REGULATION OF ZINC TRANSPORT IN THE CHOROID PLEXUS

A Thesis

by

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MASTER OF SCIENCE

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ABSTRACT

The choroid plexus epithelium forms the blood-cerebrospinal fluid barrier, but also accumulates and transports nutritive minerals, such as zinc, into and out of the cerebrospinal fluid. The goal of this thesis was to analyze interdependent regulation of zinc transporters with metallothionein as the choroid plexus epithelium adapts to increases or decreases in extracellular zinc. My first objective was to characterize time-dependent changes in zinc transporter and MT-1 expression as extracellular zinc was pharmacologically depleted or supplemented. My second objective was to characterize changes in zinc transporter and MT-1 expression in response to exposure to prolactin. My experimental approach was to analyze gene expression of ZnT1, Zip1, Zip6, MT-1 and carbonic anhydrase (CA-2) in primary cell cultures of neonatal rat choroid plexus and isolated tissues in which extracellular zinc was depleted with 10 µM diethylene triamine pentaacetic acid or supplemented with 25 µM ZnCl₂ for 48 h. Gene expression was analyzed by fluorescence quantitative real-time polymerase chain reaction.

Zinc accumulation studies indicate choroid plexus cells maintain capacity to accumulate zinc, even when zinc is chelated. In cells, zinc depletion decreased expression of MT-1 and ZnT1 at 3 h and increased Zip1 expression; Zip6 expression fluctuated. In isolated tissues, zinc depletion down-regulated MT-1 and ZnT1 expression, while up-regulating Zip1 and Zip6 expression. In cells, zinc supplementation induced MT-1, ZnT1 and Zip6 expression at 3 h. Zip1 expression decreased at 3 h. In isolated tissues zinc supplementation up-regulated MT-1 and ZnT1
expression, but did not alter Zip1 and Zip6 expression. These data indicate there is coordinated regulation of MT-1 and zinc transporters as extracellular zinc altered. Prolactin up-regulated gene expression of CA-2, MT-1, ZnT-1 and Zip6 in choroid plexus cells. The JAK/STAT inhibitor AG-490 increased CA-2 and MT-1 expression, but decreased ZnT1 and Zip6 expression. AG-490 further increased expression of CA-2 and MT-1 in prolactin treated cells. This suggests the JAK/STAT signaling pathway might tonically suppress basal expression of MT-1 and CA-2. AG-490 partially reversed up-regulation of ZnT-1 and Zip6 expression by prolactin. These data indicate there is a coordinated regulation of MT-1 and zinc transporters during extracellular zinc depletion or supplementation.
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1. INTRODUCTION

This thesis elucidates the regulation of zinc transport in the choroid plexus, which forms the blood-cerebrospinal fluid barrier. There are two primary anatomical barriers that regulate the chemical composition of the extracellular fluid of the brain; these are the blood-brain-barrier and the blood-cerebrospinal fluid (CSF) barrier (22, 29). The blood-brain-barrier consists of the endothelial cells of the brain capillaries. The choroid plexus epithelium forms the blood-CSF barrier, which is separate and distinct from the blood-brain-barrier. There are four choroid plexus tissues, one in each lateral, third and fourth ventricle of the brain. The capillaries of choroid plexus tissue are fenestrated; therefore, the tight junctions between the epithelial cells form the blood-CSF barrier (22, 29). The choroid plexus epithelium produces CSF, which is functionally continuous with the extracellular fluid that bathes the neurons and glia of the brain. The choroid plexus regulates composition of CSF by transporting ions, nutrients, drugs, and metabolites into and out of CSF. The choroid plexus synthesizes and secretes vital macromolecules, such as insulin-like growth factor-II and transthyretin, into CSF. The epithelium expresses receptors for hormones, such as prolactin, progesterone and estrogen (22). Similar to the small intestine, mammary gland and prostate, the choroid plexus is an epithelial tissue that accumulates zinc (30). The choroid plexus accumulates excess nutritive minerals, such as copper and zinc, but also heavy metals, such as cadmium, acting as a ‘sink’ for these metals and minerals. As such, it protects the brain from toxicity of heavy metals and regulates balance of nutrient minerals (37).
Zinc is an essential mineral, and biological and physiological roles of zinc are defined by its chemical characteristics. As a divalent cation zinc readily binds enzymes and proteins (32). In biological systems, zinc serves three main functions: catalytic, structural and regulatory. Because zinc contains a filled $d$ orbital, it does not participate in redox reactions but functions as a Lewis acid to accept electrons (21). As such, zinc is very stable and an ideal cofactor for enzymes that catalyze reactions requiring a redox-stable ion. Members in every class of enzymes require zinc for specific functions, and one prominent example is carbonic anhydrase. Zinc is the essential cofactor for carbonic anhydrase, which is a critical regulator of acid-base balance in the plasma as it catalyzes the reversible hydration of carbon dioxide and converting it to bicarbonate (10, 21). Zinc also facilitates enzyme function by stabilizing enzyme structure as it binds to the active site or amino acid residues (10). An important structural role of zinc is its binding to transcription factors to form zinc fingers, which interact with DNA sequences to regulate gene transcription and thus, gene expression (10). Zinc is also an essential cofactor for certain enzymes, such as copper-zinc superoxide dismutase in which copper serves the catalytic role and zinc provides structural stability (31). Zinc also regulates specific genes such as metallothionein by binding to the metal response element transcription factor (MTF1). This is consistent with the role of metallothionein as a zinc-binding protein that regulates intracellular concentration of free zinc (3, 16).

Zinc is an essential catalytic cofactor for enzymes, such as DNA and RNA polymerases and DNA ligases that mediate DNA replication and cell proliferation and glutamic acid dehydrogenase that is critical for normal central neural function.
Therefore, zinc deficiency may impair brain development and cognitive function (18). Zinc deficiency disrupts neurotransmission, attenuates enzymatic activity and reduces cell proliferation, thus impairing developmental neurogenesis (2, 23, 34). A decrease in zinc availability affects glutathione metabolism in neuronal cells (20). Glutathione (GSH) is an important thiol in the antioxidant defense system in both brain and peripheral cells; thus, zinc deficiency may lead to decreased GSH levels and induction of oxidative stress (23). Zinc deficiency not only compromises central neural function, but also other organ systems. The immune system may be compromised, and growth retardation can occur (25, 26). According to the World Health Organization (WHO), approximately one third of the world population is currently zinc deficient and an additional 17.3% are at risk for inadequate zinc intake (36). Currently, it is estimated approximately 15% of the United States population has inadequate zinc intake (6). Because zinc deficiency is prevalent worldwide, it is important to understand the physiology of zinc and how it enters the cells of the body. I seek to elucidate the adaptation of the choroid plexus to the reduced extracellular availability of zinc.

Cellular accumulation of zinc and intracellular zinc availability is regulated by specific zinc transporters and the zinc-binding protein metallothionein. Zinc transporters are categorized into two gene families, Solute Linked Carrier 30A (SLC30A) and Solute Linked Carrier 39A (SLC39A). The SLC30A family members are also referred to as Zinc Transporters (ZnT); there are 10 identified ZnT zinc transporters, numbered ZnT 1-10 (SLC30A1-10). ZnT zinc transporters are responsible for transporting zinc out of the cytosol or out of cell altogether (i.e., into extracellular compartments) or into
The SLC39A family members are also referred to as Zrt, Irt-like proteins (Zip); there are 14 identified Zip zinc transporters, numbered Zip 1-14 (SLC39A1-14). Zip zinc transporters mediate transport of zinc into the cytosol either from the extracellular space or from subcellular compartments (10). The Zn transporters regulate the total amount of zinc within the cell by transporting zinc into the cell out of cellular compartments when the availability is reduced or transporting zinc out of the cell or into specific subcellular compartments when excess zinc is present. In addition, metallothionein, a metal binding protein containing cysteine residues, also contributes to intracellular regulation of zinc. The thiol groups in the cysteine residues permit metallothionein to bind to metals. Metallothionein gene and protein expression is up-regulated in the presence of increased extracellular zinc and may help regulate intracellular zinc homeostasis by releasing the mineral during decreased availability (9, 16, 28). This is important in managing the intracellular zinc load and maintaining zinc homeostasis in order to avoid intracellular toxicity or deficiency of this essential nutrient. In cells, zinc accumulates in high amounts but the free concentration of zinc is very low at femtomolar $10^{-12}$-15$^{-15}$ M picomolar concentrations (24). Which subset of the 10 ZnT and 14 Zip transporters are expressed in a given cell vary with cell type. Still, for choroid plexus and other tissues known to accumulate zinc and/or regulate compartmentation of zinc in different extracellular fluid compartments, it is unclear which ZnTs and Zips are expressed and what is their intracellular location.
Few studies have analyzed the accumulation and transport of zinc in the choroid plexus but only a limited number of specific zinc transporters have been identified in the rodent choroid plexus. Figure 1 shows the current model of zinc transporter expression in rodent choroid plexus based on current, albeit limited literature on zinc transport in this epithelium. Zip6 has been localized at the apical membrane in the rat choroid plexus, thus mediating zinc uptake from CSF and into the cytosol of the cell (5). Expression of both Zip1 mRNA and Zip4 mRNA has been demonstrated in rat choroid plexus, but cellular localization of these transporters has not yet been determined (1). Additionally, ZnT1, ZnT4 and ZnT6 are expressed within intracellular compartments in mouse choroid plexus, and ZnT7 is localized specifically in the golgi apparati (4, 5, 35). These ZnT transporters would mediate Zn removal from the cytosol into subcellular compartments or Golgi apparatus (11, 19, 35). ZnT3 has been localized specifically in the apical membrane of the choroid plexus in mice and would thus mediate Zn efflux from the cytosol into CSF (35). Nevertheless, current data for ZnT and Zip localization in choroid plexus are not altogether consistent with the total intracellular Zn accumulation in a Zn adequate state and cannot explain reported adaptations of choroid plexus to changes in Zn intake.
Figure 1: Current model for zinc transport in the rodent choroid plexus based on published literature. Zip6 has been localized at the apical membrane, thus mediating zinc uptake from CSF and into the cytosol. ZnT1, ZnT4, ZnT6 and ZnT7 have been localized intracellularly, with ZnT7 localized specifically in the golgi apparatus; these transporters would mediate Zn transport from cytosol into subcellular compartments or Golgi apparatus. ZnT3 has been localized specifically at the apical membrane, thus mediating Zn efflux from the cytosol into CSF (1, 4, 5, 35).
In the choroid plexus of zinc deficient rats, zinc still may be accumulated in high amounts. However, based on the current model it is unclear how zinc is transported and accumulated in the choroid plexus. Figure 2 shows the proposed model of zinc transport expression in choroid plexus. I propose that zinc importers, Zip1 and Zip4 are localized at the basolateral membrane and mediate zinc uptake from blood, and the Zn exporter, ZnT1, is localized at the plasma membrane and mediates zinc efflux from the cytosol into blood. Chowanadisai et al. observed that zinc concentrations in brains of neonatal rats from dams fed diets marginally low (10 mg Zn/kg) or deficient in zinc (7 mg Zn/kg) were comparable to those in brains of neonatal rats from dams fed control diets (25 mg Zn/kg); however, zinc deficient diets did decrease plasma zinc concentrations (5). Also, the brains of neonatal rats from dams fed zinc deficient diets had a greater capacity to accumulate zinc as compared to controls. However, in the current model for zinc transport in the choroid plexus, Zip6 is the only zinc importer localized at the plasma (apical) membrane; this would indicate zinc is accumulated at the apical membrane from CSF. CSF, however, is a limited source of zinc. Instead, it is more likely the choroid plexus would accumulate zinc at the basolateral membrane from blood, a richer source of zinc, to maintain optimal CSF zinc concentrations during periods of normal and reduced zinc availability. Thus, as depicted in Figure 2, I propose that zinc importers, Zip1 and Zip4 and Zn exporter, ZnT1, are localized at the basolateral membrane. An additional discrepancy is that Chowanadisai et al. also observed in the brains of neonatal rats of dams fed zinc deficient diets ZnT1 mRNA and MT-1 mRNA expression decreased while Zip6 mRNA increased (5).
Figure 2: Proposed model for zinc transport in the choroid plexus. Zip1 and Zip4 are localized at the basolateral membrane and mediate zinc uptake from the blood into the cytosol. ZnT1 is localized at the basolateral membrane, mediating zinc efflux from the cytosol and into the blood.
Zinc transporter expression may be regulated by select hormones, such as estrogen and prolactin (22). Whether ZnT or Zip zinc transporters are regulated by these or other hormones has not been investigated. Like the choroid plexus, mammary and prostate epithelial tissues accumulate zinc to high levels, and transport zinc into breast milk and seminal fluid, respectively. In both mammary and prostate epithelia the peptide hormone, prolactin, regulates zinc transport (15). In murine mammary epithelial cells, Zip3 is localized to the basolateral membrane and likely facilitates zinc uptake into the mammary epithelial cell, whereas ZnT2 is localized to the apical membrane and mediates efflux zinc into milk (14, 27). In HC11 mammary cells treated with 1 µg/ml prolactin, ZnT2 mRNA increased as early as 8 hours and continued to increase through 24 hours (27). Similarly, in immortalized PC-3 prostate cells, pretreatment with prolactin (30 nM, 18 hours) stimulated 60-minute zinc accumulation by 33% as compared to non-treated control cells (7). The choroid plexus expresses an abundance of prolactin receptors and accumulates zinc in high amounts (22). However, the potential for prolactin to regulate zinc transporter or metallothionein expression has not been investigated.

The overall goal of this thesis project is to analyze the interdependent regulation of zinc transporters with the zinc-binding protein metallothionein as the choroid plexus epithelium adapts to increases or decreases in extracellular zinc. The two specific objectives are as follows. The first objective is to characterize the time-dependent changes in zinc transporter and metallothionein-1 (MT-1) gene expression in cultured choroid plexus as extracellular zinc is pharmacologically depleted or supplemented. I
I hypothesize that in response to pharmacological depletion of extracellular zinc, ZnT1 and MT-1 gene expression will decrease, while Zip1 and Zip6 gene expression will increase. *I hypothesize* that in response to pharmacological supplementation of extracellular zinc, ZnT1 and MT-1 gene expression will increase, while Zip1 and Zip6 gene expression will decrease. **The second objective** is to characterize the changes in zinc transporter and MT-1 gene expression in cultured choroid plexus in response to *in vitro* exposure to prolactin. *I hypothesize* that *in vitro* exposure to prolactin will induce gene expression of MT-1, ZnT1, Zip1 and Zip6 in the choroid plexus of primary cultures.
2. MATERIALS AND METHODS

2.1 Animal and tissue harvest

For preparation of primary cultures of choroid plexus epithelia, choroid plexus tissues were harvested from 2-3 day old Sprague-Dawley rats. Neonatal rats were obtained from timed pregnant dams that were purchased from an approved commercial vendor (Charles River Laboratories, Spencerville, OH) and arrived at the university vivarium at gestational day 16; dams had free access to food and water. With approval from the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University (Protocol #2011-128), choroid plexus tissues were harvested from the brains of neonatal rats using ethanol-sterilized instruments. Rats were decapitated just above shoulders, leaving cerebellum and brain stem intact. The brain was removed and placed on sterile filter paper for dissection of lateral and fourth choroid plexus. Lateral and fourth choroid plexus tissues were removed from 36-50 individual animals, pooled and held in chilled collection media.

In vitro experiments were performed in choroid plexus tissues isolated from adult and neonatal rats. Individual adult rats were asphyxiated with compressed CO₂ gas dispensed with a regulator. Following decapitation, the brain was removed and lateral and fourth choroid plexus tissues were harvested. The fourth and lateral choroid plexus tissues were collected in penicillin-supplemented DMEM/F12. Neonatal rat choroid plexus tissues were harvested as described above and collected in penicillin-supplemented DMEM/F12. Both adult and neonatal tissues were thoroughly rinsed in
artificial cerebrospinal fluid supplemented with penicillin before placement in experimental media.

2.2 Reagents

Tissue collection medium consisted of DMEM/F12 (Sigma, St. Louis, MO) and penicillin (100 U/mL; Calbiochem-EMD Millipore, Billerica, MA). Dissociation buffer contained 137 mM NaCl, 2.7 mM KCl, 0.7 mM Na₂HPO₄, 5.6 mM glucose and 10 mM HEPES (pH 7.4) and 5 U/mL protease (Sigma, St. Louis, MO) and 1,500 kU/mL DNase I (Calbiochem-EMD Millipore, Billerica, MA). Cells were preplated in penicillin-supplemented DMEM/F12 with 10% Nu-Serum IV (BD Biosciences, San Jose, CA). Initial plating medium consisted of minimum essential medium with d-valine substituted for l-valine (U.S. Biological, Swampscott, MA) with 10% Nu-Serum IV, 1.5 μM triiodo-l-thyronine, 50 ng/mL epidermal growth factor, 100 ng/mL prostaglandin E₁ and 10 μM forskolin; all growth supplements were of tissue culture grade and purchased from Sigma (St. Louis, MO). Cells were maintained in DMEM/F12 with 5% Nu-Serum IV and the growth supplements listed above at the same concentrations. Plating and maintenance media did not contain antibiotics or fungicides. Treatment medium consisted of DMEM/F12, but contained neither serum nor growth supplements. Stock solutions of approximately 4 mM zinc chloride (Sigma, St. Louis, MO) were prepared in 25 mL sterile ultra pure water; aliquots were added to serum-free DMEM/F12 to a final concentration of 10 or 25 μM for experimental in vitro treatment of cells or tissues. Stock solutions of approximately 10 mM diethylene triamine pentaacetic acid (DTPA;
TCI, Philadelphia, PA) were prepared in 10 mL of ultra pure water containing 0.5 M sodium hydroxide (NaOH); aliquots were added to serum free DMEM/F12 to a final concentration of 10, 25, or 50 µM for experimental *in vitro* treatment of cells or tissues. Stock solutions of approximately 325 µM prolactin (Sigma, St. Louis, MO) were prepared in 929 µL of ultra pure water; aliquots were added to serum free DMEM/F12 to a final concentration of 1000 nM, 100 nM, 10 nM, or 1 nM for experimental *in vitro* treatment of cells or tissues. Stock solutions of approximately 100 mM AG-490, a JAK/STAT inhibitor, (Calbiochem-EMD Millipore, San Diego, CA) were prepared in 170 µL of dimethyl sulfoxide (DMSO), and AG-490 was further diluted to 10 mM prepared in 27 µL of DMSO; aliquots were added to serum-free DMEM/F12 to a final concentration of 10 µM for experimental *in vitro* treatment of cells or tissues. Reagents used specifically for analyses of gene expression and protein expression are described in their respective section.

All chemicals were of analytical grade and purchased from commercial vendors. The following specific reagents were purchased from the respective vendors: zinc chloride (Sigma, St. Louis, MO); DTPA (TCI, Philadelphia, PA); tetrakis-(2 pyridylmethyl) ethylenediamine (Calbiochem-EMD Millipore, San Diego, CA); prolactin (Sigma St. Louis, MO); AG-490, (Calbiochem-EMD Millipore, San Diego, CA). Primers were designed by Primer Express software (PE Applied Biosystems) and purchased from DNA Technologies (Coralville, IA) and Qiagen (Foster City, CA).
2.3  *Choroid plexus epithelial cell isolation and primary culture*

Epithelial cells were dispersed from neonatal rat choroid plexus tissues following the protocol described previously with minor modifications (33). Briefly, tissues were suspended in dissociation buffer (10 mM HEPES, pH 7.4) with 5 U/mL protease and 1,500 kU/mL DNase I. The tissue-enzyme suspension was shaken at 37°C and triturated intermittently with fire polished pipettes over a 20-min period. Aliquots of released cells were filtered through a sterile nylon cell strainer (100 µm; BD Biosciences, San Jose, CA) with intermittent rinsing of the filter with penicillin-supplemented DMEM/F12-10% Nu-Serum IV to facilitate filtration of cells and dilute the enzyme concentration in the filtrate by 10- to 12-fold. The cell suspension was centrifuged and washed once with a generous volume of penicillin-supplemented DMEM/F12. Cells then were suspended in penicillin-supplemented DMEM/F12-10% Nu-Serum IV and pre-plated in a single 35-mm Petri dish for 3.5 h in a humidified atmosphere (37°C, 95% air-5% CO2). During the pre-plating period, fibroblasts rapidly attach to the plating surface such that most unattached cells are epithelial cells. Unattached cells then were collected, centrifuged, and suspended in plating medium consisting of a mixture of antibiotic-free minimum essential medium with d-valine substituted for l-valine (a nutritional modification that starves additional fibroblasts), 10% Nu-Serum IV and growth promoters (1.5 µM triiodo-l-thyronine; 50 ng/mL epidermal growth factor; 100 ng/mL prostaglandin E1; 10 µM forskolin). Cells were plated at a density of 3 x 10^5 cells/cm² on impermeable supports, e.g., polystyrene tissue culture plates or glass chambered-coverslips, and maintained at 37°C (humidified 95% air-5% CO2). Seventy-two hours post-plating,
unattached cells were removed, and the initial plating medium was replaced with maintenance medium (DMEM/F12 with 5% Nu-Serum IV and growth promoters but no antibiotics). Thereafter, medium was changed every 2-3 days. Cells grew to confluence as a differentiated monolayer within 6 days, and experiments were conducted 6-9 days post-plating.

2.4 In vitro supplementation of extracellular zinc or depletion with diethylenetriaminepentaacetic acid (DTPA) in primary cell cultures of choroid plexus and isolated choroid plexus tissues

Cells were plated in 12-well culture plates. Cells were incubated in serum-free DMEM/F12 medium 12-16 h prior to start of experimental treatment. Cells were treated with serum free DMEM/F12 that contained no agent (control), 25 µM ZnCl₂ (supplementation), 10 µM DTPA (extracellular zinc depletion) for up to 48 hours at 37°C (95% air-5% CO₂; humidified air). Neonatal choroid plexus tissues were incubated in 12-well plates with 1 ml DMEM/F12 and treated with DMEM/F12 with no agent (control), with 25 µM ZnCl₂ (supplementation) or 10 µM DTPA (depletion) for 24 hours. Additional test reagents were added to treatment media as needed. At the end of treatment, control and experimental cells and tissues were collected and processed for assay of specific parameters.
2.5 Analysis of gene expression by Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from cultured choroid plexus cells or isolated choroid plexus tissues was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). After treatment cells were rinsed briefly with phosphate buffered saline (PBS). Per manufacturer’s instructions, cells were disrupted in 500 μL of the provided RLT lysis buffer with β-mercaptoethanol (β-ME; 10 μL per 1 ml RLT lysis buffer), and the suspension was homogenized by centrifugation (maximum RPM, 2 min) through a QIAshredder™ column. Homogenates were cleared by a 1:1 dilution in 70% EtOH and applied to the RNeasy Spin Column on which each was treated with DNase (Qiagen, Valencia, CA) before final elution in RNase-free water. Total RNA was extracted from intact tissue in a similar manner with the following minor modifications. After treatment, tissues were transferred to 1500 μL microtubes and rinsed with 500 μl of PBS by inversion then centrifuged (10,000 RPM, 30 sec). PBS was removed, and tissues were triturated in 600 μl of RLT/β-ME prior to homogenization by centrifugation through a QIAshredder™ (maximum RPM, 2 minutes).

Samples of total RNA were evaluated for quality and integrity as follows. Using a Nanodrop 1000 nanospectrophotometer, the Abs260 nm:Abs280 nm ratio was determined to assess the purity of RNA; a ratio of ~2.0 is an acceptable index of pure RNA, whereas values less than 2.0 indicate unacceptable contamination by DNA and protein or other contaminants. The Abs260 nm:Abs230 nm ratio was determined as a secondary assessment of nucleic acid purity; the values were typically in the range of
values below this range may indicate contamination. Integrity of RNA was assessed using an Agilent 2100 Bioanalyzer (Santa Clara, CA) and an Agilent RNA 6000 Nano Kit (Santa Clara, CA). First-strand cDNA was synthesized from samples with RNA integrity numbers >8 using Superscript III First-Strand Synthesis System SuperMix (Invitrogen, Carlsbad, CA) with 5x iScript reaction mix and iScript reverse transcriptase. For each sample, a 5-µL aliquot of cDNA was amplified by qRT-PCR using forward and reverse primers against each gene of interest, as well as β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Gene expression in each cDNA sample was analyzed in triplicate by qRT-PCR with SYBR Green® detection (iQ™ SYBR® Green Supermix, BioRad, Hercules, CA) in a single-color real-time detection system (BioRad MyQ, Hercules, CA). Initial Denaturation: 10 min, 95°C; Amplification/Quantification (45 cycles): 15 sec, 95°C; 30 sec, 60°C; Melt Curve: 55°C-95°C. Copy number for each gene of interest was determined and normalized to those of β-actin and GAPDH. Primers for rat metallothionein-1 and carbonic anhydrase-2 and zinc transporters, ZnT1 and Zip6 were designed by Primer Express software (PE Applied Biosystems) based on GenBank registered sequences for rat β-actin, NM_031144; GAPDH, NM_017008; MT-I, NM_138826; CA-2, NM_019291.1; ZnT1/Sle301, NM_022853.1; ZIP6/Sle39a6, NM_001106708.1 and synthesized by DNA Technologies (Coralville, IA). Primer sequences are listed in Table 1. Primers for rat ZIP1 were obtained from Qiagen (Foster City, CA).
Table 1: Forward and reverse primer sequences used to analyze expression of genes that encode for rat MT-1, CA-2 and zinc transporters, ZnT1 and ZIP6 are listed as well as gene name and respective PubMed GeneBank ID number.

<table>
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<th>Gene</th>
<th>GeneBank ID</th>
<th>Forward Primer (5’ – 3’)</th>
<th>Reverse Primer (3’ – 5’)</th>
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<td>TGAGTGAGCCAGGAAGA</td>
<td>CAGACAAGACCGACATCAA</td>
</tr>
<tr>
<td>ZnT1</td>
<td>NM_022853</td>
<td>CCATTCTGGAAAGGAGGCA</td>
<td>TGATTCGGGCTGTTGTTTGTG</td>
</tr>
</tbody>
</table>
2.6 Analysis of intracellular accumulation of elemental zinc by inductively coupled plasma mass spectrometry (ICP-MS)

Total intracellular accumulation of elemental zinc was determined by inductively coupled plasma-mass spectrometry (ICP-MS). Cells were plated in 12-well culture plates and incubated with 1 mL experimental medium per well. To account for background levels of zinc, 1 mL of DMEM/F12 was placed in wells with no cells. After treatment, all experimental media were collected into pre-weighed (± 0.1 mg) 15 mL polyethylene tubes. All wells were rinsed twice with phosphate buffered saline (PBS) with 1 mM ethylenediaminetetraacetic acid (EDTA, a chelating agent that removes extracellular divalent cations), then once with PBS alone. Cells were air-dried for 48 hours. 200 μL of concentrated nitric acid was added to each treatment well and each blank well to solubilize all metals and minerals, as well cellular material. The nitric acid mixture from an individual well was collected and transferred to a pre-weighed (± 0.1 mg) 15 mL tube. The well was rinsed 3 times with ultra pure water, and each rinse was collected and placed in the same tube as the nitric acid mixture in the collection tube. Additional ultra pure water was dispensed into each tube, bringing the final total volume to approximately 10 mL, and the final weight was recorded (± 0.1 mg). The final nitric acid concentration was 0.02% (200 μL/10,000 μL). The samples were then analyzed by ICP-MS by Dr. Robert J. Taylor of the Trace Element Research Laboratory in the College of Veterinary Medicine at Texas A&M University.
2.7  *Fluorescent immunocytochemical staining for metallothionein-1 (MT-1)*

Cells were plated in 4-well chamber slides and incubated with 400 µL experimental medium. At the end of treatment, cells were cleared of experimental medium, rinsed briefly in phosphate buffered saline (PBS) and then fixed in 3.7% formaldehyde/0.1% Triton X-100/PBS at room temperature for 10 minutes while rocking. Cells were then rinsed twice with PBS prior to immersion in cold acetone (3 min, -20°C); the latter treatment permeabilizes lipid membranes and improves antibody penetration into the cell and subcellular compartments. Cells were rinsed twice in PBS, and then incubated with 1% bovine serum albumin (BSA) in PBS for 30 minutes while rocking to minimize non-specific binding of primary antibody. Cells were subsequently incubated with mouse monoclonal anti-MT-1 (ENZO, Plymouth Meeting, PA; 1:200 dilution in 1% BSA/PBS) for 1 hour while rocking. Cells were rinsed in 1% BSA/PBS, then incubated with Alexa Fluor® 350 goat anti-mouse IgG (Invitrogen, Eugene, OR) at 1:200 dilution in 1% BSA/PBS for 30 minutes while rocking. To correct for background fluorescence due to non-specific binding of secondary antibody, representative cells were incubated with only secondary antibody and no primary antibody. Cells then were rinsed in PBS. Cover slips were mounted with Prolong® Gold antifade reagent with 4', 6-diamidino-2-phenylindole (DAPI; Molecular Probes-Invitrogen, Eugene, OR) to counterstain nuclei. Cells were viewed on an inverted epi-fluorescence microscope (Zeiss Axiovert 200, Munich, Germany) fitted with 20X and 63X (oil immersion) objectives and FITC and UV filters and illuminated by a mercury lamp. Digital images were captured with Axiovision 4.7 software (Zeiss, Munich, Germany).
2.8 *Statistical analysis*

Results shown are expressed as means ± SE, except where noted. Control and experimental means from experiments that investigated the possible differences in zinc accumulation in DTPA-treated cells or zinc-supplemented cells *versus* that in control cells were compared by one-way ANOVA with a Tukey-Kramer post hoc test. Control and experimental means from experiments that evaluated time-dependent effects of DTPA treatment and extracellular zinc supplementation on gene expression in cultured choroid plexus cells were first compared by two-way analysis of variance (ANOVA) or ANOVA with the appropriate post hoc test. If significant differences in the main effect of treatment was determined, then a t-test was performed to compare control and experimental means at each time point to identify differences between control and DTPA treatment groups or between control and zinc-supplemented groups. Differences were deemed significant at $P \leq 0.05$. 
3. RESULTS

3.1 Experimental approach

Time-dependent changes in zinc transporter and metallothionein (MT-1) gene expression elicited by depletion or supplementation of extracellular zinc were characterized in vitro in primary cultures of choroid plexus epithelial cells and isolated segments of choroid plexus harvested from neonatal rats. My experimental approach was to analyze time-dependent gene expression of specific zinc transporters, i.e., ZnT1, ZIP1 and ZIP6, the zinc-binding protein, MT-1 and the zinc-dependent enzyme, carbonic anhydrase-2 (CA-2) in primary cultures of choroid plexus in which extracellular zinc was depleted or supplemented. Because carbonic anhydrase is a zinc-dependent enzyme that is critical for production of CSF, analysis of CA-2 gene expression may lend insight into how other aspects of choroid plexus biology might be altered by extracellular zinc depletion or supplementation. Experiments were performed in primary cultures of neonatal rat choroid plexus epithelium, which permits discrete and direct access to the epithelium. Cells were incubated in serum-free DMEM/F12 medium 12-16 h prior to start of experimental treatment; this would minimize the influence of the various growth factors used to promote cell differentiation and proliferation on gene expression. Cells were then treated with serum-free DMEM/F12 without any test agent (i.e., Control), 10 μM of the cell impermeant zinc chelator diethylene triamine pentaacetic acid (DTPA) (i.e., extracellular zinc depletion), or 25 μM ZnCl₂ (i.e., extracellular zinc supplementation) for up to 48 hours. Changes in gene expression were analyzed in control cells and cells in which extracellular zinc was depleted or
supplemented for both a short time course with time intervals of 3 h, 6 h, 9 h and 12 h and a long time course with time intervals of 12 h, 18 h, 24 h and 48 h. Neither supplementation with 25 μM zinc chloride (ZnCl₂) or depletion of extracellular zinc with 10 μM DTPA was toxic, permitting evaluation of cell adaptation rather than cytotoxicity. Control, DTPA-treated, and zinc-supplemented cells were collected at various timed intervals, and total RNA was extracted. mRNA levels of the genes of interest (CA-2, MT-1, ZnT-1, Zip1, Zip6) and β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were analyzed by fluorescence quantitative real-time polymerase chain reaction (qRT-PCR; SYBR Green® detection). Expression of each gene was normalized to that of β-actin and GAPDH gene expression; expression of each gene of interest in treated cells was compared to expression in the time-matched controls.

3.2 Cellular accumulation of zinc in cultured choroid plexus cells treated with extracellular zinc chelator or supplemented with zinc

To determine whether pharmacological depletion of extracellular zinc, i.e., treatment with the DTPA, or supplementation of extracellular medium with zinc altered total cellular accumulation of zinc, cellular content of elemental zinc was measured by inductively coupled plasma-mass spectrometry (ICP-MS; Trace Element Research Laboratory TAMU). Evaluation of possible changes in cellular accumulation of zinc would lend insight into the implications of changes in zinc transporter and MT-1 gene expression. Cells were incubated in serum-free medium for 48 h without zinc or DTPA (control) or with 10 μM DTPA or 25 μM ZnCl₂. Total accumulation of elemental zinc
was normalized to total cellular protein (n = 2 separate culture preparations). Cells accumulated zinc in each condition. Total zinc accumulation in control cells was 0.903 ± SD 0.042 ng/mg cellular protein. Cellular accumulation of zinc was not markedly altered after 48-h depletion or supplementation of extracellular zinc. In DTPA-treated cells total zinc accumulation was 0.856 ± SD 0.041 ng/mg cellular protein; in zinc-supplemented cells, zinc accumulation was 0.898 ± SD 0.036 ng/mg cellular protein.

3.3 Effects of pharmacological depletion of extracellular zinc on gene expression of CA-2, MT-1, and zinc transporters in cultured choroid plexus cells

Cells were treated without (control) or with 10 µM DTPA for 12 hours, and both representative DTPA-treated and time-matched control cells were collected at 3 h, 6 h, 9 h, and 12 h (Figure 3C, D). CA-2 gene expression in DTPA-treated cells was similar to that in control cells through 9 h; at 12 h CA-2 expression increased by 34% as compared to time-matched control but was not significant (p = 0.088). MT-1 expression was induced 1.2-fold at 3 h, but by 6 h and 9 h had decreased to 30% of control values (p < 0.03) and further decreased at 12 h to 10% of control values (p < 0.0005). Gene expression of the zinc exporter ZnT1 decreased by 70% at 3 h and remained low up through 12 h as compared to control. Gene expression of the zinc importer Zip1 was comparable to that in time-matched controls through 9 h; at 12 h mean Zip1 gene expression was 60% greater than in controls (p < 0.17). Similarly, gene expression of the zinc importer, Zip6, in DTPA-treated cells was significantly greater than controls by 10% and 30% at 6 h and 12 h respectively in control cells (p < 0.05).
Similar changes in CA-2, MT-1, ZnT1, Zip1 and Zip6 gene expression were observed during extended depletion of extracellular zinc with 10 µM DTPA for up to 48 h; representative DTPA-treated cells and time-matched control cells were collected at 12 h, 18 h, 24 h and 48 h (Figure 4C, D). Throughout the extended DTPA treatment CA-2 gene expression was comparable to that in controls from 12 h through 24 h, but at 48 h was slightly greater than controls (p < 0.03). In DTPA-treated cells, MT-1 expression was again markedly decreased by 90% at 12 h and remained low through 48 h (p < 0.007); in some cases MT-1 expression was decreased by 98% as compared to controls. Likewise, as compared to control, DTPA treatment decreased ZnT1 expression by 70% at 12 h through 18 h, and ZnT1 expression remained suppressed by approximately 50% at 24 h and 48 h (p < 0.02). Extended DPTA treatment also elicited changes in zinc importer gene expression. Zip1 expression fluctuated somewhat; expression decreased slightly at 12 h (p < 0.40), returned to control values at 18 h, but then decreased by 50% at 24 h (p < 0.03) and returned to control values by 48 h (p < 0.12). DTPA increased Zip6 expression by 50% at 12 h as compared to controls, (p < 0.04); however, expression levels were comparable to controls at 18 h and 48 h (p < 0.23).

3.4 Effects of direct supplementation of extracellular zinc on gene expression of CA-2, MT-1, and zinc transporters in cultured choroid plexus cells

The short term regulation of gene expression was examined in cultured choroid plexus cells treated without (control) or with 25 µM ZnCl₂ for 12 hours; zinc-supplemented cells and time-matched control cells were collected at 3 h, 6 h, 9 h, and 12 h (Figure 3A, B). As compared to controls, extracellular zinc supplementation did not
significantly alter CA-2 expression over the 12-h treatment period (p > 0.10). However, as compared to controls, MT-1 expression was induced 6-fold within 3 h of the initiation of zinc supplementation (p < 0.03), and remained elevated through 12 h. Zinc supplementation also increased gene expression of ZnT1 by 2-fold within 3 h as compared to controls and expression remained elevated through 12 h (p < 0.05). In contrast, significant changes in gene expression of zinc importers, Zip1 and Zip6, were not observed over the 12-h zinc supplementation period.

Similar changes in CA-2, MT-1, ZnT1, Zip1 and Zip6 gene expression were observed during the extended 48-h supplementation of with 25 µM ZnCl₂; representative zinc-supplemented cells and time-matched control cells were collected at 12 h, 18 h, 24 h and 48 h (Figure 4A, B). CA-2 expression was comparable to controls through 24 h, but at 48 h expression was approximately 33% greater than controls (p < 0.02). MT-1 expression also increased with extended zinc supplementation, peaking at 12 h through 18 h, but remaining markedly greater than controls by 8 and 3-fold at 24 h and 48 h respectively. Similarly, zinc supplementation induced gene expression of ZnT1 by approximately 50% at 12 h through 24 h (p < 0.0001) and at 48 h, ZnT1 expression was 2-fold greater than controls (p < 0.11). With extended zinc supplementation, Zip1 expression decreased by 40% as compared to control at 12 h (p < 0.004); however, at 18 h through 48 h Zip1 expression in zinc-supplemented cells was comparable to controls (p > 0.10). Zinc supplementation also increased Zip6 expression 40% at 12 h as compared to control (p < 0.007), but thereafter expression was comparable to controls at 18 h through 48 h (p > 0.40).
Figure 3. Time-dependent changes on gene expression of metallothionein-1 (MT-1), carbonic anhydrase-2 (CA-2) and zinc transporters ZnT1, Zip1 and Zip6 in primary cultures of neonatal rat choroid plexus epithelial cells during 12-hour direct supplementation of extracellular zinc (A, B) and pharmacological depletion of extracellular zinc with a cell impermeable zinc chelator (C, D). Cells were plated in 12-well culture plates. Experiments were conducted on confluent differentiated monolayers 7-9 days post plating. Cells were incubated in serum-free DMEM/F12 medium 12-16 h prior to start of experimental treatments. Cells were treated for 12 h with serum free DMEM/F12 that contained no agent (control, Ctrl), 25 µM ZnCl2 (supplementation; A, B), or 10 µM diethylene triamine pentaacetic acid (DTPA; depletion; C, D). Representative non-treated (Control), zinc-supplemented and DTPA-treated cells were collected at 3 h, 6 h, 9 h, and 12 h. For all samples, expression of genes encoding for MT-1, CA-2, ZnT1, Zip1 and Zip6 were normalized to expression of gene encoding for β–actin and GAPDH. Gene induction was expressed as fold-change versus respective time-matched control, as calculated by dividing normalized gene expression in experimental sample by normalized gene expression in the respective control sample. Data are the means for fold-induction at each time point as determined in 3 separate primary culture preparations, i.e., N=3. * The asterisk indicates significant difference as compared to time-matched control; p<0.05.
Figure 4. Time-dependent changes on gene expression of metallothionein-1 (MT-1), carbonic anhydrase-2 (CA-2) and zinc transporters ZnT1, Zip1 and Zip6 in primary cultures of neonatal rat choroid plexus epithelial cells during 48-hour direct supplementation of extracellular zinc (A, B) and pharmacological depletion of extracellular zinc with a cell impermeable zinc chelator (C, D). Cells were plated in 12-well culture plates, and experiments were conducted on confluent differentiated monolayers 7-9 days post plating. Cells were incubated in serum-free DMEM/F12 medium 12-16 h prior to start of experimental treatments. Cells were treated for 48 h with serum free DMEM/F12 that contained no agent (control, Ctrl), 25 µM ZnCl2 (supplementation; A, B), or 10 µM diethylene triamine pentaacetic acid (DTPA; depletion; C, D). Representative non-treated (Control), zinc-supplemented and DTPA-treated cells were collected at 12 h, 18 h, 24 h, and 48 h. For all samples, expression of genes encoding for MT-1, CA-2, ZnT1, Zip1 and Zip6 were normalized to expression of gene encoding for β-actin and GAPDH. For conditions of extracellular zinc supplementation or depletion, gene induction was expressed as fold-change versus control, as calculated by dividing normalized gene expression in experimental sample by normalized gene expression in the respective control sample. Data are the means for fold-induction at each time point as determined in 3 separate primary culture preparations, i.e., N=3. * The asterisk indicates significant difference as compared to time-matched control; p<0.05.
3.5 *Protein expression of metallothionein-1 in zinc-depleted or supplemented choroid plexus cells*

Cultured cells were plated in chamber slides and incubated for 48 h in serum-free medium alone (control), with 10 µM DTPA, or with 25 µM ZnCl₂. Cells were then fixed and immunostained for MT-1 protein using a fluorescent secondary antibody (green); cell nuclei were stained with DAPI (blue) (Figure 5). In control cells, there was a diffuse fluorescence in the cytosol with marked punctate fluorescence. In DTPA-treated cells, the diffuse cytosolic fluorescence was less intense than in control cells; punctate fluorescence also was less pronounced. In zinc-supplemented cells, both diffuse and punctate fluorescent was more intense that in control cells. These imaging data indicate that metallothionein protein expression decreased in response to DTPA treatment and is increased in response to zinc supplementation; this was consistent with the observed suppression and induction of MT-1 gene expression by the respective manipulations of extracellular zinc.
Figure 5. Fluorescent immunocytochemical staining for metallothionein-1 (MT-1) in primary cultures of neonatal rat choroid plexus epithelial cells during 48-hour direct supplementation of extracellular zinc and pharmacological depletion of extracellular zinc with a cell impermeable zinc chelator. Cultured cells were plated in chamber slides, and incubated for 48 h in serum-free medium alone (control; A1, 2), with 25 µM ZnCl₂ (B1, 2) or with 10 µM DTPA (C1, 2). Cells were then fixed and immunostained for MT-1 protein using a fluorescent secondary antibody (green); cell nuclei were stained with DAPI (blue) and fitted with 20X (A1, B1, C1) and 63X (A2, B2, C2) objectives.
3.6  Effects of in vitro pharmacological depletion and supplementation of extracellular zinc on gene expression of CA-2, MT-1, and zinc transporters in isolated choroid plexus tissues

To determine whether gene expression of zinc transporters and metallothionein might be regulated in response to changes in extracellular zinc in the intact choroid plexus epithelial tissue as they were in primary cultures of choroid plexus epithelial cells, gene expression was compared in isolated neonatal rat choroid plexus tissues subjected to similar manipulations simulating extracellular zinc depletion and supplementation. Both lateral and IVth choroid plexus tissues from 3 animals were pooled and incubated for 24 h in serum-free DMEM/F12 with 10 µM DTPA or with 25 µM ZnCl₂ (n = 3-5 sets of pooled tissue per each condition). At the end of treatment, total RNA was extracted, and mRNA levels of CA-2, MT-1, ZnT1, Zip1, Zip6, β-actin, and GAPDH were analyzed by fluorescence qRT-PCR (Figure 6A, B). In each control and treated tissue sample, expression of each test gene was normalized to that of β-actin and GAPDH; however, gene expression values in treated tissues were not subsequently normalized to those in control tissues. Although CA-2 expression in DTPA-treated tissues was comparable to CA-2 expression in control tissues (p > 0.3), metallothionein and zinc transporter gene expression levels were markedly different.
Mean MT-1 expression in DTPA-treated tissues was approximately 93% less than that in control tissues (0.015 ± SE 0.0007 vs. 0.561 ± SE 0.004, p < 0.007). Likewise, ZnT1 expression in DTPA tissues was approximately 50% less than that in control tissues (0.341 ± SE 0.062 vs. 0.688 ± SE 0.004, p < 0.005). Concurrently, gene expression of zinc importers increased in DTPA-treated tissues. Mean Zip1 expression in DTPA-treated tissues was 65% greater than that in control tissues (1.676 ± SE 0.270 vs. 1.022 ± 0.078, p < 0.04), while mean Zip6 expression was approximately twice that in control tissues (1.374 ± SE 0.093 vs. 0.602 ± SE 0.077, p < 0.0004). Zinc supplementation did not alter CA-2 expression as compared to control values (p > 0.23), but doubled mean MT-1 expression (Zinc: 1.162 ± SE 0.174 vs. Control: 0.561 ± SE 0.004, p < 0.03) and increased ZnT1 expression by 85% (Zinc: 1.280 ± SE 0.167 vs. Control: 0.688 ± SE 0.004; p < 0.02). Zinc supplementation did not significantly alter Zip1 or Zip6 expression as compared to controls (Zip1, Zinc: 1.150 ± SE 0.053 vs. Control: 1.011 ± SE 0.078, p > 0.093; Zip6-Zinc: 0.841 ± SE 0.185 vs. Control: 0.602 ± SE 0.077, p > 0.142).
Figure 6. Effects of pharmacological depletion of extracellular zinc or zinc supplementation on gene expression of metallothionein-1 (MT-1) and carbonic anhydrase-2 (CA-2) (A) or zinc transporters ZnT1, Zip 1 and Zip6 (B) in isolated primary cultures of neonatal rat choroid plexus tissues. Neonatal rat choroid plexus tissues (lateral and IVth) were harvested and pooled from 3 animals and treated for 24 h with serum free DMEM/F12 that contained no agent (control, Ctrl), 25 μM ZnCl₂ (supplementation), or 10 μM diethylene triamine pentaacetic acid (DTPA). For all samples, expression of genes encoding for MT-1, CA-2, ZnT1, ZIP1 and ZIP6 were normalized to expression of gene encoding for β−actin and GAPDH. For conditions of extracellular zinc supplementation or depletion, gene induction was expressed as the normalized gene expression in experimental sample without normalizing gene expression to the control sample. Data are expressed in triplicate measures. Data are the fold-induction as determined in 3 sets of pooled tissues, i.e., N=3. *The asterisk indicates significant difference as compared to control; p<0.05.
3.7 Potential regulation of zinc transporter and metallothionein gene expression by prolactin

Prolactin may regulate zinc transporter expression in mammary epithelia (19). Given the high density of prolactin receptors in the choroid plexus epithelium, prolactin is a reasonable candidate hormone for regulation of zinc transport in this epithelium. As a first approach to characterize the potential regulation of zinc transport in choroid plexus, I conducted a series of pilot experiments to evaluate the efficacy of prolactin to alter zinc transporter and metallothionein gene expression in concentration-dependent manner in cultured choroid plexus cells. After overnight incubation in serum-free medium, cells were incubated in serum-free medium for 24 h without additional agents (Control), with 25 µM ZnCl₂, or with 1, 10, 100, or 1000 nM prolactin. At the end of treatment; total RNA was extracted, and gene expression of CA-2, ZnT1, Zip6, and MT-1 were analyzed by fluorescence qRT-PCR and normalized to β-actin and GAPDH mRNA (Figure 7A, B). This experiment was performed in two separate culture preparations (n = 2). For these experiments, supplementation of extracellular zinc served as an internal control for the predicted changes in gene expression of zinc transporters and MT-1. Whereas zinc supplementation did not markedly alter mean CA-2 expression, prolactin increased CA-2 expression by 30% or more at all concentration. There was however, no apparent concentration-dependent effect by prolactin. Zinc supplementation increased MT-1 expression an average of 28-fold. At all concentrations, prolactin also induced MT-1 expression. As compared to controls, 1 nM and 1000 nM prolactin increased MT-1 expression by an average of 6.5-fold and 7.5-
fold, respectively, and 10 nM and 100 nM prolactin increased MT-1 expression by an average of 3.8- and 4.5-fold. Zinc supplementation increased expression of the zinc exporter, ZnT1 by 90%; prolactin also increased ZnT1 expression at all concentrations by 30%, 50%, 30%, and 70% at 1, 10, 100 and 1000 nM. Zinc supplementation increased Zip6 expression by an average of 3.4-fold as compared to controls. Prolactin also induced Zip6 expression; at 1, 10 and 100 nM prolactin each increased expression by approximately 3-fold, and 1000 nM prolactin increased expression by approximately 4.5-fold.

In the mammary gland prolactin-dependent regulation of zinc transporter gene expression is mediated through the JAK/STAT signaling pathway (19). Thus, the JAK/STAT inhibitor, AG-490, was used to determine whether this signaling pathway might also be involved in the observed effects of prolactin on zinc transporter and MT-1 gene expression. After overnight incubation in serum-free medium, cultured choroid plexus epithelial cells were treated for 24 h in serum-free medium without hormone (Control), or 10 or 100 nM prolactin alone or with 10 µM AG-490 (4-h pretreatment). This experiment was conducted in two separate culture preparations (n = 2). Total RNA was then extracted; ZnT-1, Zip6, MT-1 and CA-2 mRNA expression was analyzed by fluorescence qRT-PCR, and gene expression was normalized to β-actin and GAPDH (Figure 8A, B). As compared to control, treatment with the JAK/STAT inhibitor alone nearly doubled expression of both CA-2 and MT-1, but decreased expression of both ZnT1 and Zip6 by approximately 25%. This suggested that in absence of prolactin JAK/STAT signaling pathway might suppress basal expression of CA-2 and MT-1,
while facilitating expression of ZnT1 and Zip6. Prolactin at 10 nM and 100 nM increased expression of CA-2 by an average of 80%; in the presence of AG-490, increased expression of CA-2 persisted. Expression of MT-1 was not altered in cells treated with 10 nM prolactin, but was reduced by 20% by 100 nM prolactin. Although AG-490 did not markedly enhance the MT-1 expression induced by 10 nM prolactin, the JAK/STAT inhibitor enhanced MT-1 expression induced by 100 nM prolactin to 5-fold as compared to control. 10 nM prolactin decreased ZnT-1 expression by 30%, and AG-490 reversed this effect. Although 100 nM prolactin induced no marked change in ZnT1 expression, addition of AG-490 reduced expression by 25%. Expression of Zip6 also decreased by approximately 30% in presence of 10 nM prolactin, and AG-490 partially reversed this effect. Similarly, 100 nM prolactin decreased Zip6 expression by 55%, and again AG-490 partially reversed this effect.
Figure 7. Concentration-dependent changes on gene expression of metallothionein-1 (MT-1), carbonic anhydrase-2 (CA-2) and zinc transporters ZnT1 and Zip6 in primary cultures of neonatal rat choroid plexus epithelial cells during 24-hour direct supplementation of extracellular zinc or exposure to prolactin (A, B). Cells were plated in 12-well culture plates, and experiments were conducted on confluent differentiated monolayers 7-9 days post plating. Cells were incubated in serum-free DMEM/F12 medium 12-16 h prior to start of experimental treatments. Cells were treated for 24 h with serum free DMEM/F12 that contained no agent (control, Ctrl), 25 µM ZnCl₂ or 1-1000 nM prolactin (A, B). For all samples, expression of genes encoding for MT-1, CA-2, ZnT1 and ZIP6 were normalized to expression of gene encoding for β−actin and GAPDH. For conditions of extracellular zinc supplementation or prolactin exposure gene induction was expressed as fold-change versus control, as calculated by dividing normalized gene expression in experimental sample by normalized gene expression in the control sample. Data are expressed in triplicate measures and representative of 2 experiments.
Figure 8. Concentration-dependent changes on gene expression of metallothionein-1 (MT-1), carbonic anhydrase-2 (CA-2) and zinc transporters ZnT1 and Zip6 in primary cultures of neonatal rat choroid plexus epithelial cells during 24-hour exposure to prolactin or AG-490 + prolactin (A, B). Cells were plated in 12-well culture plates, and experiments were conducted on confluent differentiated monolayers 7-9 days post plating. Cells were incubated in serum-free DMEM/F12 medium 12-16 h prior to start of experimental treatments and cells treated with AG-490 were pretreated with the inhibitor for 4 h. Cells were then treated for 24 h with serum free DMEM/F12 that contained no agent (control, Ctrl), 10-100 nM prolactin or 10-100 nM prolactin + 10 µM AG-490 (A, B). For all samples, expression of genes encoding for MT-1, CA-2, ZnT1 and Zip6 were normalized to expression of gene encoding for β-actin and GAPDH. For conditions of prolactin exposure or prolactin + AG-490, gene induction was expressed as fold-change versus control, as calculated by dividing normalized gene expression in experimental sample by normalized gene expression in the control sample. Data are expressed in triplicate measures and representative of 2 experiments.
4. DISCUSSION

There has been limited elucidation of the coordinated regulation of zinc transporters and metallothionein in maintaining choroid plexus zinc homeostasis. The overall objective of this thesis project is to analyze the interdependent regulation of zinc transporters with the zinc-binding protein metallothionein as the choroid plexus epithelium adapts to increases or decreases in extracellular zinc. Here, in primary cultures of choroid plexus epithelial cells, pharmacological depletion of extracellular zinc with DTPA markedly decreased gene expression of MT-1 and that of the zinc exporter, ZnT1, as early as 3 hours. Expression of the genes remained suppressed throughout the duration of treatment. Pharmacological depletion of zinc also elicited a delayed, but sustained increase in gene expression of the zinc importer, Zip1. Regulation in gene expression of Zip6 was more dynamic, as its expression fluctuated. Similarly, in choroid plexus tissues isolated from neonatal rats, in vitro depletion of extracellular zinc with DTPA down-regulated gene expression of MT-1 and ZnT1, while up-regulated expression of Zip1 and Zip6. Conversely, in cultured choroid plexus cells, supplementation of extracellular zinc with zinc chloride markedly induced gene expression of both MT-1 and ZnT1 as early as 3 hours, and expression remained elevated throughout treatment. Supplementation with zinc also elicited a marked induction of Zip6 as early as 3 hours, but gene expression fluctuated throughout 48 hours. There was a delayed decrease in Zip1 gene expression, but then was similar to control for the duration of treatment. Similarly in isolated neonatal choroid plexus tissues, in vitro supplementation of extracellular zinc resulted in up-regulation of MT-1
and ZnT1 gene expression, while Zip1 and Zip6 gene expression were not significantly affected. These data indicate that there is coordinated regulation of metallothionein and zinc transporters during depletion and supplementation of extracellular zinc.

A limited number of studies have investigated changes in zinc accumulation or membrane transport of zinc in the choroid plexus under control conditions i.e., normal zinc status, conditions of zinc depletion, or conditions of zinc supplementation. Even fewer studies have characterized changes in zinc transporter and MT-1 gene or protein expression in choroid plexus as extracellular zinc is manipulated. Because small intestine is the primary site of zinc homeostasis, the majority of studies that address regulation of cellular transport of zinc have emphasized regulation of zinc transporter gene and protein expression and localization in small intestine.

My observation that chelation of extracellular zinc with DTPA induces gene expression of zinc importers in choroid plexus is consistent with reported effects of DTPA in models for small intestine. In the intestinal epithelial cell line MODE-K, expression of zinc importer, Zip4, was induced by treatment with 4 μM TPEN, and intracellular zinc chelator, as well as by treatment with 50 μM DTPA (20). This is similar to what I observed in my depletion studies; however, I did not use TPEN and used lower concentrations of DTPA. In a separate study in CACO-2 intestinal cells, Zip1 mRNA expression was not altered by TPEN, despite treatment with chelator as great as 10 μM and for extended periods (28). Nevertheless, Zip 4 mRNA expression was up-regulated in a time and concentration-dependent manner for up to 24 hours (28).
Although Zip1 was not responsive, Zip4 was regulated in response to chelation effects of TPEN, indicating differential regulation of zinc transporters by small intestine.

*In vivo* studies of zinc depletion through modifications of dietary zinc have also been observed in other epithelia. In rats fed marginally low zinc diets (15 mg Zn/kg), ZnT1 protein expression in the jejunum was reduced by 70% compared to control (25 mg Zn/kg), which was expected as less zinc will be exported out of the cell during reduced zinc availability (12). A low zinc diet (7 mg Zn/kg) up-regulated Zip4 gene expression in rat jejunum by 4-fold. However, Zip4 protein expression was reduced. This could be due to transient fluctuations in extracellular (plasma) and intracellular zinc concentrations during the first few days of dietary zinc deficiency and during compensatory periods. In the liver of rats fed the low zinc diet, Zip1 protein expression in plasma membrane was reduced by 60%, while ZnT1 protein expression was increased 2-fold (12). Again, Zip1 reduction of protein expression could have been due to fluctuations in extracellular and intracellular zinc. In jejunum of rats fed a very low zinc diet (<1 mg Zn/kg) up-regulation of Zip4 gene expression by 7-fold and ZnT1 protein expression by 1.5-fold were observed. Hepatic Zip1 mRNA was up-regulated 1.5-fold, while hepatic ZnT1 protein expression was reduced by 50% compared to control (12).

This suggests ZnT1 transporter expression in the liver may be more sensitive to the reductions in dietary zinc. That study focused on multiple zinc transporters, similar to this study in choroid plexus, and noted dynamic changes occurring in each tissue in response to varying dietary zinc concentrations. In a separate study in mice fed a low zinc diet (7 mg Zn/kg 30 d), Zip6 protein expression in the plasma membrane of the
testes was reduced by >50% compared to control (30 mg Zn/kg) (8). In choroid plexus
cells, Zip6 mRNA was not consistently responsive to zinc depletion; however, in
isolated choroid plexus tissues, Zip6 and Zip1 gene expression increased in response to
extracellular zinc depletion. Furthermore, adaptive up-regulation of Zip1 and Zip6 and
possibly other zinc importers may explain the observations in an earlier study in zinc
deficient rats. Kasarkis et al. fed rats zinc deficient diets and determined that the choroid
plexus maintained the capacity to accumulate zinc. In fact, choroid plexus in zinc
deficient rats accumulated ~80% more zinc per tissue weight compared to control (13).

Currently, there are no published data on metallothionein expression in
coordination with zinc transporters in the choroid plexus. However, coordinated
regulation of zinc transporters and metallothionein gene and protein expression, similar
to what I observed in choroid plexus, have been reported for other epithelial tissues, such
as liver and small intestine. In hepatoma cells, mRNA expression MT-1 decreased by
30% by 48-hour treatment with 50 µM DTPA as compared to control, whereas ZnT1
mRNA and protein expression remained constant in both hepatoma cells and primary
hepatocytes (9). The supposed lack of change in ZnT1 mRNA might have been due to
measurement of gene expression only at the end of zinc depletion, rather than throughout
the 48 hour treatment as performed in this study. Still, a coordinated response of
metallothionein and zinc transporters as observed in my study was observed in the
following studies. mRNA expression of MT-1 was down-regulated by 80%, while Zip4
mRNA expression was up-regulated almost 6-fold in small intestine of zinc-depleted
mice fed zinc deficient diets (<1 mg Zn/kg) as compared to those fed zinc sufficient diets
(control-30 mg Zn/kg) (20). In a separate study, in whole brains of pups that were zinc deficient *in utero* (10, 7 mg Zn/kg), ZnT1 mRNA was reduced by 20% and MT-1 mRNA by more than 20%, but Zip6 mRNA increased by >40% as compared to controls (25 mg) (5). Also in whole brains of zinc deficient pups, ZnT-1 protein expression was reduced by more than 50% and Zip6 protein expression was increased by more than 3 fold (5). Plasma zinc concentrations in pups from dams fed a marginally or moderately zinc deficient diet were markedly lower, as compared to controls. However, reductions in dietary zinc did not lower zinc concentrations in the brain, indicating retention of zinc during a deprived state. Furthermore, zinc uptake was greatest in brain of pups fed zinc deficient diets at 3 h and 8 h post-injection of $^{65}$Zn, suggesting increased expression or activity of zinc importers (5). This also indicates there is tight regulation of intracellular zinc for prevention of detrimental effects due to zinc deficiency. That *in vivo* study, nevertheless, is consistent with changes observed in this study in choroid plexus cells and tissue.

A limited number of studies have analyzed the relationship between metallothionein and zinc transporters in liver and intestinal cells in response to zinc supplementation. Lanmade et al. found that ZnT1 and MT-1 gene expression was induced as early as 3 hours with exposure to 100 µM ZnSO$_4$ in Hepa cells (17). This is similar to results in choroid plexus cells, as MT-1 and ZnT1 mRNA were also up-regulated as early as 3 hours in response to zinc supplementation. In a different study in hepatoma cells, 100 µM zinc sulfate supplementation for 48 hours enhanced ZnT1 mRNA expression by 60%, but no change was noted in primary cultures of hepatocytes.
Zinc supplementation, concurrently, increased expression of MT-1 mRNA in hepatoma cells by 35% (9). That may have been due to specific differences in zinc transporter expression in tumor cells; in addition, ZnT1 and MT-1 were analyzed at the end of treatment without analysis of time-dependent effects or adaptations to zinc. A separate study in CACO-2 cells analyzed MT-1 mRNA and ZnT-1 mRNA that were induced in a time-dependent manner by 200 μM zinc sulfate through 24 hours. MT-1 peaked at 24 hours with a 4-fold induction, and ZnT1 peaked at 18 hours with a 2.5-fold induction. In that study, 12-hour levels of MT-1 mRNA and ZnT1 mRNA were incrementally induced by exposure to 100, 150 and 200 μM zinc sulfate (28). Although that study used higher concentrations of zinc and was performed in CACO-2 cells, which are a human adenocarcinoma cell line, those results still paralleled the adaptive response of MT-1 and ZnT1 to zinc observed at different time points here in primary cultures of choroid plexus cells.

The choroid plexus has the greatest density of prolactin receptors of any tissue in the brain. Prolactin regulates zinc transport in mammary gland, which similar to choroid plexus, is also an epithelial tissue. Thus, it is a reasonable candidate hormone for the regulation of zinc transporters in choroid plexus. The pathway through which prolactin regulates zinc transporter gene expression in mammary is the JAK/STAT pathway, which is inhibited by AG-490. Therefore, AG-490 was used to determine whether JAK/STAT signaling pathway is also involved in prolactin regulation of MT-1 and zinc transporters in choroid plexus. In my pilot experiments, treatment with prolactin at 1-1000 nM up-regulated gene expression of CA-2 and MT-1 and the zinc transporters,
ZnT1 and Zip6. Interestingly, treatment with AG-490 alone also increased gene expression of both CA-2 and MT-1, but decreased expression of both ZnT1 and Zip6. Furthermore, co-treatment with prolactin and the JAK/STAT inhibitor AG-490 further increased expression of CA-2 and MT-1. This suggests that the JAK/STAT signaling pathway tonically suppresses basal gene expression of MT-1 and CA-2. However, co-treatment with AG-490 and prolactin elicited varying responses in zinc transporter gene expression, partially reversing effects of prolactin. It is important to note that currently there are no data on the effect of AG490 on metallothionein, carbonic anhydrase or zinc in other epithelial tissues. Future investigations of the effects of prolactin in choroid plexus epithelium might yield more consistent results, if the cells or tissue were primed with estrogen, which is known to up-regulate and maintain prolactin receptor expression.

In other studies done in the epithelial tissues, the mammary and the prostate, varying results to this study were observed. In HC11 mammary cells, treatment with prolactin for 24 hours, increased gene expression of ZnT2 mRNA (27). Also in that study, co-treatment of the Jak2 inhibitor AG490 (10 µM) with prolactin reduced ZnT2 mRNA from 2 to 1-fold, which may indicate a possible regulation of prolactin through the Jak2/Stat5 pathway (27). Similarly, in immortalized PC-3 prostate cells, pre-treatment with prolactin stimulated zinc accumulation by 33% (7). In addition, in that study, Zip1 gene expression in response to treatment of zinc alone was down-regulated. However, when cells were treated with $10^{-9}$ M prolactin, gene expression of Zip1 was increased, indicating a possible regulation of prolactin on zinc transport (7).
5. CONCLUSIONS AND PERSPECTIVES

This thesis addresses regulation of zinc transport in the choroid plexus. The choroid plexus epithelium forms the blood-CSF-barrier, which is separate and distinct from the blood-brain-barrier. The choroid plexus accumulates excess nutritive minerals, such as copper and zinc, but also heavy metals, such as cadmium, acting as a ‘sink’ for these metals and minerals. As such, it protects the brain from toxicity of heavy metals and regulates balance of nutrient minerals. In the described studies, I characterized gene expression of zinc transporters and the zinc-binding protein, metallothionein-1 in response to depletion and supplementation of extracellular zinc using primary cell cultures and isolated neonatal choroid plexus tissues.

Zinc homeostasis is vital for choroid plexus biology. Based on the data presented herein, I conclude that choroid plexus can regulate total intracellular zinc concentrations as well as intracellular zinc availability as it adapts to changes in extracellular zinc status. The results indicated there is a coordinated regulation of metallothionein and zinc transporters during extracellular zinc depletion or extracellular zinc supplementation. Cellular zinc accumulation studies showed that extracellular zinc depletion did not greatly reduce total intracellular zinc, and extracellular zinc supplementation did not increase total intracellular zinc as compared to control. During zinc depletion, expression levels of both MT-1 and ZnT1 mRNA are down-regulated, while Zip1 and Zip6 mRNA levels are up-regulated. Conversely, in response to zinc supplementation, MT-1 mRNA, ZnT1 mRNA, and Zip6 mRNA expression are up-regulated, while Zip1 mRNA is down-regulated. Immunocytochemical staining
indicated that MT-1 protein expression was also induced and suppressed with zinc supplementation and extracellular zinc depletion, respectively. Although I did not directly measure zinc transporter protein expression, zinc accumulation results indicate the epithelium is regulating total intracellular zinc accumulation as extracellular zinc changes.

Gene and protein expression of the full compliment of zinc importers and zinc exporters in the choroid plexus were not characterized in this study. Nevertheless, these data demonstrate a coordinated regulation of zinc-binding protein - MT-1, the zinc exporter - ZnT1, and the zinc importers – Zip1 and Zip6 as the epithelium adapts to changes in extracellular zinc. During periods of limited zinc availability, down-regulation of ZnT1 would reduce efflux (removal) of zinc from the cell, while up-regulation of Zip1 and Zip6 would transport additional zinc into the cell. Concurrently, down-regulation of MT-1 would facilitate appropriate distribution of intracellular zinc. During periods of zinc supplementation as in the case of treating zinc deficiencies, although there are minimal changes in Zip1 and Zip6 activities, zinc can still be transported into the cell. However, the free intracellular concentration of zinc would be regulated by up-regulation of ZnT1, which would facilitate removal of excess zinc from the cell, and by up-regulation of MT-1, which would bind additional zinc. This could serve a protective role for choroid plexus, in permitting sequestration and storage of zinc that would be available for use during periods of zinc deficit. Zinc transporter gene expression also seems to undergo adaptations to changes in extracellular zinc throughout the 48 hours as gene expression of zinc importers fluctuated. Preliminary data suggest
that prolactin might potentially regulate zinc transport into the cell in part by up-regulating gene expression of Zip6. However, the mechanism is unclear and further, more detailed investigation is warranted.

Finally, to my knowledge, there are no other published studies that define the coordinated regulation of zinc transporters and metallothionein in maintaining choroid plexus zinc homeostasis. Therefore, these data have provided insight into the integrated roles of zinc transporters and metallothionein in zinc biology as well as the physiology of choroid plexus. Future directions include measuring total zinc accumulation at different time points of extracellular zinc supplementation and depletion as well as analyzing protein and gene expression of all zinc importers and exporters present in choroid plexus as I did not analyze the full set of zinc transporters expressed in choroid plexus.
REFERENCES


