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Chemical codes of sensory neurons innervating the guinea-pig adrenal gland

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Abstract. Retrograde neuronal tracing in combination with double-labelling immunofluorescence was applied to distinguish the chemical coding of guinea-pig primary sensory neurons projecting to the adrenal medulla and cortex. Seven subpopulations of retrogradely traced neurons were identified in thoracic spinal ganglia T1-L1. Five subpopulations contained immunolabelling either for calcitonin gene-related peptide (CGRP) alone (I), or for CGRP, together with substance P (II), substance P/dynorphin (III), substance P/cholecystokinin (IV), and substance P/nitric oxide synthase (V), respectively. Two additional subpopulations of retrogradely traced neurons were distinct from these groups: neurofilament-immunoreactive neurons (VI), and cell bodies that were nonreactive to either of the antisera applied (VII). Nerve fibres in the adrenal medulla and cortex were equipped with the mediator combinations I, II, IV and VI. An additional meshwork of fibres solely labelled for nitric oxide synthase was visible in the medulla. Medullary as well as cortical fibres along endocrine tissue apparently lacked the chemical code V, while in the external cortex some fibres exhibited code III. Some intramedullary neuronal cell bodies revealed immunostaining for nitric oxide synthase, CGRP or substance P, providing an additional intrinsic adrenal innervation. Perikarya, immunolabelled for nitric oxide synthase, however, were too few to match with the large number of intramedullary nitric oxide synthase-immunoreactive fibres. A non-sensory participation is also supposed for the particularly dense intramedullary network of solely neurofilament-immunoreactive nerve fibres. The findings give evidence for a differential sensory innervation of the guinea-pig adrenal cortex and medulla. Specific sensory neuron subpopulations suggest that nervous control of adrenal functions is more complex than hitherto believed.

Key words: Adrenal gland – Dorsal root ganglia – Immunohistochemistry – Neurofilament – Neuronal tracing – Neuropeptides – Nitric oxide synthase – Substance P – Guinea-pig

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Introduction

It is generally accepted that autonomic nervous control is of pivotal importance for adrenal functions and integrity (for review, see Parker et al. 1993).The innervation of the adrenal cortex is described as predominantly consisting of postganglionic sympathetic (Swinyard 1937; Parker et al. 1990b) and parasympathetic fibres (Teitlebaum 1933; Coupland et al. 1989), while the majority of the medullary innervation derives from cholinergic preganglionic neurons in the thoracic spinal cord (Schramm et al. 1975; Haase et al. 1982). Intrinsic adrenal neurons provide an additional fibre supply to the adrenal cortex and medulla (Coupland 1965; Unsicker 1971). Early suggestions on the participation of primary afferent neurons in the adrenal innervation (Pines and Narowtschatowa 1931; Swinyard 1937) were supported by physiological evidence (Niijima and Winter 1968) and proven by retrograde neuronal tracing in rat (Parker et al. 1990a) and guinea-pig (Mohamed et al. 1988).

In the last decade, the occurrence and distribution of neuropeptides in nerve fibres of the mammalian adrenal gland have been the subject of an increasing number of investigations. In the guinea-pig, evidence was provided for the occurrence of nerve fibres with immunoreactivities 1) for neuropeptide tyrosine (NPY; Lundberg et al. 1986; Zentel and Weihe 1988), calcitonin gene-related peptide (CGRP; Zentel and Weihe 1988), substance P (SP; Bucsics et al. 1981), dynorphin (DYN), galanin (GAL), vasoactive intestinal polypeptide (VIP; Zentel and Weihe 1988), enkephalins (Kobayashi et al. 1985) and 2) to the enzymes of catecholamine synthesis, tyrosine hydroxylase (TH) and dopamin-β-hydroxylase (DBH; Zentel and Weihe 1988). Intramedullary nerve cell bodies were shown to contain NPY-immunoreactivity (IR; Lundberg et al. 1986).

Since SP and CGRP frequently co-occur in primary afferent neurons (Hua et al. 1985), a sensory origin of SP- and/or CGRP-ir fibres in the adrenal gland appears to be likely. Moreover, SP and CGRP were shown to

Table 1. List of primary antibodies and secondary reagents applied

Primary antibodies				
Antigen	Antibody code	Host species	Dilution	Supplier/Reference
Calcitonin gene-related peptide	020816–2	rabbit	1: 800	Peninsula, St. Helens, GB
Cholecystokinin		rabbit	1:400	INC, Stillwater, MN, USA
Dynorphin A 1–13	Paula	rabbit	1: 400	Colombo et al. 1987
Galanin	lot 020386	rabbit	1:800	Peninsula
Neurofilament - 160 kD	lot 10308434–01	mouse, monoclonal	1: 30	Boehringer, Mannheim, FRG
Nitric oxide synthase	–	rabbit	1:1500	Klatt et al. 1992
Somatostatin	lot 001–1	rat, monoclonal	1:20	AMS-Medicap, Bioggio, CH
Substance P	NC 1	rat, monoclonal	1: 300	Serva, Heidelberg, FRG
Substance P	7	rabbit	1:1000	Amersham-Buchler, Braunschweig, FRG
Tyrosine hydroxylase	lot 13274622–03	mouse, monoclonal	1:20	Boehringer
Tyrosine hydroxylase	lot 90.15 TE 101	rabbit	1:600	Eugene Tech Ridgefield Park, NY, USA
Secondary reagents	Conjugate	Host species	Dilution	Supplier
Anti-rabbit IgG	FITC	donkey	1: 120	Welcome, Beckenham, UK
Anti-rat IgG	biotin	sheep	1: 100	Amersham-Buchler
Anti-mouse IgG	biotin	sheep	1: 50	Amersham-Buchler
Streptavidin	Texas Red	–	1: 50	Amersham-Buchler

play an important role in adrenal function (Kuramoto et al. 1985). Indeed, sensory neurons projecting to the adrenal in rat were demonstrated to be capsaicin-sensitive (Parker et al. 1990a) and to contain SP (Zhou et al. 1991). Furthermore, a substantial number of other mediators found in adrenal nerve fibres may be attributed to primary sensory neurons. Predominant peptide immunoreactivities of thoracic dorsal root ganglion (DRG) cells, apart from CGRP and SP, are to preprodynorphin-derived peptides such as DYN (Weihe et al. 1985), to cholecystokinin (CCK; Gibbins et al. 1987), somatostatin (SOM; Weihe 1990) and GAL (Zentel and Weihe 1988). In addition, TH, the rate-limiting enzyme of catecholamine synthesis, but not DBH (Hökfelt et al. 1973) has been demonstrated immunohistochemically in DRG cells of rat (Price 1985) and guinea-pig (Kummer 1990). Yet, each of these "sensory" mediators apparently is also utilized by efferent autonomic neurons. As an example, SP was described as a component of preganglionic sympathetic (Krukoff et al. 1985) and parasympathetic (Baude et al. 1992) neurons as well as of postganglionic sympathetic (Bohn et al. 1984), parasympathetic (Lundberg et al. 1988; Hardebo et al. 1992) and enteric nerve cells (Costa et al. 1980).

The discovery of distinct combinations of mediators in individual cell bodies and nerve fibres rendered the neurochemical characterization of neuron populations with different functions possible (Furness et al. 1988). Making use of this advantage, we attempted to further characterize sensory neuron populations participating in the guinea-pig adrenal innervation.

Applying a combination of retrograde tracing and subsequent double-labelling immunohistochemistry, the present study was initiated 1) to analyse the chemical codes of spinal primary sensory neurons projecting to the guinea-pig adrenal medulla, and 2) to examine distribution and structural relationships of intraadrenal fibres with comparable coding.

Materials and methods

Female guinea-pigs (n=10) weighing 190–250 g were anaesthetized with i.m. injections of ketamine hydrochloride (Ketanest, Parke Davis, Freiburg, Germany, 50 mg/kg body weight - b.w.), dehydrobenzperidol (Janssen, Neuss, Germany; 5 mg/kg) and fentanyl (Janssen, 0.5 mg/kg). The left adrenal gland was exposed by a posterior small incision along the inferior chest aperture, and 4.5 µl of an aqueous solution (2%, containing 1% dimethylsulphoxide) of the retrograde tracer Fast Blue (Dr. Illing, Groß-Umstadt, Germany) were injected into the left adrenal medulla via a Hamilton syringe.

Experimental and control animals (n=4) remained undisturbed until they were sacrificed 5 days later by an overdose of sodium pentobarbital (Nembutal, CEVA, Bad Segeberg, Germany, 100 mg/kg), injected intraperitoneally. The animals were transcardially perfused with 500 ml 4% parafomaldehyde in 0.1 M phosphate buffer (pH 7.4) after a preceding short flush with 0.9% NaCl containing 2.5% polyvinylpyrrolidone, 0.5% procain hydrochloride and 20 000 IU heparin/l (pH 7.3). Adrenals and dorsal root ganglia (C1 to L5) were removed bilaterally and washed in 0.1 M phosphate buffer. The specimens were immersed overnight in 0.1 M phosphate buffer containing 18% sucrose, frozen in liquid nitrogen, sectioned on a cryostat (Frigocut E, Reichert, Nußloch, Germany) at 14 µm (ganglia) or 12 µm (adrenals) thickness and air dried.

Applying the double-labelling immunofluorescence technique (Wessendorf and Elde 1986), the sections were first covered for 1 h with phosphate-buffered saline (PBS) containing 10% normal swine serum to block nonspecific protein binding sites and 0.05% Tween 20 to enhance penetration of immunoreagents. Primary antisera (Table 1) were applied overnight as a mixture of two antibodies raised in different species. Antibody combinations that were found to be present in retrogradely traced sensory neurons were preferentially evaluated for the recognition of adrenal nerve fibres. The appropriate detection systems, each applied for 1 h

Table 2. Distribution of nerve fibres with different mediator combinations in the guinea-pig adrenal gland. *Continuous lines* Distinct fibres and fibre bundles; *dashed lines* none or very rare single fibres. *Stars* indicate mediator combinations that did not occur in dorsal root neurons

Fibre population	Subcapsular region	Zona glomerulosa	Zona fasciculata	Zona reticularis	Medulla
CGRP/SP	———	———	———	———	———→
SP/DYN	———	———	———→		
SP/CCK	———	———→	- - - - -	- - - - -	- - - - - →*
NF	———	———→	- - - - -	- - - - -	- - - - - →*
CGRP/TH*	———	———	———→		
SOM/NOS*	———	———→			

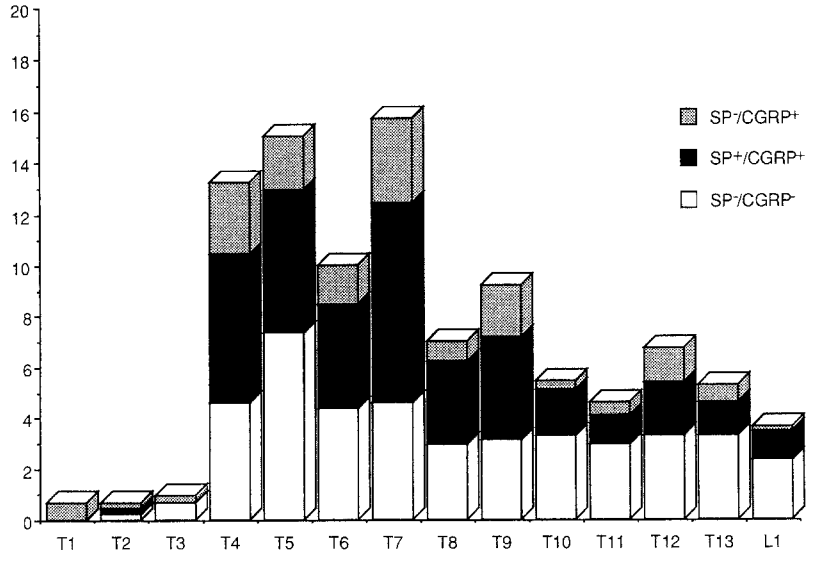


Fig. 1. Relative percentages of Fast Blue (FB)-labelled dorsal root ganglion cells in the guinea-pig, immunoreactive for CGRP and CGRP/SP, respectively, and of retrogradely traced cells, nonreactive for either peptide. *Ordinate* % of FB-labelled cell bodies; *abscissa* level of dorsal root ganglia

and preceded by washing in PBS, are listed in Table 1. Finally, the slides were rinsed in PBS and coverslipped in carbonate-buffered glycerol at pH 8.6. Specificity tests including omission of primary antisera and preabsorption of primary antibodies with the respective antigen (5–10 µg/ml diluted antiserum) indicated the specificity of the immunoreactions. Microscopic evaluation was performed with a Polyvar microscope (Reichert Jung, Nussloch, Germany) equipped for epifluorescence using appropriate filter modules for the tracer (band pass - BP 390–450 nm, long pass - LP 475 nm) and for fluorescent dyes (BP 546/10 nm, LP 590 nm for Texas Red; BP 455–490 nm, BP 515–560 nm for FITC).

The relative frequency in occurrence of two chemically identified subpopulations [SP- and CGRP-immunoreactive (-ir)] of retrogradely labelled neurons was determined in serial sections of DRG T1 to L1 from eight animals. Only nucleated cell profiles in every second section were counted. Data are presented as mean ±S.D..

Results

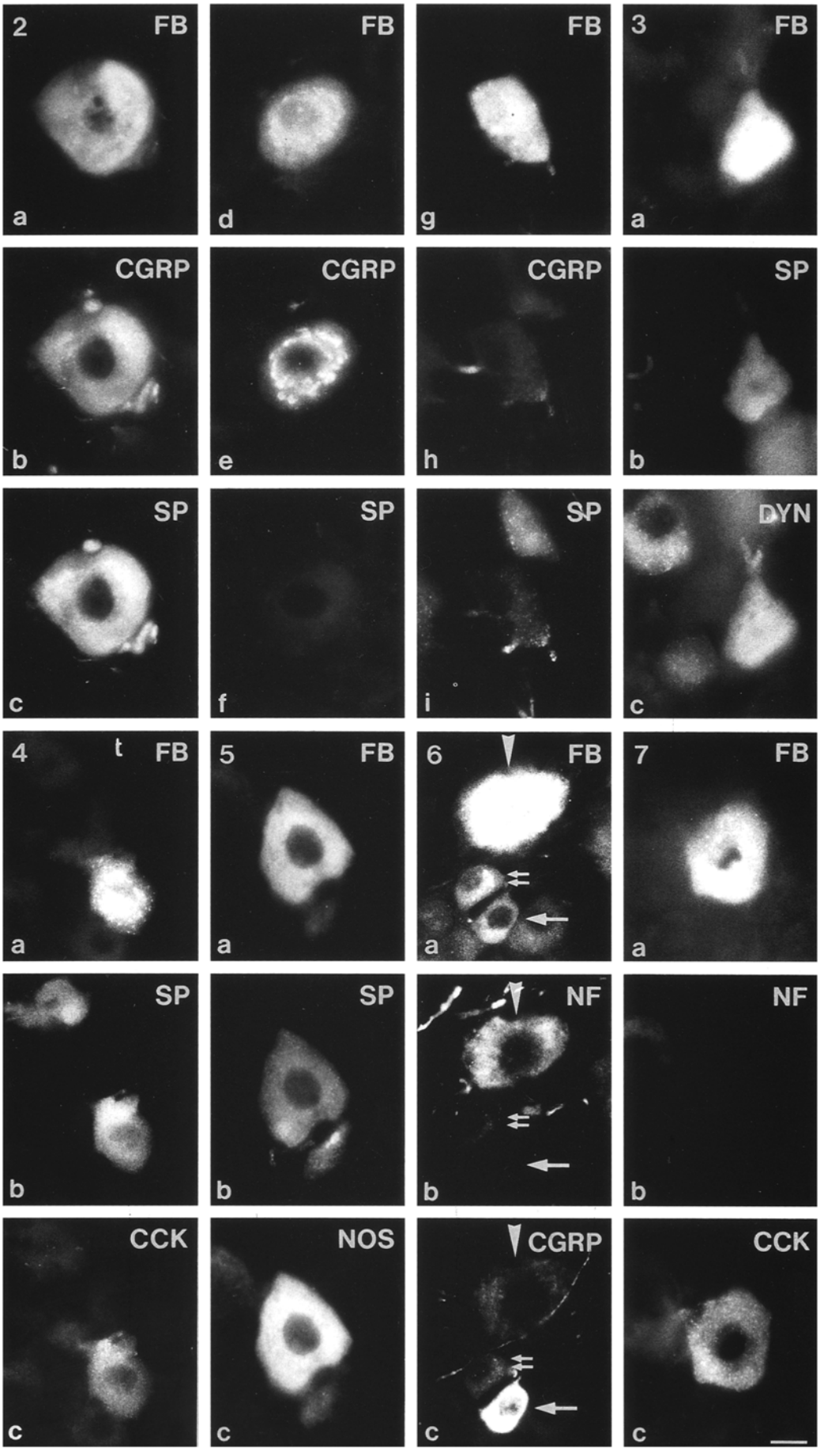
Left adrenal glands at the injection site still contained fluorescent tracer. Dorsal root ganglia of only those animals (n=8) were used for documentation of the retrograde neuronal labelling in which the tracer was substantially confined to the adrenal medulla.

Dorsal root ganglia

Fast Blue-labelled neurons were found exclusively in DRG ipsilateral to the site of injection. Retrogradely labelled cells (15–50 µm diameter) were present in DRG from T1 to L1. They showed a random distribution within the ganglia. The majority of traced neurons was detected at levels T4-T7 (51% of the total population of Fast Blue-positive cells). The percentage was 70% when including the DRG at levels T8 and T9. The average total number of retrogradely labelled cells per experiment was 150±54 (Fig. 1).

Six out of nine substances, visualized in primary sensory neurons of thoracic DRG, were immunohistochemically detectable in cell bodies that were retrogradely traced from the adrenal medulla: CGRP, SP, NF, CCK, DYN and NOS (listed according to the frequency of their occurrence). Although immunolabelling for the remaining three substances, SOM, GAL and TH, was observed in DRG cells, immunostaining for these substances was absent in Fast Blue-labelled perikarya.

Double-labelling immunofluorescence revealed a high degree of coexistence of SP- and CGRP-immunoreactivity (IR) in retrogradely traced neurons (Fig. 2a–c). Some of the Fast Blue-containing cells exhibited CGRP-IR but were nonreactive for SP (Fig. 2d–f), while the en-


2 FB
a
FB
d
FB
g
3 FB
a
CGRP
b
CGRP
e
CGRP
h
SP
b
SP
c
SP
f
SP
i
DYN
c
4 t FB
a
5 FB
a
6 FB
a
7 FB
a
SP
b
SP
b
NF
b
NF
b
CCK
c
NOS
c
CGRP
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CCK
c

tire SP-positive neuron population contained co-localized CGRP. Forty percent of the retrogradely labelled neurons exhibited SP- and CGRP-IR, 17% contained immunolabelling for CGRP but no SP, and in 43% of the cell bodies none of these peptides could be detected (Fig. 1). The distribution of $SP^+/CGRP^+$, $SP^-/CGRP^+$, and $SP^-/CGRP^-$ types of Fast Blue-containing perikarya was independent of the level of DRG.

DYN-immunoreactive (ir) neurons, which occurred rarely, always contained CGRP- and substance P-ir (Fig. 3). Similarly, a small proportion of the SP/CGRP-IR neurons exhibited co-localized CCK-IR (Fig. 4), or NOS-IR (Fig. 5). Although numerous sensory neurons were immunostained for the 160 kD subunit of the neurofilament triplet (NF), only some of the retrogradely traced neurons contained immunostaining for this structural protein. Fast Blue-labelled NF-ir cells were nonreactive for the other mediators investigated (Fig. 6) and vice versa (Fig. 7). A substantial population of retrogradely labelled cells contained none of the peptides/proteins in question (Fig. 6).

Adrenal medulla

Fibre bundles and single varicose fibres in the adrenal medulla exhibited immunolabelling for the peptides CGRP, SP, CCK, and SOM, as well as for the enzymes NOS and TH, and for the structural protein NF, respectively; DYN-IR was present only in short, nonvaricose processes. Immunostaining for the peptide GAL could not be detected in adrenomedullary nerve fibres.

CGRP-IR and SP-IR were for the most part overlapping in medullary varicose nerve fibres which enmeshed chromaffin tissue and abutted on blood vessels (Table 2; Fig. 8a, b). While most of the SP-ir nerve fibres contained co-localized CGRP-IR, a considerable fibre population was solely CGRP-ir. CGRP as well as SP-ir varicosities formed basket-like formations around aggregations of TH-ir chromaffin cells (Fig. 9). In addition, SP-ir fibres formed meshes around some nonreactive neurons (inset Fig. 8b).

CCK-IR was visible in some single nerve fibres, which were scattered between chromaffin cells. These fibres were congruent with SP-ir fibres in distribution but were less numerous. In virtually all fibres CCK-IR was co-localized with SP-IR (Table 2; Fig. 10). A particularly dense network of delicate NOS-ir varicose fibres interlaced the entire medulla. NOS-IR did not co-occur with SP-, TH-, or NF-IR (Fig. 11); NOS/SOM-ir nerve fibres could also not be identified. Co-localization of NOS with CGRP, CCK or DYN in nerve fibres was not investigated, because antibodies against these substances were all raised in rabbit. Co-localization of TH/SOM and TH/DYN will be the subject of another investigation.

Fibre bundles and a dense network of linear and varicose fibres throughout the medulla were NF-ir; such varicosities sometimes approached neuronal perikarya. NF-IR was not co-localized with any of the other substances investigated; in particular, no co-localization of NF with NOS (Fig. 11), SP, or CGRP (Fig. 12) was detected.

SOM-IR was distinguished in some scattered varicose fibres, which were associated with chromaffin tissue or neuronal perikarya. SOM-IR was not co-localized in any of these fibres with SP- or CGRP-IR (Fig. 13). SP- or CGRP-IR was also absent in short and coarse intramedullary processes which revealed DYN (Fig. 14) or TH-IR (Figs. 9, 15). The same applied for TH-ir varicose fibres at the cortico-medullary border.

Only a few intramedullary nerve cell bodies were present in the adrenal medulla. These perikarya were distinguished by their size (20–40 μm) and characteristic large nuclei. NOS-ir nerve cell bodies were most numerous (Fig. 11). Some intramedullary neurons contained TH-, CGRP-(Fig. 15), SP- (Fig. 16), or SOM-IR (Fig. 13). Despite screening a large number of sections, none of these substances appeared to be co-localized with each other.

Adrenal cortex

Nerve fibres in the adrenal cortex contained immunolabelling for CGRP, SP, CCK, DYN, SOM or TH (listed according to the frequency of their occurrence) and, rarely, for NOS or GAL. CGRP- as well as SP-IR (Fig. 17) occurred in thick fibre bundles traversing the cortex. Fine varicose fibres branched off to approach arterial and venous blood vessels and capillaries along the endocrine tissue. In co-localization experiments, CGRP- and SP-IR were congruent in approximately 80% of the nerve fibres throughout all cortical zones (Table 2; Fig. 18), the remaining fibres were solely CGRP-ir. CGRP-IR in some fibres of the external cortical zones exhibited coexistence with TH-IR (Table 2; Fig. 19), whereas such a co-existence was not detected for SP- and TH-IR. Co-localization of TH with the other peptides was beyond the scope of the present study and not further investigat-

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Figs. 2–7. Double-labelling immunofluorescence of FB-labelled primary sensory neurons in guinea-pig thoracic dorsal root ganglia. *Bar*: 10 μm

Fig. 2a–i. Large FB-labelled perikaryon (**a**) exhibits co-localized CGRP- (**b**) and SP-IR (**c**) while CGRP (**e**) but not SP-IR (**f**) is visible in another retrogradely traced nerve cell body (**d**). A third retrogradely traced neuron (**g**) contains neither SP- (**h**) nor CGRP-IR (**i**)

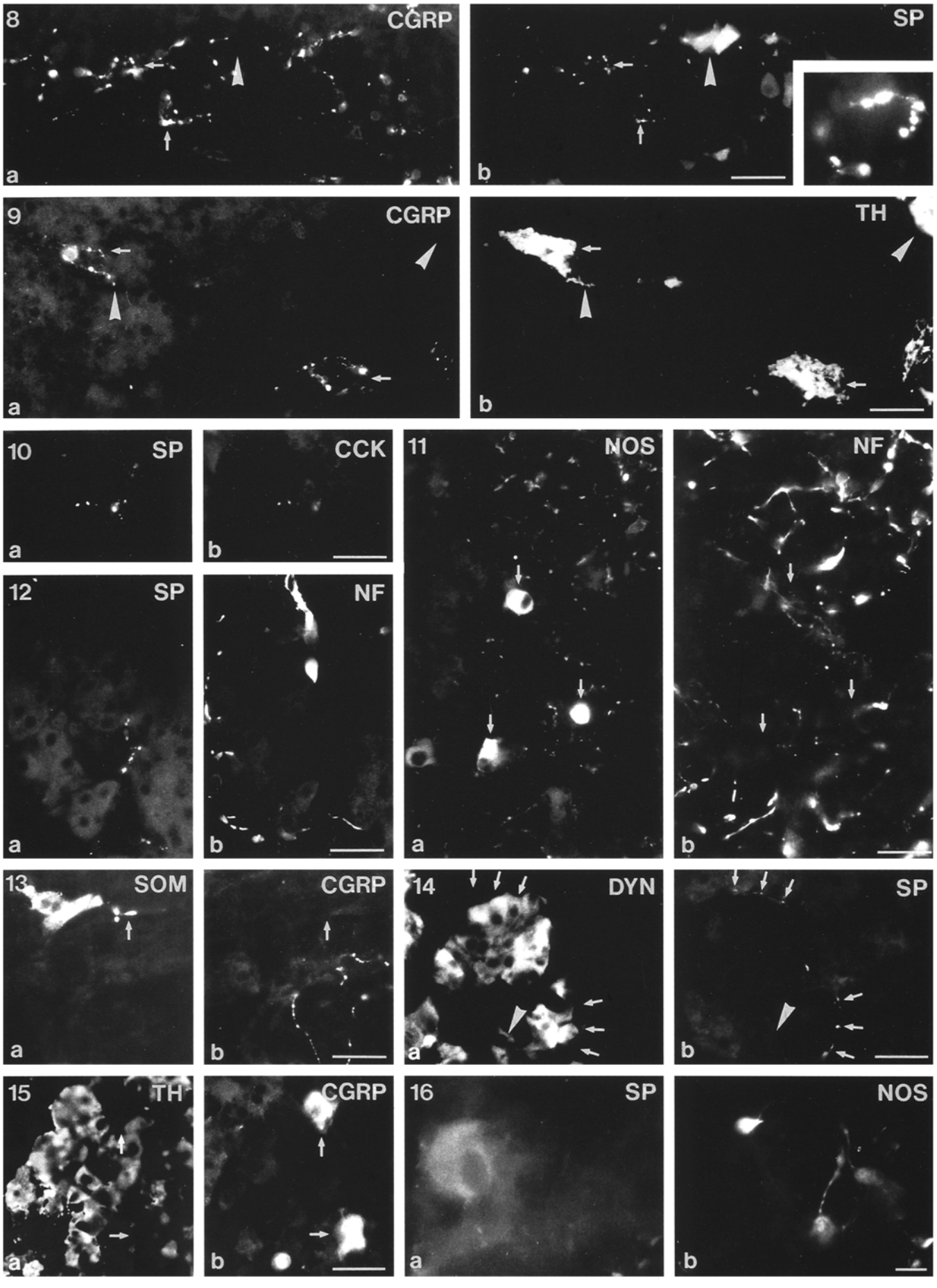
Fig. 3a–c. SP- (**b**) and DYN-IR (**c**) co-occur in a retrogradely labelled small neuron (**a**)

Fig. 4a–c. An FB-labelled small neuron (**a**) with SP- (**b**) and additional CCK-immunostaining (**c**)

Fig. 5a–c. An FB-labelled neuron (**a**) exhibits co-localization of SP- (**b**) and NOS (**c**)-IR

Fig. 6a–c. NF- (**b**) but not CGRP-IR (**c**) is visible in a large FB-labelled perikaryon (**a**, *arrowhead*). A second, small FB-labelled perikaryon (*arrow*) is CGRP-ir but lacks NF-immunolabelling, and in a third retrogradely labelled cell body (*double arrows*) NF-IR as well as CGRP-IR are absent

Fig. 7a–c. FB-labelling (**a**) is present in a perikaryon nonreactive for NF (**b**) but exhibits CCK-IR (**c**)


8
CGRP
a
SP
b
9
CGRP
a
TH
b
10
SP
a
CCK
b
11
NOS
a
NF
b
12
SP
a
NF
b
13
SOM
a
CGRP
b
14
DYN
a
SP
b
15
TH
a
CGRP
b
16
SP
a
NOS
b

ed. Infrequent CCK-ir fibres were preferably located in the subcapsular region and in the glomerular zone and were associated with endocrine tissue but not visibly connected with blood vessels. Substantially all of the CCK-fibres contained co-localized SP-IR (Table 2; Fig. 20); this, however, concerned only a small proportion of the total number of SP-ir fibres. Some SP-ir fibres in the two external cortical zones and around blood vessels in the subcapsular region revealed co-localization with DYN-IR (Fig. 21). Unlike CCK, this peptide additionally occurred in fibres distinct from SP-ir fibres. In contrast to the adrenal medulla, NOS-ir nerve fibres occurred rarely in the external cortex and were absent in the fasciculate and reticular zones. As in the medulla, NOS-ir fibres did not contain SP- or NF-IR, but, contrary to medullary NOS-ir fibres, some of them also exhibited SOM-IR (Table 2; Fig. 22). In another small fibre population of similar distribution, SOM-IR was present without NOS-IR. SOM-IR never co-occurred with CGRP-,SP-,CCK-,DYN-, or NF-IR in cortical nerve fibres. The few GAL-ir cortical nerve fibres always were SP-nonreactive. NF-ir fibres were very infrequent in the adrenal cortex. Such fibres were visible in the capsular tissue (Fig. 23) or traversed the cortical septa (Fig. 24); they did not exhibit co-staining with any of the other substances demonstrated to occur in sensory neurons (Figs. 23, 24).

Occasionally, a small ganglion consisting of 3 to 10 neurons was attached to the capsular connective tissue. Ganglionic perikarya frequently contained TH- (Fig. 25), DYN- (Fig. 26) and sometimes CGRP-IR (Fig. 27), but were nonreactive for SP, NF, or NOS. Neuronal cell bodies were enmeshed by CGRP- and SP-ir fibre basket formations (Figs. 25–27), in which CGRP-IR was predominant (Fig. 27).

Discussion

The present results are in agreement with previous findings that primary afferent neurons supplying the adrenal medulla are located in the thoracic DRG ipsilateral to the site of injection (Mohamed et al. 1988; Zhou et al. 1991). The random intraganglionic distribution and the wide segmental range of labelled cells support previous arguments against a distinct viscerotopic organization of sensory ganglia (Kuo et al. 1981). Slight incongruencies in numbers of labelled cells between an earlier description (Mohamed et al. 1988) and the present findings are probably due to technical differences, since tracing methods cannot provide accurate information as to the total number of neurons innervating an organ. Taking into account that the cortico-medullary border does not constitute a boundary for tracer diffusion from the intramedullary injection site, medullary applied FB resulted in labelling of the cortex as well, while contamination of subcapsular tissue with FB in our experiments was only confined to the track of the injection needle.

Comparison of chemical codes in FB-labelled sensory neurons and adrenomedullary structures

Previous investigations (Weihe et al. 1985; Hua et al. 1985; Gibbins et al. 1985) have identified specifically coded subpopulations of sensory neurons according to the patterns of mediator co-localization. The present results are in accordance with these findings, indicating a high degree of the simultaneous occurrence of SP and CGRP in primary sensory neurons. The discovery of a second retrogradely labelled neuron population that was immunoreactive only for CGRP but not for SP is supported by an earlier description based on single fluorescence immunohistochemistry in which the number of CGRP-ir neurons in guinea-pig thoracic ganglia exceeded that of SP-ir neurons (Gibbins et al. 1987). This pattern is well reflected by the presence of two major types of adrenal medullary fibers showing either CGRP alone or SP/CGRP-immunolabelling, as also described by Zentel and Weihe (1988). The few intramedullary fibres, which in our findings were solely SP-ir, according to these authors co-localize TH-IR, suggesting their postganglionic sympathetic origin. It is possible that our TH-antibody was insufficiently sensitive for immunolabelling of medullary SP-ir cell bodies and nerve fibres. On the other hand, SP-ir neurons in the spinal cord intermediate grey matter (Krukoff et al. 1985) may plausibly be a candidate for a preganglionic source of the exclusively SP-ir intramedullary fibres.

Comparison of pair-wise coexistence patterns or double-labelling experiments in two adjacent sections allow conclusions on triple-immunoreactive cells. Since SP-IR

Figs. 8–16. Double-labelling immunofluorescence of the guinea-pig adrenal medulla

Fig. 8a, b. SP-IR (**b**) is visible in a subset of CGRP-ir varicose nerve fibres (**a**, *arrows*); while an SP-labelled perikaryon (**b**, *arrowhead*) is CGRP-nonreactive (**a**). *Inset b* SP-ir fibre basket formation is associated with a nonreactive nerve cell body. *Bar*: 35 μm

Fig. 9a, b. CGRP-ir varicose fibres (**a**) enmesh clusters of TH-ir chromaffin cells at the cortico-medullary border (**b**, *arrows*). *Arrowheads* Short TH-ir cell process nonreactive for CGRP. *Bar*: 35 μm

Fig. 10a, b. SP-ir varicose fibre (**a**) exhibits co-localization of CCK-IR (**b**). *Bar*: 35 μm

Fig. 11a, b. NOS-IR (**a**) is present in four nerve cell bodies (*arrows*) and in numerous fine varicose fibres, which are non-identical with coarser NF-ir fibres (**b**). *Bar*: 60 μm

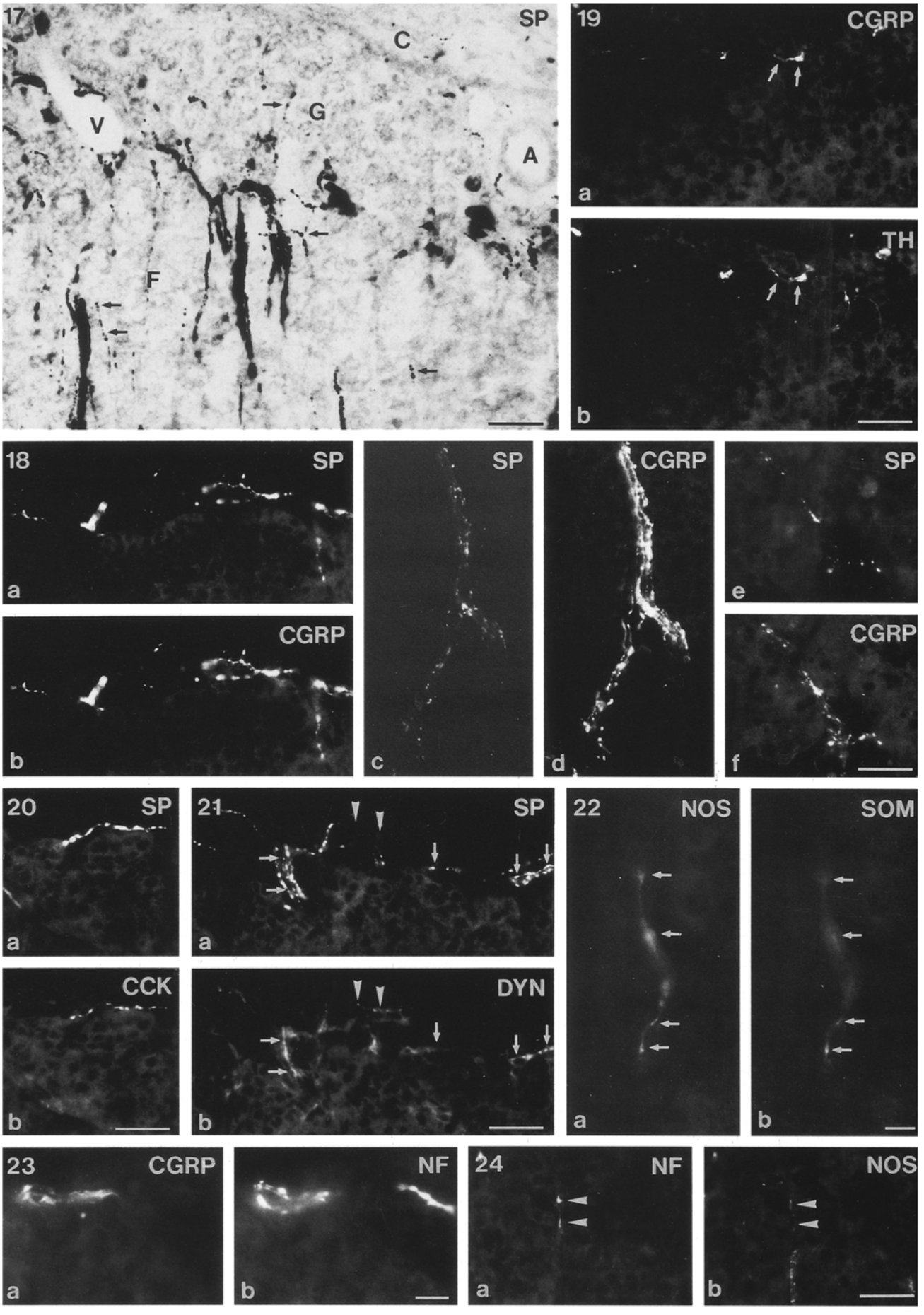
Fig. 12a, b. An SP-ir varicose fibre (**a**) is present in a medullary area, which is free of NF-ir nerve fibres (**b**). *Bar*: 35 μm

Fig. 13a, b. SOM-ir nerve cell body and an adjacent SOM-ir fibre (**a**, *arrow*) are incongruent with fine varicose CGRP-ir fibres (**b**) in the immediate vicinity. *Bar*: 35 μm

Fig. 14a, b. Aggregations of DYN-ir chromaffin cells (**a**) are surrounded by slender varicose SP-ir fibres (**b**, *arrows*). *Arrowhead* DYN-ir coarse fibre. *Bar*: 35 μm

Fig. 15a, b. TH-ir chromaffin tissue (**a**) with two interspersed CGRP-ir neuronal perikarya (**b**, *arrows*). *Bar*: 35 μm

Fig. 16a, b. SP-ir nerve cell body (**a**) is nonreactive for NOS (**b**) while a varicose NOS-ir fibre does not show co-localized SP-IR. *Bar*: 10 μm


17
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SP
e
CGRP
f
20
SP
a
CCK
b
21
SP
a
DYN
b
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NOS
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SOM
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23
CGRP
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NF
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24
NF
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NOS
b

in retrogradely labelled sensory cells in our experiments always was co-localized with CGRP- and sometimes co-existed with DYN- or CCK-IR, one can assume a third (and possibly fourth) population of DRG neurons projecting to the adrenal medulla, which contains SP/CGRP/DYN and/or SP/CGRP/CCK. (As DYN or CCK-IR was rarely observed in retrogradely labelled cells and antisera were available only from the same species, extensive screening would be needed to co-localize these two peptides in adjacent sections.) Indeed, Gibbins et al. (1987) have shown afferent fibres in the guinea-pig skin with the peptide-combination CGRP/SP/CCK/DYN, while afferents from airways and pelvic viscera contained CGRP/SP/DYN without CCK, and those from skeletal muscular blood vessels CGRP/SP/CCK without DYN. Fibres supplying peripheral ganglia were immunolabelled only for CGRP/SP. According to current knowledge, the adrenal medulla belongs to the postganglionic sympathetic system having migrated peripherally during development (Le Douarin 1982). This fact may explain our observation of a substantial population of CGRP/SP-ir fibres in the adrenal medulla associated with chromaffin tissue. The detection of retrogradely traced CGRP-/SP-ir neurons with additional CCK-IR correlates well with our finding of a small population of SP-/CCK-ir nerve fibres in the adrenal medulla, while medullary fibres, containing the "sensory" peptide combination SP/DYN, apparently were absent. Such fibres may have been masked by the multitude of DYN-ir chromaffin cells and their processes; as it will be discussed below, it is more likely that the lack of SP/DYN-ir fibres may reflect a differential innervation pattern of the two adrenal compartments. Alternatively, cleavage of the larger opioid DYN to the bioactive pentapeptide leu-enkephalin on its way to the fibre periphery may occur (Gibbins et al. 1987). Solely CGRP-ir intraadrenal fibres may not necessarily be of solely sensory origin but may also be derived from postganglionic efferents (Kummer and Heym 1991).

The combination SP/NOS was previously demonstrated in visceral afferent neurons of rat lower thoracic DRG (Aimi et al. 1991). Although CGRP/SP/NOS-positive sensory cell bodies were retrogradely labelled, intraadrenal SP/NOS- or CGRP/NOS-ir nerve fibres could not be detected in the present study. Probably, the abundant medullary, solely NOS-ir fibres are derived from non-sensory sources. Retrograde tracing, combined with immunohistochemistry has revealed NOS in sympathoadrenal projections from the rat spinal cord (Blottner and Baumgarten 1992). The moderate number of intraadrenal neurons which in rat (Bredt et al. 1990; Dawson et al. 1991), man (Heym et al. 1994) and guinea-pig (present study) exhibit NOS-IR, may also contribute to this fibre population.

Finally, a rather small population of primary afferent neurons projecting to the adrenal medulla was characterized by its immunoreactivity to NF; such neurons were nonreactive to all of the peptides tested. NF-160kD, the largest neurofilament subunit, belongs to the class of neuronspecific intermediate filaments and occurs in sensory (Price 1985) and postganglionic sympathetic neurons (Vickers et al. 1990) of selective distribution. To date, no specific association of NF with a particular transmitter system has been recognized. Price (1985) demonstrated that DRG cells reacting for this antibody did not contain substance P immunolabelling. According to the present findings, this observation can be extended to the peptides CGRP, DYN, and CCK, and additionally to the NO-generating enzyme, NOS, all of which occurred in perikarya separate from the NF-neurons. The large population of intramedullary NF-ir fibres differed completely from the peptide-containing fibres investigated, supporting the view that the NF-fibres represent a discrete chemically coded entity, the mediator of which is not yet known. However, as NF-IR was rarely found in retrogradely labelled sensory neurons and was absent from intraadrenal nerve cells, the dense intramedullary meshwork of NF-ir nerve fibres is supposed to be mainly derived from other than sensory sources (Vickers et al. 1990).

Albeit some TH-, SOM- or GAL-ir cell bodies were present in DRG, retrogradely labelled neurons exhibited neither immunoreactivity for TH nor for SOM or GAL. SOM-ir primary sensory nerve cell bodies, which in rat are rare (Tuchscherer and Seybold 1985) and as a rule do not contain SP-IR (Hökfelt et al. 1976; Nagy and Hunt 1982), apparently do not take part in the innervation of the adrenal medulla. This is corroborated by the demonstration of SOM in thermosensitive afferents (Wiesenfeld-Hallin 1986) retrogradely labelled from musculocutaneous nerves (Molander et al. 1987). Whether the observed intrinsic SOM-ir nerve cell bodies provide the main or sole source of the few intramedullary SOM-positive fibres remains to be elucidated.

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Fig. 17. Bright-field micrograph of an Epon-embedded, 30-µm-thick cryostat section of the adrenal cortex, free-floating incubated for SP-immunoreactivity according to the indirect peroxidase-antiperoxidase-technique (Sternberger and Joseph 1979). Thick SP-ir fibre bundles traverse the cortex, giving off fine varicose fibres to an arterial (*A*) and a venous blood vessel (*V*) as well as to capillaries along endocrine cell strands (*arrows*). *C* Capsule; *G* glomerular zone; *F* fasciculate zone. *Bar*: 60 µm

Figs. 18–24. Double-labelling immunofluorescence of the guinea-pig adrenal cortex

Fig. 18a–f. SP-IR (**a, c, e**) and CGRP-IR (**b, d, f**) are co-localized in the majority of nerve fibres and fibre bundles in subcapsular tissue and the glomerular zone (**a, b**) as well as in the fasciculate (**c, d**) and reticular zones (**e, f**). *Bar*: 35 µm

Fig. 19. Varicose CGRP-ir fibre (**a**, *arrow*) in the glomerular zone is also TH-ir (**b**). *Bar*: 35 µm

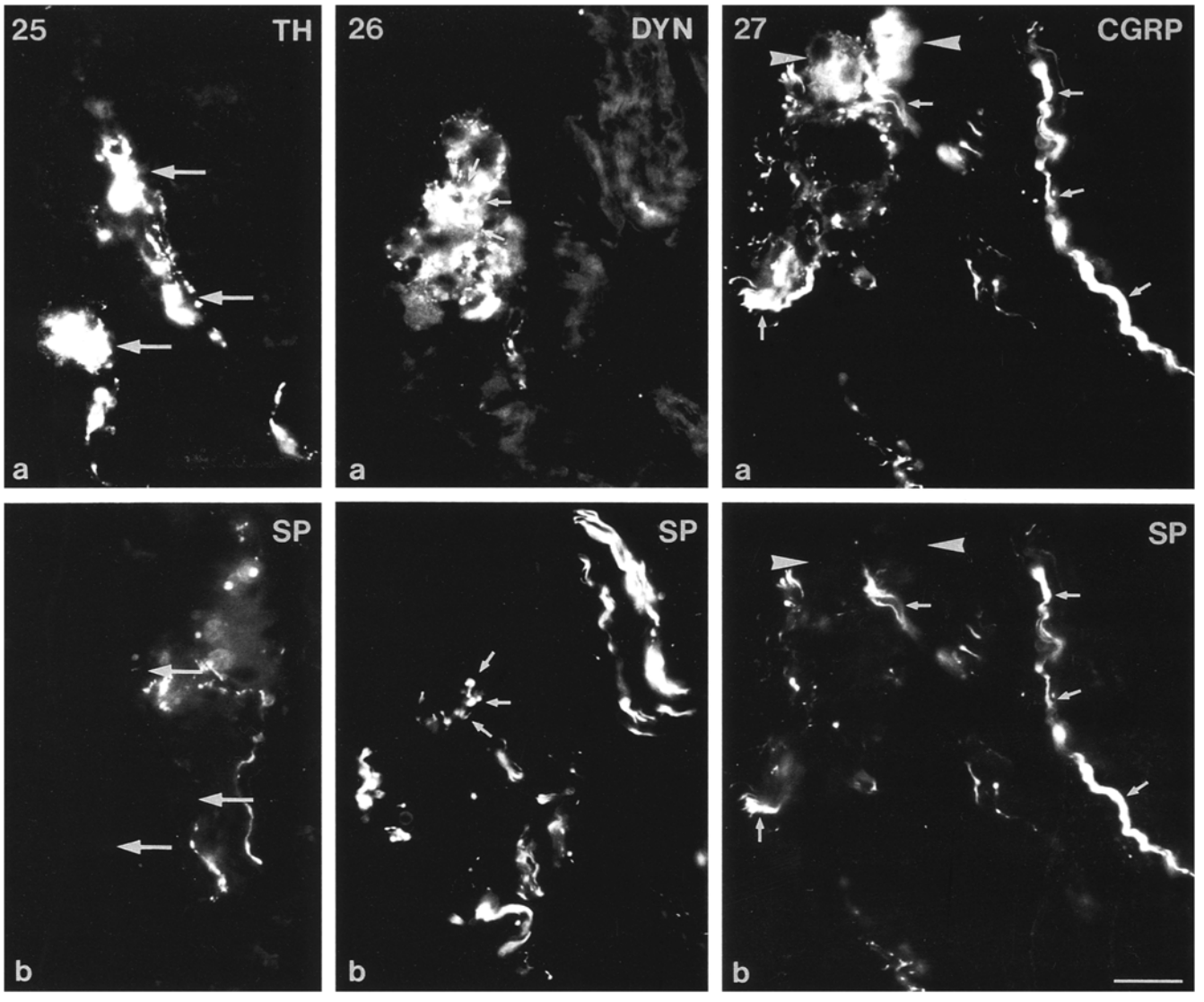
Fig. 20a, b. SP- (**a**) and CCK-IR (**b**) co-localize in a varicose fibre along a glomerular cell strand. *Bar*: 35 µm

Fig. 21a, b. Some of the subcapsular SP-ir nerve fibres (**a**) are also DYN-ir (**b**, *arrows*), while other fibres reveal either SP- or DYN-IR (*arrowheads*). *Bar*: 35 µm

Fig. 22a, b. NOS-ir nerve fibre (**a**) in the fasciculate zone apparently co-localizes with SOM-IR (**b**, *arrows*). *Bar*: 10 µm

Fig. 23a, b. CGRP- (**a**) and NF-ir nerve fibres (**b**) in the glomerular zone are similarly distributed but incongruent. *Bar*: 10 µm

Fig. 24. Solitary NF-ir nerve fibre (**a**, *arrowheads*) in juxtaposition to fasciculate cell strands differs in location to fine varicose NOS-ir fibres. *Bar*: 35 µm



Figs. 25–27. Double-labelling immunofluorescence of intracapsular small ganglia in the guinea-pig adrenal gland. *Bar*: 35 µm

Fig. 25a, b. Some of the TH-ir nerve cell bodies (**a**, *arrows*) are approached by SP-ir fibres (**b**)

Fig. 26a, b. One of the cell bodies in a small DYN-ir ganglion (**a**) is surrounded by an SP-ir fibre basket formation (**b**, *arrows*)

Fig. 27a, b. Two perikarya in a small ganglion are CGRP-ir (**a**, *arrowheads*); other nonreactive cell bodies are surrounded by CGRP-ir varicose fibres. SP-IR (**b**) co-localizes with CGRP-IR in neighbouring fibre strands (*arrows*) but not in varicose fibre basket formations

The lack of immunolabelling for any of the investigated peptides or proteins in a remaining subset of sensory neurons is highly suggestive for participation of still other, as yet undisclosed sensory mediators.

Chemical coding of adrenocortical structures and functional considerations

The presence of nerve fibres in the adrenal cortex was already demonstrated at the end of the last century (Dogiel 1894), however, these fibres were long thought to traverse the cortex on their way to the medulla. Meanwhile, morphological (Unsicker 1971, 1984; Garcia-Alverez 1972) and physiological evidence (Holzwarth et al. 1987; Edwards 1990) has been provided for a nervous control of cortical functions. The present demonstration of a highly differentiated cortical innervation supports the new understanding that not only chromaffin cell activity but also adrenal steroid secretion is directly modulated by a variety of peptides and peptide combinations. As the cortical and medullary blood supply is derived from different external sources and forms separate capillary beds (Coupland 1976), the presence of apparently sensory peptide combinations, e.g., CGRP/SP surrounding capsular and cortical as well as medullary vessels, is in favour of a second, indirect peptidergic nervous control system that regulates the blood flow of both adrenal

compartments, by this supporting neurohormonal feedback circuits (Bornstein et al. 1990; Hinson and Vinson 1990).

The distributional pattern of CGRP-containing nerve fibres in the guinea-pig adrenal cortex is similar in various mammalian species (Kuramoto et al. 1987; Pelto-Huikko 1989; Kong et al. 1989), suggesting a general principle in CGRP-innervation of steroidogenic cells. The demonstration of CGRP-binding sites in rat adrenal homogenates (Wimalawansa et al. 1987) is in support of a presumed effect of CGRP on chromaffin as well as on cortical endocrine cells (Kuramoto et al. 1987).

Similarly, our findings concerning the distribution of SP-ir fibres in the adrenal cortex correspond to previous descriptions in guinea-pig (Bucsics et al. 1981; Zentel and Weihe 1988), rat (Kondo 1985) and man (Linnoila et al. 1980). Such cortical fibres in their totality contained co-localized CGRP, indicating their sensory nature (Hua et al. 1985). While the dose-dependent inhibitory effect of SP on the catecholamine-secretion at the site of nicotinic receptors in the adrenal medulla has been the subject of extensive investigation (Livett et al. 1979, 1990; Role et al. 1981; Kuramoto et al. 1985), SP-effects on cortical functions in vivo seem to be confined to the zona glomerulosa, stimulating its secretory activity and growth (Nussdorfer et al. 1988). Furthermore, it is likely that SP/CGRP-ir fibres, associated with cortical blood vessels, exert vasomotor effects (Furchgott and Zawadski 1980; Brain et al. 1985) by increasing the blood flow in activated tissue.

The cortical distribution of NOS-ir fibres in guinea-pig is similar to that of rat (Afework et al. 1992; Dun et al. 1993). Although a number of peptides has been disclosed in the peripheral autonomic nervous system to cooperate with NO (see Yamamoto et al. 1993), SOM-IR and NOS-IR as yet have not been co-localized in nerve fibres. NO is regarded as a powerful vasodilator (see Vincent and Hope 1992), ensuring the appropriate blood supply under enhanced adrenal activity. Moreover, the NO generator, sodium nitroprusside, was shown to potentiate nicotinic transmission in the rat superior cervical ganglion, probably by the activation of guanylate cyclase coupled to the NO pathway (Briggs 1992). In this way, NO may regulate catecholamine release in the adrenal medulla in a direct manner (Dohi et al. 1983). Effects of NO on cortical functions are not yet known. Likewise, origin and functional relevance of NO in combination with SOM in the external adrenal cortex remain to be clarified.The almost complete absence of NF-ir fibres in the cortex supports previous suggestions of a separate innervation of the two adrenal compartments (see Parker et al. 1993).

The present results support the hypothesis of a separate innervation of adrenal medullary and cortical endocrine cells, both directly and indirectly via the circulation. Furthermore, the findings provide evidence for a differential participation of sensory mediator combinations in adrenal hormone regulation. It is tempting to assume specific effects of the investigated mediators on both medullary and cortical adrenal functions (Khalil et al. 1988; Bloom et al. 1989; Zhou and Livett 1990; Hinson and Vinson 1990), attributable to the activity of distinct subpopulations of spinal primary sensory neurons.

Neuronal perikarya in subcapsular ganglia revealed chemical codes that were not attributable to the neurochemical equipment of sensory neurons and, most likely, belong to the postganglionic sympathetic system (Heym et al. 1993).

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