CHARACTERIZATION OF AN UNUSUAL CATECHOLAMINE-CONTAINING CELL TYPE IN THE TOAD HYPOTHALAMUS

A Correlated Ultrastructural and Fluorescence Histochemical Study

OLIVIA C. McKENNA and JACK ROSENBLUTH

From the Departments of Physiology and Rehabilitation Medicine, New York University Medical Center, New York 10016

ABSTRACT

A nucleus of catecholamine-containing cells bordering the preoptic recess of the toad hypothalamus has been studied by both fluorescence histochemical and electron microscopic methods. The perikarya of these cells form one to three rows immediately subjacent to the ependyma. They send brightly fluorescent apical processes between the ependymal cells to the ventricular surface, and also give rise to long basal processes, the proximal portions of which are also fluorescent. These cells contain two distinctive constitutents: juxtanuclear bundles of tightly packed filaments, the members of which are separated from one another by only ~100 A, and large numbers of dense-cored vesicles (400–2200 A in diameter), which appear to arise from an agranular tubular reticulum distinct from the Golgi apparatus. Axons containing either clear vesicles alone or clear and dense-cored vesicles form synapses on the subependymal cells, but no evidence has been found that the subependymal cells themselves form presynaptic contacts, or that axons originate from them. The cytological characteristics of these catecholamine-containing cells, plus the fact that they border directly on the cerebrospinal fluid, suggest that they may be more closely related to peripheral chromaffin cells than to the other cell types intrinsic to the central nervous system, and the name "encephalo-chromaffin cells" is therefore proposed for them. The possible functions of such cells in the central nervous system are discussed.

INTRODUCTION

The presence of catecholamine-containing cells has been demonstrated in the central nervous systems of many species by means of the fluorescence histochemical method of Falck and Hillarp (11, 12). However, studies designed to characterize these cells ultrastructually have been limited, since the cells tend to be interspersed among nonfluorescent cells and therefore cannot be identified with assurance in specimens prepared for electron microscopy.

In contrast, the preoptic recess of the amphibian hypothalamus is surrounded by a well-defined layer of subependymal cells which have been shown by fluorescence microscopic studies to contain catecholamines (44), and which can be identified reliably on the basis of their location. The packing and distribution of fluorescent cells in this nucleus varies somewhat among different amphibians, but in the toad *Bufo marinus* we have found virtually pure populations of them in

some regions. The nucleus in this species, therefore, lends itself particularly well to correlative cytological studies employing both fluorescence histochemistry and electron microscopy, the results of which are reported here.

On the basis of this investigation the cells of this nucleus are characterized and compared with other cells intrinsic to the central nervous system. We find that although these subependymal cells contain catecholamines, their configuration and their complement of organelles are such that they cannot readily be classified as nerve cells, ependymal cells, or glial cells, but seem instead to be more closely related to peripheral chromaffin cells. This paper thus represents a description of an unusual type of cell in the central nervous system and includes a discussion of its possible functions and of its relationship to various cells both outside the brain and within other "circumventricular organs" (40, 43, 44) inside the brain.

MATERIALS AND METHODS

Fluorescence Histochemistry

Adult toads (*Bufo marinus*), of both sexes, weighing 150–300 g each were pithed or anesthetized with ethyl carbamate (2–3 mg/g), and their brains were removed. To obtain specimens which included the preoptic area, transverse cuts were made posterior to the optic chiasm and through the cerebral hemispheres rostral to the anterior commissure. The dorsal portion of this piece was usually removed to facilitate freezing and drying. Subsequently, the tissue was frozen in isopentane cooled by liquid nitrogen, and then dried in a tissue drier (Edwards High Vacuum, Inc., Grand Island, N. Y.) at −40°C in a vacuum of 10^{-2}–10^{-3} mm Hg for 8–10 hr. The tissue blocks were then exposed to paraformaldehyde at 80°C for 1–4 hr (13). The paraformaldehyde had been stored for at least 7 days over varying concentrations of sulfuric acid, producing paraformaldehyde of 20, 50, or 70% relative humidity (19). The tissues were embedded *in vacuo* in Araldite (Ciba Products Co., Summit, N. J.) or paraffin. Blocks embedded in paraffin were cut into sections 8–10 μ thick, and those in Araldite were cut into sections 1–4 μ thick. For control studies freeze-dried blocks of tissues were heated to 80°C without formaldehyde. Sections were mounted in paraffin or immersion oil. Consecutive sections of the plastic-embedded tissue were stained with 0.5% toluidine blue in 1% sodium borate.

The response of the formaldehyde-induced fluorescence to conditions known to reduce the fluorescence in other tissues was studied (29). In some instances tissue sections were exposed to water or to ultraviolet irradiation; in others they were treated with 0.5% sodium borohydride in 80% isopropanol for 2 min. Subsequently, the sections were again exposed to paraformaldehyde for several hours.

The effect of several drugs on the fluorescence of the subependymal cells was studied by pretreating animals with the following agents: (*a*) reserpine (25–50 mg/kg) injected intraperitoneally, intramuscularly, or subcutaneously 48 hr before sacrifice, (*b*) α-methyl-meta-tyrosine (500 mg/kg) injected intraperitoneally 24 hr before sacrifice, (*c*) nialamide (Chas. Pfizer & Co., Inc., N. Y.) (500 mg/kg) injected intraperitoneally 6 hr before sacrifice, (*d*) L-dopa (30–100 mg/kg) injected into the dorsal lymph sac 1 hr before sacrifice, or (*e*) nialamide (500 mg/kg) injected intraperitoneally, followed 6 hr later by L-dopa (100 mg/kg) injected into the dorsal lymph sac. The animal was sacrificed 45 min after administration of the second drug. Tissues were then freeze-dried and processed in the manner described above.

In an effort to trace possible processes of the subependymal cells into the adjacent tissue (9), surgical incisions were made in the preoptic area 5–7 days before sacrifice. In anesthetized animals a midline incision was made in the dorsal mucosa of the mouth, and the portion of the parasphenoid bone which covers the preoptic area was reflected. The preoptic recess was easily visualized through its thin floor. One of three types of lesions was made from the ventral surface of the brain: (*a*) midsagittal lesions through the floor of the preoptic recess, extending from the rostral end of the recess to the optic chiasm, (*b*) two parasagittal incisions approximately 0.25 mm long on either side of the recess, each approximately 1 mm deep, and (*c*) two parallel transverse incisions across the entire preoptic area, each approximately 1 mm deep, one at the rostral end of the recess and the other immediately rostral to the optic chiasm. After the operations the parasphenoid bone was replaced over the preoptic area, and the dorsal mucosa of the mouth was sutured. Three animals were subjected to each of the three procedures, so that a complete series of transverse, horizontal, and sagittal sections could be cut from the operated preoptic area.

Sections were cut with a paraffin microtome or, in the case of the plastic-embedded tissue, with glass knives on a Porter-Blum ultramicrotome. A Zeiss fluorescence microscope equipped with an Osram mercury lamp and a BG 12 exciter filter in combination with Zeiss 50 and 53 barrier filters was used for examination of the specimens.

Histological Procedures

Brains were fixed by perfusion through the heart with Zenker-formol fixative, and the hypothalamus,

including the preoptic area, was removed, dehydrated, and embedded in paraffin. Sections (8–10 μ thick) were stained by either chrome hematoxylin-phloxine or paraldehyde-fuchsin for the demonstration of neurosecretory material as described by Gabe (16).

For a study of the chromaffin reaction, brains were either perfused through the heart or pieces were immersed in buffered formol-dichromate according to Coupland (8). Tissues were washed, dehydrated, and sectioned in the usual manner. Sections were stained with hematoxylin alone.

Transverse, sagittal, and horizontal sections through the preoptic area of three fixed brains were stained by Bodian's copper protargol method for the demonstration of nerve fibers (14).

Electron Microscopy

Animals used in this part of the study were anesthetized with ethyl carbamate (2–3 mg/g) and the tissue was prepared by perfusing fixative through the heart (35), or by perfusing the fixative directly into the preoptic recess. In the latter method, a needle attached to a syringe containing the fixative was positioned in the preoptic recess by a micromanipulator. The fixative, which was perfused with a Sage pump directly into the preoptic recess at 0.1–0.2 ml/min for 30 min, flowed out through a large transverse cut in the floor of the infundibulum caudal to the optic chiasm. The opening in the infundibulum was sufficiently large so that a pressure increase would not be expected in the preoptic recess.

In some cases, primary fixation was carried out with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2–5 hr. Blocks containing the preoptic area were then dissected free from the remander of the brain and postfixed in 2% osmium tetroxide in the same buffer. In other animals, this solution of osmium tetroxide was used for primary fixation. Dalton's fixative (1% potassium dichromate buffer, 1% osmium tetroxide at pH 7.2 in balanced saline [36]) and 1–2% osmium tetroxide in acetate-Veronal buffer, pH 7.0, 7.2, or 7.4 in balanced saline, were also used as primary fixatives. After fixation with osmium tetroxide in acetate-Veronal buffer, some blocks were immersed in 0.5% uranyl acetate in acetate-Veronal buffer, pH 5.0–5.5, for 2–5 hr, and then rinsed in saline before dehydration. After perfusion of 2% glutaraldehyde in 0.1 M phosphate buffer through the preoptic recess, blocks of tissue from this area were studied by the histochemical method of Wood and Barrnett for the demonstration of catecholamine-containing granules (46). Potassium permanganate, 3%, at pH 7.0 or 7.4 in 0.1 M phosphate buffer, was perfused through the heart in some animals, or the block of tissue which included the preoptic area was immersed directly. The urinary bladder was also immersed in this fixative.

After fixation, tissues were dehydrated in increasing concentrations of methanol and embedded in Araldite or Epon. Sections 1–2 μ thick, used for orientation, were cut with glass knives and stained with 0.5% toluidine blue in 1% sodium borate. Thin sections were cut with glass or diamond knives on a Porter-Blum microtome, stained with uranyl acetate and lead hydroxide, and examined with a Philips 300 electron microscope.

RESULTS

The preoptic recess of the toad hypothalamus is a ventral midline pocket of the third ventricle which extends approximately 1 mm rostrally from the optic chiasm. Along much of the recess, just beneath the ependymal cells, are one to three rows of closely packed subependymal cells, which in turn overlie a distinct layer of neuropil (Figs. 1 and 2). This laminar group represents the subependymal portion of the nucleus periventricularis preopticus described by Frontera (15).

Histochemistry

When pieces of frozen-dried tissue from this region are exposed to formaldehyde vapors, the subependymal cells develop a bright yellow-green fluoresence readily seen by fluorescence microscopy. The cell bodies are 7–10 μ in diameter and give rise to apical processes extending between nonfluorescent ependymal cells to the ventricular surface (Fig. 6). The tips of these processes may bulge into the ventricle and form bulbous expansions. Less frequently, fluorescent basal processes are seen extending into the subjacent neuropil. When this layer of cells is seen by the fluorescence method, subependymal cells are always visible in the corresponding regions of adjacent, routinely stained sections (Fig. 4). Conversely, in areas where subependymal cells are not seen in routinely stained sections, no fluorescent cells are found in adjacent sections prepared for the demonstration of catecholamines (Fig. 5). This precise correspondence holds up throughout the preoptic recess except along the "lateral recesses," which are ventro-lateral expansions of the preoptic recess, where nonfluorescent cells are interspersed among fluorescent cells. Occasionally individual fluorescent cells also occur in the tissue lateral to the preoptic recess; however, we have not seen processes extending from them to the ventricle (44).

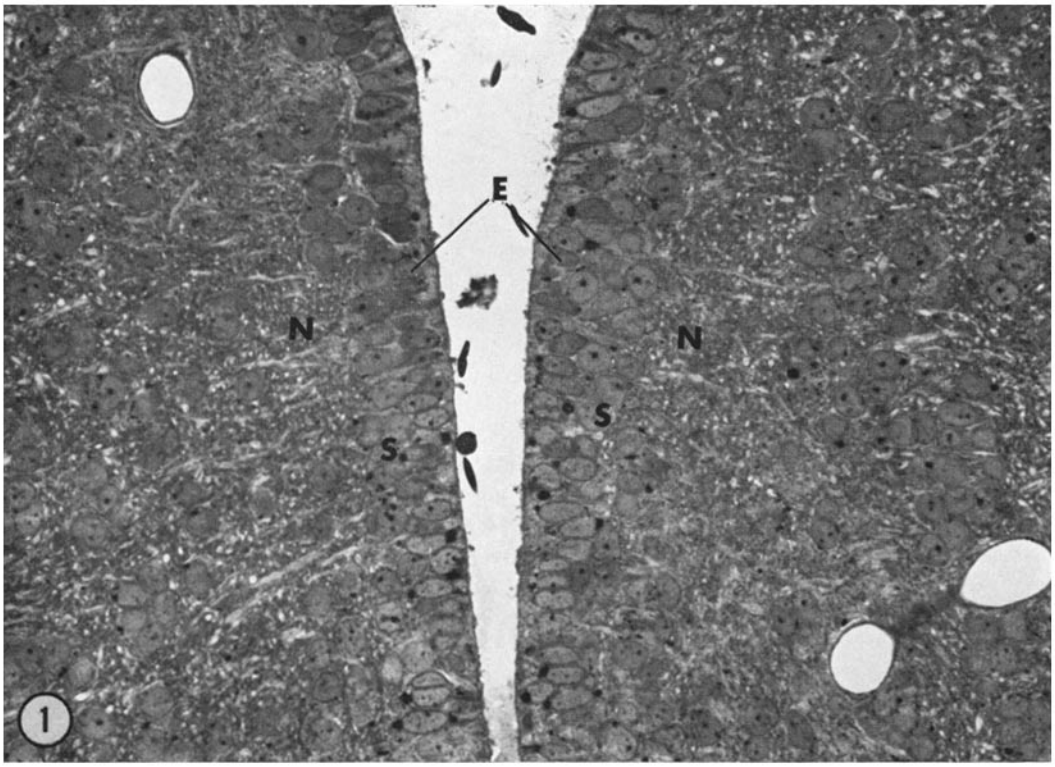


FIGURE 1 Horizontal 1 μ section through the preoptic recess showing several layers of subependymal cells (*S*) between the ependyma (*E*) and the underlying neuropil (*N*). Several extravasated blood cells are visible within the ventricle. Toluidine blue. × 480.

Although there is some variation in the distribution of the nucleus around the preoptic recess from animal to animal, a reasonably consistent pattern can be described (Fig. 3). In transverse sections the recess has the form of a narrow dorsoventrally oriented slit. Approximately midway between the rostral extremity of the recess and the optic chiasm, the ventral portion flares out to form "lateral recesses" which impart a triangular configuration to cross-sections of the third ventricle in this region. Rostrally, the fluorescent cells arch over the dorsum of the recess (Fig. 3, *A*), or occasionally the cells may form a continuous ring around the entire recess. As the nucleus extends caudally, fluorescent cells are added to the ventral edges of the nucleus until they surround the ventricle almost completely (Fig. 3, *B*). Fluorescent cells are, however, never found along the ventral floor of the recess, except occasionally at its rostral tip. Caudally, the nucleus diminishes in extent again (Fig. 3, *C*), and, at the level of the optic chiasm, only a few fluorescent cells are found dorsally.

When the tissue is exposed to formaldehyde for only 1 hr, the subependymal cells develop only a weak green fluorescence. After this exposure time, the median forebrain bundle dorsolateral to the recess contains weakly green-fluorescent fibers and in addition contains a separate population of brightly yellow-fluorescent fibers. These observations indicate that 5-hydroxytryptamine is present in the median forebrain bundle but not in the subependymal cells. When the exposure time to formaldehyde is increased to 3 hr, the fluorescence intensity of the subependymal cells becomes maximal and they appear yellow-green. No additional change in intensity or color occurs with exposure times up to 4 hr. Maximal fluorescence intensity develops in the cells when they are exposed to paraformaldehyde at a relative humidity of 50 or 70%. In untreated tissue, or tissue that has been exposed only to heat, no fluorescence develops in

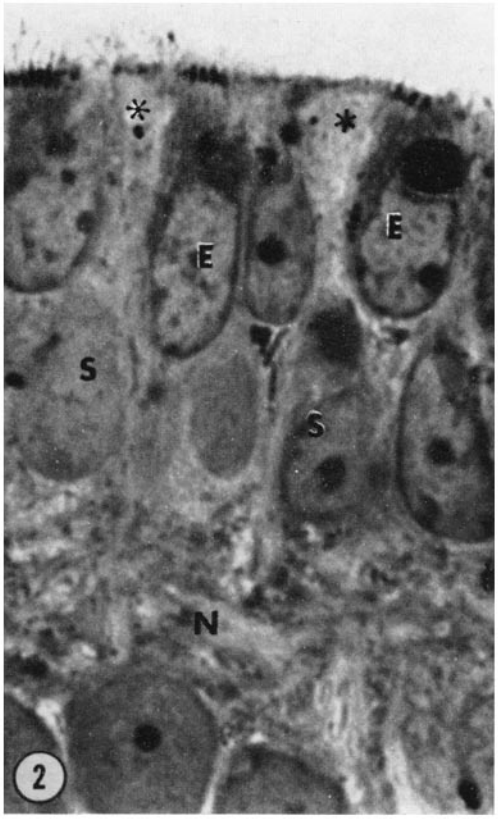


FIGURE 2 High-power light micrograph showing subependymal cells (*S*) with their apical processes (*) extending between ependymal cells (*E*) to border on the ventricle at the top. These cells are separated from deeper cells by an intervening neuropil (*N*). × 2100.

the cells, although gold-colored, autofluorescent granules are found scattered throughout the preoptic area. These granules can be easily distinguished on the basis of color from the yellow-green

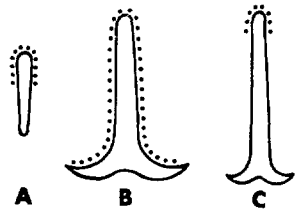


FIGURE 3 Diagram showing outlines of the preoptic recess and the distribution of the fluorescent nucleus (.....) in three transverse sections cut at intervals along a recess ~1000 μ long. The outlines seen in *A*, *B*, and *C* respectively represent sections cut ~150 μ, ~400 μ, and ~800 μ caudal to the rostral tip.

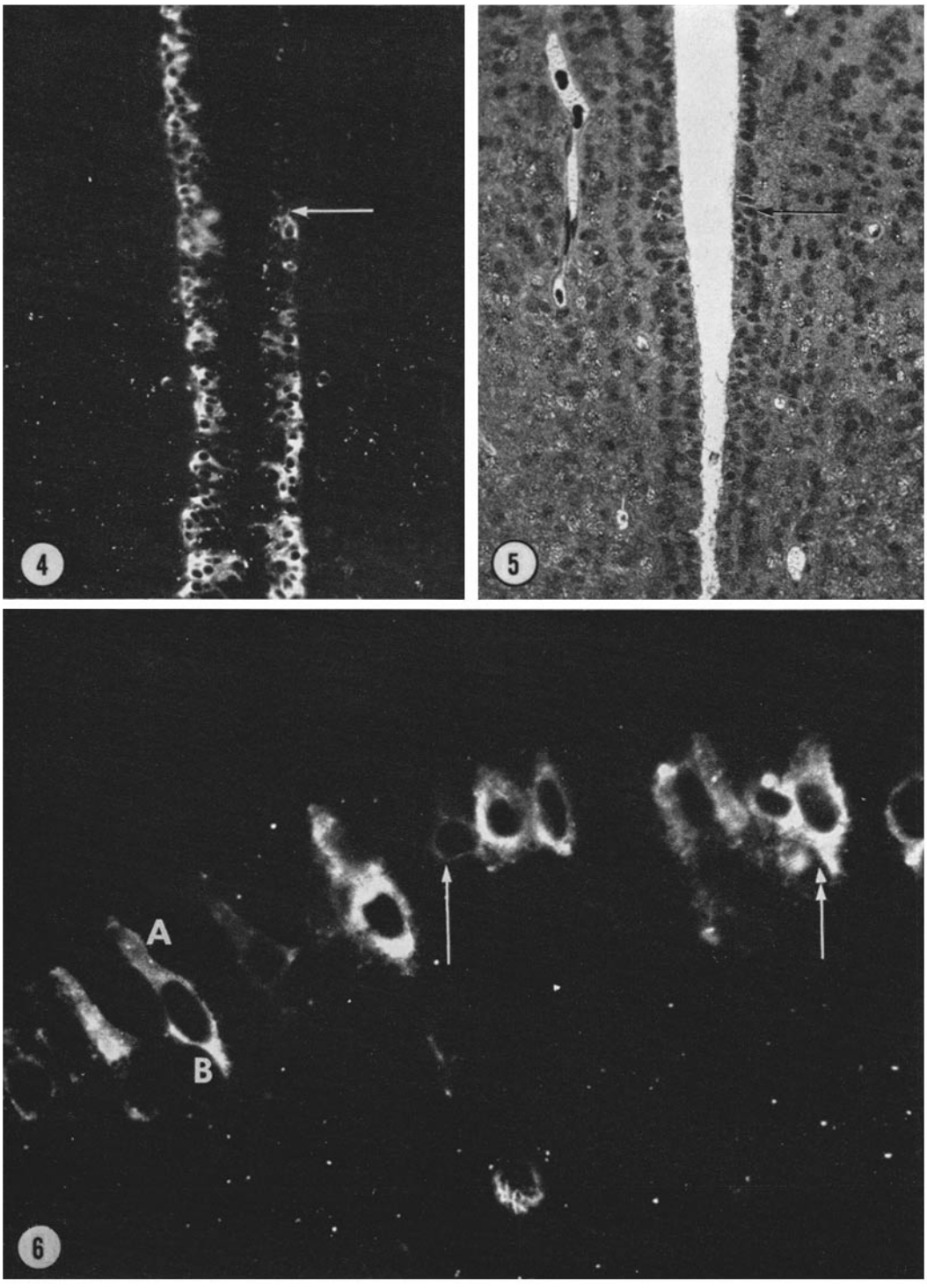
formaldehyde–induced fluorescence of the subependymal cells.

To evaluate further the fluorescence observed in these cells, the histochemical and pharmacological specificity tests of Norberg and Hamberger (29) were applied. Prolonged exposure to ultraviolet light or to water results in a reduction in the fluorescence intensity of the cells. When the sections are exposed to sodium borohydride, which chemically reduces the fluorescent compound to a nonfluorescent compound, the fluorescence disappears almost immediately from the cells. The fluorescence reappears after the sections are again exposed to formaldehyde vapor for several hours. Pretreatment of the animals with reserpine produces a reduction in the fluorescence intensity of the cells, whereas injection of α-methyl-meta-tyrosine produces a complete disappearance of the fluorescence. In contrast, the fluorescence intensity of the cells increases after the administration of the monoamine oxidase inhibitor nialamide, L-dopa, or nialamide followed by L-dopa (Fig. 7). The intensity of the fluorescent fibers in the median forebrain bundle dorso-lateral to the preoptic recess is also enhanced by these drugs.

FIGURE 4 Fluorescence photomicrograph showing a layer of fluorescent cells on either side of the preoptic recess. On the right side the fluorescent layer becomes discontinuous (arrow). × 220.

FIGURE 5 Light micrograph of section adjacent to that depicted in Fig. 4. Several layers of closely packed cells border the ventricle. On the right side, in the area where fluorescent cells are absent in Fig. 4, only a single layer of ependymal cells is found (arrow). Toluidine blue. × 220.

FIGURE 6 High magnification of fluorescent subependymal cells. Within each cell the nucleus appears dark. Brightly fluorescent apical processes (*A*) extend from the cell body to the ventricle. Several cells also possess a fluorescent basal extension (*B*). Note that the fluorescence intensity varies from weak to intense (arrows). × 940.


4
5
A
B
6

In addition to demonstrating the capacity of the cells to accumulate catecholamines, L-dopa and nialamide pretreatment was also expected to facilitate mapping of their basal processes, which can be seen readily in Golgi preparations (unpublished observations), and any other processes that might originate from these cells. However, after this treatment the fluorescence in the basal processes still ends abruptly near the cell body, and none can be traced into a fiber tract even when serial sections of the area are studied. Silver-stained sections from this area also fail to demonstrate the presence of a fiber tract originating from these cells. In addition, midsagittal, parasagittal, and transverse incisions made into the adjoining preoptic area approximately a week before sacrifice fail to enhance the fluorescence of these cells, or to demonstrate any elongated fluorescent processes emanating from them (9), confirming the impression that the cells do not give rise to long axons. In contrast, the fluorescence intensity of interrupted fiber tracts in the nearby median forebrain bundle is increased in the operated animals. An incision dorsal to the nucleus could not be made, and therefore dorsally oriented fibers could not be detected by the surgical technique. However, no evidence for fluorescent fibers in this position is seen in transverse, horizontal, or longitudinal sections (3 μ thick, embedded in plastic, or 10 μ thick, embedded in paraffin) through the area, even after treatment with nialamide and L-dopa. In contrast, the occasional fluorescent cells found lateral to the subependymal cells do display elongated fluorescent processes in some instances (Fig. 7).

When sections from the preoptic area are stained with Gomori's paraldehyde-fuchsin (or chrome-hematoxylin phloxine) method, neurosecretory material is found in cells of the nucleus magnocellularis hypothalami dorsal to the optic chiasm, and in the fibers running in the preopticohypophyseal tract. Neurosecretory material is also found in cells lateral to the preoptic recess, as well as in cells seen immediately subjacent to the neuropil which underlies the subependymal cells. However, no neurosecretory material occurs within the subependymal cells themselves, which are invariably separated from the neurosecretory

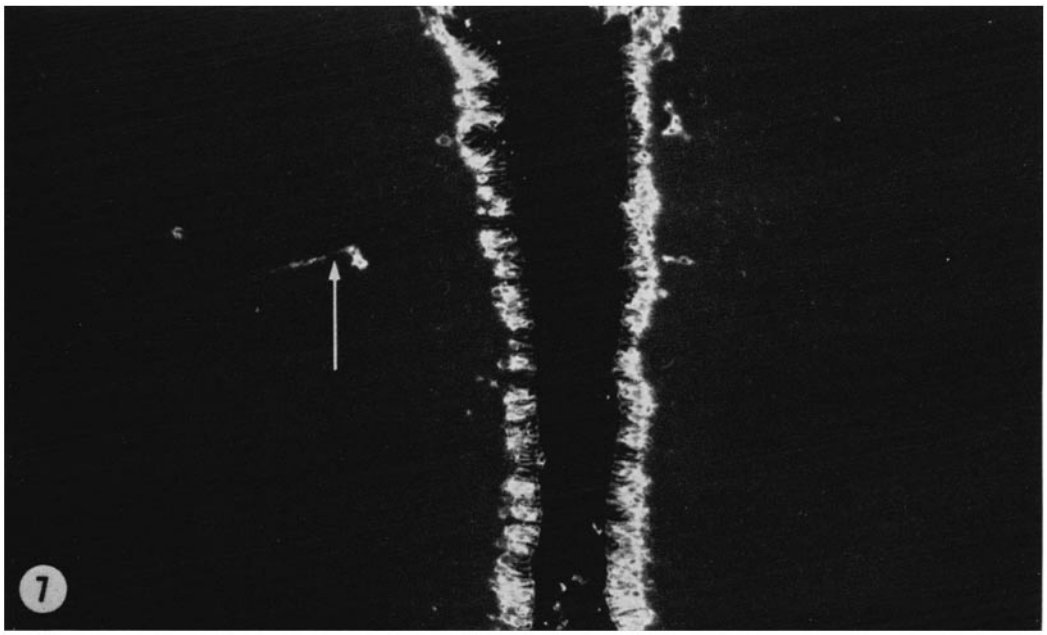


FIGURE 7 Fluorescence micrograph of preoptic area after pretreatment with nialamide and L-dopa. Fluorescence intensity of subependymal cells has increased but no fluorescent processes are seen arising from the basal surface of the cells. In contrast, a fluorescent cell found laterally exhibits a process (arrow). Other than showing several scattered fluorescent cells deep to the subependymal nucleus, the tissue lateral to the recess is devoid of fluorescent structures. × 180.

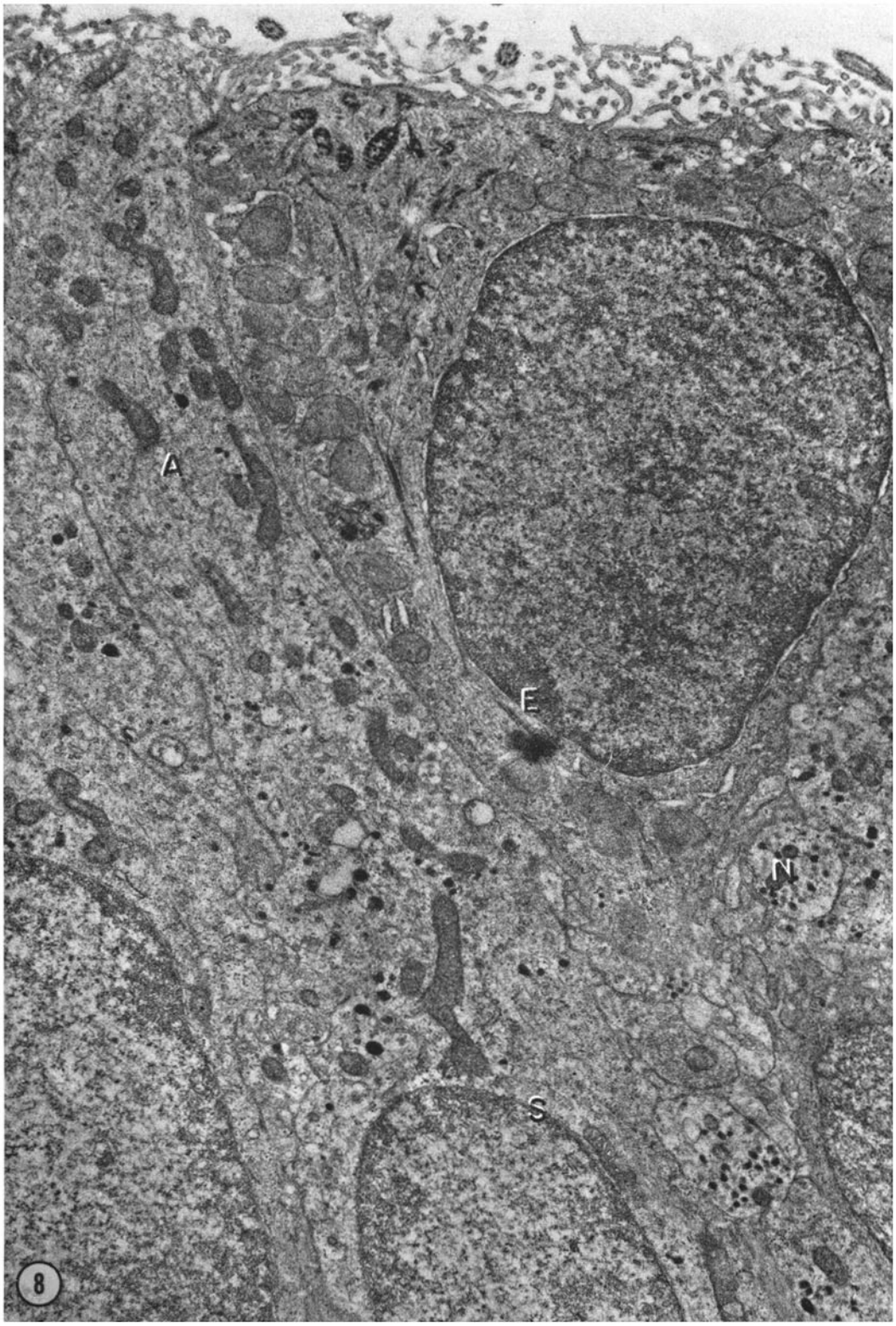


FIGURE 8 Survey electron micrograph showing a subependymal cell (*S*) and its apical process (*A*) extending to and bordering on the ventricle. A neighboring ependymal cell (*E*) contains conspicuous ciliary basal bodies. Nerve fibers (*N*) are visible among the cells. × 17,000.

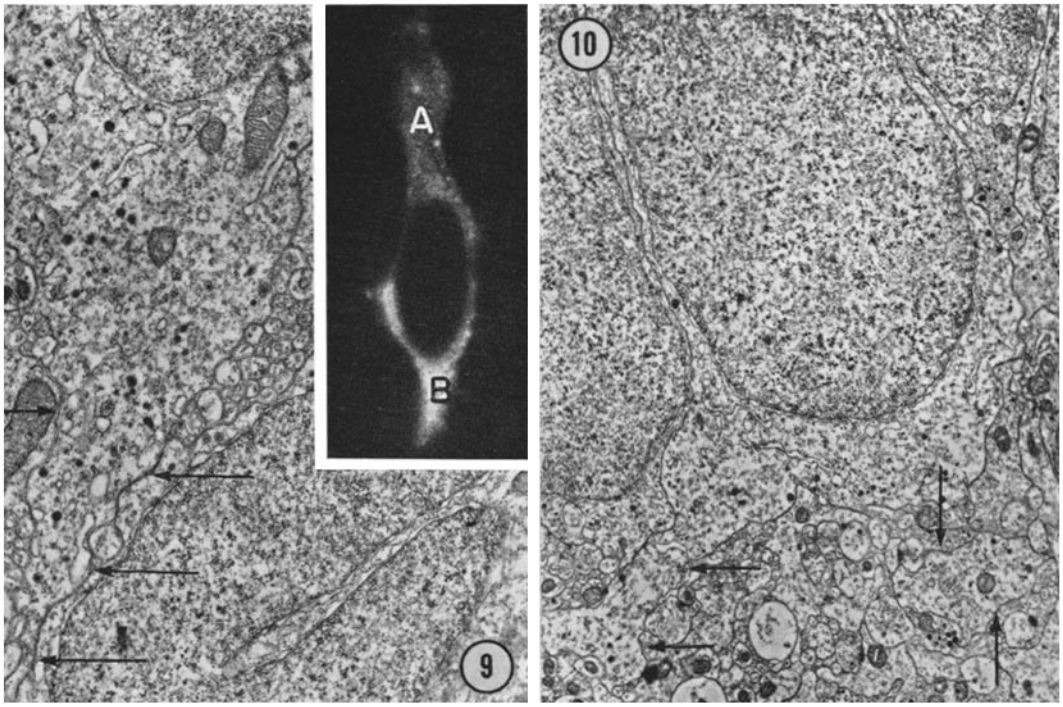


FIGURE 9 The basal portion of a subependymal cell (arrows) projecting between other cells and into the neuropil. The cytoplasmic components of this tail are similar to those in the base of the cell. × 14,000. *Inset:* An equivalent cell prepared for fluorescence histochemistry showing both apical (*A*) and basal (*B*) processes. Enlargement of a cell in Fig. 6. × 1700.

FIGURE 10 Basal portion of a subependymal cell (horizontal arrows) extending into the neuropil. A circular profile (vertical arrows) in neuropil contains equivalent cytoplasmic components and probably represents a cross-section of another subependymal cell basal process. The basal portion of another subependymal cell at the upper right is also tapering into a process. × 8000.

cells by an intervening neuropil. Clearly, the neurosecretory cells and the fluorescent subependymal cells cannot be confused, since their respective locations are quite separate from each other. The subependymal cells, like certain other catecholamine-containing cells, have so far failed to give a classic chromaffin reaction (22).

Fine Structure

The subependymal cells found along the lateral walls of the preoptic recess (Fig. 4) were chosen for the electron microscopic study since it is known from the fluorescence histochemical studies that, when a distinct subependymal layer of cells can be identified along the lateral walls, all cells in this position contain catecholamines. The lateral recesses were avoided since nonfluorescent cells may be interspersed among the fluorescent subependymal cells in this region.

The catecholamine-containing cells are easily identified by electron microscopy not only because of their subependymal position but because of their characteristic shape. As in the histochemical preparations, apical processes of the subependymal cells extend between the ependymal cells to border directly on the ventricle (Fig. 8), and basal processes are found projecting into the subjacent neuropil (Figs. 9 and 10).

The structural details of these cells are equivalent, whether the tissue is preserved by perfusion of fixative through the heart, or by perfusion through the preoptic recess. The nucleus is round or oval in profile and usually exhibits a single nucleolus. The perikaryal cytoplasm contains

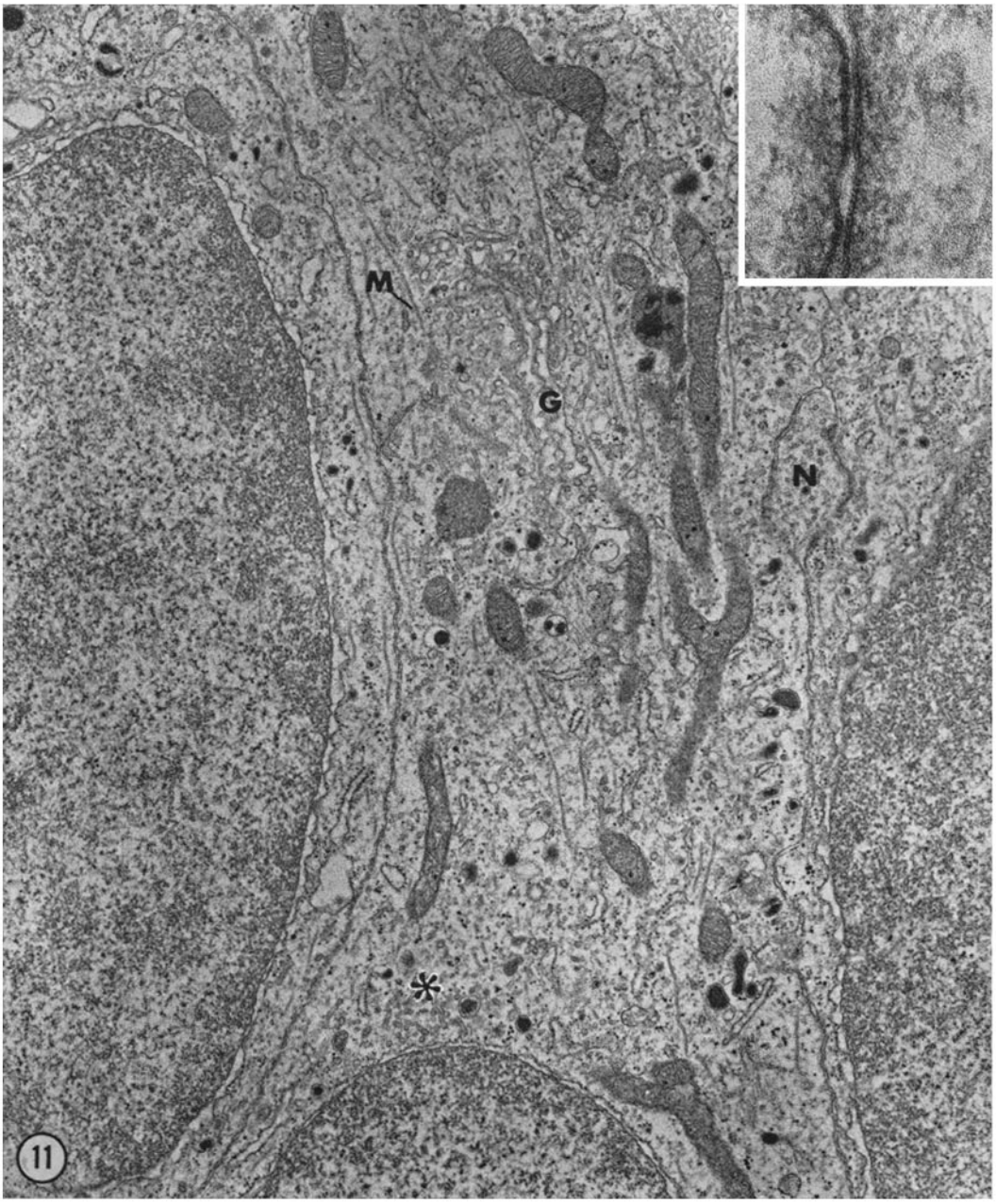


FIGURE 11 Apex of a subependymal cell containing microtubules (*M*), agranular reticulum (*), Golgi apparatus (*G*) and dense-cored vesicles. Similar subependymal cells are found on either side. A nerve fiber (*N*) is visible along this apical process. × 18,000. *Inset:* Detail of a gap junction between the apical process of a subependymal cell (left) and an ependymal cell (right) just beneath the ventricular surface. × 170,000.

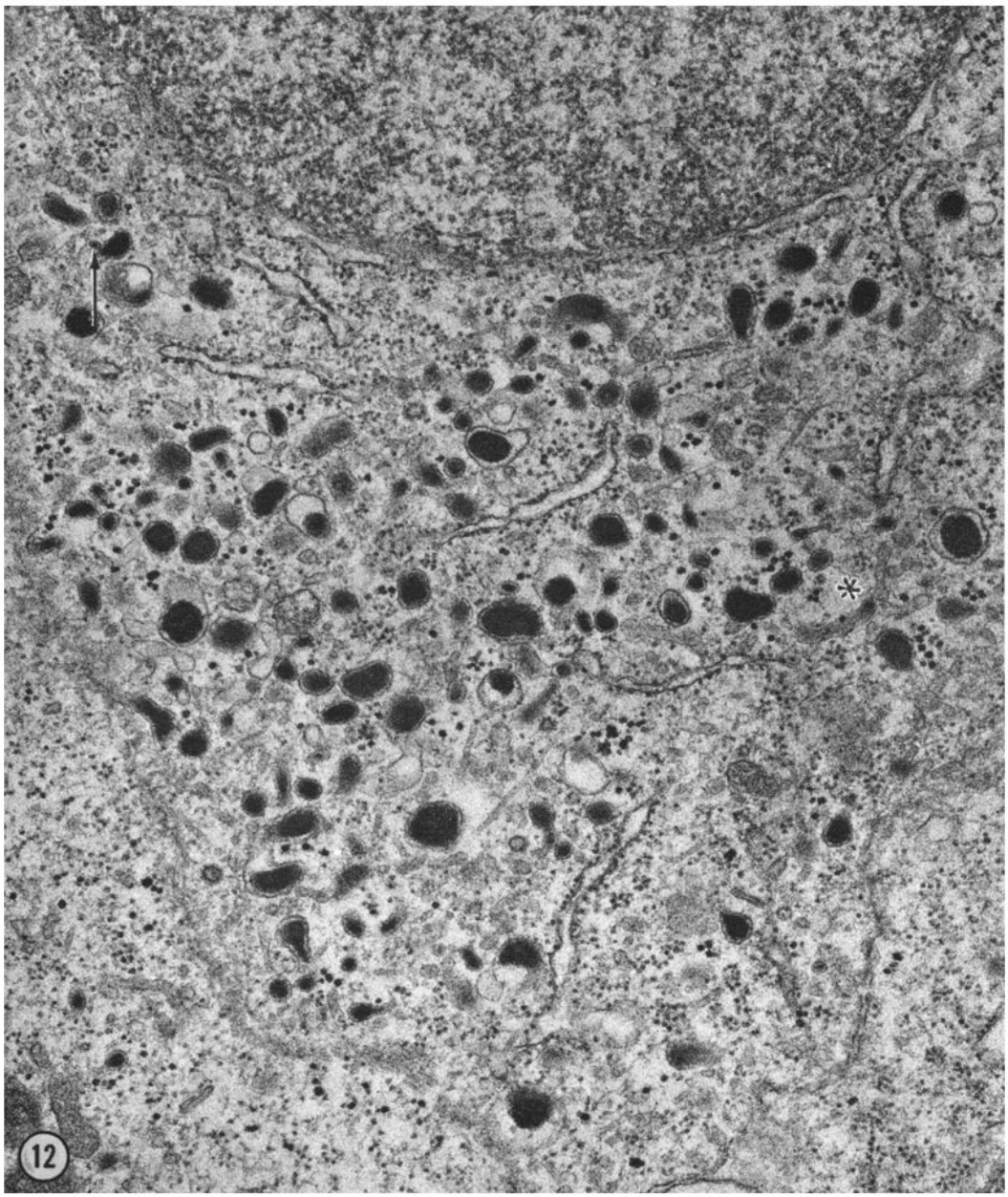
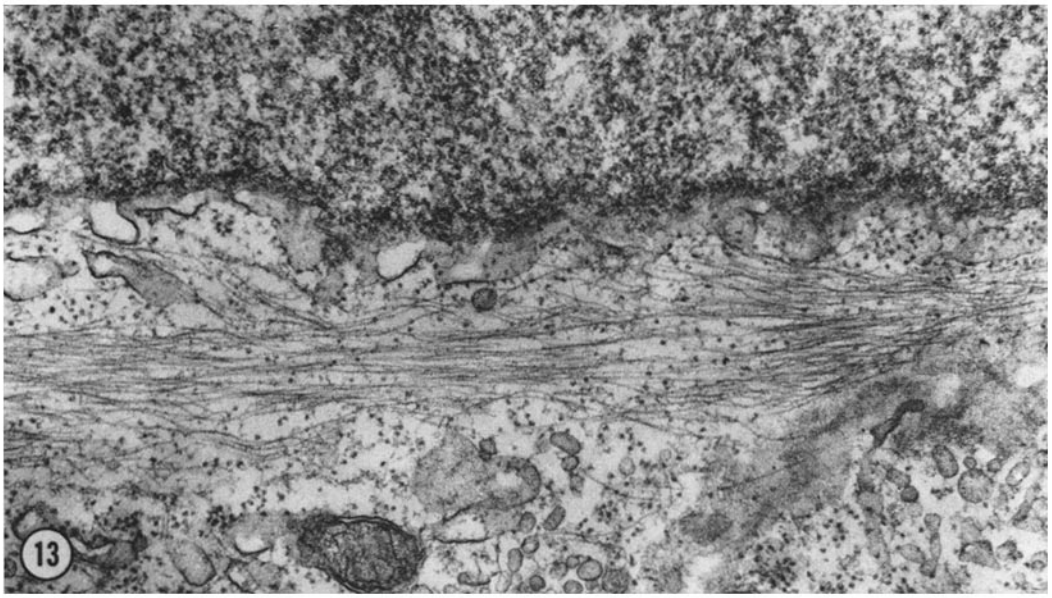


FIGURE 12 The basal cytoplasm of the subependymal cells contains scattered cisternae of granular endoplasmic reticulum, free ribosomes, some glycogen, microtubules, and a large number of dense-cored vesicles of varying size and shape, some of which are closely associated with elements of tubular reticulum (*). A very small dense-cored vesicle is present (arrow). × 37,000.

mitochondria, rough and smooth endoplasmic reticulum, free ribosomes, microtubules, filaments, cisternal and vesicular elements of the Golgi complex, glycogen particles, and dense-cored vesicles (Figs. 8, 11, and 12).

The filaments are approximately 100 A in diameter and of undetermined length (Fig. 14). Usually they are grouped into bundles of 50–60 filaments to form perinuclear sheaves resembling the filament bundles present in the ependymal



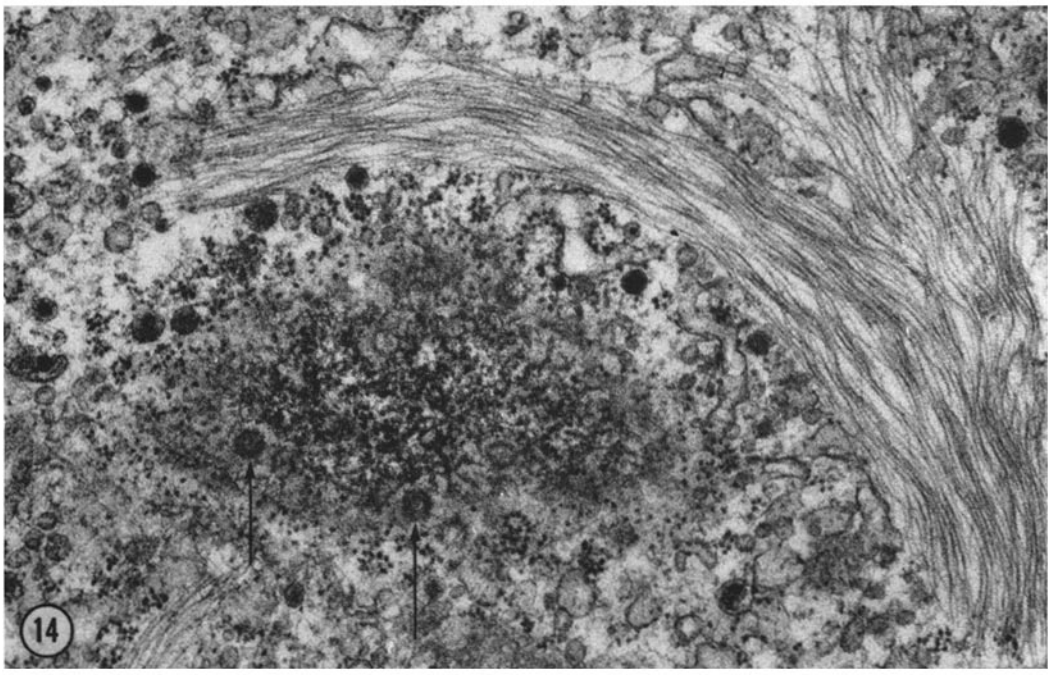


FIGURE 13 A bundle of filaments found lying adjacent to the nucleus of an ependymal cell. × 41,000.

FIGURE 14 In a subependymal cell a bundle of filaments found in close proximity to the nucleus. Nuclear pores (arrows) and numerous dense-cored vesicles are also seen. × 41,000

cells and subpial astrocytes in these animals (36). In all three of these cell types, the individual filaments are separated from one another by a distance of ~100 A, which is severalfold less than the spacing of the neurofilaments found in nerve cell bodies, dendrites, or axons. Occasionally, single filaments are seen in the apical process as well as in the basal cytoplasm of the cells. Microtubules ~250 A in diameter, which are also present in all areas of the cells, are most conspicuous in the apical processes (Figs. 8 and 11).

Granular endoplasmic reticulum can be seen

extending from the nuclear membrane as well as scattered through the cytoplasm. Occasionally, two or three parallel cisternae occur in a perinuclear position (Fig. 24). Free ribosomes, including polysomes, are dispersed through the cells.

The most striking feature of the subependymal cells is the presence of dense-cored vesicles, ranging from 400 to 2200 A in diameter, and randomly distributed throughout the cytoplasm of the cell body, the apical processes, and the proximal parts of the basal processes. The size and content of the vesicles vary within the cells and differ also depending on the fixation method used. Following glutaraldehyde fixation and osmium tetroxide postfixation, circular and irregularly shaped dense-cored vesicles are found mixed together in the cytoplasm of the subependymal cells (Figs. 12 and 18). The cores are uniformly dense, but vary in shape. In an irregularly shaped vesicle the core conforms to the shape of the limiting membrane and in some cases adheres to one side of the membrane. Occasionally, two or three cores occur within one vesicle. In the case of the vesicles having circular profiles, the cores are usually round and centrally located and they fill most of the vesicle, leaving only a narrow halo between the core and the vesicle membrane.

When, on the other hand, osmium tetroxide is used as the primary fixative (Fig. 15), the size range of the vesicles is comparable to that found in glutaraldehyde-fixed tissue, but the appearance and size distribution of the vesicles are noticeably different. Although quantification is difficult in electron micrographs, it appears that the smallest vesicles, 400–600 A in diameter with a core 180–290 A in diameter, are significantly more abundant after primary osmium tetroxide fixation than after glutaraldehyde fixation. Some vesicles ranging from 800 to 1500 A in diameter exhibit a finely granular material of medium density surrounding the eccentrically located dense core. Other vesicles in this size range, however, lack this granular material, and the dense core is surrounded instead by a clear rim. At the larger end of the scale, vesicles 1800–2200 A in diameter are filled with an electron-opaque material which may be mottled. These vesicles can be distinguished from lysosomes by their smaller size, the homogeneity of the individual cores, and by a halo which separates the core from the vesicle membrane. The size and appearance of all of the vesicles are independent of the nature of the buffer or its pH, except that in tissue preserved with Dalton's fixative some dense-cored vesicles appear ovoid.

Granular vesicles of all sizes are visible even in specimens which have not been postfixed with osmium tetroxide (Fig. 17), or which have not been stained with uranyl acetate or lead hydroxide. When the tissue from the preoptic area is exposed to the histochemical reaction of Wood and Barrnett (46) to distinguish epinephrine- from norepinephrine-containing granules, the cores in the subependymal cells are found to have considerable density after glutaraldehyde fixation alone, and to display virtually no increase in density after exposure to potassium dichromate at either pH 4.1 or 6.5. Thus "light" and "dark" populations of cores comparable to those seen in the adrenal medulla cannot be distinguished in these

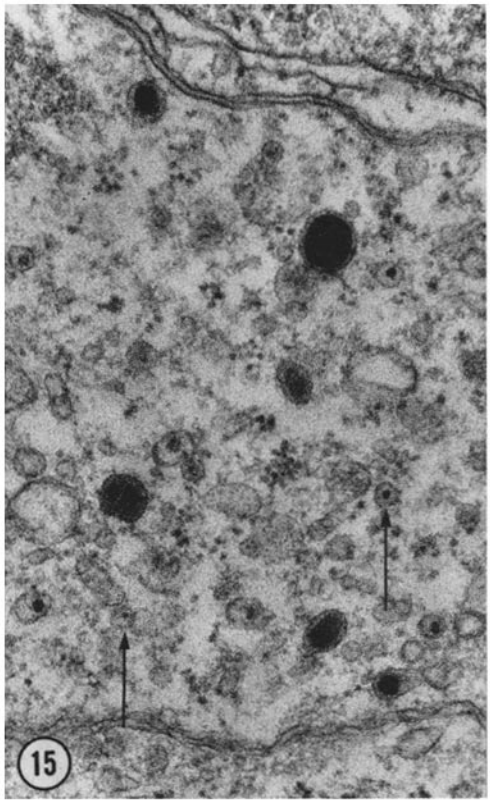
FIGURES 15–18 Appearance of dense-cored vesicles in subependymal cells after various fixation procedures.

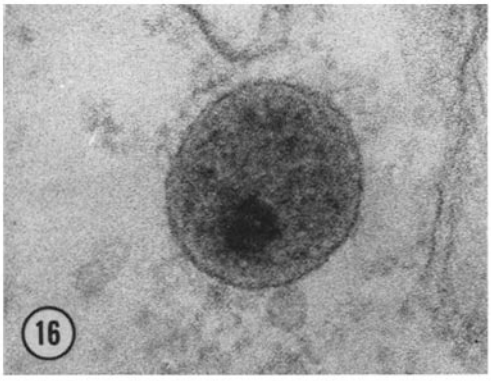
FIGURE 15 Dense-cored vesicles after primary OsO_4 fixation. The vesicles and their cores vary in size, and the density of the cores varies. Several very small dense-cored vesicles are present (arrows). × 68,000.

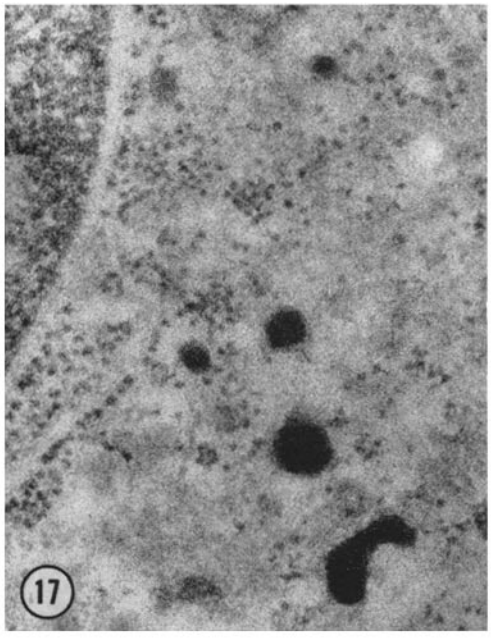
FIGURE 16 High magnification of a dense-cored vesicle after primary OsO_4 fixation showing a dark granular core and a medium-dense mottled material filling in the rest of the vesicle. × 170,000.

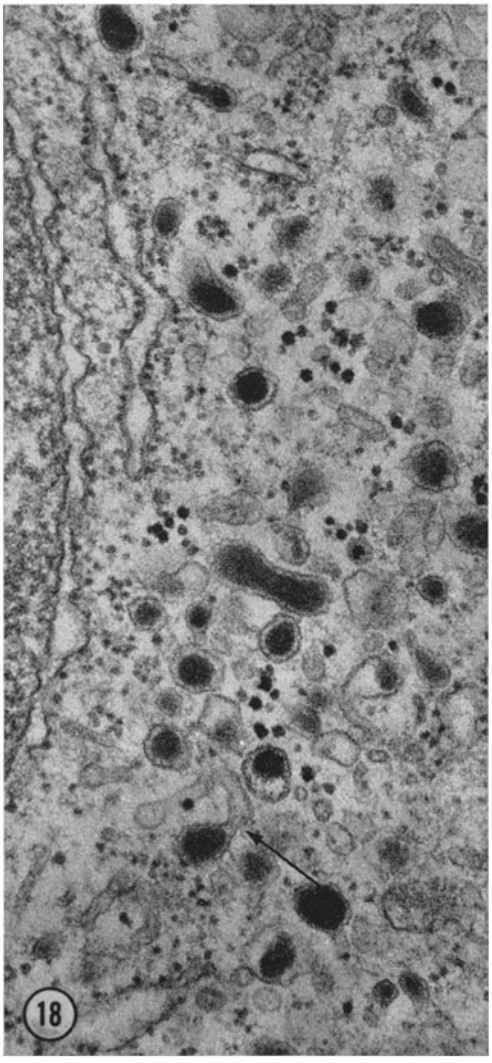
FIGURE 17 Glutaraldehyde fixation with no OsO_4 postfixation. Dense cores are preserved and can assume irregular shapes. No membranes are visualized. × 68,000.

FIGURE 18 After glutaraldehyde fixation and OsO_4 postfixation, dense-cored vesicles vary in shape. In addition to circular profiles with a central dense core, irregular shapes with eccentric cores are also found. A dense-cored vesicle can be seen joined to the agranular reticulum (arrow). Small vesicles are also present. × 68,000.


15


16


17


18

cells. When potassium permanganate is used as the fixative, either by immersion or perfusion through the heart, the dense cores are not preserved; nor are they present in axons within the urinary bladder where epinephrine-containing nerve fibers are known to occur (27). In mammals, potassium permanganate preserves dense cores in norepinephrine-containing neurons, but not in the chromaffin cells of the adrenal medulla or the carotid body (10).

In both the apical processes and perikarya of the subependymal cells, the dense-cored vesicles are closely associated with a smooth-surfaced, branching, tubular reticulum distinct from the Golgi apparatus (Figs. 19–21). Elongated densities resembling the cores of the granular vesicles may be seen within these tubules, and, in some instances, a vesicle containing a density appears to be in the act of pinching off such a tubule (Figs. 18, 20, and 21). Recently, Greenberg has also reported continuity of the limiting membrane of dense-cored vesicles with that of an agranular reticulum in stimulated adrenal medullary and pheochromocytoma cells (17). Although dense-cored vesicles can be seen near the Golgi complex (Fig. 22), which is always confined to the apical part of the cell, no densities have been found within Golgi cisternae. This is in contrast to reports of intracisternal densities in the Golgi apparatus of serotonin-containing neurons of the leech (37), in neurosecretory cells (30), and in adrenal medullary cells (8). Fig. 22 depicts the only instance seen, in this investigation, of a possible association between a dense-cored vesicle and the Golgi complex.

The apical processes of these cells, which extend to the ventricular surface (Figs. 8 and 11), contain granular endoplasmic reticulum and free ribosomes, and elongated mitochondria and microtubules, both of which are oriented parallel to the long axis of the cell containing them. Although the Golgi complex is occasionally found immediately adjacent to the apical surface of the nucleus, in most cases it is situated well within the apical process. The Golgi complex consists of four to six cisternae plus vesicles of various sizes associated with them (Figs. 11 and 22).

The ventricular surface of the apical process has many microvilli extending from it (Fig. 8), and occasionally the process can be seen to protrude into the ventricle (Fig. 26). The cytoplasm of the protrusion, which contains fewer organelles than the rest of the apical process, has many free ribosomes, some dense-cored vesicles, and a few microtubules. Rarely, a ciliary basal body and a few ciliary rootlets are found near the ventricular surface, but no "terminal web" is detectable. The plasma membrane of the subependymal cells forms gap junctions with the adjacent ependymal cells in the subapical region (Fig. 11, inset). The respective membranes, which are separated by ~30 A, and the associated cytoplasmic densities are equivalent and symmetrical in the two cell types.

In fortunate sections basal processes can be followed into the subjacent neuropil (Figs. 9 and 10). These extensions contain free ribosomes as well as granular endoplasmic reticulum, but relatively few dense-cored vesicles. The basal processes have none of the specializations associated with the initial segments of axons (31), but are rather more similar to ependymal tails (34). Presumably, subependymal cell basal processes represent one of the constituents of the subjacent neuropil along with incoming axons and perhaps the dendrites of neurons whose perikarya are located

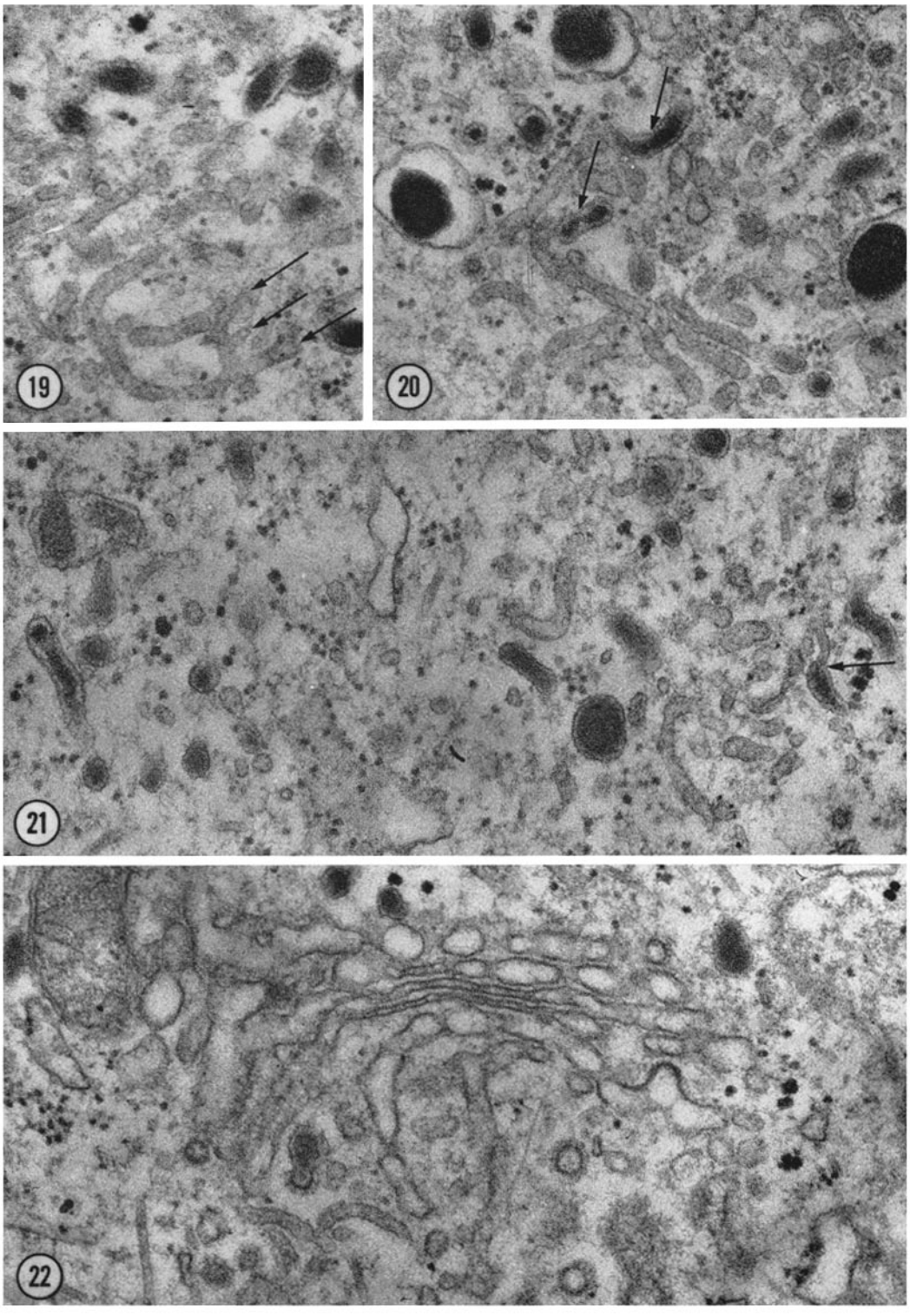
FIGURES 19–22 Agranular reticulum in subependymal cells.

FIGURE 19 Branching agranular reticulum (arrows) associated with dense-cored vesicles. × 74,000.

FIGURE 20 Several elongated membranous sacs of agranular reticulum containing densities are shown (arrows). × 74,000.

FIGURE 21 Several densities enclosed by a single membrane appear at the left. At the right a dense-cored vesicle (arrow) appears to be pinching off the agranular reticulum. × 74,000.

FIGURE 22 Golgi apparatus with several dense-cored vesicles alongside. In contrast to the tubular reticulum, the Golgi cisternae contain no elongated densities. × 74,000.


19
20
21
22

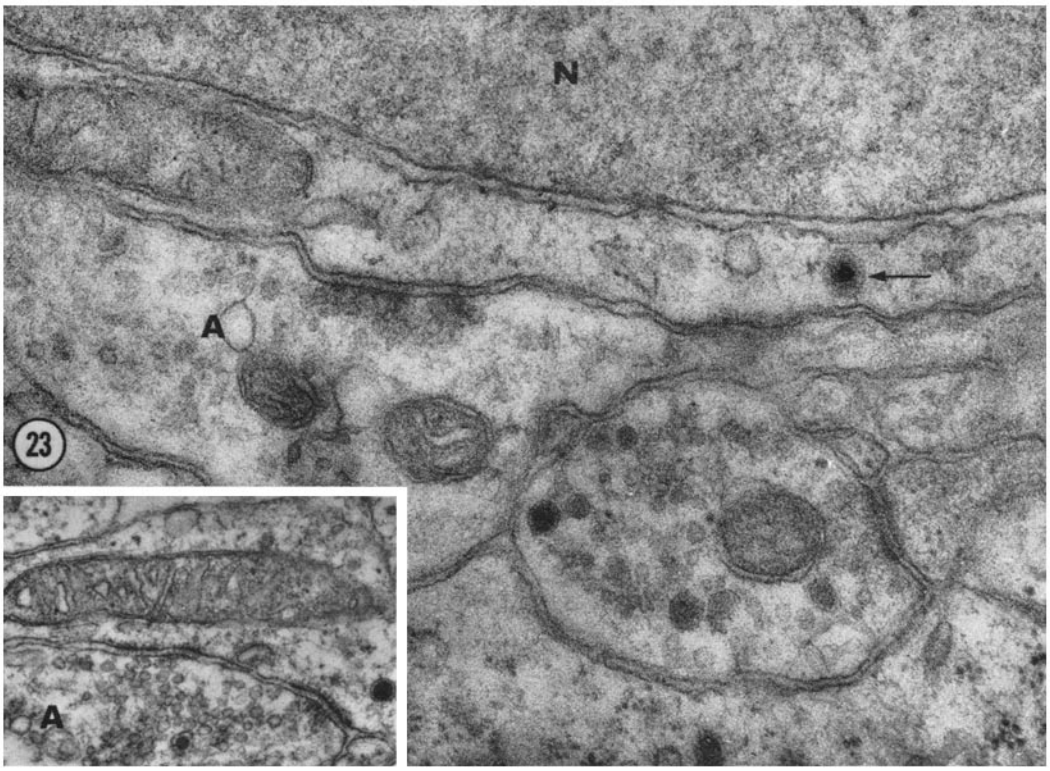


FIGURE 23 Synapse ending on the cell body of a subependymal cell whose nucleus is indicated (*N*). One dense-cored vesicle appears in the cytoplasm (arrow). Within the axon (*A*) clear vesicles are clustered against the presynaptic membrane. A slight density is found within the synaptic cleft and along the postsynaptic membrane. Dalton's fixative. × 55,000. *Inset:* Synaptic ending on a basal process of a subependymal cell. The axon (*A*) contains a dense-cored vesicle as well as clear vesicles. Synaptic cleft and postsynaptic densities are prominent. Primary OsO_4 fixation. × 41,000.

at deeper levels. Golgi-stained material (unpublished data) shows that the subependymal cells, like the neighboring ependymal cells, give rise to greatly elongated, but usually unbranched, tails which taper gradually as they extend away from the ventricle.

Synaptic junctions are found on the basal and lateral surfaces of the subependymal cell bodies, as well as on their apical and basal processes (Figs. 23 and 24). These synapses are characterized by an accumulation of vesicles along the junctional membrane of the axon terminal, by a widening of the cleft between the pre- and postsynaptic membranes to approximately 200–250 A, by the presence of a radially striated material within the cleft (Fig. 23, inset), and by a dense cytoplasmic coating on the postsynaptic (i.e. subependymal cell) membrane. The latter coating is not visualized in Dalton's-fixed specimens (Fig. 23). In one instance a synaptic junction on a "spine" was seen (Fig. 24, inset). In this case a subsynaptic density, like that which occurs in sympathetic ganglion cells (41), as well as in the habenula and interpeduncular nuclei (28), was also present. Several varieties of vesicles are found within the presynaptic axon terminals. Sometimes small (~400 A), clear vesicles are the only ones visible within the presynaptic process, but medium-sized, dense-cored vesicles (~800 A) can also be found. As in the case of the subependymal cells, these medium-sized, dense-cored vesicles are round and contain a homogeneous density surrounded by a clear halo, except in glutaraldehyde-fixed material, in which the dense-cored vesicles may be irregular in shape. In all instances it is only the clear vesicles that adjoin the presynaptic mem-

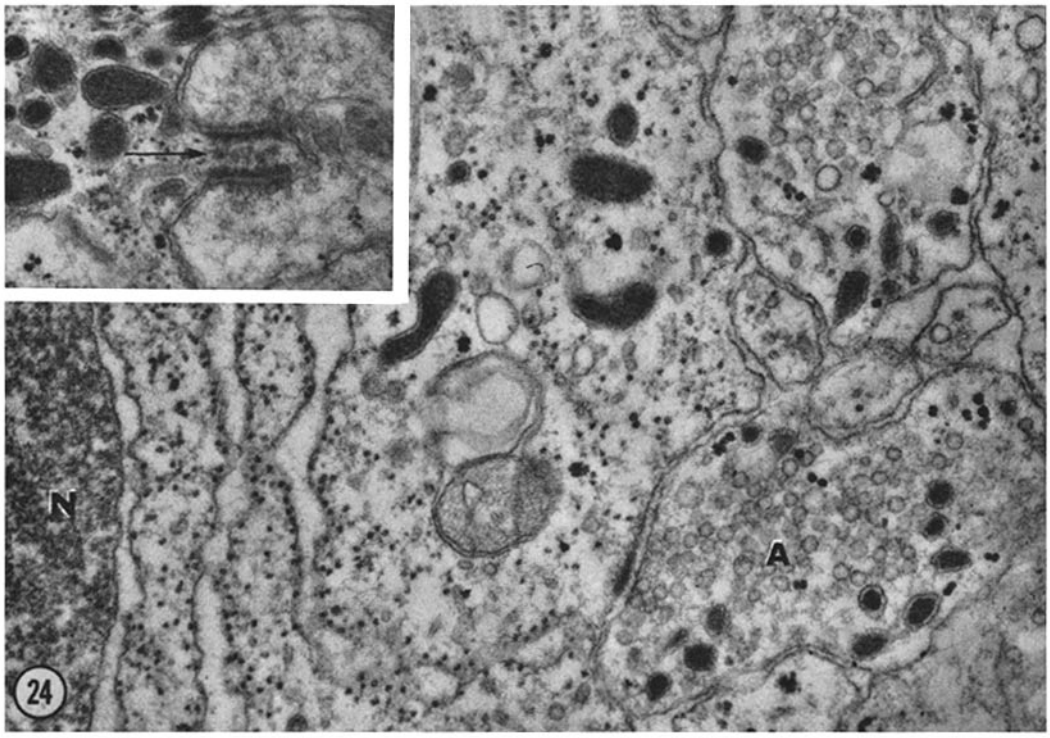


FIGURE 24 Synapse terminating on a subependymal cell whose nucleus is indicated (*N*). Within the axon (*A*) clear vesicles are clustered against the presynaptic membrane and many dense-cored vesicles are scattered within the ending. Glutaraldehyde-OsO_4 fixation. × 50,000. *Inset:* Synapse terminating on a portion of a subependymal cell which resembles a dendritic spine. A subsynaptic density (arrow) is seen between the two postsynaptic membranes of the subependymal cell. Glutaraldehyde-OsO_4 fixation. × 45,000.

brane. At no time were equivalent accumulations of synaptic vesicles found within a subependymal cell against its limiting membrane. Thus, although these cells clearly receive a synaptic input, no evidence that they give rise to a synaptic output has been found. The possibility that their basal processes form presynaptic contacts distally, as the processes of similar cells in the sympathetic ganglion do (25), cannot be excluded, however.

The ependymal cells lining the preoptic recess resemble those described previously in mammalian (6) and amphibian (34) brain, in that they have an extensive ciliary apparatus with associated basal bodies and rootlets (Fig. 25), and closely packed perinuclear bundles of filaments (Fig. 13), and may exhibit microvilli as well. Beneath the cell membrane bordering the ventricle a finely granulo-filamentous layer containing few organelles other than the ciliary apparatus can be found. The Golgi complex, mitochondria, ciliary rootlets, free ribosomes, and granular endoplasmic reticulum are situated beneath this "terminal web." Rarely, dense-cored vesicles occur in these cells. Mitochondria appear more rounded than those in the subependymal cells. Thus, although ependymal and subependymal cells are distinct from each other, it is noteworthy that they share some of the same unusual organelles, although in strikingly different concentrations. The ependymal cells contain many ciliary basal bodies, rootlets, and large numbers of filament bundles, whereas the subependymal cells contain only a rare basal body, a few rootlets, and usually a single bundle of filaments in any section. Subependymal cells contain many dense-cored vesicles; ependymal cells only a rare one. Both cells also have basal processes

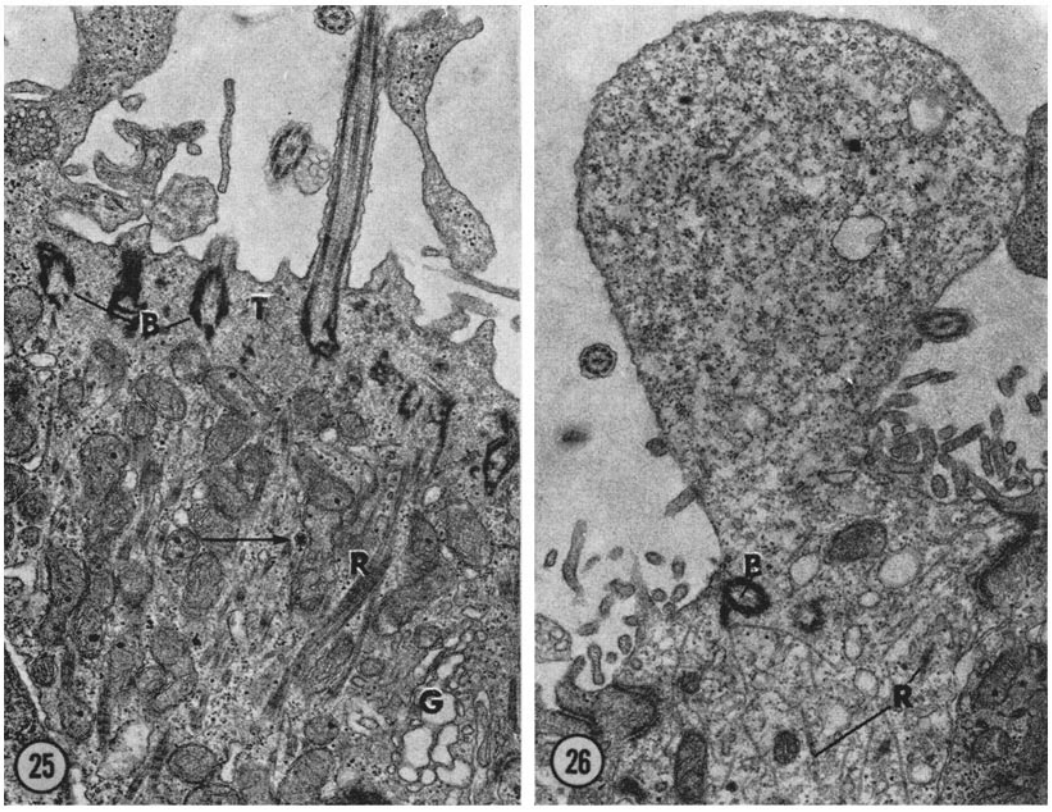


FIGURE 25 Ventricular surface of an ependymal cell showing cilia with basal bodies (*B*) and rootlets (*R*). Beneath the plasma membrane a terminal web (*T*) can be seen. Golgi apparatus (*G*) and a single dense-cored vesicle (arrow) are also present. × 13,000.

FIGURE 26 Bulblike expansion of the apical process of a subependymal cell projecting into the ventricle. The expansion contains several dense-cored vesicles and many free ribosomes. The cytoplasm of the main body of the process contains a ciliary basal body (*B*) and rootlets (*R*), as well as microtubules and dense-cored vesicles. Microvilli are visible at the ventricular surface. No terminal web is present in this cell. × 13,000.

which extend through the subjacent neuropil away from the cell bodies and ventricle.

DISCUSSION

By combining the results of fluorescence histochemical and electron microscopic studies, it has been possible to characterize a group of catecholamine-containing cells in the amphibian hypothalamus. The histochemical data indicate that epinephrine is the principal catecholamine in these cells in *Bufo marinus*, as it is in the central nervous system of amphibians in general (4). This has been confirmed by chemical analysis which measured a high level of epinephrine in pooled tissue dissected from the preoptic area of 20 toads (M. Goldstein, personal communication). The only nucleus of fluorescent cells in the tissue dissected for analysis was that of the subependymal cells. These studies do not eliminate the possibility that other catecholamines are also present in low concentrations.

Morphologically, the cells are distinguished by their apical processes, which extend directly to the ventricle, by the bundles of closely packed filaments, and by the abundant, dense-cored vesicles of various sizes which are sometimes associated with a smooth, tubular reticulum resembling that of steroid-synthesizing cells (7). Axons

containing either clear vesicles or both clear and granular vesicles form typical synaptic terminals on the subependymal cell bodies and their apical and basal processes, but no evidence that these cells are presynaptic or that axons originate from them has been found. Experiments designed to increase the catecholamine content of cellular processes by pharmacological treatment or by lesions in the surrounding tissue have also failed to demonstrate the presence of catecholamine in long processes originating from the subependymal cells. In contrast, the fluorescence of the median forebrain bundle increases after surgical interruption, and drug treatment permits visualization of processes arising from cells located lateral to the recess (44). Thus, even though long basal processes can be seen in Golgi preparations of the subependymal cells, it appears that fluorescent material does not flow into them as it does into the axons of nerve cells. Instead, the fluorescent material is restricted to the perikarya, the most proximal portions of the basal processes, and the apical processes. These findings are consistent with the view that the basal processes correspond to ependymal tails, rather than axons, and that the movement of catecholamines in these cells is toward, rather than away from, the ventricle.

Dense-cored vesicles are found in the subependymal cells in the same regions that are fluorescent. The vesicles are, however, a heterogeneous group ranging in size from 400 to 2200 A, and it is not yet clear whether all or only some of these vesicles can be correlated with the occurrence of fluorescence. In other catecholamine-containing cells, neurons and chromaffin cells, three sizes of dense-cored vesicles have been generally recognized (reviewed in references 8 and 18). The smallest vesicles are 400–600 A in diameter, those of medium size are 800–1500 A, and the vesicles of large size are over 1500 A in diameter. Adrenergic neurons, both peripheral and central, in mammals typically contain dense-cored vesicles of the small and medium sizes (20), and in amphibia the corresponding neurons contain only the medium-size vesicles (33). In neither case, however, are large-size, dense-cored vesicles present. In contrast, chromaffin cells of both vertebrate classes (3, 21, 32) typically contain all three sizes of dense-cored vesicles. Thus, in this respect the subependymal cells more closely resemble chromaffin cells than adrenergic neurons.

Whether the various sizes of dense-cored vesicles found in the subependymal cells represent a single organelle at different stages of activity or biochemical maturity, perhaps containing different precursors of epinephrine, or, alternatively, whether they include several functionally different populations, is not yet known. A number of functions might be suggested. Any of the various vesicles might represent the organelle responsible for extrusion of amine when the cell is stimulated, or the organelle exhausted of its amine after stimulation. Some vesicles might be actively synthesizing catecholamines while others are used solely for storage.

Physiology

The fact that the subependymal cells have apical processes extending directly to the cerebrospinal fluid suggests two possible functions for these cells. First, as proposed by Vigh-Teichmann et al. (43), such cells might be sensory in nature with the apical process responding to changes in the composition of the cerebrospinal fluid. The nerve endings on the cells might then have a modulating function, as has been suggested for the nerves terminating on the outer hair cells of the organ of Corti, for example (39). However, since no evidence has been found that the cells form presynaptic connections to transmit sensory information to other cells in the central nervous system, and since the fluorescence is present in the apical processes, a second possibility, that the cells are secretory, seems more likely. The axons ending upon the subependymal cells would in this case be interpreted as motor fibers which stimulate the cells to release epinephrine into the third ventricle to affect near or distant receptors. Blood vessels of the choroid plexuses and of the median eminence, for example, or neurons which border the cerebral ventricles might serve as receptors to the catecholamines in the cerebrospinal fluid. The secretion of the active compound into a circulating fluid suggests that the effect might be widespread, perhaps similar to the general alerting reaction associated with secretion from the adrenal medulla. Such secretion could be part of a homeostatic mechanism for regulating the composition of the cerebrospinal fluid (36). It may also be part of an intracerebral endocrine circuit concerned with the control of anterior pituitary activity, as suggested by a study in which injec-

tion of another catecholamine, dopamine, into the third ventricle was found to produce indirectly the release of luteinizing hormone from the pituitary (23). All such suggestions must remain speculative for the present.

Classification

As suggested in the foregoing discussion, the structure and form of the subependymal cells do not allow them to be readily classified as any of the typical central nervous components—neurons, glial cells, or ependymal cells. The relatively sparse granular endoplasmic reticulum, the bundles of closely packed filaments in the perikaryon, the direct contact of the cell with the cerebrospinal fluid, and the presence of large-size, dense-cored vesicles are all features which would be atypical in neurons. There is also no evidence from fluorescence histochemistry, electron microscopy, and silver stains that the cells form axons or are presynaptic to dendrites as some sensory cells are (39). On the other hand, the presence of catecholamines, dense-cored vesicles, and nerve endings terminating on the cells distinguish them from typical glial cells (36).

Of the cell types intrinsic to the central nervous system, the subependymal cells most closely resemble the ependymal cells adjacent to them with respect to size, shape, location, and complement of organelles. As discussed above, the only qualitative difference between the two cell types consists of the presence of synaptic junctions on the subependymal cells and their absence on ependymal cells. Even this difference, however, may not be absolute; other workers have reported the existence of "synaptoid junctions" on cells considered to be ependymal (24).

Although these fluorescent subependymal cells are not strictly comparable to the typical cells intrinsic to the central nervous system, they do correspond to a well-defined cell type that occurs *outside* the central nervous system. The latter, usually referred to as the "chromaffin cell," is represented in the adrenal medulla, carotid body, autonomic ganglia, and "paraganglia" (3, 8, 21, 22, 25, 45, 47). Such peripheral chromaffin cells, which are derived from the neural crest, contain high concentrations of catecholamines, have nerve endings terminating on them, and, in some cases at least, are known to secrete into the general circulation (8). Their endoplasmic reticulum is also relatively sparse, and, most strikingly, they contain large populations of dense-cored vesicles ranging in size from a maximum of ~3500 A in the adrenal medulla to a minimum of 400 A in glomus cells of the carotid body. In view of the facts that the subependymal cells are similar to peripheral chromaffin cells and are different from other central nervous system cells, we suggest that the term "encephalochromaffin cell" be used to refer to the catecholamine-containing subependymal cells described here[1] and any other cells in the brain which: (*a*) contain catecholamines, (*b*) structurally resemble adrenal medullary cells or other peripheral chromaffin cells, and (*c*) terminate directly at a cerebral ventricle, capillary portal system, or other fluid space in such a way as to permit secretion into a circulating compartment. This definition obviously emphasizes the secretory role of such cells but does not exclude the possibility that they may also transmit synaptically. Matthews and Raisman (25) have previously suggested the possibility of a dual role for certain catecholamine-containing cells in the superior cervical ganglion. In either case, the distinctive morphology of these cells justifies the creation of a separate category for them, just as neurosecretory cells are distinguished from typical central neurons by their affinity for certain special histological stains, and by their characteristic structure. The term "encephalo-chromaffin cell" has the advantages of being more specific and more informative than the general term "atypical ependymal cell" used previously to describe a considerable variety of specialized cells bordering the cerebral ventricles, including, for example, certain cells in the organum vasculosum hypothalami of fish, reptiles, birds, and amphibia (1, 2, 5, 38, 42) which also send processes to the ventricular surface and contain catecholamines.

The present study was originally intended to characterize a nucleus of catecholamine-containing neurons in the central nervous system. Instead, we find a group of cells which do not fit comfortably into any of the usual categories of brain cells, but

[1] According to Jacobowitz (22), the highly sensitive and specific fluorescence method, rather than the less reliable classic chromaffin reaction, should be used to demonstrate the presence of catecholamines and hence to identify "chromaffin cells." This term is therefore used in referring to the subependymal cells described in this paper even though no chromaffin reaction was obtained from them.

which belong to a class of atypical cells within the central nervous system found, characteristically, in close association with the cerebral ventricles. As discussed above, this relationship suggests that the encephalo-chromaffin cells described here, like secretory cells within other circumventricular organs (40), release their products directly into the ventricular fluid. Thus, in addition to its other functions, cerebrospinal fluid may also play an important role as a vehicle for the distribution of humoral agents to target cells within the brain comparable to the role of peripheral blood in carrying hormones to their sites of action.

A report of this investigation was presented at the annual meeting of the American Society for Cell Biology held in November, 1969 (26).

This work has been supported by postdoctoral fellowship NS-37, 650, and grants NS-07197 and NS 07495 from the United States Department of Health, Education, and Welfare.

Received for publication 13 May 1970, and in revised form 14 August 1970.

REFERENCES

1. BAUMGARTEN, H. G., and H. BRAAK. 1967. Catecholamine im Hypothalamus von Goldfisch. (*Carussius auratus*). *Z. Zellforsch. Mikrosk. Anat.* **80**:247.
2. BAUMGARTEN, H. G., and H. BRAAK. 1968. Catecholamine im Gehirn der Eidechse. (*Lacerta viridis* u. *Lacerta nuralis*). *Z. Zellforsch. Mikrosk. Anat.* **86**:574.
3. BISCOE, T. J., and W. E. STEHBENS. 1966. The ultrastructure of the carotid body. *J. Cell Biol.* **30**:563.
4. BOGDANSKI, D. F., L. BONOMI, and B. B. BRODIE. 1963. Occurrence of serotonin and catecholamines in brain and peripheral organs in various vertebrate classes. *Life Sci.* **1**:80.
5. BRAAK, H., H. G. BAUMGARTEN, and B. FALCK. 1968. 5-Hydroxytryptamin im Gehirn der Eidechse (*Lacerta viridis* und *Lacerta nuralis*). *Z. Zellforsch. Mikrosk. Anat.* **90**:161.
6. BRIGHTMAN, M. W., and S. L. PALAY. 1963. The fine structure of ependyma in the brain of the rat. *J. Cell Biol.* **19**:415.
7. CHRISTENSEN, A. K., and D. W. FAWCETT. 1961. Normal fine structure of opossum testicular cells. *J. Biophys. Biochem. Cytol.* **9**:653.
8. COUPLAND, R. E. 1965. The Natural History of the Chromaffin Cell. Longmans Green & Co. Ltd., London.
9. DAHLSTRÖM, A., and K. FUXE. 1965. Evidence for the existence of monoamine neurons in the central nervous system. II. Experimentally induced changes in the intraneuronal amine levels of bulbospinal neuron systems. *Acta Physiol. Scand.* **64**: *Suppl.* 247.
10. DUNCAN, D., and R. YATES. 1967. Ultrastructure of the carotid body of the cat as revealed by various fixatives and the use of reserpine. *Anat. Rec.* **157**:667.
11. FALCK, B. 1962. Observations on the possibilities of cellular localization of monoamines by a fluorescence method. *Acta Physiol. Scand.* **56**: *Suppl.* 197.
12. FALCK, B., N-Å HILLARP, G. THIEME, and A. TORP. 1962. Fluorescence of catecholamines and related compounds condensed with formaldehyde. *J. Histochem. Cytochem.* **10**:348.
13. FALCK, B., and C. OWMAN. 1965. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic amines. *Acta Univ. Lund. Sect. 2 MM* No. 7.
14. FOLEY, J. O. 1943. A protargol method for staining nerve fibers in frozen or celloidin sections. *Stain Technol.* **18**:27.
15. FRONTERA, J. G. 1952. A study of the anuran diencephalon. *J. Comp. Neurol.* **96**:1.
16. GABE, M. 1966. Neurosecretion. Pergamon Press Ltd., Oxford. 10.
17. GREENBERG, R. 1970. Fine structural arrangement observed in tissues with accelerated synthesis of catecholamines. *Fed. Proc.* **29**:679.
18. GRILLO, M. A. 1966. Electron microscopy of sympathetic tissues. *Pharmacol. Rev.* **18**(1):387.
19. HAMBERGER, B., T. MALMFORS, and C. SACHS. 1965. The standardization of paraformaldehyde and certain procedures for histochemical demonstration of catecholamines. *J. Histochem. Cytochem.* **13**:147.
20. HÖKFELT, T. 1968. *In vitro* studies on central and peripheral monoamine neurons at the ultrastructural level. *Z. Zellforsch. Mikrosk. Anat.* **91**:1.
21. ISHII, K., and T. OOSAKI. 1969. Fine structure of the chemoreceptor cell in the amphibian carotid labyrinth. *J. Anat.* **104**:263.
22. JACOBOWITZ, D. 1967. Histochemical studies of the relationship of chromaffin cells and adrenergic nerve fibers to the cardiac ganglia of several species. *J. Pharmacol. Exp. Ther.* **158**: 227.
23. KAMERI, I. A., R. S. MICAL, and S. C. PORTER. 1969. Luteinizing hormone-releasing activity in hypophysial stalk blood and elevation by dopamine. *Science* (*Washington*). **166**:388.
24. KOBAYASHI, H., and T. MATSUI. 1967. Synapses in the rat and pigeon median eminence. *Endocrinol. Jap.* **14**:279.
25. MATTHEWS, M. R., and G. RAISMAN. 1969. The

ultrastructure and somatic efferent synapses of small granule-containing cells in the superior cervical ganglion. *J. Anat.* **105**:255.

26. McKENNA, O. C., and J. ROSENBLUTH. 1969. Fluorescence histochemical and ultrastructural characterization of a discrete catecholamine-containing nucleus in the amphibian hypothalamus. *J. Cell Biol.* **43**(2, Pt. 2):89 *a*. (Abstr.)
27. McLEAN, J. R., and G. BURNSTOCK. 1966. Histochemical localization of catecholamines in the urinary bladder of the toad. *J. Histochem. Cytochem.* **14**:538.
28. MILHAUD, M., and G. D. PAPPAS. 1966. Postsynaptic bodies in the habenula and interpeduncular nuclei of the cat. *J. Cell Biol.* **30**: 437.
29. NORBERG, K.-Å, and B. HAMBERGER. 1964. The sympathetic adrenergic neuron. *Acta Physiol. Scand.* **63**: *Suppl.* 238.
30. PALAY, S. L. 1960. The fine structure of secretory neurons in the preoptic nucleus of the goldfish (*Carassius auratus*). *Anat. Rec.* **138**:417.
31. PALAY, S. L., C. SOTELO, A. PETERS, and P. M. ORKAND. 1968. The axon hillock and the initial segment. *J. Cell Biol.* **38**:193.
32. PIEZZI, R. S. 1967. Chromaffin tissue in the adrenal gland of the toad, *Bufo arensum* Hensel. *Gen. Comp. Endocrinol.* **9**:143.
33. RODRIQUEZ, E. M. 1969. Ultrastructure of the neurohaemal organ of the toad median eminence. *Z. Zellforsch. Mikrosk. Anat.* **93**:182.
34. RODRIQUEZ, E. M. 1970. Ependymal specializations. I. Fine structure of the neural (internal) region of the toad median eminence, with particular reference to the connections between the ependymal cells and the subependymal capillary loops. *Z. Zellforsch. Mikrosk. Anat.* **102**:153.
35. ROSENBLUTH, J. 1966. Redundant myelin sheaths and other ultrastructural features of the toad cerebellum. *J. Cell Biol.* **28**:73.
36. ROSENBLUTH, J. 1968. Functions of glial cells. *In* The Central Nervous System. O. Bailey and D. Smith, editors. The Williams & Wilkins Company, Baltimore, Md. 21.
37. RUDE, S., R. E. COGGESHALL, and L. S. VAN ORDEN. 1969. Chemical and ultrastructural identification of 5-hydroxytryptamine in an identified neuron. *J. Cell Biol.* **41**:832.
38. SHARP, P. J., and B. K. FOLLETT. 1968. The distribution of monoamines in the hypothalamus of the Japanese quail. *Coturnie japonica. Z. Zellforsch. Mikrosk. Anat.* **90**:245.
39. SMITH, C. A., and F. S. SJÖSTRAND. 1961. Structure of the nerve endings on the external hair cells of the guinea pig cochlea as studied by serial section. *J. Ultrastruct. Res.* **5**:523.
40. STERBA, B., editor. 1969. Zirkumventrikulare Organe und Liquor. VEB Fischer, Jena, East Germany.
41. TAXI, J. 1962. Étude au microscope électronique de synapses ganglionnaire chez quelque Vertébrés. *Proc. 4th Int. Congr. Neuropathol.* **2**:197.
42. VIGH, B. 1969. The paraventricular organ, its structure and function. *In* Zirkumventrikulare Organe und Liquor. G. Sterba, editor. VEB Fischer, Jena, East Germany.
43. VIGH-TEICHMANN, I., P. ROHLICH, and B. VIGH. 1969. Licht- und elektronenmikroskopische Untersuchungen am Recessus praeopticus-Organ von Amphibien. *Z. Zellforsch. Mikrosk. Anat.* **98**:217.
44. VIGH-TEICHMANN, I., P. ROHLICH, and B. VIGH. 1969. Fluorescence histochemical studies on the preoptic recess organ in various vertebrates. *Acta Biol. Acad. Sci. Hung.* **20**:473.
45. WILLIAMS, T. H., and S. L. PALAY. 1969. Ultrastructure of the small neurons in the superior cervical ganglion. *Brain Res.* **15**:17.
46. WOOD, J. G., and R. J. BARRNETT. 1964. Histochemical demonstration of norepinephrine at a fine structural level. *J. Histochem. Cytochem.* **12**: 197.
47. YATES, R. D., and J. A. MASCORRO. 1970. Electron microscopic studies of sympathetic paraganglia. *Anat. Rec.* 166(2):400.