as we have, a distinct lack of VIP-immunoreactive nerves in the gland parenchyma, a finding which was also demonstrated in the nipple of non-lactating animals (Thulesen et al., 1994). Apart from differences in experimental procedures, these discrepancies may also reflect species and hormonal status variations. In any case, we found no morphological evidence that VIP-containing fibres are directly involved in regulating ductal tone in the canine mammary gland as suggested for the human, rat and porcine gland (Eriksson et al., 1996a; Franke-Radowiecka, 2003). VIP is a potent vasodilator in most vascular beds (see Fahrenkrug, 1993) and may induce, through local vasodilation, secretory effects in exocrine glands (Lundberg et al., 1980). However, the present findings that the secretory parenchyma of the canine mammary gland lacks VIP-immunoreactive fibres and that the density of perivascular fibres stained for this neuropeptide is very low does not suggest any relevant vasoactive and/or secretagogue role for VIP.

PHI derives from the same precursor molecule as VIP and in fact it colocalizes with VIP in many peripheral autonomic neurons (see Fahrenkrug and Hannibal, 2004). In the dog mammary tissues, nerve fibres immunoreactive for PHI are rare, exhibiting a similar distribution to that of VIP-containing neurons, with immunoreactivity confined to larger arteries. As for VIP, the functional role of PHI in mammary tissues of the dog is probably negligible.

Somatostatin immunoreactivity was not demonstrated in mammary tissues of the dog. A previous study has reported the presence of a moderate number of somatostatin-immunoreactive nerve fibres in the porcine mammary nipple (Franke-Radowiecka et al., 2002). The reason for this discrepancy is uncertain, but interspecies differences and/or methodological factors may be involved.

In conclusion, our study demonstrated that the innervation of canine mammary tissues is mainly affiliated with the arterial vasculature and comprises peptidergic nerves which may be involved in the regulation of local blood flow. The presence of sensory neuropeptides in nerves supplying the mammary nipple also suggests that these peptides may play a role in the afferent pathway of the milk ejection reflex. Further investigations are needed to clarify the contribution of peptidergic nerves to the regulatory mechanisms in

canine mammary tissues.

Acknowledgements. We are grateful to Dr. Vitor Major of the Oeiras County Animal Shelter (Portugal) for his cooperation in obtaining tissue specimens. This work was supported by CIISA, Faculty of Veterinary Medicine, Lisbon, Portugal, Grant No. 64. Peptidérgica.

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Innervation of canine mammary gland

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Innervation of canine mammary gland

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Accepted April 20, 2007