ELSEVIER

Brain Research Bulletin 71 (2006) 91–96

BRAIN RESEARCH BULLETIN

www.elsevier.com/locate/brainresbull

Localization of ZnT7 and zinc ions in mouse retina—Immunohistochemistry and selenium autometallography

Xin Wang [a], Zhan-You Wang [a,*], Hui-Ling Gao [a], Gorm Danscher [b], Liping Huang [c]

[a] *Department of Histology and Embryology, China Medical University, Shenyang 110001, PR China*
[b] *Department of Neurobiology, University of Aarhus, DK 8000 Aarhus C, Denmark*
[c] *Western Human Nutrition Research Center, Agriculture Research Service, United States Department of Agriculture, USA*

Received 15 June 2006; received in revised form 11 July 2006; accepted 7 August 2006
Available online 31 August 2006

Abstract

Zinc transporter 7 (ZnT7, Slc30a7), a member of the Slc30 family, is involved in mobilizing zinc ions from the cytoplasm into the Golgi apparatus. In the present study, we examined the distribution and localization of ZnT7 and the labile zinc ions in the mouse retina using immunohistochemistry and in vivo zinc–selenium autometallography ($ZnSe^{AMG}$). Our results showed that ZnT7 is abundantly expressed in the ganglion cells and pigment epithelial cells of the mouse retina. ZnT7 is also expressed in the amacrine cells and the layer of optic fibers of the mouse retina, but to a lesser extent. Weak staining of ZnT7 was detected in the inner plexiform layer, outer plexiform layer, and outer segment of the photoreceptors. However, ZnT7 was not detected in the outer nuclear layer and inner segment of the photoreceptors. A high level of labile zinc pool was detected in the pigment epithelial cells, the inner segment of the photoreceptors, and the marginal region of the inner nuclear layer. Less amount of labile zinc ions were detected in the ganglion cells of the retina. These observations strongly suggest that ZnT7 may play critical roles in retinal zinc homeostasis and that chelatable zinc pools may have multiple functions in the retina.

Keywords: Zinc; ZnT7; Zinc transporter; Retina; Mouse

1. Introduction

Zinc is an essential trace metal which plays critical roles in biological processes of the body. In brain, approximately 90% of total zinc is tightly bound in metalloenzymes and other zinc-containing proteins, where zinc serves as a cofactor for enzymatic activities or for maintaining the three-dimensional structure of proteins [6,4]. The remaining 10% of total zinc is called chelatable zinc, because it presents as free or loosely bound ions. These chelatable zinc ions, most of which are localized in a population of synaptic vesicles of the so-called zinc enriched (ZEN) terminals, can be detected by both fluorescence and autometallographic (AMG) techniques. The fluorescence techniques including TSQ and Zinquin staining are suitable for low magnification approaches while the AMG techniques are excellent for detecting nM levels of chelatable zinc ions in tissues [17,10,51,16,9].

The telencephalon contains large amount of zinc enriched neuronal somata and terminals. Neocortex and hippocampal regions of brain contain the highest zinc enriched neuronal terminals in the human brain. These zinc enriched neuronal terminals are glutaminergic [31,32,12]. During synaptic vesicle exocytosis, vesicular zinc is co-released with glutamate into the synaptic space [3,21,2]. It has been suggested that these zinc ions modulate the activities of the excitatory *N*-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors localized on the postsynaptic membrane ([49,35]). In addition, studies also showed that most zinc enriched terminals in the spinal cord and cerebellum are either GABAergic or glycinergic [44–46].

Zinc is abundant in the mammalian retina [20]. Several studies have demonstrated that cellular zinc ions were localized in the inner segment of the photoreceptors, the outer and inner plexiform layers, and inner nuclear layer [1]. However, the ultrastructural evidence of the exact zinc localization is

* Corresponding author. Tel.: +86 24 23256666 5305; fax: +86 24 23256666 5305.
E-mail address: wangzy@mail.cmu.edu.cn (Z.-Y. Wang).

0361-9230/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.brainresbull.2006.08.002

not strong. These shortcomings make the final proof of where the zinc ion pools are localized incomplete which obscures understanding of the biological roles of zinc in the retina [14,15,18–20,23–26,33,34,40–42,50]).

Recent studies have indicated that the zinc transporter (ZnT) family plays critical roles in maintaining intracellular zinc homeostasis [7,29]. Since 1995, eight members in ZnT family, namely ZnT1-8, have been cloned and functionally characterized. All members analyzed so far have similar membrane topology with six transmembrane domains and a histidine-rich loop between domains IV and V, where zinc has been presumed to be bound by histidines and subsequently transported across the membrane. Although ZnT1-8 proteins resides in different subcellular compartments, they function as zinc efflux transporters to reduce the cytoplasmic zinc concentrations by transporting zinc directly out of the cell or into intracellular compartments [27]. The detailed distribution of ZnT3 in the light-adapted mouse retina has been described using immunohistochemical techniques. Immunostaining indicated that the ZnT3 protein is abundant in the regions of the outer limiting membrane and the inner segment of photoreceptors. The ZnT3 immunoreactivity was also found in the outer plexiform, inner nuclear, inner plexiform, and ganglion cell layers [36]. It is well known that ZnT3 is localized on the synaptic vesicle membrane and is required for transporting zinc into synaptic vesicles from cytosol [28,48,5,11]). The abundant expression of ZnT3 in the retina suggests that ZnT3 may be important for synaptic zinc transportation and for maintaining zinc metabolism in the mouse retina.

ZnT7 is abundantly expressed in the mouse small intestine and lung [27]. Double immunofluorescent labeling has demonstrated that the ZnT7 protein resides in the Golgi apparatus as well as an unknown vesicular compartment. Over expression of ZnT7 in Chinese hamster ovary (CHO) cells induced accumulation of zinc in the Golgi apparatus when cells were exposed to a higher concentration of zinc [27,13,22,29]. In this study, we examined the distribution and localization of ZnT7 and chelatable zinc ions in the mouse retina to elucidate the retinal zinc physiology.

2. Materials and methods

2.1. Experimental animals

Adult male CD-1 mice, weighing approximately 40–50 g, were used as experimental animals in this study. They were housed in a 12 h light/dark cycle with food and tap water available ad libitum. All experimental procedures were performed in an agreement with the ethical standards of China Medical University.

2.2. Immunohistochemical staining for ZnT7

Three adult male CD-1 mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 50 ml isotonic saline, followed by 200 ml 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The entire eye balls were removed and postfixed using the same fixative at 4 °C for 4 h.

The fixed eye balls were placed in a 30% sucrose solution made in PB at 4 °C overnight. Anteroposterior axes sections (10 μm) of retinas were prepared on a cryostat and placed on glass slides. An affinity-purified rabbit anti-ZnT7 polyclonal antibody was used for immunohistochemical detection of the ZnT7 protein in the retina sections [27]. The immunostaining procedures were performed in accordance with the standard ABC method. Briefly, sections were rinsed in 0.1 M Tris-buffered saline (TBS, pH 7.4) and endogenous peroxidase activity was blocked by treating sections with 3% hydrogen peroxide (H_2O_2) in pure methanol for 10 min. Sections were rinsed three times in TBS and treated with 5% bovine serum albumin (BSA) and 3% goat serum in TBS for 1 h to reduce nonspecific staining. Sections were then rinsed in TBS for 30 min and incubated in TBS containing 3% goat serum, 1% BSA, 0.3% Triton X-100, and 1:100 diluted anti-ZnT7 antibody at 4 °C overnight. After rinsing in TBS, sections were incubated in biotinylated goat anti-rabbit IgG (1:200) at room temperature for 1 h. The ABC Kit (DAKO) was applied at room temperature for 1 h in order to visualize the immunoreaction sites. Color was developed by rinsing sections in 0.1 M Tris buffer (pH 7.6) and incubating sections in 0.025% 3,3-diaminobenzidine (DAB) with 0.0033% H_2O_2 at room temperature for 10 min. Sections were then rinsed with Tris buffer to stop the DAB reaction. Some sections were counterstained with 1% toluidine blue. All sections were mounted and examined under a light microscope equipped with an image analysis system.

Control sections were incubated with normal sera instead of the primary anti-ZnT7 antibody followed by all subsequent incubations as described above. No distinct staining was observed.

2.3. Zinc–selenium autometallography

Three mice were used for analyzing the distribution of chelatable zinc ions by zinc–selenium autometallography in the mouse retina. Deeply anesthetized mice (50 mg/kg pentobarbital) were injected intraperitoneally with sodium selenite (30 mg/kg) and kept in cages for 40 min. Animals were then perfused transcardially with 100 ml isotonic saline, followed by 100 ml 2.5% glutaraldehyde in 0.1 M PB. The entire eye balls were removed and immediately immersed in the same fixative at 4 °C for 4 h.

For cryoprotection, all samples were placed into 30% sucrose at 4 °C overnight. After frozen, 10-μm-thick cryostat sections were cut and placed on glass slides pre-treated with Farmer solution in order to get rid of contamination. Sections were incubated in the AMG developer in a 26 °C water bath for 1 h as described by Danscher ([8]). The AMG developer consists of 60 ml gum Arabic, 10 ml citrate buffer, 0.85 g hydroquinone in 15 ml distilled water, and 0.12 g silver-lactate in 15 ml distilled water. After AMG developer incubation, sections were immersed in a 5% thiosulphate solution to stop the AMG reaction. Sections were then placed under running tap water (37–40 °C) for 20 min in order to remove the gelatine membrane, dipped in distilled water and counterstained with 1% toluidine blue. The sections were analyzed and photographed with a light microscope.

3. Results

3.1. Expression of ZnT7-immunoreactivity in the mouse retina

Seven layers above the pigment epithelial layer (PEL) were distinguished in the mouse retina sections incubated with an anti-ZnT7 antibody (Fig. 1a). They were: (1) the photoreceptor layer (OS and IS), (2) the outer nuclear layer (ONL), (3) the outer plexiform layer (OPL), (4) the inner nuclear layer (INL), (5) the inner plexiform layer (IPL), (6) the ganglion cell layer (GC), and (7) the nerve fiber layer (NFL).

The somata of the ganglion cell layer and the pigment epithelial layer showed the strongest ZnT7 immunoreactivity (Fig. 1; Table 1). The nerve fiber layer and inner nuclear layer exhibited moderate ZnT7 reactions (Fig. 1a; Table 1), suggesting that both the axons and dendrites of ganglion cells contain ZnT7. A moderate reaction of ZnT7 was also observed in the outer segment (OS) but not in the inner segment (IS) of the photoreceptors (Fig. 1a and d; Table 1). The inner plexiform layer and outer plexiform layer showed weak ZnT7 reactions (Fig. 1a; Table 1).