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Cytological Evidence for Different Types of Cerebrospinal Fluid-Contacting Subependymal Cells in the Preoptic and Infundibular Recesses of the Frog

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Summary. Blue-green fluorescent subependymal cells with intraventricular processes were shown by the fluorescent histochemical method to be distributed from the preoptic recess to the infundibular recess of the frog hypothalamus. Electron microscopy revealed at least two types of CSF-contacting subependymal cells, type 1 containing large dense granules (about 100–200 nm in diameter) and type 2 containing small dense core vesicles (about 60–100 nm in diameter). Subsequent to fixation in permanganate solution, the small dense core vesicles in type 2 cells reacted with the fixative and consistently showed a dense content. However, the large granules in type 1 cells were mostly pale or less dense after this fixation.

Two hours after intraventricular injection of ^{3}H-dopamine, a large number of silver grains appeared only in the cytoplasm of intraventricular processes possessing dense core vesicles (type 2 cells). A few grains were also found in the perikarya. It is concluded that type 2 cells are catecholamine-storing cells. It is suggested that type 1 cells in the infundibular recess are peptidergic neurons which may secrete some hypothalamic regulating hormones of the anterior pituitary. Most of these cells in the preoptic recess belong to the neurosecretory cells of the preoptic nucleus, while some cells probably function similarly to those in the infundibular recess.

Key words: CSF-contacting subependymal cells – Frog hypothalamus – Fluorescence microscopy – Autoradiography – Ultrastructure.

Introduction

Since the demonstration of catecholamine-containing cells by means of the fluorescent histochemical method (Falck et al., 1962), several studies have been

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reported on the distribution of fluorescent subependymal cells possessing fluorescent apical processes in contact with the cerebrospinal fluid (CSF) in the amphibian diencephalon (Vigh-Teichmann, 1969; McKenna and Rosenbluth, 1971; Parent, 1973; Chacko et al., 1974). Ultrastructural aspects of these neurons in Amphibia have been presented by many authors (Vigh-Teichmann et al., 1969; Peute, 1969, 1971; Vigh and Vigh-Teichmann, 1971; McKenna and Rosenbluth, 1971, 1974; Dierickx et al., 1972; Peute and Meij, 1973; Chacko et al., 1974).

Since Richardson (1966) first demonstrated the small granular vesicles containing noradrenaline in the peripheral sympathetic neurons at the ultrastructural level using potassium permanganate as a fixative, it is now possible to identify the storage sites of noradrenaline in the various granular and small dense core vesicles in central nervous tissue (Hökfelt and Jonsson, 1968; Hökfelt, 1968; Nakai, 1971).

In the present study, the classification of catecholamine-fluorescent subependymal cells is discussed, based on microspectrofluorometry. For the purpose of identification of the chemical nature of the different intracytoplasmic dense granules and dense core vesicles in the CSF-contacting subependymal cells, potassium permanganate was used as fixative, and electron microscopic autoradiography using ^{3}H-dopamine was performed for a correlative study.

Material and Methods

Forty-five adult frogs, *Rana nigromaculata* and *Rana catesbeiana*, of both sexes were used. The preoptic and the infundibular recesses were removed for subsequent fluorescence histochemistry and electron microscopy.

Fluorescence Microscopy

Specimens of the whole brain of normal frogs were prepared after the formaldehyde-induced fluorescent technique (Falck et al., 1962). Paraffin-embedded whole brains were sectioned frontally as well as sagittally. Deparaffinized 10 μm thick sections were examined under a FL microscope (Nikon) equipped with an improved filter system for monoamines (Ochi and Hosoya, 1974). Some slightly deparaffinized sections were subjected to microspectrofluorometric measurement by an instrument which consists of a transmitted-light dark field microscope and the MSA system (Farrand). Due to insufficient performance of the instrument for detection of excitation maximum under 350 nm, the excitation peak of catecholamine at 320 nm could hardly be obtained, and presented values here are all uncorrected instrument readings.

Simple differentiation between dopamine and noradrenaline: Model experiments using formaldehyde-treated, dried droplets containing dopamine or noradrenaline (1 mg/ml of 2% bovine serum albumin solution buffered at pH 7.4) demonstrated that the excitation and emission maxima of the catecholamine are at 415 nm and at 480 nm, respectively. HCl vapor exposure for only three to five seconds shifted the excitation maximum of both dopamine and noradrenaline fluorescence to 380 nm. After additional HCl vapor treatment for up to five minutes, the excitation maximum of dopamine fluorescence remained at 380 nm, whereas that of noradrenaline could no longer be detected at that wave length.

Electron Microscopy

(1) Thirty frogs were sacrificed by decapitation. The exposed brain was immediately covered with several drops of cold 2% glutaraldehyde fixative and then removed. Small blocks containing the

walls of the preoptic and infundibular recesses were fixed for 1 h in cold 2% glutaraldehyde solution buffered with 0.1 M phosphate (pH 7.4), and then postfixed for 1 h in 1% osmium tetroxide solution buffered with 0.2 M phosphate (pH 7.4).

(2) Whole brains of twelve frogs were immediately removed and immersed in either cold 3% $KMnO_4$, 9% $LiMnO_4$ or 9% $NaMnO_4$ fixatives buffered with 0.1 M phosphate (pH 7.0) for 2 h. The hypothalamic regions were removed from the whole brain in cold 50% acetone solution.

(3) The skulls of three frogs were opened under anesthesia with MS-222 (meta aminobenzoic acid ethylester methane sulfonate). Dopamine-H^3 (2–3 μCi/g body weight, specific activity 9.3 Ci/mM) was injected into the third ventricle via the posterior region of the pineal organ with the aid of a stereotaxic instrument. Two hours after the intraventricular injection, the frogs were sacrificed by decapitation. The hypothalamus was fixed for 1 h in cold 2% glutaraldehyde solution and then postfixed for 2 h in cold 1% osmium tetroxide solution.

All tissues fixed in glutaraldehyde followed by osmium tetroxide or in permanganate solutions were dehydrated in a graded series of ethanol, or acetone, and embedded in Epon 812. Sections were cut with glass knives on a Porter-Blum microtome.

The sections for autoradiography picked up on uncoated grids were coated with carbon and then with Ilford L_4 emulsion by the wire-loop technique. Following exposure for 1 month at 4° C, the autoradiograms were developed in Microdol X and fixed with 20% sodium thiosulfate.

Ultrathin sections were doubly stained with uranyl acetate and lead hydroxide and examined with Hitachi HS-9 and HU-12 electron microscopes.

Results

Fluorescence Microscopy

The blue-green fluorescent cells were clearly visible among and beneath the non-fluorescent ependymal cell proper in the walls of the preoptic and lateral recesses. The blue-green fluorescent material was uniformly distributed throughout the cytoplasm of the cell bodies and intraventricular processes in contact with the cerebrospinal fluid. The apices of the intraventricular processes were often the most intensely fluorescent (Fig. 1). Fluorescent basal processes were seldom found extending into the subjacent neuropil of the brain. The number of fluorescent cells in the preoptic recess appeared to gradually diminish from the rostral to the caudal region. In the infundibular recess, fluorescent cells were scarce. No yellow fluorescent cells were found in either the preoptic or infundibular recesses.

Catecholamine fluorescence in the apical enlargements of the ventricular processes showed excitation/emission maximum at a wavelength of 415/480 nm. Upon HCl vapor exposure for three to five seconds, the excitation peak shifted to 380 nm with about twofold increase of fluorescence intensity. The shifted peak remained unchanged after further treatment for up to five minutes, in spite of an accompanying slight decrease in fluorescence intensity (Fig. 2). This suggests that the demonstrated catecholamine was dopamine.

Electron Microscopy

1. Glutaraldehyde and OsO_4 Fixation

At least two types of CSF-contacting subependymal cells, which were located beneath the ependymal cells and possessed short club-like intraventricular

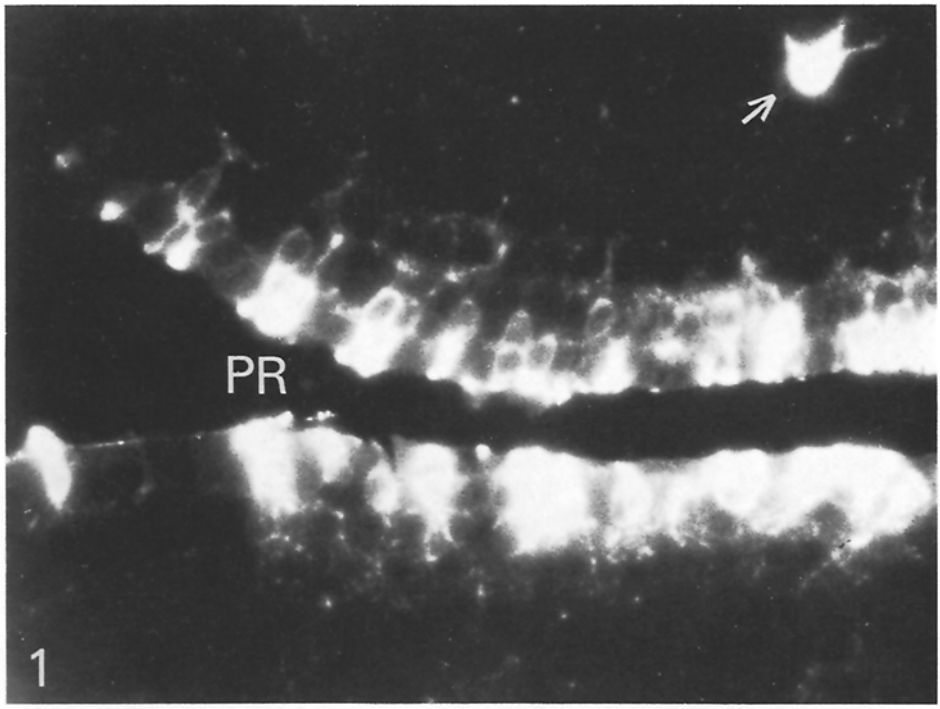


Fig. 1. Transverse section through the preoptic recess. Fluorescence micrograph showing the fluorescent subependymal cells on both sides of the preoptic recess (*PR*). Within each cell the nucleus appears dark. A fluorescent cell is seen in the deeper portion (*arrow*). ×400

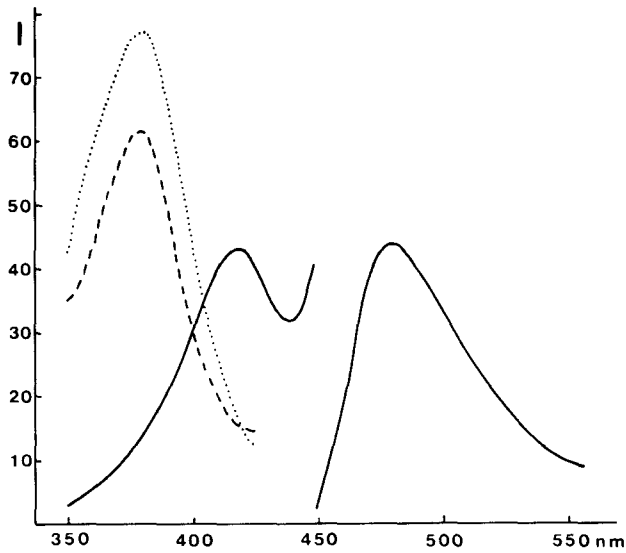


Fig. 2. Excitation (left) and emission (right) spectra of formaldehyde-induced fluorescence in a subependymal cell of the frog preoptic recess. *I* indicates arbitrary units of relative fluorescence intensity. All curves are obtained from uncorrected instrument values. —— after formaldehyde treatment only; ···· after a subsequent treatment with HCl vapor for only 5 s; ----- after an additional treatment with HCl vapor up to 5 min

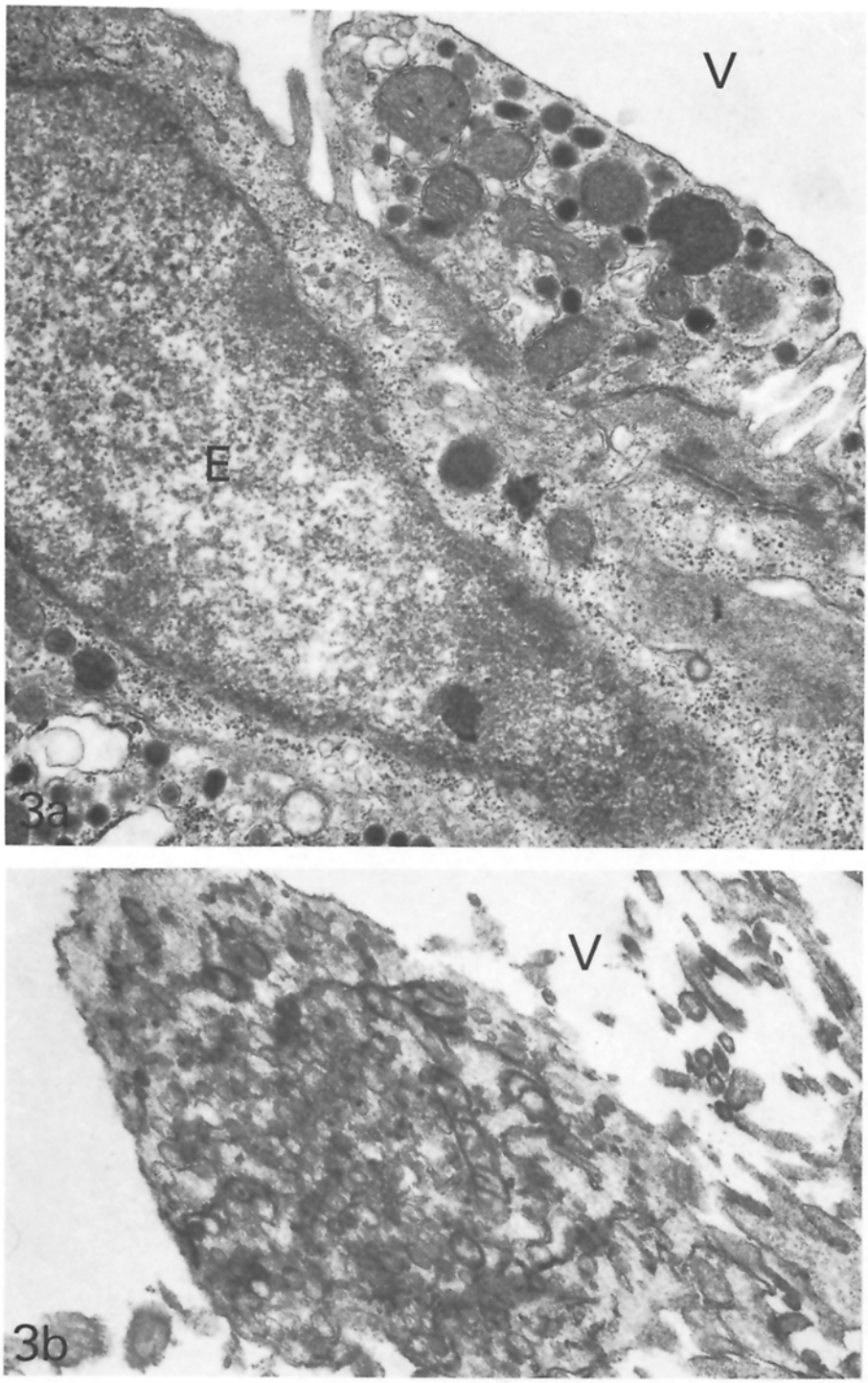


Fig. 3a. The processes of a type 1 subependymal cell on and beneath the ependymal cell (*E*) in the infundibular recess. Many large dense granules are seen in the cytoplasm of the processes on the ependymal cell surface and beneath the ependymal cell. *V* third ventricle. Glutaraldehyde and OsO_4 fixation. ×25,000. **b** An intraventricular process of type 1 cell in the preoptic recess. Many large electron-lucent or less dense granules are seen in the cytoplasm. *V* third ventricle. $LiMnO_4$ fixation. ×22,000

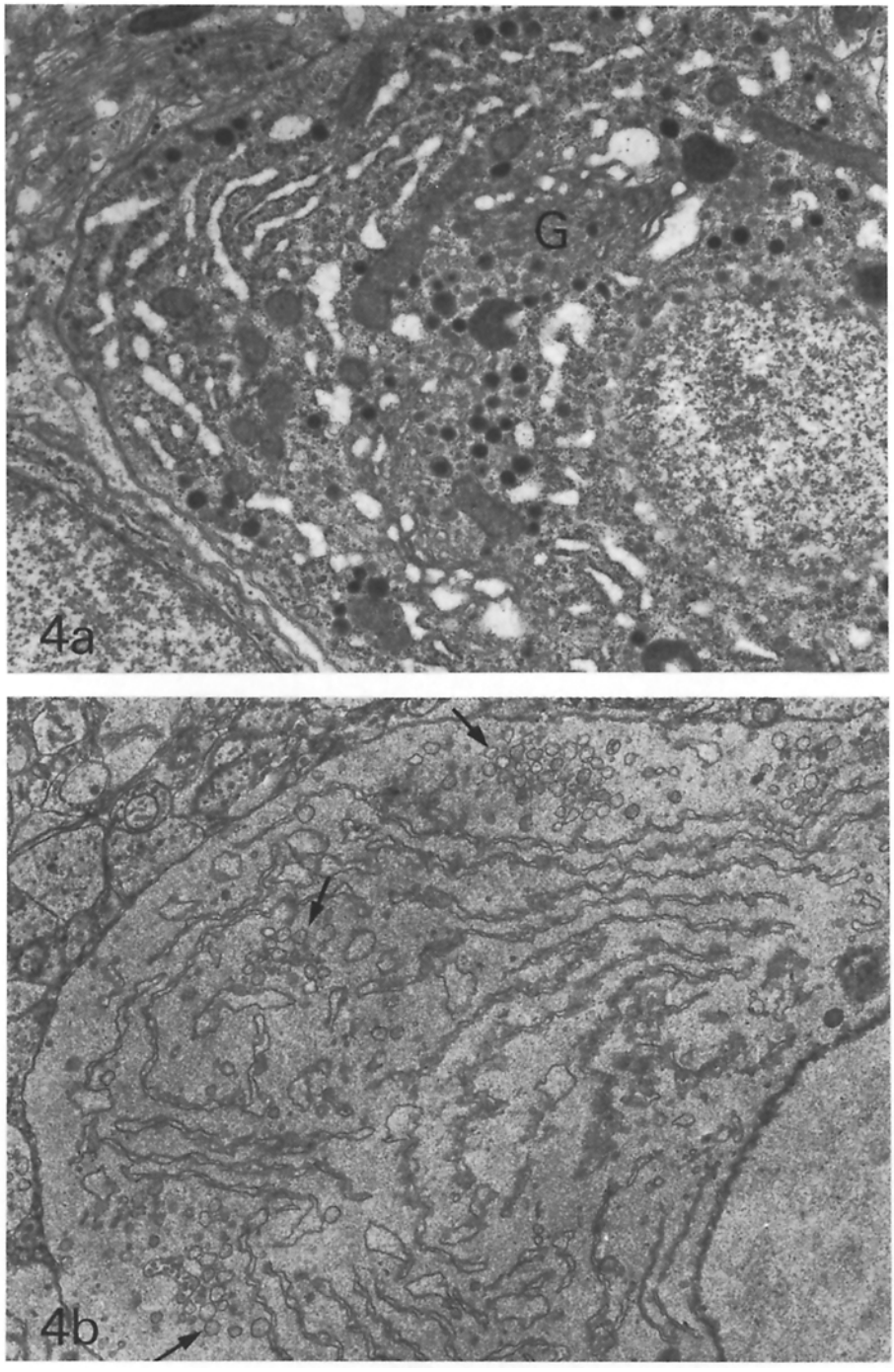


Fig. 4a and b. Type 1 cells beneath the ependyma in the infundibulum. **a** Many large dense granules are seen in the perikaryon. *G* Golgi apparatus. Glutaraldehyde and OsO_4 fixation. ×13,000. **b** Many large electron lucent or less dense granules (*arrows*) are seen especially in the periphery of the perikaryon. $LiMnO_4$ fixation. ×11,000

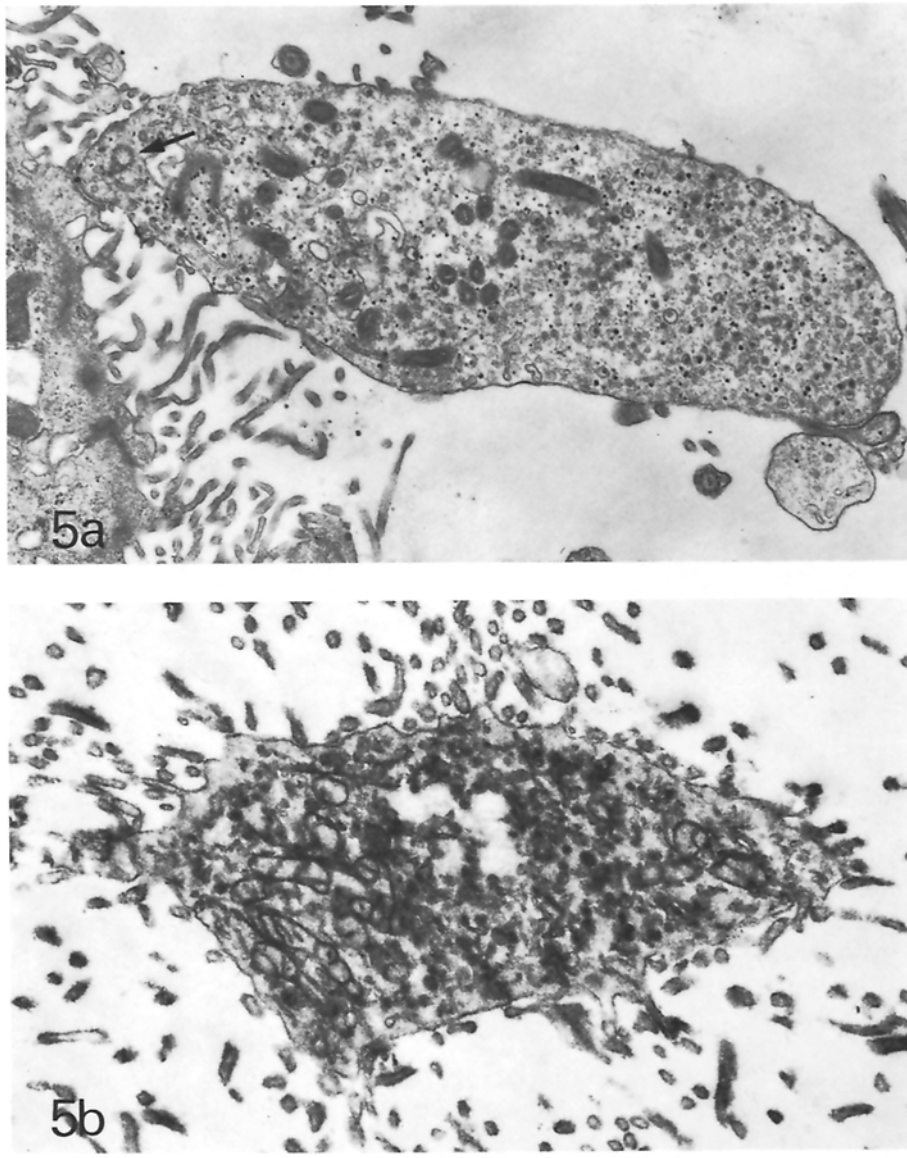


Fig. 5a. An intraventricular process of a type 2 subependymal cell in the infundibular recess. Many small dense core vesicles, mitochondria, glycogen particles and a basal body (*arrow*) are seen in the cytoplasm. Glutaraldehyde and OsO_4 fixation. $\times 3{,}600$. **b** Cross section of an intraventricular process of a type 2 cell in the preoptic recess. Microvilli are present on the surface of the intraventricular process and in the third ventricle. Many small dense core vesicles and mitochondria are seen in the cytoplasm. $NaMnO_4$ fixation. $\times 21{,}000$

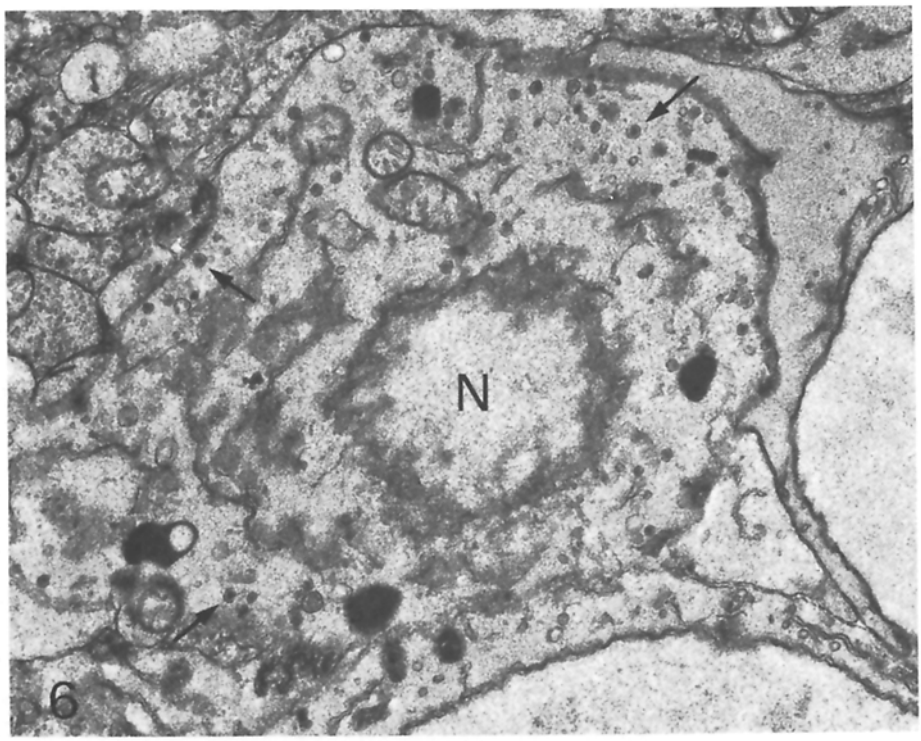


Fig. 6. Type 2 subependymal cell in the infundibulum. Many small dense core vesicles (*arrows*) are seen in the perikaryon. *N* nucleus. $LiMnO_4$ fixation. ×18,000

processes in contact with the cerebrospinal fluid, could be classified by the size and the structure of the dense granules and the dense core vesicles present in the cytoplasm of the cell body and the intraventricular processes. Two different types of subependymal cells were observed in the ventro-lateral walls of both the preoptic and the infundibular recesses.

Type 1 Cells. This type was characterized by the presence of numerous large and round dense granules about 100–200 nm in diameter in the perikaryon as well as in the intraventricular process (Figs. 3a, 4a). Most of the granules contained a homogeneous dense content with a very thin outer halo (Figs. 3a, 4a) and were very similar to the neurosecretory elementary granules in the frog neurohypophysis. These cells were abundantly present in the rostral part of the infundibular recess, but were scattered diffusely in the region of the preoptic recess. Generally, they appeared to increase in number from the preoptic region to the infundibular region.

In addition to the above described dense granules, mitochondria, well developed rough endoplasmic reticulum, numerous free ribosomes, microtubules, microbodies and Golgi apparatus consisting of cisternal and vesicular elements were present in the perikaryal cytoplasm (Fig. 4a). Immature small dense gran-

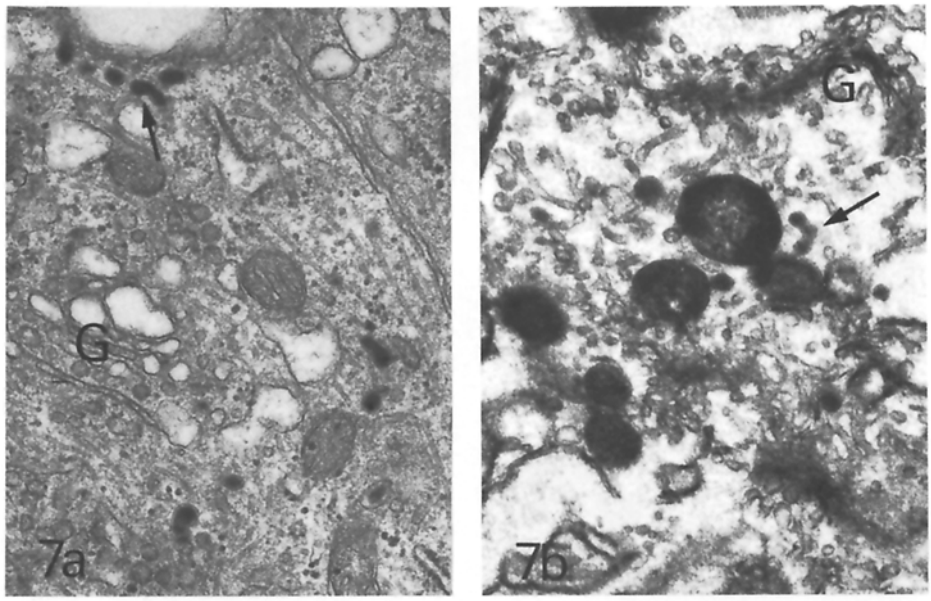


Fig. 7a and b. The perikarya of subependymal cells in the infundibulum. Rod-shaped dense granules (*arrows*) are seen. *G* Golgi apparatus. **a** Glutaraldehyde and OsO_4 fixation. ×29,000. **b** $NaMnO_4$ fixation. ×29,000

ules were occasionally observed at the extremities of the cisternae of the Golgi lamellae.

Type 2 Cells. This type was characterized by the presence of small dense core vesicles (60—100 nm in diameter) with a wider electron lucent zone between the dense core and the limiting membrane. The dense core vesicles were found in the perikarya as well as in the intraventricular club-like processes (Fig. 5a). In most of the dense core vesicles the core itself was located eccentrically (Fig. 5a). Cells belonging to this type were almost equally distributed throughout both regions of the preoptic and the infundibular recesses.

In addition to the above described two types of CSF-contacting subependymal cells, intermediate types of cells were often recognized in the infundibular recess. One intermediate cell type contained round and moderately dense granules ranging mostly between 100 and 130 nm in diameter. Another type of CSF-contacting subependymal cell contained a large number of rod-shaped granules of high density and a few round dense core vesicles which were similar to those of type 2 cells (Figs. 7a, 8). These rod-shaped granules were about 200 × 100 nm in size, and an electron lucent space occurred between the limiting membranes and the dense core (Fig. 7a). This cell type was also characterized by the presence of numerous mitochondria in the cytoplasm of the intraventricular processes. The cells of this type were observed in the rostral region of the infundibular recess and their intraventricular processes were often crowded together (Fig. 8).

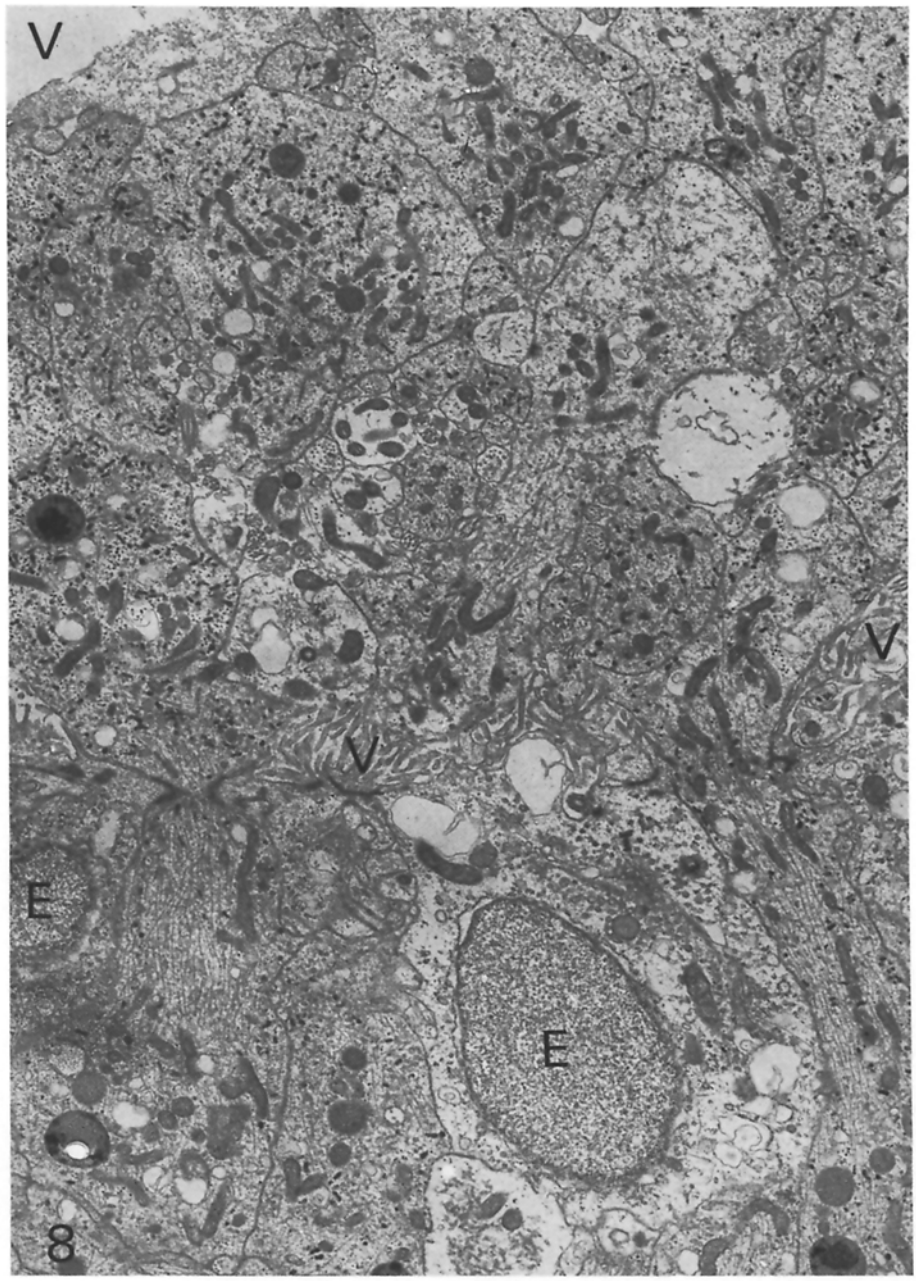


Fig. 8. Aggregation of many intraventricular processes of subependymal cells containing both rod-shaped and round dense granules in the infundibular recess. *E* ependymal cells, *V* third ventricle. Glutaraldehyde and OsO_4 fixation. × 7,000

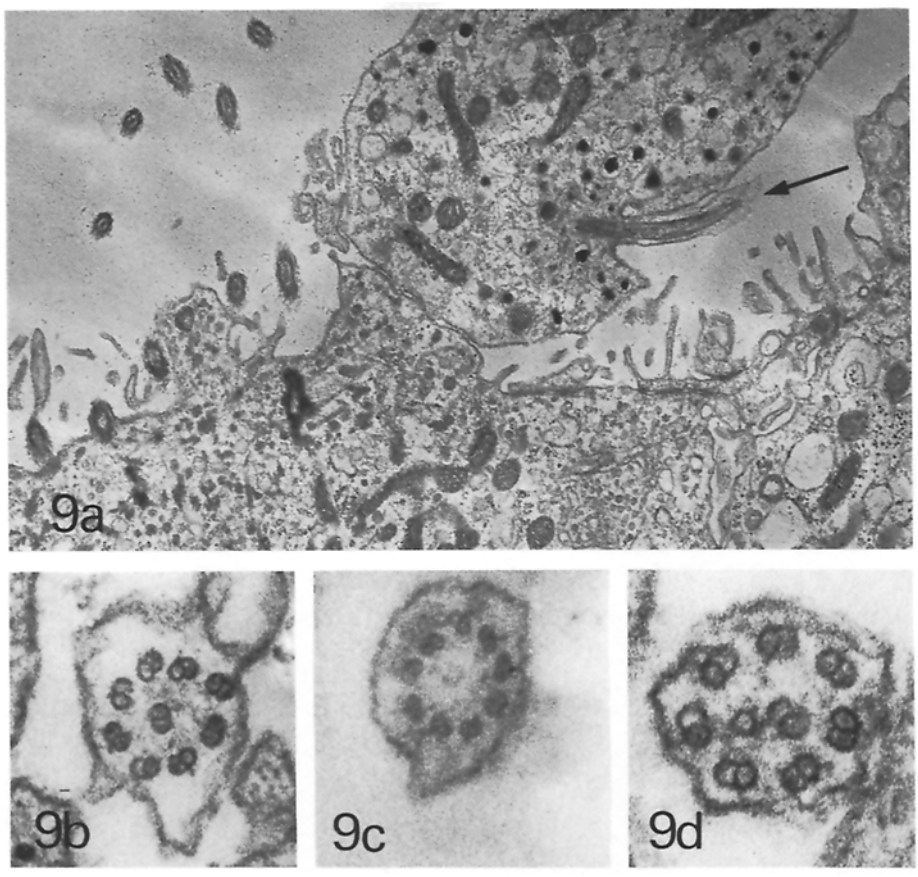


Fig. 9a. A cilium (*arrow*) in the intraventricular process of a type 2 cell. Glutaraldehyde and OsO_4 fixation. ×12,000. **b** Cross section of type 8+1 cilium. ×100,000. **c** Cross section of type 9+0 cilium. ×80,000. **d** Cross section of type 7+1.5 cilium. ×100,000

The intraventricular processes of both cell types possessed cilia (Fig. 9a). Cross sections of the cilia revealed an atypical 8+1 distribution of paired microtubules (Fig. 9b) and the 9+0 arrangement was occasionally present (Fig. 9c). Rarely, an atypical type of cilium consisting of seven pairs of peripheral microtubules and one pair plus a single central microtubules (7+1.5) was observed (Fig. 9d).

2. Permanganate Fixation

The small dense core vesicles of type 2 cells observed in material fixed with glutaraldehyde-OsO_4 reacted with the permanganate fixatives and revealed an increased electron density. These small dense granules (about 100 nm in diameter) were observed in the cytoplasm of both the cell bodies and the intraventricu-

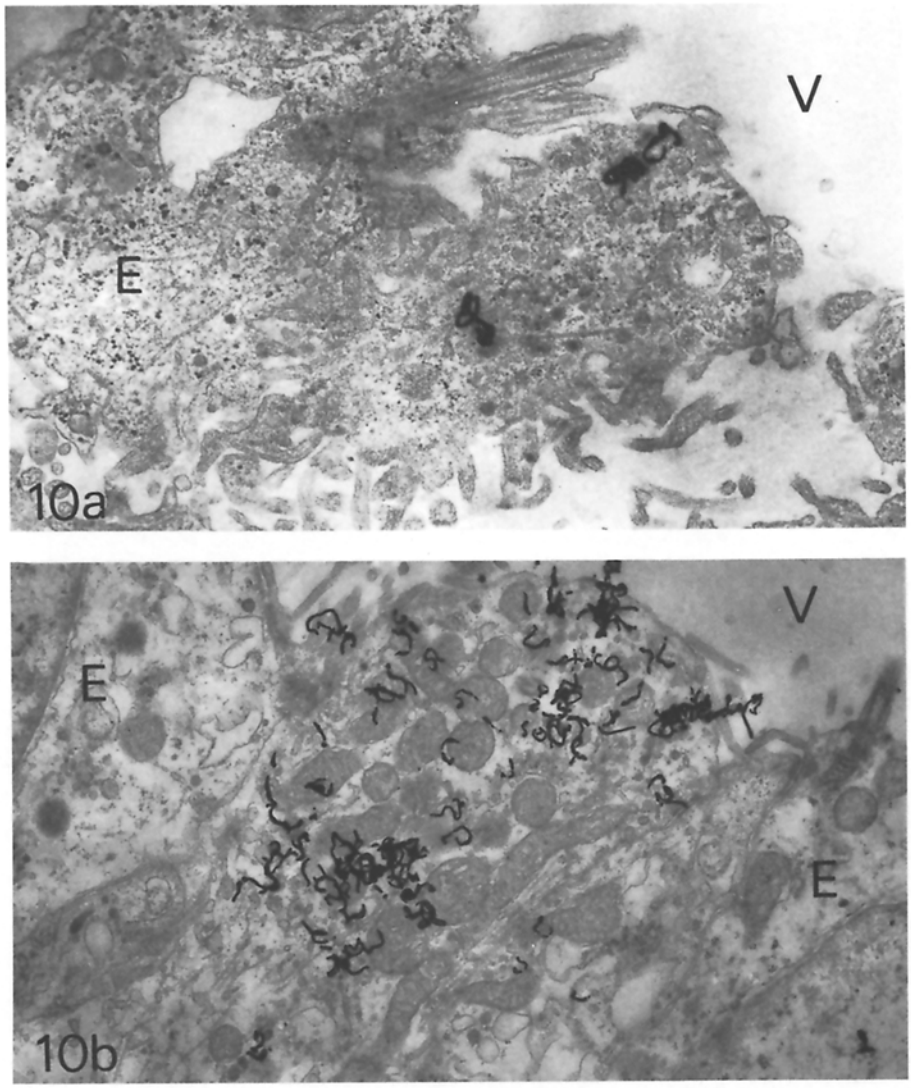
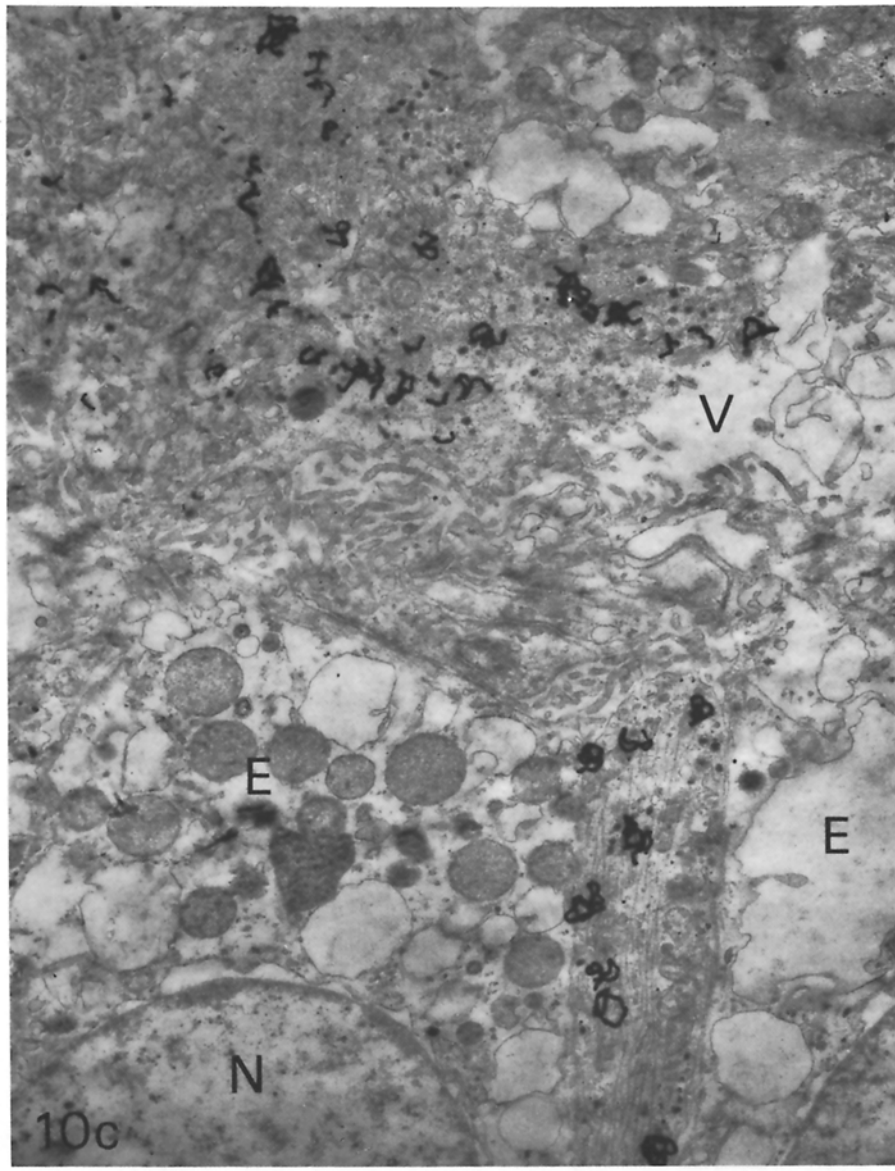


Fig. 10a–c. The preoptic recess 2 h after intraventricular injection of ^{3}H-dopamine. **a** Silver grains are seen over the dense granules in the intraventricular process of a type 2 cell. *E* ependymal cell, *V* third ventricle. ×14,000. **b** Many silver grains are scattered over the intraventricular process of a type 2 cell containing a large number of mitochondria and small dense granules. *E* ependymal cells, *V* third ventricle. ×16,000. **c** The infundibular recess 2 h after intraventricular injection of ^{3}H-dopamine. Aggregation of many intraventricular processes of subependymal cells containing small dense core vesicles and rod-shaped dense granules. Cell process between ependymal cells (*E*). Many silver grains are seen over the intraventricular processes and the process between the ependymal cells (*E*), but no silver grains are seen in the ependymal cells. *N* nucleus of ependymal cell, *V* third ventricle. ×25,000



lar processes of type 2 cells (Figs. 5b, 6). After fixation with 9% $NaMnO_4$ or 9% $LiMnO_4$ the electron density of the small granules was more intense than that of granules in cells fixed with 3% $KMnO_4$. On the other hand, the large dense granules found in the type 1 cells for the most part did not react with the permanganate fixatives. Although the large dense granules of type 1 cells were mostly found as electron lucent vesicles or granules of very low electron density, a few granules contained a content of moderate electron density

(Figs. 3b, 4b). The rod-shaped granules were reactive with the permanganate fixatives and showed almost the same density as the granules of type 2 cells (Fig. 7b).

3. Autoradiography

Two hours after intraventricular injection of ^{3}H-dopamine silver grains accumulated in type 2 CSF-contacting subependymal cells. Most of the silver grains were distributed within the cytoplasm of the intraventricular processes and were observed to be in close association with the small dense core vesicles (Fig. 10a, c). A large number of silver grains were also present in the intraventricular processes containing a large number of mitochondria and small dense core vesicles, which were covered by clusters of silver grains (Fig. 10b). The subependymal cells containing both rod-shaped and round dense granules were labeled by a relatively large aggregation of silver grains (Fig. 10c). Silver grains were not detected in the intraventricular process nor in the perikaryon of the type 1 CSF-contacting subependymal cells containing large dense granules. Silver grains were not found in ependymal cells.

Discussion

As to the intraventricular neural processes of the neurosecretory nuclei, Scharrer (1933) first observed in *Bufo vulgaris* that the neurons of the preoptic nucleus send their processes into the third ventricle. Later, Okada et al. (1955) reported similar findings in the frog hypothalamus.

Many electron and fluorescence microscopic studies have been reported in various vertebrates on the CSF-contacting subependymal cells. Based on the direct contact of the apical cell processes with the CSF, these subependymal cells were first termed liquor-contacting or CSF-contacting neurons by Vigh (1969) and Vigh et al. (1969). On the other hand, McKenna and Rosenbluth (1971) observed catecholamine-containing cells bordering the preoptic recess of the toad hypothalamus by both fluorescence and electron microscopy and proposed the name "encephalo-chromaffin cells" based on the cytological characteristic that the subependymal cells themselves do not form presynaptic contacts with the neurons and the fact that they border directly on the cerebrospinal fluid. In the present study we have used the term "CSF-contacting subependymal cell" which underlies the ependymal cell and sends apical processes into the third ventricle between adjacent ependymal cells.

In the frog diencephalon the presence of green fluorescent catecholamine-containing cells bordering the third ventricle with bright fluorescent club-like intraventricular protrusions has been demonstrated by fluorescent microscopy (Vigh-Teichmann et al., 1969; McKenna and Rosenbluth, 1971, 1974; Parent, 1973; Peute and Meij, 1973). The present study demonstrates an abundance of fluorescent subependymal cells in the preoptic recess which gradually decreases towards the infundibular recess. Terlou and Ploemacher (1973) observed two types of fluorescent cells in the paraventricular organ of tadpoles of *Xenopus*

laevis; green cells, containing a catecholamine and yellow-orange cells, presumably containing a tryptamine. In this study, apart from the paraventricular organ, the latero-ventral wall of the infundibular recess was observed and no yellow-orange fluorescent cells were found.

In order to clarify the physiological function of fluorescent subependymal cells, it is essential to identify the nature of the catecholamine contained in them. The excitation peak shift of the catecholamine fluorescence from 420 nm to 380 nm even after HCl vapor treatment for five minutes indicates that the fluorescence is due to dopamine (Björklund et al., 1972), in accordance with the finding on paraventricular organs of *Xenopus laevis* tadpoles by Terlou and van Kooten (1974) as well as that on preoptic recess organs of *Rana temporaria* by Prasada Rao and Hartwig (1974).

The ultrastructure of the CSF-contacting neurons of the preoptic nucleus was first observed in the Pacific Tree Frog by Smoller (1964). Later, similar findings were observed in an urodele (Vigh-Teichmann et al., 1970; Vigh and Vigh-Teichmann, 1973). Neurosecretory elementary granules with a mean diameter of 150–250 nm in the ventricular process stained positively with chromalum gallocyanine (Vigh and Vigh-Teichmann, 1973). Chacko et al. (1974) showed two types of aminergic CSF-contacting neurons in the preoptic recess of *Bufo poweri*. They have shown that electron lucent neurons contain dense core vesicles in the perikaryon ranging in diameter from 80 to 100 nm and larger vesicles (150–180 nm) in the intraventricular protrusions. In addition, electron dense neurons contained many free ribosomes and similarly sized dense core vesicles (80–100 nm in diameter) in both the cytoplasm of the perikaryon and the intraventricular process. McKenna and Rosenbluth (1971) reported that subependymal cells in the preoptic recess of the toad show green fluorescence and possess dense core vesicles, ranging in diameter from 40 to 220 nm in the cytoplasm of the cell body and ventricular process when fixed in glutaraldehyde and osmium tetroxide. They also described the presence of juxta-nuclear bundles of tightly packed filaments.

In the amphibian infundibular recess the neurons forming the intraventricular endings have been observed electron microscopically (Pehlemann, 1969; Dierickx et al., 1972; Vigh and Vigh-Teichmann, 1973; Peute, 1973; Peute and Meij, 1973). Peute (1973) classified two cell types of CSF-contacting neurons in the nucleus infundibularis dorsalis of *Xenopus laevis*, namely, type I cells containing light electron dense core vesicles (60–100 nm in diameter) and type II cells containing round to dumb-bell shaped vesicles (90–140 nm in diameter) with a dense core of greater electron density as well as an increase in polysomes and mitochondria compared with type I cells.

Two different types of CSF-contacting subependymal cells were also recognized in the present study in the infundibulum fixed with glutaraldehyde and osmium tetroxide: type 1 cells containing large, round dense granules (100–200 nm in diameter) and the type 2 cells containing small dense core vesicles (60–100 nm in diameter). After fixation in permanganate solution small dense core vesicles in type 2 cells react positively, whereas the large granules of type 1 cells are only slightly reactive or negative to permanganate. These results seem to suggest that the small dense core vesicles of type 2 cells correspond

to catecholamine-containing vesicles and the large granules of type 1 cells probably contain a substance of peptidergic nature. Although the intraventricular processes containing large dense granules (100–200 nm in diameter), which are very similar in size and structure to the neurosecretory elementary granules in the preoptic nucleus, are found in the preoptic recess, they are also much more abundant in the infundibular recess. These findings do not allow a definite conclusion as to whether these large dense granules are identical with Gomori-positive neurosecretory elementary granules, or whether they contain some other peptidergic component. It may be considered that some intraventricular processes containing large electron lucent vesicles or less dense granules in the preoptic recess after permanganate fixation belong to the preoptic nucleus; however, similar large dense granules in the intraventricular processes of the infundibular recess probably contain another secretory product of peptidergic nature. The latter are different from the neurosecretory elementary granules having affinity for Gomori's aldehyde fuchsin. Large dense granules in the intraventricular processes of the infundibular recess are very similar to the granules in some nerve endings in the frog median eminence visualized when fixed with glutaraldehyde-OsO_4 or permanganate (Nakai, 1971). This evidence seems to suggest that the cells which contain large dense granules in the infundibular recess secrete regulating hormones (releasing or inhibiting hormones) affecting endocrine cells in the pars distalis into the cerebrospinal fluid. Biochemical evidence for the presence of thyrotropin releasing hormone in the cerebrospinal fluid has been given by Knigge and Joseph (1974) and Ishikawa (1973). Dierickx (1965) postulated in lesion experiments on *Rana temporaria* that aldehyde fuchsin-negative neurosecretory cells in the lobus infundibularis are involved in the regulation of the gonadotropic activity of the pars distalis.

After intraventricular injection of ^{3}H-dopamine, silver grains accumulated in the cytoplasm of the intraventricular processes of some type 2 cells and in cells containing rod-shaped granules in CSF-contacting subependymal cells in the frog preoptic and infundibular recesses. From autoradiographic findings and fluorescent microscopy it can be concluded that type 2 cells are catecholamine-containing cells. This seems to indicate that exogenous dopamine has a strong affinity for the intraventricular processes of subependymal cells, which may play an important regulatory role between the cerebrospinal fluid and some of the deeper neurons which have synaptic contacts with the subependymal cells.

Several atypical types of cilia have been reported in the intraventricular protrusions of the subependymal neurons of Amphibia by many authors; 8+1, 9+0, 10+1, 20+0 (Vigh-Teichmann et al., 1969), 8+1 (Peute, 1969), 8+1, 9+0 (Peute and Meij, 1973), and others. In the present study atypical 8+1 type cilia are the most frequently encountered and atypical 9+0 and typical 9+1 cilia are sometimes present in both types of the subependymal cells. However, an atypical 7+1.5 type is also rarely found in type 2 cells. It is not yet known whether the 8+1 type cilia have special function(s). Peute (1969) suggested that, in *Xenopus laevis*, these cilia-bearing cells display a secretory activity. Nevertheless, the possibility remains that they might also have a receptor function.

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