Golgi apparatus localization of ZNT7 in the mouse cerebellum

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Summary. We have recently reported that four members of the zinc transporter (ZNT) family, ZNT1, ZNT3, ZNT4, and ZNT6, are abundantly expressed in the mouse cerebellum. In the present study, we reported that ZNT7 was present throughout the cerebellar cortex. ZNT7 immunoreactivity was predominately present in the somas and primary dendrites of the Purkinje cells. ZNT7 was also present in the Bergmann glial cell bodies as well as their radial processes, which extended into the molecular cell layer. Confocal immunofluorescence results demonstrated that the expression of ZNT7 overlapped with that of TGN38 in the somas of the Purkinje cells and granule cells. Immuno-electron microscopic study showed that ZNT7 was localized to the membrane of the Golgi apparatus in the somas of the Purkinje cells, Bergmann glial cells, and granule cells. Western blot analysis demonstrated that a considerable amount of ZNT7 was expressed in the cerebellum. These findings suggest a significant role of ZNT7 in zinc homeostasis in the mouse cerebellum.

Key words: Zinc transporter, Cerebellum, Purkinje cells, Bergmann cell, Mouse

Introduction

Zinc transporter (ZNT) proteins are required for cellular zinc homeostasis, since zinc cannot cross the plasma membranes freely. Recently, a ZNT family (SLC30) is believed to participate in transporting zinc across membranes in mammalian cells (Cousins and McMahon, 2000; Kambe et al., 2004; Seve et al., 2004).

To date, at least seven members of the ZNT family, ZNT1-7, have been cloned and well characterized (Palmiter and Findley, 1995; Palmiter et al., 1996a,b; Huang and Gitschier, 1997; Cole et al., 1999; Murgia et al., 1999; Liuzzi et al., 2001; Huang et al., 2002; Inoue et al., 2002; Kambe et al., 2002; Michalczyk et al., 2002; Kirschke and Huang, 2003). They are predicted to have six transmembrane domains with a large histidine-rich intracellular loop, except for ZNT5, which has extra six transmembrane domains at the N-termini of the protein. ZNT1 is localized to the plasma membrane and is responsible for pumping zinc out of the cell, whereas ZNT2-7 are localized to intracellular membranes and are involved in zinc sequestration into different intracellular compartments (Kambe et al., 2004).

ZNT7 is a 42 kDa membrane protein that is highly expressed in the mouse nervous system including brain, spinal cord and dorsal root ganglion, retina and choroids plexus (Kirschke and Huang, 2003; Chi et al., 2006, 2008; Wang et al., 2006; Zhang et al., 2007), and the senile plaques in the brains of Alzheimer patients and APP/PS1 transgenic mice (Zhang et al., 2008a,b). A recent report from our laboratory has demonstrated that the expression of ZNT7 overlapped with that of TGN38 (a Golgi complex marker) in the mouse spinal cord and dorsal root ganglia neurons (Zhang et al., 2007). Immuno-electron microscopic analysis indicated that ZNT7 protein is located to the forming face of the Golgi apparatus in the choroid plexus epithelial cells (Chi et al., 2006). Further, over-expression of ZNT7 in Chinese hamster ovary cells led to zinc accumulation in the Golgi apparatus when the cells were exposed to high zinc (Kirschke and Huang, 2003). These data suggest that ZNT7 may function in zinc accumulation in the Golgi complex (Kirschke and Huang, 2003; Chi et al., 2006; Zhang et al., 2007).

Recent studies have shown that chelatable zinc ions

are dispersed throughout the cerebellar cortex (Wang et al., 2002; Wall, 2005) and they are stored in inhibitory synaptic vesicles in the molecular and granule cell layers (Wang et al., 2002). We have recently reported that four members of the ZNT family, ZNT1, ZNT3, ZNT4 and ZNT6, are abundantly expressed in mouse cerebellum (Wang et al., 2005). To gain further insight on the involvement of the ZNT family in zinc homeostasis in the cerebellum, the present study has been undertaken to determine the cellular and subcellular localization of a new member of the ZNT family, ZNT7, in the mouse cerebellum.

Materials and methods

Experimental animals

A total of 19 adult CD-1 mice about 35g in weight were used as experimental animals in this study. All experiments were carried out in agreement with the ethical standards of China Medical University. Efforts were made to minimize both the suffering and number of animals used in this study.

Immunohistochemical procedures

Five mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). They were then transcardially perfused with saline followed by 4% paraformaldehyde in phosphate buffer (pH7.4). Brains were dissected, postfixed in the same fixative for 4 h at 4°C, and cryoprotected in 20% sucrose until they sank. They were then frozen with CO_2 and cryostat sections (50 μm) were prepared. The free floating immuno-staining of the sections was carried out. Briefly, sections were preincubated with 5% bovine serum albumin and 3% goat serum in 0.1 M Tris-buffered saline (TBS, pH 7.4) for 1 h at room temperature. They were then incubated with ZNT7 antisera (an affinity-purified rabbit antibody specific for ZNT7, 1:100) overnight at 4°C. The source and specificity of the antibody was reported in our previous study (Kirschke and Huang, 2003). After rinsing, sections were incubated with biotinylated goat anti-rabbit IgG (1:200) for 1 h at room temperature. Sections were rinsed and incubated with reagents from an ABC Kit (DAKO) for 1 h at room temperature. Sections were then rinsed in TBS and treated with 0.025% 3,3'-diaminobenzidine (DAB) in the presence of 0.033% H_2O_2 in TBS for about 10 min. Sections were dehydrated through graded alcohols, cleared in xylene, covered with coverslips, and examined by a light microscope. Some sections were counterstained with hematoxylin.

For electron microscopy, vibratome sections (50 μm) of mouse cerebellum were prepared. The free floating immunostaining of ZNT7 was carried out as mentioned above. After DAB treatment, the vibratome sections were fixed for 30 min in 1% osmium acid, and were embedded in Epon 812. Ultrathin sections were collected on mesh nickel grids, stained with uranyl acetate for 10 min, and examined in an electron microscope (JEM-1200EX).

As a negative control, some cryostate and vibratome sections were incubated without primary antibody and processed as described above. Non-specific staining was not observed.

Double immunofluorescence staining and confocal laser scanning microscopy

Double labeling of ZNT7 and TGN38 was performed to analyze the colocalization of the two proteins. Briefly, cryosections were prepared as above. They were preincubated with normal donkey serum (1:20) for 1 h and then incubated with a mixture of ZNT7 antiserum (1:50) and TGN38 monoclonal antibody (1:50, Oncogene Research Products) overnight at 4°C. After rinsing, sections were incubated with fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (1:50, Jackson ImmunoResearch Laboratories) and Texas Red-conjugated donkey anti-mouse IgG (1:50, Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Sections were mounted with an anti-fading mounting medium and examined in a confocal laser scanning microscope (SP2, Leica).

Western blot analysis

Nine mice were killed by over-dose sodium pentobarbital intraperitoneal injection. Cerebella, hippocampi and cerebral cortices were isolated and homogenized in an ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.25% sodium deoicolate, 0.1% SDS, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, and 10mg/ml leupeptin), phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF). The resulting homogenate was centrifuged at 12,000g for 30 min at 4°C. The supernatant was collected and total protein levels were determined using a BCA protein assay kit (Pierce). Proteins (20 μg) were separated on 10% SDS-polyacrylamide gels and transferred onto PVDF membranes in a wet electron transfer device (45 V, 15 h). Membranes were preincubated in nonfat milk for 3h and incubated with either a ZNT7 polyclonal antibody (1:1000) or a GAPDH monoclonal antibody (1:12000, KC-5G5, Kang Chen) overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:5000, Santa Cruz) against either the rabbit or the mouse, respectively, for 2 h. After washing, membranes were reacted with reagents in an enhanced chemiluminescence kit (ECL, Pierce). Finally, specific protein bands were visualized by exposure of the membranes to Kodak-XAR films. After development, the intensities of protein bands were quantified using Image-pro Plus 6.0 analysis software. Statistical significance of the difference between the mean values was evaluated by applying one-way

analysis of variance test (ANOVA) and analyses by a post hoc test (SPSS 13.0). $p < 0.05$ was accepted as statistically significant.

Results

Expression of ZNT7-immunoreactivity in the mouse cerebellum

ZNT7 immunoreactivity was present throughout the cerebellar cortex (Fig. 1a,b). In the Purkinje cell layer, ZNT7 immunoreactivity was predominately present in the somas and primary dendrites of most, if not all, Purkinje cells (Fig. 1a,b). ZNT7 immunoreactivity was also present in the Bergmann glial cell bodies and processes. At high magnification, it was shown that the positive radial processes of Bergmann glia extended into the molecular layer (Fig. 1b). A few ZNT7-positive neuronal somas were seen in the molecular cell layer (data not shown). In the granule cell layer, ZNT7 immunoreactivity displayed as puncta or semilunar structures (Fig. 1b).

Colocalization of ZNT7 and TGN38 in the mouse cerebellum

TGN38 was used as a marker for the Golgi apparatus (Reaves and Banting, 1992). Double
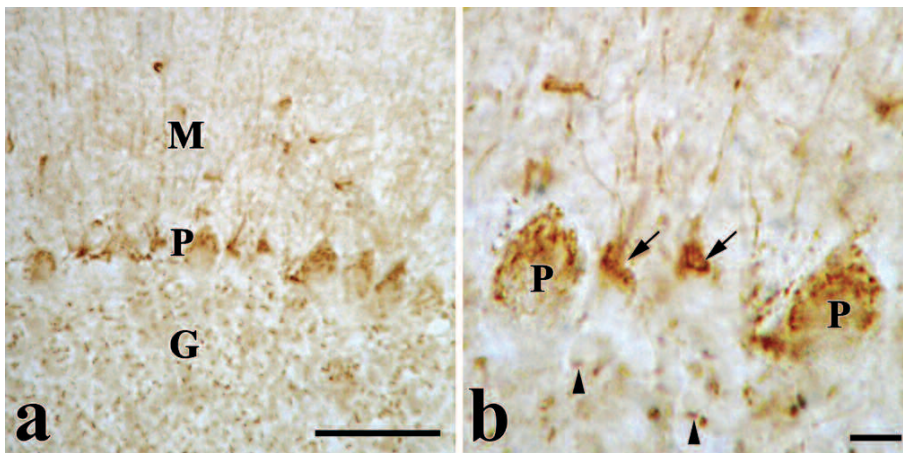

Fig. 1. Light micrographs illustrating the cellular localization of ZNT7 immunoreactivity in the mouse cerebellar cortex. **a and b.** ZNT7 immunoreactivity is present in the Purkinje cell bodies (P) and primary dendrites, the Bergmann glial cell bodies (arrows in b) and processes, and the granule cell bodies (arrowheads in b). M: molecular layer, P: Purkinje cell layer, G: granule cell layer. Bars: 50 μm (a) and 10 μm (b).
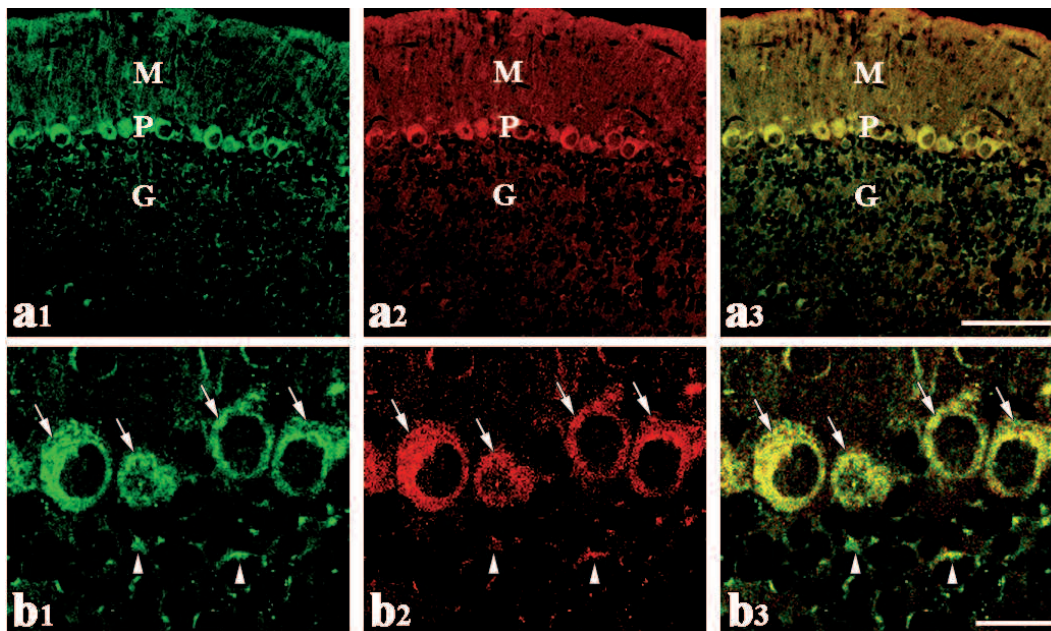

Fig. 2. Confocal laser scanning microscopic images showing ZNT7 (**a1, b1**) and TGN38 (**a2, b2**) immunoreactivities in the mouse cerebellar cortex. Colocalization of ZNT7 and TGN38 (**a3, b3**) is evident in the somas of Purkinje cells (arrows in b1-3) and granule cells (arrowheads in b1-3). M: molecular layer, P: Purkinje cell layer, G: granule cell layer. Scale bars: 75 μm (a1-3) and 7.5 μm (b1-3).
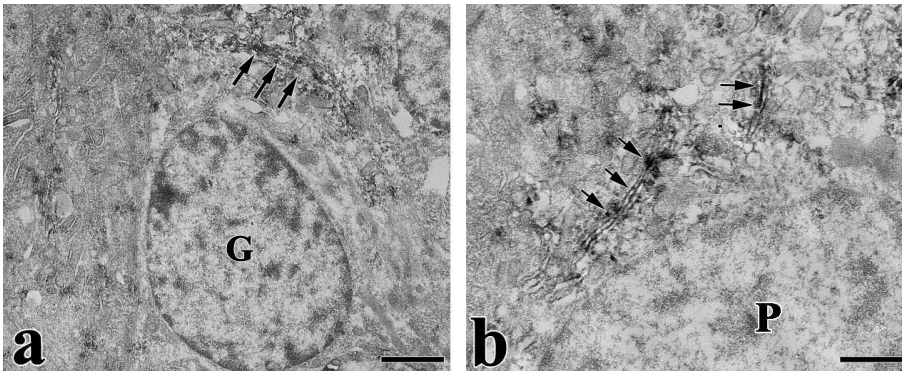
Fig. 3. Electron micrographs showing the subcellular localization of ZNT7 immunoreactivity in the mouse cerebellar cortex. ZNT7 immunoprecipitates can be found in the Golgi apparatus in the cytoplasm of a granule cell (G) (arrows in **a**). ZNT7 immunoprecipitates are clearly shown on the membranes of the Golgi apparatus in the Purkinje cells (P) (arrows in **b**). Bars: 0.5 μm.
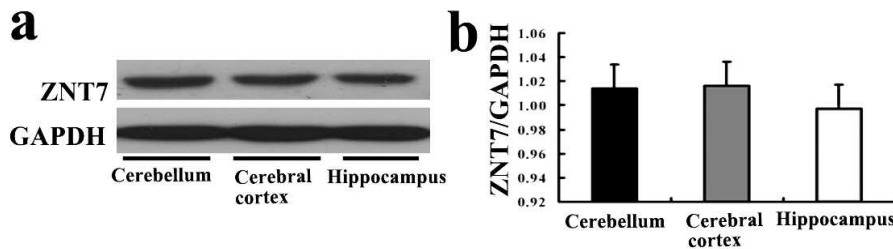

Fig. 4. Western blot analysis showing that the ZNT7 protein is expressed as a 42 kDa polypeptide in the cerebellum, cerebral cortex, and hippocampus (**a**). The densitometry analysis showing that the expression of ZNT7 is equivalent in the cerebellum, cerebral cortex and the hippocampus (**b**). Results are analyzed by one-way analysis of variance test (p>0.05).

immunofluorescence staining demonstrated a colocalization of ZNT7 and TGN38 in the somas of Purkinje cells (Figs. 2a1-3, b1-3). Structures positive to both ZNT7 and TGN38 staining were also distributed in the granule cell layer. These double-stained puncta or semilunar structures were the Golgi apparatus in the cytoplasm of granule cells (Fig. 2b1-3).

Electron microscopic immunolocalization confirmed the presence of ZNT7 in the Golgi apparatus

The Golgi apparatus localization of ZNT7 in the somas of granule and Purkinje cells was further confirmed by immuno-electron microscopy (Fig. 3a,b). The ZNT7 immunoreactive puncta or semilunar structures in the granule cell layer seen under the light microscope were confirmed to be the Golgi apparatus (Fig. 3a,b). In the Purkinje cell body, ZNT7 immunoprecipitates were clearly seen on the membranes of the Golgi apparatus (Fig. 3a,b).

Expression of ZNT7 protein in the mouse cerebellum, cerebral cortex and hippocampus

The amount of ZNT7 protein expressed in the mouse cerebellum, cerebral cortex, and hippocampus was examined using Western blot analysis. The ZNT7 antibody recognized a ZNT7-specific band of 42 kDa in these tissues (Fig. 4a), which is in agreement with the

previous study (Kirschke and Huang, 2003). Quantification of ZNT7 in these brain tissues was carried out using densitometry analysis and the expression level of ZNT7 was normalized by the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As shown in Fig. 4b, the expression of ZNT7 in the cerebellum was not different from that in the cerebral cortex and the hippocampus (P>0.05).

Discussion

In the present study, we showed that ZNT7 was localized in the Golgi apparatus of the Purkinje and granule cells in the mouse cerebellar cortex, judging from the colocalization of ZNT7 and TGN38, as well as the distribution of electron-dense ZNT7 immunoprecipitates on the membranes of the Golgi apparatus. Together with our previous study indicating that another four members of the ZNT family, ZNT1, ZNT3, ZNT4 and ZNT6, were abundantly expressed in the cerebellar cortex (Wang et al., 2005), the present study supports the notion that ZNT family members are involved in zinc homeostasis in the cerebellum.

ZNT family members have been demonstrated to reside in different subcellular compartments (Kambe et al., 2004). The functional role of ZNT family members is believed to be the transport of zinc from the cytosol to the extracellular space or store zinc in the intracellular

organelles (Colvin et al., 2003; Kambe et al., 2004). When the cellular zinc level rises, ZNT1 reacts to pump zinc out of the cell, while the others, ZNT2-7, are responsible for sequestering zinc into different intracellular organelles to decrease the zinc concentration in the cytosol (Kambe et al., 2004). Thus, ZNT proteins may play similar roles in different cellular compartments to maintain zinc levels at physiological levels in a living cell (Palmiter and Findley, 1995; Palmiter et al., 1996a,b; Murgia et al,, 1999; Nitzan et al., 2002; Kirschke and Huang, 2003; Asano et al., 2004; Kambe et al., 2004; Yu et al., 2007). It is well known that excess of zinc in the cytoplasm of neurons causes cell death (Weiss et al., 2000). The evidence that many ZNT family members are highly expressed in the cerebellar cortex suggests that ZNT proteins might play a neuroprotective role in prevention of zinc from accumulation in the mouse cerebellum (Wall, 2005).

Our previous study has shown that ZNT1, ZNT3, ZNT4 and ZNT6 were highly expressed in the Bergman glial cell bodies, as well as their radial processes, which extended into the molecular layer in the cerebellar cortex (Wang et al., 2005). Interestingly, the present data indicate that Bergmann glial cells also express ZNT7. Thus, the presence of many ZNT proteins in Bergmann glia suggests that the zinc homeostatic mechanisms are complicated in Bergmann glial cells of the cerebellar cortex.

Our double labeling of ZNT7/TGN38 and immuno-electron microscopic studies have revealed that ZNT7 is predominantly present in the Golgi apparatus of the Purkinje cell, granular cell, and Bergmann glial cell. The finding of ZNT7 in the Golgi apparatus is in agreement with previous studies reporting that ZNT7 is localized in the perinuclear regions of Chinese hamster ovary cells, and cells in the mouse choroid plexus and retina, as well as dorsal root ganglion (Kirschke and Huang, 2003; Chi et al., 2006; Wang et al., 2006; Zhang et al., 2007). It has been demonstrated that the cerebellum contains a considerable amount of metallo-proteins, such as metallothionine, matrix metalloproteases etc (Yanagitani et al., 1999; Beltramini et al., 2004; Luo, 2005; Williams et al., 2006). The Golgi complex is the center for protein modifications, sorting, and packing after proteins are synthesized. Therefore, colocalization of ZNT7 and TGN38 in the Golgi apparatus suggests that ZNT7 may function in pumping zinc from the cytosol into the Golgi apparatus for zinc to be incorporated into zinc-containing proteins in the neurons and glial cells of the cerebellar cortex.

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