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Zinc transporter 7 is located in the *cis*-Golgi apparatus of mouse choroid epithelial cells

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The cellular localization of zinc transporter 7 protein in the mouse choroid plexus was examined in this study. Zinc transporter 7 immunoreactive cells were detected in the third, lateral, and fourth ventricles of CD-1 mouse brain. Distinct zinc transporter 7 immunoreactivity was concentrated in the perinuclear regions of the positive cells. The results from zinc autometallography showed that zinc-positive grains were also predominantly located in the perinuclear areas. Ultrastructural localization showed that

zinc transporter 7 immunostaining was predominantly present in the membrane and cisternae of the *cis*-Golgi networks and some vesicle compartments. The results support the notion that zinc transporter 7 may participate in the transport of the cytoplasmic zinc into the Golgi apparatus, and may be involved in local packaging of zinc-binding proteins in the mouse choroid plexus. *NeuroReport* 17:1807–1811 © 2006 Lippincott Williams & Wilkins.

Keywords: autometallography, choroid plexus, Golgi apparatus, immunohistochemistry, zinc, zinc transporter

Introduction

In mammalian cells, zinc transport across cell membranes is mediated by zinc transporters. Zinc transporters are classified into two families, ZIP (ZRT, IRT-like protein, Slc39) and ZnT (zinc transporter, Slc30), on the basis of their amino acid similarities [1,2]. ZIP family members function in zinc influx into the cytosol from extracellular spaces and the lumen of intracellular organelles, whereas ZnT family members serve to transport zinc in the opposite direction [1,2]. ZnT family members are predicted to have six transmembrane domains and a large histidine-rich intracellular loop, which is thought to be the zinc-binding site. Since 1995, eight ZnT zinc proteins, named ZnT1–ZnT8, have been cloned and characterized [3–11]. These ZnT proteins share a high degree of amino acid sequence and structural similarities, but they reside in different subcellular compartments in the cell, suggesting that they participate in the regulation of cellular zinc homeostasis in various cellular compartments [4–11]. Among the ZnT proteins, ZnT1 is the only transporter that is responsible for transporting zinc from the cytosol to the extracellular spaces. The others (ZnT2–ZnT8) are involved in pumping zinc into different intracellular organelles when the cellular zinc level is elevated [2].

Zinc autometallographic studies have shown that the choroid plexus is involved in the biodynamics of zinc ions in the central nervous system [12]. Immunohistochemical

studies have also demonstrated that many ZnT proteins (ZnT1, ZnT3, ZnT4, and ZnT6) are expressed in the choroid epithelium [13]. Localization of ZnT proteins and free zinc ions within the choroid plexus suggested that the choroid plexus may participate in zinc transport to or from cerebrospinal fluid [12,13].

We have recently reported that ZnT7 is expressed in many organs including the brain [4]. Immunofluorescence microscope observations revealed that ZnT7 is localized in the Golgi apparatus. Zinc exposure to the ZnT7-overexpressing Chinese hamster ovarian (CHO) cells caused an accumulation of zinc in the Golgi apparatus, indicating that ZnT7 facilitated zinc transport from the cytosol into the Golgi complex [4]. In the choroid plexus, however, the involvement of ZnT7 in zinc metabolism is obscure. The present study was therefore undertaken to examine the expression of ZnT7 in mouse choroid plexus with the emphasis on its subcellular localization.

Materials and methods

Animals

Male CD-1 mice (8–10 weeks old, 30–35 g) were used in the present study. They were housed under a 12-h light/dark cycle, with water and food available *ad libitum*. All procedures were performed in agreement with the ethical standards of China Medical University.

Immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneal), and were perfused transcardially with isotonic saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4), or 4% paraformaldehyde and 0.025% glutaraldehyde in 0.1 M PB for electron microscopy. The brains were carefully removed and further postfixed by immersion into the corresponding fixatives for 3 h at 4°C.

For light microscopy, samples were rinsed in PB and placed in 30% sucrose until they sank to the bottom of the container. After rinsing, samples were cut into 10- μ m coronal sections in a cryostat and mounted on glass slides. The sections were rinsed in Tris-buffered saline (TBS, pH 7.4), and endogenous peroxidases were inactivated by treatment with 3% hydrogen peroxide in pure methanol for 10 min. After rinsing, sections were treated with 5% bovine serum albumin (BSA) and 3% goat serum in TBS for 1 h to reduce nonspecific staining. They were then incubated for 18 h at 4°C with anti-ZnT7 antibody [4], diluted 1:100 in TBS containing 3% goat serum, 1% BSA, and 0.3% Triton-X 100. Sections were rinsed and incubated in biotinylated goat anti-rabbit IgG (diluted 1:200) for 1 h at room temperature (RT). The ABC Kit (Boster, Wuhan, China) was then applied for 1 h at RT to visualize the immunoreactive sites. The ABC solution was diluted 1:100 in TBS. A brown color appeared in the sections after incubation in 0.025% 3,3'-diaminobenzidine with 0.033% hydrogen peroxide for 10 min at RT. The sections were dehydrated and covered with coverslips for light microscopy.

For electron microscopy, samples were cut into 50 μ m thick slices in a vibratome. Immunostaining was performed on free floating vibratome slices. Briefly, samples were incubated at 4°C with anti-ZnT7 antibody, diluted 1:50 in TBS containing 1% BSA and 0.25% dimethylsulfoxide for

18 h. After rinsing, the slices were incubated in biotinylated goat anti-rabbit IgG (diluted 1:200) for 12 h at 4°C. The ABC solution was then applied for 1 h at RT. A brown color appeared in the slices after 3,3'-diaminobenzidine colorization. Samples were further processed for electron microscopy by postfixation in 1% osmium tetroxide in 0.1 M PB for 1 h at RT. After alcohol dehydration and propylene substitution, slices were flat-embedded in Epon812 (Serva Biochemicals, Heidelberg, Germany). Semithin sections were prepared with an ultramicrotome and stained with 0.1% toluidine blue for light microscope examinations. Ultrathin sections were cut perpendicular to the plane of the vibratome slices, stained with uranyl acetate for 10 min, and examined at 80 kV in an electron microscope (JEM-1200EX; JEOL, Tokyo, Japan).

To assess nonspecific staining, a few sections in every experiment were incubated with normal sera instead of the primary antibody. This procedure always resulted in a complete lack of immunoreactivity.

Zinc selenide autometallography

Two mice were used for analyzing the distribution of chelatable zinc ions by zinc selenide autometallography (ZnSe^{AMG}) in the choroid plexus. They were injected intraperitoneally with sodium selenite (20 mg/kg). After 1.5 h survival time, they were deeply anesthetized with pentobarbital (50 mg/kg) and perfused transcardially with isotonic saline, followed by 2.5% glutaraldehyde in 0.1 M PB. The brains were carefully removed and further postfixed by immersion into the same fixatives for 3 h at 4°C.

For cryoprotection, all samples were placed in a 30% sucrose solution overnight at 4°C. After rinsing, samples were cut into 10- μ m coronal sections on a cryostat and mounted on glass slides pretreated with Farmer solution to

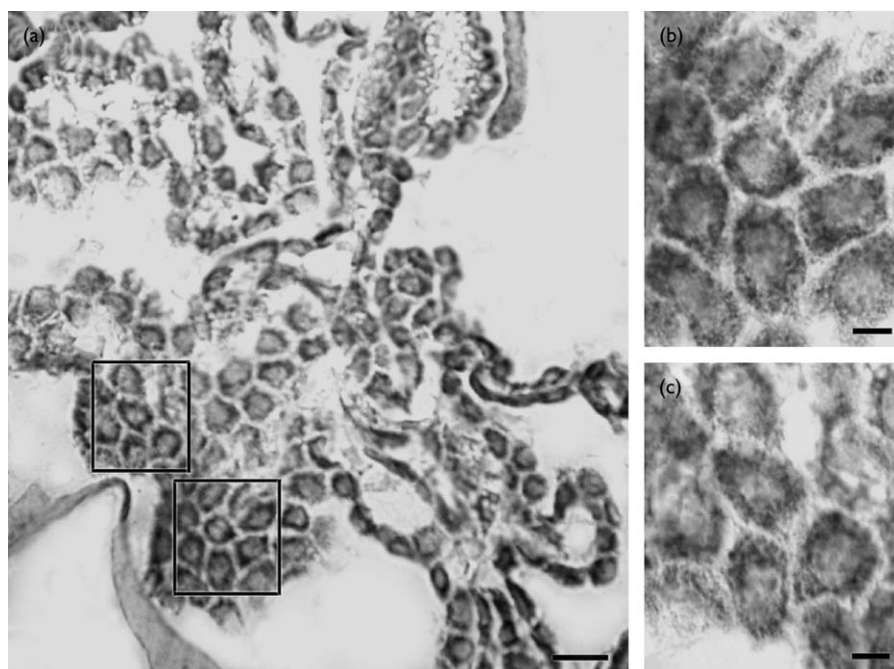


Fig. 1 Immunohistochemical analysis of zinc transporter ZnT7 in the third ventricle of the mouse choroid plexus. (a) At low magnification, strong immunoreaction of ZnT7 was present throughout the epithelial cells of the choroid plexus. Bar=20 μ m. (b and c) Higher magnification pictures showing strong ZnT7 immunoreactivity predominantly located in the perinuclear region of the cell. Bar=5 μ m.

avoid contamination. Sections were incubated in the autometallography (AMG) developer in a 26°C water bath for 1 h as described by Danscher [14]. After incubation in the AMG developer, sections were immersed in a 5% thiosulfate solution to stop the AMG reaction. Sections were then placed under running tap water for 20 min to remove the gelatine membrane. The sections were then dehydrated and covered with coverslips for light microscopy.

Results

ZnT7 immunoreactivities were observed in the epithelial cells lining the choroid plexus in the third, lateral, and fourth ventricles (Fig. 1). No visible differences were seen in the intensity of immunoreactivity or the staining pattern among the cerebral ventricles. So, only the immunocytochemical localization of ZnT7 immunoreactivity in the third ventricle is shown in Fig. 1. In general, a distinct immunoreactivity of ZnT7 was observed throughout the cytoplasm of epithelial cells of the choroid plexus. At a higher magnification, the ZnT7 immunoreactivity was predominantly seen in the perinuclear regions of the epithelial cells, suggesting a Golgi apparatus localization (Fig. 1b and c).

Electron microscopic immunolocalization confirmed the presence of ZnT7 in the Golgi apparatus of the choroid epithelial cells of the lateral, third, and fourth ventricles. At a low magnification, strong ZnT7 immunostaining in the Golgi apparatus of the cell was clearly observed (Fig. 2a). At a higher magnification, ZnT7 was predominantly detected in the *cis*-face or forming face of the Golgi apparatus (Fig. 2b). Moreover, ZnT7 immunoreactivity could also be found in some vesicle compartments adjacent to the Golgi apparatus (Fig. 2b). ZnT7 immunostaining was not detected in other subcellular organelles, including rough endoplasmic reticulum (ER), mitochondria, and lysosomes.

ZnSe^{AMG} staining was used to determine the distribution of zinc ions in the mouse choroid epithelial cells. A dispersed granular staining pattern of AMG grains was revealed in the choroid epithelial cells (Fig. 3a). At a high magnification, the ZnSe^{AMG} staining was predominantly noticed in the perinuclear regions of the choroid epithelial cells, which is similar to the ZnT7 immunostaining (Fig. 3b and c).

Discussion

The epithelium of the choroid plexus controls the exchange of certain ions and molecules between the brain tissue and the cerebrospinal fluid. The choroid plexus is believed to be involved in the biodynamics of zinc ions in the brain [12,13]. We previously reported that four members of the zinc transporter family, ZnT1, ZnT3, ZnT4, and ZnT6, are abundantly expressed in the brain including the choroid plexus [13]. Accumulation of ⁶⁵Zn in the choroid plexus after an intravenous injection of ⁶⁵ZnCl₂ has been demonstrated by autoradiography [12,15]. The initial high levels of zinc in the choroid plexus gradually decreased while increasing in other brain regions, suggesting that the brain parenchyma receives zinc ions from the cerebrospinal fluid [12]. On the other hand, the choroid epithelium also deposits zinc out to the cerebrospinal fluid compartments [16].

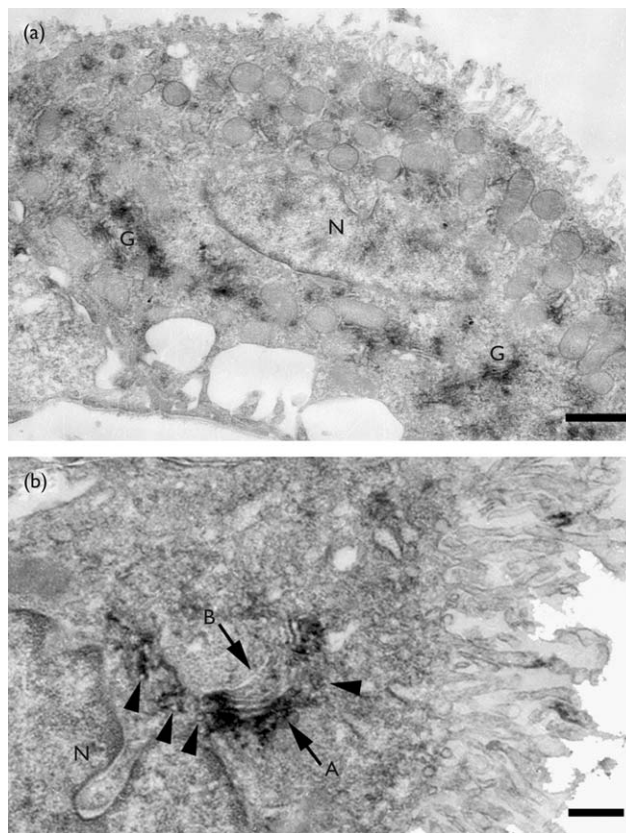


Fig. 2 Electron micrographs showing zinc transporter 7 immunoreactivity in the choroid epithelial cells. (a) At low magnification, ZnT7 was observed in the Golgi apparatus (G). Bar=1 μ m. (b) Higher magnification picture showing strong ZnT7 immunoreactivity predominantly located in the *cis*-face or forming face of the Golgi apparatus (arrow A). The *trans*-face or secreting face appeared to be devoid of ZnT7 immunostaining (arrow B). Some vesicle compartments also showed strong ZnT7 immunoreactivity (arrowheads). N, nucleus. Bar=500 nm.

In this study, we demonstrate that ZnT7 is expressed in epithelial cells of mouse choroid plexus. We also show that ZnT7 predominantly resides in the *cis*-Golgi stacks of the choroid epithelial cells. This is in good agreement with our earlier study showing that ZnT7 is localized in the Golgi apparatus of CHO cells [4]. The main functions of *cis*-Golgi stacks are to sort ER-resident proteins back to the ER and to modify proteins exported from the ER. It is also known that metallo-proteins acquire trace metals, such as zinc, during their itinerary in the biosynthetic-secretory pathway in the Golgi apparatus. Therefore, it is reasonable to suggest that ZnT7 may function to transport the cytoplasm zinc into the *cis*-Golgi apparatus for incorporation of zinc into zinc-containing proteins and/or for zinc storage in the choroid epithelial cells.

Compared with previous immunofluorescent microscopic data that showed the localization of ZnT7 in the Golgi apparatus [4], the result of the present immunoelectron microscopic study demonstrates the more precise localization of ZnT7 in the *cis*-Golgi apparatus, where proteins and lipids are imported into the Golgi apparatus from the ER, sorted, and modified. Moreover, the ZnSe^{AMG} staining indicates that the chelatable zinc ions are predominantly

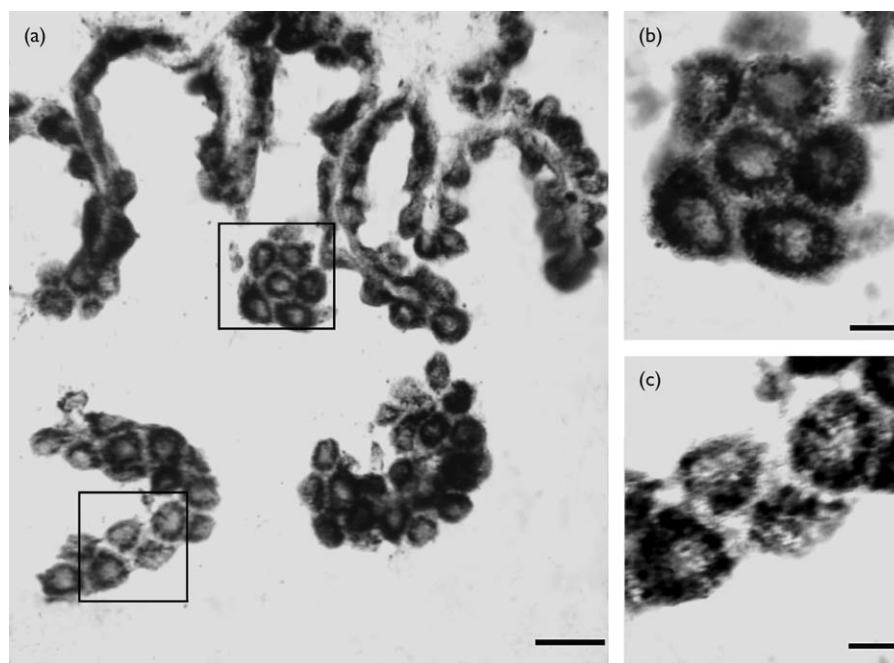


Fig. 3 Distribution of autometallographic (AMG) grains in the mouse choroid plexus. (a) At low magnification, a dispersed staining pattern of AMG grains in the choroid epithelial cells could be seen. The ZnSe^{AMG} staining exhibited a granular staining pattern. Bar=20 μm . (b and c) At high magnification, the ZnSe^{AMG} staining was located in the perinuclear region of the cell, which is similar to the ZnT7 immunostaining pattern. Bars=5 μm .

in the perinuclear region of the choroid epithelial cells, which is similar to the ZnT7 immunoreactive staining. This study provides morphological evidence that ZnT7 is probably involved in protein processing and zinc storage in the Golgi apparatus.

Our previous immunofluorescent data showed that there was a punctate staining pattern of ZnT7 in the cytoplasm, an indication of cytoplasmic vesicle localization of ZnT7 [4]. The present immunoelectron microscopic study confirmed that some vesicle compartments were ZnT7 positive in the choroid epithelial cells. The function of ZnT7 protein located in these vesicle compartments is, however, not known. This requires further study to clarify it.

The functional significance of zinc in the choroid plexus is not well understood. Our previous studies have shown that both free zinc ions and ZnT3 protein are localized in the perinuclear Golgi complex areas in neurons of rodent superior cervical ganglion [17]. Other members of the ZnT family, such as ZnT4 and ZnT6, are also present in the Golgi apparatus in many tissues [9,10]. These data indicate that ZnT7 is not the only zinc transporter to pump zinc from the cytosol into the Golgi apparatus. Therefore, it is essential to identify the proper locations of individual transporters in the Golgi apparatus and the mutual relationship between these zinc transporters.

Conclusion

ZnT7 is highly expressed in the epithelium of the lateral, third, and fourth ventricles of the mouse brain. Electron microscopic immunolocalization indicates that ZnT7 is expressed in the *cis*-Golgi apparatus of choroid epithelial cells. ZnT7 may participate in zinc storage and incorpora-

tion of zinc into zinc-binding proteins in the Golgi apparatus of the choroid epithelium.

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