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Zinc Distribution and Expression Pattern of ZnT3 in Mouse Brain

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Abstract To explore the relationship between the zinc distribution and zinc transporter 3 (ZnT3) mRNA expression in the mouse brain, zinc contents and its distribution were determined by synchrotron radiation x-ray fluorescence (SRXRF), and ZnT3 mRNA expression was examined by reverse-transcription polymerase chain reaction and in situ hybridization. The results showed that the zinc contents were not distributed evenly in various brain tissues. The zinc contents in cerebral cortex and hippocampus were nearly 5–10 times higher than that in other neural locations. Correspondingly, ZnT3 mRNA expression was observed in high abundance in the cerebral cortex, hippocampus, and testis, but was not detected in other organs and tissues. In the nervous system, ZnT3 mRNA was detected mainly in hippocampus, cerebral cortex, and spinal ganglion. The present results show the coincident distribution of zinc and ZnT3 mRNA in mouse brain. The high zinc contents might be determined by the high expression of ZnT3. More meaningfully, the results showed the feasibility of applying of SRXRF in examining the distribution of minerals in different organs and tissues. In addition, it was observed for the first time that ZnT3 mRNA was expressed in the facial nucleus. The function of ZnT3 in facial nucleus awaits further study.

Keywords Zinc · Zinc transporter 3(ZnT3) · Synchrotron radiation x-ray fluorescence (SRXRF) · Reverse transcription polymerase chain reaction (RT-PCR) · In situ hybridization (ISH)

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Introduction

Zinc, an essential trace element, is involved in many cellular processes, such as a cofactor for many enzymes and forming complexes with numerous nuclear factors and with hormones and playing a role in their activation. Large amount of zinc are present in the brain. Approximately 10% of the total brain zinc is in a histochemically reactive chelatable pool, particularly, in hippocampal fibers [1]. Zinc is essential for normal brain development, as evidenced by the fact that zinc deficiency in lactating mothers was characterized by a high incidence of neuroanatomical malformations and functional abnormalities in suckling offspring [2]. In our previous studies, we used a different model, in which maternal zinc deficiency was initiated on the first day of pregnancy and continued throughout the period of lactation. We found impairments in learning and memory and reductions in long-term potentiation (LTP) in adult mice. The abnormalities were not reversed by dietary zinc supplementation after weaning [3]. Further study showed that the nestin protein levels in the developing brain of zinc-deficient groups were lower than those of zinc-supplemented groups, and the level correlated with the amount of dietary zinc consumed by the mother [4]. These findings suggested that zinc deficiency suppresses development of neural stem cells, an effect which might lead to neuroanatomical and behavioral abnormalities in adults.

Zinc homeostasis results from a coordinated regulation by different proteins involved in uptake, excretion, and intracellular storage/trafficking of zinc [5]. These proteins are metallothioneins (MTs) and transmembrane transporters, belonging to the Zrt, Irt-like protein (ZIP) and cation diffusion facilitator (CDF) families. Metallothioneins belong to a family of low-molecular-weight, cysteine-rich intracellular proteins that bind transition metals, including zinc and cadmium [6], and their biological roles were considered to be the detoxification of harmful metals, the homeostasis of essential metals, and the protection of cells against damage induced by oxidative stress and alkylating agents [7]. The ZIP family plays prominent roles in zinc uptake, transporting zinc from outside the cell into the cytoplasm. ZIP transporters have also been found to mobilize stored zinc by transporting the metal from within an intracellular compartment into the cytoplasm. They were first discovered in plants and yeast and several members were later described in mouse and human [8, 9]. The CDF family transports zinc in the direction opposite to that of the ZIP proteins, promoting zinc efflux or compartmentalization by pumping zinc from the cytoplasm out of the cell or into the lumen of an organelle [10]. In mammalian cells, 10 homologous SLC30 proteins, named zinc transporter 1 (ZnT1) to 10, have been described [11]. These proteins are members of the CDF family. The murine ZnT3 gene was cloned by virtue of its homology to the ZnT2 gene and shared 52% amino acid identity with ZnT2. Human ZnT3 cDNAs were also cloned; the amino acid sequence is 86% identical to murine ZnT3. The mouse ZnT3 gene has eight exons and maps to chromosome 5. Northern blot and reverse transcription polymerase chain reaction (RT-PCR) analyses demonstrated that murine ZnT3 expression was restricted to the brain and testis. In the brain, ZnT3 mRNA was most abundant in the hippocampus and cerebral cortex [12].

Herein, synchrotron radiation x-ray fluorescence (SRXRF) microprobe was applied to study the zinc distribution in mouse brain. ZnT3 mRNA expression was measured by RT-PCR and in situ hybridization (ISH). The aims of the study were to provide scientific basis for research of the relationship between the distribution of zinc and the expression of ZnT3 in the brain and to enrich the rationale of the application of SRXRF in the examination of minerals contents and distribution in organs.

Materials and Methods

Animals

Animal protocols were approved by the animal care and use committee of the Second Military Medical University (SMMU). A total of 10 male ICR mice (17–20 g; aged 4 weeks) were purchased from the animal center of SMMU and housed on a 12-h light/dark cycle in stainless steel hanging cages. The mice had free access to deionized water and a commercial diet (AIN93G, Dyets, Bethlehem, PA, USA) containing 30 mg zinc/kg diet.

Preparation of Slices

Mice were anesthetized with Nembutal (100 mg/kg, i.p.) after 3 weeks. Five mice were killed and the brain, heart, liver, kidney, lung, small intestine, and testis were removed immediately and kept in liquid nitrogen. Coronal frozen sections of mouse brain (10 μm in diameter) were prepared, the slices then mounted on a 6-μm polyethylene film and applied to SRXRF after air-drying.

Another five mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. The brain, spinal cord, and spinal ganglion were removed, post fixed for 24 h at 4°C, and placed in 30% sucrose/phosphate-buffered saline before preparing 10-μm frozen sections (brain and spinal ganglion for coronal sections, spinal cord for transverse sections).

Determination of Zinc Contents in Mouse Brain by XRF

The determination of trace elements in mouse brain was carried on the 4W1B station of the Beijing Synchrotron Radiation Facility by using XRF technique. An ionization monitor chamber filling with nitrogen under normal pressure in front of collimation system was used to measure the intensity of exciting radiation. The exposure time was 100 s at a photon energy of 3.5–35 KeV for each sample, and the length of the scanning step in the experiments was 0.15 mm. The scanner was driven by a motor driver, which was controlled by a computer. The fluorescent radiation was detected by a 30-mm^2 Si (Li) detector with a resolution of 145 eV (full width at half-maximum) at MnKα 5.9 KeV. The detector was located at 90° with respect to the beam direction. Because the synchrotron radiation is plane polarized with the electric vector in the plane of electron orbits in the storage ring, there is a minimum Compton scattering background when the detector is located at 90°. The associated electronics was comprised of a pulsed optical preamplifier, spectroscopy amplifier, and pulse pile-up rejecter. Then, the signals were connected to an Oxford multichannel analyzer for data acquisition and analysis. Data of spectra were processed by use a program AXIL and the concentrations were obtained by comparing the elemental XRF intensity of the sample with that of the standard [13].

Determination of Zinc Distribution in Mouse Brain Slice by SRXRF Microprobe

The scanning XRF microprobe analysis of bone slices was performed using a synchrotron radiation from the Beijing Electron Positron Collider storage ring (2.2 GeV, 66–85 mA) at Beijing Institute of High Energy. When the x-ray beam produced by a synchrotron radiation accelerator was focused to a micron dimension, it became an SRXRF microprobe. A tiny aperture with four knives imported from Japan was mounted at the beam terminal. Adjusting the width of the slot by electronics, the incidence light became a 0.2×0.2-mm

microbeam. A sample platform of three dimensions controlled by computer was installed. The whole scanning analysis system was operated by an online computer to collect spectrum fully automatically. The details of the same experimental set up were described above and can be obtained elsewhere [13]. Each sample was routinely placed on the three-dimensional moving platform and scanned along interesting range by incidence x-ray. A profiling of elemental image was performed using a manner of point by point.

Measurement of ZnT3 mRNA Expression in Mouse Tissues

Total RNA was isolated from tissues with the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture's instructions and stored at −70°C in 75% ethanol until required. RT-PCR was performed in a single-step reaction using ready-to-go RT-PCR beads (Amersham Pharmacia Biotech, Buckinghamshire, UK) with each RNA sample (500 ng) and the following primer sequences: ZnT3 (NM_011773) 5′ GGA GGT GGT TGG TGG GTA TTT AGC 3′(forward) and 5′ GAT GGA GAT CAT GGG TTG CTC G 3′ (reverse); β-actin 5′ CAA GGT GTG ATG GTG GGA ATG G 3′ (forward) and 5′ GCT CAT AGC TCT TCT CCA GGG AGG 3′ (reverse). The RT-PCR reaction was in a final volume of 50 μl, which contained 2.0 units of *Taq* DNA polymerase, 10 mM Tris–HCl, 60 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, Moloney murine leukemia virus reverse transcriptase, RNAguard™ ribonuclease inhibitor and stabilizers, and 12.5-pmol primers. The RT-PCR reactions of ZnT3 and β-actin were conducted in the same reaction tube. The cDNA was produced by incubation at 42°C for 30 min. PCR was performed by 28 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, followed by a final single extension at 72°C for 10 min in a PTC-100 thermal cycler (MJ Research, Waltham, MA, USA). PCR products were stained with ethidium bromide on a 2% agarose gel and visualized by UV fluorescence.

In Situ Hybridization

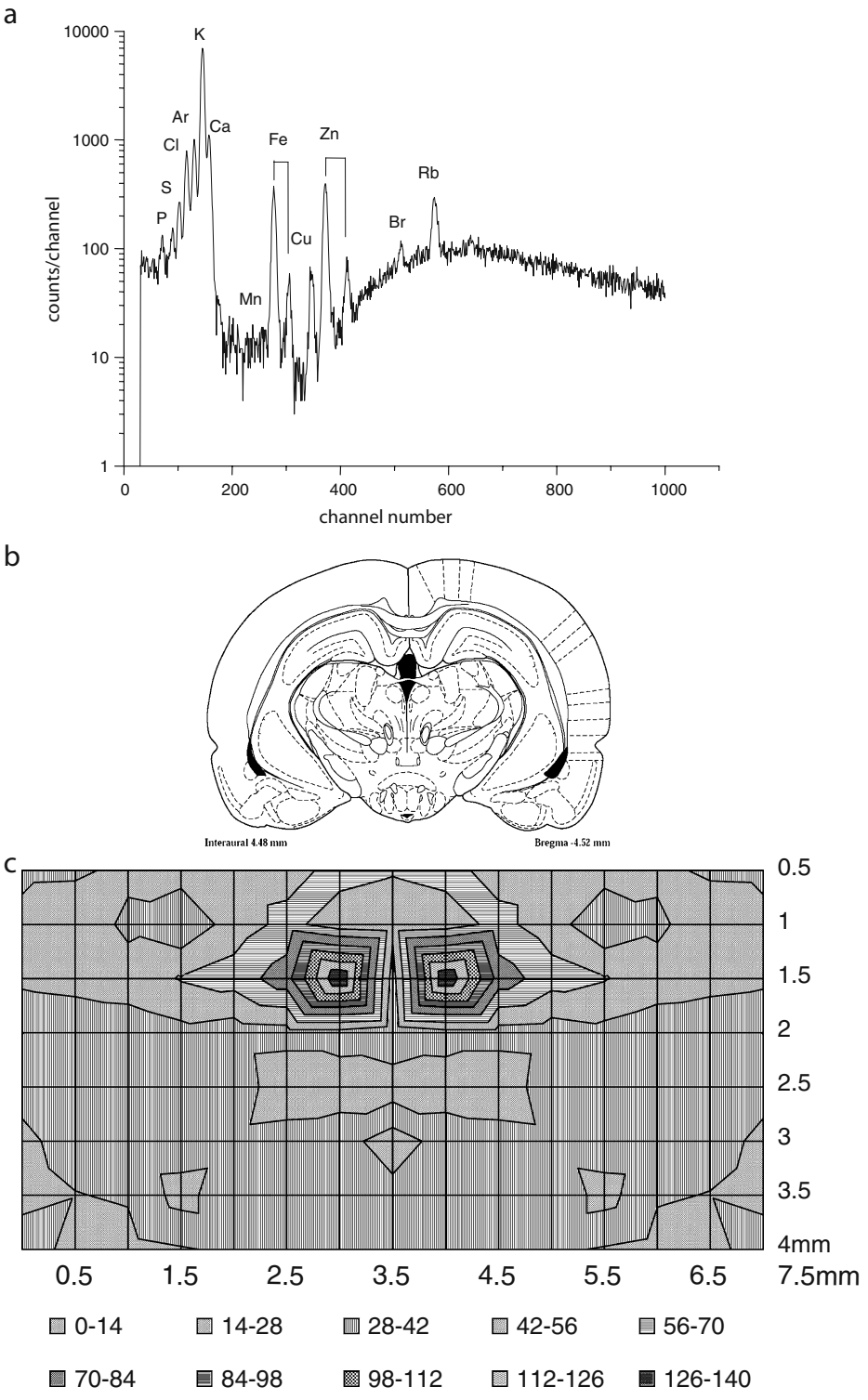
The PCR product of was ligated into the pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant was then transformed into competent *Escherichia coli* DH10B cells. A transformant was chosen and grown in Luria-Bertani-ampicillin medium for plasmid preparation. The plasmid containing the PCR product was purified and restricted with *Eco*RI. The insert was sequenced to confirm its identity as ZnT3.

Linear *ZnT3* DNA template was obtained by cutting circled *ZnT3* plasmid with *Xba*I (Promega). The concentration of the linear *ZnT3* DNA template was 0.5 μg/μl. Then antisense riboprobes were generated by in vitro transcription of mouse cDNA encoding *ZnT3* with T7 RNA polymerase (Promega) and nucleotide triphosphate mix including 10 mM ATP, cytidine triphosphate and GTP, 6.5 mM uridine 5′-triphosphate (UTP), and 3.5 mM digoxigenin-16-UTP (Promega), in which ZnT3 cRNA probes were labeled with digoxigenind-16-UTP. The ISH was performed as described previously [14]. Control of hybridization with sense strand labeled riboprobes was proceeded at the same time.

Results

Zinc Content in Mouse Brain

Using internal standard method and AXIL software, the quantitative results of some elements in a whole brain of mouse could be obtained. The Zn, Cu, Fe, Ca, and K contents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84-98
98-112
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in mouse brain were 37.6±6.7, 10.8±3.0, 240±35, 9423±847, and 302±41 μg/g of dry matter, respectively.

Zinc Distribution in Mouse Brain Slice

The subtle distribution of zinc in a mouse brain slice could be obtained by SRXRF microprobe. Figure 1a is a typical energy spectrum of a brain slice sample excited by SRXRF microprobe. The sketch map of brain slice and corresponding zinc distribution are shown in Fig. 1b and c, respectively. As shown in Fig. 1c, the zinc content was not distributed evenly and higher in the limbic system, cortical part, cingulated gyrus, dentate gyrus, and hippocampus. The zinc content in hippocampus and cerebral cortex was about 1 mg/g, nearly 5–10 times higher than that in other locations, such as corpus callosum, centrum ovale, or crus of fornix.

ZnT3 mRNA Expression in Tissues

RT-PCR showed that ZnT3 mRNA was detected in brain and testis, but it was not found in heart, liver, spleen, lung, kidney, and small intestine (Fig. 2a). Further analysis of ZnT3 mRNA expression in the brain demonstrated that ZnT3 expression was restricted to the cortex and hippocampus. ZnT3 mRNA was not detected in the cerebellum or olfactory bulb (Fig. 2b).

Expression of ZnT3 mRNA in Mouse Nervous System

The ISH study showed that ZnT3 mRNA was mainly distributed in hippocampus as shown by its presence in granular cells at dentate gyrus and pyramidal cells at CA1–CA4 areas (Fig. 3a), and also in the small cells of pyriform cortex and the posterior cortex of cingulum (Fig. 3b). Compared with the reticular formation of the brain stem, ZnT3 mRNA was expressed much more in the facial nucleus (Fig. 3c, d). Moreover, ZnT3 mRNA was strongly expressed in most neurons of the spinal ganglion (Fig. 3e). Some large cells in the anterior horn of the spinal cord were ZnT3 mRNA-positive (Fig. 3f). The control of hybridization with sense strand labeled riboprobes showed no hybridization signal (data not shown).

Discussion

The accurate elemental analysis at the microscopic/submicroscopic level is of growing importance in many areas of scientific research and characterization at the submicron/nanometer level in materials technology. The elements localization and heavy metal tolerance mechanisms in plants have been studied by electron dispersive x-ray spectroscopy, electron energy-loss spectroscopy, and proton induced x-ray analysis. When compared with these methods, however, SRXRF microprobe is more sensitive and less injurious to cells and also has excellent characteristics such as very high brightness, collimation, polarization, and lower bremsstrahlung, which is the electromagnetic radiation

◀ **Fig. 1** Zinc distribution in mouse brain slice by SRXRF. **a** The typical SRXRF spectrum of mouse brain slice excited by SRXRF microprobe. **b** The sketch map of mouse brain slice. **c** The zinc distribution in mouse brain slice (μg/g)

produced by an accelerated electrically charged subatomic particle. The SRXRF microprobe is extensively applied in many fields, such as materials science, life science, medical science, and environmental science [15]. The amount of Zn^{2+} in the mammalian brain averages about 10 μg/g (wet weight), and a previous study showed that the zinc amount in the brain is fairly uniform over different regions or when comparing the gray and white matter [16]. The present study detected by SRXRF revealed that the zinc content was not distributed evenly and higher in the limbic system, cortical part, cingulated gyrus, dentate gyrus, and hippocampus. The zinc content in hippocampus and cerebral cortex was about 1 mg/g, nearly 5–10 times higher than that in other locations, such as corpus callosum, centrum ovale, or crus of fornix. Zn^{2+} concentration in the serum and extracellular fluid are estimated to be 0.15 μM [17]. Neurons, like most cells, accumulate Zn^{2+}. The total Zn^{2+} in neurons may be as high as 150 μM [17]. Most of this Zn^{2+} is bound with high affinity to MT. The intracellular free Zn^{2+} in neurons, estimated to be ~1 nM using fluorescent probes, is dependent on their intracellular concentration [18].

There was little research about ZIP family in neurons. It has been established in studies using RT-PCR that ZIP1 mRNA is abundant in the brain [19], and LIV-1 (a member of the ZIP family of proteins) mRNA also occurs in the brain [20]. Compared to ZIP, the function,

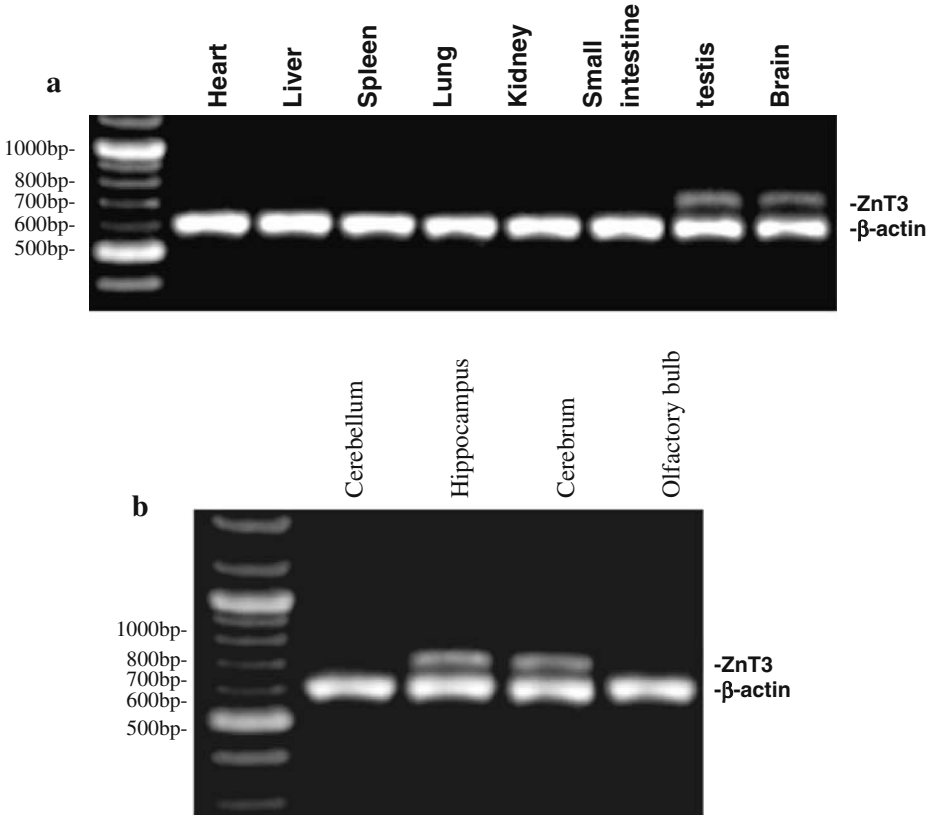

Fig. 2 ZnT3 mRNA expression in tissues by RT-PCR. **a** The ZnT3 mRNA expression in different mouse tissues. **b** The ZnT3 mRNA expression in different regions of the mouse brain

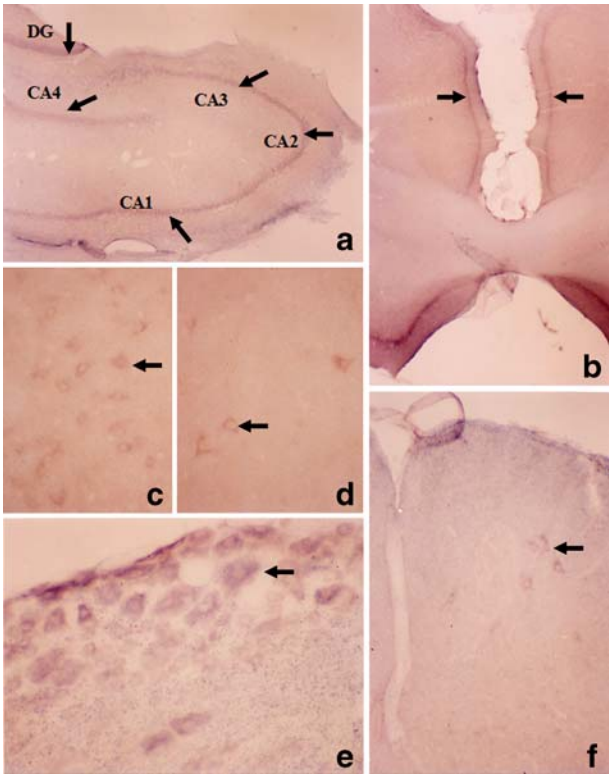

Fig. 3 Detection of ZnT3 mRNA in the nervous system. ZnT3 mRNA was detected in the brain, spinal cord, and spinal ganglion by ISH as described in the "Materials and Methods" section. **a** Hippocampus, wherein *DG* denotes detate gyrus and *CA1–4*, cornu ammonis subfield 1–4. **b** Posterior cortex of cingulum. **c** Facial nucleus. **d** Reticular formation of the brain stem. **e** Spinal ganglion. **f** Spinal cord. *Arrows* show the locations of ZnT3 mRNA in the various tissues

tissue location, and mechanism of Zn^{2+} transport by CDF family members were best studied for the ZnT3 in the brain. Recently, the tissue location of ZnT3 was getting wider. ZnT3 was observed related in particular to motor neuronal somata and big dendrites in the ventral horn in mouse spinal cord, the rodent superior cervical ganglia, and the ependyma of the mouse spinal cord [21–23]. ZnT3 might play roles in these locations. It was accepted generally that the ZnT3 played a role in the regulation of zinc level in brain. Histochemically reactive zinc was undetectable, and total zinc levels in the hippocampus and cortex of these mice were reduced by about 20% in the brains of mice with targeted disruption of the ZnT3 gene [24].

There was a coincident distribution of zinc content and expression pattern of ZnT3 in the brain, which had higher content and expression in cortex and hippocampus, demonstrated by SRXRF, RT-PCR, and ISH in the present study. The high content of zinc in cortex and hippocampus might be relevant to the high expression of ZnT3 in these two areas. Although the present results were identical to most previous studies, it would enrich the understanding of ZnT3 mRNA and zinc in the brain. In addition, to our knowledge, our study was the first to demonstrate the expression of ZnT3 mRNA in high amounts in the facial nucleus by ISH. Whether ZnT3 mRNA in the facial nucleus plays a role in the zinc homeostasis awaits further study. In addition, SRXRF was available in the examination of materials science, life science, medical science, and so on.

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