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Immunocytochemical distribution of serotonin and neuropeptide Y (NPY) in mouse adrenal gland

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Summary. By the use of immunocytochemical staining methods, we studied the morphology and distribution of 5HT and NPY immunoreactive cells and fibres in the mouse adrenal gland. The 5HT-immunoreactive cells were numerous and widely localized in the medullar tissue. These cells were arranged in three cellular types with regard to their morphological and immunocytochemical features. One of them showed cells with polygonal shape, being intensified like the typical medullary chromaffin cells. These immunoreactive cells were observed arranged in medullar islets. The second 5HT-immunoreactive celular type was constituted by cells with polygonal shape and strong immunoreactivity. The third one was formed by cells with immunoreactive prolongations. We found some islets of chromaffin non-immunoreactive cells surrounded by immunostained cells. We also observed some 5HT-immunoreactive nerve fibres in the medullar tissue. NPY-like immunoreactivity was detected in both chromaffin and ganglion cells in adrenal medulla. NPY-like immunoreactivity was also detected in nerve fibres at cortical level. In a few cases, we observed medullar 5HT- and NPY-immunoreactive tissue in the adrenal cortex (monotremas).

Key words: 5HT, NPY, Immunocytochemistry, Adrenal gland, Chromaffin cells

Introduction

Many studies indicate that adrenal cells have the capacity to produce, store and release biologically-active amines and peptides. However, the localization and concentrations of neuropeptides and amines exhibit marked interspecific heterogeneity. Not only does the quantity or quality of chromaffin cell immunoreactivity to different peptides and amines vary remarkably in different species, but also their presence in cells and nervous structures is often different (Pelto-Huikko, 1989). This suggests that the functions of these substances may be different in separate species. Serotonin (5HT) and neuropeptide Y (NPY) are two neuroendocrine mediators present in adrenal gland with wide distribution in mammals, fundamentally in central and peripheral nervous system.

5HT is a biogenic amine largely studied in mammals in differents organs and systems (Lauweryns et al., 1973; Fujita and Kobayashi, 1981; Steinbusch, 1981; Griffith and Burnstock, 1983; Grönblad et al., 1983; Koevary, 1983; Humphrey, 1984; Petrovic and Bell, 1984; Appel et al., 1986). Although its presence in the chromaffin cells of the adrenal gland has been described by many authors in different mammals (Csaba and Sudar, 1978; Sudar and Csaba, 1979; Berhofstad and Jonsson, 1983; Holzwarth et al., 1984; Holzwath and Brownfield, 1985; ...Holzwarth and Sawetawan, 1985; Brownfield et al., 1985; Bacon and Smith, 1988; Delarue et al., 1988a; Soinila et al., 1988, 1989; Kong et al., 1989), little is known about its existence and location in the mouse adrenal gland.

NPY is a 36-aminoacid peptide originally isolated from porcine brain by Tatemoto et al. (1982), but now know to be widely distributed throughout the mammalian central and peripheral nervous systems (Lundberg et al., 1982; Adrian et al., 1983b; Allen et al., 1983b; Morley, 1986). Furthermore, NPY coexists with norepinephrine in certain sympathetic nerves (Everit et al., 1984; Fried et al., 1985; Cadieux et al., 1989; Chalmers et al., 1989). In adrenal medulla of different animals the occurrence of NPY immunoreactivity in chromaffin cells and nerve fibres has been demonstrated (Varndell et al., 1984; Pelto-Huikko, 1989), while in human, NPY has been detected in phaeochromocytomas and ganglioneuroblastomas (Adrian et al., 1983a). However, from the discovery by RIA of NPY-like immunoreactivity in this gland (Allen et al., 1983a), the references about its distribution in mice show significative discrepancies. In view of this, our study was performed in order to establish the location,

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distribution and morphology of the 5HT-like and NPY-like immunoreactive structures in the mouse adrenal gland, providing an anatomical description of these neuroendocrine mediators at this level.

Materials and methods

50 adrenal glands obtained from adult male mice (Swiss OF-1) (b.w. = 25-35 gr.) were studied. Under anaesthesia (Ketamine 0.1 mg/gr b.w. and valium 0.05 mg/gr b.w.) the animals were perfused with heparinized (1 U.I./ml) physiological saline serum, followed by the perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2 (150 ml). Ketamine and valium were employed in order to reduce the stressful stimuli which might otherwise affect adrenal gland. The adrenal glands were rapidly removed and postfixed by immersion in the same fixative for four hours at 4 °C. The adrenal glands were also fixed by immersion for five hours in the same fixative at the same temperature. After fixation, they were rinsed in buffer (0.05 M TRIS-HCl pH 7.6 containing 0.1% Triton X-100). Triton X-100 at this concentration was used in order to facilitate the antibodies' access through membranes. After dehydration and embedding in paraffin, the sections were cut at 6-8 µm thickness and processed for immunocytochemistry by peroxidase-antiperoxidase (PAP) (Sternberger et al., 1970), indirect immunoperoxidase (IP) (Nakane, 1968) or streptavidin-biotin (Bonnard et al., 1984) methods. Prior to incubation with the primary antisera, the sections were exposed to 0.3% H_2O_2 in methanol for 30 min in order to eliminate the endogenous peroxidase activity (Streefkerk, 1972) and were rehydrated. The sections were treated with normal swine serum diluted 1:20 in 0.05 M TRIS-HCl buffer, pH 7.6 for 30 min. Then, sections were incubated in a moist chamber at 4 °C for 48 h with the primary antiserum against 5HT (Immunonuclear Corporation) or NPY (CRB), both raised in rabbit, diluted 1:1000 and 1:500 respectively in 0.05 M TRIS-HCl buffer, pH 7.6. After rinsing in the same buffer (2 x 5 min), sections were incubated sequentially with 1:50 diluted antiserum against rabbit IgG raised in swine (Dako) for 45 min at room temperature and, after rinsing in the same buffer (2 x 5 min), they were incubated with 1:50 diluted PAP complex raised in rabbit (Dako) for 30 min at room temperature. In the indirect immunoperoxidase method, the sections were incubated with normal goat serum diluted 1:20 for 30 min at room temperature. After incubation in the primary antiserum for 48 h under the same conditions and rinsing in buffer (2 x 5 min), the sections were incubated with 1:50 diluted peroxidase-labelled antiserum against rabbit IgG raised in goat (Dako). In the streptavidin-biotin method, after incubation in primary antiserum and rinsing in buffer (2 x 5 min.), the sections were incubated sequentially with biotinylated-IgG (Sigma) for 30 min and then with the streptavidin-biotin-peroxidase complex (Sigma) for 20 min at room temperature, previous to a rinse in buffer (2 x 5 min.). In the three methods the peroxidase activity was demonstrated by exposure of the sections to a fresh solution of 0.05% 3,3'-diamonobenzidine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide in 0.05 M TRIS-HCl buffer, pH 7.6, under microscopic control for five minutes. The sections were counterstained, dehydrated, cleared in xylene and mounted with DePeX (Serva). In order to establish the specificity of immunostaining, adjacent sections were incubated with control sera and processed in parallel with experimental sections. Furthermore, control was carried out with the incubation of the diluted 5HT antiserum overnight with an excess of 5-HT (10 µg/ml of diluted antiserum).

Results

The use of different immunocytochemical techniques revealed 5HT-like immunoreactivity (5HT-IR) and NPY-like immunoreactivity (NPY-IR) in a high percentage of chromaffin cells in mouse adrenal medulla (40-60%) (Figs. 1, 9). The three immunocytochemical methods gave similar results, although the immunostaining background was less and the immunoreactive cells were best visualized with Sternberger's unlabelled antibody enzyme technique (PAP method).

Results for 5HT

The 5HT-immunostained chromaffin cells, which showed polygonal morphology, were observed widely distributed in the medullar tissue and were located in immunoreactive medullar clusters (Fig. 1). However, we also found solitary 5HT-immunoreactive chromaffin cells in the non-immunostained medullar tissue. These cells showed a high nucleo-cytoplasmic ratio, with a large nuclei (Fig. 2). We also observed some islets of chromaffin non-immunoreactive cells surrounded by immunostained cells. The immunostaining showed a marked intensity in the peripheral medullar tissue, and this observation was less prevalent in the innermost zone of the adrenal medulla (Fig. 3). Furthermore, areas with low number of immunoreactive cells were intermingled with areas of densely-packed immunostained cells. In addition to these 5HT-immunoreactive chromaffin cells we observed a second serotoninergic subpopulation cellular type characterized by their strong immunoreactivity, the majority of them being close to blood vessels. The morphology of these cells was polygonal, similar to the chromaffin cells and they were scarce in number (Fig. 4). The third cellular type was formed by cells with immunoreactive prolongations, being sparsely distributed in the medullar tissue. These cells were also scarce in number (Fig. 5). Single serotonin immunoreactive fibres were also seen in the medullary adrenal parenchyma. Some of them ended in the proximity of chromaffin cells, suggesting a synaptic contact (Fig. 6). In a few cases, we observed immuno-reactive medullar

tissue in the adrenal cortex (monotremas) (Fig. 7).

Results for NPY

NPY-IR was identified in both adrenal medulla and adrenal cortex. In the medullar tissue the immunostaining was fundamentally observed in chromaffin cells. These cells were widely distributed in the medullar tissue and they appeared both as single immunoreactive cells and forming immunostained cellular clusters (Fig. 8). The NPY-immunoreactive cells were polygonal in shape and occasionally showed cytoplasmic processes (Fig. 9). The intensity of the immunoreaction varied from cell to cell, and the immunoreactive material within the cytoplasm appeared granular. We could also observe some immunoreactive cells with topographical relations to central veins. In a few cases, NPY-IR ganglion neurons showing characteristics prolongations were also

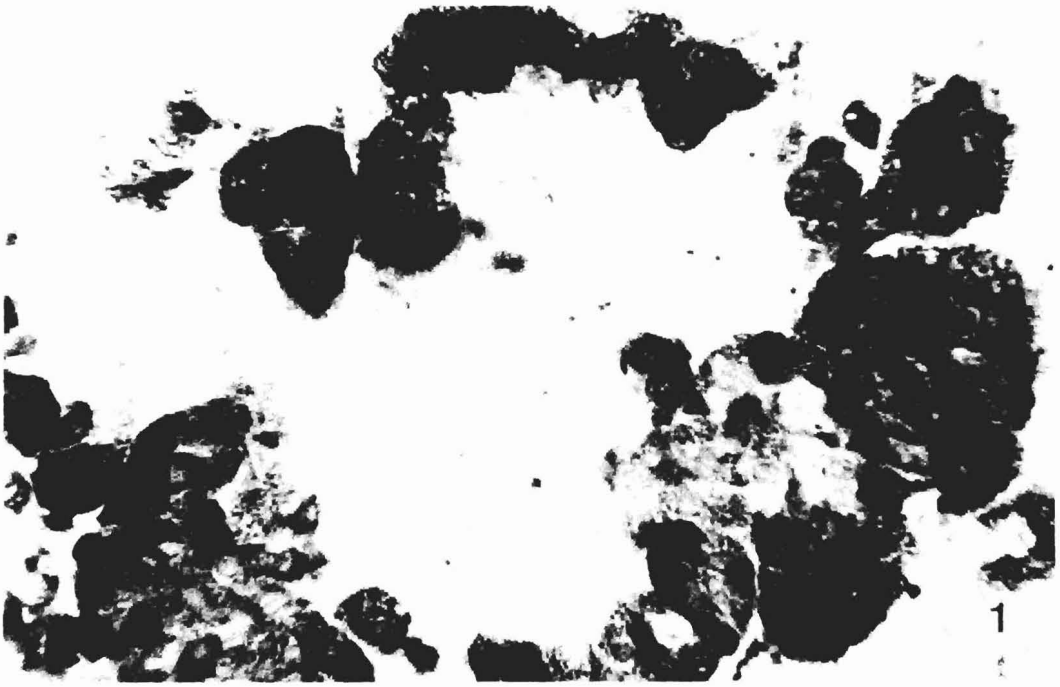

Fig. 1. 5HT-like immunoreactivity in chromaffin cell clusters. Perfusion. Streptavidin-biotin method. x 250

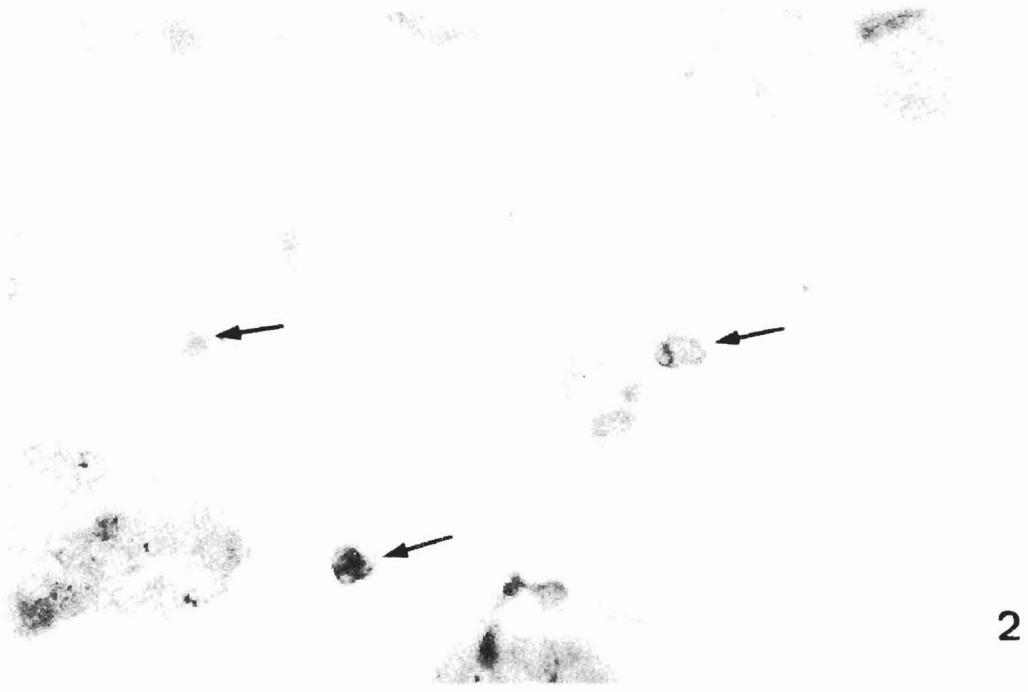

Fig. 2. Isolated serotoninergic chromaffin cells in adrenal medulla (arrows). Immersion. PAP method. x 250

observed (Fig. 10). The NPY-IR in the adrenal cortex was localized in nerve fibres. The plexus of NPY-IR nerve fibres with varicosities was observed around the small blood vessels, which penetrated the capsule and coursed in the subcapsular regions of the adrenal gland (Fig. 11). These nerve fibres extended into the cortical zones where they surrounded blood vessels and cortical cells. Single varicose nerve fibres were sparsely distributed in the fasiculata and reticularis zone of the cortex. In a few cases, we observed NPY-like immunoreactive medullar tissue in the adrenal cortex (monotremas). These structures could be observed as immunoreactive cell groups in the adrenal cortex (Fig. 12). Some of them were in relation to blood vessels and in the vecinity of the origin of the suprarrenal vein. These could be observed without any topographical

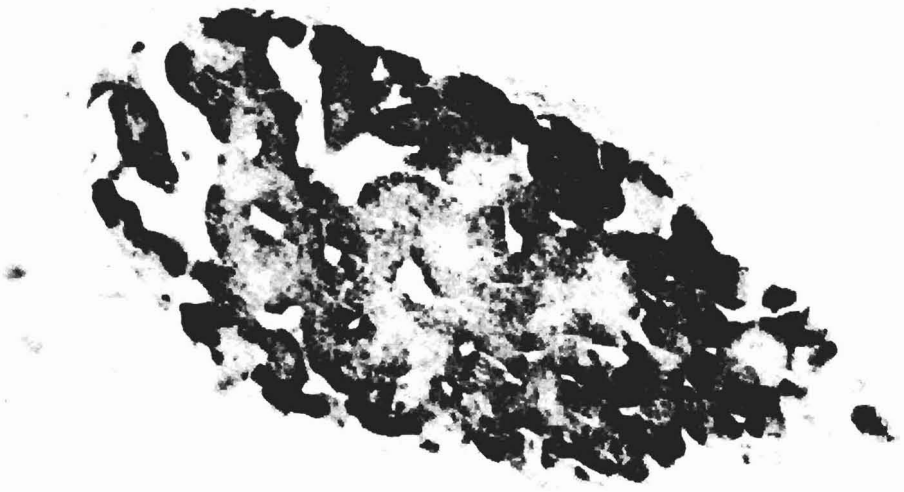

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Fig. 3. A more intensive 5HT-like immunostaining adrenal medulla periphery. Perfusion. I.P. method. x100

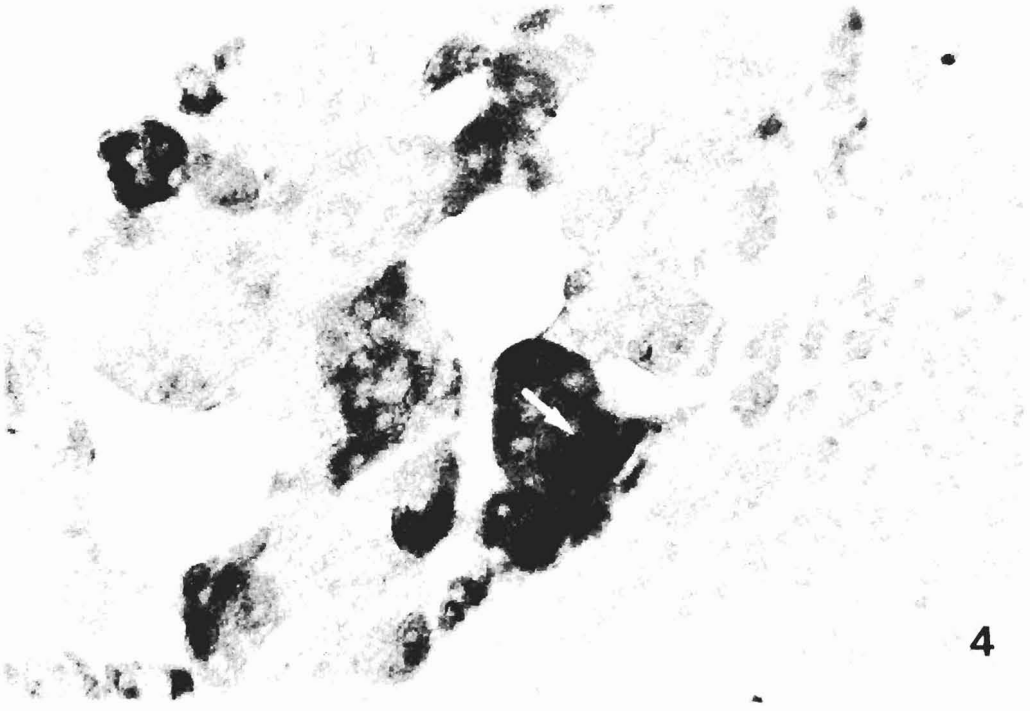

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Fig. 4. 5HT-like immunoreactive medullar cells intensely stained (arrow). Perfusion. PAP method. x 250

relation to the blood vessels. Within these immunoreactive monotremas, the immunostained cells were variable from cell to cell.

Discussion

In our study we distinguished three serotoninergic subpopulations in the mouse adrenal medulla. The first one was constituted by the typical 5HT-like immunoreactive chromaffin cells, which have been identified by many authors in other animals, such as rat (Marshall et al., 1975; Csaba and Sudar, 1978; Sudar and Csaba, 1979; Berhofstad and Jonsson, 1983; Brownfield et al., 1985; Holzwarth and Sawetawan, 1985; Holzwarth and

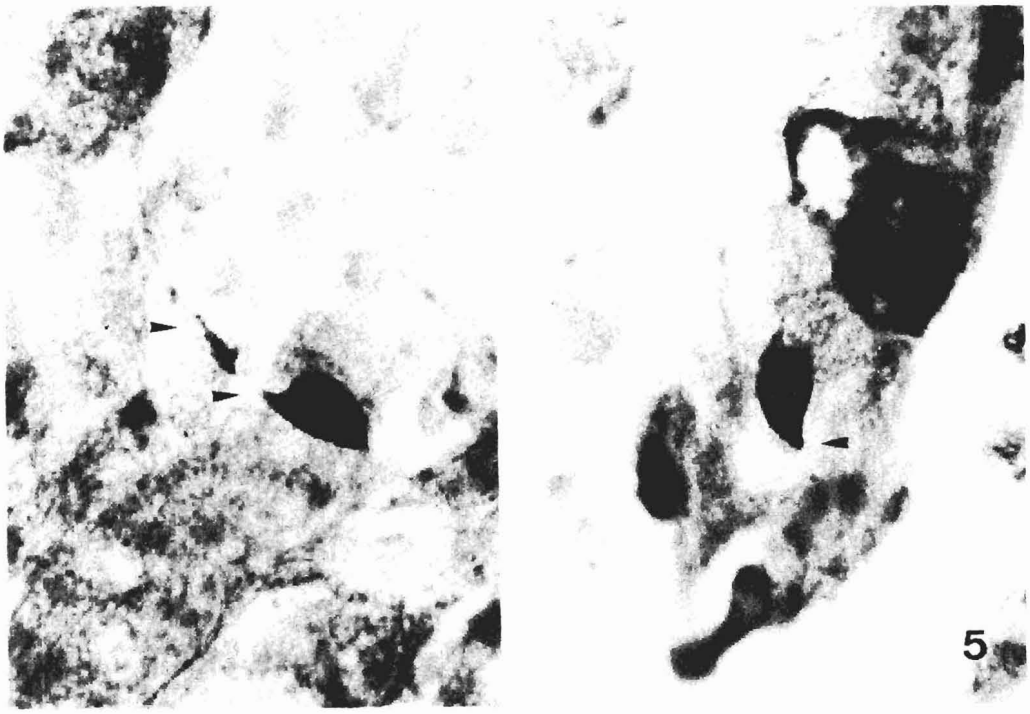

Fig. 5. 5HT-like immunoreactive medullar cells with immunostained prolongations (arrowheads). Immersion. I.P. method. x 1,000

Fig. 6. 5HT-like immunoreactive medullar tissue in the adrenal cortex (monotremas) (arrows). Perfusion. Streptavidin-biotin method. x 250

Brownfield, 1985), rabbit (Prada et al., 1976), pig (Kong et al., 1989), frog (Delarue et al., 1988a), and in human pheochromocytomas (Nilsson et al., 1986). However, we have not found references to serotonin existence and distribution in mouse adrenal gland. In rats, the best animal studied in this field, 5HT IR has been identified in adrenergic chromaffin cells (Holzwarth and Brownfield, 1985). Ultrastructurally, this amine was located in the core and next to the membrane chromaffin granules, when the fenil-atanolamin-N-methil-transferase (PNMT) was disposed in eccentric position (Brownfield et al., 1985). Equally, in the frog interrenal bodies, 5HT IR has been demonstrated in almost all epinephrine-producing cells, which represent about 90% of the total chromaffin cells. In this study, 5HT was visualized in secretory vesicles, essentially located in the

Fig. 7. 5HT-like immunoreactive nerve fibre towards serotoninergic chromaffin cell (arrow). Possible synapsis (arrowhead). Perfusion. Streptavidin-biotin method.
x 1,000

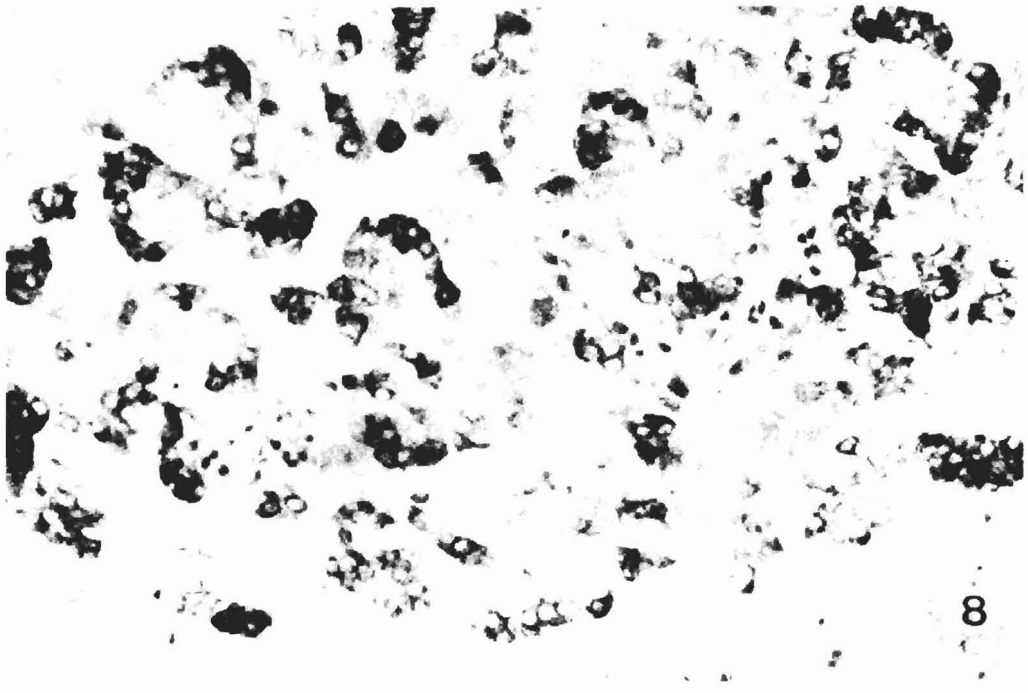

Fig. 8. NPY-like immunoreactive cells in mouse adrenal gland. Immersion. Streptavidin-biotin method. x 100

cellular periphery (Delarue et al., 1988a). On the contrary, in the pig (*Sus scrofa*) most 5HT IR has been identified coinciding with noradrenergic histofluorescence (Kong et al., 1989), which slow clear interspecific differences.

With regard to our second 5HT-like immunoreactive cellular type subpopulation, characterized by its strong immunoreactivity and polygonal morphology, we have not found references in the studies carried out by other authors in other animal species. These cells would be a specific cellular type in this animal, which, because of its strong immunoreactivity, would have a larger degree of amine synthesis or a lesser secretion capacity.

Our third 5HT-like immunoreactive cellular subpopulation type was formed by cells with immunoreactive prolongations. The occurrence in several mammals of an adrenal chromaffin cell population differentiated to ganglion cells and characterized by

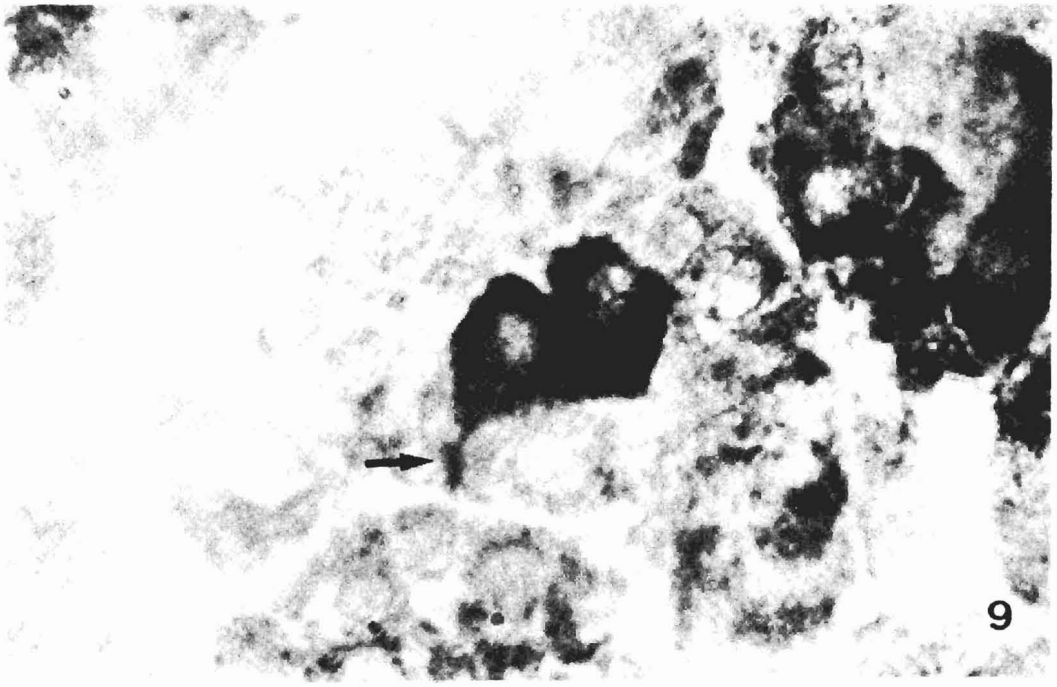

Fig. 9. Polygonal NPY-like immunoreactive cells. One of them lacks cytoplasmic process to vessel. Perfusion. PAP method. x 1,000

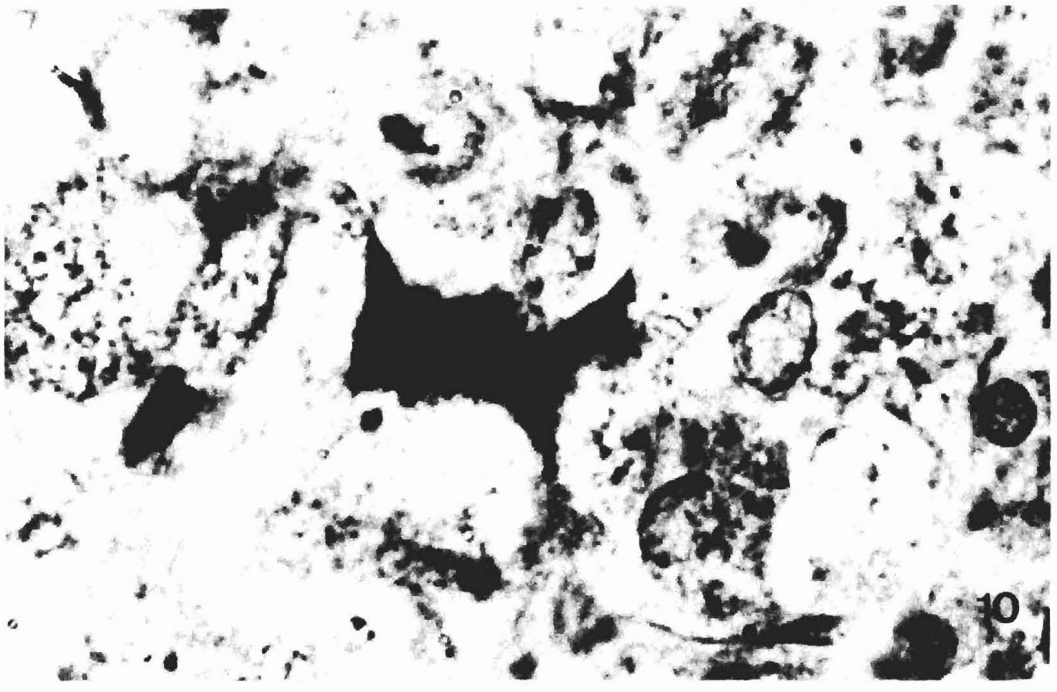

Fig. 10. NPY-like immunoreactive ganglion cells in adrenal medulla. Immersion. PAP method. x 1,000

cellular prolongations has been signalled by different authors (Kobayashi and Coupland, 1977; Kajihara et al., 1978; Marinkovic et al., 1978; Unsicker et al., 1978). These cells have been identified as a transient form between neurons and paraneurons (Serizawa and Kobayashi, 1980) and it has been suggested that they may be a special morphological representation at this level of SIF (small intensely fluorescent) cells. SIF cells have been identified as the storage site for serotonin in the mouse sympathetic ganglia (Eränkö and Härkönen, 1965; Soinila et al., 1989). With regard to this last affirmation, and based on the analogy between sympathetic ganglia and adrenal medulla, we suggest that our 5HT-like immunoreactive cells with prolongations would represent serotoninergic SIF medullary cells.

In the mouse adrenal medulla, we found single serotonin-immunoreactive fibres, in some cases,

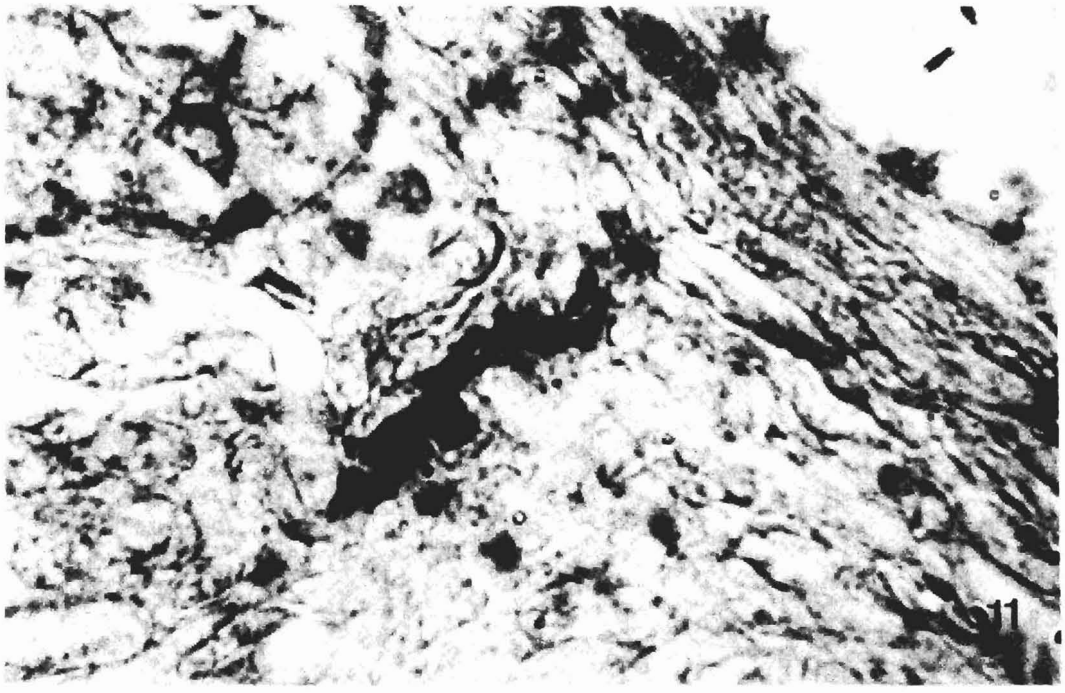

Fig. 11. NPY-like fibre nerve immunoreactivity in glomerular zone of adrenal cortex. Perfusion. Streptavidin-biotin method. x 1,000

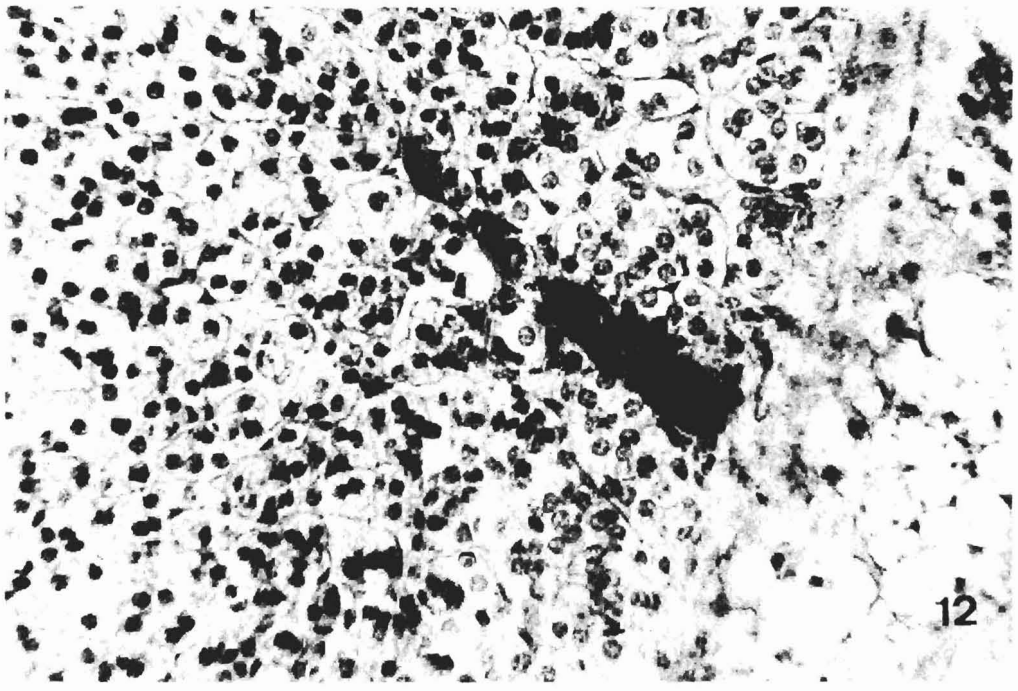

Fig. 12. NPY-like medullar tissue immunoreactivity in glomerular and reticular zones of adrenal cortex (monotrema). Immersion. PAP method. x 250

adjacent to serotoninergic cells (Fig. 6). Serotoninergic fibres have not been seen in adrenal medulla to date. In this animal, serotonin-containing nerve fibres may enter the gland via splanchnic nerves, which represent the main neuronal input to the adrenal gland, or through other routes known to contain fibres innervating the adrenals, such as fibres entering via the sympathetic chain. However, these serotoninergic fibres may also possess an intrinsic origin. Further studies are necessary to elucidate these questions. They would be implicated in a serotoninergic self-modulation mechanism. Whether the amine localized in nerve terminals acts directly on chromaffin cells as modulator/transmitter, or modulates the receptors of other transmitters is not known to date, and a presynaptic function must be kept in mind. The origin of these immunoreactive fibres remains to be established.

With regard to functional aspects, Holzwarth et al. (1984) studied the distribution and response of serotonin in the rat adrenal medulla to pharmacological manipulations, suggesting the existence of a regulatory mechanism in the release of serotonin at this level. Moreover, serotonin has been recently reported as a potent stimulator of corticosterone and aldosterone secretion and this action has been demonstrated to be mediated through a new non-classical 5HT receptor (termed 5HT-4) (Delarue et al., 1988b; Leboulenger et al., 1988; Idres et al., 1989, 1991). It would be convenient to study a possible «feed-back» mechanism by which a decrease in cortical hormones would stimulate the 5HT synthesis and release in medullar cell chromaffin. This would be possible thanks to adrenal microcirculatory characteristics, in the same way that the glucocorticoids stimulate the medullar adrenalin synthesis (Poorecky and Wurtman, 1971). The elevated plasmatic 5HT levels would close this feed-back mechanism by the enhancement of corticosterone and aldosterone release.

From the discovery of NPY by Tatemoto et al. (1982) many authors have related this neuropeptide with the noradrenergic system. Furthermore, NPY has been considered as a cotransmitter with this amine, and the coexistence has been revealed in the same sympathetic postganglionic neurons (Everit et al., 1984; Fried et al., 1985; Cadieux et al., 1989; Chalmers et al., 1989). The removal of the cervical superior ganglion leads to the disappearance of NPY-immunoreactivity in the tissue innervated by this ganglion. On the other hand, the parallel distribution of this peptide with the amine-synthesizing enzymes like cathecolaminase has also been demonstrated (Cadieux et al., 1989; Dagerlind et al., 1990). Furthermore, its corelease with norepinephrine during sympathetic activation and stress has been observed by some authors (Allen et al., 1984; Lundberg et al., 1986a; Zukowska-Grojec et al., 1988; Chalmers et al., 1989; Russell et al., 1989; Briand et al., 1990; Mormede et al., 1990; Stoddard et al., 1992). Recently the localization and regulation of the messenger molecules adrenaline, noradrenaline and neuropeptide tyrosine (NPY) within the cells of the sympathetic nervous system and the adrenal medulla of rat by *in situ* hybridization and Northern blot analysis has been studied (Schalling et al., 1991). In the adrenal gland, NPY was described for the first time by HPLC techniques in different mammalian species (Allen et al., 1983a) and at the same time in human ganglioneuroblastomas and phaeochromo-cytomas (Adrian et al., 1983a). However, Lundberg et al. (1986b) adduce the first demonstration of the peptide in this organ. These authors demonstrated the presence of Pancreatic Polypeptide (PP) in the adrenal medulla previous to the discovery of NPY by Tatemoto (Lundberg et al., 1980). At this time, we know that the PP immunoreactivity described by Lundberg et al. (1980) corresponds at least in a large measure, to NPY. The first immunocytochemical description of NPY in the adrenal gland was made by Varndell et al. (1984) showing immunoreactive structures in horse, cat, rat, hamster, dog and mouse. The authors identified NPY-like immunoreactivity in medullar norepinephrine cells and in a varicose nervous fibre populations, more prominent in dogs and rats. Similar nerve fibres in the cortical and reticular zone have also been described. The distribution of these fibres was very similar to norepinephrine fibres demon-strated by tyrosine-hydroxylase-like immunoreactivity and DBH-immuno-reactivity. The results obtained for these authors in this animal are similar to ours. However, they did not observe any cells with prolongations to medullar level or NPY-like immunoreactive monotremas. Furthermore, they identified medullar NPY-like immunoreactive fibres, although with regard to this, our results were negative in mice. Ultrastructurally, Kuramoto et al. (1986) localized these NPY-like immunoreactive fibres in the glomerular zone of the rat adrenal gland, surrounding blood vessel and cortical cells, and also in the fascicular, reticular and medullar zones. These findings are similar to our results in the mouse adrenal cortex, but in our work, the fibres were predominant at the glomerular level. These authors also described a population of numerous clear vesicles (45-50 nm diametre) next to two NPY-like immunoreactive granular populations. One of them showed a similar size to the clear vesicles; the other one was composed of large granular vesicles of 90-100 nm diameter. NPY-like immunoreactivity was also detected in the axoplasm surrounding the described vesicles and mitochondria. These NPY-like immunoreactive fibres in the rat adrenal gland, which are localized around vascular component, correspond to noradrenergic postganglionic axons whose neuronal bodies are localized in the celiac ganglion. This relation suggests a motor role of the neuropeptide Y, acting as a cotransmitter with norepinephrine. On the other hand, the fibres associated to the cortical cells could have an endocrine function. However, the role of the immuno-reactive fibres related to the chromaffin cells is unknown to date (Kuramoto et al., 1986). With

regard to these findings an inhibitory effect of NPY findings on the function of rat zona glomerulosa and perhaps of the zona fasciculata has been recently suggested (Lesniewska et al., 1990). It is obvious, in spite of the interespecific differences, that the authors are in agreement about the existence and distribution of NPY immunoreactive fibres in the adrenal gland, and NPY immunoreactivity in chromaffin cells, in function to the analyzed animals (Pelto-Huikko, 1989). However, this does not occur with regard to the chromaffin cellular type producing and storing the neuropeptide Y in rat adrenal medulla (Varndell et al., 1984; Kuramoto et al., 1986) and in the presence of NPY-like immunoreactive ganglion neurons. In mouse, our experimental animal, the NPY-like immunoreactive material has been described in norepinephrine chromaffin cells by concomitant demonstration of PNMT (Lundberg et al., 1986b; Pelto-Huikko, 1989). However, in apposition to our results, in these works immunoreactive ganglionar cells in the adrenal medulla or monotremas in the cortical parenchyma showing NPY-like immunoreactivity were not observed in mouse adrenal gland. The ganglionar NPY-immunoreactive cells have been described in rat and hamster adrenal medulla (Pelto-Huikko, 1989). Most of these ganglion cells in the rat adrenal medulla are noradrenergic (Dagerlind et al., 1990). Up to date, we have not found any studies referring to the morphology of different cellular populations with NPY-immuno-reactivity in the adrenal gland. For this reason, we can conclude that the polygonal chromaffin cells showing short prolongations towards vascular structures, could constitute a special type of chromaffin cell in this animal. The presence of islets or prolongations of medullar tissue in the adrenal cortex (monotremas) suggests that in the organogenesis of this gland a kidnapping of medullar adrenal outline included in the cortical parenchyma could occur.

In relation to the functional aspects, the NPY is released in the blood vessels after medullar sympathetic stimulation of the splacnic nerve (Lundberg et al., 1986a; Gaumann et al., 1989) and in response to acute stress stimuli (Castagne et al., 1987; Zukowska-Grojec et al., 1988; Mormede et al., 1990) or during physical exercise (Pernow et al., 1988). On the other hand, this neuropeptide can act in the cathecolaminergic medulladrenal release producing a dependent-concentration inhibition of the release of epinephrine and norepinephrine mediated by nicotinic receptor, suggesting that the modulatory effect could be produced throughout the activation of the specific receptors (Y_1 or Y_2) (Higuchi et al., 1987; Sheikh et al., 1989). The investigations of Kuramoto et al. (1986), Lundberg et al. (1986b) and Hexum et al. (1987) can lead to various possible mechanisms of action of this neuroendocrine mediator in the adrenal gland:

1.- A paracrine or endocrine release of NPY from medulloadrenal chromaffin cells after acetylcholinergic preganglionic stimulation.

2.- An NPY release from postganglionic nerve fibres, performing its action on the medullar or cortical glandular cells.

3.- An NPY postganglionic release performance together with the norepinephrine on the vascular smooth muscle, controlling the vasomotor tone of the zone and consequently the intradrenal microcirculation.

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