Evidence Suggesting the Presence of Serotonergic Nerve Terminals on Catecholamine-Containing Subependymal Cells in the Preoptic Recess Organ of the Bullfrog (*Rana catesbeiana*)

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SUMMARY

The innervation of serotonergic nerve fibers to catecholamine (CA)-containing subependymal cells (cerebrospinal fluid-contacting neurons) was studied in the preoptic recess organ of the bullfrog brain by immunohistochemistry together with CA fluorescence histochemistry. Microspectrofluorometric analysis indicated that fluorescent subependymal cells in the preoptic recess organ contained mainly dopamine. By light microscopy, a number of serotonin (5HT)-immunoreactive fibers and terminals were distributed in subjacent neuropils of the subependymal layer. Double staining using the same section indicated that 5HT-immunoreactive punctate structures resembling axon terminals were situated around subependymal perikarya showing tyrosine hydroxylase (a marker enzyme for CA-containing neurons)-like immunoreactivity. A correlative analysis of the same specimen with fluorescence and electron microscopes revealed that CA-containing subependymal cells possessed numerous electron-dense granules (80-150 nm in diameter) within the perikaryal cytoplasm. Finally, immunoelectron microscopic examination confirmed that 5HT-positive nerve terminals were located closely adjacent to subependymal somata that contained similar granules to those of CA-containing cells. Although a typical synaptic feature has not yet been observed between the two structures, the present results strongly suggest that CA-containing subependymal cells receive input of serotonergic neurons via axo-somatic synapses.

INTRODUCTION

The presence of monoamine-containing subependymal cells has been well established in frog brains using Falck-Hillarp's fluorescence

histochemical methods (Chacko et al., 1974; Prasada Rao and Hartwig, 1974; Tohyama et al., 1977; McKenna and Gorski, 1979; Shimizu et al., 1982). In the preoptic recess organ, catecholamine (CA) appears to be mainly responsible for monoamine-containing subependymal cells (Prasada Rao and Hartwig, 1974), although serotonin (5HT)-containing subependymal cells were also located in the paraventricular organ and nucleus infundibularis dorsalis (Shimizu et al., 1983). Such cells possessed a club-like apical process contacting the cerebrospinal fluid of the third ventricle, and also possessed a few basal processes extending to the brain parenchyma. Based on these morphological characteristics, some Golgi-impregnated cells in the preoptic recess organ seemed to correspond to CA-containing subependymal cells (McKenna et al., 1973; Shimizu et al., 1982) and ultrastructural analyses revealed that CA-containing subependymal cells contained numerous electron-dense granules and were occasionally terminated with several types of nerve fibers (Chacko et al., 1974; Nakai et al., 1977; McKenna and Gorski, 1979). However, there is no direct evidence showing the presence of electron-dense granules in CA-containing fluorescent subependymal cells and no neurochemical information has been provided about the nerve axons which terminate at the CA-containing subependymal cells.

The present study demonstrated that CA-containing fluorescent subependymal cells contain electron-dense granules and receive serotonergic nerve fiber innervation using fluorescence histochemical and immunohistochemical methods at the light and electron microscopic levels.

MATERIALS AND METHODS

Adult bullfrogs (*Rana catesbeiana*) of both sexes weighing 300-450 g were obtained between April and October. The animals were anesthetized by ether and the brains were processed for the following histochemical examinations.

Microspectrofluorometry

Three brains were rapidly removed and freeze-dried according to Falck-Hillarp's formaldehyde-induced fluorescence histochemistry (Falck et

al., 1962). Ten-micrometer-thick sections on quartz slides were lightly deparaffinized, mounted in liquid paraffin and covered with quartz slips. In order to differentiate monoamines, excitation and emission spectra of monoamines in the sections were analyzed by a Nikon microspectrofluorometer (improved SPM-RFL system, Ochi et al., 1979a). Some sections were exposed to HCl vapor for 3 sec to 3 min to differentiate catecholamines.

Immunohistochemistry

Ten animals were perfused with a solution containing 4 % paraformaldehyde and 0.2 % picric acid in 0.1 M sodium phosphate buffer (PB, pH 6.9), and the brains were post-fixed for 2 days in the same fixative and cut into 20-μm-thick coronal sections in a cryostat. The free-floating sections were incubated in a solution containing rabbit anti-5HT (1:500; Immuno Nuclear Corp., Stillwater, MN, USA) and sheep anti-tyrosine hydroxylase (1:500; Chemicon International, Inc., Temecula, CA, USA) for 48 h. Then the sections were further incubated in a solution containing fluorescein-conjugated donkey anti-rabbit IgG (1:100; Chemicon) and biotinylated donkey anti-sheep IgG (1:500; Chemicon), and finally incubated with Texas Red avidin D (1:100; Vector Laboratories, Burlingame, CA, USA). Dilution and washing were all performed with 0.1 M PB containing 0.9 % saline, 0.3 % Triton X-100 and 1 % bovine serum albumin (PBST-BSA, pH 7.4). After a final wash in PBST-BSA the sections were observed with a fluorescence microscope equipped with an ultraviolet excitation filter system for fluorescein, or with a green excitation filter system for Texas Red. To detect 5HT-like immunoreactivity at the electron microscopic level, post-fixed brains were cut into 20-μm-thick coronal sections in a cryostat and incubated with anti-5HT (1:500), anti-rabbit IgG (1:20; Chemicon) and rabbit peroxidase anti-peroxidase complex (1:100; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA). After detection of enzyme activity in a 3, 3'-diaminobenzidine hydrochloride (DAB) solution, the sections were fixed in 1 % OsO_4 in 0.1 M PB, dehydrated and embedded in Quetol-812 (Nisshin EM, Tokyo, Japan).

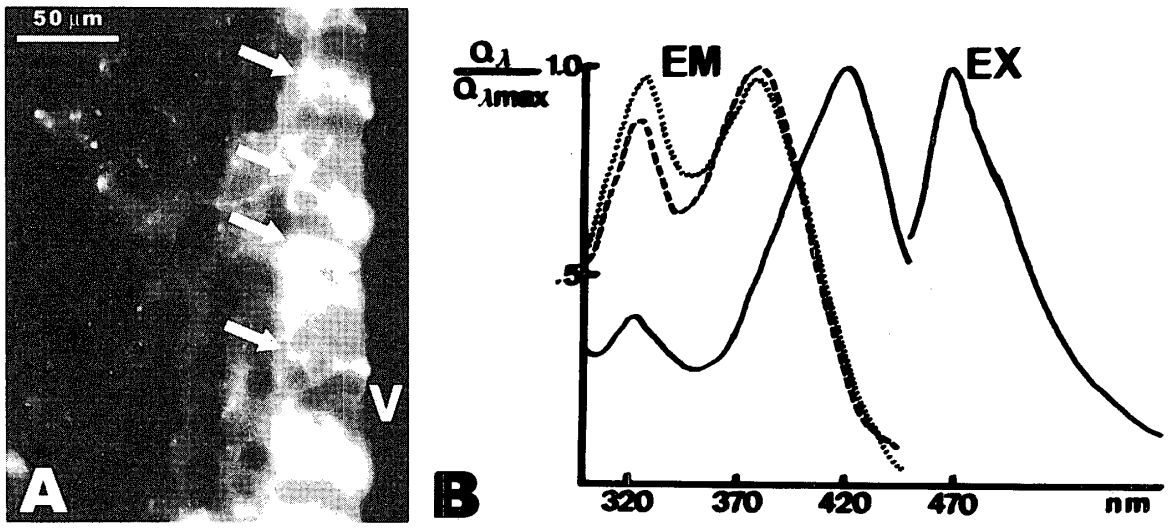

Fig. 1A. Fluorescence micrograph of the lateral wall of the preoptic recess treated with paraformaldehyde vapor. Each blue-green fluorescent cell (arrows) extends a thick club-like apical process to the preoptic recess of the third ventricle (V) and a few fine basal processes to the subjacent neuropil. Bar: 50 µm. **B.** Emission (EM) and excitation (EX) spectra of blue-green fluorescent cells seen in A. The spectra were automatically corrected instrumental values expressed as relative quanta versus wavelength (nm). Three excitation spectra were recorded from the non-treated specimen (solid line), the specimen treated with HCl vapor for 3 sec (broken line), and the specimen treated with HCl vapor for 3 min (dotted line), respectively.

CA fluorescence histochemistry and electron microscopy in the same section

These methods were performed according to the previous papers (Kataoka et al., 1979; Yamamoto et al., 1983). Briefly, three animals were perfused with a solution containing 4 % paraformaldehyde and 0.5 % glutaraldehyde in 0.1 M PB (pH 7.4). The brains were excised and cut into small pieces. The brain pieces containing the preoptic recess region were post-fixed overnight in the same solution at 4 ^{o}C, and dehydrated at 70 ^{o}C in a graded series of ethanol diluted with 8% paraformaldehyde. After washing in propylene oxide the samples were embedded in Quetol-812. Semi-ultrathin sections (ca. 0.2-µm thick) on a reference grid were observed under a fluorescence microscope. Subsequently, the same sections on the grid were observed under an electron microscope.

RESULTS

In the specimens exposed to paraformaldehyde vapor, blue-green fluorescent subependymal cells were observed along the lateral wall of the preoptic recess (Fig. 1A). Such fluorescent cell bodies gave rise to a thick

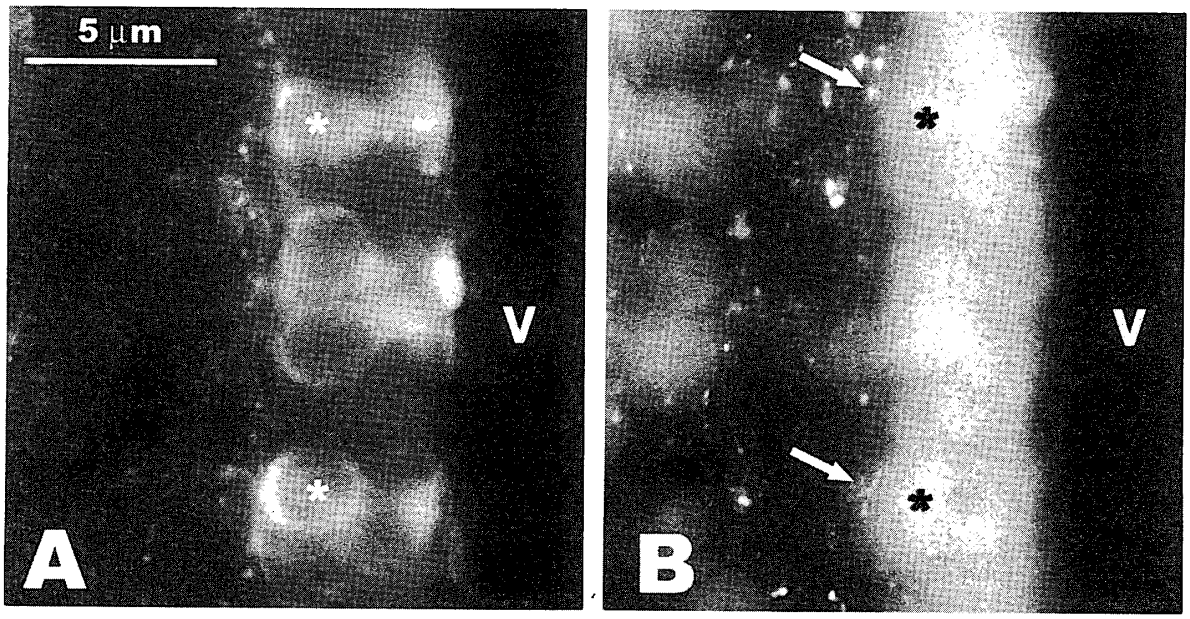

Fig. 2. Fluorescence micrographs showing TH-like immunoreactivity (**A**) and 5HT-like immunoreactivity (**B**) in the preoptic recess organ of the same section. Note that some fluorescent subependymal cells (asterisks in **A**) seem to be terminated by beaded 5HT-immunoreactive fibers (arrows in **B**). Asterisks in **B** indicate the same somata seen in **A**. Abbreviation: V, third ventricle. Bar: 5 μm.

apical process running among non-fluorescent ependymal cells and reaching the third ventricular surface. Basal processes of the cell were occasionally seen extending and ramifying into the underlying layers. Spectrum analysis of the fluorescent subependymal cells indicated that these cells contained CA. This was because emission spectra showed a peak at 470 nm, while excitation spectra had two peaks (Fig. 1B): a larger peak at 420 nm and smaller one at 320 nm. Treatment with HCl vapor for 3 sec changed the two excitation peaks into two large peaks with a similar height of 370 nm and 320 nm. No further change was observed by an additional treatment with HCl vapor for 3 min (Fig. 1B). The results indicated that the CA fluorescence was mainly due to dopamine.

Tyrosine hydroxylase (TH)-immunoreactive subependymal cells showed similar profiles to those of CA-containing subependymal cells visualized by fluorescence histochemistry (Fig. 2A). 5HT-immunoreactive nerve fibers and dot-like structures representing varicose fibers were scattered in the subjacent layer of the subependymal cell layer (Fig. 2B). Some 5HT-immunoreactive varicoses seemed to contact with the somata of subependymal cells showing TH-like immunoreactivity (Fig. 2A, B).

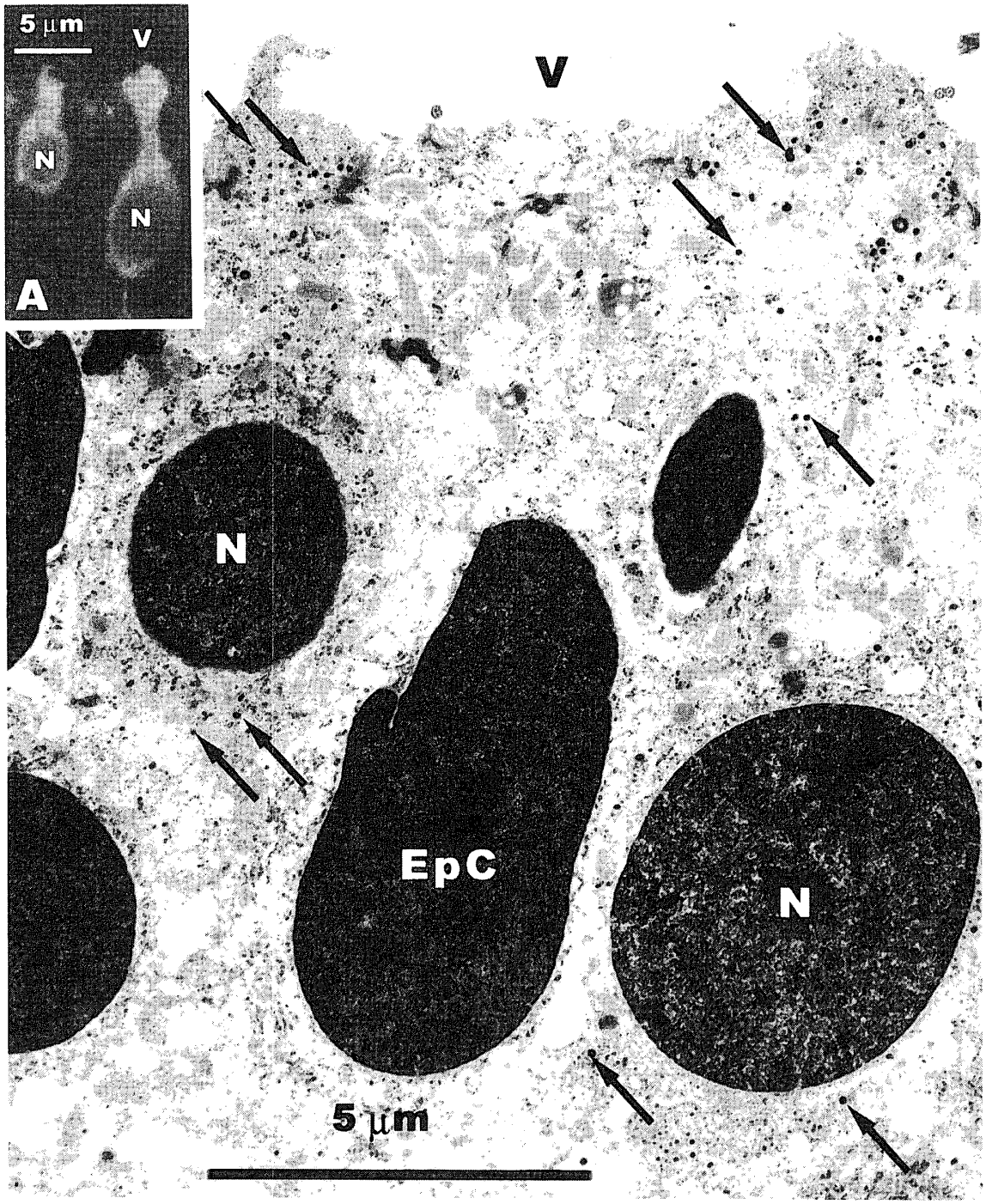

Fig. 3A. Fluorescence micrograph of a semi-ultrathin section on a reference grid. Note that two fluorescent subependymal cells (N) extend apical processes to the third ventricle (V). Bar: 5 µm. **B.** Electron micrograph of the same section in A. Note that the same fluorescent cells (indicated by N) contain spherical nuclei and many electron-dense granules (arrows). In contrast, a non-fluorescent cell (EpC) corresponds to an ependymal cell which contains an ovoid nucleus and does not contain electron-dense granules. Bar: 5 µm.

The CA fluorescent subependymal cells were also visible in Quetol-812-embedded sections (Fig. 3A), so they were examined with both light and electron microscopes. By correlative analysis, it was proven that the blue-green CA fluorescent cells possessed spherical nuclei and electron-dense

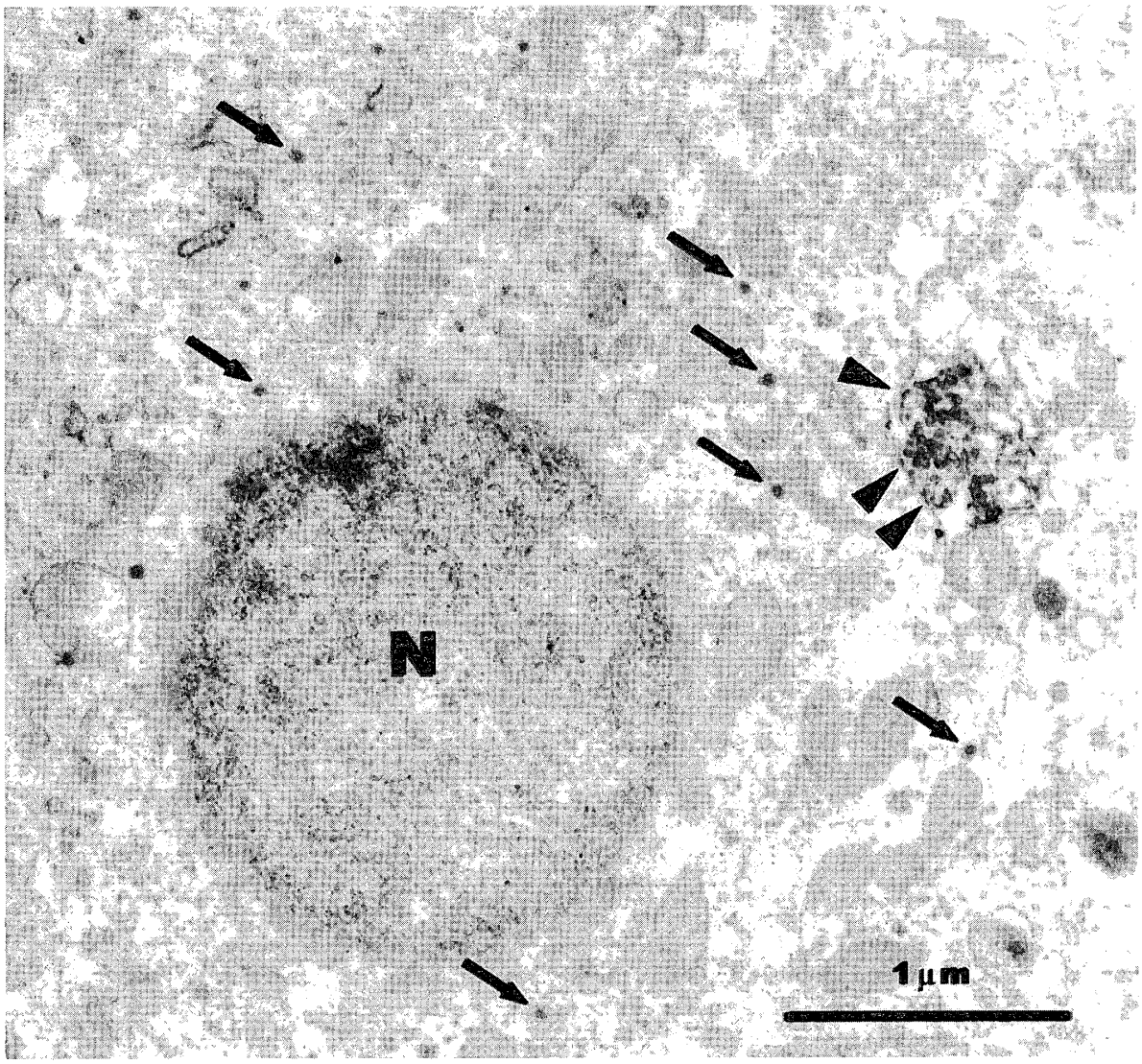

Fig. 4. Immunoelectron micrograph showing 5HT-like immunoreactivity in the preoptic recess organ. Note that an immunopositive terminal (arrowheads) is seen in the vicinity of a cell which contains a spherical nucleus (N) and immunonegative granules similar to those seen in Fig. 3B. Direction for the top is toward the third ventricle. Bar: 1μm.

granules (80-150 nm in diameter). The apical processes of such CA-containing cells extended to the ventricular surface (Fig. 3B). On the other hand, non-fluorescent cells (the so-called ependymal cells) did not contain electron-dense granules and their nuclei were oval in shape (Fig. 3B).

By immunoelectron microscopy, DAB deposition indicating 5HT-like immunoreactivity was located in some nerve terminals of the preoptic recess region. The reaction products occurred around the unit membrane of small synaptic vesicles and in the adjacent matrix. A few cores of the small vesicles seemed to be immunoreactive for 5HT (Fig. 4). Subependymal somata having spherical nuclei were sometimes apposed to the 5HT-

immunoreactive terminals and the cells contained immunonegative electron dense granules (80-150 nm in diameter). These non-immunoreactive granules had a similar size to those seen in the CA fluorescent subependymal cells.

DISCUSSION

The microspectrofluorometric analysis showed that the blue-green fluorescent subependymal cells in the preoptic recess organ mainly contained dopamine on referring to our model experiments of artificial droplets containing various catecholamines (Ochi et al., 1979b). This finding was in agreement with the result of Chaco et al. (1974).

The immunohistochemical technique using anti-5HT serum has allowed to demonstrate the precise distribution of cell bodies and fibers of serotonergic neurons to be elucidated in the brain of various animals (Steinbusch 1981; Sano et al., 1982, 1983; Takeuchi et al., 1982a, b). In the present study, many 5HT-positive nerve fibers were demonstrated in the wall of the preoptic recess. Moreover, using combined techniques we assumed that the 5HT-immunopositive terminals would probably make synaptic contacts with TH-immunoreactive subependymal cells. The assumption was partly supported by the results of immunoelectron microscopy in which 5HT-immunoreactive terminals were juxtaposed to the subependymal cells containing electron-dense granules. Due to limitations of morphological techniques, however, a synaptic specialization was not visualized.

In agreement with previous results (Chacko et al., 1974; Nakai et al., 1977; McKenna and Gorski, 1979), the present electron microscopy provided direct proof that CA-containing subependymal cells possessing electron-dense granules. On the other hand, substance-P- or calcitonin-containing subependymal cells also contained electron-dense granules (Gaudino and Fasolo, 1980; Inagaki et al., 1981; Yui et al., 1981). Therefore, direct examination is indispensable to distinguish CA-containing subependymal cells from peptide-containing ones by electron microscopy. The possible coexistence of CA and peptides should also be examined in the subependymal

cells.

An essential feature of CA-containing subependymal cells seemed to be that their apical processes contact the cerebrospinal fluid. Cerebrospinal fluid-contacting cells or neurons have been found in various animals. Although it is unclear whether these cells belong to neurons or glia, they are thought to have a role in chemoreceptive and/or neurosecretory functions (McKenna and Gorski, 1979; Vigh-Teichmann and Vigh, 1981). If a similar mechanism is present in the CA-containing subependymal cells of the frog preoptic recess organ, the present results imply the possibility that serotonergic neurons have an important influence on the mechanism.

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