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Authors

Wang, Zhan-You
Stoltenberg, Meredin
Jo, Seung Mook
[et al.](#)

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Dynamic zinc pools in mouse choroid plexus

Zhan-You Wang,^{1,2} Meredin Stoltenberg,¹ Seung Mook Jo,⁵ Liping Huang,³ Agnete Larsen,¹ Annica Dahlström^{4,CA} and Gorm Danscher¹

¹Department of Neurobiology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C, Denmark; ²Department of Histology and Embryology, China Medical University, Shenyang 110001, PR China; ³Western Human Nutrition Research Center, Agriculture Research Service, US Department of Agriculture, University of California at Davis, Davis, USA; ⁴Department of Anatomy and Cell Biology, Gothenburg University, Box 420, SE-40530 Gothenburg, Sweden; ⁵Department of Anatomy, College of Medicine, Hallym University, Okchon-I-Dong, Chunchon 200-702, Korea

^{CA}Corresponding Author: annica.dahlstrom@anatcell.gu.se

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We examined the presence of Zn-transporters (ZnT1, ZnT3, ZnT4, and ZnT6) proteins and zinc ions in rat choroid epithelium with immunohistochemistry and zinc selenide autometallography (ZnSe^{AMG}). The four ZnT proteins were all expressed in the choroid epithelial cells. ZnT3 immunostaining was found in vesicle membranes in the apical part of the cells, associated to the microvillus membrane. Correspondingly, the ZnSe^{AMG} technique revealed

zinc ions in small vesicles, in microvilli, and multivesicular bodies in the epithelial cells. Traceable zinc ions were also found in lysosome-like organelles of fenestrated endothelial cells, but here no corresponding ZnT3 immunostaining was seen. The observations suggests that the choroid plexus is instrumental to regulation of the level of zinc ions in the cerebrospinal fluid. *NeuroReport* 15:1801–1804 © 2004 Lippincott Williams & Wilkins.

Key words: AMG; Zinc-enriched cells (ZEN); Zinc transporter

INTRODUCTION

Subpopulations of glutaminergic neurons everywhere in the CNS and GABAergic neurons in the cerebellum, brain stem, and spinal cord have been found to contain zinc ions in a fraction of their synaptic vesicles [1–5]. These zinc-enriched (ZEN) neurons release zinc ions into the synaptic clefts in a calcium-dependent manner [6,7]. The concentrations of ZEN terminals are dense in the hippocampus, amygdala, cerebral neocortex, and olfactory bulb. However, most part of the CNS is populated with myriads of ZEN terminals [8,9].

Several autoradiographic studies have suggested that the choroid plexus is involved in the biodynamics of zinc ions in the CNS [10,11]. It has been hypothesized that the choroid epithelium that covers the capillaries of the plexus and constitutes the blood–CSF barrier secretes protein(s) involved in zinc transportation [12].

Zinc transporter (ZnT) proteins are believed to be involved in zinc homeostasis by transporting zinc ions across the plasma membranes and the membranes of intracellular compartments [10]. Seven members of the ZnT family (ZnT1–ZnT7) have been cloned and characterized since 1995. In general, ZnTs share a similar membrane topology with six transmembrane domains and a histidine-rich loop, which has been hypothesized as a zinc binding region. Differences in activity and localization of ZnTs have been characterized in some detail and their roles in zinc homeostasis have been suggested [10,13]. ZnT3 has been studied extensively in the brain due to its specific and abundant expression in the CNS [14–17]. The present study adds to the hypothesis that the level of zinc ions in the CSF is controlled by the choroid plexus.

MATERIALS AND METHODS

BALB/c mice (M & B Breeding Center, Denmark), weighing about 30 g, were used in this study. The animals were housed at 21–22°C in a 12:12 h light:dark cycle, and with free access to food and tap water. All procedures were performed in agreement with the ethical standards of the University of Aarhus and Danish law.

Zinc transporter immunohistochemistry: Four ZnT antibodies were used in this study. They were affinity-purified rabbit antisera specific for each ZnT protein. (1) ZnT1 antiserum (dilution 1:500) was provided by Dr W.F. Silverman, Department of Physiology, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Israel. (2) ZnT3 antiserum (dilution 1:100) was provided by Dr R.D. Palmiter, Department of Biochemistry, University of Washington, USA. (3) ZnT4 antiserum (dilution 1:20) and (4) ZnT6 antiserum (dilution 1:25) were provided by Dr L. Huang, US Department of Agriculture, Davis, California, USA. Normal goat serum (NGS), biotinylated goat anti-rabbit IgG (dilution 1:200), and fluorescein (DTAF)-conjugated streptavidin (dilution 1:800) were purchased from Jackson ImmunoResearch, PA, USA.

Eight mice were anaesthetized with pentobarbital (50 mg/kg, i.p.), transcardially perfused with 50 ml normal saline, followed by 200 ml fixative (4% paraformaldehyde and 0.01% glutaraldehyde) in 0.1 M phosphate buffer (PB). Brains were removed and postfixed with the same fixative for 3 h (4°C).

Four brains were cryoprotected in 30% sucrose and frozen with CO₂ gas. Coronal sections (30 µm) were prepared on a cryostat and preincubated in NGS for 1 h. Incubations with rabbit anti-ZnT1, ZnT3, ZnT4 or ZnT6 were performed overnight at room temperature. As secondary antibody biotinylated goat anti-rabbit IgG was applied for 2 h, followed by DTAF-conjugated streptavidin for 2 h. The immunofluorescence-labeled sections were examined in a confocal laser scanning microscope with a krypton/argon laser (MRC 1024, BIO-RAD). The excitation filters for DTAF (488 nm) were selected. Images were collected using the Kalman mode scanning (10 scans) and processed using Adobe Photoshop 5.0.

Routine floating ABC immunocytochemical procedures were used. Endogenous peroxidase activity was quenched with 3% H₂O₂ in 20% methanol for 15 min. The sections were incubated for 24 h (4°C) with ZnT3 antiserum, and after washing in TBS with 1% Triton X-100 the sections were further incubated for 1 h (22°C) in biotinylated goat anti-rabbit IgG, incubated in streptavidin-HRP (1:100) for 1 h, in 0.05% 3,3'-diaminobenzidine (DAB) with 0.033% H₂O₂ for 10 min, and finally washed in TBS to stop the reaction when a clearly positive reaction was visible, dehydrated, and mounted with DPX.

Four brains for electron microscopy (EM) were sectioned at 100 µm on a vibratome. The sections were handled as described above, except that no Triton X-100 was used in the washing procedures and 0.5% DMSO was added to the incubation solutions. The sections were further processed by postfixation in 1% osmium acid in 0.1 M PB for 1 h (22°C) followed by dehydration and embedding in Epon. Semi-thin sections were cut and observed by LM. Selected areas were re-embedded and ultrathin sections were prepared, stained with uranyl acetate and lead citrate and observed by EM (Philips 208, Eindhoven, The Netherlands) at 80 kV.

In both immunofluorescence and immunoperoxidase experiments, control sections were incubated with normal sera instead of the primary antibodies, followed by all subsequent incubations as described above. Control sections showed a complete lack of immunoreactivity.

Zinc selenide autometallography: Twelve mice were randomly divided into two groups. They were anaesthetized and injected with sodium selenide (10 mg/kg, i.p.) and sodium selenite (20 mg/kg, i.p.), respectively. After 1.5 h survival, they were re-anaesthetized and transcardially perfused with 3% glutaraldehyde in 0.1 M PB. The brains were removed and postfixed in the same fixative for 3 h. For LM, three brains from each group were rinsed in PB and cryoprotected in 30% sucrose for 24 h. They were then frozen with CO₂ gas. Cryostat sections (30 µm) were cut and placed on Farmer rinsed slides. For EM, the remaining brains were sectioned at 100 µm on a vibratome. Both the cryostat and the vibratome sections were incubated in the AMG developer in a 26°C water bath. The AMG developer contained gum arabic, citrate buffer, hydroquinone and silver lactate. The sections were developed in the dark for 60 min at 26°C, washed with distilled water and finally counterstained with 0.1% toluidine blue. The AMG stained vibratome sections were treated by the routine EM technique described in detail previously [4].

Four mice not injected with sodium selenide or sodium selenite served as blank controls. The effect on the staining

pattern of prior treatment with the metal chelating agent diethyldithiocarbamate (DEDTC) was examined in four mice (divided in two groups representing the sodium selenide and sodium selenite groups, respectively). These mice were injected i.p. with 1000 mg/kg DEDTC 1 h before the sodium selenide or sodium selenite injection (for details regarding DEDTC and autometallography, see [18]).

RESULTS

Immunofluorescence and confocal laser scanning microscopy were used to determine the distribution of ZnT1, ZnT3, ZnT4, and ZnT6 in the choroid plexus. Sections of the choroid plexus from the lateral, third, and fourth ventricles were probed with the four tested antibodies. At the confocal microscopic level, ZnT1, ZnT3, ZnT4 and ZnT6 showed a similar staining pattern in all the cerebral ventricle choroid plexus. In general, a distinct immunofluorescence of ZnT1, ZnT3, ZnT4 or ZnT6 was observed throughout the epithelial cells of the choroid plexus while the nuclei were void of staining. The sections with ZnT3 and ZnT4 antibodies showed more grainy staining than ZnT1 and ZnT6 (Fig. 1a–d).

Routine floating ABC immunocytochemical observations revealed a distinct ZnT3 immunostaining in the coronal sections of all the choroid plexus of the mouse brain (Fig. 2a). However, the intensity of immunostaining was weak compared to the hippocampal mossy fibres in CA3 (data not shown). At higher magnification, ZnT3 immunostaining was found to be located primarily in the apical part of the choroid epithelial cells. Most, if not all, epithelial cells were ZnT3-positive, though stained with varying intensity. At EM levels, a particularly intense ZnT3 immunostaining was observed in the plasma membrane of the 'finger-like' microvilli and in the membranes of apical vesicles (Fig. 3a). A diffuse faint staining for ZnT3 was seen in the cytoplasm.

ZnSe^{AMG} staining was used to determine the distribution of zinc ions in the mouse choroid plexus. At low magnification, the ZnSe^{AMG} staining pattern was similar to the ZnT3^{IH} staining. At high magnification, ZnSe^{AMG} grains were observed primarily in the apical part of the epithelial cells located either free in the cytoplasm of the microvilli or in vesicles (Fig. 2b). AMG control sections from mice not treated with selenium, or treated with DEDTC before being exposed to selenium were void of AMG staining in the epithelial cells of the choroid plexus. Ultrastructurally, ZnSe^{AMG} grains were observed in the choroid epithelial cells, epiplexus cells, basement membranes, and the endothelial cells. Most of the ZnSe^{AMG} grains in the choroid epithelial cells were located in the organelles resembling multivesicular bodies within the cell body; only few isolated grains were found in the microvilli and the cytosol (Fig. 3b).

DISCUSSION

In the CNS, histochemically detectable zinc (~10–15% of total brain zinc) is primarily located in the synaptic vesicles of a subset of neurons [19]. Neurons that contain free zinc ions in the vesicles of their presynaptic boutons have been termed zinc-enriched (ZEN neuron). Studies from the hippocampus, amygdala and neo-cortex revealed that ZEN neurons in the forebrain represent a subgroup of

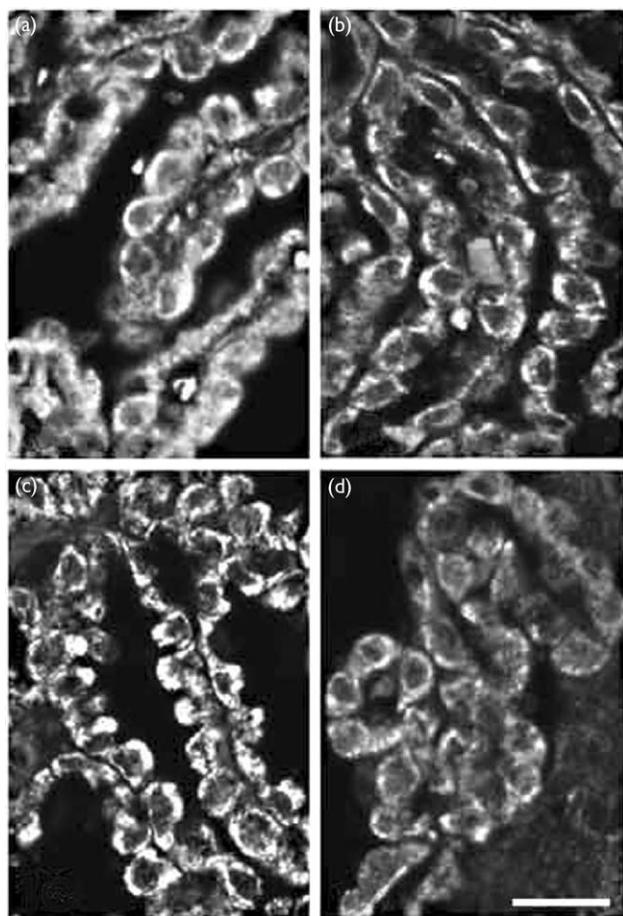


Fig. 1. Confocal micrographs showing the immunofluorescence localization of ZnT1 (a), ZnT3 (b), ZnT4 (c), and ZnT6 (d) in mouse choroid plexus. ZnT1, 3, 4, and 6 are expressed in choroid epithelial cells from the third ventricle. It seems that ZnT3 and ZnT4 antibodies exhibit a granular staining pattern. Bar=50 μ m.

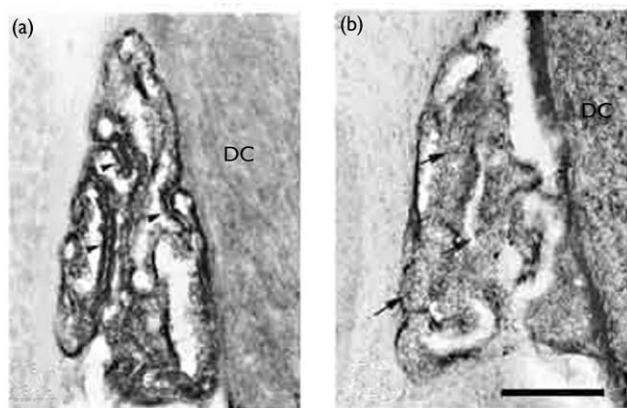


Fig. 2. Light micrographs taken from a 30 μ m coronal section of the mouse brain stained by ZnT3 immunocytochemistry and zinc selenium autometallography (ZnSe^{AMG}). Arrowheads and arrows indicate the choroid plexus showing strong ZnT3 immunostaining (a) and a granular pattern of AMG staining (b) in a lateral view of the fourth ventricle (4V), respectively. Note the abundant ZnSe^{AMG} stained ZEN neurons in the dorsal cochlear nucleus (DC). Bar=50 μ m.

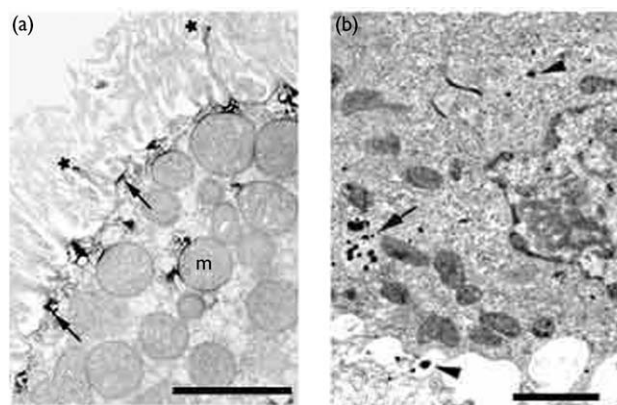


Fig. 3. Electron micrographs showing ZnT3 immunoreactivity (a) and AMG grains (b) in the choroid epithelial cells. ZnT3 immunostaining is observed in the apical vesicles (arrows), the 'finger-like' microvilli (asterisks) and in the cytosol. Most of the ZnSe^{AMG} grains in the choroid epithelial cells are located in organelles resembling multivesicular bodies (arrow) within the cell body, and only a few isolated grains are found in the intercellular space and the cytosol (arrowheads). N, nucleus; m, mitochondria. Bars=20 μ m.

excitatory glutamatergic neurons [3,20]. However, recent studies have shown that inhibitory GABAergic ZEN neurons exist in the spinal cord and the cerebellum [2,4,5,21]. The significance of zinc ions in the synaptic transmission is far from well understood, though a common notion is that zinc ions released into the synaptic clefts of ZEN terminals may be neuromodulators working on postsynaptic receptors [22]. Recently identified mammalian zinc transporters (ZnTs) are believed to participate in zinc ion trafficking across membranes in living cells. ZnT3 is expressed in the nervous system [5,15,17], and one of its important functions is presumably to sequester zinc ions into presynaptic vesicles of ZEN neurons, since no histologically detectable zinc can be found in ZnT3 knock-out mouse brains.

The epithelium of the choroid plexus controls the exchange of certain ions between brain tissue and CSF [23]. Accumulation of ⁶⁵Zn in the choroid plexus after an intravenous injection of ⁶⁵ZnCl₂ has been demonstrated by high resolution autoradiography [23]. The initial high levels of zinc in the choroid plexus gradually decreased while increasing in other brain regions, suggesting that brain tissue takes up zinc ions from the CSF [11]. Kasarskis *et al.* [24], on the other hand, have suggested that the choroid plexus transports zinc out of the cerebrospinal fluid compartment.

CONCLUSION

Localization of ZnT proteins within mouse choroid plexus is of particular interest as previous works have suggested that the choroid plexus may participate in zinc transport to or from CSF [11,25]. It has been suggested that MT-III, a zinc-binding protein, is involved in neuromodulation based on the observation of the abundant expression of MT-III mRNA in neurons with ZEN terminals [25]. The present data therefore further support the notion that the choroid plexus plays an important role in regulation of zinc homeostasis in the brain.

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