Ultrastructural Localization of ZnT3 and Zinc Ions In Mouse Choroid Plexus

Yong Kuk Kim, Soo Jin Kim[1], Sung Joo Kim, Young Gil Jeong[2], Seung Mook Jo
Department of Anatomy, Hallym University,
[1]Department of Biology, Hallym University
[2]Department of Anatomy, Konyang University

Introduction

In the mammalian brain, two pools of zinc ions were confirmed so far. One pool is bound firmly in proteins or are vital to the function of enzymes (Ebadi, 1991,1994), another comprises loosely bound or free zinc ions found in synaptic vesicles of what have been called zinc enriched (ZEN) neurons. The pattern of ZEN terminals can be studied by zinc sulphide or selenide autometallography (ZnSAMG or ZnSeAMG) at both light and electron microscopic levels (Danscher 1981, 1982, 1996) or by TSQ fluorescence staining (Frederichson et al. 1987) and is most impressive in hippocampus, amygdala, cerebral neocortex, brainstem and spinal cord (Slomianka et al1990; Christensen 1992; Schrøder et al. 2000). In the brain, ZEN neurons are believed to be a subset of glutamatergic neurons and the zinc ions have been suggested to serve both as a glue for proteins/peptides being transported in a pool of synaptic vesicles and as a modulator of post synaptic receptors such as N-methyl-D-aspartate NMDA (Frederickson and Danscher 1990 Slomoianka et al 1990).

Except the above two main pools of zinc ions which have been reported in detail, the non-neuronal cells in the brain also contain free zinc ions, such as the glial cells and the choroid epithelial cells (Danscher G, 1981). Several lines of evidence from autoradiography suggest that the choroid plexus may participate in cerebral zinc transportation (Kasarskis, 1984; Takeda et al., 1994). It has been suggested that the choroid plexus secretes protein(s) which play a role in zinc transportation but no morphological evidence have until now supported the hypothesis.

Recently, a murine zinc transporter called ZnT3 was cloned and it was shown that the transporter is located to synaptic vesicles in ZEN terminals throughout the brain (Cole et al., 1999). The immunohistochemical staining pattern for ZnT3 in mouse and monkey

hippocampus were almost identical to that seen with the Timms staining. We present data showing that in the choroid plexus ZnT3 and zinc ions are related to the choroid epithelial cells and fibroblast-like cells. The results support the notion that the choroid plexus play an important role for cerebral zinc homeostasis.

Material and Method

1. Experimental animals

Twelve BALB/c mice, weighting about 30g, were served as experimental animals in this study. The mice were housed in cages with the following conditions: 12 hr light/dark cycle, 21-22 °C, laboratory chow and tap water ad libitum.

2. ZnT-3 immunohistochemistry

Mice were anaethetized with pentobarbital (50mg/kg, i.p.), transcardially perfused with 50ml normal saline, followed with 200 ml mixed fixative which contained 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1M phosphate buffer. The brains were removed and postfixed with the same fixtive for 3 hr (4°C). Four brains used for light microscopy (LM) were cryoprotected with 30% sucrose until they sank to the bottom of the jars and then frozen with CO2 gas. 30ìm coronal sections were prepared on a cryostat. Three brains for electron microscopy (EM) were cut in 100ìm sections with a vibratome. All sections were rinsed in 0.1 M Tris buffered saline (TBS) before immuno-staining.

The routine floating ABC immunocytochemical procedures were used. After rinsed in TBS the sections were treated with 1% hydrogen peroxide in pure methanol for 15 min to inactivate the endogenous peroxidases, then rinsed in 1% bovine serum albumin (BSA), 3% normal goat serum (NGS) in TBS to reduce nonspecific immuno-staining. Sections were incubated for 48 hr (4°C) with ZnT-3 antiserum (an affinity-purified rabbit antibody specific for ZnT-3, provided by Professor R.D. Palmiter), diluted 1:100 in TBS containing 1% BSA and 3% NGS. After rinsed in TBS for 45 min, the sections were incubated for 1 hr (22°C) in biotinylated goat anti-rabbit IgG, diluted 1:200 in TBS containing 1% BSA and 3% NGS. Sections were incubated for 1 hr (22°C) in ABC solution (DAKO), diluted 1:100 in TBS with 1% BSA. Sections were rinsed in Tris-HCl buffer (TB), then incubated in 0.025% 3,3'-diaminobenzidine with 0.0033% H2O2 for about 15 min, stopped the reaction by rinsing them in TB until the positive reaction could be seen clearly on the sections.

The stained cryostat sections for LM were treated by alcohol dehydration, xylene clearance and DPX mounting. Vibratome sections for EM were further processed by

postfixed in 1% osmium acid in 0.1 M PB for 1 hr (22°C), followed by alcohol dehydration and embedded in Epon 812. Semithin sections were cut and observed under a LM. Selected immunoreactive areas were re-embedded and cut in 80 nm ultrathin sections, stained with uranyl acetate and lead citrate, observed and photographed under an EM (Philips 208, Eindhoven, The Netherlands) at 80 kV.

Control sections for evaluated the specificity of the immunostaining were treated as above except incubated in solution without primary antibody, and resulted in a complete lack of immunoactivity.

3. ZnSeAMG

Six mice were anaesthetized and intraperitoneally (i.p.) injected with sodium selenide (10mg/kg). After 1.5 hours survival, the animals were re-anaesthetized and transcardially perfused with saline followed with 3% glutaraldehyde in 0.1M phosphate buffer. The brains were removed, placed in the same fixative for three hours, three brains were rinsed in the phosphate buffer and placed in 30% sucrose about 24 hours until they sank to the bottom of the containers. They were then frozen with CO 2 gas. Cryostat sections in 30 μm thickness were cut and placed on Farmer rinsed object glasses, dipped in a 0.5 % gelatine solution and placed in vials. Three brains were cut in 80 μm vibratome sections. The AMG developer was poured into the developing vials placed in a 26°C water bath, and finally the whole setup was covered by a light tight top as described by Danscher (Danscher et al. 1997). The AMG developer contains gum arabic, citrate buffer, hydroquinone and silver lactate, and the sections were developed in the dark for 60 min at 26°C. Development was stopped by replacing the AMG developer with the AMG stop bath, 5% thiosulphate solution. The vials were then placed under running tap for 10 minutes in order to remove the gelatine membrane, dipped in distilled water and finally counterstained with 0.1% toluidine blue. The AMG stained vibratome sections were treated with routine EM technique as described above.

Results

1. Immunohistoshemical localization of ZnT-3 in the choroid plexus

In the coronal sections of mouse brain stained with antiserum to ZnT-3, intense immunostaining was detected in the choroid plexus of all cerebral ventricles of the brain, except abundant in zinc-enriched terminals that had been shown previously, such as the hippocampal formation, amygdaloid complex, cerebral cortex and so on. Compared with the

mossy fibers in hippocampus, the immunostaining in the choroid plexus was weak. At the lateral ventricle level, the staining degree of the choroid plexus was similar to that in the amygdala, but it was stronger than that in the other brain areas. At higher magnification, the immunostaining was located in the membrane of the choroid epithelial cell and the connective tissue of the capillary. Almost all of the choroid epithelial cells were stained, but the staining degree was different. The immunostaining was void in the cavities of capillaries and ventricles. ZnT-3 immunopositive reactivity of the mossy fiber terminals was consistent with previous descriptions (Wenzel et al., 1997).

At the EM level, ZnT-3 immunoreactivity with high electron dense was selectively located in the vesicle membrane in the apical side of the mouse choroid epithelial cells. However, the immunostaining of ZnT-3 was completely lost in the finger-like microvilli and cilia in the apical membrane, the basolateral plasma membrane and other organelles. ZnT-3 immunopositive reactivity also could be observed in the fibroblast-like cells, but nothing in the endothelial cells and the epiplexus cells. The distribution of ZnT3 in the membrane of the synaptic vesicles in mossy fibers was consistent with previous descriptions (Wenzel et al., 1997).

2. Histochemical localization of zinc ions in the choroid plexus

After silver enhancement, the choroid plexus of all cerebral ventricles were stained with ZnSeAMG grains at low magnification. The fine ZnSeAMG grains, at high magnification, were observed in both the membrane and cell body of the epithelial cells of the choroid plexus and connective tissue, but they were void in the cavity of ventricle and capillary. Ultrastructuraly, ZnSeAMG grains were observed in the choroid epithelial cells, basement membranes, connective tissue and the endothelial cells. In the choroid epithelial cells, most ZnSeAMG grains were found in the cell bodies, located in the multivesicular body-like organelles, few isolated ZnSeAMG grains were found in the microvilli and the cell body. Its striking that a huge amount of ZnSeAMG grains were located in the connective tissue. The fibroblast-like cells in the connective tissue, which showed a strong ZnT3 immunoreactivity as described above, also contained ZnSeAMG grains in their cell bodies. The epiplexus cells also contained ZnSeAMG grains.

Discussion

ZnT3 immunoreactivity has been shown to be an alternative marker of ZEN terminals in the CNS to autometallographic detections of zinc ions. Here we found there was a good

correlation between ZnT3 immunohistochemistry and ZnSeAMG in the mouse choroid plexus. This result suggests that some of the non-neuronal zinc containing cells also can be detected with both the immunohistochemisty for ZnT and autometallography for zinc ions. It had been reported that MT-III might participate in the utilization of zinc as a neuromodulator, and there was marked correspondence between the neurons that are rich in MT-III mRNA and those neurons that store zinc in their terminal vesicles. A similar colocalization of MT-III mRNA and zinc also occured in the choroid plexus (Masters BA et al., 1994).

The choroid plexus epithelium controls the exchange of certain ions between the brain tissue and cerebrospinal fluid (CSF). Localization of ZnT3 within the mouse choroid plexus is of particular interest, because previous works have shown that the choroid plexus maybe participate in the zinc transport to or from CSF (Kasarskis, 1984; Takeda et al., 1994, 1997). By using high resolution autoradiography, Takeda et al has reported that 65Zn was largely concentrated in choroid plexus after 65ZnCl2 injected intravenously, and then gradually decreased here while increasing in the different brain regions. This suggested that the brain tissue take zinc ions from CSF (Takeda et al, 1994). In contrast, Kasarskis has also suggested that the choroid plexus were involved in cerebral zinc homeostasis by transporting zinc out of the cerebrospinal fluid compartment (Kasarskis, 1984).The present study showed the presences of ZnT-3 and zinc ions in the choroid plexus at the LM level, suggesting a role of ZnT3 in the regulation of zinc ion-transporting functions of these specialized cells.

Previous work has shown that the glial cells in the brain contain loosely bound or free zinc ions, which can be detected with AMG technique, and suggested that the glial cells is another pool of zinc ions in the brain, except the pools of zinc-bounding protein and the zinc containing synaptic vesicles of ZEN neurons. To our experience, only very a few free zinc ions exist in the glial cell. In the present study, we reported that there were a huge number of free zinc ions exist in the choroid plexus, especially in the connective tissue. Therefore, we suggest that the choroid plexus should be confirmed as a non-neuronal pool of zinc ions in the brain, although the functional significance of this pool is not very clear.

Its striking that the fibroblast-like cell in the connective tissue of the choroid plexus showed a high concentration of ZnT3 immunoreactivity and zinc ions, except the choroid epithelial cell. The endothelial cell of the fenestrated capillary within the choroid plexus, however, contained only few zinc ions and no ZnT3 immunoreactivity. These results suggest that both the choroid epithelial cell and fibroblast-like cell may play an important mutual

role for zinc transportation between the CSF and the brain tissue.

References

· Christensen MK, Frederickson CJ, Danscher G. Retrograde tracing of zinc-containing neurons by selenide ions: a survey of seven selenium compounds. J Histochem Cytochem 1992; 40(4):575-579

· Cole TB, Wenzel HJ, Kafer KE et al. Elimination of zinc from synaptic vesicles in the intact mouse brain by disruption of the ZnT3 gene. Proc Natl Acad Sci U S A 1999; 96(4):1716-1721

· Danscher G, Juhl S, Stoltenberg M et al. Autometallographic silver enhancement of zinc sulfide crystals created in cryostat sections from human brain biopsies: a new technique that makes it feasible to demonstrate zinc ions in tissue sections from biopsies and early autopsy material. J Histochem Cytochem 1997; 45(11):1503-1510

· Danscher G, Stoltenberg M, Juhl S. How to detect gold, silver and mercury in human brain and other tissues by autometallographic silver amplification. Neuropathol Appl Neurobiol 1994 ; 20(5):454-467

· Danscher G. Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. Histochemistry 1981; 71(1):1-16

· Danscher G. Exogenous selenium in the brain. A histochemical technique for light and electron microscopical localization of catalytic selenium bonds. Histochemistry. 1982; 76(3): 281-293.

· Danscher G. Histochemical demonstration of heavy metals. A revised version of the sulphide silver method suitable for both light and electronmicroscopy. Histochemistry. 1981; 71(1):1-16.

· Danscher G.The autometallographic zinc-sulphide method. A new approach involving in vivo creation of nanometer-sized zinc sulphide crystal lattices in zinc-enriched synaptic and secretory vesicles. Histochem J. 1996; 28(5):361-373.

· Ebadi M, Elsayed MA, Aly MH. The importance of zinc and metallothionein in brain. Biol Signals 1994; 3(3):123-126

· Ebadi M., Metallothioneins and other zinc-binding proteins in brain. Methods Enzymol 1991; 205:363-387

· Frederickson CJ and Howell GA. merits and demerits of zinc dithizone histochemistry, In: The neurobiology of zinc, part A. Physiochemistry, Anatomy, and Techniques (Frederickson

CJ, Howell GA and Kasaeskis EJ, eds.). Alan R. Liss, New York, pp. 289-303

· Frederickson CJ, Kasarskis EJ, Ringo D et al. A quinoline fluorescence method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in the brain. J Neurosci Methods. 1987 ; 20(2):91-103.

· Frederickson CJ. Neurobiology of zinc and zinc-containing neurons. Int Rev Neurobiol. 1989; 31:145-238.

· Kasarskis EJ. Zinc metabolism in normal and zinc-deficient rat brain. Exp Neurol. 1984 ; 85(1):114-127.

· Masters BA, Quaife CJ, Erickson JC et al. Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles. J Neurosci 1994 Oct; 14(10):5844-57

· Palmiter RD, Cole TB, Quaife CJ et al. ZnT-3, a putative transporter of zinc into synaptic vesicles. Proc Natl Acad Sci U S A 1996; 93(25):14934-14939

· Slomianka L, Danscher G, Frederickson CJ. Labeling of the neurons of origin of zinc-containing pathways by intraperitoneal injections of sodium selenite. Neuroscience 1990; 38(3):843-854

· Takeda A, Akiyama T, Sawashita J et al. Brain uptake of trace metals, zinc and manganese, in rats. Brain Res 1994; 640(1-2):341-344

· Takeda A, Sawashita J, Okada S. Localization in rat brain of the trace metals, zinc and manganese, after intracerebroventricular injection. Brain Res 1994; 658(1-2):252-254

· Wenzel HJ, Cole TB, Born DE et al. Ultrastructural localization of zinc transporter-3 (ZnT-3) to synaptic vesicle membranes within mossy fiber boutons in the hippocampus of mouse and monkey. Proc Natl Acad Sci U S A 1997; 94(23):12676-12681