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Innervation of human adrenal gland and adrenal cortical lesions

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Abstract The innervation of the human adrenal gland and of cortical lesions was studied in sections of cortical tissue (*n*=10), hyperplastic cortical tissue (*n*=3), and tissue from cortical adenomas (*n*=5) and carcinomas (*n*=6). The presence and distribution of nerve structures containing neuronal markers indicating sympathetic and parasympathetic innervation were studied by immunohistochemistry and the co-existence and co-localization patterns of the different markers by immunofluorescence. The cortex and hyperplastic cortical tissue had a moderate to rich supply of nerve structures containing the typical neuronal markers: protein gene product 9.5 (PGP 9.5), neuron-specific enolase (NSE), small vesicle synaptic protein type 2 (SV2), and nerves showing immunoreactivity to the adrenergic marker tyrosine hydroxylase (TH). All these immunoreactive nerves were located predominantly adjacent to blood vessels, but also among parenchymal cells. The cortex showed numerous nerve structures containing the neuropeptide substance P (SP), neuropeptide Y (NPY) and vasoactive intestinal protein (VIP), but few nerves containing these peptides were seen in hyperplastic cortical tissue. Typical markers were occasionally observed in cortical adenomas but were not found in carcinomas, except in a few cases where PGP 9.5 and NSE were present, but only adjacent to necrotic areas. Nerves containing NPY and VIP occurred in varying numbers in both adenomas and carcinomas. NPY- and VIP-immunoreactive nerve structures were seen mostly alongside blood vessels. There were several types of co-existence. For instance, NSE/VIP-, TH/VIP- and TH/NPY-immunoreactive nerve structures were often seen in the same trunk, but were only partly co-localized.

Abstract Adrenal cortex · Adrenal tumour · Innervation · Immunohistochemistry · Neuronal markers

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Introduction

The adrenal gland is richly innervated. Its capsule contains nerve plexuses of fibres originating from the greater splanchnic nerve and associated abdominal plexuses of the sympathetic autonomic nervous system, combined with parasympathetic contributions from the phrenic and vagal nerves [5, 21]. From these plexuses, nerve bundles traverse the cortex, mostly in association with blood vessels and connective tissue trabeculae. The vast majority of these nerves, which may be pre- or postganglionic, terminate either in the medulla or in the smooth muscles of the adrenal vessels, but also among cortical cells.

Neuropeptides are known to influence hormonal secretion and/or blood flow in different endocrine glands, and endocrine cell systems in nonendocrine organs. The involvement of neuropeptides in the innervation of the adrenal gland was discovered by Schultzberg et al. [26, 27], who identified the met-enkephalin-immunoreactive (ir) nerve structures in the adrenal medulla of rat, guinea pig and cat. So far, there have been few studies on the human adrenal gland, particularly the cortex. Using silver impregnation techniques, Mikhail and Amin [20] reported in 1969 that there is a rich innervation of all three zones of the adrenal cortex. However, such techniques could not identify the nerve types, or the transmission substances. McNicol et al. [19] studied the innervation of human adrenal cortex in 1994 using protein gene product (PGP) 9.5, a ubiquitin carboxyl-terminal hydrolase, which at present seems to be the most appropriate nerve marker. It is not certain, however, whether this marker identifies all nerves collectively. In previous studies, some peptides and enzymes associated with nerve structures of the human adrenal gland have been

reported, such as substance P (SP), vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), cholecystokinin, somatostatin, dynorphin, neuron-specific enolase (NSE) and nitric oxide synthase [2, 10, 17]. In the present study the innervation of the human adrenal cortex – and particularly of cortical lesions – was investigated by using neuronal markers mainly associated with sympathetic and parasympathetic innervation. Furthermore, the co-existence and co-localization of the markers in the different nerve structures was examined.

Materials and methods

The following surgical tissue samples were included in the study: cortex from adrenal glands, obtained in two cases from patients operated on for renal carcinoma not involving the adrenal gland and in eight cases from tissue adjacent to cortical neoplasms (n=10), hyperplastic cortex (n=3), and tissue from cortical adenomas (n=5) and carcinomas (n=6). The hyperplasia was very slight in one case (no. 52; weight 6.8 g) but extensive in another (no. 18; 25 g). The weight in a third case (no. 19) is not known. Its cortical thickness was 3 mm, measured on the fixed, deparaffinized section; its weight was estimated to be about 10 g, corresponding to moderate hyperplasia. The cancer diagnosis was based on infiltrative growth (one case) or on Weiss' histopathological criteria (five cases) [33]. Tissue specimens from cortex included head, body and tail [15] as well as cortical extrusions, nodules and cortical cell nests in the medulla. All tissue specimens were fixed in 4% buffered formaldehyde solution for 18–24 h at room temperature (RT) and processed routinely to paraffin.

Deparaffinized sections 5 µm thick were stained with haematoxylin and eosin, or immunostained, to demonstrate PGP 9.5, NSE, small vesicle synaptic protein type 2 (SV2), tyrosine hydroxylase (TH), neuropeptide Y (NPY), VIP, SP, galanin, CGRP, met-enkephalin, ACTH, and serotonin immunoreactivity. The avidin–biotin peroxidase complex (ABC; Elite kit) method was used with diaminobenzidine as chromogen. The sections were pretreated with 0.3% hydrogen peroxide in methanol for 30 min to suppress the endogenous peroxidase. Microwave pretreatment of the sections was used for the SV2 and TH immunostaining (see below). The sections were incubated overnight at room temperature (RT), with the primary antibody and then with biotinylated IgG followed by avidin–biotin complex (Table 1), each for 30 min at RT. Each incubation step was followed by careful rinsing with PBS.

As positive controls we used human adrenal medulla, which is known to contain PGP 9.5-, NSE-, SV2-, TH-, NPY-, VIP-, and met-enkephalin-ir cells; human small intestine, which contains galanin-ir nerves and serotonin-ir cells; brain tissue and thyroid medullary carcinoma tissue, which contains SP- and CGRP-ir cells, respectively, and pituitary, which contains ACTH-ir cells. Subsequently, negative controls were obtained by omitting the primary antibody, which was replaced with an irrelevant monoclonal (insulin) antibody or with a nonimmune rabbit serum. Control immunostaining was performed on buffered paraformaldehyde-fixed, paraffin-embedded tissue using the ABC and fluorescence techniques. Neutralization tests were carried out by preincubating the NPY, VIP, and SP antibodies with the corresponding antigen (0.5, 2 or 10 nmol per ml diluted antibody solution) at 4°C, overnight. The antigens were from SIGMA (St Louis, Mo., USA). These tests showed that the intensity of the immunostaining was reduced by 0.5 nmol of the antigen and was quenched by both 2 and 10 nmol.

Sections immunostained with SV2 and TH antibodies were pretreated in a microwave oven (Philips Whirlpool, Sweden) using a citrate buffer, pH 6.0, at 750 W for 2×5 min followed by the hydrogen peroxide treatment.

The numbers of ir-nerve fibres and terminals were estimated semiquantitatively and graded as: sparse, present in moderate numbers, or numerous.

Co-existence studies were performed on consecutive sections, using the ABC technique. In the co-localization studies on neuronal markers and neuropeptides, a double-immunofluorescence technique was used. The sections were incubated with two antibodies (one monoclonal + one polyclonal), overnight at RT–> bio-

Table 1 Summary of the antibodies used

Antibody	Code	Raised in	Dilution		Source
			ABC	Fluorescence	
Chromogranin A	LK2H10	Mouse	1:1000		Boehringer-Mannheim, Mannheim, Germany
Protein gene product 9.5	13C4	Rabbit	1:1000	1:100	UltraClone, Wellow, Isle of Wight, England
Neuron-specific enolase	A589	Rabbit	1:2000	1:100	DAKO, Glostrup, Denmark
Neuron-specific enolase	M0873	Mouse	1:200	1:50	DAKO
Synaptic vesicle protein 2		Mouse	1:100	1:50	Dr R.B. Kelly, Dept. of Biochemistry and Biophysics, University of California, San Francisco, USA
Tyrosine hydroxylase	2/40/15	Mouse	1:50	1:25	Boehringer-Mannheim
Substance P	SP1-11 87-3T	Rabbit	1:2000	1:200	BMC, Dept. of Cell Biology, Uppsala, Sweden
Neuropeptide Y	RIN7172	Rabbit	1:2000	1:100	Peninsula Laboratories, Belmont, Calif.
Vasoactive intestinal polypeptide	B34-1	Rabbit	1:3000	1:400	Euro-diagnostica, Malmö, Sweden
Galanin	B49-1	Rabbit	1:500		Euro-diagnostica
Met-enkephalin	B15-1	Rabbit	1:1600		Euro-diagnostica
ACTH	A571	Rabbit	1:6000		DAKO
Calcitonin gene-related peptide	B-GP 640-1	Guinea-pig	1:2000		Euro-diagnostica
Serotonin	YC5/45	Rat	1:100		Medicorp, Montreal, Canada
Biotinylated anti-rabbit IgG	E0432	Goat	1:200	1:40	DAKO
Biotinylated anti-mouse IgG	E0354	Rabbit	1:200	1:40	DAKO
Biotinylated anti-guinea-pig IgG	BA7000	Goat	1:200		VECTOR Laboratories, Burlingame, Calif.
Biotinylated anti-mouse IgG	BA2001	Horse		1:40	VECTOR Laboratories
Biotinylated anti-rabbit IgG	E0353	Swine	1:200	1:40	DAKO
FITC-conjugated anti-rabbit IgG	F0511	Goat		1:10	Sigma Chemical Co., St. Louis, Mo.
FITC-conjugated antimouse IgG	F2012	Goat		1:10	Sigma Chemical Co.
Elite ABC kit	PK-6100		1:100		VECTOR Laboratories
Texas Red streptavidin	SA 5006			1:100	VECTOR Laboratories

tinylated horse anti-mouse IgG, 30 min at RT–> a mixture of TXRD (TXRD)-conjugated streptavidin and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, 30 min at RT. Before application of the appropriate primary antibody, the sections were incubated with nonimmune serum from the animal species producing the secondary antibody, diluted 1:10. Between staining steps, the sections were always carefully rinsed with PBS. To ascertain whether the outcome of the initial immunostaining affected the second, and vice versa, a single immunostaining with the appropriate antibody was performed.

When two primary polyclonal antisera raised in the same species (rabbit) or two monoclonal antibodies were to be used, the staining procedure was as follows: initially the sections were incubated overnight at RT with the first primary antibody, followed by incubation for 30 min with biotinylated porcine anti-rabbit IgG or biotinylated horse anti-mouse IcG, respectively, and TXRD-conjugated streptavidin, each at RT. Thereafter the sections were exposed to paraformaldehyde vapour for 4 h at 80°C [32] and then incubated overnight with the second primary antibody, followed by incubation with FITC-conjugated goat anti-rabbit or goat anti-mouse IgG for 30 min at RT.

The sections were examined in a Vanox AHBS3 fluorescence microscope (Olympus, Tokyo, Japan) with filters for viewing FITC and TXRD fluorescence alternately and a double-band filter for simultaneous visualization of the two fluorochromes.

Results

The findings in normal cortical tissue and the different cortical lesions are summarized in Table 2.

Cortex

The thickness of the cortex was fairly even in all cases except two, which contained a few small cortical nodules, indistinctly encapsulated. The cortex, associated with the adenomas causing Cushing's syndrome, showed slight atrophy, whereas cortex associated with the other adenomas was of normal thickness. Use of an Elite ABC kit visualized more nerve structures than did the fluorescence method.

Cortex, including capsule, was richly innervated by nerve trunks, bundles and fibres (Table 3). The trunks and bundles often traversed the cortex to medulla, generally following the blood vessels. Some nerve fibres were seen among parenchymal cells, with a more horizontal distribution pattern.

In the head and body PGP 9.5 was the most useful neuronal marker, i.e. visualized most nerve structures in all cases (Fig. 1a). NSE demonstrated rather fewer nerve structures than PGP 9.5, and SV2 even fewer. As seen in Table 3, PGP 9.5- and NSE-ir nerves occurred as nerve trunks, bundles, and fibres deriving from the capsule, entering and passing through the cortex more or less radially, whereas SV2 immunoreactivity appeared mainly in nerve fibres. PGP 9.5- and NSE-containing nerve bundles and fibres were seen, particularly close to vessels but also among cortical cells, whereas SV2-ir nerves (Fig. 2) were more frequently observed among cortical cells than adjacent to vessels. TH-ir nerve structures were observed, mainly as bundles and fibres. The fibres (Fig. 1b) were located predominantly adjacent to vessels (Table 3) but were also seen among the parenchymal cells. The TH-ir bundles and fibres were most numerous in the outer cortex. The nerve trunks of the capsule also contained TH-ir nerve structures. NPY, VIP, and SP immunoreactivity were seen mainly in cortical nerve fibres, but also in trunks and/or bundles (Table 3). The NPY- and VIP-ir nerves were sparse to moderate in number and observed mainly in the outer cortical zones. VIP- and NPY-ir nerves were located particularly close to blood vessels (Fig. 1c), and sometimes close to smooth muscle cells, whereas SP-ir nerve fibres were seen mainly among the cortical cells and were manifestly numerous in cortex associated with Conn's adenomas.

In the tail there were some nerves, but fewer than in the head and body, particularly for the SV2- and TH-ir nerves. The nerve structures occurred as fibres, some of which were adjacent to vessels, others among the cortical parenchymal cells (Table 3).

Between the two cortices, PGP 9.5-, NSE- and SP-ir (Fig. 3) nerve fibres were observed.

Pericapsular groups of cortical cells (extrusions) were seen in three of our cases. In these extrusions, nerve fibres (but no nerve trunks or bundles) were identified. These nerve fibres were visualized mainly by PGP 9.5, NSE and SV2. Very few TH-ir fibres were found. VIP-ir nerve fibres varied from sparse to moderate in number, but only a few fibres displayed NPY immunoreactivity. SP-ir nerves were lacking in two cases, but numerous in the third case, which was linked with a Conn's adenoma.

The nodules mentioned above contained a few nerve structures, mostly appearing as fibres. These fibres were identified with PGP 9.5, NSE and SV2 as well as with the neuropeptides NPY and VIP.

Small groups of cortical cells were seen within the medulla, sometimes in apposition to the central vein. No nerve structures were seen within these cell groups.

Cortical hyperplasia

Nerve trunks, bundles and fibres visualized with PGP 9.5, NSE and TH markers were seen in all cases, but SV2-ir nerves, usually identified as trunks or fibres, were observed only in the two cases with slight and moderate hyperplasia. On the whole, in these latter cases the frequency and distribution of the nerve structures were consistent with those of 'normal' cortex (Table 3). In the case with extensive hyperplasia they were very sparse. There were few NPY-, VIP- or SP-ir nerve fibres in the hyperplastic cortex. The nerves were mainly associated with vessels, but sometimes with parenchymal cells (Table 3).

In adenomas and carcinomas, only nerve bundles and fibres were observed (Table 3). Only one of the adenomas contained PGP 9.5-ir nerve fibres, and they were few. No NSE- or SV2-ir nerves were seen, and nor were any TH- or SP-ir structures. NPY- and VIP-ir nerves in the form of bundles and fibres were observed in three of the adenomas (Fig. 4), but they were few in number and

Table 2 Immunohistochemical identification and semiquantification of nerves with antibodies to protein gene product (*PGP*) 9.5, neuronspecific enolase (*NSE*), synaptic vesicle protein 2 (*SV2*), tyrosine hydroxylase (*TH*), neuropeptide Y (*NPY*), vasoactive peptide (*VIP*), and substance P (*SP*) in adrenal cortex and cortical lesions: *1* few fibres, *2* moderate number of fibres; *3* numerous nerve fibres (*Hyperpl* hyperplasia, *Non-funct* nonfunctioning, *Ad* adenoma, *Ca* carcinoma, *Phaeo* phaeochromocytoma)

Case no.	Histopathological diagnosis	Clinical syndrome	Adjacent tumour	PGP 9.5	NSE	SV2	TH	NPY	VIP	SP
38	Normal[h]		Kidney Ca	3	2	1	3	1	1	1
39	Normal[h]		Kidney Ca	2	1	1	3	1	1	1
23	Normal[h]		Ad (Cushing)	1	1	0	1	1	1	1
5	Normal[f,h]		Ad (Conn)	3	3	2	2	1	1	3
7	Normal[fh]		Ad (Conn)	2	2	1	2	1	2	3
41	Normal[f,h]		Ad (Conn)	3	2	3	3	0	1	3
13	Normal[h]		Ad (Non-funct)	1	1	1	1	1	2	1
10	Normal[h]		Ca[c] (virilism)	2	0	0	2	1	3	2
12	Normal[h]		Ca[b] (non-funct)	1	0	0	1	0	2	0
40	Normal[f,h]		Phaeo[d]	2	1	2	3	1	1	1
18	Hyperpl	Cushing		1	1	0	1	0	1	0
19	Hyperpl	Conn		3	2	1	2	0	0	1
52	Hyperpl	Susp.phaeo[e]		3	2	3	3	1	1	0
23	Ad	Cushing		1	0	0	0	1	1	0
24	Ad	Cushing		0	0	0	0	0	0	0
5	Ad	Conn		0	0	0	0	2	3	0
7	Ad	Conn		0	0	0	0	1	3	0
13	Ad	Non-funct		0	0	0	0	0	0	0
30	Ca[b]	Cushing		0	0	0	0	0	2	0
36	Ca[b]	Conn		0	0	0	0	0	0	0
35	Ca[b]	Virilism		0	0	0	0	0	2	0
10	Ca[c]	Virilism		0	0	0	0	1	3	0
27	Ca[a]	Non-funct		++	++	0	0	2[g]	3[g]	0
12	Ca[b]	Non-funct		0	0	0	0	1	1	0

[a] Invasive growth
[b] Malignancy according to Weiss' histopathological criteria
[c] Mild suspicion of malignancy
[d] Malignant
[e] Diagnosis of phaeochromocytoma could not be confirmed histopathologically
[f] Tissue sections contained head/body and tail
[g] Nerve fibres were seen both among the neoplastic cells and close to necrotic areas
[h] Specimens were taken from head/body of cortex

Table 3 Distribution of nerve markers and peptides in different nerve structures in cortex, cortical hyperplasias, adenomas and carcinomas and their relation to parenchymal cells (+ nerve structures identified; – nerve structures not identified, *P* nerve structures seen predominantly among parenchymal cells, *V* nerve structures seen predominantly adjacent to vessels)

	PGP 9.5	NSE	SV2	TH	NPY	VIP	SP
Cortex							
Head and body							
Trunk	+	+	–	+	+	+	–
Bundle	+V	+V	–	+	+	+	+P
Fibre	+V	+V	+P	+V	+V	+V	+P
Tail							
Trunk	–	–	–	–	–	–	–
Bundle	–	–	–	–	–	–	–
Fibre	+P,V	+P,V	+P,V	+P,V	+	+	+
Hyperplasia							
Trunk	+	+	+	+	+	+	–
Bundle	+V	+V	–	+	+	+	+P
Fibre	+V	+V	+P	+V	+V	+V	+P
Adenoma							
Trunk	–	–	–	–	–	–	–
Bundle	–	–	–	–	+V	+V	–
Fibre	+	–	–	–	+V	+V	–
Carcinoma							
Trunk	–	–	–	–	–	–	–
Bundle	+	+	–	–	+	+	–
Fibre	+	+	–	–	+V	+V	–

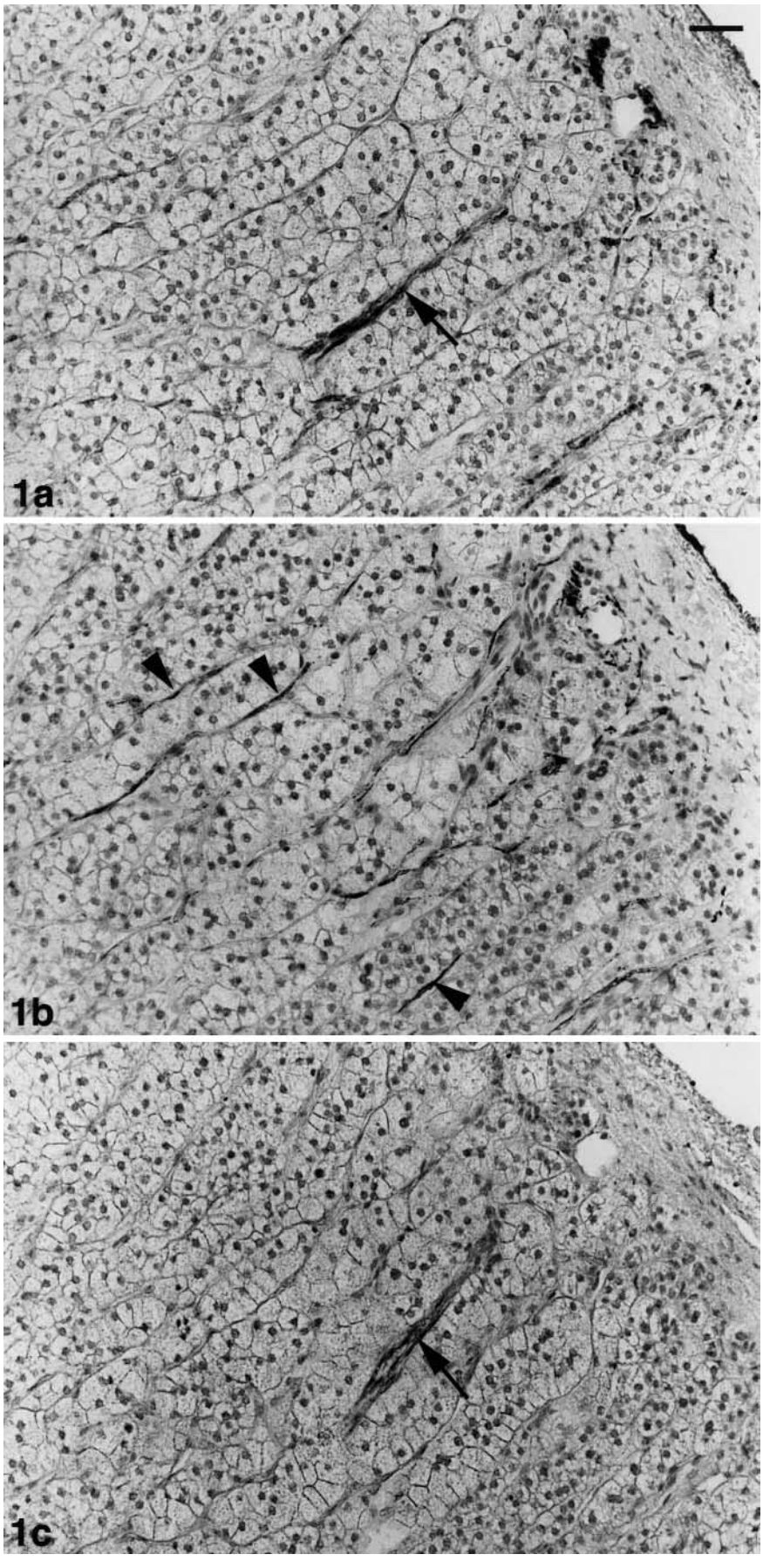
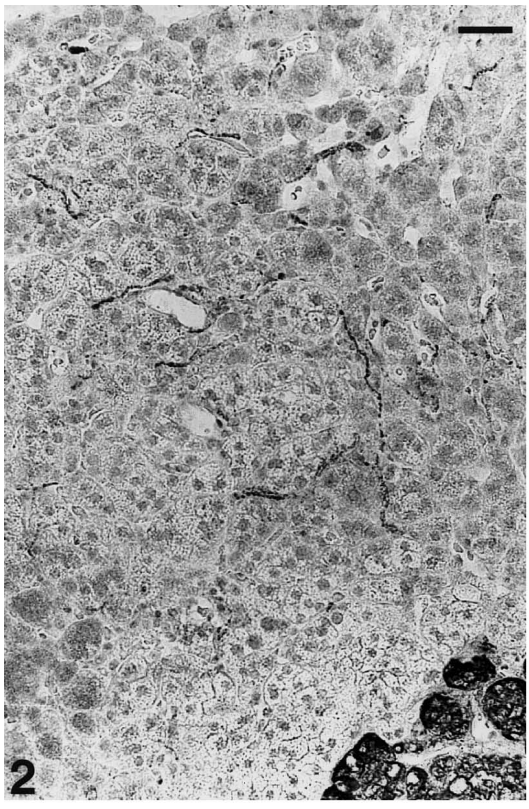
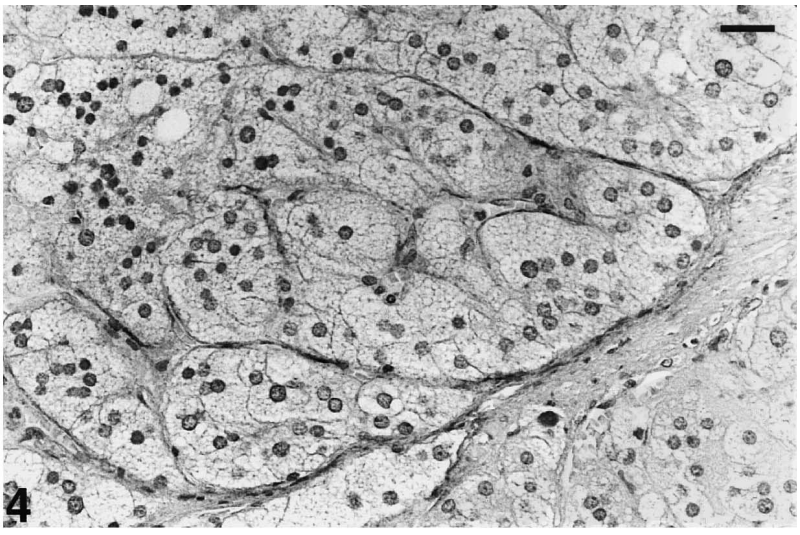
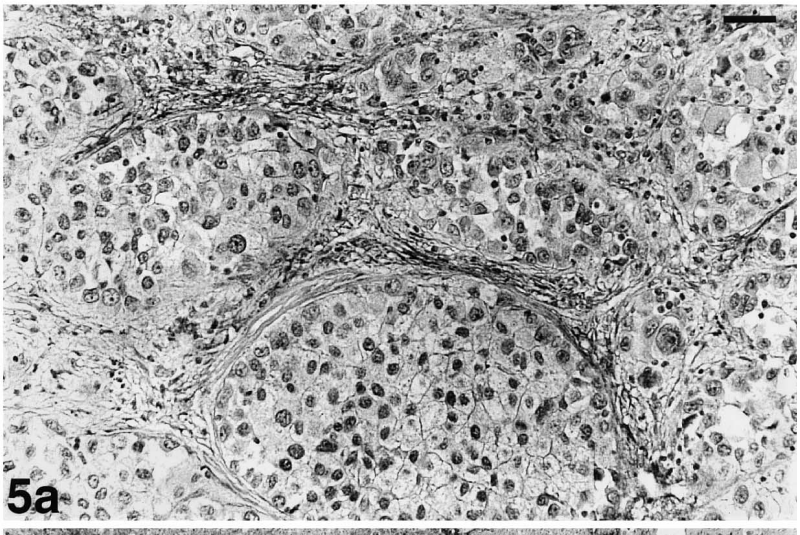
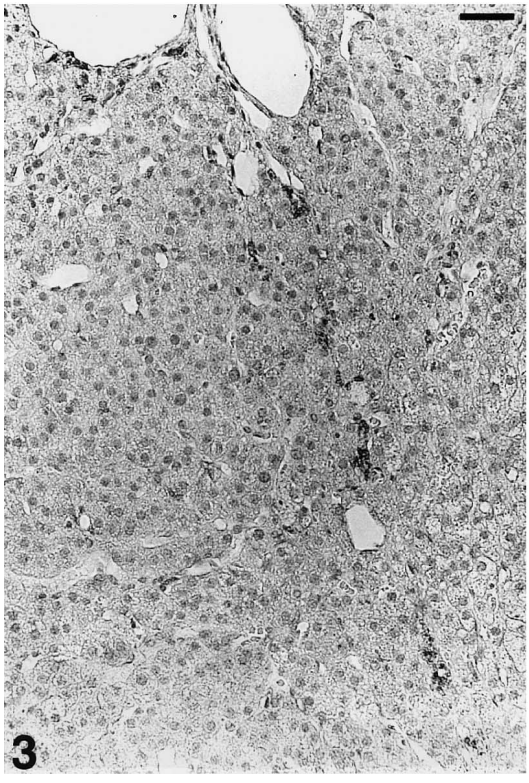


Fig. 1a–c Consecutive sections from cortex immunostained for **a** PGP 9.5, **b** tyrosine hydroxylase (TH) and **c** vasoactive intestinal protein (VIP). The sections contain a nerve bundle showing PGP 9.5- and VIP-immunoreactive fibres (*arrows*) and several individual fibres displaying particularly TH-immunoreactivity (*arrowheads*). ABC method, *bar* 50 µm









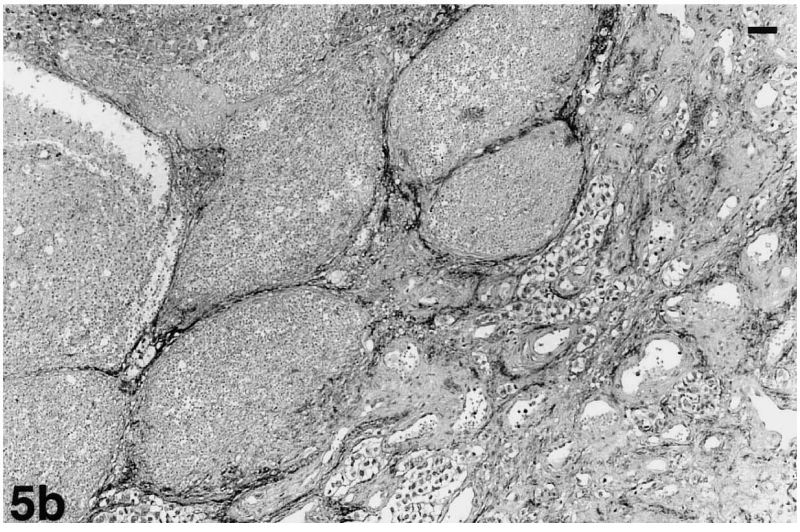


Fig. 2 Microphotograph of cortex, showing synaptic vesicle protein 2 (SV2)-immunoreactive nerve fibres with varicosities localized among parenchymal cells. Part of medulla is seen in *lower right corner*. ABC method, *bar* 50 µm

Fig. 3 Microphotograph of cortical tail, showing substance P (SP)-immunoreactive nerve fibres between the two cortical layers. ABC method, *bar* 50 µm

Fig. 4 Microphotograph of an adenoma, showing VIP-immunoreactive nerve fibres branching out from a slender nerve bundle adjacent to a fibrous septum. Occasionally the nerve fibres partly embrace parenchymal cell nests. ABC method, *bar* 50 µm

Fig. 5a, b Cortical carcinoma containing **a** VIP-reactive and **b** neuropeptide Y (NPY)-immunoreactive nerves. The NPY-immunoreactive nerves embrace dilated vessels located within partly necrotic areas. ABC method, *bar* 50 µm

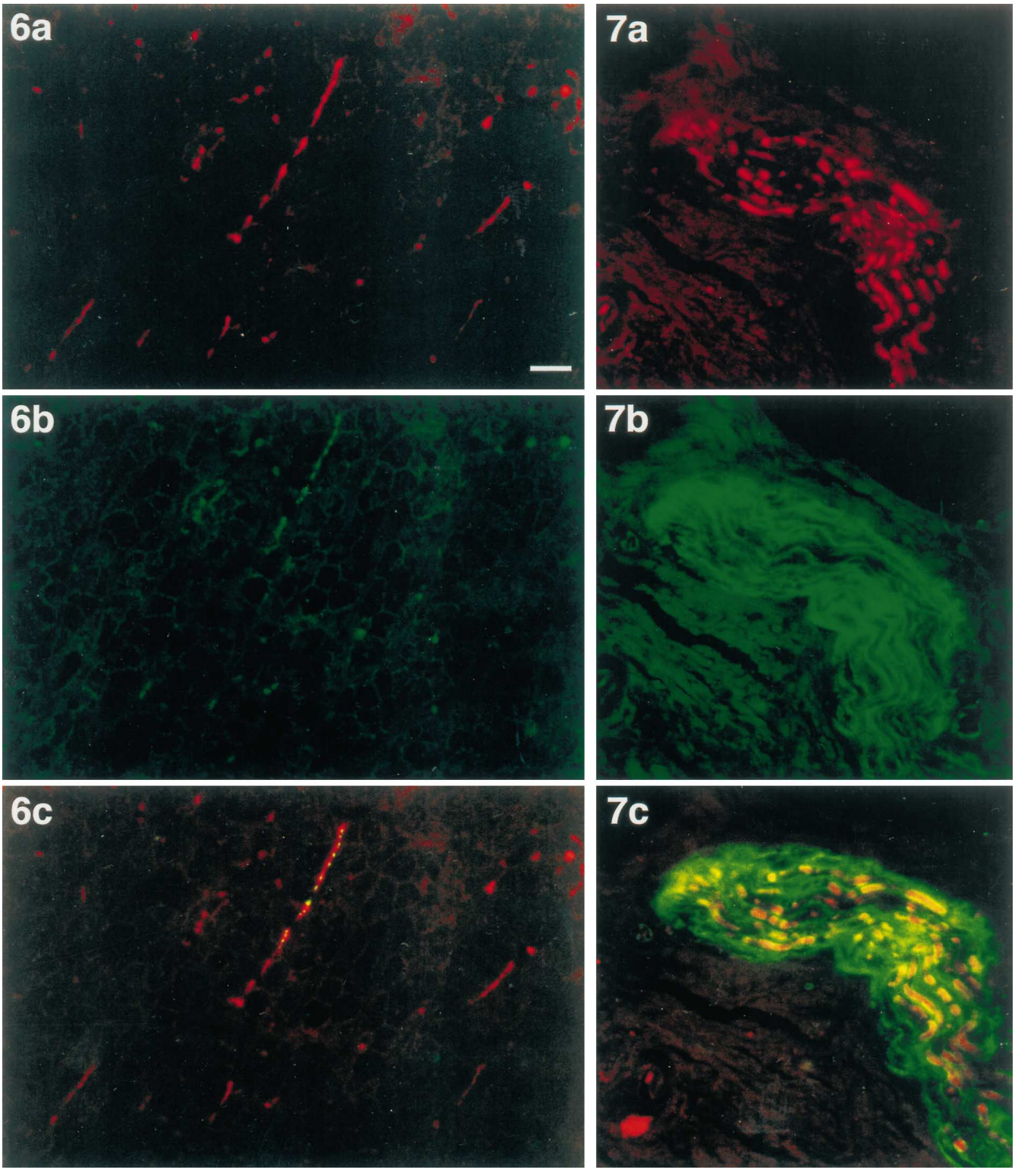


Fig. 6a–c Microphotographs of a cortex double immunostained for **a** TH and **b** substance P (SP). Few SP-immunoreactive fibres are visible (**a**), but there are several TH-immunoreactive ones (**b**). Immunoreactivities are evident in varicosities. **c** Co-localization of SP and TH (*yellow colour*) visualized using a double-band filter for TXRD and FITC. Co-localization of SP and TH occurs in some (but not all) varicosities. **a** TXRD, **b** FITC, *bar* 50 µm

Fig. 7a–c Microphotographs of the capsule region double immunofluorescence stained for **a** neuron-specific enolase (NSE) and **b** VIP. A nerve trunk is seen containing both NSE- and VIP-immunoreactive nerve fibres. **c** These substances are partly co-localized (*yellow*), as visualized with a double-band filter for TXRD and FITC. **a** TXRD, **b** FITC, *bar* 50 µm

were associated mainly with vessels and to some extent also among neoplastic cells and within stroma. These neuropeptide-containing fibres appeared to be different from the PGP 9.5-ir nerves (Table 3).

Only one of the carcinomas contained PGP 9.5- and NSE-ir bundles and fibres, which were occasionally found close to necrotic areas, surrounding dilated vessels to a greater or lesser degree. VIP-ir bundles and fibres were observed in five of six carcinomas (Fig. 5a), and NPY-ir in three; these neuropeptide-containing nerves often appeared in broad neurofibrous nets, sometimes surrounding vessels (Fig. 5b). No SV2-, TH- or SP-ir nerves were demonstrated.

No nerve structures displaying galanin, CGRP, enkephalin, ACTH or serotonin immunoreactivity were observed in cortex or cortical lesions.

Nerve trunks, bundles and individual fibres contained varying numbers of varicosities. When identified with the different neuronal markers, they were found to contain one or more of the neuropeptides being studied.

Co-existence and co-localization of neuronal markers

In the cortex the nerve trunks, bundles and fibres in both the capsule and the cortex showed various combinations of the different markers co-existing. These were located in the same nerve trunks, bundles, fibres or varicosities in varying proportions (Figs. 6, 7). Various combinations were also co-localized, the most common being PGP 9.5/TH and NPY/VIP. NPY and VIP were also observed in TH nerves, but co-localization of NPY and SP was not seen.

In the two cases of slight hyperplasia, the coexistence and co-localization of the different markers showed a pattern similar to that observed in 'normal' cortex. In the case with extensive hyperplasia, the nerves were too few to permit of any evaluation.

In adenomas, nerve structures were sparse or absent, which is why relevant co-localization studies could not be undertaken. One of the carcinomas contained PGP 9.5- and NSE-ir nerves, and these enzymes seemed to be coexisting to some extent. In the carcinomas where there were NPY- and VIP-ir nerves these appeared, to some extent, in the same nerve fibres.

Discussion

ABC and immunofluorescence techniques were both used to identify the nerve structures; with the sensitive ABC technique (ABC Elite kit), more nerve structures could be demonstrated than with the fluorescence method, which was mainly used for the co-localization studies. The lower sensitivity of the latter staining technique hampered the co-localization analyses to some extent, but this was compensated by studies on consecutive sections using the ABC method.

Most previous innervation studies on the adrenal gland have been performed on fixed cryosections, but some have also been done on fixed paraffin-embedded tissue specimens. In pilot studies we tested the fluorescence technique on cryosections, but this method did not prove any better than the ABC technique used on deparaffinized sections. However, the application of double fluorescence to cryosections will probably elicit further information about co-localization. In pilot studies we also compared the staining results obtained from 5-µm sections with those obtained in thicker ones, but as the staining intensity was more distinct in the thin sections these were used throughout our studies.

The adrenal gland is innervated by the autonomous nervous system. In 1994, McNicol et al. [19] described three types of nerve structure in the human adrenal cortex: large nerve trunks traversing the cortex to the medulla; a complex, branching network of slender nerve bundles; and individual nerve fibres. These authors [19] also identified nerve fibres in the muscular wall of the central vein. Our results confirmed their findings, as do our recent results [1, 7]. The present study, however, has produced more detailed knowledge about the occurrence and distribution of the peptide- and amine-containing nerve structures in the adrenal cortex and in cortical lesions. Of the different anatomical regions of the adrenal cortex, the head and body seemed to have a better nerve supply than the tail; this was most apparent in the two outer zones. The absence of visible nerve structures in the cortical cell nests in the medulla was rather surprising, as nerve trunks and most nerve bundles traversing cortex enter the medulla, indicating that it receives a rich sympathetic and parasympathetic innervation.

The nerve structures displayed a variety of distribution patterns, in both cortex and cortical hyperplasias. The rich nerve supply to the cortex suggests an important hormonal regulatory role for the nervous system, not only via the vascular system but also directly at the parenchymal cell level. Our findings also indicate that nerve bundles – and particularly fibres – play a more local role than the trunks, as the bundles and fibres were often closely associated with vessels and/or parenchymal cells. Many of the nerves displayed varicosities, which further indicate a neurotransmitter release and a local site of cortical action [19]. In hyperplasia, nerve density does not increase in parallel with the increase in the parenchymal cell mass, thus indicating that the regulatory role of the nerves probably decreases inversely with the increase in hyperplasia. Nerve structures were seen in some neoplasms only, which indicates that the nervous regulation must have a less important role.

The present results further confirm that PGP 9.5 is a good nerve marker in cortex and cortical hyperplasia, although it does not visualize all nerve structures; for instance, in some cases the frequency of VIP- and SP-ir nerves exceeded that of PGP 9.5-ir nerves. This last finding was confirmed by double immunostaining. The NSE marker also visualized nerve trunks, bundles and fibres, but seemed at least in some cases to have fewer nerve structures than did PGP 9.5. The SV2, an integral membrane glycoprotein present in synaptic vesicles, appeared

mainly in the nerve fibres seen among the cortical cells and is therefore less suitable as a 'general' nerve marker.

As TH is the first enzyme in the biosynthetic pathway of catecholamines, it can be used as an adrenergic marker. TH-ir nerve fibres and ganglion cells have been reported in rat adrenal gland, where the fibres occurred mainly in the vicinity of blood vessels [23]. From the present study it was also apparent that human cortex and cortical hyperplasias contain TH-ir nerves, which are mainly adjacent to blood vessels but also appear as fibres among the cortical cells. The TH-ir nerves were most numerous in the outer cortical zone. As far as we are aware, this is the first report of TH-ir nerve fibres in human adrenal gland, and our results support the proposed role of the sympathetic nerve system in hormonal regulation of the adrenal gland [5].

Nerve fibres containing neuropeptides have been demonstrated in the adrenal cortex of several species (rat, mouse, guinea pig, cat, horse) [12, 13, 18, 22, 30], including human [17]. Our results confirm the constant presence of NPY-, VIP-, and SP-ir nerve structures, mainly in the form of fibres, in cortex, where they were sparse or moderate in number. The NPY- and VIP-ir fibres occurred mostly in the close vicinity of small vessels. This intimate relation indicates that these neuropeptides are probably involved in regulating the local blood flow. SP has been shown to modulate catecholamine secretion in isolated rat chromaffin cells and in perfused rat adrenal gland [35–37]. In 1994, Hinson et al. [11] reported that SP also stimulated corticosterone secretion in perfused rat adrenal gland. In the present study, SP-ir nerves were found in the cortex, mainly among the parenchymal cells. Like other authors [2], we too found SP-ir nerves in the medulla, but our observation of SP-ir nerves in the cortex suggests that SP may be involved in the regulation not only of catecholamines but also of corticosteroids. The cortices associated with Conn's adenomas contained a remarkable quantity of SP-ir nerve structures, but the significance of this finding is unclear. NPY is widely distributed in the body and is known to occur primarily in a certain population of sympathetic adrenergic neurons, but also in nonadrenergic neurons [28]. This peptide is a vasoregulator of the sympathetic nervous system and is thought to cooperate with noradrenaline [3]. In the present study, NPY was observed mostly in TH-ir nerves, thus confirming the adrenergic nature of NPY-containing nerves [12].

Our findings further corroborate the importance of VIP in the cortical function. Previous animal studies have shown that VIP stimulates corticosterone secretion [16]. The effect of VIP on steroidogenic secretion is ACTH dependent, but also directly affects – at least in the rat – the adrenal glands [1]. These results suggest that cortical parenchymal cells may be stimulated independently of the hypothalamus-pituitary-adrenal axis, or via a neuroadreno-cortical axis. In this regulatory pathway, VIP may act as a potent stimulatory factor, by innervating the adrenal cortex [4]. VIP acutely increases TH activity in cultures of dispersed normal adult rat chromaffin cells and of a PC 12 rat phaeochromocytoma cell line [29]. In our study it was also apparent that VIP and TH often occurred in the same nerve trunk and were also often co-localized in the same fibres.

CGRP-ir nerve fibres have been found in the adrenal glands of rat [12, 14], guinea pig [9], flat snake [24], frog [6] and human [10]; galanin-ir nerve fibres in rat, guinea pig, pigeon, chicken, phodopus [34] and frog [8], but not in duck [34]. Serotonin-ir nerve fibres have been observed in adrenal gland of human [10] and mouse [7]. In our study, however, no galanin-, CGRP-, enkephalin-, ACTH-, or serotonin-ir nerves were visualized in any of the cases. These results suggest that the human adrenal gland lacks these ir-nerve fibres, or that these neuropeptides occur in amounts too small to be detected with the methods used.

In our study, PGP 9.5 and NSE were common nerve markers in normal cortical tissues and hyperplasias, but were almost totally absent in adenomas and carcinomas. Neither SV2 nor TH was found in our neoplasms. The findings suggest that PGP 9.5, NSE and SV2 can be helpful in histopathological diagnosis to distinguish hyperplasia and hyperplastic nodules from true adrenal tumours, but further studies seem necessary to answer this question.

However, NPY- and/or VIP-containing nerve fibres were seen in most tumours; VIP-ir fibres were particularly numerous in some of the carcinomas. These VIP-ir nerve fibres, as well as the NPY-ir nerves, were found especially between or along blood vessels, but they were also located within the fibrous tissue of the tumours. Our results suggest that blood vessels within the tumour are at least partly innervated by NPY- and VIP-ir nerves, which may exert some influence on the blood flow within the tumour, concerning vasoconstriction and vasodilatation, respectively. One can also speculate whether these two neuropeptides may influence the angiogenesis and in this way affect the tumour growth. It has also been suggested that nerve structures use tumour blood vessels as a substrate for axonal guidance, maybe mimicking a normal event in development, but that tumour vessels do not respond to neurally derived signals [25]. The NPY- and VIP-ir nerve fibres seen between the parenchymal cells may also directly affect the tumour cell growth expressed by the action of various growth factors [31]. Our studies corroborate the importance of the nervous system in cortical functioning and the hypothesis that adrenal neuropeptides may be involved in regulating glucocorticoid and mineral-corticoid secretion in cortex [5, 11, 22], and perhaps also in hormonal regulation in carcinomas.

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