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Protein markers in the microcyst of the posteroventral cochlear nucleus of the gerbil

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Summary. Microcysts are most evident in the posteroventral and anteroventral cochlear nuclei (PVCN and AVCN) of the Mongolian gerbil. The origin and contents of the microcyst are not elucidated at present. The present study investigated the possible inclusions in the microcyst by employing immunocytochemical labeling to localize the existence of various protein markers. Thirty and 100 µm thick sections were used to substitute and reconstruct between 6 and 20 paraffin serial sections, respectively. In 30-µm-thick slice sections, immunoreactivity of glial fibrillary acidic protein (GFAP-IR), mitochondria inner membrane (MCA-In-IR), S-100 (S-100-IR), serotonin (5-HT-IR), myelin proteolipid protein (PLP-IR) and substance P (SP-IR) abutted on the perimeter of the microcyst. The immunolabeled SP-positive cells were adjacent to the evagination of the microcyst. In 100-µm-thick slice sections, immunoreactivity of nitric oxide synthase (NOS-IR) and somatostatin (SOM-IR) mainly precipitated as flocculent structures in the small to medium-sized microcysts. 5-HT-IR also precipitated as an elongated flocculent stalk adjacent to the large microcyst or randomly distributed in the neuropil. The findings suggest that GFAP, MCA-In, S-100, 5-HT, PLP, SP, NOS and SOM may be involved in modulating the physiological functions and maintaining micro-environmental homeostasis of the microcyst in the cochlear nucleus of the gerbil.

Key words: Glial fibrillary acidic protein, Microcyst, Nitric oxide synthase, Somatostatin, Posteroventral cochlear nucleus

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Introduction

Microcysts are also known as microcystic lesions containing large holes, cavities or vacuoles. They are most evident in the posteroventral and anteroventral cochlear nuclei (PVCN and AVCN) of the Mongolian gerbil. The presence of microcysts in the cochlear nucleus of the gerbil was first reported by Morest et al. (1986). These microcysts are small, fluid-filled cavities in the posterior ventral cochlear nucleus and visible between 5 weeks and 36 months of age (Morest et al., 1986; Ostapoff et al., 1987; Czibulka and Schwartz, 1991; Yu and Ke, 1992). These microcystic lesions are shown as a neurodegenerative disorder in the cochlear nucleus of the gerbil (Morest et al., 1986; Ostapoff et al., 1987; Statler et al., 1990) and described as a spongioform lesion (McGinn and Faddis, 1987; Faddis and McGinn, 1993). The gerbils exposed to high-frequency noise had fewer spongioform lesions than the gerbils exposed to low-frequency noise (McGinn and Faddis, 1994). The number and size of the microcysts are greatly reduced by conductive hearing loss induced by ligation of the external auditory canal (McGinn and Faddis, 1987, 1988; Faddis and McGinn, 1993). However, the microcysts do not cause major changes in neuronal populations in the posteroventral cochlear nucleus (PVCN) (Schwartz and Karnofsky, 1988). There is no correlation between an increase in the number of microcysts and a decrease in the number of the cells (Czibulka and Schwartz, 1991). Results of quantitative measurements also indicate that age and acute unilateral deafening do not cause any change in the number or density of the astrocyte distribution (Czibulka and Schwartz, 1993). Therefore, microcyst formation appears to be a dynamic process related to the degree of auditory stimulation. Immunocytochemical localization of different markers, for example, antisera to gamma-aminobutyric acid (GABA), glycine, glial fibrillary

acidic protein (GFAP), S-100 and Rip (for oligodendrocyte) (Wenthold, et al., 1986; Oberdorfer et al., 1988; Yu, 1992; Czibulka and Schwartz, 1993; Faddis and McGinn, 1993; Yu and Du, 1997) have been demonstrated in the cochlear nucleus. Previous findings indicate that GFAP-immunoreactivity is not found in the microcysts (Faddis and McGinn, 1993). However, immunolabeled GFAP-positive reaction product can be located in the microcysts (Czibulka and Schwartz, 1993). The formation, nature and content of these microcysts are still unsolved and remain controversial. We have therefore raised the question of whether other neurotranmitters or protein markers are expressed in the microcyst. The aim of the present study was to reexamine and investigate the possible inclusions of the microcyst in the gerbil cochlear nucleus by using immunoperoxidase techniques to label a series of protein markers and employing different thicknesses of vibratome sections in order to minimize the loss of the internal content in the microcyst. In 30-µm-thick slice sections, immunoreactivity of GFAP(GFAP-IR), mitochondria inner membrane (MCA-In-IR), S-100 (S-100-IR), serotonin (5-HT-IR), myelin proteolipid protein (PLP-IR) and substance P (SP-IR) were mainly localized on the perimeter of the microcyst. In 100-µm-thick vibratome sections, immunoreactivity of nitric oxide synthase (NOS-IR), somatostatin (SOM-IR), and serotonin (5HT-IR) were predominantly localized in the small to medium-sized microcysts or found in an elongated flocculent structure.

Materials and methods

Vibratome slice preparation: Immunoperoxidase staining

The Mongolian gerbils, *Meriones unguiculatus*, were received at 6-months old from the National Breeding and Research Center of Laboratory Animals in the National Taiwan University and housed in standard vivarium facilities with room temperature (24-26°C) and lighting schedule (light on 0500-1900 h daily). Animals were anesthetized with sodium pentobarbital (30 mg/kg, i.p.) and sacrificed by cardiac flush with saline nitrite solutions (0.9% NaCl, 0.1% $NaNO_2$). Animals were fixed in a modification of McLean and Nakane's paraformaldehyde-lysine-periodate mixture fixative (1974) in 0.1 M phosphate buffer. The brains were removed from the skull, immersed in the same fixative overnight, embedded in agar and sliced at 30 (n1=3) or 100 (n2=4)µm with DTK-1000 Microslicer. The number (n1=3 or n2=4) in the brackets indicated the number of animals sectioned at 30 or 100 µm thickness, respectively.

Brains were sectioned in a coronal plane throughout the rostrocaudal extent of the nucleus. The sliced sections including the PVCN were used for this study. Slices (30 or 100 µm thickness) of each animal were placed in eight individual scintillation vials and were processed for immunocytochemical staining (Hsu et al., 1981) to localize the following eight different protein markers. Immunocytochemical staining was performed as follows: (1) incubation in rabbit antiserum to GFAP (GFAP, diluted 1:75, Zymed, CA, USA), mitochondria inner membrane (MCA, diluted 1:100, Serotec, USA), S-100(S-100, diluted 1:100, Zymed, CA, USA), serotonin (5-HT, diluted 1:100-800, Zymed, CA, USA), myelin proteolipid protein (PLP, diluted 1:100, Serotec, USA), substance P (SP, diluted 1:100, Biogenex Lab., CA, USA), nitric oxide synthase (NOS, diluted 1:800, Transduction Laboratories, Kentucky, USA), and somatostatin (SOM, diluted 1:3000, Peninsula Laboratories, Inc., CA, USA) for 14-16 hours; (2) incubation in goat anti-rabbit biotinylated gamma-globulin (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1-2 hours; (3) incubation in ABC complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) for 1-2 hours ; (4) treatment with 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.3 mg/ml, Sigma, USA) in 0.05 M Tris buffer, pH 7.6, containing 0.002% H_2O_2, until the reaction site was visible. All incubations were carried out at room temperature. Finally, the slices were dehydrated in ethanol, cleared in xylene, and coverslipped. In control samples, the first antibody was replaced by (1) normal rabbit or (2) PBS.

Results

Vibratome slice preparation: Immunoperoxidase staining

In 30-µm-thick slice sections, immunoreactivity of GFAP(GFAP-IR), mitochondria inner membrane (MCA-In-IR), S-100 (S-100-IR), serotonin (5-HT-IR), myelin proteolipid protein (PLP-IR) and substance P (SP-IR) partially abutted on the perimeter of the microcyst in the posteroventral cochlear nucleus (PVCN) (Fig. 1A-F). The immunoreactivity of nitric oxide synthase (NOS-IR) and somatostatin (SOM-IR) were less discernibly aggregated in clumps as shown in Figures 2A and 2C, respectively. In addition, GFAP-IR was randomly localized in the neuropil of the PVCN (Fig. 1A). MCA-In-IR was localized in the small to medium-sized cells adjacent to the microcyst (Fig. 1B). S-100-IR abutted on the perimeter of the microcyst and bulged into its lumen (Fig. 1C). 5-HT-IR was localized in small to medium-sized cells. The blood vessel was frequently connected with the microcyst (Fig. 1D). PLP-IR was localized in small to medium-sized cells. Some of the immunolabeled PLP-positive cells were in close association with the microcyst (Fig. 1E). SP-IR was localized in small to medium-sized cells. The immunolabeled SP-positive cells were adjacent to the evagination of the microcyst (Fig. 1F).

In 100-µm-thick slice sections, NOS-IR (Fig. 2A-B) and SOM-IR (Fig. 2C-D) were aggregated more discernibly than those in the 30-µm-thick slice sections. The patterns of GFAP-IR, MCA-In-IR, S-100-IR, 5-HT-IR, PLP-IR and SP-IR were similar to those in the 30-µm-thick slice sections. NOS-IR was mainly precipitated as a flocculent structure in the small to medium-sized

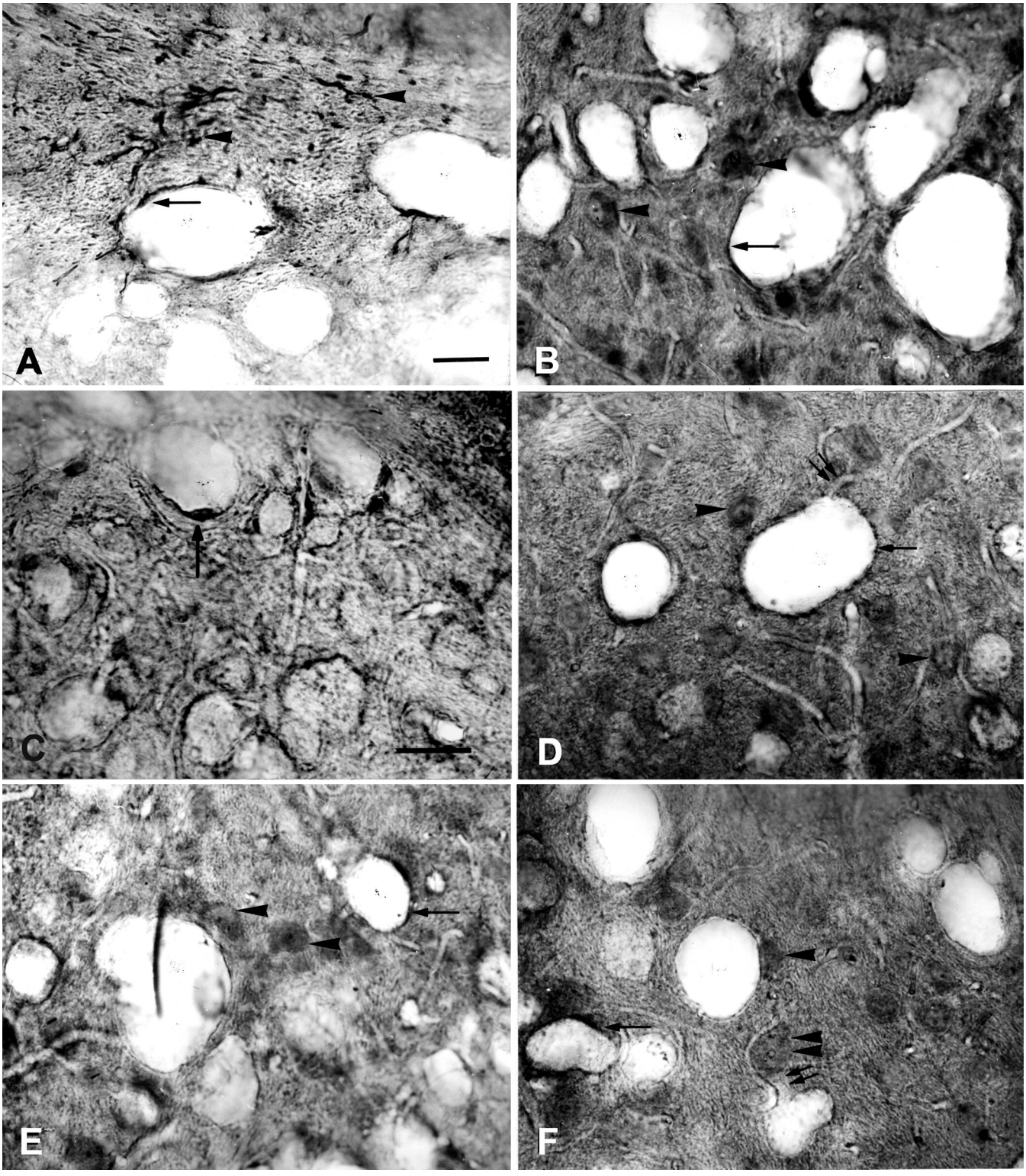

Fig. 1. Light micrographs of the PVCN in 30-µm-thick slice sections. **A.** GFAP-IR abutted on the perimeter (arrow) of the microcyst and the neuropil (arrowheads) in the PVCN. **B.** MCA-In-IR abutted on the perimeter (arrow) of the microcyst and the small to medium-sized cells (arrowheads). **C.** S-100-IR abutted on the perimeter (arrow) of the microcyst and bulged into its lumen. **D.** 5-HT-IR abutted on the perimeter (arrow) of the microcyst and the small to medium-sized cells (arrowheads). Note that a blood vessel (double arrows) connected with the microcyst. **E.** PLP-IR abutted on the perimeter (arrow) of the microcyst and the small to medium-sized cells (arrowheads). **F.** SP-IR abutted on the perimeter (arrow) of the microcyst and was localized in the small to medium-sized cells (single and double arrowheads). Such SP immunolabeled cells (single arrowhead) frequently abutted on the microcyst. Note that a SP immunolabeled cell (double arrowheads) was adjacent to the evagination (double arrows) of the microcyst. Scale bar: 25 µm.

Immuno-labeled microcysts in the PVCN

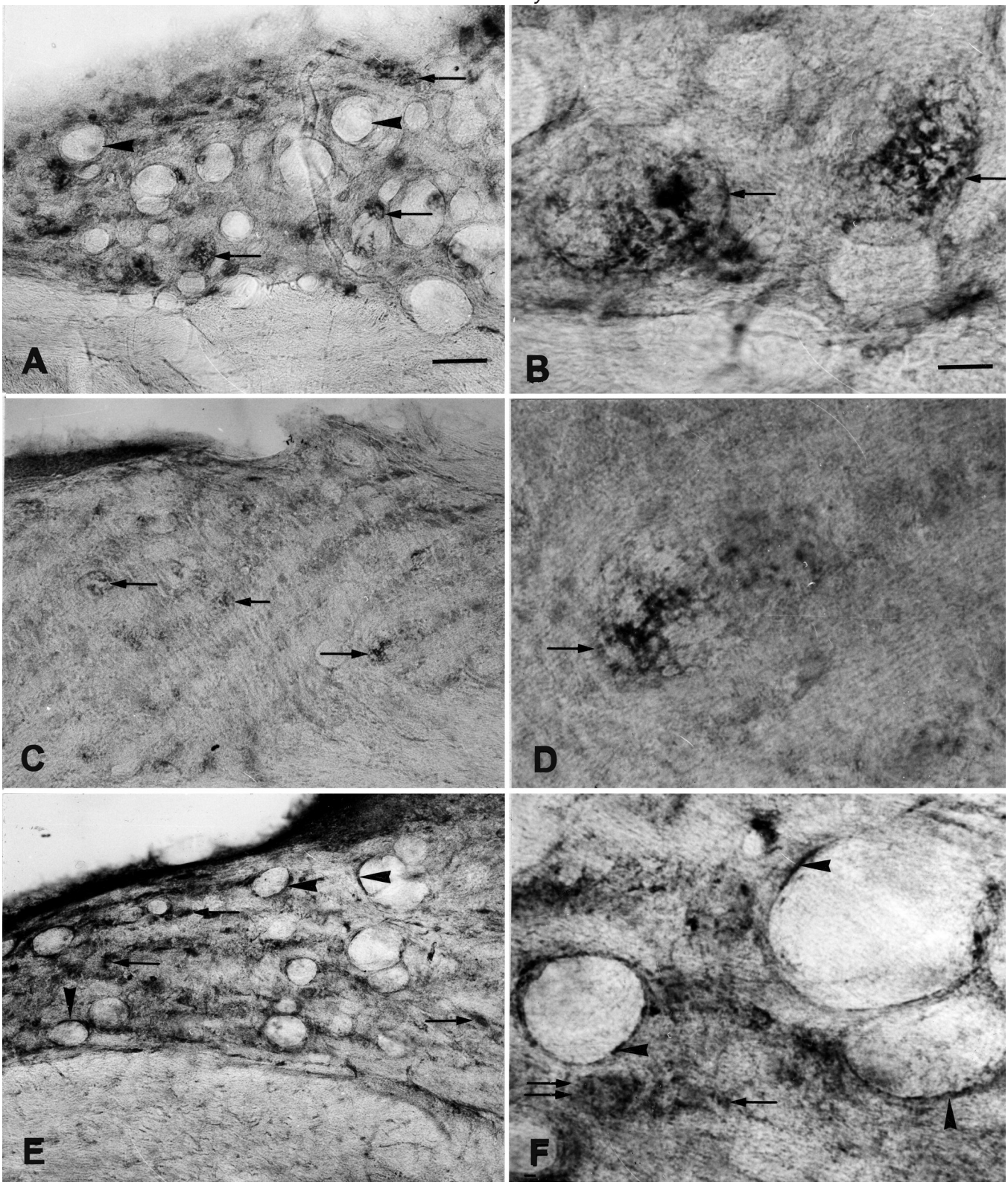

Fig. 2. Light micrographs of the PVCN in 100-µm-thick slice sections. **A.** NOS-IR (arrows) mainly precipitated as a flocculent structure in the small to medium-sized microcyst. There was no discernible NOS-IR in the large microcyst (arrowheads). **B.** At higher magnification, NOS-IR abutted on the perimeter (arrows) of the microcyst and evaginated a narrow stalk similar to a bulb-like structure. **C.** SOM-IR (arrows) formed flocculent precipitates in the small to medium-sized microcysts with an extended stalk. **D.** At higher magnification, SOM-IR abutted on the perimeter (arrow) of the microcyst and mainly precipitated as a flocculent structure. **E. 5**-HT-IR mainly abutted on the perimeter (arrowheads) of the large microcyst. 5-HT-IR precipitated as a flocculent structure with an elongated extension (arrows). **F.** At higher magnification, note that 5-HT-IR abutted on the perimeter (arrowheads) of the microcyst and located in the small to medium-sized cell (double arrows) as well as elongated flocculent stalk (arrow). Scale bars: A, C, E, 5 µm; B, D, F, 25 µm.

microcysts of the PVCN (Fig. 2A). In addition, NOS-IR abutted on the perimeter of the large microcyst. However, no discernible NOS-IR was found in these large microcysts. Immunolabeled NOS-positive microcysts evaginated a narrow stalk similar to a bulb-like structure (Fig. 2B). SOM-IR formed flocculent precipitates in the small to medium-sized microcysts with an extended stalk (Fig. 2C-D). Discernible 5-HT-IR abutted on the perimeter of the large microcyst similar to that in the 30-µm-thick slice section. 5-HT-IR mainly precipitated as an elongated flocculent stalk adjacent to the large microcyst or randomly distributed in the neuropil (Fig. 2-F).

Discussion

Although the microcyst is a common finding in the central auditory system of the Mongolian gerbil, it has been suggested to be a progressive neurodegenerative disorder. Microcystic formation is considered to be the result of a pathological process (Ostapoff and Morest, 1989). Previous investigations have demonstrated that microcysts are not histologically stained with basic or acidic dyes, or the Sudan black B stain (Ostapoff and Morest, 1989), or with glial fibrillary acidic protein (GFAP) (Faddis and McGinn, 1993). However, we found the immunoreactive product of NOS, somatostatin and 5-HT in the small to medium-sized microcyst in this study by employing 30- and 100-µm-thick slice sections for immunocytochemical staining.

There are two advantages of using 30- or 100-µm-thick slice sections to substitute 6 or 20 paraffin sections (5µm in thickness), respectively. First, it provides the microcyst retaining the internal content and intact perimeter when the diameter of the microcyst is less than 30 or 100 µm in length. The immunolabeled perimeter of the microcyst is not to be likely revealed distinctly in the paraffin sectioned tissues. Second, it allows the immunoreactive product to detect the protein markers for substituting and reconstructing a series of sequentially immunolabeled paraffin sections. We had shown that both GFAP-IR and S-100-IR distinctly abutted on the perimeter of the microcyst. Our findings thus confirm the previous reports (Czibulka and Schwartz, 1993) utilizing the monoclonal antibody to GFAP and S-100 and demonstrating the immunolabeled microcysts on the 2-µm-thick plastic sections and 100 µm vibratome sections. It should be mentioned, however, that no discernible flocculent precipitate was found in our GFAP and S-100 immunolabeled slice sections.

A striking finding was that MCA-In-, 5-HT-, PLP-, and SP-IR were localized in the small to medium-sized cells. MCA-In is a marker for mitochondria inner membrane and PLP is a myelin proteolipid protein. Both immunolabeled markers also abutted on the perimeter of the microcyst. We considered that the small to medium-sized immunolabeled positive cells might be the internuncial cells for externally modulating the physiological functions of the microcyst and maintaining the homeostasis of the microenvironment in the cochlear nucleus. In a previous study, normal appearing mitochondria are trapped between the trabeculae within an entirely membrane-bounded spongioform lesion (Faddis and McGinn, 1993). Moreover, it has been postulated that the microcyst is formed within an axon or its myelin sheath (Czibulka and Schwartz, 1993). Previous observations clearly indicate that microcysts are presumably derived from the detachment of the myelin sheath of the retracted axons, protrusion of the myelin sheath, and disruption of the myelin sheath (Yu and Du, 1997). The present immunolabeled evidence therefore supports the suggestion that mitochondria and myelin may be involved in regulating the development and differentiation of the microcyst.

In this study we showed another striking finding that immunolabeled NOS-, SOM- and 5-HT-positive flocculent structures were localized in the small to medium-sized microcysts. This new evidence was explored by using 100-µm-thick slice sections in the immunocytochemical staining. It is possible that the existence of NOS, somatostatin and 5-HT might be involved in internally modulating the physiological functions of the microcyst. Although the small to medium-sized microcyst was immunolabeled, the large microcyst was not immunolaeled at all. A likely explanation is that the large microcyst is cut open by vibratome sectioning and the small to medium-sized microcysts (less than 100 µm thick) remain intact in situ. For this reason, the internal contents in the large microcyst were completely lost and in the small to medium-sized microcyst were retained in situ. The scarcity of internal contents in the large microcyst could also account for the possible existence of a leakage route or channel between the blood vessel and the microcyst (Yu and Du, 1997). Although there is no direct evidence that there is a release of immunolabeled protein markers into the blood vessel, our present result indicating that the microcyst is connected with a blood vessel is consistent with our previous findings(Yu and Du, 1997). In this study, we have shown that a series of protein markers abutted on the perimeter of the microcyst. The above-mentioned protein markers may be involved in externally and internally modulating the physiological functions and maintaining microenvironmental homeostasis of the microcyst. Ultrastructural localization of immunolabeled reactive products is under investigation.

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