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Localization of zinc transporter-3 (ZnT-3) in mouse retina

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Abstract

Studies of the central nervous system have localized the zinc-transporter-3 (ZnT-3) protein to synaptic vesicles containing glutamate and zinc. We have examined the distribution of the ZnT-3 protein in the light-adapted mouse retina using immunohistochemical techniques. Light microscopic analysis of 15–30-μm retinal sections revealed a rich band of ZnT-3 protein in the region of the outer limiting membrane and photoreceptor inner segments. ZnT-3 reactivity was also present in the outer plexiform, inner nuclear, inner plexiform, and ganglion cell layers. The outer nuclear layer and photoreceptor outer segments did not exhibit ZnT-3 immunoreactivity. In the light-adapted murine retina, ZnT-3 appears localized in regions which have been found reactive for ionic zinc.

Keywords: Retina; Zinc; ZnT-3; Zinc transport; Rodent

1. Introduction

Zinc is receiving increasing attention as a factor in neuronal function and disease (Age-Related Eye Disease Study Research Group, 2001; Ritchie et al., 2003). It is well known that zinc homeostasis is essential for neural cellular and nuclear functioning (Dineley, Votyakova, & Reynolds, 2003; Frederickson, Suh, Silva, & Thompson, 2000; Tate, Miceli, Newsome, Alcock, & Oliver, 1995; Ugarte & Osborne, 2001). It has been estimated that zinc levels in the central nervous system (CNS) are ~200 ng/mg protein (Dineley et al., 2003) and that approximately 95% of zinc in the CNS is bound by peptides and proteins (Frederickson et al., 2000). On the other hand, histochemically reactive or ionic zinc comprises approximately 5% of CNS zinc (Frederickson et al., 2000). Vesicles of zinc-enriched hippocampal terminals have estimated zinc concentrations of up to 1.4 mmol/L (Frederickson et al., 2000) and histochemical reactivity for zinc ions in neural tissue appears predominately in terminals containing glutamatergic vesicles (Frederickson et al., 2000; Wenzel et al., 1997).

The hydrophilic cation zinc cannot pass through membranes via passive diffusion and requires an active transport protein (Wang, Danscher, Dahlstrom, & Li, 2003). In the central and peripheral nervous system, co-localization of histochemically reactive zinc with the zinc transporter-3 (ZnT-3) protein has been shown on synaptic vesicles (Palmiter, Cole, Quaife, & Findley, 1996; Wang et al., 2003). The ZnT-3 protein is in the SLC30 family of transporters involved in ionic zinc homeostasis (Palmiter & Huang, 2004). The murine 388 amino acid ZnT-3 protein has six transmembrane domains and localizes primarily to membranes of zinc-enriched terminal vesicles in neural tissue (Palmiter et al., 1996). The murine ZnT-3 gene has 8 exons and maps to chromosome 5 (Palmiter et al., 1996). In a murine knockout model, ZnT-3 was shown to be responsible for the sequestering and concentration of ionic zinc in

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mouse mossy fiber boutons of hippocampal neurons (Cole, Wenzel, Kafer, Schwartzkroin, & Palmiter, 1999).

Recent studies show increasing numbers of central and peripheral nervous system regions exhibiting co-localization of reactive zinc with the ZnT-3 protein (Danscher et al., 2003; Wang et al., 2003; Wenzel, Cole, Born, Schwartzkroin, & Palmiter, 1997). Zinc-enriched neurons have been located in entorhinal cortex, the amygdala, and hippocampus of mouse and primate (Wenzel et al., 1997). In glutamatergic hippocampal mossy fiber pyramidal neurons, light and electron microscopy revealed a 60–80% co-localization of ionic zinc with the ZnT-3 protein on synaptic vesicles (Wenzel et al., 1997). In all levels of mouse spinal cord, ependymal cells of the central canal have shown antibody reactivity for the ZnT-3 protein (Danscher et al., 2003). Based on that study, Danscher et al. (2003) proposed that the ZnT-3 protein in mouse spinal cord serves to regulate zinc homeostasis between cerebral spinal fluid and gray matter. It has been shown that the majority of zinc-enriched neurons are glutamatergic (Wenzel et al., 1997). Recently, however, the tyrosine hydroxylase positive post-synaptic superior cervical ganglions of mouse have demonstrated positive staining for zinc vesicular terminals as well (Wang et al., 2003). Approximately 5% of the somata and processes of these ganglia exhibit ZnT-3 protein antibody reactivity (Wang et al., 2003). The investigators speculated that superior cervical ganglion cell vesicular zinc was functioning as a peripheral nervous system neuromodulator (Wang et al., 2003).

In the rat retina, using the Timm's silver sulfide method, ionic zinc has been visualized in retinal pigment epithelium and photoreceptor inner segment regions as well as in the outer plexiform, inner plexiform, inner nuclear, and ganglion cell layers (Ugarte & Osborne, 2001). In the light-adapted rat retina, ionic zinc localized strongly to the region of photoreceptor inner segments. In dark-adapted rat retinas, however, ionic zinc was visualized in outer nuclear layer somata, but not in the region of the photoreceptor inner segments (Ugarte & Osborne, 2001). In an earlier analysis of retinal zinc localization in tiger salamander, it was proposed that zinc released from photoreceptors feeds back to decrease the tonic release of glutamate in the dark-adapted retina (Wu, Qiao, Noebels, & Yang, 1993). A histochemical study in skate retina also revealed evidence of a highly concentrated band of ionic zinc in the proximal region of the outer nuclear layer (Qian, Malchow, Chappell, & Ripps, 1996).

As a step towards further understanding retinal zinc physiology, we have analyzed the localization of the ZnT-3 protein in the retina of light-adapted mouse. The presence of ZnT-3 in neural retina could indicate the transport of zinc into photoreceptor glutamatergic synaptic vesicles and a mechanism for retinal zinc homeostasis.

2. Methods

Six male mice C57BL/6J (Taconic Labs) ages 12–18 weeks were euthanized using carbon dioxide gas. The immunoperoxidase amplification protocol and ZnT-3 antibody used have been described (Wenzel et al., 1997). Modifications for retinal immunohistochemistry are described below. Palmiter et al. (1996) using Western blots of total cell proteins from control BHK cells and those transfected with constructs expressing ZnT-1, ZnT-2 or ZnT-3 showed that only the cells expressing ZnT-3 reacted with the ZnT-3 antibody, indicating that it does not react with the homologous proteins.

Eyes were removed and placed in 4% paraformaldahyde in 0.1 M sodium phosphate buffer (PBS), pH 7.4 for 4 h at 4 °C. Eyes were then rinsed in PBS and cryoprotected, first in 10% sucrose for 1 h and second in 30% sucrose for 12 h, followed by further protection in frozen specimen embedding resin (Cryomatrix, Shandon) for 10–15 min. Next, the eyes were placed in a cryostat and allowed to freeze for 30 min at −20 °C. They were then transversely sectioned at 15–30 μm using a cryostat microtome (Bright Instruments). Sections ($N = 42$) were placed into 0.2 ml PBS in 0.2–0.4 ml chamber slide wells (Lab-Tek). The sections were rinsed 2×with PBS and then rinsed again 2× with 0.1 M Tris.HCL buffer solutions (TBS) at pH 7.4. Endogenous peroxidases were inactivated through section incubation in 1.0% H_2O_2 in TBS for 2 h. Sections were then incubated in a blocking buffer of 3% BSA, 3% goat serum, and 0.25% dimethyl sulfoxide in TBS (0.05 M TBS/0.15 M NaCl, pH 7.4) for 1 h and rinsed for 15 min 2× in TBS.

Thereafter, the sections were incubated in an affinity-purified rabbit antibody specific for ZnT-3 diluted 1:20–1:100 in 2% BSA, 1% goat serum, and 0.25% dimethyl sulfoxide in TBS for 20–40 h at 4 °C. This was followed by rinse for 15 min 4× in TBS. Sections were then incubated in biotinylated goat anti-rabbit IgG (Sigma) diluted 1:20–1:200 in 2% BSA, 1% goat serum, and 0.25% dimethyl sulfoxide in TBS for 24 h at 4 °C, rinsed for 15 min 4× in TBS incubated in ABC (Elite ABC Kit: Vector Labs) diluted 1:200 in 2% BSA, 1% goat serum, and 0.25% dimethyl sulfoxide in TBS for 24 h at 4 °C, and rinsed 2× in TBS pH 7.6. Finally, sections were incubated in DAB enhanced liquid substrate (Sigma) for 15 min and then in fresh DAB for 5–10 min followed by a rinse 2× in TBS pH 7.6 and a rinse 1× in PBS. Slides were covered and sealed for light microscopic analysis.

3. Results

Eight slices were processed for immunohistochemistry from each of six mouse eyes. Once successful para-

meters had been established, 42 of the 15–30 μm slices incubated with ZnT-3 antibody showed a consistant robust reaction for protein localization. Antibody-labelled sections demonstrated elevated reactivity for the ZnT-3 protein in the photoreceptor inner segment region as well as in the outer plexiform, inner nuclear, inner plexiform, and ganglion cell layers (Fig. 1). Control retinal slices processed in the absence of the ZnT-3 antibody showed no distinct staining (Fig. 1, inset). The regions of the outer nuclear layer and the outer segments of photoreceptors showed no ZnT-3 reactivity.

The horizontal, bipolar, and amacrine somata regions of the inner nuclear layer showed consistent ZnT-3 reactivity slightly weaker than the reactivity in the region of inner segments. The inner plexiform layer, the synaptic region of amacrine, bipolar, and ganglion cells, demonstrated weak reactivity for the ZnT-3 protein. The ganglion cell layer and inner limiting membrane region showed ZnT-3 reactivity with slightly more intensity than either of the plexiform layers. These observations were confirmed through more detailed examination at higher magnification (Fig. 2).

We also observed that the proximal region of the inner nuclear layer where amacrine cell bodies predominate showed stronger immunoreactivity than the more distal region. We note that Wang et al. (2003) reported the presence of ZnT-3 protein in superior cervical ganglia synapses containing tyrosine hydroxylase positive vesicles so that association of ZnT-3 protein with dopaminergic amicrine cells should be considered in addition to the usual association of ZnT-3 with glutamatergic pathways. We also note that although most cell bodies in the ganglion cell layer demonstrated strong labelling, it appeared that a small number of cells in this region were not immunoreactive for the ZnT-3 antibody.

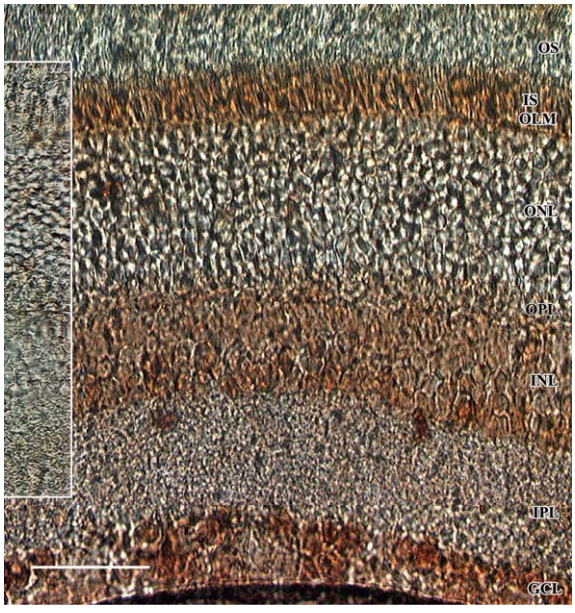

Fig. 1. Mouse retinal ZnT-3 localization. The 40× light microscopic image shows a ganglion cell layer (GCL) with dark reactivity for ZnT-3 protein. The inner plexiform layer (IPL) shows weak granular reactivity. The inner nuclear layer (INL) soma and outer plexiform layer (OPL) region of synaptic junctions present moderate levels of ZnT-3 reactivity. The outer nuclear layer (ONL) remains clear of reactivity. The inner segments and outer limiting membrane region (IS, OLM) show strong reactivity. Outer segments (OS) were clear of reactivity. The left inset is a control slice processed in absence of antibody. Bar: 50 μm.

4. Discussion

In general, ZnT-3 reactivity was found in regions of the mouse retina which have been found reactive for ionic zinc in prior studies of murine retinas described above (Ugarte & Osborne, 2001). This is consistent with the possibility that ZnT-3 mediated zinc transport may be related to zinc retinal neuromodulation as suggested by a number of studies. Already, a variety of vertebrate retinal cells have demonstrated zinc sensitivity. Müller cell $GABA_A$ receptor currents increased when 10 μM zinc and 1 μM GABA were co-applied under voltage-clamp conditions compared with 1 μM GABA alone (Qian et al., 1996). $GABA_A$ and $GABA_C$ receptor responses of bipolar cells have shown concentration-dependent sensitivity to ionic zinc as well (Qian, Li, Chappell, & Ripps, 1997). Retinal ganglion cell glycine receptors have also shown zinc sensitivity (Han & Wu, 1999). Furthermore, removal of extracellular free zinc by chelation resulted in an increased ERG b-wave (Redenti & Chappell, 2002).

It appears that localized retinal ZnT-3 may be involved in ionic zinc sequestering and vesicular storage in both plexiform layers. The ZnT-3 protein in the outer plexiform layer is co-localized in a region of zinc reactivity associated with glutamate in photoreceptor synaptic vesicles. It has been suggested that synaptically-released zinc may modulate photoreceptor transmitter release (Wu et al., 1993). Similarly, ZnT-3 is localized in the inner plexiform layer, the region of synaptic interaction among bipolar, amacrine, and ganglion cell processes. It is possible that some amacrine cells may contain zinc and ZnT-3 in their terminals as seen in tyrosine hydroxylase positive cells of the superior cervical ganglia (Wang et al., 2003). The localization of ZnT-3 observed in several retinal layers suggests ionic zinc transport and localization may be a factor in normal functioning of the neural retina.

The localization of ZnT-3 immunoreactivity in the region of the photoreceptor inner segments and outer limiting membrane is of special interest. This is not a region of synaptic activity where synaptic vesicles are known to be concentrated. It coincides, however, with the location of a dense band of zinc found in other studies for

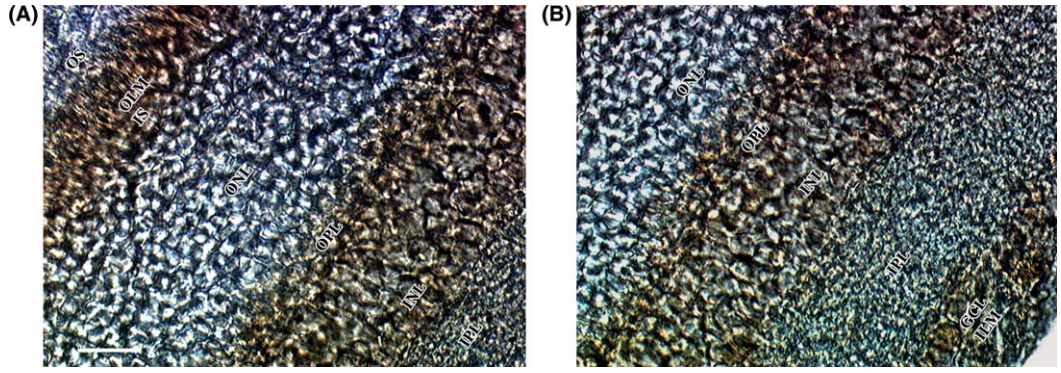

Fig. 2. Mouse outer and inner retinal layer ZnT-3 localization. The 60× light microscopic images confirm the pattern of ZnT-3 localization consistent with that observed at lower power. (A) *Outer retina*: Inner segments and outer limiting membrane region (IS, OLM) show strong ZnT-3 localization. Photoreceptor outer segments (OS) and outer nuclear layer (ONL) soma remained completely clear of reactivity. The outer plexiform layer (OPL) synaptic region shows consistent reactivity. (B) *Inner retina*: The inner nuclear layer (INL) shows consistent reactivity. The inner plexiform layer (IPL) exhibited weak granular reactivity. The ganglion cell layer and inner limiting membrane region (GCL, ILM) show consistent reactivity. Bar: 20 μm.

light-adapted, but not dark-adapted, murine retinas (Ugarte & Osborne, 1999). Rhodopsin and cGMP phosphodiesterase utilize zinc in outer segments (Palsgard, Ugarte, Rajta, & Grime, 2001). It has also been proposed that in the light adapted outer retina, higher amounts of zinc localize to an organelle (Palsgard et al., 2001). Approximately 60–75% of mouse retinal mitochondria can be found in photoreceptor inner segments (Perkins, Ellisman, & Fox, 2003). Photoreceptor mitochondrial respiration has been shown to decrease in light-adapted retina (Perkins et al., 2003) and it has been shown that zinc inhibits mitochondrial ATP production (Perkins et al., 2003). In addition, zinc interferes with mitochondrial glycolysis, the tricarboxcylic acid cycle, and electron transport (Perkins et al., 2003). Consequently, we suggest that in light-adapted inner segments, ZnT-3 transported zinc may have a functional significance relative to mitochondrial respiration.

In closing, we take note of the fact that Müller cells project apical villi in the region of the photoreceptor inner segments where ZnT-3 protein is localized. We suggest the possibility that the Müller cell apical villi in the region of the photoreceptor inner segments and the outer limiting membrane may utilize the ZnT-3 protein for zinc homeostasis. Mammalian Müller cells have already been shown to regulate extracelluler K+, glutamate and GABA in the neural retina (Reichenbach et al., 1997). It seems possible that ZnT-3 transporter associated with Müller cells may be involved in zinc distribution in light and dark adapted states. Müller cell associated ZnT-3 transporter could also account for the distribution of weak ZnT-3 immunoreactivity throughout the neural retina except for the region of the photoreceptor outer segments which are free of Müller cell processes. If so, the Müller cells may also provide a system for zinc homeostasis throughout the neural retina. Such an hypothesis is consistent with evidence for zinc sensitivity of isolated retinal Müller cells previously reported (Qian et al., 1996).

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