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A Golgi-Electron Microscopic Study of Anterior Preoptic Neurons in the Bullfrog and the Toad

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ABSTRACT — The structures of certain neurons in the anterior part of the preoptic nucleus (APON), which is a presumed center for anuran mating behavior, were studied using Golgi-electron microscopic and scanning electron microscopic techniques. The examined locus contains cerebrospinal fluid (CSF)-contacting neurons and blood capillary (BC)-contacting neurons. Gold-toned CSF-contacting neurons were examined by scanning electron microscopy after removal of surrounding tissues with hydrochloric acid and collagenase. The somata of these neurons bear debris of nerve terminals on their surfaces, while their processes protrude into the preoptic recess. It is probable that these neurons receive neuronal inputs on their somata and detect ventricular hormonal inputs on their intraventricular end bulbs. Further, when unstained semithin sections of gold-toned neurons were examined by scanning transmission electron microscopy, it was found that the BC-contacting neurons sent their dendrites laterad toward the white matter. There, many axon terminals form synapses on the dendritic spines of these neurons. The BC-contacting neurons probably detect changes in titers of blood-born hormones, and receive neuronal inputs through dendritic synapses. Thus, the present study shows that a portion of the APON neurons have the proper anatomical features for integrating both neural and hormonal signals concerned with the initiation of sex behavior.

INTRODUCTION

The preoptic area plays an important role in the evocation of sexual behavior in many vertebrate species [1–6]. In the anuran brain, this region is concerned with male mate calling [2] and female orientation to conspecific mating calls [7]. Because of its conspicuousness, the neuroendocrine control mechanisms of mate calling have been studied more extensively than female sexual behavior; e.g., mate calling has been induced by electrical stimulation of the anterior part of the preoptic nucleus (APON) [2]. Intracranial implantation of testosterone into this area enhanced mate calling which was evoked acoustically by play-back of tape-recorded conspecific mating calls in *Rana pipiens* [8]. Wada and Gorbman [9] further localized the rostroventral part of the APON (rv-APON) as the best locus for evoking male mate calling. This area

contained many testosterone accumulating neurons in *Xenopus laevis* [10] and *Rana pipiens* [11]. These facts suggest that the rv-APON is testosterone sensitive, and that the activity of the rv-APON neurons is responsible for mate calling which has been evoked acoustically. This hypothesis is supported by the fact that electrical activity of many rv-APON units was excited by auditory stimulation in *Rana pipiens,* and that injections of pituitary homogenate significantly increased the percentage of rv-APON units excited by *pipiens* mating calls [12].

The previous studies mentioned above thus indicate that neuronal activity of the rv-APON is controlled by both neural and hormonal input signals. However, it is not clear whether or not individual rv-APON neurons have appropriate anatomical features for receiving these two different kinds of signals. In this study, the structures of rv-APON neurons, which were determined by scanning electron microscopic and Golgi-electron microscopic techniques, are described in relation to the above question.

Such information would permit further understanding of the neural and hormonal control mechanisms of sexual behavior in anurans.

MATERIALS AND METHODS

Adult bullfrogs (*Rana catesbeiana*) and toads (*Bufo japonicus*) of both sexes were used as experimental animals. They were purchased from an animal supplier between April and October, and were kept in plastic basins in a room at a regulated temperature (18–21°C) with controlled lighting (12L-12D) for 1 week to 3 months before use. They were fed live crickets during this time. Prior to the electron microscopic studies, Nissl-stained paraffin sections and hematoxylin-eosin stained celloidin sections were prepared as reference sections. The fixation procedure for brain tissues was the same in the various electron microscopic techniques utilized in this study. The animals were anesthetized by injection of MS222 (tricaine methanesulfonate, 0.1 mg/g B.W.) into the dorsal lymph sac. After brief transcardial perfusion with frog Ringer's solution, they were perfused with 30 ml of a fixative which contained 2% glutaraldehyde, 2% paraformaldehyde and 0.03% calcium chloride in 0.08 M cacodylate buffer (pH 7.2). The brains were removed, further fixed overnight by immersion in the same fixative at 4°C, and were washed in 0.15 M cacodylate buffer for 15 to 20 min.

Scanning electron microscopy (SEM) of the APON ventricular wall

Serial transverse sections (200–300 μm) of the fixed brains were cut at the level of the preoptic area on a vibratome. Each section was halved along the midline, and then postfixed in a 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 1 hr. They were washed twice in cacodylate buffer for 15 min each, immersed twice in 1% tannic acid in deionized water (DW) for 30 min each, and were washed again in the buffer for 10 min to remove free tannic acid. Thereafter, the sections were dehydrated through graded ethanols and isoamyl acetate, dried by the critical point method, mounted on specimen holders, and were sputtered with platinum-palladium. Then, the ventricular wall of the

preoptic recess was observed using JEOL 100CX-ASID4 in the SEM mode. Nomencratorial identification of the observed loci was performed by examining the transverse plane of the APON in the same specimen.

Scanning electron microscopy of freeze-fractured brains

After fixation followed by washing in cacodylate buffer, the whole brains were dehydrated through graded ethanols. They were then frozen by immersion in liquid nitrogen, and were fractured at the level of the APON with a razor blade. The fractured brain tissues were dried by the critical point method, mounted on specimen holders, sputtered with platinum-palladium, and were observed by scanning electron microscopy.

Rapid Golgi-staining and gold-toning

For Golgi-staining and gold-toning, the rapid Golgi method of Peters [13] was adopted with a slight modification. In brief, the whole brain which was fixed and washed as mentioned above was immersed in a osmium-dichromate solution including 0.2% osmium tetroxide and 2.4% potassium dichromate in DW. A week later it was rinsed in 0.75% silver nitrate and stored in a fresh solution of silver nitrate for 2 days. After silver impregnation, the tissue block was transferred through a graded series of glycerol, superficially embedded in 7% agar, and was cut at 200 μm on a vibratome. Sections were transferred back through a descending series of glycerol, rinsed in 0.05% hydrogen tetrachloroaurate, and gold-toned in a fresh gold chloride solution at 0°C for 40 min. They were then immersed in a 0.05% oxalic acid solution at 0°C for 6 min to reduce the gold chloride to metallic gold, rinsed in DW, and were deimpregnated in a 1% sodium thiosulphate solution at room temperature for 90 min. Afterward, the sections were rinsed in DW at room temperature.

Golgi-scanning electron microscopic study

The gold-toned vibratome sections, in which many liquor contacting cells were stained, were digested first with 8 N hydrochloride (60°C, 1 hr) and then by treatment with collagenase (Sigma type 2, 1 mg/ml, 37°C, 4 hr) to remove tissues surrounding the gold-toned neurons. Then, the sections were washed in phosphate buffer (0.05 M,

pH 7.2), and processed further for SEM observation by a procedure similar to the one mentioned above.

Golgi-scanning transmission electron microscopic (STEM) study

After gold-toning, the thick vibratome sections were immersed in 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) for 30 min at room temperature, rinsed in 0.15 M cacodylate buffer, and dehydrated through graded ethanols. They were then embedded in Epon-Araldite mixture in flat trays so that the thick sections could be studied under the light microscope. Stained neurons were drawn with camera lucida, sectioned with glass knives at 0.1–0.2 μm, and were mounted on collodion carbon-coated slot grids. These semithin sections were then observed with the JEOL 100CX-ASID4 in the STEM mode without uranium- and lead-staining. Frequently, 1 micron sections were cut and stained with toluidine blue for light microscopy to determine the exact location of the gold-toned neurons in the semithin sections.

RESULTS

The anterior part of the preoptic nucleus surrounds the preoptic recess as a densely packed mass of neurons (Fig. 1). This region can be divided into the laminar periventricular part, the non-laminar medial part, and the lateral part of the white matter. Along the dorsal-ventral axis, the APON can be divided into the dorsal part where neurons containing melanin pigments are abundant and the ventral part where such neurons are few.

Scanning electron microscopic observation

The SEM study showed the presence of many rv-APON neurons which contact either the cerebrospinal fluid (CSF) or the blood capillaries (BC) of the brain. They are referred to as the CSF-contacting neurons and the BC-contacting neurons, respectively.

The ventricular wall of the dorsal part of the APON is ciliated as are the surfaces of the ventricular walls in many other brain loci; however, the wall of the ventral part is sparsely ciliated (Figs. 2–4). The sparsely ciliated area includes almost all the portions of the ventricular wall of the rv-APON. The dominant surface structures of this area are bulbous protrusions of two types: large bulbous protrusions (2.85±0.095 μm in diameter, mean±S.E., N=49) often with a wrinkled surface, and small bulbous protrusions (0.67± 0.026 μm, N=112) usually with a smooth surface (Fig. 3). A comparison of the magnitudes of protrusion sizes with those in STEM photographs of the rv-APON indicates that the large bulbous protrusions are cytoplasmic extensions of ependymal and hypendymal cells, and that the small bulbous protrusions are dendritic end bulbs of CSF-contacting neurons. Although seasonal changes in the surface structures of the APON ventricular wall were not examined in this study, large bulbous protrusions were numerous in the toad killed in the summer.

Examination of cross sections of fractured bullfrog APON showed the presence of neurons located very close to blood capillaries (Fig. 5). Such neurons were frequently found in the non-laminar medial part of the rv-APON near the boundary of the laminar periventricular part. They appear to contact the blood capillaries with their somata or proximal dendrites (Fig. 6). Occasionally some rv-APON neurons are apposed to a soma or a dendrite of another rv-APON neuron (Fig. 6). This observation suggests that the rv-APON neurons have somato-somatic or dendro-somatic synapses in this region.

Light microscopy of Golgi-stained APON neurons

The periventricular part of the APON is mainly composed of unipolar and bipolar cells in both bullfrogs and toads. The CSF-contacting neurons mainly belong to the bipolar neurons (Fig. 7). Various types of CSF-contacting neurons in the rv-APON are illustrated at the lower left of Figure 11. Their processes, antipodal to the CSF-contacting dendrites, bifurcate occasionally. Poorly arborizing processes of unipolar neurons generally run toward the white matter lateral to the neuronal mass of the APON (Fig. 11).

The medial part of the rv-APON includes multipolar and pyriform cells in addition to unipolar and bipolar cells (Figs. 8 and 11). Their processes are usually running mediad or laterad perpendicular to the ventricular wall. Although
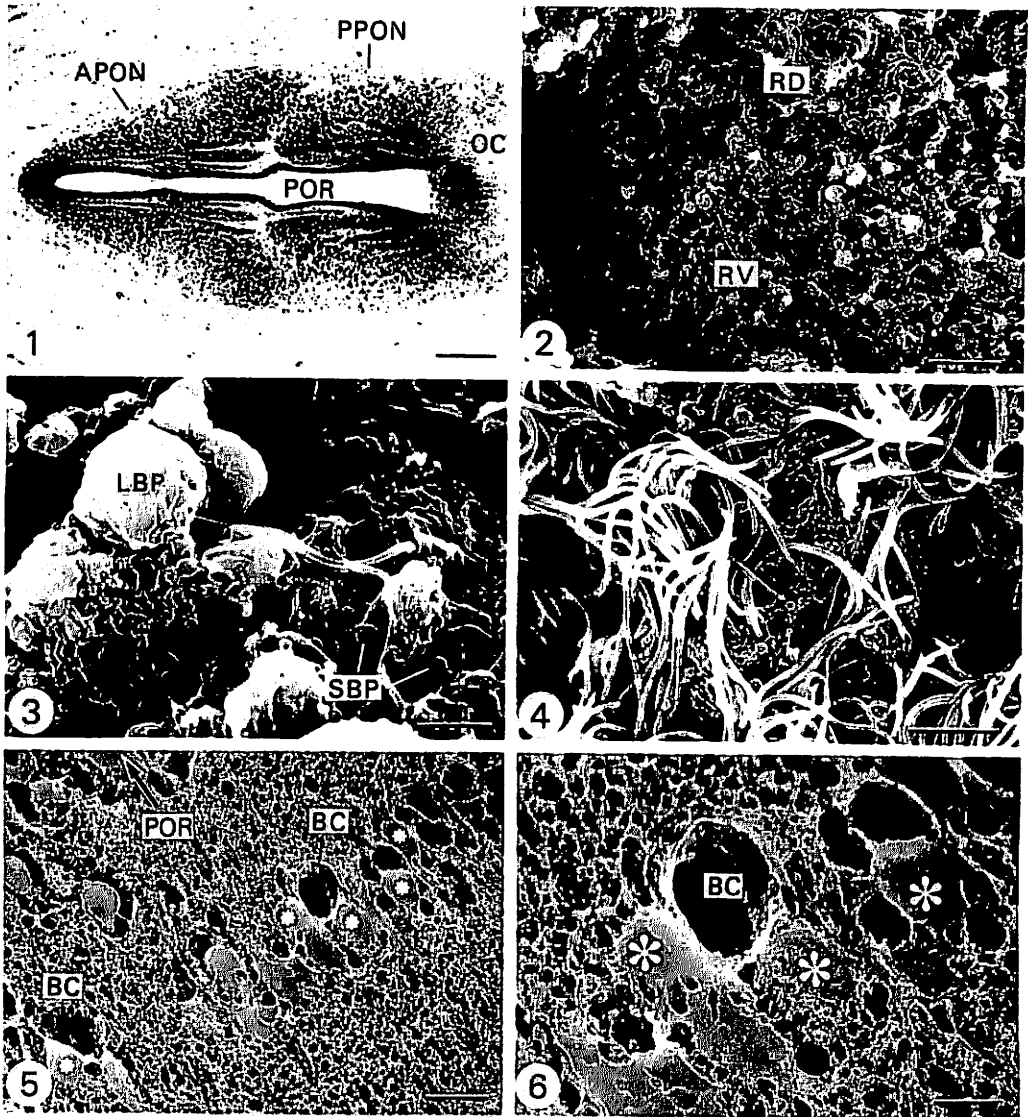
FIG. 1.   LM-photograph of the bullfrog preoptic nucleus which is divided into the anterior part (APON) and the posterior part (PPON).   OC, optic chiasma; POR, preoptic recess.   Hematoxylin-eosin stained horizontal section.   Scale, 200 μm.

FIG. 2.   Surface structure of the ventricular wall of the preoptic recess at the level of the APON in the toad.   RD, rostro-dorsal ciliated part;   RV, rostro-ventral sparsely ciliated part.   SEM-photograph; scale, 20 μm.

FIG. 3.   Large (LBP) and small (SBP) bulbous protrusions of the ventricular wall of the rv-APON.   SEM-photograph; scale, 2 μm.

FIG. 4.   The ciliated dorsal part of the ventricular wall of the APON.   Numerous microvilli are also shown.   SEM-photograph; scale, 4 μm.

FIG. 5.   A cross section of the fractured bullfrog APON.   Note the presence of neurons (*) located very close to blood capillaries (BC).   SEM; scale, 10 μm.

FIG. 6.   Higher magnification of the fractured APON.   A portion of neurons (*) located very close to blood capillaries are apposed to a soma or a dendrite of another neuron (arrows).   SEM; scale, 4 μm.
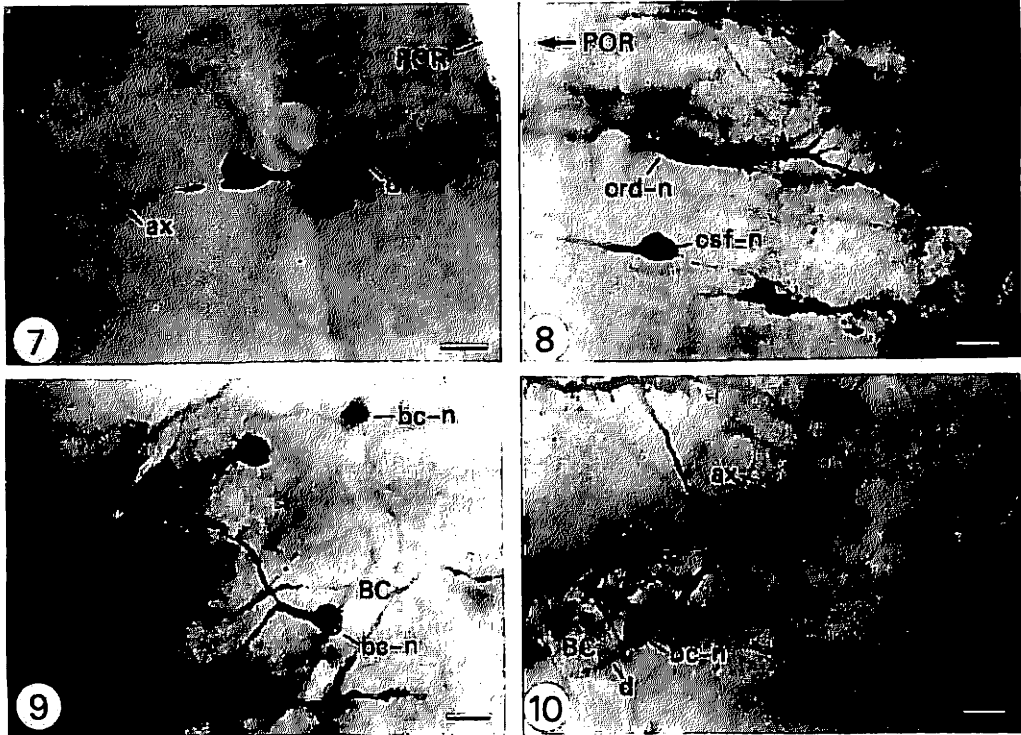
FIG. 7.   A bipolar CSF-contacting neuron in the rv-APON of the bullfrog.   ax, axon; d, dendrite;
        POR, preoptic recess.   Rapid Golgi-stained specimen; scale, 20 μm.
FIG. 8.   An ordinary pyriform cell (ord-n) in the medial part of the rv-APON.   A cerebrospinal fluid-
        contacting neuron (csf-n) is also shown.   Rapid Golgi staining; scale, 20 μm.
FIG. 9.   Presence of rv-APON neurons (bc-n) located very close to a blood capillary (BC).   Rapid Golgi
        staining; scale, 20 μm.
FIG. 10.   A pyriform APON neuron (bc-n) whose proximal dendrite seems to contact a blood capillary
        (BC).   ax, axon; d, dendrite.   Rapid Golgi-stained specimen; scale, 20 μm.

arborization is rather poor, the dendrites which proceed toward the white matter usually bifurcate several times, and intermingle with the processes of medial amygdala neurons (Fig. 12). A considerable number of rv-APON neurons in the medial part are located very close to blood capillaries (Figs. 9 and 10). Their processes behave similarly to those of the ordinary neurons mentioned above.

Golgi-SEM study

Observation of stripped rv-APON neurons with SEM revealed a tendency for CSF-contacting neurons in the medial part to bear many debris, but for those in the periventricular part to have only a few on their somata and processes. Figure 13 shows two CSF-contacting neurons whose surrounding tissues were removed. In spite of the violent digesting procedure, gold-toned neurons kept their shape almost intact. One of them, the bipolar neuron whose soma is located at some distance from the ventricular wall, sends processes towards the ventricular wall and the white matter. Higher magnification of this neuron shows that the surfaces of the soma and the proximal dendrite are studded with many debris (Fig. 14) as are ordinary neurons (Fig. 15). The sizes of the debris are compatible with those of the nerve endings. On the contrary, the other neuron in Figure 13 whose soma lies in the periventricular part has a smooth surface. Figure 16 shows a higher magnification of another bipolar CSF-contacting neuron which is also located in the periventricular part adjacent to the ventricular wall. The soma and processes of this neuron
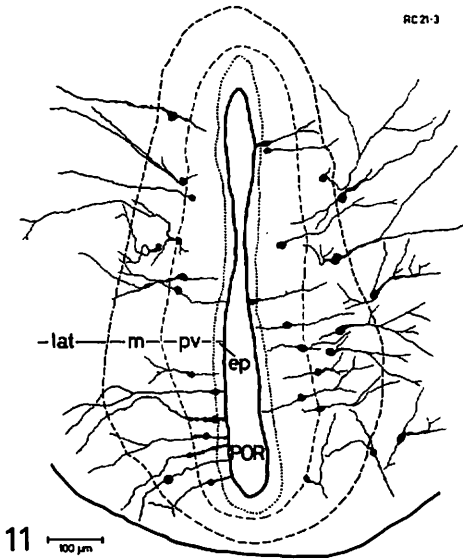
FIG. 11. Camera lucida-drawing of Golgi-stained cells in the APON of the bullfrog. Various types of CSF-contacting neurons in the rv-APON are illustrated at the lower left. ep, ependymal layer; lat, lateral part of the APON; m, medial part of the APON; pv, periventricular part of the APON; POR, preoptic recess.
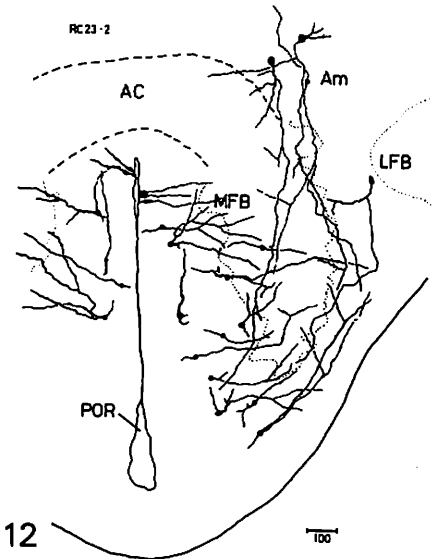

FIG. 12. Drawing of Golgi-stained neurons in the amygdala medialis (Am) and the APON. Processes of Am and APON neurons intermingle with each other in the white matter where the fibers of the medial forebrain bundle (MFB) run through. AC, anterior commissure; LFB, lateral forebrain bundle. Scale, 100 μm.

are fairly smooth, and bear a few debris.

Golgi-STEM study

The main advantage of the use of the Golgi-electron microscopic method is that the overall shapes of neurons can be determined light-microscopically before thin-sectioning. However, the method has a serious limitation, in that, it is unpredictable which neurons will be impregnated by the rapid Golgi staining. Another problem is that, without a diamond knife, serial sectioning of single gold-toned neurons is practically impossible. In spite of these limitations, I have succeeded in observing a single BC-contacting neuron at various levels along its dendrites and soma. This gold-toned rv-APON neuron which is fairly large and bipolar contacts a blood capillary with an intervening basement membrane (Figs. 17 and 20). One of the dendritic processes proceeds toward the white matter, and bears many spines in this region (Fig. 17). Various types of nerve terminals and processes are main structural components of this region. Many nerve terminals are connected with the dendrite and spines of the BC-contacting neuron in which fine gold particles are deposited (Fig. 18). The proximal part of this dendrite, which is located in the medial part of the rv-APON, forms a gap junction with the soma of another neuron (Fig. 19). This junction lacks intercellular space and synaptic density. Further, the axon which contains electron dense granules emerges out of the same dendrite. Axo-axonic, axo-dendritic and dendro-dendritic synapses are observed near this axon emerging point. Since the soma contains well developed rough endoplasmic reticulum, Golgi apparatus, and 200 nm electron dense granules (Fig. 20), this BC-contacting neuron should be peptidergic. Examination of several other neurons, whether gold-toned or not, confirms that the fine structure of the BC-contacting neuron described above is general in the rv-APON.

DISCUSSION

The present study in the bullfrog and the toad showed that the APON, a presumed center for anuran mating behavior, contains CSF- and BC-contacting neurons especially in its ventral
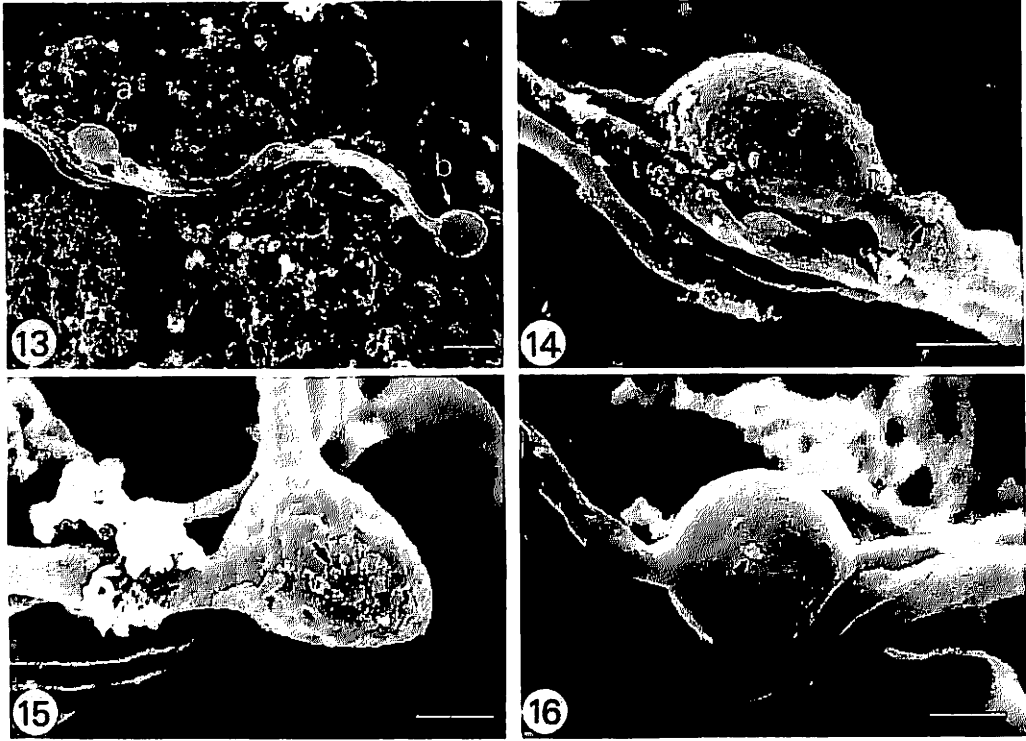
FIG. 13. SEM-photograph of rv-APON neurons whose surrounding tissues were removed. a, a bipolar CSF-contacting cell located at a distance from the preoptic recess; b, a CSF-contacting cell near the ventricular wall. Scale, 5 μm.

FIG. 14. Higher magnification of the bipolar CSF-contacting cell seen in Fig. 13 (cell a). Note debris on the surfaces of the soma and the proximal dendrite (arrows). Scale, 2 μm.

FIG. 15. SEM-photograph of an ordinary rv-APON neuron whose surface bears debris (arrows) probably of nerve terminals. Scale, 2 μm.

FIG. 16. Higher magnification of a CSF-contacting cell whose soma lies near the ventricle. Note rather few debris on the surfaces of the soma and the dendrite (arrow).

part. These neurons form synapses with nerve terminals of other neurons. Occasionally, dendro-dendritic synapses and gap junctions are found mainly in the non-laminar medial part. Since the cytoarchitecture of the APON is similar in bullfrogs and toads, the presence of CSF- and BC-contacting neurons in the APON seems to be a common feature of this nucleus among many anuran species.

The CSF-contacting neurons

The presence of CSF-contacting cells in the preoptic and infundibular recesses has been reported in many amphibian species [14, 15]. A topographic SEM study on the specialization of the wall of the third ventricle in *Rana temporaria* showed that the most ventral part of the wall of the preoptic recess is studded with numerous large bulbous protrusions [16]. These protrusions were divided into two types: intraventricular dendritic end bulbs of secretory neurons and large cytoplasmic extensions of ependymal cells. These observations by other authors coincide well with the present result that the dominant surface structure of the ventricular wall of the rv-APON is bulbous protrusions, and that two types of protrusions are discriminable.

In the APON of the *Xenopus* brain, sex-steroid concentrating cells were localized throughout the dorsal-ventral extent in the rostral part; however, such cells were found ventrally in the caudal part [10, 17]. This distributional pattern of sex-steroid concentrating APON neurons corresponds
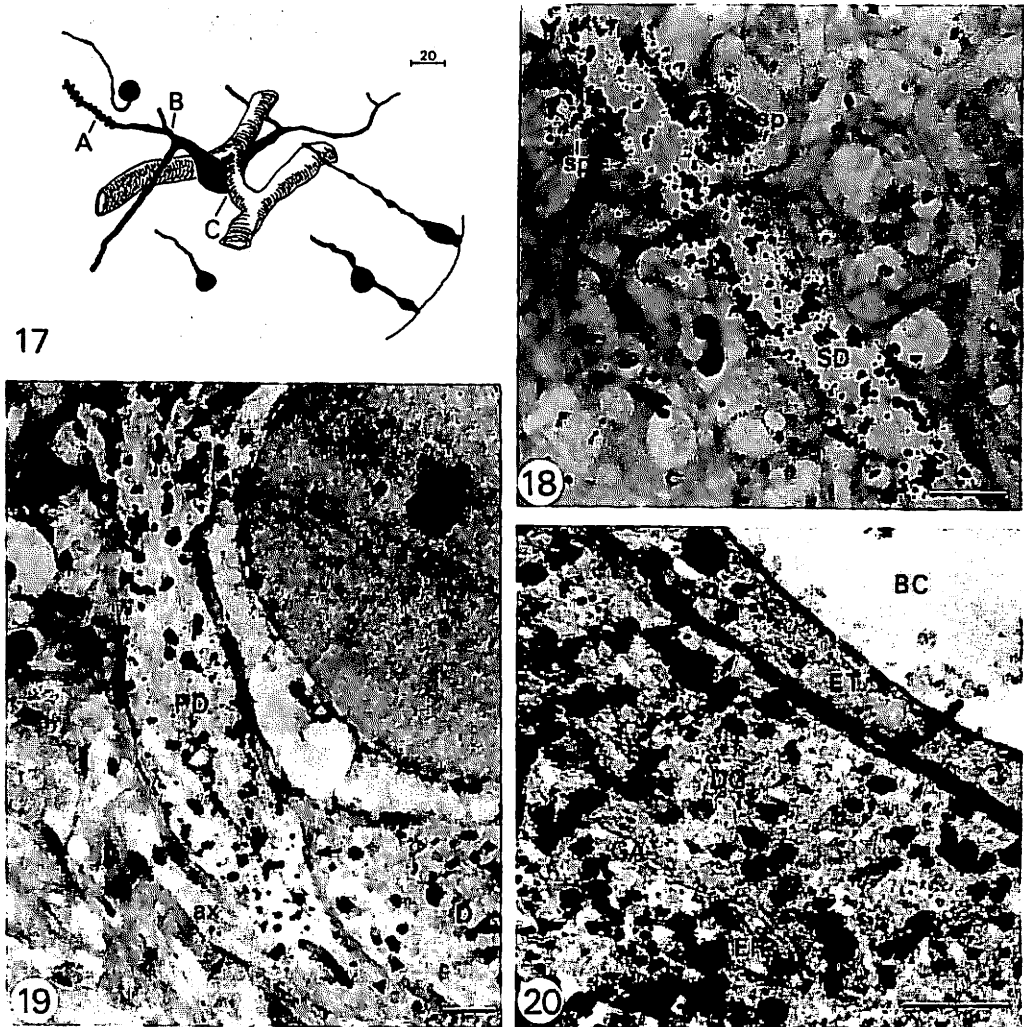
FIG. 17.   Drawing of an overall shape of a large BC-contacting neuron in the rv-APON which is success-
fully Golgi-stained and examined at various levels along its dendrites and soma.   The STEM
photographs corresponding to level A to C are Fig. 18, 19 and 20, respectively.

FIG. 18.   STEM-photograph of the gold-toned spined dendrite (SD) of the neuron seen in Fig. 17 (at
level A).   Note various types of nerve terminals around this dendrite in the white matter.   sp, spine.
Dark deposits are metallic gold.   Scale, 2 μm.

FIG. 19.   Proximal dendrite (PD) of the neuron seen in Fig. 17 (at level B).   This dendrite forms gap
junctions (arrow heads) with other dendrite (D) and soma, and also synapses (arrows).   ax, axon.
Scale, 1 μm.

FIG. 20.   Soma of the neuron seen in Fig. 17 (at level C).   This neuron contacts an endothelial cell
(ET) of a brain blood capillary (BC) only with an intervening basement membrane (arrows).   DG,
dense electron granule; ER, rough endoplasmic reticulum; GA, Golgi apparatus.   Scale, 1 μm.

to the localization pattern of ventricular bulbous
protrusions described in this study, although the
species of experimental animals are different.
Since castration or estrogen treatments induced
changes in ventricular surface structures of the
median eminences of rats [18], the structures of
CSF-contacting neurons also can be modified by
sex-steroid hormones in amphibians.

The CSF-contacting neurons in the medial part of the APON bear many debris on their somata and proximal dendrites after digestion treatments. Since the debris and nerve terminals have similar sizes, and the adhesion between two synaptic membranes withstands many drastic treatments [19], these debris may be the nerve terminals which form synapses on the CSF-contacting neurons. Then, neuronal activity of these cells can be modulated by various signals transmitted at the synapses on their surfaces. On the other hand, Smoller [20] suggested that the dendrites projecting into the preoptic recess from preoptic neurosecretory cells are equipped to serve both secretory and sensory functions. The CSF-ventricular system is thought to distribute biologically active molecules within the brain, since many authors have found various hormonal substances in the CSF, such as luteinizing hormone-releasing hormone and thyrotropin-releasing hormone [21], oxytocin and vasopressin [22], melatonin [23], and so on. The concentrations of these hormones in the CSF are either increased or decreased according to various physiological conditions. Therefore, the CSF-contacting neurons whose dendritic processes protrude into the preoptic recess may detect changes in ventricular hormonal status through their intraventricular end bulbs.

The BC-contacting neurons

Blood capillaries in the vertebrate brain are generally surrounded by astrocytic endfeet with an intervening basement membrane, so that brain neurons, even hypothalamic neuroscretory cells in fish, are separated from the vascular endothelium [24]. The present finding that the peptidergic rv-APON neurons contact blood capillaries only with an intervening basement membrane is thus incompatible with the general concept of the relations between brain neurons and capillaries. However, the presence of neurosecretory cells which directly contact blood capillaries was previously shown in the toad preoptic nucleus by ordinary transmission electron microscopy [25]. Moreover, in this study, the presence of BC-contacting neurons was exhibited by use of two different electron microscopic techniques, the SEM of freeze-fractured brains and the Golgi-STEM method. These facts strongly support the claim that the present finding of the presence of BC-contacting neurons was not artifactual. In addition, the Golgi-STEM study showed that the same gold-toned cell contained characteristic electron dense granules and Golgi apparatus, contacted a blood capillary, and formed synapses with various nerve terminals. This BC-contacting cell therefore can not be an astrocyte, but a peptidergic neuron.

The single BC-contacting neuron that was observed in this study forms various synapses, such as axo-spinous, axo-dendritic, axo-axonic and dendro-dendritic chemical synapses and a gap junction between its own dendrite and a soma of another neuron. Since Halpern [26] noted that terminal degeneration by the telencephalic lesions was located along the lateral edges of cell masses in the frog hypothalamus, the nerve terminals which contact the spined dendritic process in the white matter may have a partly telencephalic origin. A previous horseradish peroxidase study in *Rana pipiens* has shown that the brain loci which send axons to the APON are the limbic area including the amygdala pars medialis, the magnocellular part of the preoptic nucleus, several thalamic nuclei, the reticular formation, and some tegmental nuclei (Urano and Gorbman, unpublished). The BC-contacting neurons may receive various neuronal signals of both extrahypothalamic and intranuclear origin in addition to humoral signals conveyed through the BC-contacting surfaces of their plasma membranes.

Physiological significance of the CSF- and BC-contacting neurons

Seasonal changes found in concentrations of various hormones in toad plasma including sex-steroids showed intimate relations to breeding activity [27, 28]. Further, electrical activity of APON units was modulated by injections of pituitary homogenate in *Rana pipiens* [12], and an intraventricular injection of luteinizing hormone-releasing hormone induced changes in amplitudes and frequency of electroencephalogram in hibernating toads (Urano, unpublished data). These facts strongly indicate that blood-born hormones and hormones in the CSF act on

the APON neurons to modulate their electrical activity, although direct experimental evidence is scarce. The present study gives anatomical evidences that the CSF- and BC-contacting neurons have appropriate structures to accept such hormonal influences. Thus, they could integrate neuronal and hormonal signals which concern initiation of sex behavior, since Schmidt [29] proposed the APON as the triggering center for mate calling.

The BC-contacting neurons can have another physiological role in sex behavior. Sex-steroids may be conveyed rapidly through the thin basement membranes to the BC-contacting neurons. The activational effects of sex-steroids then first operate on these cells to alter the neuronal circuitry of the APON, and induce seasonal changes or sexual dimorphism in the APON volume which has been found in the toad brain (Takami and Urano, in preparation).

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