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Ultrastructural Localization of ZnT3 and Zinc Ions in the Mouse Choroid Plexus

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생쥐 맥락얼기에 분포하는 ZnT3 및 zinc 이온의 조직화학적 동정

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ABSTRACT

We have detected the murine zinc transporter, ZnT3, and zinc ions in the mouse choroid plexus by immunocytochemistry (ICC) and zinc selenium autometallography ($ZnSe^{AMG}$), respectively. BALB/c mice served as experimental animals. Routine floating ABC immunocytochemical procedures were used for the ZnT3 immunocytochemistry, and the mice were injected intraperitoneally (i.p.) with sodium selenide (10 mg/kg) for the zinc selenium autometallography. The choroid plexus showed weak immunoreactivity (Ir) for ZnT3. At high magnification, ZnT3 Ir was seen to be located in the choroid epithelium and the connective tissue of the capillaries. At the EM level, a high electron density of ZnT3 immunoreactivity was restricted to vesicle membranes as well as microvilli in the apical membrane. In contrast, immunostaining of ZnT3 was completely absent in the basolateral plasma membrane and other cell organelles. After silver enhancement, fine $ZnSe^{AMG}$ grains were observed in both the epithelial and endothelial cells of the choroid plexus. Few $ZnSe^{AMG}$ grains present in the cell bodies of the choroid epithelial cells were located in multivesicular bodies. It is striking that very many $ZnSe^{AMG}$ grains were observed in the endothelial cells of the capillaries.

These findings establish the choroid plexus as a non neuronal pool of zinc ions in the brain, although the functional significance of this pool is not clear. The choroid epithelium, however, may play an important role in the transportation of zinc between the CSF and brain tissue.

Key words : Autometallography, Choroid plexus, Immunocytochemistry, Mouse, Zinc ions, ZnT3

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INTRODUCTION

Two pools of zinc ions have been demonstrated to date in the mammalian brain. One is tightly complexed with proteins or vital for the functioning of enzymes (Ebadi, 1991,1994), the other consists of loosely bound or free ions in the synaptic vesicles of what have been called zinc enriched (ZEN) terminals. The pattern of ZEN terminals can be studied at both the light and electron microscopic levels by zinc sulphide or selenide autometallography (ZnS^{AMG} or $ZnSe^{AMG}$) (Danscher 1981, 1982, 1996), or by TSQ fluorescence staining (Frederichson et al., 1987). It is most impressive in the hippocampus, amygdala, cerebral neocortex, brainstem and spinal cord (Slomianka et al., 1990; Christensen, 1992; Schrøder et al., 2000). The ZEN neurons in the brain are believed to be a subset of glutamatergic neurons, and it has been suggested that the zinc ions serve both as a glue for proteins/peptides being transported in synaptic vesicles and as a modulator of post-synaptic receptors such as N-methyl-D-aspartate NMDA receptors (Frederickson & Danscher, 1990; Slomoianka et al., 1990).

In addition to these two major pools of zinc ions that have been investigated in detail, non-neuronal cells of the brain, such as glial cells, also contain free zinc ions (Danscher, 1981). Several lines of autoradiographic evidence suggest that the choroid plexus participates in cerebral zinc transportation (Kasarskis, 1984; Takeda et al., 1994), possibly by secreting protein (s) involved in zinc transportation, but no morphological support has been presented for this hypothesis.

A murine zinc transporter called ZnT3 has been cloned recently and shown to be located in the synaptic vesicles of ZEN terminals throughout the brain (Cole et al., 1999). The immunohistochemical ZnT3 staining patterns in the mouse and monkey hippocampus were almost identical to those seen with Timm's stain. We present data show here that ZnT3 and zinc ions in the choroid plexus are associated with epithelial and fibroblast-like cells. Our observations support the notion that the choroid plexus plays an important role in cerebral zinc homeostasis.

MATERIAL AND METHODS

1. Experimental animals

Twelve BALB/c mice, weighing about 30 g, served as experimental animals. They were housed in cages under the following conditions: 12 hr light/dark cycle, 21～22 °C, laboratory chow and tap water ad libitum.

2. ZnT3 immunocytochemistry

Mice were anaesthetized with pentobarbital (50 mg/kg, i.p.), transcardially perfused with 50 ml normal saline followed by 200 ml mixed fixative consisting of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed with the same fixative for 3 hr (4°C). Four brains to be used for light microscopy (LM) were cryoprotected in 30% sucrose until they sank to the bottom of the container, and then frozen with CO_2 gas. 30 μm coronal sections were prepared on a cryostat. Three brains for electron microscopy (EM) were cut in 100 μm sections with a vibratome. All sections were rinsed in 0.1 M Tris buffered saline (TBS) before immunostaining.

Routine floating ABC immunocytochemical procedures were used. After rinsing in TBS, the sections were treated with 1% hydrogen peroxide in pure methanol for 15 min to inactivate endogenous peroxidases and then rinsed in 1% bovine serum albumin (BSA), 3% normal goat serum (NGS) in TBS to reduce nonspecific immuno-staining. They were incubated for 48 hr (4°C) with ZnT3 antiserum (an affinity-purified rabbit antibody specific for ZnT3, provided by Professor R.D. Palmiter) diluted 1 : 100 in TBS containing 1% BSA and 3% NGS. After rinsing in TBS for 45 min, they were further incubated for 1 hr (22°C) in biotinylated goat anti-rabbit

IgG diluted 1 : 200 in TBS containing 1% BSA and 3% NGS. Thereafter sections were incubated for 1 hr (22°C) in ABC solution (DAKO) diluted 1 : 100 in TBS with 1% BSA, rinsed in Tris–HCl buffer (TB), and incubated in 0.025% 3, 3′–diaminobenzidine (DAB) with 0.0033% H_2O_2 for about 15 min. The reaction was stopped by rinsing in TB until a positive reaction could be clearly seen.

Stained cryostat sections for LM were treated by alcohol dehydration, xylene clearance and DPX mounting. Vibratome sections for EM were further processed by postfixation in 1% osmium acid in 0.1 M PB for 1 hr (22 °C), followed by alcohol dehydration and embedding in Epon 812. Semi thin sections were cut and observed by LM. Selected immunoreactive areas were re–embedded and cut in 80 nm ultrathin sections, stained with uranyl acetate and lead citrate and observed by EM (Philips 208, Eindhoven, The Netherlands) at 80 kV.

Control sections, treated as above but without primary antibody, showed a complete lack of immunoreactivity.

3. $ZnSe^{AMG}$

Six mice were anaesthetized and intraperitoneally (i.p.) injected with sodium selenide (10 mg/kg). After 1.5 hours, they were re–anaesthetized and transcardially perfused with saline followed by 3% glutaraldehyde in 0.1 M phosphate buffer. Their brains were removed and placed in the same fixative for 3 hours. Three of the brains were rinsed in the phosphate buffer and placed in 30 % sucrose for about 24 hours until they sank to the bottom of the containers. They were then frozen with CO_2 gas. 30 μm cryostat sections were cut and placed on Farmer rinsed slide glasses, dipped in a 0.5% gelatin soluti-

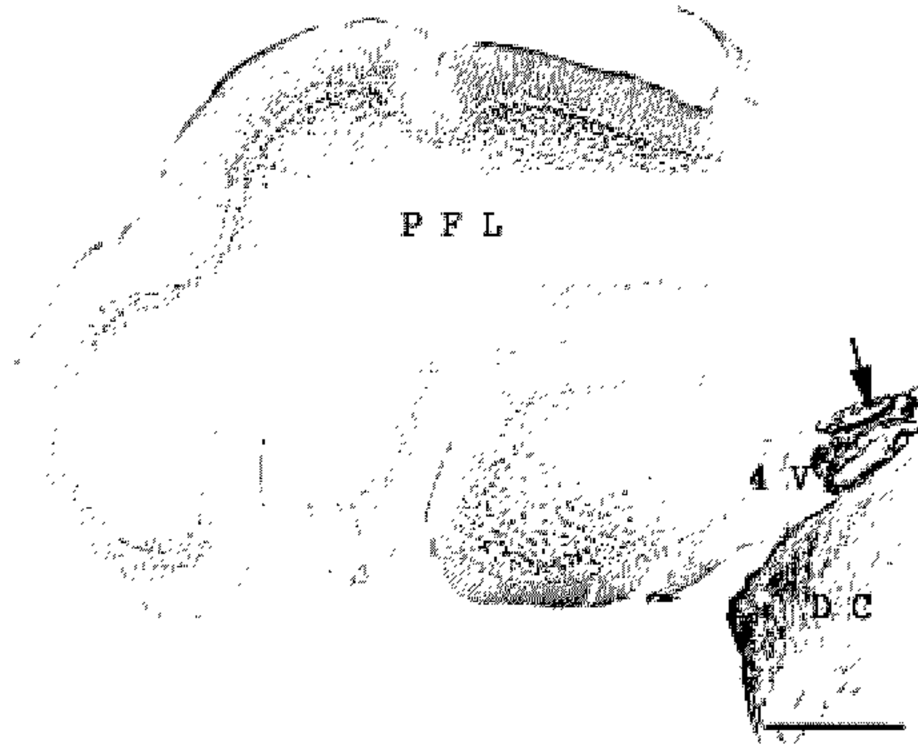

Fig. 1. Light micrograph of a coronal section immunostained for ZnT3. The arrow indicates the choroid plexus showing strong ZnT3 immunostaining in the lateral recess of the fourth ventricle (4 V). DC, dorsal cochlear nucleus; PFL, paraflocculus of the cerebellum. Scale bar represents 200 μm.

on and placed in vials. The other three brains were cut in 80 μm vibratome sections. AMG developer was poured into the developing vials placed in a 26°C water bath, and the whole setup covered with a light-tight lid as described by Danscher et al. (1997). The AMG developer contained gum arabic, citrate buffer, hydroquinone and silver lactate, and sections were developed in the dark for 60 min at 26°C. Development was stopped by replacing the developer with the AMG stop bath, 5% thiosulphate solution. The vials were then placed under running tap water for 10 minutes in order to remove the gelatin membrane, dipped in distilled water and finally counterstained with 0.1% toluidine blue. The AMG stained vibratome sections were treated by the routine EM technique described above.

RESULTS

1. Immunocytochemical localization of ZnT3

Strong ZnT3 immunostaining was detected in the coronal sections of the choroid plexus of all cerebral ventricles of the mouse brain (Fig. 1). However the intensity of immunostaining was weak compared with the mossy fibers in the hippocampus. At the lateral ventricle level, the intensity of staining was similar to that in the amygdala, but stronger than in the other brain areas. At higher magnification, the immunostaining was located to the membrane of the choroid epithelial cells and the connective tissue of capillaries. Almost all of the choroid epithelial cells were stained, but the intensity varied. Immunostaining was absent in the cavities of capillaries

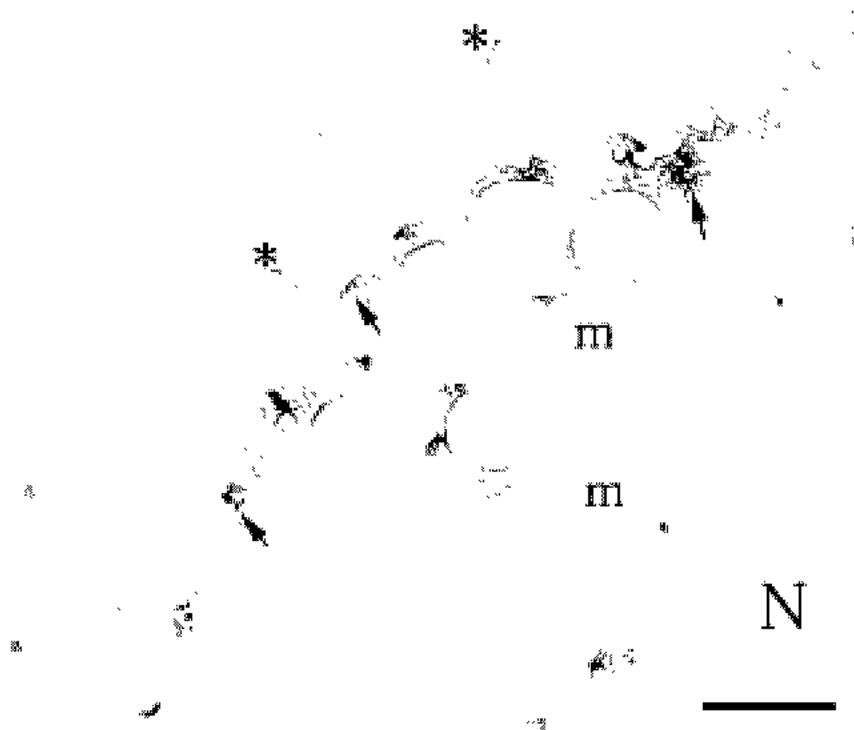

Fig. 2. Electron micrograph showing ZnT3 immunoreactivity in a choroid epithelial cell. Immunostaining is restricted to the apical vesicles (arrows) and the 'finger-like' microvilli (asterisks). m, mitochondria; N, nucleus. Scale bar: 1 μm.

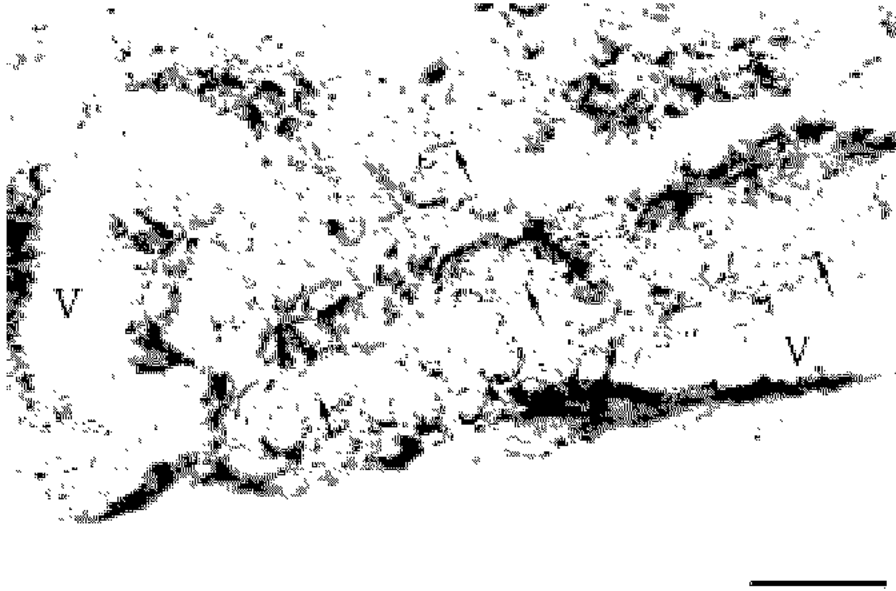

Fig. 3. Light micrograph illustrating the autometallographic detection of zinc ions by $ZnSe^{AMG}$. Arrows indicate $ZnSe^{AMG}$ grains found in the choroid plexus of the fourth ventricle. Note that grains are observed in both epithelial cells of the choroid plexus and connective tissue including capillaries, but not in the cavity (V) of the ventricle. Scale bar: 50 μm.

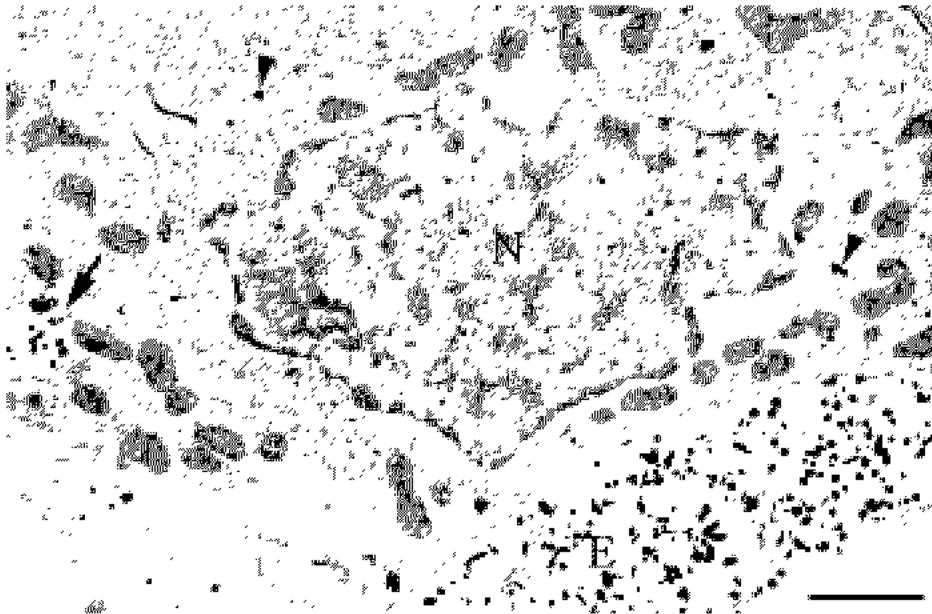

Fig. 4. Electron micrograph of choroid epithelium and endothelium containing $ZnSe^{AMG}$ grains. Most of the $ZnSe^{AMG}$ grains in the choroid epithelial cells are located in organelles resembling multivesicular bodies (arrow) within the cell body, and only a few isolated grains are found in the microvilli and the cytosol (arrowheads). Note the many $ZnSe^{AMG}$ grains in the endothelial cell (E) of the capillaries. N, nucleus. Scale bar: 2 μm.

and ventricles.

At the EM level, strong ZnT3 immunoreactivity was restricted to the apical vesicle membranes of the choroid epithelial cells and the 'finger-like' microvilli and cilia of the apical membranes (Fig. 2). A positive reaction was also observed in the fibroblast-like cells, but not in the endothelial cells.

2. Autometallographical dectection of zinc ions

After silver enhancement, the choroid plexus of all cerebral ventricles stained with $ZnSe^{AMG}$ grains at low magnification. At high magnification, fine $ZnSe^{AMG}$ grains were observed in both epithelial cells of the choroid plexus and connective tissue including the capillaries, but not in the cavity of ventricles (Fig. 3).

Ultrastructurally, $ZnSe^{AMG}$ grains were observed in the choroid epithelial cells, basement membranes, connective tissue and endothelial cells. Most of the $ZnSe^{AMG}$ grains in the choroid epithelial cells were located in organelles resembling multivesicular bodies within the cell body; only a few isolated grains were found in the microvilli and the cytosol. It is striking that very many $ZnSe^{AMG}$ grains were observed in the endothelial cells of the capillaries (Fig. 4).

DISCUSSION

ZnT3 immunoreactivity has been shown to be an alternative to autometallographic detection of zinc ions as a criterion for ZEN terminals in the CNS. Here we found a good correlation between ZnT3 immunoreactivity and $ZnSe^{AMG}$ staining in the mouse choroid plexus, suggesting that some non-neuronal zinc-containing cells can also be detected by both ZnT3 immunohistochemistry and zinc ion autometallography. It has been suggested that MT-III participates in zinc utilisation, since there is a close correspondence between the neurons rich in MT-III mRNA and those that store zinc in their terminal vesicles. A similar colocalization of MT-III mRNA and zinc has also been observed in the neurons (Masters et al., 1994).

The epithelium of the choroid plexus controls the exchange of certain ions between brain tissue and cerebrospinal fluid (CSF). Localization of ZnT3 within the mouse choroid plexus is of particular interest because previous work has indicated that the choroid plexus may participate in zinc transport to or from CSF (Kasarskis, 1984; Takeda et al., 1994, 1997). Using high resolution autoradiography, Takeda et al. reported that ^{65}Zn was concentrated in the choroid plexus after intravenous injection of $^{65}ZnCl_2$. Thereafter it gradually decreased at this location while increasing in other regions of the brain, suggesting that brain tissue takes up zinc ions from the CSF (Takeda et al., 1994). In contrast, Kasarskis (1984) has suggested that the choroid plexus transports zinc out of the cerebrospinal fluid compartment. The present study revealed the presence at the LM level of ZnT3 and zinc ions in the choroid plexus, suggesting a role of ZnT3 in the zinc ion-transporting functions of these specialized cells.

Previous work has shown that the glial cells of the brain contain loosely bound or free zinc ions, which can be detected with the AMG technique. This has led to the notion that glial cells may represent a pool of zinc ions in the brain additional to protein-bound zinc, and zinc within the synaptic vesicles of ZEN neurons. In our experience, only a very small number of free zinc ions exist in the glial cells. However, we detected very numerous zinc ions in the choroid plexus, especially in the connective tissue. We therefore suggest that the choroid plexus represents a non-neuronal pool of zinc ions in the brain, although its functional significance is not clear. These observations suggest that the choroid epithelial cells may play important roles in the transportation of zinc between the CSF and brain tissue.

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＜국문초록＞

본 연구는 BALB/c 생쥐 백악얼기내 ZnT3 및 zinc 이온을 ZnT3 항혈청을 이용한 면역세포화학법(ABC법)과 autometallography(ZnSe^{AMG})로 각각 동정하였다.

저배율에서 백락얼기내 ZnT3 면역반응은 미약하였으나, 고배율에서는 백락상피와 백락조직에 국한된 뚜렷한 면역반응이 관찰되었다. 전자현미경에서 관찰된 ZnT3 면역반응은 주로 백락상피의 자유면쪽 미세융모 및 막성 소기관에 국한되었던 반면 백락조직내 모세혈관의 내피세포에서는 면역반응이 전혀 관찰되지 않았다.

AMG 염색결과 백락상피와 백락조직에서 강한 AMG 과립이 관찰되었으며, 특히 모세혈관의 내피상피에서 가장 많이 AMG 과립이 분포하고 있었다. 백락상피내 AMG 과립은 주로 다소포체에서 관찰되었으며, 소수는 특정 세포질소기관과 상관없이 사이토졸에 산재해 있었다.

이러한 본 연구의 결과를 바탕으로 저자들은 백락얼기가 일종의 zinc pool의 역할이 있을 것이며, 최소한 뇌척수액과 뇌실질간에 zinc의 이동에 중요한 역할을 담당할 것으로 믿는다.