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ORIGINAL RESEARCH
ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ

Rat adrenal medulla modular organization

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Abstract. *Relevance*. The concept of the tissue morpho-functional units (modules) of the adrenal medulla is currently not fully developed for adrenaline-storing (A-) cells and completely undeveloped for noradrenaline-storing (NA-) cells. *Aim.* Separately for A- and NA-cells, establish modules in adrenal medulla based on criteria developed by fundamental histology. *Materials and Methods.* The study used serial, semithin, and ultrathin sections of the adrenal glands, 7—9 μm thick, from 6 adult male Wistar rats (weight 335 ± 25 g). The sections were stained according to the Honoré method with additional staining with toluidine blue, which allows one to reliably distinguish between A and HA cells in the medulla. A cells are stained blue and HA cells are stained green. Light and electron microscopy was used to visualize serial, semithin, and ultrathin sections of the adrenal glands of adult male rats with A- and HA-cell differentiation. *Results and Discussion.* A-cells formed round clusters, in which they were located in one layer on the basement membrane. Their lateral sides closely adjoined each other, while the inner sides (the central part of the complexes) formed intercellular expansions, microprotrusions, and primary cilia. Less firmly pressed NA-cells formed polyhedral beams. Both types of cell complexes were associated with auxiliary components (stromal, nervous, circulatory, etc.). The central expansions of A-cell round clusters apparently to serve to retain some of the already produced adrenaline, which increases the readiness of the medulla to rapidly release large amounts of adrenaline in case of hyperacute stress. Accordingly, the adherence of A-cell complexes to a rounded shape is determined by the need to create such central isolated storage expansions. NA-cells are located more freely and do not form isolated intercellular expansions. This allows NA-cells to wedge between stably round A-cell complexes and form polyhedral beams as a result. *Conclusion.* It was found that the rat adrenal medulla contains two logically and morpho-functionally distinct types of specific modules. A-module are A-cells rounded cluster and NA-module is polyhedral NA-cells beam, both associated with auxiliary components.

Keywords: adrenal medulla, chromaffin cells, adrenalocyte, noradrenalocyte, modules, morpho-functional units

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Introduction

From the point of view of fundamental histology, specific tissue modules of an organ (hereinafter simply modules) are its morpho-functional units, which are the smallest (elementary) repetitive tissue systems capable of performing a function specific to this organ. Such modules consist of a main component that ensures the performance of a function specific to an organ, and a complex of auxiliary (supporting, trophic, regulatory, and immune) structures that create the necessary conditions for its functioning [1—3]. In turn, auxiliary components can be mandatory and optional. Mandatory auxiliary components are an integral part of the module both morphologically and functionally. The presence of optional components is of an irregular nature and is not of fundamental importance for the organization of the module.

Concept of modules implies the structure and function of individual cells strongly depend on their role and place in the module. The importance of the modular concept is confirmed by the fact that the study of organs, the idea of modules in which has long been established (kidney nephrons, lung acini, liver lobules, etc.), invariably implies an appeal to the state of these important structural complexes, both in the norm and in pathology. At the same time, the idea of the modules of the adrenal medulla has developed only partially. Usually, when describing its structure in the scientific and educational literature, modules are either not mentioned at all or vague and highly varying formulations are used about the existence of certain cellular complexes in it. Such a simplifying approach impoverishes the understanding of both the structure and function of the adrenal medulla, which is an important part of the sympathoadrenal system and plays a key role in the body's adaptation to stress. Accordingly, the question of the modules of the adrenal medulla needs significant improvement. A convenient object in this respect is the medulla of adult laboratory rats, since their medulla has been studied in the most detailed and versatile way.

Main component of the adrenal medulla is chromaffin tissue. This tissue makes up about 60 % of the adult rat medulla. Blood vessels occupy about 25 % (mainly due to extensive thin-walled venous sinuses) in the medulla, and connective tissue, nerve and other components divide the remaining volume [4, 5]. Two main subpopulations of chromaffinocytes form rat chromaffin tissue: adrenaline-storing and noradrenaline-storing cells (A-cells and NA-cells). A third type of chromaffin cells, small granule containing (SGC) cells, has also been described in the rat adrenal medulla. Perhaps these cells are an intermediate form between neurons and chromaffinocytes or a variant of small intensely fluorescent (SIF) ganglion cells [6—10]. One way or another, these cells in the adrenal medulla of the rat are very few in number and they cannot serve as the structural basis for the required modules.

Hillarp [11] is believed to have first raised the question of the functional units of the adrenal medulla.

He drew attention to the fact that the chromaffinocytes of the rat adrenal medulla reacted to the introduction of insulin by degranulation not singly, but in complexes. Some complexes remained non-degranulated even under very strong exposure. With partial transection of the celiac nerve, some cell complexes degranulated only partially. Hillarp suggested each neuron innervates a limited number of cells, which combine into one or possibly several complexes and thus can respond as functional units. Later, the reaction according to the type of functional units was confirmed by electrophysiological methods by Iijima and coauthors [12], and Kajiwara awith coauthors [13]. The authors determined the morphological parameters of these units. Their diameter is about 80 μm. Each contains approximately 100 cells and 4 nerve fibers. Each cell has 1 to 4 synapses. Martin, showed that chromaffinocytes are united into functional units not only by common innervation, but also by gap junctions that transmit impulses within it [14]. Unfortunately, in these very interesting works, complexes of A- and NA-cells, which differ very significantly both functionally and morphologically, were not differentiated. Also, the ultrastructural principles of the organization of chromaffinocytes into modules are poorly covered in the literature and there are no explanations for the reasons for the modular organization of the adrenal medulla.

The present work is devoted to the establishment of modules in the adrenal medulla of rats on the basis of clear criteria developed by fundamental histology. We will also pay more attention to the differences between the complexes of A- and NA-cells.

Materials and methods

In the spirit of modern trends in the protection of animals and the environment, collection preparations were used for histological examination: serial sections of 7—9 μm thick adrenal glands of 6 adult male Wistar rats (8 months old, 335 ± 25 g weight). These sections were stained according to the method of Honoré [15] with additional staining with toluidine blue, which makes it possible to reliably distinguish between A- and NA-cells in the medulla. A-cells are stained blue, and NA-cells are green. The preparation procedure for preparations of this collection is described in detail in our recent publication [16].

For transmission electron microscopy (TEM), we used 6 adult male Wistar rats with similar parameters (8 months old, 343 ± 18 g weight). The handling of animals was in accordance with national and international legal standards: Order of the Ministry of Health of the Russian Federation No. 199n dated 01.04.2016 and Directive 2010/63/EU of the European Parliament and Council dated 22.09.2010. After euthanasia of the animals with the anesthetic zoletil, we removed the adrenal glands, fixed with 2.5 % glutaraldehyde and 1 % osmium tetroxide, embedded in epon, and prepared semi-thin and ultra-thin sections.

We stained the semithin (1 μm) sections for light microscopy with 1 % methylene blue and contrasted the ultrathin sections for TEM with uranyl acetate. Classical for TEM fixation of samples in glutaraldehyde and post-fixation in osmium tetroxide allows reliable differentiation of different types of chromaffin cells. A-cells contain homogeneous granules of moderate optical and electron density, while NA-cells contain granules with an asymmetrically located core of high optical and electron density [17, 18]. We used a transmission electron microscope JEM-1011, JEOL Ltd.

We loaded photographs of randomly selected sections into ImageJ (freeware), detected complexes of A- and NA-cells and calculated their sizes: linear dimensions and cross-sectional area (40 measurements for each of 6 animals).

Results and Discussion

The adrenal medulla contained complexes of chromaffin cells and auxiliary components. The structure of these complexes for A- and NA-cells differed significantly both at the light-optical and ultrastructural levels.

A-cells formed round clusters (RCs) separated by thin layers of connective tissue (Figs. 1, 2, 3). The area of such RCs was 2316.9 ± 356.6 μm^2.

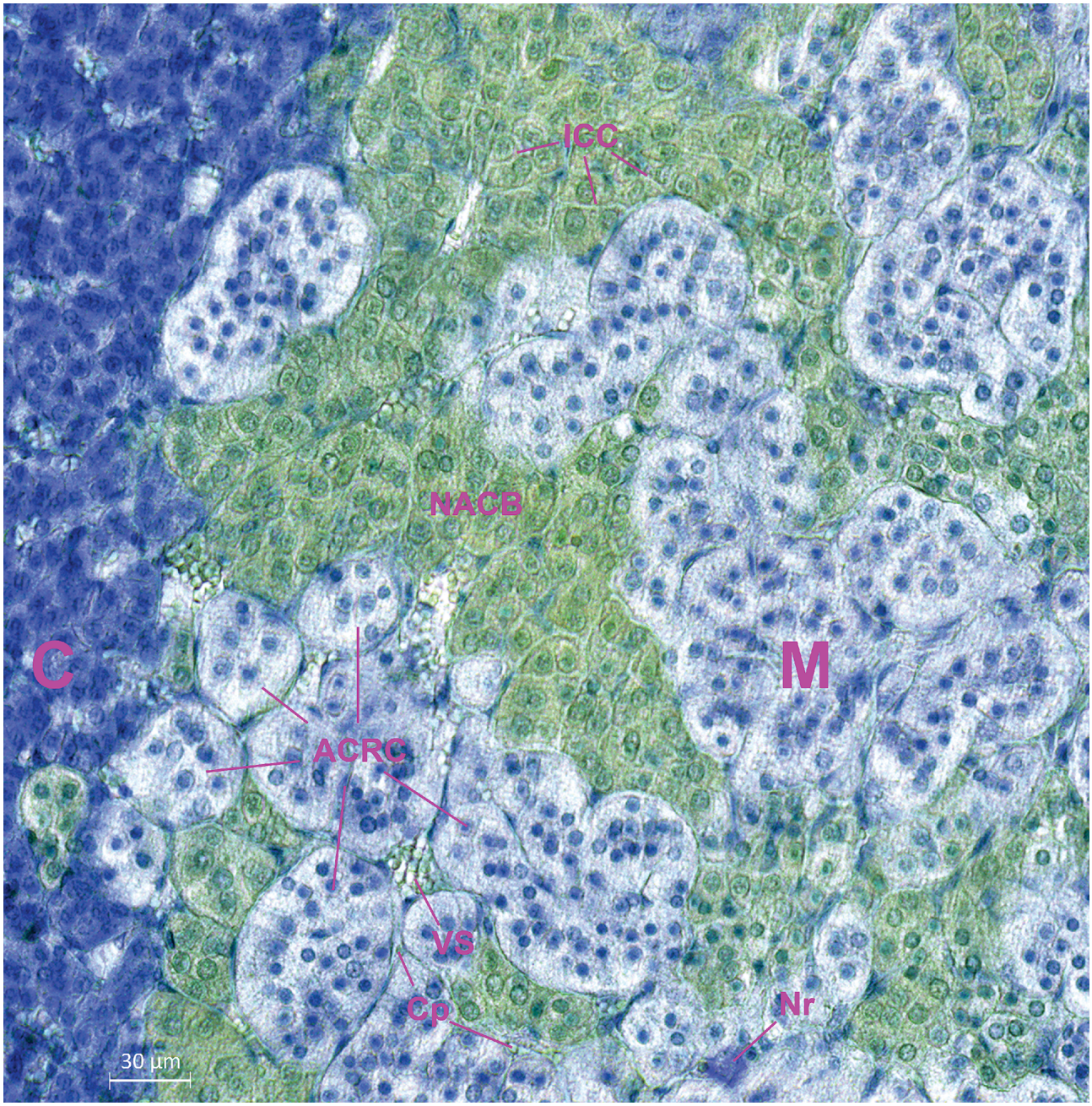

Fig. 1. Fragment of a full-size photograph (scan) of one of the serial sections of an adult rat adrenal gland. C — cortex, M — medulla, ACRC — adrenalocyte rounded clusters, NACB — noradrenalocyte beams, VS — venous sinus, Cp — capillaries, Nr — neuron, ICC — intercellular contacts. Honoré staining, 20× objective High-resolution scan shown in Figure 1: https://drive.google.com/file/d/10DKasT976MJiQKUvqj0juaQLLWUI6fBb/view?usp=sharing

Tracing the contours of individual RCs on serial sections and looks of RCs at different angles showed that their shape in volume tended to be ellipsoidal. Many of them formed anastomoses with each other. In any form of RC, the distance from its periphery to the center is equal to the size of one A-cell. The shape of A-cells is predominantly pyramidal or columnar. Their outer side was oriented to the periphery of the RC and was in contact with the basement membrane (Figs. 4a, 7a), and their inner side was oriented to the

center of the RC and was in contact with the inner sides of other A-cells. The lateral sides of A-cells were connected by interdigitations and desmosomes (Fig. 4b, c). In the central part of the RC, slit-like spaces and expansions with a diameter of 0.5 to 2 μm formed between neighboring A-cells. The transition between such expansions and simple intercellular junctions was quite sharp, so the expansions on sections had angular outlines (Figs. 3, 4c, 7b). Cellular protrusions, nerve fibers, and synaptic endings could be found in the expansions (Fig. 4c, 7b). Some cells were provided with cilia. The cilia had a basal body with many laterally oriented basal peduncles. The rods of the cilia were oriented to the intercellular spaces into which their terminal parts protruded. The bases of the cilia shafts are located in membrane pockets (Fig. 4c). By structure, these were primary cilia. Thus, A-cells had a polar structure.

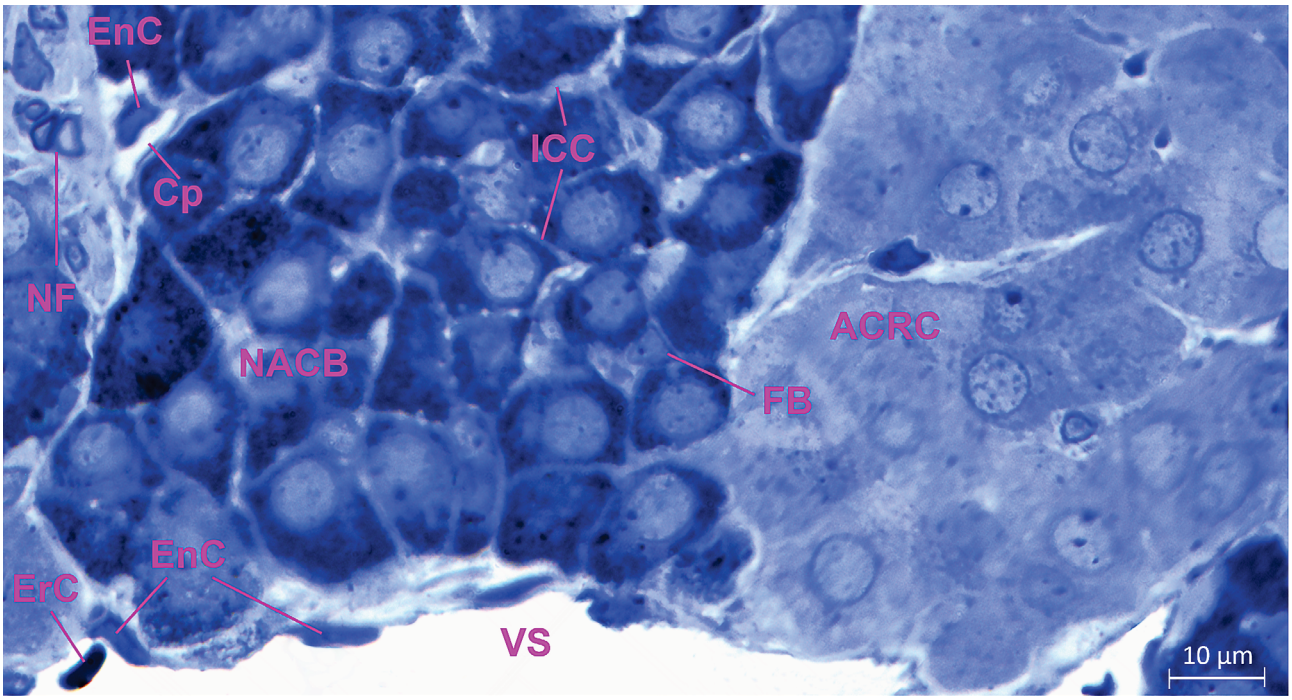

Fig. 2. Semi-thin (1 μm) section of the adrenal medulla of an adult rat. ACRC — adrenalocyte rounded clusters, NACB — noradrenalocyte beams, VS — venous sinus, Cp — capillaries, FB — fibroblasts, NF — nerve fibers, EnC — endotheliocyte, ErC — erythrocyte, ICC — intercellular contacts. Methylene blue staining, 100× oil immersion objective

Complexes of NA-cells on sections looked like angular islands (Fig. 1, 2).

Even light microscopy, especially on semithin sections, showed that NA-cells differed from A-cells in a lower density of bonds, and also that the shape of NA-cells was multifaceted, without polarity in structure (Fig. 1, 2). TEM shows this even more clearly (Fig. 5). The sites of contact between NA-cells were an alternation of simple junctions, desmosomes, slit-like spaces, and characteristic ampulla-shaped extensions 0.5—0.8 μm in diameter. In intercellular spaces, often very wide, one could find elongated cellular protrusions, nerve fibers and synaptic endings. Some groups of NA-cells are separated from others by thin layers of loose connective tissue, which are associated with fibroblasts that lie in the thickness of the islets.

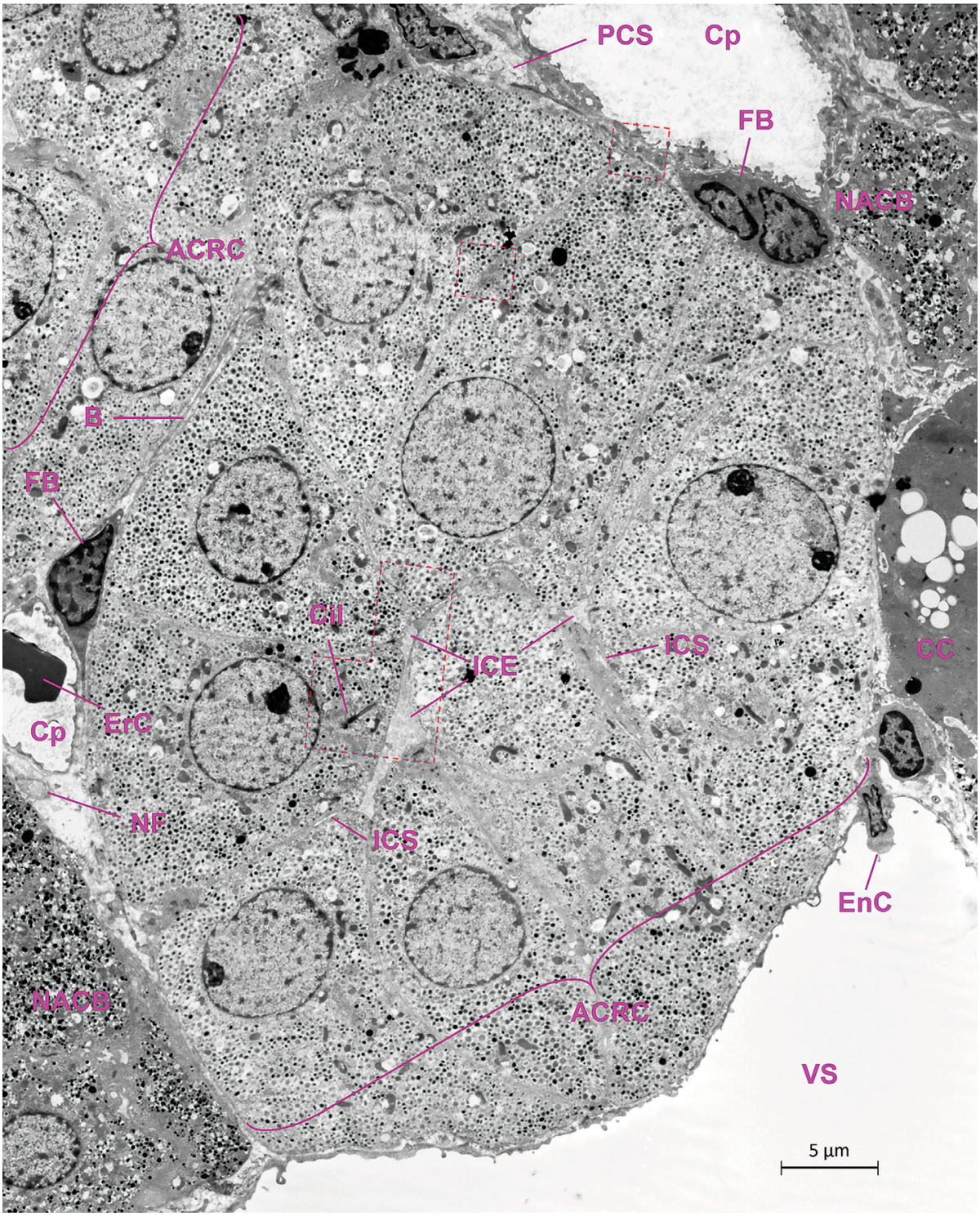

Fig. 3. Transmission electron microscopy of an adrenaline module of the rat adrenal medulla. ACRC — adrenalocyte rounded clusters, NACB — noradrenalocyte beams, VS — venous sinus, Cp — capillaries, FB — fibroblasts, NF — nerve fibers, EnC — endotheliocyte, ErC — erythrocyte, ICE — intercellular expansions, ICS — intercellular slits, Cil — cilium, B — boundary between neighboring ACRCs, CC — cortical cell

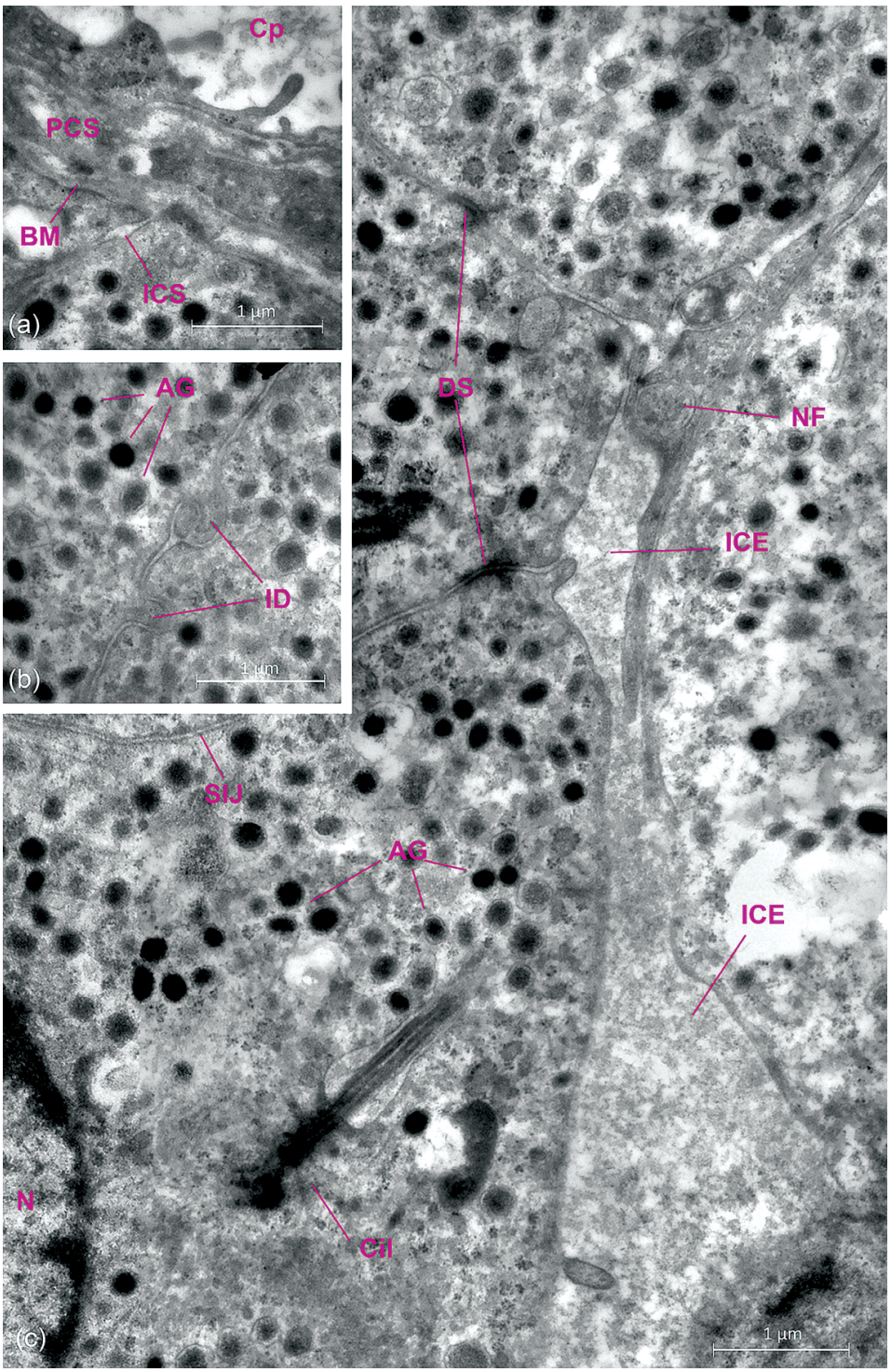

Fig. 4. Transmission electron microscopy of the adrenalocyte rounded cluster in the area indicated with red dotted lines on the Figure 3. Cp — capillary, NF — nerve fibers, N — nucleus, ICE — intercellular expansions, ICS — intercellular slits, Cil — cilium, BM —basement membrane, PCS — pericapillary space, AG — adrenaline granules, ID — interdigitations, DS — desmosomes, SIJ — simple intercellular junctions The area of such islands was 2707.7 ± 402.9 µm^2. Tracing the islets on serial sections showed that they looked like irregularly shaped polyhedrons and formed anastomoses with each other.

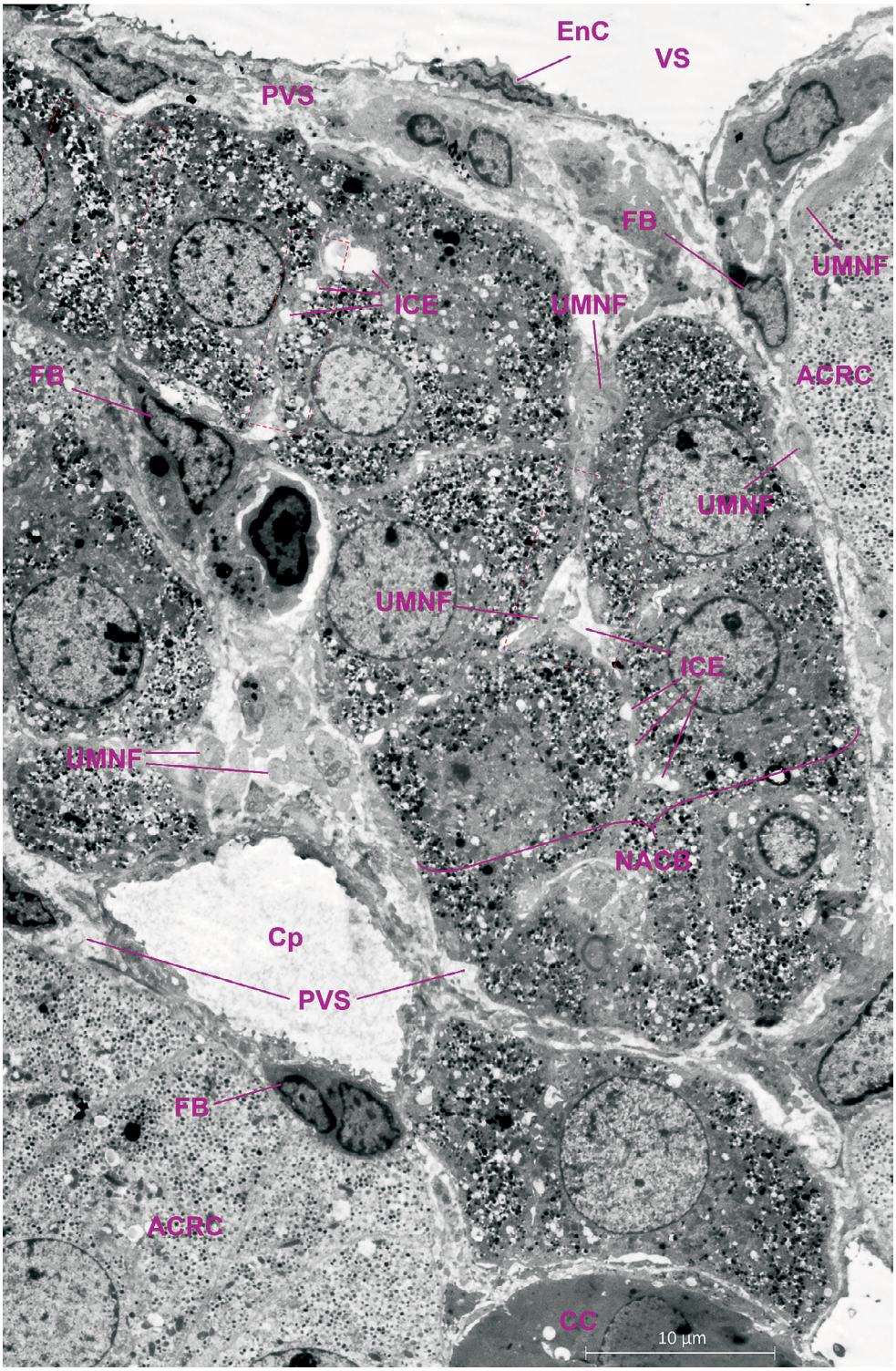

Fig. 5. Transmission electron microscopy of a noradrenaline module of the rat adrenal medulla

NACB — noradrenalocyte beam, ACRC — adrenalocyte rounded clusters, Cp —capillaries, VS — venous sinus, FB — fibroblasts, UMNF — unmyelinated nerve fibers, EnC —endotheliocyte, ICE — intercellular expansions, CC — cortical cell, PVS —perivascular spaces.

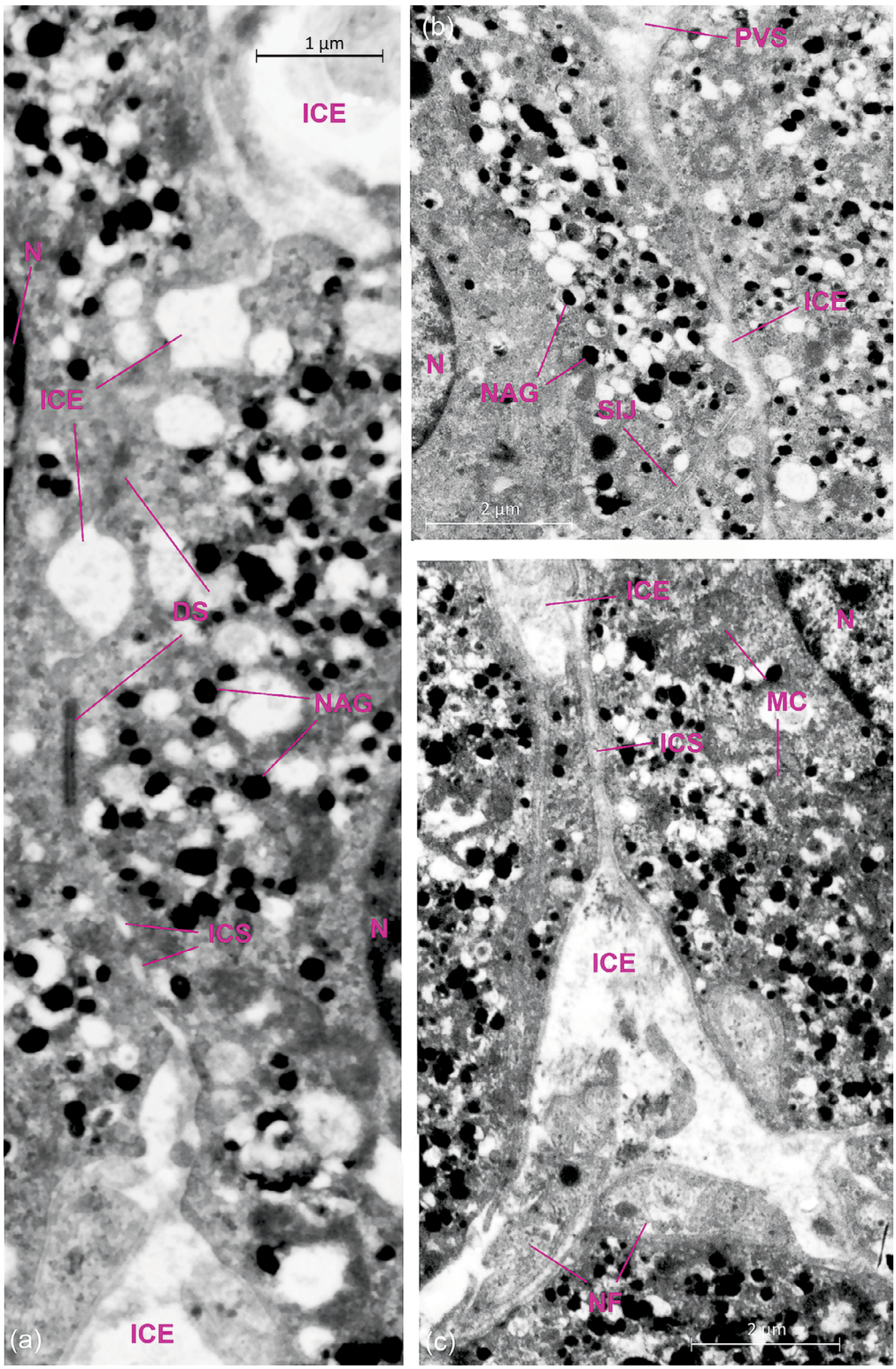

Fig. 6. Transmission electron microscopy of the noradrenalocyte beam in the area indicated with red dotted lines on the Figure 5. NF — nerve fibers, N — nuclei, ICE — intercellular expansions, ICS — intercellular slits, PVS — perivascular space, NAG — noradrenaline granules, DS — desmosomes, SIJ — simple intercellular junctions, MC — mitochondria

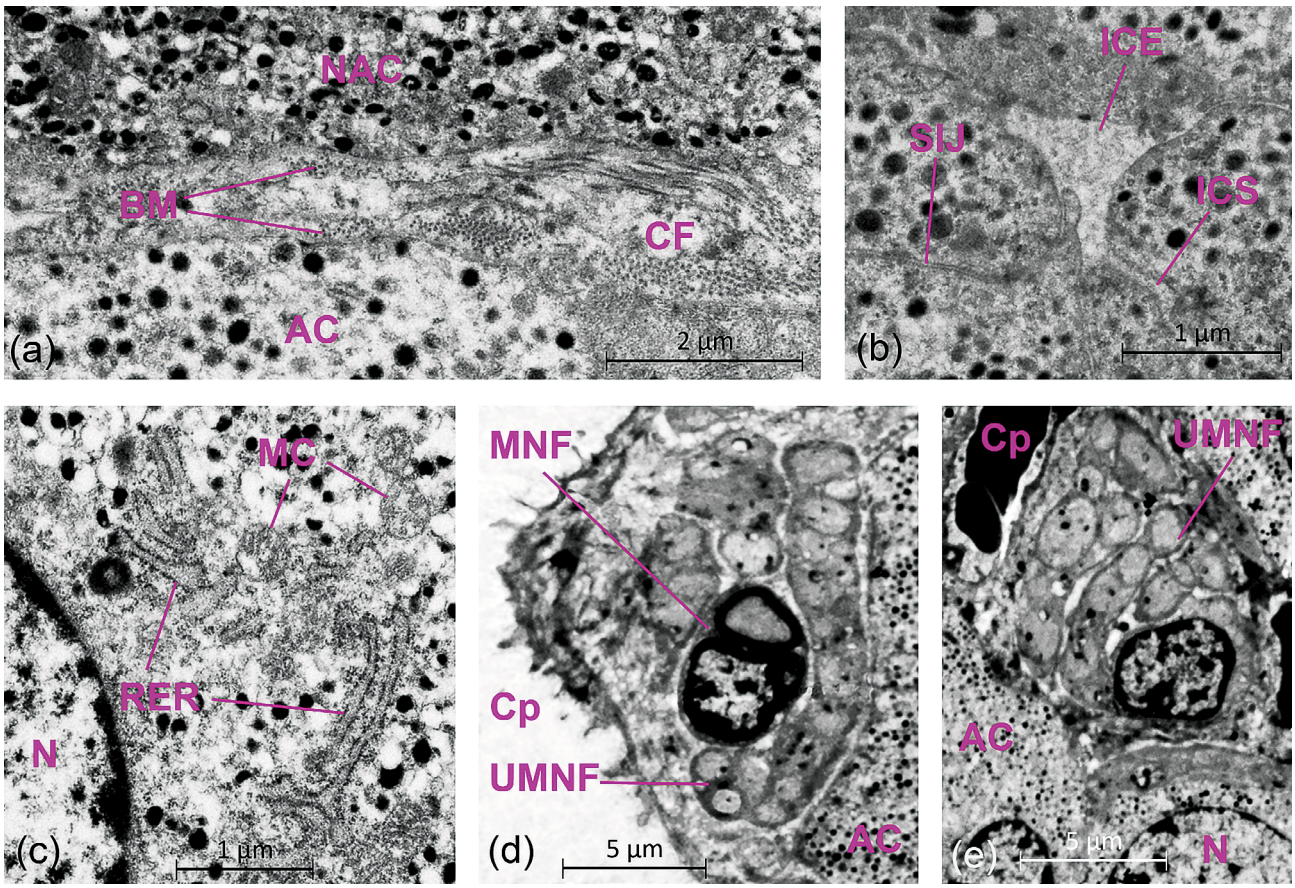

Fig. 6. Transmission electron microscopy of some additional structural details of adrenaline and noradrenaline modules of the rat adrenal medulla. AC — adrenalocytes, NAC — noradrenalocytes, Cp — capillaries, FB — fibroblasts, MNF — myelinated nerve fibers, UMNF — unmyelinated nerve fibers, ICE — intercellular expansions, ICS — intercellular slits, N — nuclei, ICS — intercellular slits, BM — basement membrane, CF — collagen fibers, SIJ — simple intercellular junctions, MC — mitochondria, RER — rough endoplasmic reticulum

Consideration of the tissue components at the angle of their role and place in the module makes it possible to form a more complete picture of the modular organization of the adrenal medulla.

Capillaries and venous sinuses were located on the periphery of the complexes of chromaffin cells. The perivascular spaces were well developed. Microvessels braid the modules from the outside, but do not penetrate inside. Numerous myelinated and non-myelinated nerve fibers also lay between the complexes of chromaffin cells (Fig. 2, 3, 5, 7d, e).

The cytoplasm of A- and NA-cells, in addition to the usual cellular organelles (rough and smooth endoplasmic reticulum, mitochondria, Golgi apparatus, vesicles, vacuoles, etc.), contained numerous specific chromaffin granules. Adrenaline granules had a regular symmetrical shape and medium or high electron density, while noadrenaline granules had the form of bubbles with eccentrically located clots of high electron density. The endoplasmic reticulum was located near the nuclei, the rest of the organelles and secretory granules were evenly distributed throughout the cytoplasm (Fig. 4, 6, 7a).

Based on the above definition of the module, in order to establish the fact that a particular tissue complex is a module of a given organ, it is necessary to check it for compliance with three basic criteria: specificity, repeatability and elementarity. Specificity of module also determines what is the main and what is the auxiliary.

Checking for compliance with the criteria of specificity and repeatability are formal: specific

repetitive structures in the medulla are individual chromaffin cells or their complexes (RCs of A-cells and beams of NA-cells). On the contrary, compliance with the elementarity criterion requires justification. This criterion implies the desired module should consist of the minimum functionally complete specific cells complex. Thus, it is necessary to find out whether the RCs of A-cells and beams of NA-cells are elementary complexes or their parts are such complexes.

It is easier to reveal the elementarity for RC of A-cells. A-cells within their RC have common structures that either are formed by cells together (intercellular expanded spaces) or are present only in some cells (cilia). Accordingly, a group of A-cells smaller than the RC cannot be a self-sufficient full-functional basis of the adrenaline module (A-module).

It is somewhat more difficult to substantiate the elementarity of bundles of NA-cells. However, the very fact that single NA-cells are not actually found in 3D reconstruction of the medulla from serial sections [16] unequivocally indicates that only the beams are self-sufficient full-functional basis of the noradrenaline module (NA-module). The internal boundaries of the NA-modules, apparently, be considered the places where between the NA-cells there are layers of connective tissue and fibroblasts associated with them (Fig. 5).

Mandatory auxiliary components of the module of the medulla are the connective tissue frame, microcirculatory blood vessels and nerve fibers with endings.

Kikuta et al. clearly showed that the stroma around the chromaffinocyte complexes in the rat medulla has the form of rounded «baskets», most of which communicate with each other by «windows» [19]. This fully corresponds to the outline RCs of A-cells: they themselves are located in the baskets, and the anastomoses between them are located in the windows. For the NA-module, its own stromal scaffold is not clearly defined. Obviously, it repeats the outlines of the beams, i. e. has the appearance of angular chambers.

Small branches of venous sinuses and capillaries are most closely in contact with modules. The capillaries of the medulla are a continuation of the arterial vessels passing through the cortex. The venous sinuses collect blood from the capillaries of the medulla and from the capillaries of the cortex at its border with the medulla [20—22].

A simple solution is to assume that the capillaries feed the modules and take in metabolites and catecholamines, while the venous sinuses bring corticosteroids important for the synthesis of adrenaline. However, no differences were found between complexes of A- and NA-cells in interaction with different types of vessels and in localization relative to them [21—24]. Therefore, the way corticosteroids enter the A-module is obviously somewhat more complicated. It can be assumed that well-developed perivascular spaces are of great importance. The importance of the vascular component of the modules of the medulla increased even more after the discovery of the ability of chromaffinocytes to their own (not from nerves) excitability in response to humoral stimuli [25].

The modules of the adrenal medulla are richly innervated. Both myelinated and unmyelinated nerve fibers are involved in this. [13, 26, 27]. We observed the same with our preparations. The fact that the boundaries of the functional areas obtained by direct or indirect nerve stimulation clearly coincide with the morphological boundaries of the modules clearly shows the important organizing role of the nerve component in organization of these morpho-functional units [11—13].

Optional auxiliary components of the adrenal medulla module in intact adult rat are, for example, macrophages (found often) and mast cells (found very rarely). There is no information about any of their roles in the organization of the module.

With all of the above in mind, we present a drawing of typical A- and NA-modules of the rat adrenal medulla (Fig. 8). The figure combines all the main structural features of the modules based on our data and those of other authors. Since the asymmetry of noradrenaline granules is a consequence of the specific interaction of noradrenaline with glutaraldehyde and is not detected in other fixation variants (Coupland, 1965a), we depicted them without this artifact.

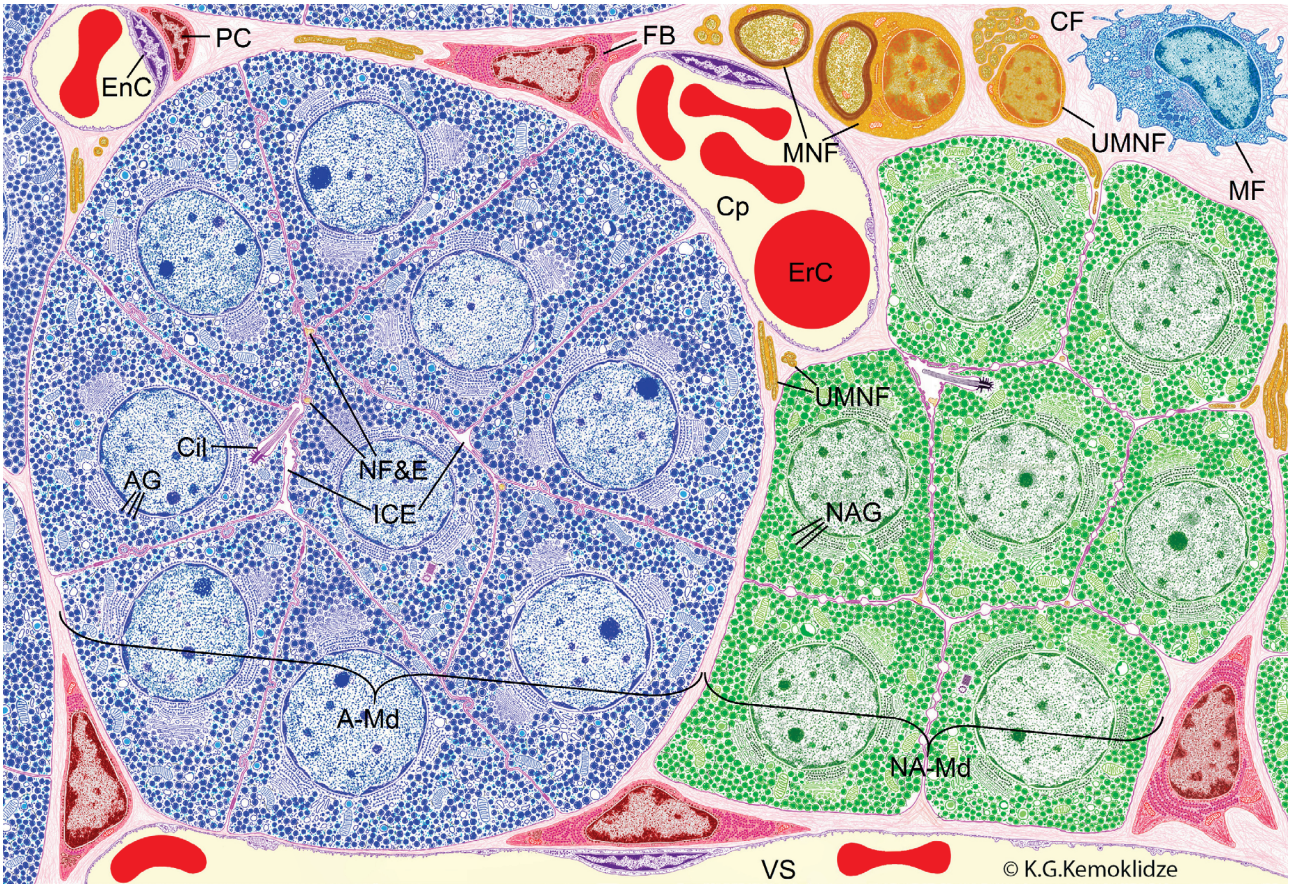

Fig. 8. Schematic drawing of the adrenaline and noradrenaline modules of the rat adrenal medulla.

A-Md — adrenaline module, NA-Md — noradrenaline module, AG — adrenaline granules, NAG — noradrenaline granules, ICE — intercellular expansions, Cil — cilia, Cp — capillaries, VS — venous sinus, FB — fibroblasts, MNF — myelinated nerve fibers, UMNF — unmyelinated nerve fibers, NF&E — nerve fibers and synaptic endings, EnC — endotheliocytes, ErC — erythrocytes, PC — pericyte, MF — macrophages, CF — collagen fibers. High-resolution drawing shown in Figure 8 https://drive.google.com/file/d/1M6ZNOjavTdryBI6Rs4DhuEZyflxLEbFv/view?usp=sharing

Differences in the structure of A- and NA-modules stimulate the search for their causes and connection with the function.

An acute selective effect on the A-cells of the rat medulla leads to an increase in the amount of adrenaline in the blood plasma against the background of a slow depletion of these hormones in the medulla. While a similar hyperacute effect leads to a rapid and many times more powerful increase in adrenaline in the blood plasma against the background of an equally rapid decrease in its amount in the medulla. NA-cells do not show the same super-powerful release of noradrenaline during extremal selective exposure [28—30]. This clearly indicates that during hyperacute exposure adrenaline is released into the blood according to the principle of rapid release of the finished product from extracellular depots. The place of reservation of ready-made adrenaline, obviously, is the characteristic isolated expansions of the intercellular spaces in the central part of the A-modules. The expansions are quite voluminous and there are several of them in the module (Fig. 3). Accordingly, the adherence of A-modules to a rounded shape is determined by the need to create isolated storage expansions in their central part. NA-cells that do not form RCs with central expansions and are less densely packed do not show the same

ability. Accordingly, the effect of the wedging of NA-module beams between A-modules is the result of a freer arrangement of NA-cells: in the growing medulla, NA-cells occupy the free spaces between the stable rounded A-modules.

The foregoing also applies to the explanation of the reasons for the modular organization of the adrenal medulla. In addition to common reasons for all organs [3], we clearly see the specific one for the appearance of special A-modules. This is the need to store ready adrenaline in case of a sudden life-threatening and requiring an immediate and strong (fight or flight) reaction.

Conclusion

It was found that the rat adrenal medulla contains two logically and morpho-functionally distinct types of specific modules. A-module are A-cells rounded cluster and NA-module is polyhedral NA-cells beam, both associated with auxiliary components.

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Модульная организация мозгового вещества надпочечника крысы

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Аннотация. *Актуальность.* В настоящее время представление о тканевых морфофункциональных единицах (модулях) мозгового вещества надпочечников не полностью сформировано для клеток, запасающих адреналин (А-), и совершенно не разработано для клеток, запасающих норадреналин (НА-). *Цель.* Отдельно для А- и НА-клеток установить модули в мозговом веществе надпочечников на основе критериев, разработанных фундаментальной гистологией. *Материалы и методы*. В исследовании использовали серийные, полутонкие и ультратонкие срезы надпочечников толщиной 7—9 мкм 6 взрослых крыс-самцов линии Вистар (масса 335 ± 25 г). Срезы окрашивали по методу Оноре с дополнительным окрашиванием толуидиновым синим, позволяющим достоверно различать А- и НА-клетки в мозговом веществе. А-клетки окрашены в синий цвет, а НА-клетки — в зеленый. Использовали световую и электронную микроскопию для визуализации серийных, полутонких и ультратонких срезов надпочечников взрослых самцов крыс с дифференцировкой А- и НА-клеток. *Результаты обсуждение.* А-клетки образовывали округлые скопления, в которых располагались в один слой на базальной мембране. Их боковые стороны плотно прилегали друг к другу, а внутренние стороны (центральная часть комплексов) образовывали межклеточные расширения, микровыпячивания и первичные реснички. Менее плотно расположенные НА-клетки образовывали многогранные балки. Оба типа клеточных комплексов были связаны с дополнительными компонентами (стромальными, нервными, сосудистым и др.). Центральные расширения округлых скоплений А-клеток, по-видимому, служат для удержания части уже образованного адреналина, что повышает готовность мозгового вещества к быстрому высвобождению больших количеств адреналина в случае сверхострого стресса. Соответственно, приверженность скоплений А-клеток к округлой форме определяется необходимостью создания таких центральных изолированных накопительных расширений. НА-клетки располагаются более свободно и не образуют изолированных межклеточных расширений, что позволяет НА-клеткам вклиниваться между стабильно круглыми скоплениями А-клеток,

в результате чего формируются их многогранные балки. *Выводы.* Установлено, что мозговое вещество надпочечников крыс содержит два логически и морфофункционально различных типа специфических модулей. А-модуль представляет собой округлое скопление А-клеток, а НА-модуль — многогранная балка из НА-клеток, оба связаны со вспомогательными компонентами.

Ключевые слова: мозговое вещество надпочечников, хромаффинные клетки, адреналоциты, норадреналоциты, модули, морфофункциональные единицы

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