

Expression of ZnT and ZIP Zinc Transporters in the Human RPE and Their Regulation by Neurotrophic Factors

Kar Wah Leung,¹ Mugen Liu,² Xuming Xu,² Magdalene J. Seiler,³ Colin J. Barnstable,^{1,2} and Joyce Tombran-Tink^{1,2}

PURPOSE. Zinc is an essential cofactor for normal cell function. Altered expression and function of zinc transporters may contribute to the pathogenesis of neurodegenerative disorders including macular degeneration. The expression and regulation of zinc transporters in the RPE and the toxicity of zinc to these cells were examined.

METHODS. Zinc transporters were identified in a human RPE cell line, ARPE19, using a 28K human array, and their expression was confirmed by PCR, immunocytochemistry, and Western blot analysis in primary human RPE cultures and ARPE19. Zinc toxicity to ARPE19 was determined using monotezazolium, propidium iodide, and TUNEL assays, and Zn²⁺ uptake was visualized with Zinquin ethyl ester. The effect of various growth factors on zinc transporter expression also was examined.

RESULTS. Transcripts for 20 of 23 zinc transporters are expressed in fetal human RPE, 16 of 23 in adult human RPE, and 21 of 23 in ARPE19. Zn transporter proteins were also detected in ARPE19. ZnT5 expression was not observed, whereas ZnT6, ZIP1, and ZIP3 were the most abundantly expressed in all RPE samples. The addition of low concentrations of Zn²⁺ to cultures resulted in a dose-dependent increase in intracellular Zn²⁺ content in ARPE19, and >30 nM Zn²⁺ induced necrosis with an LC₅₀ of 117.4 nM. Brain-derived neurotrophic factor, ciliary neurotrophic factor, glial-derived neurotrophic factor (GDNF), and pigment epithelial-derived neurotrophic factor (PEDF) increased ZIP2 expression, GDNF and PEDF increased ZnT2 expression, and PEDF increased ZnT3 and ZnT8 expression. These neurotrophic factors also promoted Zn²⁺ uptake in the RPE.

CONCLUSIONS. The array of zinc transporters expressed by the RPE may play a key role in zinc homeostasis in the retina and in ocular health and diseases. (*Invest Ophthalmol Vis Sci*. 2008;49:1221-1231) DOI:10.1167/iovs.07-0781

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Disruption in zinc homeostasis is strongly implicated in the pathophysiology of many chronic neurodegenerative diseases and acute neural injuries. Excessive and inadequate levels of bioavailable zinc are detrimental to the health of neurons.¹ For example, increased concentration of Zn²⁺ is associated with aggregation of β -amyloid protein in patients with Alzheimer disease.²⁻⁵ Zinc enrichment in the cerebrovasculature may be an underlying factor in the development of cerebral amyloid angiopathy, a condition characterized by β -amyloid deposits in perivascular spaces of the brain.⁶ Intense presynaptic activity in epilepsy, ischemia, or traumatic lesions can trigger the release of Zn²⁺ to neurotoxic levels in surrounding tissues. This release may account for the unusually high Zn²⁺ content in the somata of neurons degenerating after severe episodes of ischemia or seizure activity.^{2,7-14}

A deficiency in Zn²⁺, on the other hand, promotes brain malformations during development and has other adverse consequences in the nervous system.¹⁵ Depleted pools of intracellular zinc in primary retinal cell cultures induce the caspase-dependent death of photoreceptors and other retinal neurons¹⁶ in the eye, and decreased levels of zinc in the retina contribute to the pathogenesis of age-related macular degeneration (AMD). The zinc-AMD connection is supported by the findings that retinas from monkeys with early-onset macular degeneration contain fourfold less Zn²⁺ than monkeys with normal vision^{17,18} and that levels of zinc in drusen and sub-RPE deposits¹⁹ are increased, suggesting that the risk for or severity of AMD increases with the depletion of available intracellular zinc pools in the retina. Newsome et al.²⁰ were the first to report that replenishing Zn²⁺ by oral administration has a positive effect on AMD. The 2004 AREDS report and other studies confirm that replacing zinc with a dietary supplement has beneficial effects against AMD.²¹⁻²³

Normal ocular tissues contain relatively high levels of zinc, ranging from approximately 25 μ g/g wet weight in the RPE/choroid to 100 μ g/g dry weight in the retina,^{24,25} with a high percentage of this localized in the photoreceptors and RPE cells. Recent studies show that intracellular localization of Zn²⁺ pools in photoreceptors changes with light exposure, with the greatest intensity of zinc staining observed in the perikarya of photoreceptors of dark-adapted retinas and in the inner segments of light-adapted retinas.²⁵ Zn²⁺ movement between RPE and photoreceptors is also light dependent, suggesting that Zn²⁺ is critical to normal visual function.

The importance of Zn²⁺ in biological processes is not restricted to the nervous system. It is the second most abundant trace element in the human body and is critical to housekeeping roles in physiology, cellular metabolism, protein structure, and gene expression. It provides structural stability to the Zn²⁺ finger domains of many DNA-binding proteins and is a cofactor for more than 300 metalloenzymes, in which it is an essential element for the catalytic site of the enzymes or serves in a structural capacity to facilitate enzymatic function.²⁶ For example, mutations in the copper-zinc superoxide dismutase (SOD1), an anti-oxidative Zn-binding enzyme, promote loss of

TABLE 1. ZnT Transporters Expressed in RPE Cells from Microarray Analyses and EST Database Mining

Gene	GenBank Accession No.	Primer Sequences		Amplicon Size (bp)
		Forward	Reverse	
<i>ZnT1</i>	NM_021194	GCATCAGTTTATGAGGCTGGTCCT	CAGGCTGAATGGTAGTAGCGTGAA	287
<i>ZnT2</i>	NM_032513	CTGGCCTTTGCCTTTATGAATCTG	GGACATCTCAGGGCAACAGAAAGT	257
<i>ZnT3</i>	NM_003459	ATGGTCACTGGCATCTCCTGTA	AGATGGAGAAGAGGAATGCTGA	251
<i>ZnT4</i>	NM_013309	TGTTAACTGACCTAAGCGCCATCA	GGTTACGTTTACACCCAGAACCTC	254
<i>ZnT5</i>	NM_022902	GATCCTAGGAACTAGTGGGAAACTGAG	GTCCACAAAAGAGTGAGGCCAAAAA	250
<i>ZnT6</i>	NM_017964	CCCATGAGTGTGTACAGTGGGAAA	GCATTGGGATTACGTGATGATCTG	250
<i>ZnT7</i>	NM_133496	AGAGGGTACAGCAGTTGCAAGGAG	AAAGCATGAACGGGCTGACTCTACC	274
<i>ZnT8</i>	NM_173851	TACGATGCACTCAGTCACCATTCA	AGCTGTACTTCGGCTCCACTCAG	264
<i>ZnT9</i>	NM_006345	TTGCGTGGATTTATACCGGTTTCAG	CCGAGCATTCCTACGAAGTTGATT	291
<i>ZIP1</i>	NM_014437	GATTGGGGAAGACACTTGACTGCT	GAAAGAGGAAGGGGATTTGTTGG	263
<i>ZIP2</i>	NM_014579	CCCTGTCTCTTGTGTCACTCT	AGCTCCCGTGAAGAATTTCTAGG	250
<i>ZIP3</i>	NM_213568	GTGGAGATATGAGGACCCCTGTT	GATGAACTCAGCGTAAACCGATCT	273
<i>ZIP4</i>	NM_017767	AGACTGAGCCAGAGTTGAGGCTA	TGTCGCAGAGTGCTACGTAGAGGA	272
<i>ZIP5</i>	NM_173596	GAGCAGGAGCAGAACCATTACCTG	CAATGAGTGGTCCAGCAACAGAAAG	290
<i>ZIP6</i>	NM_012319	CATAGCCATGAAGAACCAGCAATG	GAGAATCAAAGTGGGAGGGCTCTT	284
<i>ZIP7</i>	NM_006979	ACTGAAGGAGGAGCAGTGGACAGT	AGGCCCTAATGCCAAAGTAAACCAT	252
<i>ZIP8</i>	NM_022154	CCTCGGATTTGATTTGACTCCACT	AGCAGGATTTGCATAGCATGTCCAC	258
<i>ZIP9</i>	NM_018375	GCCTAAAGAACTGGAAAGCCCACT	GTGTTTCACTTGGTGGTGTGTT	250
<i>ZIP10</i>	NM_020342	TAGCCGTCTTCTGTATGAACTGC	TCATAGAGGGCAATCACCAGCATA	251
<i>ZIP11</i>	NM_139177	TCTCCTAAGCATTGTTGGTGGCCTA	TCTCTTCTTCCACAGGGCTCACT	255
<i>ZIP12</i>	NM_152725	CAACCCTCAAGAAGCCTCATCAA	AAGTACTGCCTGGTAAAAGCCAAAG	253
<i>ZIP13</i>	NM_152264	AAGAAGATCGGGCTCTGACAAAC	GAGAACAGCACCATTCACACAGATG	273
<i>ZIP14</i>	NM_015359	CATTTGGTTTCAACCTCTGGAAG	TTTCAGCCAGTAGCAAGCACTCTG	270

These primers were all used in RT-PCR experiments at an annealing temperature of 58°C for 35 cycles to confirm expression of the genes in human primary cultures of fetal and adult RPE cells and in ARPE19.

zinc and copper ions from the enzyme, resulting in protein destabilization and the formation of neurotoxic SOD1 aggregates.^{27,28} This condition is found in approximately 20% of familial cases of amyotrophic lateral sclerosis (ALS) and 2% to 7% of sporadic cases.

Fluctuations in intracellular labile zinc are mediated by influx and efflux transport mechanisms. The major influx and efflux routes of Zn²⁺ are through two classes of multipass transmembrane proteins, ZnT and ZIP, which are encoded for by two solute-linked carrier (SLC) gene families, *SLC30* and *SLC39*, respectively. At least nine ZnT and 14 ZIP transporters have been identified in human cells. These transporters exhibit tissue-specific expression and have unique responses to dietary zinc, hormones, and cytokines. They are located on plasma and vesicular membranes,²⁹ and the two families have opposing functions in mediating zinc homeostasis. ZnT transporters are efflux transporters that reduce cytoplasmic Zn²⁺ concentrations by promoting zinc efflux from the cytoplasm to the extracellular compartment or into intracellular vesicles. ZIP transporters, on the other hand, are the influx transporters that mediate Zn²⁺ uptake into the cytoplasm from extracellular or vesicular sources.²⁹ Alterations in the expression and function of the Zn transporters have severe consequences for Zn²⁺ homeostasis. At least five zinc efflux transporters, ZnT1–4, and 6 are implicated in protein aggregation, amyloid plaque formation, and the early progression of Alzheimer disease,^{3,30} suggesting that these transporters are also candidate genes in the pathogenesis of other neurodegenerative diseases, including AMD.

In this study, we found that most of the Zn transporters (ZnT1–9 and ZIP1–14) are expressed in human RPE and that neurotrophic factors can alter their expression. We also show that neurotrophic factors can modulate Zn²⁺ uptake by RPE cells and that extracellular Zn²⁺ concentrations greater than 30 nM are toxic to the cells. These novel findings are an important first step in understanding how alterations in zinc metabolism, transport, and regulation in the retina contribute to AMD and the role the RPE plays in this process.

MATERIALS AND METHODS

Materials

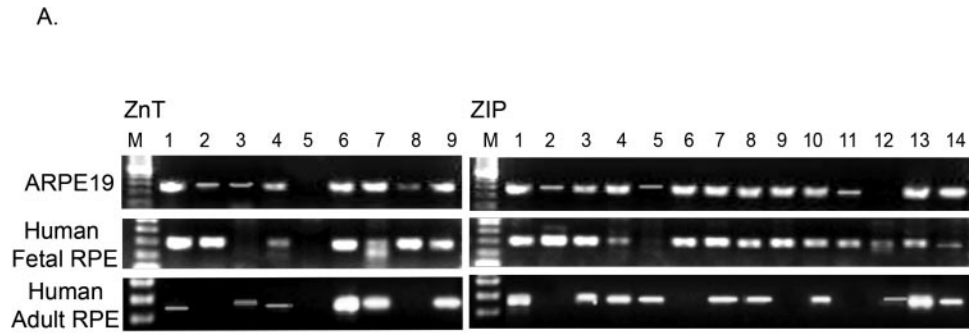
Specific primers for the zinc transporters were designed using specialized software (OligoPerfect Designer Software; Invitrogen, Carlsbad, CA) and were synthesized. Goat-anti-human ZnT antibodies (ZnT1 [sc-27501], ZnT2 [sc-27506], ZnT4 [sc-27511]) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit-anti-human ZIP antibodies (ZIP1 [ZIP11-A], ZIP2 [ZIP22-A], ZIP3 [ZIP31-A], ZIP4 [ZIP42-A], ZIP5 [ZIP51-A], ZIP6 [ZIP62-A], ZIP7 [ZIP71-A]) were obtained from Alpha Diagnostic International, Inc. (San Antonio, TX). The catalog number for each is listed in parentheses. The ZnT3 polyclonal antibody was a generous gift from Victor Faundes (Emory University, Atlanta, GA).³¹ Antibodies against ZnT5–9 and ZIP8–13 were not commercially available. Tissue culture reagents and TUNEL assay kits were purchased from Invitrogen. Propidium iodide (PI), zinc sulfate (ZnSO₄), and a specific zinc fluorescence probe, Zinquin ethyl ester, were obtained from Sigma (St. Louis, MO).

Primary Human RPE Cell Culture

Fetal human RPE cells were isolated from three retinas obtained from normal eyes at gestational ages 13, 15, and 18 weeks in accordance with approved institutional protocols. These RPE cells were provided by Magdalene Seiler (Doheny Eye Institute, Los Angeles, CA). Human RPE cells from adults (50–60 years old) were provided by Janice Burke (Medical College of Wisconsin, Milwaukee, WI). Primary cultures were maintained in RPMI-1640 medium supplemented with 2% FBS and 100 ng/mL penicillin-streptomycin-neomycin (PSN). At the second passage, the cells were harvested for RNA extraction.

ARPE19 Cell Culture

Human ARPE19 cells (derived from a normal eye of a 19-year-old man as a spontaneously arising cell line) were obtained from American Type Culture Collection (Manassas, VA) and were used at passages 12 to 17. The cells were routinely cultured in RPMI-1640 medium supplemented with 2% FBS and 100 ng/mL PSN and were grown to a confluent monolayer (approximately 5 × 10⁴ cells/cm²) before they were har-



B.

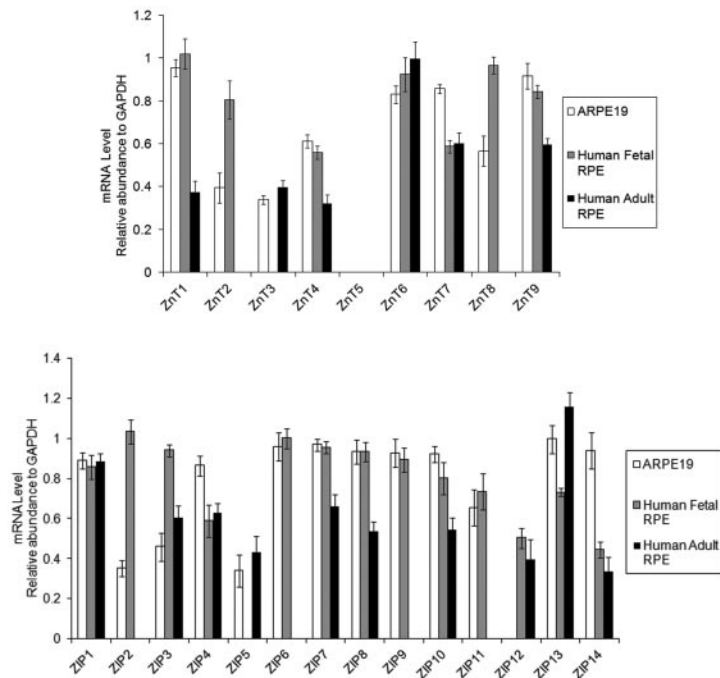


FIGURE 1. Expression of zinc transporter mRNA in human RPE cells. **(A)** Expression of zinc transporter mRNA was examined in the human ARPE19 cell line and in human fetal and adult RPE primary cultures using RT-PCR and the primers listed in Table 1. PCR amplification reactions were carried out for 35 cycles, and the products were resolved by agarose gel electrophoresis. **(B)** Expression of these transporters in the three samples was quantified using real-time PCR. The histogram represents the abundance of each transporter relative to GAPDH. Expression of GAPDH was arbitrarily set at 1. $n = 3$ (\pm SD).

vested or treated. For all experimental conditions, the serum-containing medium was removed from the cultures and replaced with zinc-free, serum-free MEM.

Zn²⁺ Uptake by Cultured RPE Cells

RPE cells were incubated with 0 to 18 nM Zn²⁺ for 60 minutes in serum-free MEM that contained no detectable levels of Zn²⁺. After removal of extracellular Zn²⁺ from the cultures, the cells were loaded with 2.5 mM zinc fluorescent sensor, Zinquin ethyl ester, for 30 minutes, and the fluorescent Zinquin-Zn²⁺ complex was visualized using fluorescence microscopy at 364 nm excitation and 385 nm emission.

Regulation of Transporters and Zinc Uptake with Growth Factors

ARPE19 cultures were treated with 50 ng/mL brain-derived neurotrophic factor (BDNF), 25 ng/mL ciliary neurotrophic factor (CNTF), 50 ng/mL glial-derived neurotrophic factor (GDNF), or 100 ng/mL pigment epithelial-derived neurotrophic factor (PEDF) in serum-free medium for 48 hours to study the regulation of zinc transporter expression with these factors. Representative cultures were harvested for

RNA isolation and protein extraction or were fixed for immunocytochemistry.

After 48 hours of treatment with the growth factors, various doses of Zn²⁺ (0–18 nM) were then added to some cultures for 60 minutes to study the concentration of Zn²⁺ taken up by the cells and the level of Zn²⁺ toxicity with increasing extracellular doses.

Three fields were photographed for each treatment, and each study was conducted in triplicate in three separate trials. Fluorescence intensity of each cell was measured with NIH Image J software, and data were presented as the mean (\pm SD) of three trials.

Microarray Analysis

Confluent RPE cells were homogenized (QIAshredder; Qiagen, Valencia, CA), and total RNA was extracted from the cell pellet and purified by the RNeasy mini kit (Qiagen). RNA concentration was measured with a spectrometer (GeneSpect III; Hitachi, Tokyo, Japan). RNA at a 280/260 ratio greater than 1.9 was used for array hybridization without amplification.

The basic method for this experiment has been described.^{32,33} Briefly, RNA was labeled using a DNA array kit (DNA Array 900 Kit; Genisphere, Hatfield, PA) for the dye-swap experiment. Cy3- and Cy9-

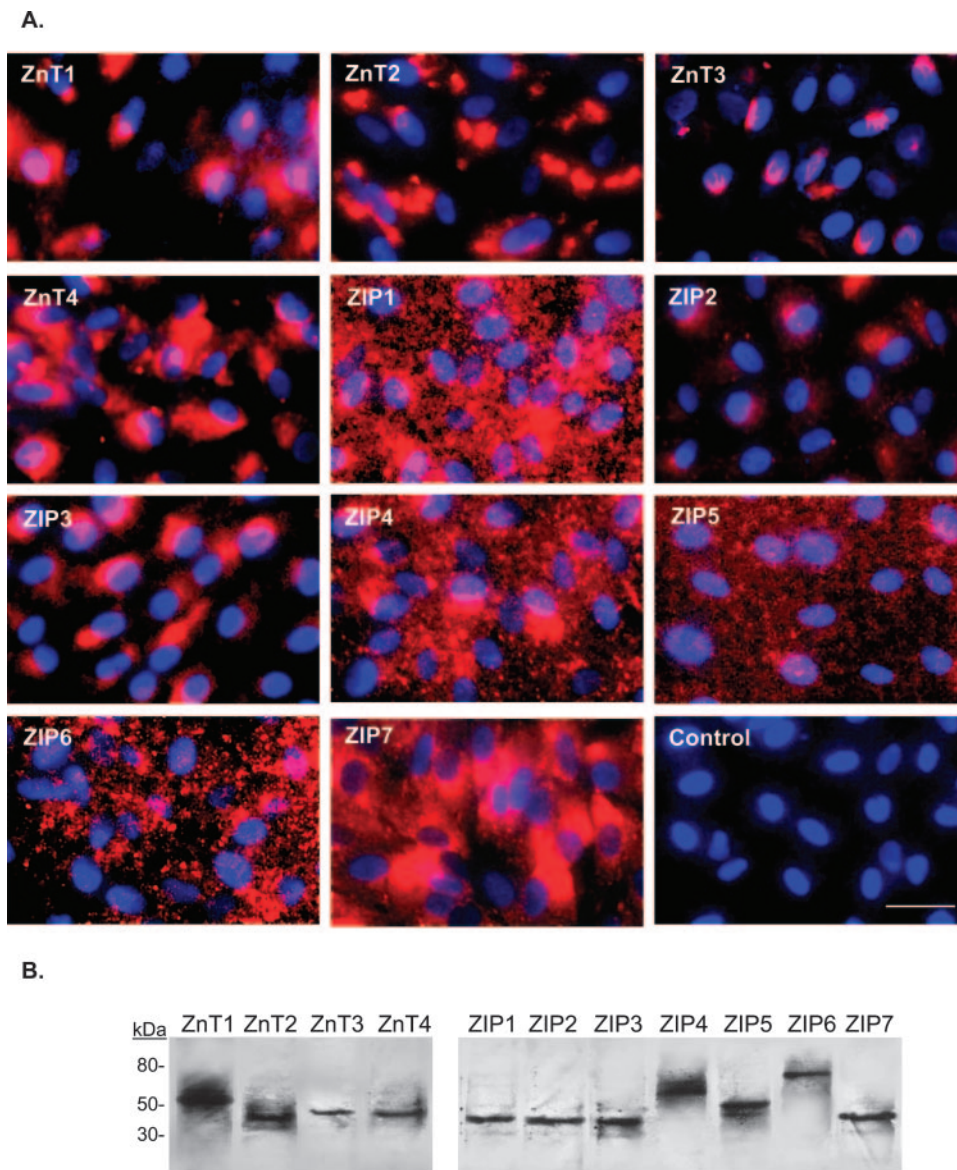


FIGURE 2. Expression of zinc transporter proteins in ARPE19. **(A)** Fixed RPE cells were immunolabeled with the polyclonal antibodies against ZnT1 to ZnT4 and ZIP1 to ZIP7 and were colabeled with DAPI. Control samples were treated with the secondary antibody only (Alexa Fluor 568) and DAPI. Expression of the zinc transporter proteins showed a trend in abundance similar to that of their mRNA levels in the RPE cells. Scale bar, 50 μ m (all micrographs). **(B)** Proteins separated by SDS-PAGE were labeled with the polyclonal antibodies against ZnT1 (60 kDa), ZnT2 (35 kDa), ZnT3 (40 kDa), ZnT4 (43 kDa), ZIP1 (34 kDa), ZIP2 (32 kDa), ZIP3 (34 kDa), ZIP4 (71 kDa), ZIP5 (56 kDa), ZIP6 (86 kDa), and ZIP7 (51 kDa). Zinc labeling was diffused throughout the cytoplasm, in perinuclear locations, or as discrete punctate fluorescent inclusions in the RPE cells.

labeled RNA were hybridized to QuantArray 28K human OHU28K oligo microarray slides (provided by the Yale Keck Microarray Center). Dye swap comparison experiments were performed on RPE samples. The DNA array kit (DNA Array 900 Kit; Genisphere) was used for labeling. Slides were scanned by a GenePix 4000a scanner (Axon). Microarray analysis software (GeneSpring 7.2, demonstration version; Silicon Genetics, Redwood City, CA) was used for the initial data normalization.

RT-PCR and Real-Time PCR

Total mRNA from primary human RPE cells samples and the ARPE19 cultures were isolated (RNeasy kit; Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized (iScript cDNA synthesis kit; Bio-Rad, Hercules, CA), and RT-PCR was performed (iTaq polymerase; Bio-Rad) at an annealing temperature of 58°C for 35 cycles for all ZnT and ZIP primers. Accession number, primer sequence, and PCR amplification product size for each gene are listed in Table 1. GAPDH was used as the internal RNA loading control, and samples for which no reverse transcriptase was added to the PCR experiments were used as negative controls to ensure that amplification was RNA dependent. PCR products were resolved by 1% agarose gel electrophoresis. For quantitative real-time PCR, the two-step amplifying pro-

cedure was used with supermix solution (iQ SYBR green; Bio-Rad). Melting curve and gel electrophoretic analyses were used to determine amplicon homogeneity and data quality.

Immunocytochemistry

RPE cells, grown on coverslips, were fixed with 4% paraformaldehyde and labeled with a specific ZnT or ZIP zinc transporter antibody at the manufacturer's recommended concentrations. Cells were incubated with the primary antibodies for 3 hours in the presence of normal goat serum and 0.1% Triton X-100. After incubation with IgG secondary antibody (Alexa Fluor 568; Invitrogen) for 1 hour, coverslips were mounted with medium containing DAPI (Calbiochem, San Diego, CA), and micrographs were captured by epifluorescence microscopy using identical parameters and settings.

Western Blot Analysis

RPE cells were lysed with lysis buffer (CytoBuster; Novagen, San Diego, CA), and protein concentrations in the supernatant were estimated (Dc Protein Assay Kit; Bio-Rad). Fifty micrograms of protein was separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5% (wt/vol) dried milk, each membrane was

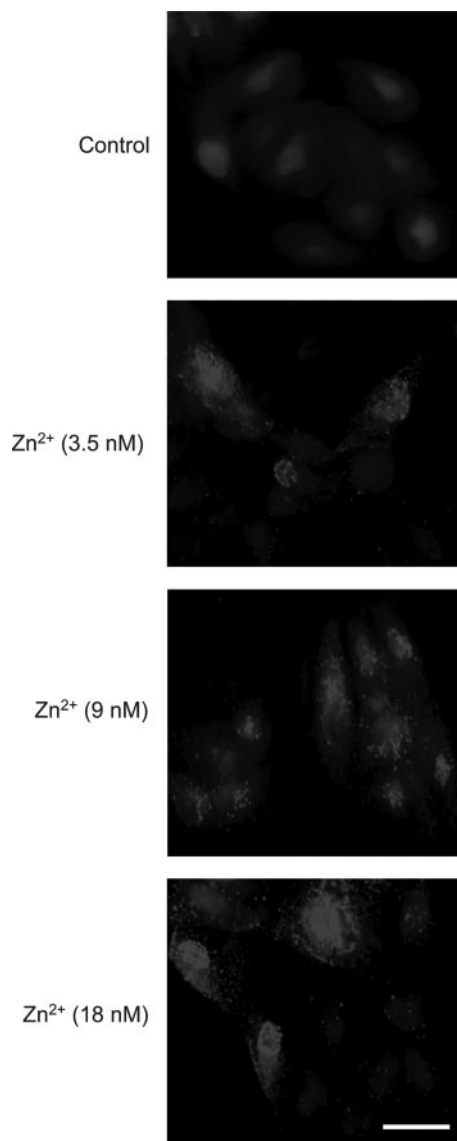


FIGURE 3. Uptake of Zn^{2+} by ARPE19. RPE cells were incubated in various concentrations of Zn^{2+} (3.5–18 nM) for 60 minutes, and intracellular Zn^{2+} levels were visualized with the fluorescent Zn^{2+} probe, Zinquin ethyl ester. Increased addition of Zn^{2+} to the cultures resulted in increased intracellular Zn^{2+} levels in the cells, as detected by the fluorophore. Zinc labeling was diffused throughout the cytoplasm or as discrete punctate fluorescent inclusions in the RPE cells. $n = 3$. Scale bar, 20 μm (all micrographs).

incubated with a primary antibody against one of the zinc transporters for 3 hours; this was followed by washing and subsequent incubation with the appropriate horseradish peroxidase-conjugated IgG secondary antibody for 1 hour. Bound antibody was determined with an ECL detection system (Bio-Rad).

Cytotoxicity Assay

MTT assay was used to test the viability of RPE cells after treatment with various doses of Zn^{2+} . The cells were cultured in 96-well plates at a density of 1×10^4 cells/well. After 48-hour treatment with Zn^{2+} (0–870 nM) diluted in serum-free medium, the cells were incubated with 5 mg/mL MTT solution for 4 hours in a 37°C humidified incubator and then solubilized with 150 μL dimethyl sulfoxide. Formazan (formed during cellular respiration in the mitochondria of live cells) was measured spectrophotometrically using OD₄₉₀. Data are expressed

as percentages of cell death in experimental samples to untreated controls.

PI Staining and TUNEL Assay

RPE cells were treated with Zn^{2+} at various concentrations (0–180 nM) for 48 hours. Some of the cells were incubated with 4 $\mu g/mL$ PI (diluted in PBS) for 60 minutes at room temperature, and others were fixed for 1 hour in cold 70% ethanol for TUNEL assay according to the manufacturer's instructions. Images of PI- and TUNEL-positive cells were captured using epifluorescence microscopy.

Statistical Analysis

Numerical results were analyzed using one-way ANOVA with Duncan post hoc test. Values shown are mean \pm SD of triplicate assays obtained from three independent experiments. Differences were considered statistically significant at a value of $P \leq 0.05$.

RESULTS

Expression of Zn Transporters in RPE Cells

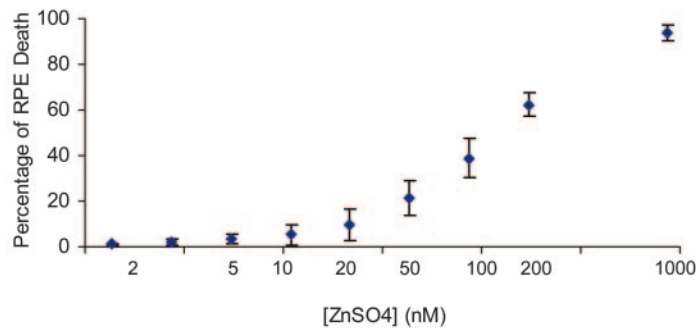
Because of the importance of zinc in the pathology and treatment of AMD, we sought to determine whether zinc transporters are present in the human RPE and examined the range of Zn^{2+} concentrations that were tolerated by these cells. We performed an initial assay using microarray analysis to identify genes expressed in a human RPE cell line, ARPE19. We found that almost all the zinc transporters were expressed in these cells and designed primers to the human genes to confirm their expression by PCR in primary cultures of human fetal and adult RPE cells and the cell line. Human ZnT and ZIP transporter primer sequences, GenBank accession numbers, and PCR amplification product sizes are given in Table 1.

Most ZnT and ZIP Zn^{2+} Transporter mRNAs Are Expressed in Human RPE Cells

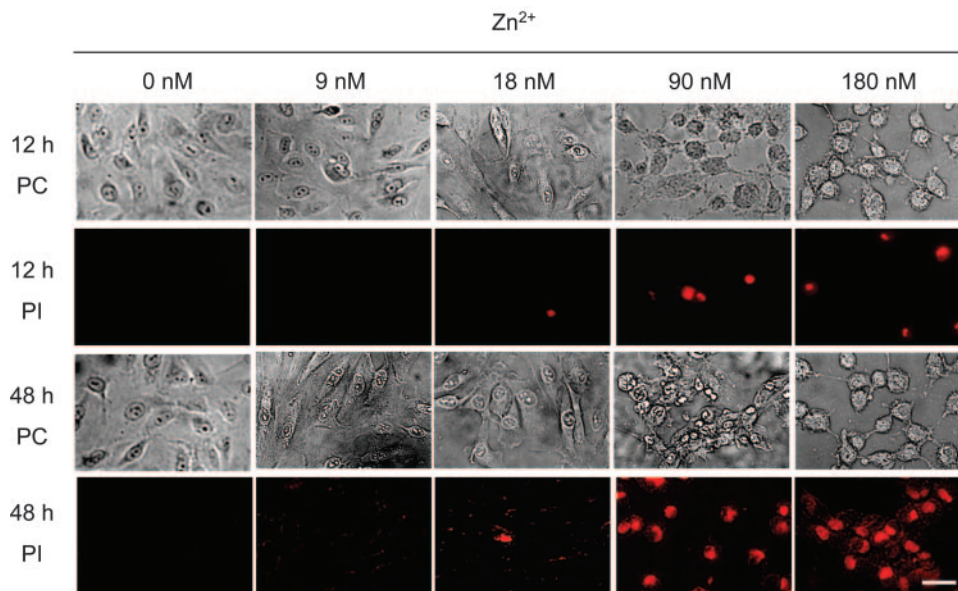
We extracted mRNA from human adult RPE, human fetal RPE, and confluent monolayers of the RPE cell line ARPE19 to analyze the expression of nine ZnT and 14 ZIP transporters identified by microarray analysis in the RPE cell line. With the use of RT-PCR, we showed that mRNAs for 21 of 23 transporters were expressed in ARPE19, 20 of 23 in fetal human RPE, and 16 of 23 in the adult RPE (Fig. 1A). ZnT5 was not detected in any of the RPE samples analyzed.

The transporters ZnT2, ZnT8, ZIP2, ZIP6, ZIP9, and ZIP11 were detected in ARPE19 and the fetal RPE cells but not in the adult cells. ZnT3 and ZIP5 were expressed in ARPE19 and the adult human RPE but not in the fetal cells. ZIP12 was the only transporter found in the primary human RPE cultures but not in the cell line. Overall, mRNA expression for most of the transporters was weaker in the human adult RPE than in the fetal RPE or the cell line. Samples in which the reverse transcriptase was omitted were negative (data not shown), indicating that amplification of the transporters was RNA dependent. A quantitative comparison of the gene expression profile of these transporters using real-time PCR is shown in Figure 1B. RPE was isolated from three human eyes and three separate cultures of ARPE19. Real-time PCR analyses were performed in triplicate for each of the six samples, and the results obtained were similar to the semiquantitative RT-PCR data in Figure 1A. ARPE19 cells showed expression patterns of zinc transporters that were sometimes similar to those of fetal cells and at other times were similar to those of adult primary cultures, which is perhaps not surprising given that the ARPE19 cells were derived from a young adult. Because the ARPE19 cells shared so many properties of the primary cultures of human RPE cells

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B.



C.

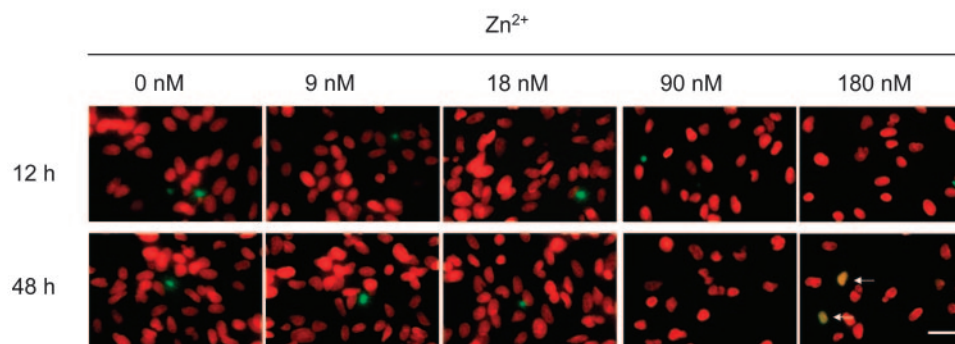


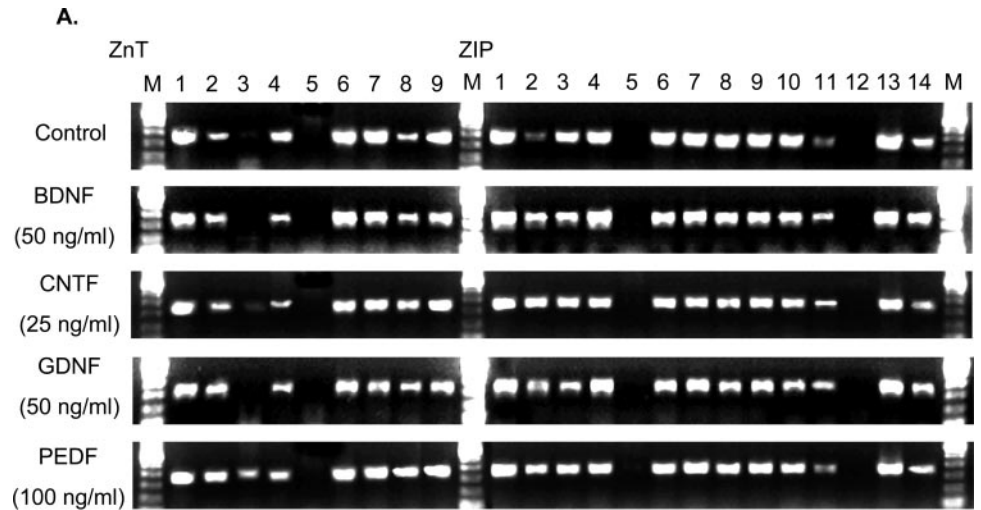
FIGURE 4. Zn^{2+} concentration greater than 30 nM are toxic to RPE cells. (A) Cytotoxicity of Zn^{2+} was determined by MTT assay. Zn^{2+} (1.4–870 nM) caused dose-dependent death of ARPE19 cells at $LC_{50} = 117.4$ nM (± 3.11 nM). (B) PI staining of unfixed cells after 12 and 48 hours of Zn^{2+} treatment. Addition of increasing concentrations of Zn^{2+} to the medium, between 90 and 180 nM, significantly increased PI staining of the cells at 12 hours and 48 hours. RPE cells change morphologically from a healthy appearance (0–18 nM Zn^{2+}) to more rounded, detached, unhealthy looking cells with the increased addition of Zn^{2+} to the medium, as shown in the phase-contrast (PC) micrographs. (C) Few apoptotic cells (arrows) are seen in the cultures with high Zn^{2+} concentrations at 48 hours, as demonstrated by TUNEL assay. In the TUNEL assay, PI staining of the fixed cells was used to identify the total numbers of cells in the cultures. Scale bar, 50 μ m (all micrographs).

with respect to zinc transporter expression, we used the more available ARPE19 cell line to conduct subsequent experiments.

Zinc Transporter Proteins Have Expression Levels Similar to Those of Their mRNA in RPE Cells

Using all the commercially available antibodies, we showed that the zinc transporter proteins are expressed in the RPE cell line (Fig. 2). The immunolabeling pattern for ZIP1, ZIP4, ZIP5, ZIP6, and ZIP7 is punctuate in appearance in the

cytoplasm of the cells, suggesting that these are located on vesicular structures where they may be involved in Zn^{2+} sequestration in the RPE cells. The immunolabeling pattern observed for ZnT1, ZnT2, ZnT3, and ZIP3 was that of a distinct perinuclear cap. The intensity of labeling for the transporters shows a good qualitative correlation with their abundance at the mRNA level. Expression of the zinc transporters was also analyzed by Western blot to confirm specificity of the antibody for the target protein (Fig. 2B). Each antibody examined gave a single major band of the expected



B.

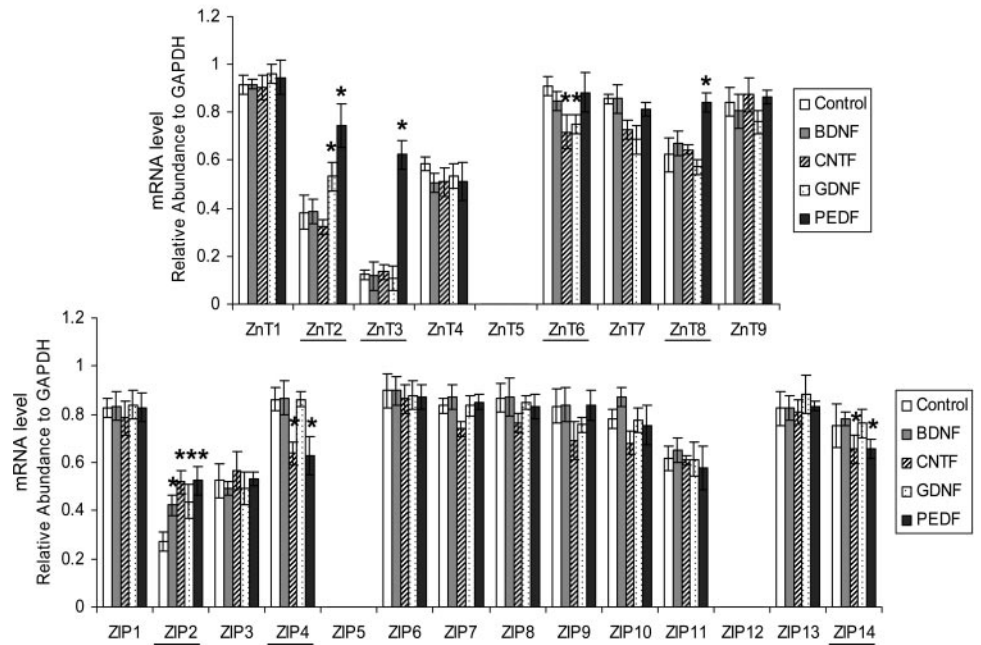


FIGURE 5. Changes in zinc transporter mRNA levels in RPE cells after treatment with neurotrophic factors. RPE cells were treated with the neurotrophic factors BDNF, CNTF, GDNF, and PEDF at the indicated concentrations in serum-free medium for 48 hours. Cells were then harvested, and total RNA was extracted for (A) RT-PCR and (B) real-time PCR. ZIP2 expression was significantly increased by all the neurotrophic factors, and ZnT3 expression was altered by PEDF alone. Significant changes were also seen in the expression of a ZnT2, ZnT6, ZnT8, ZIP4, and ZIP14. The histogram represents the relative abundance of each transporter to GAPDH. Expression of GAPDH was arbitrarily set at 1. *n* = 3 (± SD). **P* ≤ 0.05.

size. Together the two sets of data indicated that each of the 11 transporters examined was expressed as a full-length protein in the RPE cells.

Zn²⁺ Uptake by RPE Cells Is Concentration Dependent

We next examined whether the zinc transporters were active in the RPE cells. To do this, we added Zn²⁺ to the cultures at various concentrations between 3 and 18 nM for 60 minutes and then detected Zn²⁺ uptake with Zinquin ethyl ester, a membrane-permeable fluorescent compound with excitation and emission maximums at 368 nm and 490 nm, respectively (Fig. 3). An increase in Zn²⁺ concentration in the culture medium (0, 3.5, 9, 18 nM) led to an increase in intracellular Zn²⁺ levels. There was only a weak endogenous diffuse labeling of Zn²⁺ in the cytoplasm and nucleus of nontreated cultures compared with the strong fluorescence signal seen after Zn²⁺ treatment. After uptake, Zn²⁺ labeling was obvious in discrete bodies in the cytoplasm, in the matrix of the cytoplas-

mic compartment, and in the nuclei of the treated cells, suggesting that Zn²⁺ was taken up by plasma membrane and vesicular zinc transporters on cultured RPE cells and that some of it was sequestered in intracellular vesicles. These transporters may also have been the mechanism of zinc trafficking into the nuclei of the RPE cells.

Zn²⁺ Is Cytotoxic to RPE Cells at Concentrations Greater Than 30 nM

Although Zn²⁺ is essential to the physiological function and health of cells, Zn²⁺ overload is cytotoxic to many neuronal cells in the brain. The normal plasma level range of Zn²⁺ is between 8 and 23 nM and, in the retina, has approximately 10 μmol/g wet mass. Our next experiment was to analyze the cytotoxicity of Zn²⁺ to the RPE cells using the MTT assay. As shown in Figure 4, RPE cells are sensitive to Zn²⁺ concentrations higher than the 30-nM concentration range. Incubation of the cells with Zn²⁺ (1.4–870 nM) for 48 hours resulted in dose-dependent RPE cell death, with 50% lethal concentration

(LC_{50}) at 117.4 nM (SD = \pm 3.11; Fig. 4A). Less than 10% cell death was observed at doses less than 30 nM; this was followed by a rise in the percentage of death with increasing doses, until approximately 95% of cells were killed by 870 nM. We then used PI, which labels necrotic cells, and TUNEL assay, which labels apoptotic cells, to examine the mechanism of cell death induced by toxic levels of zinc ions. Live Zn^{2+} -treated RPE cells were increasingly PI positive with increasing doses of Zn^{2+} treatment ranging from 9 to 180 nM after 12 and 48 hours of incubation (Fig. 4B). In contrast, only a few TUNEL-positive cells were seen at 48 hours, suggesting that necrosis is the mode of action for Zn^{2+} toxicity in RPE cells (Fig. 4C).

Neurotrophic Factors Regulate Expression of Zinc Transporters in RPE Cells

Some neurotrophic factors are known to improve the general health of the retina in many pathologic conditions, including those caused by ischemic injury and light damage. In this experiment we examined whether neurotrophic factors can alter the expression levels of the ZnT and ZIP transporters in RPE cells. We treated cultured RPE cells for 48 hours with BDNF, CNTF, GDNF, and PEDF at their effective doses. RT-PCR (Fig. 5A) and real-time PCR (Fig. 5B) measurements indicated that these factors are effective modulators of specific zinc transporters. For example, each of the four factors upregulated the mRNA levels for the Zn^{2+} influx transporter, ZIP2. CNTF and PEDF independently decreased ZIP4 and ZIP14 expression, and PEDF induced a strong increase of ZnT3 and ZnT8 mRNA levels. Treatment with PEDF or GDNF increased ZnT2 expression, whereas CNTF and GDNF independently decreased ZnT6 expression. The addition of Zn^{2+} alone to cells at the various concentrations had negligible effect on the expression of zinc transporter mRNA levels (data not shown). The increased protein expression of ZIP2 by each of the neurotrophic factors and ZnT3 by PEDF confirmed the increased in mRNA levels of the transporters by these factors (Fig. 6). ZnT3 protein showed perinuclear localization, whereas ZIP2 was diffused throughout the cell.

Neurotrophic Factors Increase Intracellular Zinc Pools in RPE Cells

All four neurotrophic factors increased intracellular Zn^{2+} levels in the RPE cells, proportional to the concentrations of Zn^{2+} added to the medium (Fig. 7). BDNF and PEDF were more effective than CNTF and GDNF in increasing intracellular pools of Zn^{2+} in the RPE cells at low concentrations. In addition, BDNF increased the Zn^{2+} fluorescence signal in the control, no-zinc treatment cultures grown in serum-free medium, suggesting it may induce the release of bound zinc from intracellular stores.

DISCUSSION

Metal ion homeostasis is an important area of research in metalloneurochemistry because many neurologic disorders are linked to metal ion imbalances in the nervous system. Zinc, an essential metal ion, plays a significant role in antioxidative,^{34,35} anti-inflammatory,^{36,37} and cell survival mechanisms^{38,39} in the central nervous system. Too much zinc, however, is highly toxic to neurons, a finding corroborated by the ability of Zn^{2+} chelators to reduce neuronal death.

In mammalian tissues, cytoplasmic Zn^{2+} concentration is maintained within a narrow range in the cells. Cells homeostatically adjust to Zn^{2+} excess by sequestering the metal ion in cytoplasmic vesicles or by increasing its efflux and, in conditions of zinc deficiency, by increasing its influx. These adjustments to zinc distribution and homeostasis involve complex

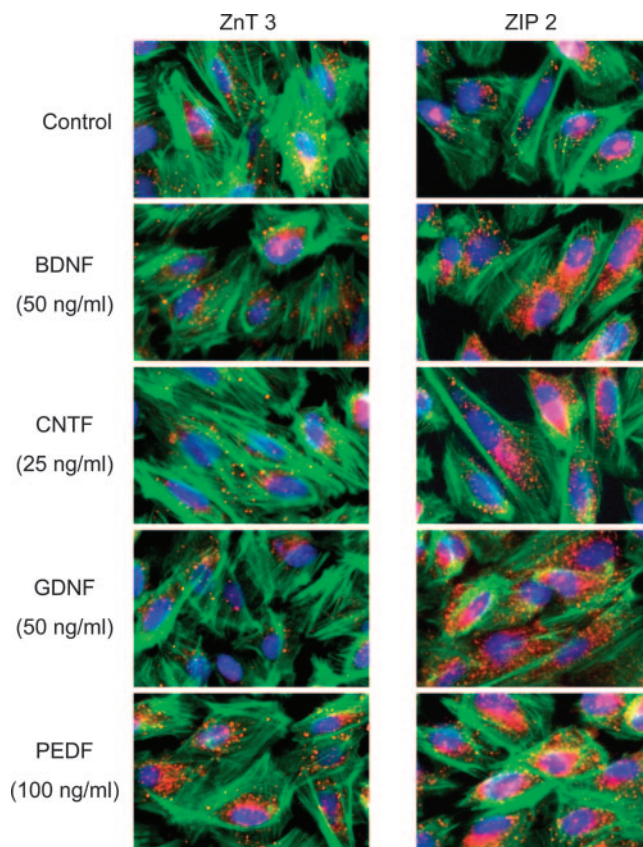


FIGURE 6. Neurotrophic factors increased the protein levels of ZnT3 and ZIP2 zinc transporters in RPE cells. After treatment with BDNF, CNTF, GDNF, and PEDF, ARPE19 cells were fixed and immunolabeled with ZnT3 or ZIP2 polyclonal antibodies (red fluorescence) and colabeled with β -actin (green fluorescence) and DAPI (blue fluorescence). ZnT3 localization was perinuclear; ZIP2 protein was distributed throughout the cell.

cellular mechanisms, which rely on many integral membrane transporters and metallochaperones to maintain a strict balance of intracellular zinc. Modulations in this balance are often detrimental to cells as seen in neuronal cells, where free Zn^{2+} concentrations reaching levels of 400 to 600 nM trigger widespread death in cultures.^{40,41} Although there are high levels of zinc in the circulation, approximately 98% of plasma Zn^{2+} is protein bound in normal physiological conditions.⁴²

The recent finding in the retina that there is a pool of free Zn^{2+} in drusen is intriguing¹⁹ and implies that zinc contributes to plaque-like drusen formation and is a marker for AMD. This is not surprising because loosely bound or free zinc promotes the formation of β -amyloid plaques in the brains of patients with Alzheimer disease.⁴³ Paradoxically, long-term dietary Zn^{2+} supplementation slows the progression of AMD,⁴⁴ suggesting zinc deficiency in the retinas of AMD patients. This apparent inconsistency in zinc levels in the retina must be explored more carefully. Is zinc accumulation in drusen caused by a pathologic efflux from the RPE or a release of zinc by degenerating RPE cells, or is it a result of Zn^{2+} deposition by circulating plasma?

The role of the RPE cells in maintaining retinal zinc pools through zinc transporters has not yet been examined. In this study, we provide evidence that the RPE is equipped with almost all the zinc influx and efflux transporters, is likely to be the key cell type regulating zinc ion homeostasis in the retina, and may contribute to retinal dysfunction by pathologic control of retinal zinc levels. Based on our findings and those of

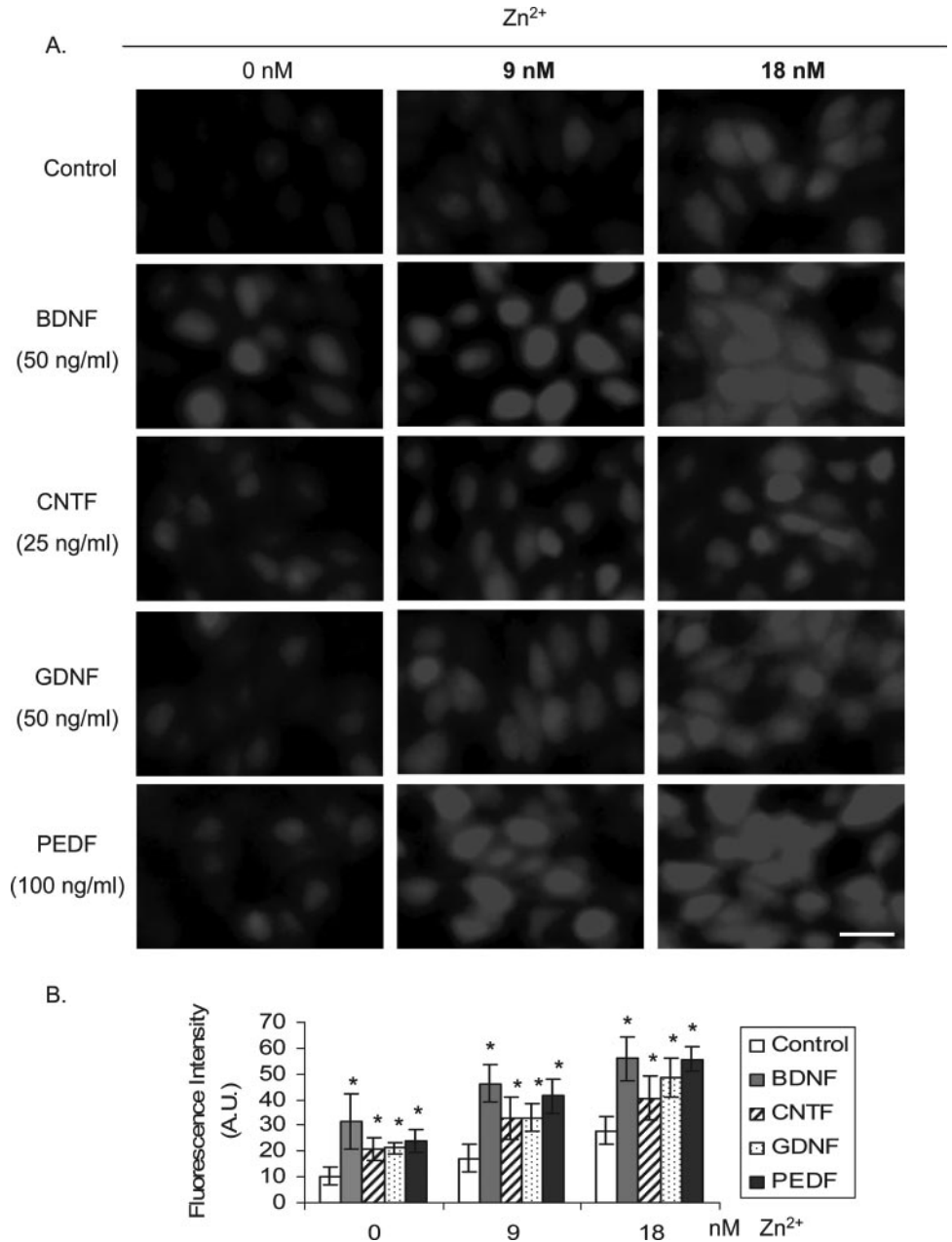


FIGURE 7. Neurotrophic factors increased Zn²⁺ uptake in RPE cells. Cells were treated for 48 hours with the neurotrophic factors BDNF, CNTF, GDNF, and PEDF in zinc-free, serum-free medium, and Zn²⁺ was added to the medium at various concentrations for 60 minutes. **(A)** Intracellular Zn²⁺ concentration was visualized with the Zn²⁺ fluorescence probe, Zinquin ethyl ester. **(B)** Fluorescence intensity was measured by NIH Image J software and expressed in arbitrary units in the histogram. *n* = 3 (± SD). Scale bar, 20 μm. **P* ≤ 0.05.

Lengyel et al.,¹⁹ we speculate that zinc accumulation in drusen reflects malfunction in zinc transporter mechanisms in the RPE because levels of free ionic zinc in the circulating plasma are likely to be low; if deposited by circulating plasma, dietary free zinc would only add to the existing pool in drusen. Zinc transporter defects in the RPE could result in a steady state basal efflux of zinc ions from these cells and increase free zinc pools in drusen. This, in turn, could deplete the retina of this important trace element, compromise normal retinal function, and eventually lead to retinal degeneration. Dietary zinc treatment may therefore, compensate for the excess Zn²⁺ efflux and provide the retina with just enough zinc to delay the progression of AMD.

Using a fluorescent sensor to track zinc uptake, we show that zinc ion is taken up by cultured RPE cells and that excess zinc is toxic at concentrations greater than 30 nM. This finding suggests that at least some of the zinc transporters expressed by RPE cells are active, but their distribution and function on the RPE cells are yet to be elucidated. The cellular localization

and function of some transporters have been described.²⁹ For example, ZnT1, which we have shown to be abundantly expressed in human RPE, is predominantly expressed on the plasma membrane of cells⁴⁵ and crucial in early embryonic development.⁴⁶ ZnT2, which is also highly expressed by the human RPE, aids in Zn²⁺ sequestration into the endosomal/lysosomal compartment of cells.⁴⁷ The ZnT3 transporter accumulates zinc in synaptic vesicles and is implicated in neuromodulatory functions,^{31,48,49} whereas the ZnT6 and ZnT7 transporters facilitate zinc transport from the cytoplasm to the Golgi apparatus.⁵⁰ ZIP1 and ZIP3 are critical in dietary Zn²⁺ uptake,⁵¹ and ZIP2 and ZIP4 form a functional unit for synapsis initiation during meiosis.⁵²

These transporters play a key role in many other disorders. To mention a few, defects in ZIP4 are associated with poor absorption of dietary zinc in acrodermatitis enteropathica.⁵³ There is altered expression of ZIP4 and ZIP6 with cognitive impairment in early and late Alzheimer disease.³⁰ ZnT3 imbalance correlates with neurodevelopmental damage.⁵⁴ Modula-

tion in the expression of ZnT4 and ZIP1, ZIP4, ZIP14 is linked to asthma; ZnT1, ZnT2, and ZnT5 are linked to diabetes; and ZnT1, ZnT3, ZnT4, and ZnT6 are linked to cognitive dysfunction.⁵⁵

We also present evidence that neurotrophic factors modulate the expression of zinc transporters in RPE cells and increase zinc uptake by the cells. In most cases, the increase in intracellular Zn²⁺ levels may be linked to transporter activity rather than to the release of free Zn²⁺ from bound zinc pools in the cells because the phenomenon correlates with increasing Zn²⁺ doses in the medium. What is interesting is the regulation of ZIP2 transporter by all four neurotrophic factors. This is an influx transporter localized on the apical membranes of cells⁵⁶ and could be a candidate protein involved in Zn²⁺ uptake by the RPE from the subretinal space. Other transporters are also regulated by the neurotrophic factors. There were significant decreases in the expression of ZIP4 and ZIP14 by CNTF and PEDF and of ZnT6 by CNTF and GDNF. Both PEDF and GDNF promoted higher levels of ZnT2 in the RPE cells. Expression of ZnT3, considered the primary mechanisms of zinc loading in the brain, is increased by PEDF but not by the other neurotrophic factors examined. The increase in zinc uptake in the RPE cells by these factors may be linked to increased expression of these specific transporters in the RPE after treatment with the neurotrophic factors.

Taken together, these studies suggest that the RPE plays a major role in facilitating zinc fluxes in the outer retina. Identifying these zinc transporters in the RPE cells is an important first step toward understanding the relevance of zinc in the eye and the role of the RPE cells in maintaining zinc homeostasis in normal visual function and in retinal diseases.

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