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Immunocytochemical and Ultrastructural Analysis of Opsin- and Vasoactive Intestinal Peptide (VIP)- like Immunoreactive Neurons in the Lateral Septum of the Pigeon

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Summary

CSF-contacting neurons of the lateral septum are considered as a putative deep brain photoreceptor in the avian brain. By means of immunocytochemistry using antibodies to a visual pigment (RET-P1, a monoclonal antibody against opsin, Barnstable, 1980) and vasoactive intestinal peptide (VIP), I demonstrated clusters of immunoreactive small neurons in the lateral septum of the pigeon. Opsin-like immunoreactive neurons and VIP-like immunoreactive neurons have similar morphological features. Their perikarya accumulate in the subependymal regions of the ventromedial walls of both lateral ventricles. The labelled neurons are multipolar or bipolar with pyriform or spindle-shaped cell bodies. Immunoreactive CSF-contacting neurons contact the CSF via a process that penetrates the ependyma and terminates in a single knob-like swelling. Immunoreactive fibers originating in the group of cell bodies seem to give rise to dense terminal-like structures in the septal area. Immuno-electron-microscopic investigations of these neurons revealed an accumulation of VIP- and opsin immunoreactive dense-core vesicles (100-150 nm in diameter) in ventricular terminals, perikarya and neuronal terminal-like structure with VIP- and RET-P1-immunolabelling respectively. Based on these evidence it seems clear that VIP-and opsin-like immunoreactive neurons of this study are the same as the neurons that express both opsin- and VIP-like immunoreactivity in the ateral septum of the ring dove (Silver *et al*., 1988). In this study double immunolabelling using VIP and RET-P1 antibodies shows the coexistence of VIP and opsin in the same dense vesicles.

Introduction

Immunohistochemical studies reveal small neurons with an accumulation of VIP-like immunoreactive (ir) in the lateral septum in avian brains (Hirunagi *et al*., 1995; Hof *et al*., 1991; Korf and Fahrenkrug, 1984; Kuenzel and Blähser, 1994; Silver *et al*., 1988; Yamada *et al*., 1982), some of which are cerebrospinal fluid (CSF)-contacting. Recently, this type of small neuron has been considered as a candidate for the deep encephalic photoreceptor of avian and reptilian brains. First, Silver *et al*. demonstrated opsin-like immunoreactivity in this type of neurons of the lateral septum of birds using an opsin antibody, RET-P1, raised against a membrane fraction of rat retina (Barnstable, 1980; Silver *et al*., 1988). In that study, they established that some VIP-like ir neurons of the lateral septum co-express RET-P1 immunoreactivity with an immuno-fluorescent double labelling method. More recently, opsin-like immunoreactivity has been reported in similar CSF-contacting neurons of the identical region of the lateral septum of the lizard *Anolis carolinensis* (Foster *et al*., 1993). Furthermore, VIP-like ir CSF-contacting neurons have been demonstrated in the same region of the lateral septum of several reptilian species (Hirunagi *et al*., 1993). With regard to the subcellular localization of VIP-like antigen, Hirunagi *et al*. showed that VIP-like ir CSF-contacting neurons have VIP-like ir dense vesicles (approximately 100-150 nm diameter) in

the perikarya with dendritic processes and axon terminals of the duck (Hirunagi *et al*., 1995). This result suggests that VIP-like ir neurons contain VIP-like neuropeptide in these electron-dense vesicles. However, until now, the ultrastructural localization of opsin-like antigen has remained elusive. In the present study we demonstrate the ultrastructural localization of opsin- and VIP-like antigens in the neurons of the lateral septum of the pigeon. I used single labelling immunocytochemistry for RET-P1 and VIP and a dual-labelled method using both ABC labelling and immunogold-silver labelling at the electron microscopic level.

Material and Methods

Male and female adult pigeons were perfused transcardially with 0.75% NaCl followed by a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer at pH 7.4. Brains were rapidly removed and a coronal block containing the lateral septum area post-fixed for overnight. Vibratome sections (50 μm thick) were cut and treated for the demonstration of neurons with RET-P1 and VIP immunoreactivities as described below. (1) Single label immunostaining for VIP; Sections were incubated in polyclonal porcine VIP antibody (1:1000, Cambridge Research Biochemicals, UK) for 72 hours at 4°C. For visualizing of immunoreaction, a PAP method was used. The details of immunostaining for VIP and the specifity of VIP antibody was described previously (Hirunagi *et al*., 1993; Hirunagi *et al*., 1995). (2) Single label immunostaining for RET-P1; Sections were incubated in monoclonal opsin antibody (RET-P1) (1: 20000) diluted in 0.1MPBS containing 1% BSA and 0.3% Triton X-100 for 72 hours at 4°C. The sites of the antibody-antigen binding were visualized with an avidin-biotin-peroxidase (ABC) procedure (Elite ABC kit, Vector Labs, Burlingame, CA) according to the protocol. Other sections were incubated in monoclonal opsin antibody (RET-P1) (1: 20000) for 72 hours at 4°C. Sections were then incubated in goat anti-mouse IgG bound to 1nm colloidal gold (AuroProbe One, Amersham, UK) diluted 1:50 in 0.1% gelatin PBS, BSA. Sections were fixed in 2% glutaraldehyde for 10 minutes. Silver intensification of gold was processed by the light-insensitive IntenSE-M Kit (Amersham, UK) following the manufacture's protocol. (3) Dual label immunostaining for VIP and RET-P1; Following incubation with RET-P1 as the first primary antisera, immunoreactivity was visualized with an ABC procedure as shown in (2). Sections were incubated in polyclonal porcine VIP antibody (1:1000, Cambridge Research Biochemicals, UK) as second primary antisera. Sections were then incubated in goat anti-rabbit IgG bound to 1 nm colloidal gold (AuroProbe One, Amersham, UK) diluted 1:50 in 0.1% gelatin PBS, BSA. Sections were fixed in 2% glutaraldehyde for 10 minutes. Silver intensification of gold was processed by the light-insensitive IntenSE-M kit (Amersham, UK), as above. For electron microscopy immunostained sections were osmicated and flat-embedded in Araldite. The details of these procedures have been described previously. Following contrast staining with uranyl acetate and lead citrate, ultra-thin sections were examined with a JEOL JEM 1210 electron microscope.

Results

Using polyclonal antibody against VIP and monoclonal antibody, RET-P1, against rat opsin, an accumulation of immunorective neurons is found in a highly circumscribed region of the lateral septum /nucleus accumbens with each label. Light- and electron-microscopic morphological features of VIP-like and opsin-like ir neurons are identical in the lateral septum of the pigeon. We show results of opsin-like and VIP-like ir neurons and double labelling with RET-P1 and VIP in Fig. 1, 2 respectively. Opsin-like ir perikarya accumulates in the subependymal regions of the ventromedial walls of both lateral ventricles at the level of the pars medialis of the lateral septal

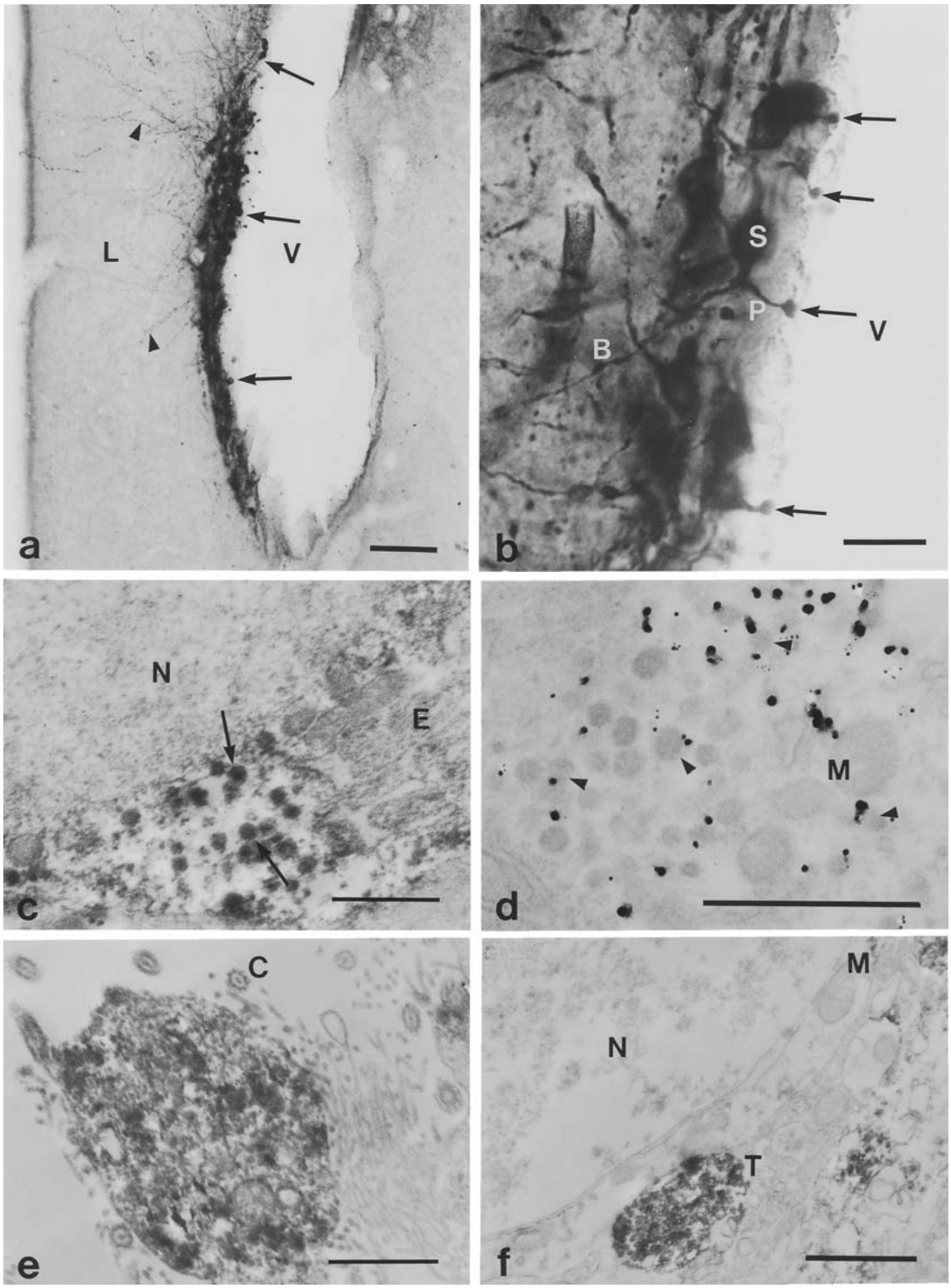

Fig 1. **a.** Accumulation of opsin-ir neurons in the lateral septum Arrows: Immunoreactive neurons Arrowheads: Basal processes V: Lateral ventricle Bar: 100 μm **b.** Opsin ir CSF-contacting neurons in the lateral septum Arrows: ventricular processes of opsin ir CSF-contacting neurons B: Basal process P: Ventricular process S: cell body V: Lateral ventricle Bar: 20 μm **c.** Opsin ir dense vesicles in immunoreactive neuron Arrows: immunoreactive vesicles E: Endoplasmic reticulum N: Nucleus Bar: 1 μm **d.** Opsin ir vesicles identified by 1 nm immunogold-silver enhanced method Arrowheads: Immunoreactive dense vesicles M: Mitochondria Bar: 1 μm **e.** Opsin ir ventricular process of the CSF-contacting neuron in the lateral septum C: Cilium in lateral ventricle Bar: 1 μm **f.** Opsin ir neuronal terminal near small neuron in the lateral septum M: Mitochondria N: nucleus of immunonegative neuron T: Opsin ir neuronal terminal Bar: 1 μm

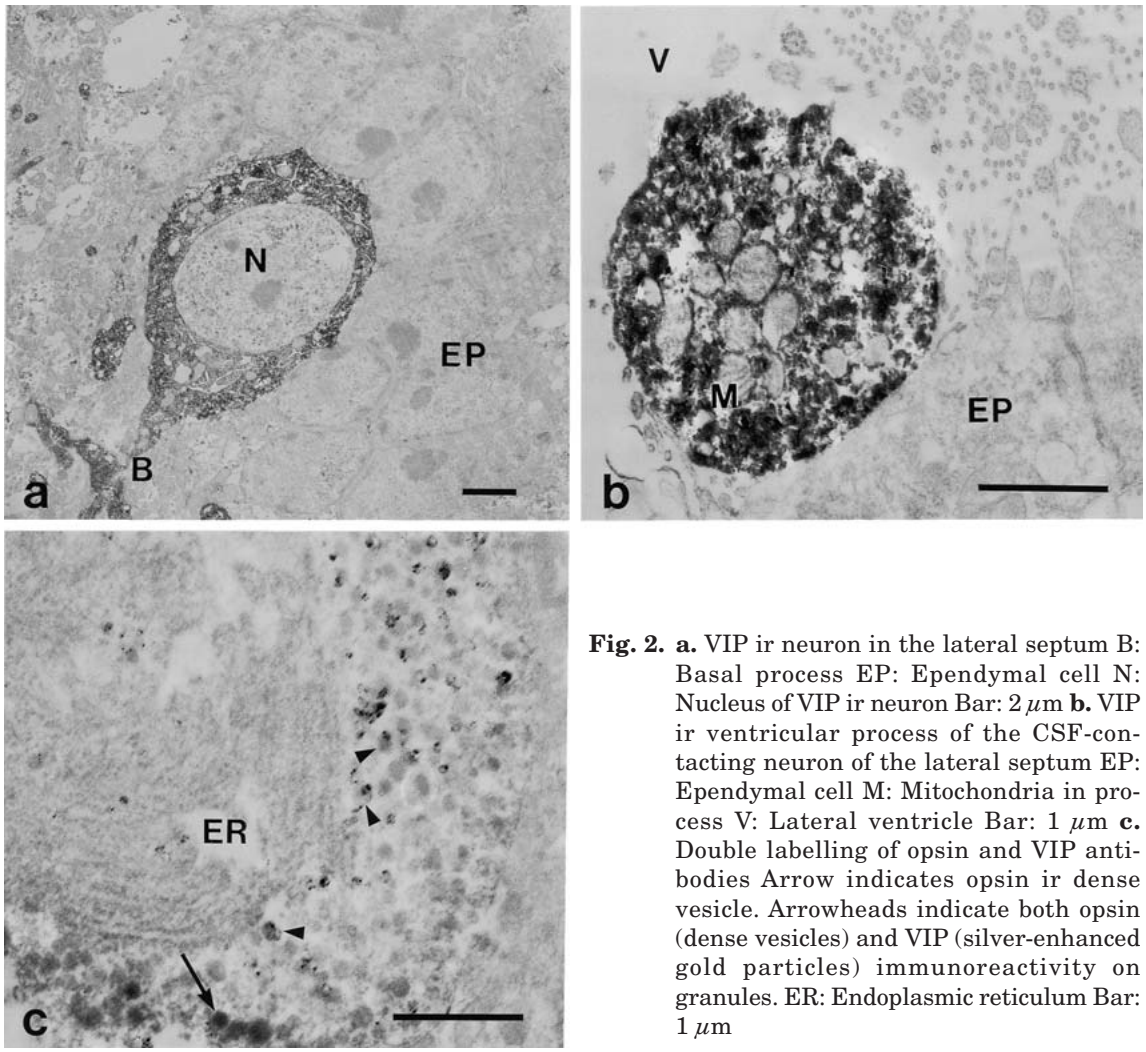

Fig. 2. a. VIP ir neuron in the lateral septum B: Basal process EP: Ependymal cell N: Nucleus of VIP ir neuron Bar: 2 μm **b.** VIP ir ventricular process of the CSF-contacting neuron of the lateral septum EP: Ependymal cell M: Mitochondria in process V: Lateral ventricle Bar: 1 μm **c.** Double labelling of opsin and VIP antibodies Arrow indicates opsin ir dense vesicle. Arrowheads indicate both opsin (dense vesicles) and VIP (silver-enhanced gold particles) immunoreactivity on granules. ER: Endoplasmic reticulum Bar: 1 μm

organ (Kuenzel and Blähser, 1994) (Fig. 1a). The labelled neurons are multipolar or bipolar with pyriform or spindle-shaped cell bodies. Some of these are CSF-contacting neurons, with a process that penetrates the ependyma and terminates in a single knob-like swelling (Fig. 1b). In addition to the cluster of cell bodies, ir fibers originating in the group of cell bodies course into deeper layers of the septal area. Some of them appear to be beaded axons (Fig. 1a). Using pre-embedding methods of ABC and 1 nm immunogold, electron- microscopic studies reveal that opsin-like neurons have an accumulations of dense-core vesicles with diameter of 100-150 nm in their perikarya and ventricular terminal. An ABC labelling demonstrates RET-P1-labelled dense vesicles near the endoplasmic reticulum in the cytoplasm (Fig. 1c) and in the ventricular terminal (Fig. 1e). With a 1nm immunogold silver labelling, silver-enhanced gold particles, which indicate the site of RET-P1 antigen, are frequently associated with dense vesicles (Fig. 1d). In the lateral septum, terminal formations with VIP or RET-P1 immunoreactivity are distributed. VIP-and RET-P1-labelled terminals lie in the same regions. Most prominent immunoreactive terminal formations are observed in the area of the nucleus septi lateralis. In this area opsin-like ir terminals form dense networks. They appeared as intensely immunoreactive spots at light-microscopic observations as VIP immunoreactive terminals that were reported our previous paper (Hirunagi *et al*., 1994). Electron-microscopic observations show that opsin-like ir terminals are observed near immunonegative small neurons in the lateral septum (Fig. 1f). VIP immunocytochemistry shows an accumulation of VIP ir neurons in the subependymal region of the lateral septum. This type of neurons project VIP-like ir

basal processes (Fig. 2a) and ventricular processes in the lateral ventricle (Fig. 2b). In ventricular swellings, dense vesicles and mitochondria are frequently observed (Fig. 2 b).

A double-labelling survey, combined ABC method for RET-P1 antibody and immunogold-silver method for VIP antibody, reveals that RET-P1-labelled dense-core vesicles appear to coexpress VIP-like immunoreactivity. Silver-enhanced gold particles which indicate the localization of VIP antigen are preferentially associated with RET-P1-immunolabelled dense core-vesicles near the endoplasmic reticulum in the cytoplasm (Fig, 2c) . Control sections in which second primary antibody (VIP antibody) was omitted from the protocol showed ABC immunolabelling corresponding to the first primary antibody alone.

Discussion

There is a lot of experimental evidence for the existence of a deep encephalic photoreceptor within avian brain (Kuenzel, 1993; Oksche, 1991; Perera and Follett, 1992). However, to date there is no morphological evidence to support the existence of photoreceptor-like structures such as the outer segment of photoreceptor of retina in the avian forebrain (Oksche, 1991). The sites of deep brain photoreceptors of birds remain unidentified. According to recent opsin-immunohistochemical studies, the lateral septum is a candidate for a photoreception site in birds (Silver *et al*., 1988). The present immunocytochemical observations clearly indicated that opsin-like and VIP-like ir CSF-contacting neurons are important components of the lateral septum region in the pigeon. Electron microscopic observations revealed that opsin- and VIP-like ir CSF-contacting neurons have similar ultrastructural features. This type of small neurons have a ventricular bulbous terminal that contains numerous mitochondira and large dense vesicles. Similar electron microscopic features have been reported in a recent study of rudimentary photoreceptor cells in the pineal organ of a marsupial, *Didelphis albiventris* (del C. González and Affanni, 1995).

In reptilian and avian brains, VIP- and opsin-like ir CSF-contacting neurons are reported in the identical regions of several species. A CSF-contacting neuron of the lateral septum is a putative deep encephalic photoreceptor of the avian and reptilian species, mainly because of its expression of opsin-like immunoreactivity using immunohistochemical method in reptilian and avian species. This study demonstrated that a CSF-contacting neuron located in the lateral septum has opsin-like immunoreactivity in the pigeon. However, immunohistochemical results regarding the existence of opsin-like immunoreactive neurons in the avian lateral septum are controversial. Using a monoclonal anti-opsin antibody RET-P1, Silver *et al*. (1988) described neurons with opsin-like immunoreactiveity in the septal and infundibular hypothalamic region of doves, ducks, quail, sparrows and junco (Silver *et al*., 1988). Furthermore, it appears that the same cells in doves were stained with Cos-1 (a monoclonal anti-opsin antibody raised against a chicken cone visual pigment) (Silver, 1990). On the other hand, Garcia-Fernández and Foster (1994) have failed to identify any opsin-immunopositive cells in the identical regions of the quail or chick brain, although they could show opsin-like ir hypothalamic CSF-contacting neurons in the the larval lamprey using the antibodies (García-Fernández and Foster, 1994). Furthermore, no specific staining of opsin and retinal S-antigen was detected in the lateral septal cells and hypothalamic cells in birds (Foster and Korf, 1987). Recently, rhodopsin cDNA was cloned from the pigeon lateral septum fraction. An antibody (RhoN) which recognizes the amino-terminal region of pigeon rhodopsin was prepared for use in an immunohistochemical study to identify photoreceptor cells in the lateral septum of the pigeon. Immunoreactive CSF-contacting neurons were detected in the lateral septum of the pigeon (Wada *et al*., 1998). It is difficult to say whether the disagreement between these result depends on some species differences or different types of opsin antibodies used.

RET-P1 ir cells of the lateral septum coexpress VIP-like immunoreactivity in the brains of ring dove, Japanese quail and duck (Silver *et al*., 1988). In the present study, we confirmed the colocalization of RET-P1 and VIP in neurons of the lateral septum in the pigeon at the electron microscopic level using a dual labeling method. Our electron microscopic results suggested that RET-P1 and VIP antigens colocalize in a dense core vesicle which is found in the perikarya and terminals. With regard to the subcelluar localization of VIP-like specific antigen, electron microscopic analysis has shown that VIP-like ir neurons of the lateral septum have an accumulation of immunostained large dense core vesicles (approximately 150 nm in diameter) in their cytoplasm (Hirunagi *et al*., 1995). Similar VIP immunoreactive core vesicles were reported in the terminal formations of the lateral septum of duck (Hirunagi *et al*., 1995) and pigeon (Hirunagi *et al*., 1994). About the subcellular localization of RET-P1 antigen, electron microscopy revealed the distribution of RET-P1 antigen in the rat retina (Fekete and Barnstable, 1983). Immunoreactivity of RET-P1 antibody can be seen in the plasma membranes of both outer segments and inner segments of rod photoreceptors. And a more diffuse reaction product was reported within the peripheral cytoplasm of the inner segments. In this study, RET-P1 immunoreactivity has been observed in the entire neuronal elements including axon and dendritic processes of CSF-contacting neurons. Ventricular bulbus terminals of CSF-contacting neurons have RET-P1 immunoreactivity. However, no lamellar membrane structures, similar to those found in the outer segments of retinal photoreceptors, have been identified electron-microscopically in the CSF-contacting neurons of lateral septum of birds. Confirmation of these CSF-contacting neurons as photoreceptors awaits physiological evidence.

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ハト外側中隔でのオプシン、VIP 免疫陽性ニューロンの免疫細胞化学および電子顕微鏡による研究

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外側中隔の髄液接触ニューロンは鳥類の脳深部に存在すると考えられている光受容細胞の候補の一つである。視物質（RET-P1）と血管作用性小腸ペプチド（vasoactive intestinal peptide）（VIP）の抗体を用いた免疫細胞化学法により、ハトの外側中隔で、それぞれ免疫陽性の小型ニューロンの集団を検出した。この種のニューロン細胞体は、両側の側脳室の腹内側で上衣下に集合する。両者の免疫陽性ニューロンは類似した形態であり、細胞のタイプは双極もしくは多極性である。一方の突起を脳室方向にのばし、その先端が脳室内に終わる髄液接触ニューロンである。もう一方の突起は細胞体から出て脳実質にのび、中隔領域に密度の高い神経終末の集合野を形成するようである。RET-P1とVIP抗体を使った免疫電子顕微鏡で観察すると、免疫陽性の顆粒（直径が 100 ～ 150 nm）が、脳室内の終末構造、核の周囲部と神経終末内に認められる。これらの所見から、VIP陽性ニューロンとRET-P1免疫陽性ニューロンは同一の細胞であると考えられる。本研究では、さらに、電子顕微鏡レベルの二重免疫染色法により、VIPとオプシンが同一の顆粒に共存する可能性を示唆した。