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Immunohistochemical characterisation of dorsal root ganglia neurons supplying the porcine mammary gland

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Summary. The present study investigated the chemical coding of mammary gland-projecting dorsal root ganglia (DRG) neurons using double-labelling immunohistochemistry. Earlier investigations revealed the presence of Fast blue - positive (FB+) neurons in Th9-Th12 DRG after injection of the tracer into the second, right thoracic mamma. Neurons projecting to the last right abdominal mamma were found in L1-L3 DRG. In the present study, the cryostat sections from these ganglia were stained for calcitonin gene-related peptide (CGRP), substance P (SP), nitric oxide synthase (NOS), galanin (GAL) and pituitary adenylate cyclase activating polypeptide (PACAP). Immunohistochemistry revealed that the vast majority of FB+ mammary gland-projecting neurons contained immunoreactivity to CGRP (68.87±0.7%), SP (63.4±0.9%), NOS (32.47±0.9%), GAL (16.28±0.8%) and less numerous nerve cells stained for PACAP (5.87±0.5%). The present results largely correspond with findings dealing with immunohistochemical characterization of nerve fibres supplying porcine mammary gland structures described earlier.

Key words: Mammary gland, Sensory innervation, Neuropetides, Pig

Introduction

The mammary gland is a modified, cutaneous gland of the exocrine, tubuloalveolar type (Cathcart et al., 1948; Cowie, 1974; Daniel and Silberstein, 1987). It is in a physiological relationship with the reproductive organs and undergoes conspicuous changes during pregnancy, lactation and after lactation period (Cathcart et al., 1948; Cowie, 1974; Daniel and Silberstein, 1987; Marchetti and Labrie, 1990; Shillingford and Henninghausen, 2001).

The mammary gland of the pig usually comprises 14 mammary complexes, arranged in two rows on the ventral side of the thorax and abdomen (Bragulla and König, 2006). Each complex consists of two or three mammary units. Each unit opens with a separate orifice at the tip of the teat (Bragulla and König, 2006). The histological structure of the porcine mammary gland is similar to that found in different breeding animal species and humans.

Earlier studies dealing with sensory innervation of the mammary gland have revealed that the nipple of the gland is one of the most highly innervated tissues of the human body (Belonoschkin, 1933; Cathcart et al., 1948; Cowie, 1974). This finding strongly suggests that nerve endings in the nipple may play an important role in the activation of the milk ejection process (Cross, 1961; Findlay and Grosvenous, 1969; Haller, 1985). Afferent nerves supplying the mammary gland can convey impulses from sensory receptors, such as receptors of pressure, touch, temperature and pain to the hypothalamus (Cross, 1961; Eriksson et al., 1996). Stimulation of the paraventricular and supraoptic nucleus of the hypothalamus causes release of oxytocin into the bloodstream (Lincoln and Paisley, 1982; Meyer et al., 1987; Eriksson et al., 1996; Uvnäs-Moberg and Eriksson, 1996; Tsingotjidou and Papadopoulos, 1996; Javier et al., 2000; Marnet and McKusick, 2001; Stern et al., 2002). Circulating oxytocin reaches the mammary gland and binds with specific receptors which are in myoepithelial cells surrounding the alveoli and small milk ducts, and then the milk ejection reflex is activated (Cross, 1961; Findlay and Grosvenor, 1969; Uvnäs-Moberg and Eriksson, 1996).

Less data have been gained dealing with peptidergic innervation of the mammary gland and the role of biologically active substances in its functioning in

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humans and different domestic animal species. Some studies have revealed the presence of a variety of neurotransmitters within sensory nerves supplying the mammary gland in woman (Erikkson et al., 1996), rat (Traurig et al., 1984; Thulesen et al., 1994; Erikkson et al., 1996; Skakkebæk et al., 1999), dog (Pinho and Gulbenkian, 2007) and pig (Franke-Radowiecka et al., 2002).

The knowledge of the distribution and immunohistochemical characteristics of neurons supplying the mammary gland is very limited. The literature in the field contains only fragmentary data dealing with the localization and neurochemical properties of sensory neurons supplying the mammary gland in laboratory mammals (Tasker et al., 1986, 1988; Erikkson et al., 1996). There is some evidence that the sensory innervation of the rat mammary gland originates from dorsal root ganglia (DRG) (Tasker et al., 1986, 1988; Erikkson et al., 1996) and nodose ganglion (Erikkson et al., 1996). The first, comprehensive study on the localization of sensory neurons supplying the mammary gland using retrograde tracing method was performed in the pig (Franke-Radowiecka, 2007). In this study, fluorescent tracer Fast Blue (FB) was injected into the second, right thoracic mamma and FB+ neurons were then observed in ipsilateral Th9-Th12 DRG. The neurons projecting to the last right abdominal mamma were found in ipsilateral L1-L3 DRG.

It should be noted that no information is available on the chemical coding of neurons supplying the porcine mammary gland. Therefore, the aim of the present study was to investigate the chemical coding of mammary gland-projecting DRG-neurons using double-labelling immunohistochemistry.

Materials and methods

Twelve immature female piglets (30-40 kg body weight, age of aprox. 4 months) of the Large White Polish breed obtained from a commercial fattening farm were used in the experiment. All the animals were housed and treated in accordance with the rules approved by the local Ethics Commision, permision number 4/200/D (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education). All the pigs were pretreated with atropine (polfa, Poland; 0.01 mg/kg b. W., s.c.) and azaperone (Stresnil, Janssen, Belgium; 8 mg/kg b.w., i.m.). The main anaesthetic, sodium pentobarbital (Vetbutal, Biovet, Poland; 20 mg/kg b.w.) was given i.v. A Hamilton microsyringe equipped with 26 gauge needle was used to inject 5% FB suspension into the nipple (four injections, each injection 2.5 μl; total volume 10 μl) or the parenchyma of the mammary gland (eight injections, each injection 2.5 μl; total volume 20 μl) as described earlier (Franke-Radowiecka 2007). The pigs were assigned into four experimental groups (g) and FB was injected into the nipple (gI, n=3) or parenchyma (gII, n=3) of the right, second thoracic mamma or into the nipple (gIII, n=3) or parenchyma (gIV; n=3) of the right, last abdominal mamma. After a survival period of 4 weeks, all the animals were reanaesthetized and transcardially perfused with buffered (pH 7.4) paraformaldehyde. After perfusion, DRG, paravertebral ganglia with the stellate ganglion, prevertebral ganglia (adrenal ganglion, aortico-renal ganglion, ovarian ganglion, inferior mesenteric ganglia), uterine cervix with paracervical ganglia, nodose ganglion, bilateral jugular ganglion, right second thoracic and the last abdominal mamma were collected from all the pigs studied (Franke-Radowiecka 2007). After short postfixation by immersion in the same fixative (20 min), the tissues were washed with phosphate buffer (0.1M., pH 7.4, at 4°C) and finally transferred to and stored in 18% buffered (pH 7.4) sucrose solution until further processing.

The control of specifity of tracer staining has been checked by analysing thirty-μm-thick cryostat sections of the nipple and parenchyma under the fluorescence microscope for the correctness of the injection sites of FB. No FB contamination of the skin and subcutaneous tissues was found. There is some evidence that FB is taken up easily by nerve terminals and basically it should not be absorbed in amounts giving the apparent staining by undamaged nerve fibres (axons or dendrites; Bentivoglio et al., 1980). Moreover, our earlier investigations have not revealed thicker bundles of smooth nerve fibres (which potentially could contain the fibres passing through and supplying the nipple) within the parenchyma of the gland. Such structures are distributed at the periphery of the mamma under the skin, but the tracer was injected to the central area of the parenchyma and did not diffuse to the margin.

Ten μm-thick cryostat sections containing retrogradely labelled neurons from the ipsilateral ganglia (after injection of the tracer into the second, right thoracic mamma - Th9-Th12 DRG, after injection of the tracer into the last right abdominal mamma - L1-L3 DRG) were processed for double-labelling immunofluorescence. Briefly, after air-drying at room temperature (rt) for 30 min the sections were preincubated with a blocking mixture containing 10% normal horse serum, 1% bovine serum albumine and 0.05% Tween 20 in PBS (1h, rt). Next, the sections were incubated in a humid chamber with a mixture of two primary antisera raised in different species (overnight, rt). Consecutive sets comprising 3 adjacent sections were stained for the following combinations of the antigens (one section stained for one combination): CGRP/SP, CGRP/NOS and SP/GAL, or CGRP/SP, SP/PACAP and SP/GAL, or CGRP/SP, CGRP/VIP and GAL/PACAP or GAL/PACAP, SP/PACAP and SP/LENK (Leu5-enkephalin), CGRP/SP, GAL/NOS and VIP/NOS, respectively. Afterwards, the sections were incubated with an appropriate biotinylated antiserum (1h, rt) and then with a mixture of Cy3-conjugated streptavidin and FITC-conjugated secondary antiserum (1h, rt). The primary and secondary antisera used are listed in Table

1. Finally, the sections were mounted with carbinate-buffered glycerol (pH 8.6). Each step of immunolabelling was followed by rinsing the sections with PBS (3x5 min, pH 7.4).

Preabsorbtion of the diluted antiserum with 20 μg/ml of the appropriate antigen completely abolished the specific immunoreactions. Additionally, the primary antisera were omitted or replaced by non-immune sera or by PBS in order to check the method specify.

The sections stained for the same combination of the antigens assigned to quantitative investigations were separated by at least 100 μm to avoid double-analysis of the neuronal somata. Only those perikarya profiles containing nuclei were counted. To determine the relative number of DRG-projecting neurons stained for a particular combination of the antigens, the neurons were counted in 12 sections, including 4 sections from the lower, middle and upper one third of each ganglion studied. Calculation sheet MS Excel 2000, the part of integrated MS Office package was used for the calculation, analysis and graphic presentation purposes. The final results of the analysis were expressed as means ± S.D. The sections labelled were viewed under a Zeiss Axiophot fluorescence microscope equipped with epifluorescence and an appropriate filter set for FB (U1 block), fluorescein isothiocyanate (FITC) or Cy2 (B block) and Texas red (G1 block). The colocalisation patterns of the substances within the FB+ neurons were analyzed and quantified either directly by interchanging filters or indirectly by means of comparison of the consecutive sections.

Results

An earlier study revealed neurons projecting to the second, right thoracic mamma in ipsilateral Th9-Th12 DRG (nipple) and Th10-Th12 DRG (parenchyma). Neurons innervating the nipple and parenchyma of the last right abdominal mamma were found in ipsilateral L1-L3 DRG. FB+ neurons were not observed in the vagal ganglia (Franke-Radowiecka, 2007).

Double-labelling immunochemistry revealed that the DRG FB+ neurons supplying both the second thoracic and the last abdominal mamma were positive to CGRP, SP, NOS, GAL or PACAP. Because there were no significant differences in the chemical coding of FB+ perikarya between neurons projecting to the nipple and those innervating the parenchyma of the mammary gland, the data were pooled together (Tables 2, 3).

CGRP-immunoreactive (IR) neurons

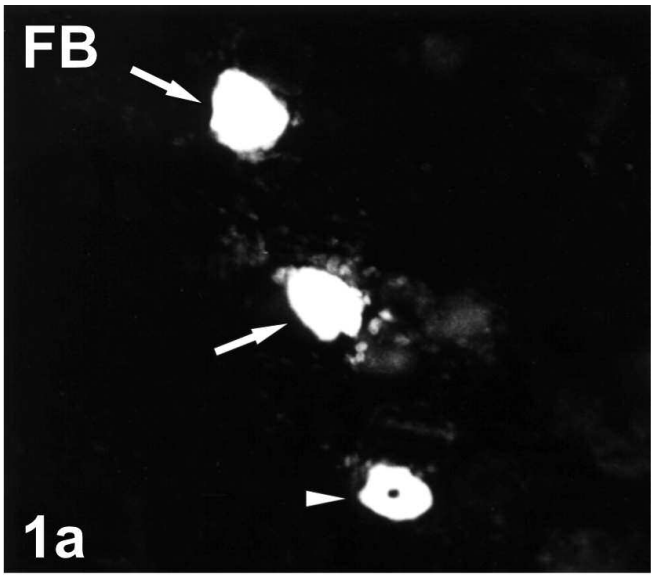
The vast majority (68.87±07%) of DRG neurons supplying the mammary gland (both nipple and parenchyma of the second thoracic and last abdominal mamma) contained immunoreactivity to CGRP. Double-labelling immunofluorescence revealed that 72±1.3% of FB+/CGRP-IR neurons also stained for SP (Figs. 1, 2), 34±1.3% for NOS (Fig. 2), 14.1±1.2% for GAL and single FB+/CGRP-IR perikarya were PACAP-positive (5.1±0.7%). Comparison of the consecutive sections revealed that FB+/CGRP neurons expressed immunoreactivity for SP and NOS (24.9±2.0%) (Fig. 2), less numerous were SP- and PACAP- (4.2±0.8%) or NOS- and GAL- (4.4±0.7%) immunoreactive. Single FB+/CGRP-IR nerve cell bodies were NOS- and PACAP- or GAL- and PACAP-positive.

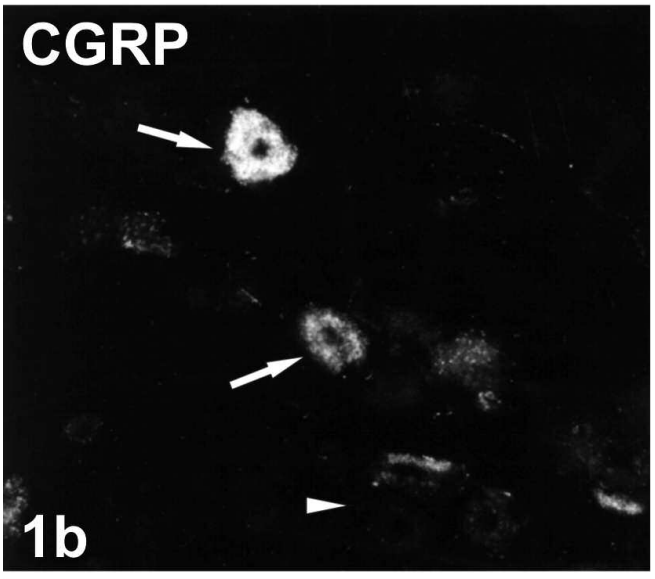
SP-IR neurons

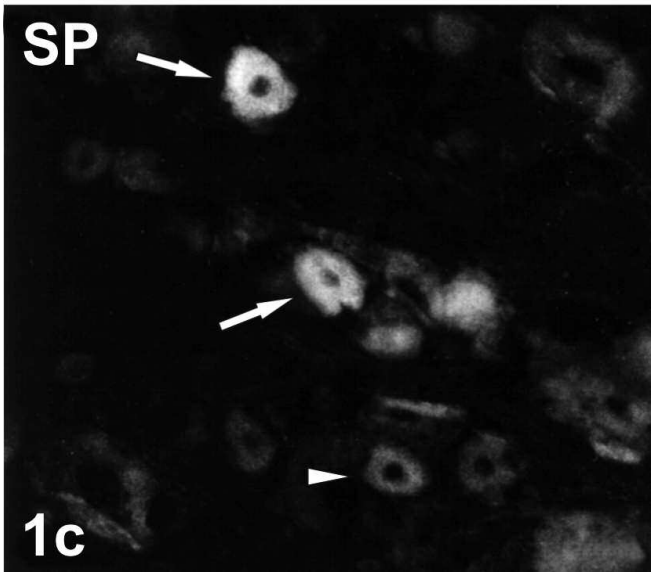
Another large group of FB+ neurons were those expressing SP (63.4±0.9%). A prominent proportion of FB+/SP neurons also contained immunoreactivity to CGRP (78.6±1.6%). Less numerous nerve cells were

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

Antigen	Code	Dilution	Species	Supplier
PRIMARY ANTIBODIES				
CGRP	RPN. 1842	1:500	rabbit	Amersham Int.
GAL	RIN-7153	1:800	rabbit	Peninsula Laboratories, Inc
LENK	RPN 1552	1:700	rabbit	Amersham Int.
LENK	4140-0355	1:500	mouse	Biogenesis
NOS	B 220-1	1:2000	rabbit	Euro Diagnostica
NOS	16	1:5000	mouse	BD Bioscences
PACAP	IHC 8922	1:10 000	rabbit	Affiniti
SP	NS1	1:400	rat	Serva
VIP	MaVIP	1:1000	mouse	East Acres
SECONDARY REAGENTS				
FITC-conjug. anti-rabbit		1:400	goat	Jackson Immun. Lab.
FITC- conjug. anti-mouse IgG		1:400	goat	Jackson Immun. Lab.
FITC conjug. anti-rat IgG		1:400	goat	Jackson Immun. Lab.
Biotynylated anti-rabbit IgG		1:400	goat	Jackson Immun. Lab.
Biotynylated anti-mouse IgG		1:100	goat	Jackson Immun. Lab.
Biotynylated anti-rat IgG		1:400	rabbit	Jackson Immun. Lab.
Streptavidin-conjug. Cy3		1:4000	goat	Dianova







SP/NOS (31.5±1.7%), SP/GAL (13.3±1.0%) (Fig. 2) or SP/PACAP-IR (5.5±1.1%). Comparison of the consecutive sections revealed that FB+/SP-IR neurons expressed immunoreactivity for CGRP and NOS (27.29±2.0%), CGRP and GAL (10.6±1.2%), CGRP and PACAP (4.5±0.9%) or NOS and GAL (3.7±0.8%). Single FB+/SP-IR nerve cell bodies were NOS/PACAP- or GAL/PACAP- positive.

NOS-IR neurons

32.47±0.9% of retrogradely labelled neurons displayed immunoreactivity to NOS-IR. The majority of these nerve cells were also positive to CGRP (74±3.0%) or SP (63±3.9%) (Fig. 2). Less numerous FB+/NOS-IR perikarya contained immunoreactivity to GAL (15.6±1.9%) or PACAP (3.0±2.1%). Comparison of the consecutive sections revealed that the majority of FB+/NOS-IR neurons expressed simultaneously immunoreactivity to CGRP and SP (54.1±5.1%). Small

Table 2. Percentages of neuronal populations in DRG (± SD) supplying the nipple and parenchyma of the second thoracic mamma in the pig (-: no neurons found).

Substances and their colocalization	Th10 %	Th11%	Th12%
CGRP	62.61±31.58	67.45±8.75	27.89±30.73
CGRP/SP	58.09±28.75	72.6±5.44	42.07±46.9
CGRP/NOS	28.66±15.62	40.13±12.65	9.81±15.46
CGRP/GAL	10.55±6.89	13.48±7.51	1.75±4.28
CGRP/PACAP	6.98±4.12	2.51±1.96	0.54±1.32
SP	56.4±27.68	63.52±4.18	38.77±44.33
SP/CGRP	64.27±32.05	76.94±9.29	31.03±35.01
SP/NOS	26.77±15.37	34.75±8.65	7.7±12.09
SP/GAL	12.16±7.25	14.13±5.02	1.98±4.86
SP/PACAP	6.57±3.46	2.62±2.13	0.66±1.62
NOS	26.47±14.08	34.47±7.54	9.62±14.9
NOS/CGRP	67.8±35.18	77.8±15.62	20±31.62
NOS/SP	56.23±28.07	63.77±4.5	17.5±27.16
NOS/GAL	12.25±9.13	16.19±5.51	3.06±7.48
NOS/PACAP	0.64±1.57	-	-
GAL	12.23±7.18	17.18±5.55	4.65±7.2
GAL/CGRP	52.07±26.88	57.12±28.88	7.74±18.95
GAL/SP	58.07±34.49	55.92±25.67	8.93±21.87
GAL/NOS	26.37±20.26	34.07±14.05	6.55±16.04
GAL/PACAP	2.78±6.8	4.17±10.21	-
PACAP	6.05±3.3	3.27±1.85	0.73±1.78
PACAP/CGRP	71.67±36.97	50±44.72	7.41±18.14
PACAP/SP	62.78±33.61	50±44.72	9.26±22.68
PACAP/NOS	3.33±8.16	-	-
PACAP/GAL	5.56±13.61	16.67±40.82	-

Fig. 1. FB+ mammary gland-projecting neurons (**a**, arrows, arrowhead) found in L1 DRG. Two FB+ neurons (**a**, arrows) were positive to CGRP **(b)** and SP **(c)**. The third FB+ neuron (**a**, arrowhead) was CGRP-negative (**b**) but stained for SP **(c)**. Scale bar: 25 μm.

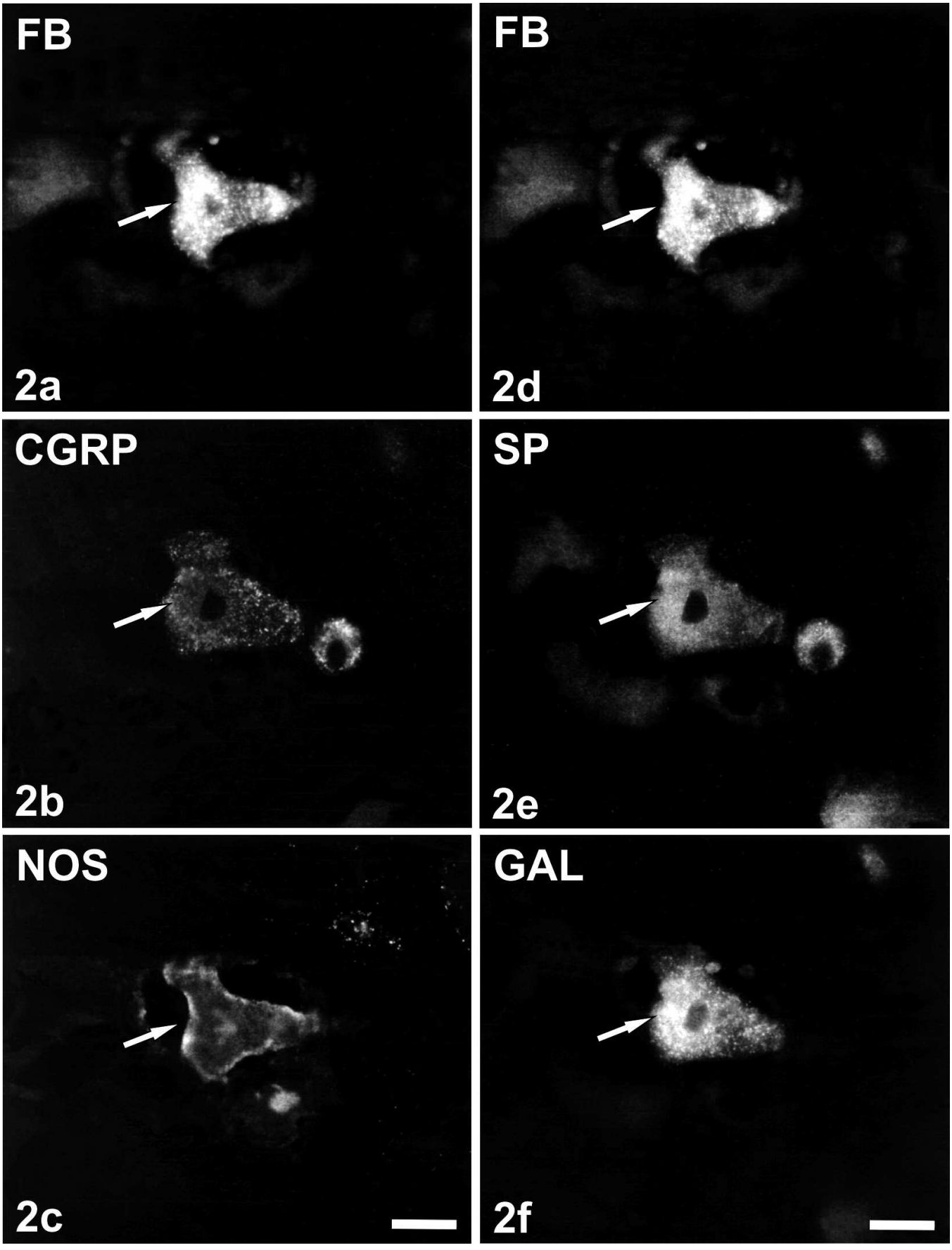


Fig. 2. Consecutive sections with the same FB–positive (FB+, **a, d)** primary sensory neuron projecting to the nipple of the second right thoracic mamma found in Th10 DRG. **a-c.** The section stained for CGRP **(b)** and NOS **(c)**. **d-f.** The section stained for SP **(e)** and GAL **(f)**. FB+ neuron contained immunoreactivity for all the substances studied. Scale bar: 50 μm.

numbers of these perikarya were CGRP/GAL-IR (9.5±1.3%) or SP/GAL-IR (7.4±1.4%). Single FB+ nerve cell bodies were NOS/CGRP/PACAP- or NOS/SP/PACAP-IR.

GAL-IR neurons

Some FB+ neurons contained immunoreactivity to GAL (16.28±0.8%). Double-labelling revealed that the majority of FB+/GAL-IR neurons were also positive to CGRP (60.7±4.7%), SP (52.6±4.9%) or NOS (30.8±4.5%) (Fig. 2). A small number of FB+/GAL-IR showed immunoreactivity to PACAP (4.3±4.5%). Comparison of the consecutive sections revealed that FB+/GAL-IR neurons expressed immunoreactivity for CGRP and SP (41.9±5.0%), CGRP and NOS (18.8±3.1%) or SP and NOS (14.5±3.7%). Single FB+/GAL-IR nerve cell bodies were also CGRP/PACAP- or SP/PACAP-positive.

Table 3. Percentages of neuronal populations in DRG (± SD) supplying the nipple and parenchyma of the last abdominal mamma in the pig (-: no neurons found).

Substances and their colocalization	L1%	L2%	L3%
CGRP	74.94±3.66	50.48±25.66	37.5±41.42
CGRP/SP	71.85±7.01	57.82±28.84	27.22±33.36
CGRP/NOS	34.38±5.72	30.24±16.17	14.44±26.81
CGRP/GAL	9.99±5.54	17.47±11.04	5.56±13.61
CGRP/PACAP	7.68±4.89	5.48±3.73	-
SP	64.4±7.14	47.94±23.99	20.83±26.74
SP/CGRP	83.82±6.4	60.91±30.96	50±54.77
SP/NOS	35.41±8.9	28.7±14.42	20.83±40.05
SP/GAL	12.21±4.44	16.18±9.37	-
SP/PACAP	8.23±7.17	5.99±6.3	-
NOS	31.39±4.16	27.63±14.51	11.11±20.18
NOS/CGRP	82.78±14.77	55.61±29.53	33.33±51.64
NOS/SP	74.31±23.69	50.61±26.36	25±41.83
NOS/GAL	8.7±11.21	16±10.68	8.33±20.41
NOS/PACAP	4.17±10.21	1.73±3.64	-
GAL	12.91±2.81	17.13±10.76	13.89±22.15
GAL/CGRP	59.01±34.52	51.73±26.24	8.33±20.41
GAL/SP	62.62±24.17	50.06±33.49	-
GAL/NOS	21.35±29.14	25.39±13.24	8.33±20.41
GAL/PACAP	-	6.21±10.43	-
PACAP	6.16±3.65	5.95±3.71	-
PACAP/CGRP	94.44±13.61	44.88±22.92	-
PACAP/SP	66.67±51.64	47.15±40.34	-
PACAP/NOS	16.67±40.82	9.21±20.09	-
PACAP/GAL	-	21.93±40.28	-

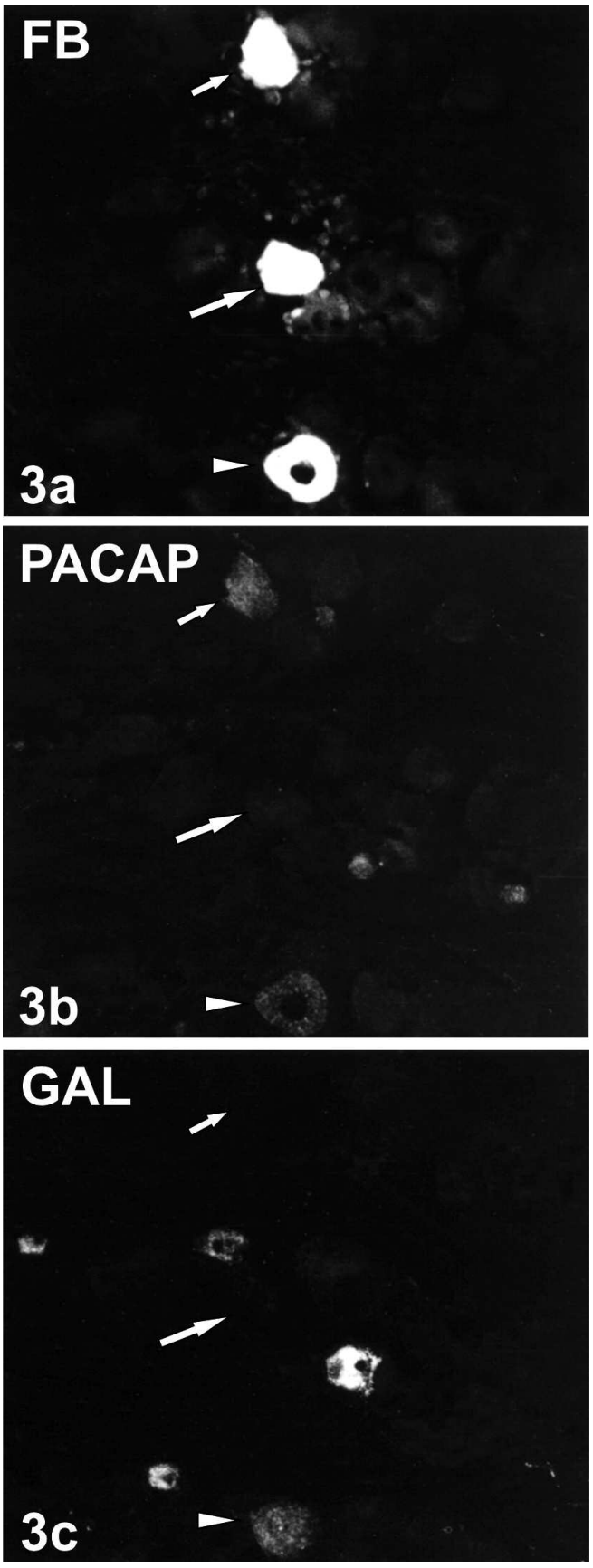


Fig. 3. Consecutive sections with the same FB+ (**a**, small arrow, large arrow, arrowhead) neurons found in L1 DRG. One section was stained for PACAP (**b**), another one for GAL (**c**). One FB+ neuron was PACAP-positive (**b**, small arrow) but GAL-negative (**c**). Another FB+ neuron (**a**, large arrow) contained immunoreactivity neither to PACAP **(b)** nor to GAL (**c**). The third FB+ neuron (**a**, arrowhead) was PACAP- (**b**) and GAL-positive (**c**). Scale bar: 25 μm.

PACAP-IR neurons

Some FB+ perikarya expressed immunoreactivity to PACAP (5.87±0.5%). Double-labelling immunofluorescence revealed that 69.2±4.5% of FB+/PACAP-positive neurons displayed immunoreactivity to CGRP. The majority (66.2±5.8%) of them also expressed SP. Less numerous FB+/PACAP-IR perikarya were GAL- (13±5.8%) (Fig. 3) or NOS-immunoreactive (5.2±8.4%). Comparison of the consecutive sections revealed that the majority of FB+/PACAP-IR neurons also expressed immunoreactivity to CGRP and SP (54.5±5.0%). Single FB+ nerve cell bodies were PACAP/CGRP/NOS-, PACAP/CGRP/GAL-PACAP/SP/NOS- or PACAP/SP/GAL-IR.

Retrogradelly labelled neurons were immunonegative to VIP or LENK.

Some DRG FB+ perikarya supplying the porcine mammary gland were negative to all the substances investigated.

Discussion

The present study has revealed that the vast majority of FB+ DRG neurons displayed immunoreactivity to CGRP. The largest subpopulation of these nerve cells also contained SP. This observation corresponds with results obtained in the rat mammary gland (Eriksson et al., 1996). CGRP and/or SP have been found in sensory neurons and fibres supplying many organs in different mammalian species (Lembek and Zetler, 1962; Dalsgaard et al., 1982; Gibbins et al., 1987; Gibson at al., 1984; Wiesenfeld-Hallin et al., 1984; Merighi et al., 1990; Hökfelt, 1991). Thus, it is tempting to assume that DRG is the major, if not the only, source of CGRP+/SP+ and CGRP+ or SP+ nerve fibres. An earlier study (Franke-Radowiecka et al., 2002) revealed CGRP+ and/or SP+ nerve fibres supplying the porcine mammary skin, subcutaneous tissue and parenchyma of the gland. CGRP-IR nerve fibres were observed in close vicinity to smooth muscle fibres of the nipple and surrounded lactiferous ducts of the parenchyma. CGRP- and SP-IR fibres were also associated with blood vessels of the gland. These results corroborate findings reported by other authors. The distribution of CGRP- and/or SP-IR nerve fibres found in the porcine gland was similar to that described in woman (Eriksson et al., 1996; Uvnäs-Moberg and Eriksson, 1996), rat (Thulesen et al., 1994; Traurig et al., 1984; Eriksson et al., 1996; Uvnäs-Moberg and Eriksson, 1996) and dog (Pinho and Gulbenkian, 2007). It has been revealed that CGRP as a transmitter in sensory nerve fibres (Fuller et al., 1987) dilates the blood vessels (McMahon, 1986). However, SP belongs to the neurokinin group and acts as a neurotransmitter and/or neuromodulator in DRG neurons (Otsuka and Konishi, 1976; Hökfelt et al., 1982; Salt and Hill, 1983; Gibbins et al., 1987). This peptide has been found to exert a vasodilatatory effect, although not so intense as that caused by CGRP (Lembek and Holzer, 1974). It is therefore possible that CGRP- and SP-positive nerve fibres influence vascular smooth muscle tone, induce a prolonged vasodilatation and then increase blood flow through the mammary gland (Lembek and Zelter, 1962; Lembek and Holzer, 1974). The consequences of this might be growing inflow of oxytocin to the gland and activation of the milk ejection reflex. It is also possible that CGRP-IR nerve fibres supplying non-vascular smooth muscles of the gland might be involved in the regulation of the muscle tone of the nipple and the resistance of the lactiferous ducts (Eriksson et al., 1996; Uvnäs-Moberg and Eriksson, 1996; Skakkebæk et al., 1999).

In this study, comparison of the consecutive sections revealed that some CGRP/SP-IR neurons contained immunoreactivity to NOS. It is known that NOS-IR DRG neurons transmit sensory information from mechano-, chemo- or thermoreceptors (Haley et al., 1992; Meller et al., 1992). These neurons are also involved in nociceptive mechanisms (Haley et al., 1992; Meller et al., 1992). Nitric oxide in cooperation with VIP has a dilatatory influence on blood vessels of the reproductive tract (Morris, 1993). Onoda and Inano (1998) have suggested that NO may participate in the regulation of morphological and functional features of the rat mammary gland. Previous studies have revealed that the porcine mammary gland is moderately supplied with NADPHd-positive nerve fibres (Franke-Radowiecka et al., 2004). These fibres are distributed in the nipple and parenchyma of the gland. They supply vascular and non-vascular tissue (Franke-Radowiecka et al., 2004). The data concerning the origin of NADPHd innervation of the gland and distribution of the fibres may suggest its role in motor function and control of the local blood flow in the porcine mammary gland.

The present study revealed that some FB+ DRG neurons displayed immunoreactivity to GAL (16.28±0.75%). Galanin was found in CGRP- and/or SP-positive perikarya. Previous investigations have revealed that some nerve fibres supplying the skin of the nipple and blood vessels of the whole gland express GAL (Eriksson et al., 1996; Franke-Radowiecka et al., 2002). In woman and rat mammary gland, GAL-containing fibres were associated with non-vascular smooth muscle cells of the nipple and lactiferous ducts (Eriksson et al., 1996). There is some information suggesting that GAL contained in sensory neurons plays a role in pain conduction (Liu and Hökfelt, 2002). Because, as already mentioned, GAL is considered to be a potent contractor of the muscular layer in reproductive organs (Ekblad et al., 1985; Stjernquist et al., 1988; Hökfelt, 1991; Papka and McNeill, 1992; Crawley, 1995) it is very likely that this peptide plays a similar role in the muscular structure of the mammary gland.

The smallest population of mammary gland-projecting DRG neurons found in this study were those expressing PACAP (5.87±0.51%). The majority of PACAP-positive DRG neurons projecting to the porcine mammary gland also contained immunoreactivity to

CGRP. In the rat (Skakkebæk et al., 1999) and pig (Franke-Radowiecka, unpublished data) immunoreactivity to PACAP was observed in many nerve fibres supplying the skin and subcutaneous tissue, and less numerous fibres were associated with the muscular layer of blood vessels and smooth muscle cells of the nipple. Skakkebæk et al. (1999) have found that PACAP-positive fibres supplying the rat mammary gland arise from DRG and nodose ganglion. However, neurons which contain PACAP and supply the porcine mammary gland originate only from DRG. There is no information dealing with the physiological role of PACAP-positive innervation of the mammary gland, thus the function of this peptide can only be a matter of speculation. According to Skakkebæk et al. (1999), the abundance of intraepithelial PACAP-immunoreactive nerve fibres and terminals that also contained CGRP suggests that these nerves are involved in the afferent transmission of suckling stimuli. Moreover, these authors observed a high concentration of PACAP in the nerve fibres in lactating rats (Skakkebæk et al., 1999).

In conclusion, the present study has revealed for the first time the chemical coding of mammary gland-projecting primary sensory neurons in a mammalian species, the pig. The greatest population of DRG neurons supplying the gland consists of CGRP/SP-IR perikarya. Moreover, these neurons co-express simultaneously immunoreactivity to other biologically active substances. A prominent proportion of FB+/CGRP/SP-IR neurons also contained immunoreactivity to NOS, while less numerous nerve cells were CGRP/SP/GAL- or CGRP/SP/PACAP-positive.

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