EFFECTS OF DIETARY ZINC-DEPRIVATION AND PAIR-FEEDING ON WATER MAZE PERFORMANCE AND PROTEIN EXPRESSION IN YOUNG MALE RATS

by

YE CHU

(Under the direction of Arthur Grider)

ABSTRACT

Zinc deprivation in young rats has been shown to negatively affect short-term memory in a water maze. This study explores further the behavioral performance of rats in the maze. A revised short-term memory testing procedure was used and no short-term memory deficit was observed in zinc-deprived rats. This observation was different from previous results and may lie in differences in the methodological approach used for determining the short-term memory. Long-term memory and learning ability were also measured. No dietary effects on long-term memory were found, but the pair-fed rats exhibited less effective learning in the consecutive and reversal-learning tests compared with the control group. Both the pair-fed and zinc-deprived animals exhibited thigmotaxic (wall-hugging) behavior in the learning test but to different degrees. Thigmotaxic behavior in the zinc-deprived rats was less severe than that of the pair-fed rats. The stress response of these rats before and after the swim tests indicated that the zinc-deprived and pair-fed rats were stressed by the dietary treatment. However, the stress level of pair-fed rats was elevated earlier than the zinc-deprived group. The stress responses in these rats may contribute to their anxiety-related thigmotaxic behavior, but may not be the only cause of the behavioral differences. Neurological changes in the cerebellum and the cholinergic systems may also be involved. Interestingly, the learning deficits observed in consecutive swim tests were not obvious in the learning tests with probing test insertions, suggesting that the consecutive swim test is a more sensitive measure of learning performance. Differential protein expression in the hippocampal region of these rats was determined using two-dimensional gel electrophoresis. The changes in protein profiles suggest that zinc deprivation and pair feeding affect protein expression differently. Twenty differentially expressed protein spots were tentatively identified using the program TagIdent and the SWISS-PROT database. The results indicate that dietary treatment affects the expression of a wide range of proteins.

INDEX WORDS: Learning, Short-term memory, Long-term memory, Thigmotaxia, Hippocampus, Two-dimensional gel electrophoresis

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YE CHU

B.S., Wuxi University of Light Industry, P.R. China, 1992

M.S., Tianjin University of Light Industry, P.R. China, 1995

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YE CHU

Approved:

Major Professor: Arthur Grider

Committee: Julie Coffield
Roger Dean
Joan Fischer
Ruth B. S. Harris

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

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CHAPTER 1

INTRODUCTION

Since the discovery of zinc as an essential trace element for human health, the functional roles for this element have been extensively explored. The wide spectrum of biological pathways that zinc participates in suggests the importance of this trace element as an indispensable nutrient.

The current Dietary Reference Intakes for zinc are listed in Table 2.1. Zinc exhibits low toxicity. Reduced copper status is considered the critical effect for determining the tolerable upper intake level (UL) for zinc (Food and Nutrition Board and Institute of Medicine 2002). However, cases of zinc-deficiency in humans have been reported more frequently than that of toxicity, especially during stages of rapid growth i.e. infancy, adolescence, pregnancy, and lactation. Typical symptoms of zinc-deficiency include stunted growth, anorexia, and suppressed immune response. Cognitive impairment has also been associated with low zinc status in humans and animals. Malformations of the brain have been found in infants whose mothers exhibited low zinc status (Jameson 1976). Experimental animals experiencing acute zinc deficiency exhibited less than optimal performance in different sets of behavioral tests such as associative learning and spatial cognition (Halas et al. 1983, Halas et al. 1979, Halas et al. 1986, Hesse et al. 1979). Zinc supplementation improves the growth, activity, motor skills and reasoning ability of children (Friel et al. 1993, Sazawal et al. 1996). In addition, the presence of ionic zinc in glutaminergic synaptic vesicles in the hippocampal

region suggests a possible role for zinc in regulating neuronal plasticity for memory formation in that brain region. However, recent studies with transgenic animals do not support the mnemonic role of zinc in the brain. ZnT-3 knockout mice, devoid of vesicular zinc in the hippocampus do not exhibit any learning impairment (Cole et al. 2001). MT-III knockout mice with normal amounts of vesicular zinc, but reduced zinc levels in other brain regions, are able to perform memory tests without significant defects (Erickson et al. 1997).

The Morris water maze (MWM) is used to evaluate the cognitive integrity of rats. In this test, a rat is highly motivated to escape from the water by locating an invisible platform underneath the water, eliminating any food reward. Since zinc deprivation and pair feeding alter the appetite for food, cognitive tests with food rewards can be confounded by the motivation of animals. Therefore, the MWM was chosen for the behavioral studies in this project. Recently, the MWM has been used to test the short-term memory of rats zinc-deprived at different ages (Keller et al. 2000, Keller et al. 2001). The studies indicated that only young rats exhibited short-term memory deficits, and the impairment was reversed upon dietary zinc supplementation. The first aim of the current research project was to examine in more depth the effect of zinc-deprivation and pair-feeding in the MWM performance of young rats. Different memory components including short-term memory, long-term memory and learning ability were tested. Considering the aversive nature of swimming in the water maze, the stress responses of animals before and after the maze tests were also monitored.

Most transcriptional factors possess a ‘zinc finger’ motif which functions to recognize transcriptional elements of genes. Thus, zinc has a regulatory role in protein

expression. Dietary zinc deprivation has been shown to affect gene expression in intestinal cells and lymphocytes (Cousins 1994). It has been suggested that the hippocampal zinc level decreases during severe dietary zinc deprivation (Keller et al. 2000, Takeda et al. 2000). However, the consequences of zinc deprivation on protein expression need to be explored further. Therefore, the second aim of this research was to compare the protein expression pattern of the rat hippocampus by two-dimensional gel electrophoresis, a proven method for detecting differentially expressed protein levels in biological systems. Although at this stage, the specific pathways responsive to dietary zinc depletion cannot be determined, the identification of differentially expressed proteins upon dietary treatment provides protein candidates for further studies on the role for zinc in the hippocampus.

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CHAPTER 2

LITERATURE REVIEW

2.1 Importance of zinc as a nutrient

Zinc is an essential trace element necessary for the growth and development of all organisms. Human zinc deficiency was first identified in the Iranian patients of Dr. Prasad and his colleagues (Prasad et al. 1961). These patients exhibited anemia, dwarfism, hypogonadism, dry skin, and mental lethargy. Supplementation with iron ameliorated the clinical symptoms. However, iron deficiency could not fully account for all of the symptoms since the iron-deficient animals did not exhibit the retarded growth or gonadal failure, symptoms that occurred in zinc-deprived animals. Subsequent treatment trials on patients in Egypt established that zinc deficiency was the primary cause of these symptoms (Prasad et al. 1963, Sandstead et al. 1967). Two major findings from these early studies support a role for zinc in human health. First, zinc concentrations in the plasma, hair, erythrocytes, urine and stool were decreased whereas the ^{65}Zn turnover rate was increased in these growth-retarded patients compared to healthy subjects. Secondly, patients treated with supplemental zinc exhibited a growth rate faster than those who received iron supplementation or an animal protein diet (Prasad 1995). Numerous studies have since characterized the role zinc plays in cellular metabolism.

Zinc is present in more than 300 enzymes belonging to all six enzyme classes including oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases (Vallee and Falchuk 1993). Among these enzymes, zinc plays three roles i.e. catalytic,

co-catalytic and structural (Vallee and Falchuk 1993). The catalytic function of zinc is defined by its direct participation in enzyme catalysis, as in carbonic anhydrase (Liljas et al. 1972). When more than one zinc-containing subunit constitutes the catalytic structure, the additional zinc site is co-catalytic, e.g. alkaline phosphatase (Kim and Wyckoff 1989). Zinc also provides the structural motif of some enzymes by forming a tetrahedral coordination with cysteine or histidine ligands. Zinc in protein kinase C resides in the non-catalytic domains (Hubbard et al. 1991). These zinc metalloenzymes catalyze more than 50 important biochemical reactions (Prasad 1995). In addition, zinc plays a key role in gene expression. Virtually all DNA-binding proteins are zinc finger proteins encoded by approximately 1% of the human genome (Choo and Klug 1997). Zinc finger proteins share a common DNA recognition helix motif, which is formed by zinc binding to histidine and/or cysteine on the peptide. These transcription factors alter the rate of transcription and the cellular abundance of certain mRNAs. Ionic zinc also regulates metallothionein gene transcription via interaction with metal response elements (MRE) in the promoter (Cousins 1994). Since zinc is present in numerous cellular pathways, many physiological systems may be affected by zinc status. There is limited information however on the specific enzymes or processes altered by abnormal zinc status and how these lead to impairment in developmental events.

2.1.1 Pathological consequences of zinc deficiency

Zinc deficiency in animals often adversely affects those organ systems that have the highest turnover and proliferation rate e.g. dermatological, intestinal, immune, and reproductive systems. The central nervous system is usually affected by zinc deficiency

in the stages of early development. Effects include deficits in motor skill, cognition and emotionality. These aspects are discussed in detail in section 2.4.

Severe zinc deficiency in humans causes prominent skin lesions around the body orifices and extremities i.e. acrial. In zinc-deficient rats, non-scarring alopecia, diminished subcutaneous tissue, and nude skin have been documented (Follis et al. 1941). Parakeratosis, i.e. increased parakeratotic plaques and skin fragility, is found in pigs, calves and lambs (Apgar and Fitzgerald 1985).

Diarrhea is another clinical feature of zinc deficiency. Plausible mechanisms include immune depression and reduced water and electrolyte permeability in intestinal mucosal cell membranes (Ghishan 1984). Zinc deficiency-induced diarrhea can be lethal if untreated. The loss of zinc from the intestine increases during the diarrhea state, worsening the disease. Zinc supplementation to children in developing countries has been shown to significantly reduce the prevalence and incidence of diarrhea (Bhutta et al. 1999).

Zinc deficiency negatively affects the immune system. Besides the above-mentioned lesions in the physical barriers, compromised immune function during zinc deficiency increases vulnerability to a wide range of viral, bacterial and fungal infections. The mortality rate of zinc-deprived mice after infection with *Typanosoma Cruzi* was seven-fold greater than in food-restricted, infected mice (Fraker et al. 1982). Zinc deficient diet depresses immune-resistance to tuberculosis in guinea pigs (McMurray et al. 1990). HIV seropositive patients usually exhibit abnormally low serum zinc levels (Bogden et al. 1990, Falutz et al. 1988). Randomized, double blinded, placebo-

controlled studies suggest that zinc supplementation significantly decreases the duration of the common cold in human subjects (Godfrey et al. 1996, Mossad et al. 1996).

Zinc deficiency suppresses both acquired cellular and humoral immunity in the host (Shankar and Prasad 1998). Atrophy of the thymus, loss of cellularity in peripheral blood and lymphatic tissues were observed in zinc deficient animals and humans (Ripa and Ripa 1995). The thymus is the site for the maturation of T-lymphocytes into helper T cells (CD_4^+: CD_8^-) and cytotoxic T-cells (CD_4^-: CD_8^+). The zinc deficiency-induced reduction in size and cellularity of the thymus is mainly observed in the cortex where the maturation of thymocytes takes place (Salgueiro et al. 2000). In addition, thymulin activity depends on plasma zinc concentration (Prasad et al. 1988). Thymulin is a nine-peptide hormone secreted by thymus epithelial cells. The active form of thymulin requires zinc association at a 1:1 ratio (Cung et al. 1988). It binds to receptors on the T lymphocytes to promote the maturation of these cells (Dardenne et al. 1982). Inactive thymulin has been detected in the serum of zinc-deficient patients (Prasad et al. 1988). Consequently, T-cell differentiation and function is negatively affected by zinc deficiency. Lymphopoiesis in the bone marrow is also adversely affected by zinc deficiency. The pre-B cell population is greatly reduced in zinc deficient animals (Fraker et al. 1995) and the antibody responses of B-lymphocytes are inhibited (DePasquale-Jardieu and Fraker 1984). Likewise, the immunological functions of natural killer cells, neutrophils, monocytes, and macrophages are impaired during zinc deficiency (Shankar and Prasad 1998).

Zinc deficiency also adversely affects reproduction in both males and females. Delayed delivery, parturition difficulty, and excessive bleeding in zinc deficient pregnant

rats have been reported (Apgar 1985). Several metabolic alterations contribute to these delivery problems such as a poor response to estrogen and reduced catalytic activity of 20-alpha-hydroxysteroid dehydrogenase, which stimulates parturition (King 1999). Zinc status also affects fetal growth and pregnancy outcome. Low plasma zinc in mothers is associated with delivery complications and retarded fetal growth. Overt zinc deficiency is associated with teratogenesis (Sandstead 1985). Zinc deficient males exhibit hypogonadism and delayed sexual maturation (Prasad et al. 1961).

2.1.2 Pathological consequence of excessive zinc

Zinc is essentially non-toxic i.e. not carcinogenic, mutagenic or teratogenic. Ingestion of a large amount of zinc causes lightheadedness, slight ataxia, lethargy, and headache, but not any long-term negative effects such as diarrhea, neurological dysfunction, or hepatic inflammation (Bertholf 1988, Lewis and Kokan 1998). There is no clinical data to indicate that chronic ingestion of zinc compounds increases susceptibility to diseases (Barceloux 1999). In fact, intestinal homeostatic mechanisms regulating zinc absorption and retention are very effective in preventing the excessive circulating concentration of zinc in the body. One of these mechanisms is the induction of thionein, a low-molecular weight peptide, which binds to seven zinc atoms per peptide and serves as the scavenger of excessive zinc supply (Vallee and Falchuk 1993). However, a risk of long-term excessive zinc supplementation is copper deficiency and anemia (Patterson et al. 1985). Since copper has a higher affinity for metallothionein than zinc, the induction of intestinal metallothionein by excessive zinc intake reduces copper absorption. This phenomenon has been exploited for treating patients with the

copper accumulation disease, Wilson disease. The intestinal copper metallothionein complex is subsequently excreted into the feces (Klevay 1975).

Inhalation of zinc oxide fumes from processing of zinc ores induces 'metal fume fever' with symptoms similar to influenza. Typical symptoms include fatigue, chills, fever, myalgias, cough, dyspnea, leukocytosis, thirst, metallic taste, and salivation. The disease is not fatal and if the patient is removed from the fumes for 36-48 hours, the clinical symptoms disappear (McCord 1960). The exact mechanism of zinc fume fever is not clear, however, zinc oxide probably induces inflammation and pyrogenic cytokine secretion in the respiratory tract (Fine et al. 1997). The dose-dependent release of pulmonary tumor necrosis factor-alpha and interleukin-8 upon zinc oxide exposure has been observed in *in vitro* studies (Kuschner et al. 1998). The observed immune-response is not accompanied by an elevation of plasma zinc. In fact, zinc oxide is relatively insoluble in aqueous solutions. Metal fume fever is not only induced by zinc ore. Similar symptoms have been reported among workers in other metal smelting operations such as copper, iron, magnesium, and manganese (Barceloux 1999).

2.2 Metabolic aspects of zinc

Since zinc is so important to animal welfare, the dietary sources of zinc, metabolic pathways that use zinc, and the dynamic distribution of zinc in the body have been well studied.

2.2.1 Dietary sources and factors affecting zinc absorption

The zinc content of foods varies greatly, ranging from 0.02 to 100 ppm. Oyster, beef, cheese, liver and other red meats are good sources of zinc. The zinc content in whole grain cereals, nuts and legumes is relative high among plants. The recommended

daily allowance (RDA) of zinc for different age groups is listed in Table 2.1 (Food and Nutrition Board and Institute of Medicine 2002). Adequate intake and efficient absorption of dietary zinc are critical for a healthy zinc status. Several dietary factors affect zinc absorption. Phytic acid (myoinositol hexaphosphate) is commonly present in plants. It binds to cations such as zinc, forming insoluble complexes that are excreted in the stool. Populations that consume a high phytate diet, e.g. corn, cereal, rice, or legume, are at high risk for zinc deficiency (Hambidge 1997). Fiber has been implicated in reduced zinc absorption since most high fiber foods also contain high phytate (Knudsen et al. 1996). Fiber however has little, or no, effect on zinc absorption. Fermentation of whole grain bread significantly reduced the phytate content and considerably increased zinc absorption (Navert et al. 1985). Purified fiber such as alpha-cellulose does not reduce zinc absorption in young men (Turnlund et al. 1984). Copper and iron have similar physicochemical characteristics to zinc, and may compete with zinc for absorption. The effect of iron on zinc absorption depends on the ratio of these two elements with a high iron to zinc ratio (26:1) inhibiting zinc absorption. The presence of zinc binding ligands, such as histidine, counterbalances the negative effect of iron (Lonnerdal 2000). Iron fortified food, or long-term administration of iron supplements do not negatively affect zinc absorption (Davidsson et al. 1995, Yip et al. 1985). Excessive ingestion of copper is very unusual and modest increases in copper intake do not inhibit zinc absorption (Lonnerdal 2000). Low-molecular weight chelators such as amino acids, and organic acids improve zinc bioavailability. The positive effect is associated with the increased solubility of zinc by chelation with these compounds (Lonnerdal 2000). Increased dietary protein is positively correlated with zinc absorption

(Sandstrom and Cederblad 1980). Released amino acids from protein digestion may counteract the inhibitory effect of phytate (Lonnerdal 2000).

In addition, several host factors such as age, gender, physiologic state and pathologic conditions also influence zinc status (Krebs 2001). For example, poor sanitary conditions increase the occurrence of diarrhea and further aggravate a zinc deficient state. It has been shown that childbearing-age women, infants and children are at high risk for zinc deficiency (Black 1998, Hambidge 1997, Krebs 2000, Sandstead 1995). At these stages of life, the rates of growth and metabolism are high, increasing the requirement for zinc. The primary source of zinc during early infancy is human milk. After seven months, appropriate complementary foods are required to meet the needs of the infant for zinc (Krebs 2000).

2.2.2 Zinc absorption, circulation, and excretion

Absorption of a nutrient refers to its translocation into the enterocyte from the lumen, passage through the basolateral membrane, and transport into the portal circulation. Exogenous zinc absorption occurs in the jejunum and ileum (Lee et al. 1989). Zinc ions are hydrophilic and unlikely to cross cell membranes via passive diffusion (Cousins and McMahon 2000). Carrier mediated diffusion occurs in a time, pH, and temperature dependent manner. Intestinal zinc transport has been characterized (Reyes 1996). Candidate zinc transporters involved in transcellular transport are discussed in Section 2.2.3. These transporters likely function during low to normal levels of zinc intake. Paracellular transport occurs with high levels of zinc intake. As zinc saturates the transcellular transport mechanism, it simply diffuses across the tight junctions. It has been shown that zinc absorption increases as the requirement for zinc in

the body increases (Davies 1980), which is indicative of homeostatic mechanisms present for regulating zinc absorption. Metallothionein and cysteine-rich intestinal proteins (CRIP) are postulated to have a role in transmucosal zinc transport but further studies are needed to substantiate this hypothesis (Hempe and Cousins 1991). Identification of the structure of CRIP suggests that this protein belongs to the LIM (cysteine-rich motif of leu-11, isl-1, and mec-3 genes) family with a double zinc finger motif (Birkenmeier and Gordon 1986). Besides its presence in the intestinal cells, this protein is also abundantly expressed in the immune system including peritoneal macrophages, peripheral blood mononuclear cells, spleen, and thymus (Fernandes et al. 1997). A regulatory role for CRIP in immune cell maturation and differentiation has been suggested (Cousins and Lanningham-Foster 2000).

Plasma is the major exchange pool for systemic zinc requirements. Virtually all zinc in this pool is associated with proteins or low-molecular weight ligands. Approximately 30% of the total zinc in the plasma binds to alpha 2-macroglobulin (Parisi and Vallee 1970). Zinc binds to this protein tightly, preventing the exchange of zinc with other tissues. The major exchangeable zinc pool is serum albumin with two zinc-binding sites of high affinity (Simons 1993). Transporter-mediated endocytosis of zinc and albumin has been suggested for zinc acquisition in endothelial cells (Rowe and Bobilya 2000). However, albumin zinc may not be essential for zinc transport into the brain. It has been shown that ^{65}Zn transport and distribution in the brain is not changed in analbuminemic mutant rats (Takeda et al. 1997). Histidine and/or cysteine bound zinc is another source of exchangeable zinc in the plasma. Infusion of histidine increases ^{65}Zn uptake relative to the plasma zinc concentration in the body (Aiken et al. 1992). ^{65}Zn

transport across the blood brain barrier is enhanced by L-histidine during short cerebrovascular perfusions (Buxani-Rice et al. 1994). Several zinc permeable channels allowing for zinc influx have been identified in neuronal cultures including zinc and calcium-permeable voltage-gated channels, N-methyl-D-aspartate (NMDA)-gated channels, and alpha-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA)/kainic acid (KA)-gated channels (Frederickson et al. 2000).

The majority of dietary zinc is excreted through the gastrointestional tract via pancreatic secretions. Urinary loss of zinc is around 500-800 μg/day which is 10-fold less than that lost in the gastrointestional tract (Abu-Hamdan et al. 1981). Intestinal epithelial layer turnover, desquamation of skin, and sweat account for further loss of zinc.

2.2.3 Mammalian zinc transporters

A number of mammalian zinc transporters have been identified within the past decade. These transporters can be classified into two major families by their similarities in topology, sequence, and functional properties (Table 2.2). The cation diffusion facilitator (CDF) family consists of transporters mainly responsible for zinc efflux or intracellular sequestration. CDF family members usually share a characteristic membrane topology including a six transmembrane domains and a cytoplamic histidine-rich loop between membrane spanning regions IV and V, which may serve as a zinc-binding site. ZnT-1 (zinc transporter 1) was the first cloned zinc transporter in the CDF family. It is expressed in a wide variety of tissues (Palmiter and Findley 1995). In the intestine, this protein is localized to the basolateral surface of enterocytes and its expression is regulated by dietary zinc supplementation (McMahon and Cousins 1998). Metal response element-binding transcription factor-1 (MTF-1) can bind to the metal

response element (MRE) in the ZnT-1 promoter region and mediates the zinc induced ZnT-1 expression in mouse embryo (Langmade et al. 2000). ZnT-1 is responsible for zinc excretion from cells (Palmiter and Findley 1995). Thus this transporter may facilitate zinc efflux into the portal circulation. ZnT-1 has also been found in the basalateral membrane of the renal tubules consistent with its functioning to conserve zinc by renal re-absorption (Cousins and McMahon 2000). In the brain, ZnT-1 is colocalized with chelatable zinc in the hippocampus. This transporter is also distributed in brain regions devoid of chelatable zinc such as the cerebellum, olfactory bulb, and hypothalamus. Therefore, ZnT-1 may play a protective role in neurons (Sekler et al. 2002).

ZnT-2 is localized in the intracellular vesicles and facilitates zinc transport into endosomal or lysosomal compartments (Palmiter et al. 1996a). ZnT-3 is exclusively expressed in the brain and testes. In the brain, it is responsible for zinc accumulation into synaptic vesicles (Palmiter et al. 1996a). ZnT-4 is found in intestinal epithelial cells (Murgia et al. 1999) and mammary gland epithelial cells (Huang and Gitschier 1997). This transporter is critical for depositing zinc from the cytoplasm into the secretory vesicles in the mammary gland (Huang and Gitschier 1997). A nonsense mutation of ZnT-4 is expressed in lethal milk mice (see section 1.3.2.). Constitutive expression of a human orthologue of ZnT-4 (hZnT-4) has been identified in human epithelial cells (Michalczyk et al. 2002). Localization of ZnT-4 at the basolateral membrane of Caco-2 cells and rat small intestinal cells suggests that ZnT-4 may participate in transporting zinc into the portal circulation as ZnT-1 (Murgia et al. 1999). ZnT-5 is abundantly expressed in pancreatic beta cells and localized on the membrane of secretory insulin granules.

This transporter is thought to transport zinc into these granules and facilitate insulin secretion (Kambe et al. 2002). Interestingly, ZnT-5 null mice suffer from osteopenia and death due to cardiac failure which suggests an important role of ZnT-5 in osteoblast maturation and cardiac conduction (Inoue et al. 2002). ZnT-6 is another intracellular zinc transporter that is responsible for the trafficking of zinc between the trans- Golgi apparatus and cytoplasmic vesicles. The distribution of ZnT-6 in the intracellular organelles is regulated by extracelluar zinc concentration (Huang et al. 2002).

HZTL-1 (human ZnT-like transporter 1) shares 34% sequence identity to ZnT-1. Although it is classified in the CDF family, the topology of this protein is different from other members of this family. The number of predicted transmembrane domains is twelve for hZTL-1 whereas other members in the CDF family have six transmembrane domains. The functional activity of hZTL-1 is to facilitate zinc uptake. The localization of this protein in the apical membrane of Caco-2 cells suggests that it is a candidate transporter for zinc uptake (Cragg et al. 2002)

Another major zinc transporter family is the ZIP (ZRT-zinc regulated transporter, IRT-iron regulated transporter-like proteins) family. These proteins typically have eight membrane-spanning domains and an intracellular loop with a histidine rich region between transmembrane domains II and IV (Guerinot 2000). To date, four mammalian zinc transporters (hZIP-1 to hZIP-4) have been identified. ZIP proteins appear to function either by transporting zinc into cells or facilitating intracellular zinc movement. Both hZIP-1 and hZIP-2 increased zinc uptake of human K562 erythroleukemia cells when they were respectively transfected into these cells (Gaither and Eide 2000, Gaither and Eide 2001). Both transporters were localized on the plasma membrane of the K562

cells, however, hZIP-1 was found in intracellular vesicles in adherent cells such as COS-2 and other epithelial cells (Milon et al. 2001). HZIP-4 has been identified in a Jordanian family as the AE gene whose mutation leads to acrodermatitis enteropathica (section 2.3.2). This transporter is proposed to be involved in the process of intestinal zinc absorption (Wang et al. 2002).

Beside the above specific zinc transporters, nonspecific metal-ion transporters such as divalent cation transporter 1 (DCT-1) also participate in the maintenance zinc homeostasis. This protein is found in the crypts and villi of the duodenum and facilitates the absorption of a broad range of divalent metal ions including Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} etc. Iron deficiency upregulates the expression of DCT-1 (Gunshin et al. 1997). A schematic illustration of the postulated functions of these zinc transporters in a cell is shown in Fig. 2.1.

2.3 Prevalence of zinc deficiency

2.3.1 Malnourishment induced zinc deficiency

Zinc deficiency has been regarded as a general public health problem with women and children being especially prone to low zinc status (Sandstead 1995). In developing countries, populations that subsist on high starch vegetables and cereal proteins have a limited zinc consumption. Thus, they are at high risk for zinc deficiency. On the other hand, in developed countries, the variety and abundance of the food supply seems to prevent zinc deficiency. However, sub-optimal zinc status still presents in these nations due to inappropriate food selection (Hambidge 1997).

Reports of food consumption from the United States Department of Agricultures (USDA) indicate that the average zinc intake in children from age 1-10 years is 7.5 mg

which is lower than the RDA (10 mg) in the United States (Sandstead 1995). Thus many children may be at risk for zinc deficiency if their ability to utilize zinc is less than optimal. Reports from other countries suggest that zinc deficiency in children is common throughout the world. Seventy percent of school children assessed in northeast Thailand had low serum zinc concentrations (Udomkesmalee et al. 1990) and vitamin A and zinc supplementation synergistically improved the visual abilities of these children (Udomkesmalee et al. 1992). Supplementation of Chinese pre-school children with zinc increased their growth rate (Chen et al. 1992). The height-for-age Z score in zinc supplemented children from Ecuador was significantly higher than that of a placebo group (Dirren et al. 1994). In urban India, daily zinc supplementation reduced the incidence of diarrhea among children with low plasma zinc concentrations (Bhandari et al. 2002, Sazawal et al. 1997). Similarly, zinc supplementation also significantly reduced the percentage of children suffering from diarrhea among rural Guatemalan children (Bhutta et al. 1999, Ruel et al. 1997). Pooled analysis of randomized control studies suggests that zinc supplementation substantially reduces the incidence of diarrhea and pneumonia in developing countries (Bhutta et al. 1999). It is estimated that millions of children worldwide have compromised health that is related to zinc deficiency (Black 1998).

Pregnant women are another group of people at risk of zinc deficiency. An additional 375 mg zinc is required to meet the needs of fetal growth (Prasad 1996). Therefore, the RDA for pregnant women is 15-19 mg. Nevertheless eight out of 10 studies indicated an average zinc intake of 9-11 mg in pregnant women from a variety of cultural backgrounds such as Lebanese, Mexican-American, vegetarians and middle-

income Americans (Apgar 1985). Many congenital malformations have been associated with zinc deficiency. For example, plasma zinc levels in women bearing fetuses with neural tube defects were lower than those in women with healthy fetuses (Cavdar et al. 1988). Furthermore, randomized controlled zinc treatment trials indicate that zinc deficiency maybe common in women of childbearing age. Reduced incidence of premature delivery, hypertension, perinatal death, and spontaneous abortions have been reported in several zinc supplementation studies (Cherry et al. 1989, Hunt et al. 1984).

2.3.2 Genetic disorder-related zinc deficiency

Besides malnourishment-induced zinc deficiency, two types of rare autosomal recessive genetic mutations affect zinc status. One of them is acrodermatitis enteropathica (AE) diagnosed in humans, cattle, and dogs (Barnes and Moynahan 1973, Jezyk et al. 1986, Machen et al. 1996). Clinical symptoms of this disorder are very similar to severe dietary zinc deficiency including acute dermatitis, alopecia, respiratory tract infection, diarrhea, and arrested growth (Van Wouwe 1989). Manifestation of this disorder usually occurs during early infancy. Large doses of zinc ameliorate the clinical symptoms (Weismann et al. 1979). Defective zinc absorption in the intestine of AE patients has been reported (Lombeck et al. 1975, Weismann et al. 1979) and fibroblast cells from AE patients also exhibit reduced zinc uptake and low zinc content (Grider and Young 1996). Recently, genomic studies of consanguineous families with AE patients located the mutant genes to the telomeric region of chromosome 8q24.3 (Wang et al. 2001). A candidate gene, hZIP-4, has been identified in this region. Eight missense mutations of this gene were discovered in AE patients (Wang et al. 2002). Sequence alignment of hZIP-4 and other human zinc transporters indicate that it is a potential zinc-

uptake protein. Localization of this protein at the apical side of mature mouse enterocytes further supports a role for hZIP-4 in zinc uptake (Wang et al. 2002).

The lethal milk (lm) mutation arose spontaneously in C57BL/6J mice. Pups consuming the milk of *lm/lm* dams developed characteristic zinc deficient symptoms, and died before weaning (Lee et al. 1992, Piletz and Ganschow 1978). Administration of zinc to either the dams or the pups alleviates the symptoms and reduces lethality (Erway and Grider 1984). Over eight month of age, *lm/lm* mice exhibit systemic zinc deficiency. Congenital absence of utricular otoconia, i.e. calcium carbonate crystals in the inner ear is also found in *lm/lm* mice (Erway and Grider 1984). The utricular defect of the mutant mice may be caused by reduced zinc supply to the inner ear (Huang and Gitschier 1997). Carbonic anhydrase is the enzyme critical for generating calcium carbonate, a major component of otoconia. The activity of this enzyme depends on the availability of zinc. Reduced zinc supply in the mutant mice may decrease the activity of carbonic anhydrase which in turn, interferes with otoconia development (Purichia and Erway 1972). The mutant gene has been mapped to chromosome 2 and identified as ZnT-4, a transport protein responsible for zinc deposition into milk (Huang and Gitschier 1997). The lethal milk mutant has a nonsense mutation that introduces a stop codon in the place of arginine.

2.4 Zinc and mental health

Zinc actively participates in neurological processes. Several neurodegenerative disorders are negatively correlated with zinc metabolism in the brain such as Alzheimer's disease. The localization of chelatable zinc in the hippocampal region and the presence of cognitive disorders in zinc deficient humans and animals suggest a functional role for

zinc in memory and cognition. However, it is unclear how zinc participates in cognitive processes, and the limitations of some behavioral measures suggest that more rigorous studies are needed to define the aspects of memory that are responsive to nutritional zinc status.

2.4.1 Zinc distribution in the brain

Zinc is unevenly distributed in the brain with the olfactory bulb and the hippocampus containing the highest concentrations of zinc (Ono and Cherian 1999, Wallwork et al. 1983). Dynamic age-related changes in brain zinc levels have been reported (Kalinowski et al. 1983, Palm et al. 1990). Most of zinc in the brain is associated with proteins and forms the protein-metal complex pool. In this pool, zinc participates in both metabolic (e.g. catalytic) and non-metabolic (e.g. structural) reactions (Cuajungco and Lees 1997). In addition, approximately 5% of total brain zinc is in glutaminergic synaptic vesicles (Frederickson 1989), but not all glutaminergic neurons are zinc-containing (Frederickson et al. 2000). Neurons containing these synaptic vesicles, namely gluzinergic neurons, are histochemically reactive. Both TSQ (N-(6-methoxy-8-quinolyl-p-toluenesulfonamide)) fluorescent probe and neo-Timm's staining can sensitively detect loosely bound zinc in these synaptic vesicles (Danscher et al. 1985, Frederickson et al. 1987). Somata of these gluzinergic neurons are located in the cerebral cortex and amygdala nuclei (Casanovas-Aguilar et al. 1998, Christensen and Geneser 1995). The hippocampus is densely populated with gluzinergic boutons (including hilus of the dentate gyrus and stratum lucidum), lateral amygdala and the perirhinal cortex. No detectable gluzinergic neurons were found in the brain stem or cerebellum (Frederickson et al. 2000). ZnT-3 is exclusively expressed in the brain and is present in all zinc-

containing neurons. It is identified as the transporter responsible for sequestering zinc into the synaptic vesicles (Palmiter et al. 1996b). Metallothionein III (MT-III), a metal-sequestering protein expressed only in the brain, is found in the gluzinergic neurons at high density (Masters et al. 1994). However, this protein does not participate in transferring zinc into the synaptic vesicles since the vesicular zinc does not change in the MT-III knockout mice (Erickson et al. 1997).

2.4.2 Physiological function of zinc in the brain

The actual physiological function of synaptically released zinc is unknown. However, acute modulation of the activity of several neurotransmitter receptors such as glutamatergic, GABAergic, glycinergic, and purinergic receptors and voltage-sensitive cation channels (Na^+, K^+, and Ca^{2+}), and the Na^+/K^+-ATPase has been reported (Harrison and Gibbons 1994, Smart et al. 1994). Among them, two types of receptors are of particular interest, NMDA (N-methyl-D-aspartate) receptor and GABA (γ-aminobutyric acid) receptor. The close proximity of these receptors to the gluzinergic boutons in the hippocampus allows for released zinc ions to bind to them before dissipating (Vallee and Falchuk 1993). Zinc is co-released with glutamate and strongly modulates the post-synaptic NMDA receptor response. In cortical neuron cell culture, Zn^{2+} at low concentrations (<10 uM) affects the opening frequency of the NMDA receptor; at high Zn^{2+} concentration (10-100 uM), Zn^{2+} induces a fast block of NMDA channels (Christine and Choi 1990). This phenomenon may be associated with the binding of zinc at either high affinity or low affinity binding sites on the receptor (Vogt et al. 2000). Chelation of zinc enhances NMDA receptor-mediated excitatory postsynaptic current (EPSC) (Molnar and Nadler 2001). In addition, the GABA receptor is sensitive to micromolar amounts of

zinc (Frederickson et al. 2000). Endogenously released zinc suppresses the activity of the GABA receptor in the dentate gyrus of the hippocampus (Berger et al. 1998, Gordey et al. 1995). Besides the modulatory effect of zinc on postsynaptic receptors, zinc has been postulated to regulate the storage of glutamate in the vesicle by either increasing its storage capacity or by slowing down the rate of release. Another possible role for zinc is to regulate the metabolism of postsynpatic neurons following uptake (Frederickson et al. 2000). These functions are not firmly established due to the lack of direct evidence.

2.4.3 Zinc and neuro-toxicity

Cytosolic accumulation of zinc in degenerating hippocampal neurons has been reported during ischemia, seizures and brain trauma (Choi 1996, Frederickson 1989, Tonder et al. 1990). Vesicular zinc has been postulated to contribute to neuronal injures. Firstly, the depletion of zinc from the presynaptic vesicles was observed after excitotoxic injuries (Sloviter 1985, Suh et al. 1999). Secondly, the appearance of zinc surrounding the dying neurons was confirmed (Suh et al. 2000). Thirdly, the chelation of extracellular zinc during and after ischemia and brain injury was neuroprotective (Koh et al. 1996, Suh et al. 2000). Finally, vesicular zinc translocation into the postsynpatic neurons has been observed in electrically stimulated hippocampal neurons (Li et al. 2001b). However, the vesicular zinc cannot be the only source of zinc. ZnT-3 knockout mice (devoid of vesicular zinc) and wild type mice responded similarly to kainate-induced seizure. Accumulation of zinc in damaged neurons was also detected in ZnT-3 knockout mice (Lee et al. 2000) and was suggested that the zinc might have come from extracellular pools.

In addition, zinc is implicated in Alzheimer's disease (AD). The senile plaques and cerebrovascular β-amyloid deposits in AD patients are rich in loosely bound zinc (Constantinidis 1990). In vitro studies indicate that zinc can induce aggregation of synthetic Aβ proteins (Bush et al. 1994, Huang et al. 1997). Chelation of zinc by metal chelators increased the solubility of Aβ precipitates (Gnjec et al. 2002). Heterogeneous Aβ protein precipitates are found in the cerebral cortex of AD patients. Thus it seems that abnormal mobilization of cellular zinc in the brain contributes to the formation of AD. On the other hand, Aβ amyloid accumulation has been associated with elevated oxidative stress surrounding the plaques (Hensley et al. 1994). Zinc has been shown to quench the excessive production of H_2O_2 by the amyloid proteins. Therefore, the actual role of zinc in the development of AD needs to be further clarified (Huang et al. 2000).

2.4.4 Vesicular zinc and cognition

Numerous studies have shown that the hippocampus is responsible for processing and encoding spatial information (Brandeis et al. 1989, Hodges 1996). Patients with hippocampal lesions are unable to form new memories, yet old memories remain intact (Squire and Zola-Morgan 1991). Selective removal of the hippocampus by ibotenic acid injection in rats impairs their performance in tasks that require the utilization of spatial and contextual information (Jarrard 1993). NMDA receptor agonist and GABA receptor antagonists improve memory processing in the hippocampus (Farr et al. 2000). Activation of long-term potentiation (LTP) is necessary for information storage (Miller and Mayford 1999). The modulatory role of zinc in these receptors has been discussed previously. The dependence of LTP formation on zinc in the CA3 region of the hippocampus has been reported (Li et al. 2001a). Thus, the special positioning of

vesicular zinc and the regulatory role of zinc in the hippocampus suggests that zinc may participate in cognitive function.

In situ binding of vesicular zinc with nanomolar amounts of diethyldithiocarbamate or sodium sulfate disrupts the spatial-working memory of rats in a time-dependent manner (Frederickson et al. 1990). This experiment supports a role for vesicular zinc in cognitive function. However, these drugs have significant side effects, such as inactivating metal-dependent enzymes. Consequently the observed behavioral defects could be associated with these side effects, making the conclusion tentative. ZnT-3, a zinc transporter, from the cation diffusion facilitator family, sequesters zinc into the synaptic vesicles (Palmiter et al. 1996b). ZnT-3 knockout mice exhibit the absence of histochemically reactive zinc in the brain. Although one would expect significant impairment of their cognitive function, their spatial learning, memory and sensorimotor functions are not affected (Cole et al. 2001). In addition, the spatial memory of metallothionein III deficient mice is not impaired. The histochemically reactive zinc level of these knockout mice is normal but the zinc concentrations of the hippocampus, cortex, brainstem, thymus, and spinal cord are reduced (Erickson et al. 1997). The absence of an effect of reduced zinc on cognition in these mice suggests that compensatory mechanisms may be involved in maintaining normal brain function. Consequently, the specific roles for zinc in cognitive function have yet to be determined.

2.4.5 Zinc and cognition in humans

Mental health disturbances associated with zinc deficiency in human subjects have been extensively reviewed (Sandstead 2000). Cerebellar dysfunction, lethargy and short-term memory impairment have been reported in patients excreting large amounts of

zinc following histidine treatment (Henkin et al. 1975). Patients that are zinc-deprived due to parenteral feeding exhibited abnormal behaviors (Kay et al. 1976). A Saudi boy suffering from acrodermatitis enteropathica exhibited cerebellar atrophy (Ohlsson 1981). These disorders were alleviated by zinc administration. Although these severe zinc deficiency cases are rare among the population, they suggest a correlation of zinc status and mental health.

Supplementation of zinc in very-low-birth weight infants improves their growth rate and motor development (Friel et al. 1993). Studies on malnourished infants and toddlers in India and Guatemala indicate that their motor skills are improved after zinc supplementation (Bentley et al. 1997, Sazawal et al. 1996). Recognition memory, reasoning and psychomotor functions were improved in 6 to 9 year-old first graders treated with zinc and micronutrients in China (Penland et al. 1997, Sandstead et al. 1998). On the other hand, it has been reported that no obvious beneficial effect of zinc supplementation on cognition was found in prenatal (Shah and Sachdev 2001), infant (Hamadani et al. 2001), or school age children (Cavan et al. 1993). Discrepancies in human zinc studies may be due to complications with other nutrient deficiencies (e.g. iron and iodine etc.), insensitive tests to assess zinc status, differences in the social context in which the children were tested between studies, and sensitivity of cognitive methodology among different studies (Black 1998).

2.4.6 Zinc deficiency and animals

Although human and molecular biology studies support a potential role for zinc in cognitive function, careful evaluation of the published results has revealed problems that confound the interpretation of the data. These problems are related to (i) dietary

treatments, (ii) the nature of the tests, and (iii) the analysis of results. Hence, more rigorous studies are needed to define the relationship between zinc and memory.

2.4.6.1 Dietary treatment

In animal behavioral studies, appropriate controls are critical for the judgment of behavioral abnormality. Zinc deficiency causes anorexia in experimental animals (Shay and Mangian 2000). Therefore, a pair-feeding group (PF) is often used in addition to an ad libitum control (CT) to determine if any behavioral alterations are due to the lack of zinc itself or to caloric restriction. The general practice of pair-feeding is to feed rats the amount of diet consumed by the zinc-deprived group (ZD) one night before. However, the feeding schedule of food restricted animals affects the circadian rhythm of their corticosterone pattern (Gallo and Weinberg 1981). Moreover, the severity and length of food restriction determines the degree of their corticosterone elevation (Garcia-Belenguer et al. 1993, Gursoy et al. 2001, Stewart et al. 1988). Zinc deprivation also elevates corticosterone in rodents (DePasquale-Jardieu and Fraker 1979, Park et al. 1989). However, the corticosterone elevation in the ZD animals maybe independent of the reduced food intake since corticosterone elevation was observed before food intake decreased in mice (DePasquale-Jardieu and Fraker 1979) and force fed ZD rats exhibited corticosterone elevation (Park et al. 1989). Elevated stress impairs cognitive performance in animals (Bedi 1992, McLay et al. 1998, Nishimura et al. 1999). Therefore, the stress responses of the ZD and PF animals in the behavioral test should be considered. However, most of the animal behavior studies with dietary zinc restriction have not considered the stress response. Furthermore, several studies only use either the PF group which has limited access to diet with normal zinc levels (Caldwell et al. 1970,

Caldwell et al. 1973, Hesse et al. 1979) or the CT group as the control (Massaro et al. 1982, Takeda et al. 2001).

2.4.6.2 Nature of the tests

The behavioral test results for dietary zinc-deprived rodents are summarized in Table 2.3. Since all cognitive behavior tests require that the animal response to a certain stimuli, its emotional state and physical activity affect its performance. The open field test has been widely used to test the emotionality of rodents. In this test, an animal is placed in the center of a large bright arena for a fixed amount of time. The floor is usually marked with large squares to define the movement of rats. Locomotion is measured by a number of parameters such as total distance moved, time spent in the center or outer area and the number of fecal boli left. Post-weanling young rats that have been deprived of zinc for 5-6 weeks consistently exhibit reduced movement and traverse the squares less frequently than controls (Caldwell et al. 1970, Gordon 1984, Hesse et al. 1979). These observations seem to suggest that ZD rats exhibit increased emotionality since it was believed that the fear of novelty correlates with reduced ambulation (Broadhurst 1969). However, this conclusion is debatable. Firstly, lethargy was observed in ZD rats, which contributes to reduced mobility (Caldwell et al. 1970, Hesse et al. 1979). Secondly, the validity of using ambulation in the open field as a measure of anxiety was challenged since this parameter gave paradoxical results with different types of anxiolytic and anxiogenic drugs (Ramos and Mormede 1998). The movement of a rat in the peripheral area relative to the central area (thigmotaxia) was suggested to be a better measure of anxiety (Ramos and Mormede 1998). Another experiment tested the immobility of zinc-deprived rats after air-puff into the eyes and found the zinc deprived

rats freeze much longer than the pair-fed controls (Hesse et al. 1979). The hyper-responsiveness of zinc-deprived rats to the mild stress suggests either an increase in sensitivity to the stimulus or an increased level of emotionality in these rats. Regardless of the lack of a clear delineation of emotional state of these rats, it is obvious that ZD rats exhibit reduced physical activity in open field tests.

In studies using previously zinc-deprived rats (PZD), zinc deprivation was given during gestation and/or lactation and behavioral tests were performed on the pups after they were repleted by consumption of a zinc-supplemented diet. Only two studies in the literature recorded their open field activity. One study found that ambulation was reduced in PZD rats mildly deprived of zinc during gestation and lactation (Caldwell et al. 1973). The other study did not find any open field behavioral changes in PZD rats that were zinc-deprived during the third phase of gestation (Halas et al. 1977). The lack of consistency of these observations may be caused by the difference in the length of zinc deprivation and repletion. However, the paucity of information prevents a definitive conclusion concerning the physical activity and emotional state of PZD rats.

The active avoidance test requires that a rat learns to associate a sound or light, i.e. conditioning stimulus (CS) with an electric shock, i.e. unconditioning stimulus (US). The level of active response measures learning ability (e.g. jumping onto a platform to avoid the subsequent US when the CS is given). In the passive avoidance test, a rat is shocked upon entering a naturally preferred location such as a dark chamber. The level of inhibition of the natural tendency after training is a measure of learning ability. Both types of test have been applied in zinc deprivation studies (Halas and Sandstead 1975, Hesse 1979, Takeda et al. 2000, Tinius et al. 1986). Since electric shock aversively

motivates the learning performance of the rat, its sensitivity and emotionality towards the shock needs to be determined before the test (Sprott and Stavnes 1975). None of the previous avoidance tests took the threshold of the shock response into consideration. Various levels of current ranging from 0.5 mA to 3 mA were used by different studies but the electrical intensity was fixed for all rats within each study. Physical inactivity of the ZD rats was observed during the habituation phase (Halas and Sandstead 1975) which was consistent with the observations of the open field studies. Therefore, the reduced responsiveness of the ZD group in the avoidance test cannot be solely explained by learning deficits (Caldwell et al. 1973, Halas and Sandstead 1975). In addition, the passive avoidance studies yielded contradictory results. Two studies reported that the ZD group exhibited a longer latency in the test (Hesse et al. 1979, Tinius et al. 1986) whereas a shorter latency of the ZD rats was reported by a more recent study (Takeda et al. 2000). Paradoxical reports and potentially confounding factors during the avoidance tests suggest that the effect of zinc deprivation on avoidance learning is inconclusive. A more stringent experimental design is required to control for the motivation of different dietary groups and to determine the effect of zinc deprivation on avoidance learning.

The Skinner box is an associative learning test with appetitive incentive i.e. food reward. If a rat presses a bar following the correct audio or visual cue, it is rewarded with a food pellet. In a concurrent zinc deprivation study, i.e. the animals were tested when they were on zinc deficient diet, food pellets with normal zinc concentration were given as the reward for bar-pressing behavior (Massaro et al. 1982). This practice certainly disrupted the zinc status of the ZD group. The degree of change due to the behavioral test however was unknown since non-tested animal tissues were utilized for assessing the

zinc status in that study (Massaro et al. 1982). In the behavioral tests, discrimination ability was assessed by counting the number of correct bar pressing following visual and audio signals. This test requires an animal to learning the association between the signals and food reward. Transferring test measures the ability of utilizing the learned food-cues association to solve an auditory discrimination task. The behavioral test indicates that the ZD rats performed inferior to the CT group in the transferring test but superior to the CT group in the discrimination test. The transferring test results were interpreted by the authors as learning impairment due to zinc deprivation whereas the superior performance in the discrimination test was suggested to be an increment of food motivation in the ZD rats. The justification seems dubious considering that the total duration of all tests was seven days and normally zinc deprivation causes an anorexic, rather than orixigenic, effect. The validity of applying this type of behavioral test for concurrent zinc-deficient studies is questionable. Another application of the Skinner box was to test the long-term memory of a tone-shock association established during lactation, the stage of life when zinc deprivation was applied to dams (Halas et al. 1979). The pups were given a sound followed by a electric shock at the age from 11 to 20 days. They were tested as young adults (53-62 days), if they remembered the contingency of the tone-shock then they would refrain from pressing the bar in the Skinner box upon hearing the tone. The ZD pups exhibited less associative responses in adulthood that controls when the tone-shock training was given at either 17 or 19 day of age. Whether this observation was truly caused by long-term memory deficits in the ZD rats is debatable. Normal rats did not show any memory responses for the association until the training was given after day 17 of age. A rat has to be mature enough to process the tone-shock associative information.

It has been well established that zinc deficiency retards growth. Therefore, the conclusion of impaired long-term memory deficit in these PZD rats cannot be the sole interpretation of their behavioral alteration. An alternative explanation could be that these PZD rats were unable to establish the tone-shock association early in life. In addition, the integrity of hearing ability was not tested in the PZD rats. Thus a hearing deficit could be another explanation for their non-responsiveness.

The Lashley III water maze requires a rat to swim through a complex maze containing cul de sacs and T choices (Denenberg et al. 1990). The fewer number of errors, the better the learning ability. One study tested gestational zinc-deprived rats at 45 days of age. These PZD rats did not behave significantly different from the CT group (Caldwell et al. 1973). Another zinc deprivation study found that the ZD group made more errors than the PF group in the maze learning (Caldwell et al. 1970). However, in the same test, swim latency was significantly increased in the ZD group. Thus, the possible explanation for the inferior performance of the ZD rats should not be limited to cognitive impairment. Reduced motivation could also contribute to the altered behavior of these rats.

Tolman-Hozik maze (Lokken et al. 1973) and T-water maze (Gordon 1984) have also been used to test the learning ability of zinc deficient animals. In the T-water maze, a rat needs to establish the association between the color of the arm and the location of the platform. The performance of the ZD rats in the T-water maze was poorer than that of controls suggesting that associative learning was impaired in these animals (Gordon 1984). However, this behavioral test was used in only one zinc depletion study, and the results need confirmation. Furthermore, analysis of the data from Tolman-Hozik maze is

susceptible to the 'pooling fallacy', discussed in section 2.4.6.3. It is questionable whether the behavioral influence of zinc deficiency in this study is significant.

Spatial cognition has been evaluated by the radial armed maze (RAM) and the Morris water maze (MWM). Although both tests are sensitive measures of hippocampal integrity, the range of cognitive processes involved in these tests may not be the same. Allocentric visual cues beyond the water pool are utilized by a rat to form a cognitive map in the MWM, whereas RAM allows the animal to use associative learning and other strategies to solve the maze (Hodges 1996).

The RAM uses food pellets as the reward. The design requires equal food motivation to perform the experiment. In the maze, half of the arms are baited with food pellets whereas the rest are empty. Reentries into the arms already visited within a trial provide the measure of short-term memory. Entries into the never-baited arms are counted as long-term memory error (Halas et al. 1983). Cyclic anorexia of the ZD rats and increased appetite of the PF rats during dietary treatment has been well-documented (Cannon et al. 1988, Selvais et al. 1997), and this test is not applicable for concurrent zinc deprivation studies. For PZD studies, food motivation increases in both the ZD and PF rats (Halas et al. 1980, Peters 1979). Consequently the radial maze performance is complicated by altered food motivation.

The MWM test was recently used to test the short-term memory of ZD rats (Keller et al. 2000, Keller et al. 2001). Short-term memory deficit in ZD rats was age-dependent. Young rats (44 to 47 day of age) fed a zinc-deficient diet for three weeks exhibited significantly longer retrieval latency for a submerged platform compared to the CT and PF groups. Zinc supplementation corrected the water maze impairment. Zinc

deprivation started at an older age induced minor or no behavioral impairment. In these studies, two releasing points were used and one of them was used more often than the other. This practice could train the rats to effectively use a response strategy such as always turning right from the starting location to find the escape platform (Gallagher et al. 1993). Therefore, the experimental design compromises the measurement of spatial cognition. In addition, the MWM is an aversively motivated behavioral test and is stressful to the rats. Elevated anxiety or stress can substantially affect water maze performance, which is independent of cognitive mechanisms that underlie spatial navigation (Hodges 1996). Although fecal boli were used as an indicator of stress, the stress responses were not quantified previously.

2.4.6.3 Analysis of results

An important assumption of statistical analysis is that data points are independent observations. One subject contributes only one data point when the treatment effect is evaluated (Martin and Bateson 1993). Unfortunately, this basic assumption of independence was violated by several zinc deprivation and cognition studies (Halas et al. 1983, Halas et al. 1986, Halas and Sandstead 1975, Halas and Sandstead 1980, Lokken et al. 1973). For instance, in the radial armed maze studies, the degree of freedom (df) associated with variation among dietary treatment changed along with the number of trials pooling into a block (Halas et al. 1983, Halas et al. 1986). In one experiment, the total number of rats was 24 with 8 rats in each dietary group. When 3 trials were pooled into one block for the reference memory measurement, the df associated with treatment was 69 (calculated from 24 x 3 – (3-1) –1) which suggests the three repeated trials of a rat were treated as three independent observations after pooling. When 10 trials were pooled

for the working memory measurement, the df was 237 (calculated from 24 x 10 –(3-1)-1) i.e. 10 repeated trials of a rat were pooled as if they were 10 different rats. The appropriate statistical analysis should use repeated measure analysis of variance and the df of dietary treatment should consistently equal to 21 (calculated from 24-(3-1)-1). This malpractice is called 'pooling fallacy'. Computer simulations suggest that this 'pooling' practice increases the probability of falsely rejecting a true null hypothesis i.e. type I error. The probability may increase by 10 times in many cases (Machlis et al. 1985). Thus, data from these reports have to be reanalyzed with the appropriate statistical analysis before their conclusions can be accepted. Since the information on df is not available in every paper, a great chance of erroneous statistical analysis exists. Considering that 5 out 19 research articles on zinc and cognition using the rodent model failed to apply the appropriate statistical analysis, the question of how zinc affects cognition has not been conclusively addressed.

In summary, behavioral studies demonstrate that zinc deprivation alters the behavior of a rat in cognitive tests. However, problems associated with inappropriate control groups, inadequate consideration of confounding factors such as stress and motivation, and erroneous statistical analyze prevent a definitive conclusion on the role of zinc deprivation in animal cognition.

2.5 Zinc and gene regulation

Zinc is involved in different aspects of genetic stability and gene expression in cell metabolism. Up-to-now, many gene products participating in a variety of physiological processes are known to be regulated by zinc. Metallothionein (MT) is the earliest discovered protein whose expression is highly regulated by zinc in the body

(Heuchel et al. 1994). The mechanism of zinc-induced transcription of MT is well established. Zinc binds to metal-transcription factor-1 (MTF-1) which recognizes metal response element (MRE) on the promoter region of MTs and activates the transcription of MT (Cousins 1994, Searle 1990). MTs are responsible for detoxification of heavy metals and free radicals (Min et al. 1991, Thomas et al. 1986).

Another zinc responsive gene is macrophage colony stimulating factor (M-CSF). Zinc increases the mRNA expression of M-CSF in a dose-dependent manner and this process is mediated through MT expression (Kanekiyo et al. 2002). M-CSF controls the maturation of monocytes and macrophages and modulates the inflammatory response of host cells (Wang et al. 1988). Zinc depletion reduces the expression of insulin-like growth factor-I (IGF-I), growth hormone receptor (GHR), and growth hormone binding protein (GHBP) in rats (Ninh et al. 1995). The suppressive effect of zinc on these gene products is related to the subnormal linear growth observed in zinc deficient animals and children (Giugliano and Millward 1984, Prasad 1988).

Leptin and neuropetide Y (NPY) gene expression is regulated by zinc status. It was found that the abundance of leptin mRNA is reduced in the adipose tissue of the ZD rats whereas hypothalamic NPY was increased in these animals (Lee et al. 1998, Ott and Shay 2001). The reduction of leptin has been suggested to be associated with reduced fat depot in the ZD animals (Ott and Shay 2001). NPY is a potent orixigenic peptide. Reduced circulating leptin promotes NPY synthesis in ZD animals (Lee et al. 1998). The apparent conflict between the elevation of NPY and anorexia has been described as 'NPY resistance'. This phenomenon may be related to the reduced sensitivity of NPY receptors.

The expression levels of several zinc transporters such as ZnT-1, 2 and 4 are differentially regulated by dietary zinc (Liuzzi et al. 2001, McMahon and Cousins 1998). Their regulation implies that zinc homeostasis in organs and tissues is possibly maintained through these zinc-sensitive transporters. In the brain, dietary zinc deficiency decreases the concentration of NMDA receptors on the cortical synaptic membrane (Browning and O'Dell 1995).

A global search for zinc responsive genes has been recently carried out. Differential display uses RT-PCR to generate cDNA from mRNA templates and identifies the genes that increase or decrease in quantity under various zinc conditions. In one study, 47 differentially expressed mRNAs were found in intestinal cells of dietary zinc-deficient rats. Three genes regulated by zinc were confirmed i.e. cholecystokinin, uroguanylin, and ubiquinone oxidoreductase (Blanchard and Cousins 1996). A microarray has been used to search for differential gene expression in the thymus of ZD mice. Several gene products, including myeloid cell leukemia sequence-1, DNA damage repair and recombination protein 23B, the mouse laminin receptor, and the lymphocyte-specific protein tyrosine kinase (LCK) were identified (Moore et al. 2001).

Proteomics is another technical approach that can be used to reveal the regulatory role of zinc in gene expression is utilization of. This method involves quantitative comparison of protein profiles from experimental samples. Two-dimensional gel electrophoresis is used to separate the proteins. The proteins that are differentially expressed are excised and digested with trypsin. Mass spectrophotometry is used to identify these proteins. The effect of dietary zinc depletion on the protein profile of the

hippocampus could provide informative clues to understanding the role for zinc in the hippocampus.

2.6 Research project

Two major aims were pursued in the current research project. The first aim was to 1) Extend the behavioral studies on MWM performance of young CT, PF and ZD rats to include the testing of different memory components, short-term memory, long-term memory and learning ability. Considering the aversive nature of swimming in the water maze, the stress responses of animals before and after the maze tests were also monitored. The second aim was to compare the protein expression pattern of rat hippocampi from different dietary groups using two-dimensional gel electrophoresis and to identify the candidate proteins that are regulated by dietary treatment.

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Table 2.1 Recommended daily allowance (RDA) of zinc for human (Food and Nutrition Board and Institute of Medicine 2002).

Age group	RDA of zinc (mg/day)
Infant, 0-6 months	2.0 *
Infant, 7-12 months	3.0
Children, 1-3 years	3.0
Children, 4-8 years	5.0
Children, 9-13 years	8.0
Boys, 14-18 years	11.0
Girls, 14-18 years	9.0
Men, >19 years	11.0
Women, >19 years	9.0

* Adequate Intake

Table 2.2 Mammalian zinc transporters

Transporters	Species	Distribution	Localization	Function
CDF family				
ZnT-1	rat/ mouse	Ubiquitous	Plasma membrane	Zinc efflux (Palmiter and Findley 1995)
ZnT-2	rat/ mouse	Intestine, kidney, testis	Intracellular vesicles	Zinc compartmentalization within cells (Palmiter et al. 1996a)
ZnT-3	mouse	Brain, testis	Synaptic vesicles	Sequester zinc into synaptic vesicles (Palmiter et al. 1996b)
ZnT-4	rat/ mouse /human	Mammary gland, heart, liver	Intracellular vesicles	Zinc compartmentalization within cells; Zinc deposition into milk (Huang and Gitschier 1997, Michalczyk et al. 2002, Murgia et al. 1999)
ZnT-5	human	Ubiquitous	Intracellular vesicles	Zinc transport into secretory granules of pancreatic beta cells (Kambe et al. 2002)

ZnT-6	Mouse/ human	Liver, kidney, brain, small intestine	Trans Golgi apparatus	Zinc transport between trans Golgi apparatus, and cytoplasmic vesicles (Huang et al. 2002)
hZTL-1	Human/ mouse	Ubiquitous	Plasma membrane	Zinc uptake (Cragg et al. 2002)
ZIP family				
hZIP1	human	Ubiquitous	Plasma membrane	Zinc uptake (Gaither and Eide 2001)
hZIP2	human	Prostate, uterine, epithelial cells	Plasma membrane	Zinc uptake (Gaither and Eide 2000)
hZIP4	human	Small intestine, kidney, colon	Plasma membrane	Zinc uptake (Wang et al. 2002)
*Nramp family**				
DCT-1	rat	Ubiquitous	Plasma membrane	Divalent cation absorption (Gunshin et al. 1997)

* Nramp family: natural-resistance-associated macrophage protein family

Table 2.3 Behavioral tests of zinc deprived rodents

ZD stage	Time of test	Behavioral consequence	Author's conclusion
Open field test			
PW	Day 33 of ZD	Traverse of square: PF>ZD	ZD rats performed like hyper-corticosterone rats (Hesse et al. 1979)
PW	Day 48 of ZD	Traverse of square: PF>ZD	ZD rats has increased emotionality (Caldwell et al. 1973)
PW	Day 49 of ZD	Latency to leave center: ZD>PF=CT Traverse of square: ZD<CT<PF	No comments (Gordon 1984)
A	Day 105 of ZD	Rearing: ZD<CT<PF Latency to leave center: ZD>PF=CT	
G	Day 45 for all three progenies	Traverse of square: ZD<PF=CT Grooming: ZD<PF<CT	ZD rats had increased emotionality (Caldwell et al. 1973)
G	75 or 105 days of age	Traverse of square, entrance to center and peripheral square, urine spots, fecal	No comments (Halas et al. 1977)

		boli: N/S	
Defensive immobility test			
PW	Day 33 of ZD	ZD immobilized longer than PF upon air puff into the eyes	ZD rats are hyper-responsive to the mild stress (Hesse et al. 1979)
Active avoidance test			
G	Day 65 for 2nd and 3rd progenies	Avoidance latency: ZD>CT % Avoidance response: ZD=CT in 2nd progenies; ZD<CT in 3rd progenies	Impairment in ZD rats (Caldwell et al. 1973)
G	60 days of age	Avoidance conditioning response: CT>PF>ZD, latency: CT=PF<ZD; Extinction response: CT<PF=ZD, latency: CT<PF=ZD Inter-trial response: CT>PF=ZD, latency: ZD>PF	Physical activity and learning ability of ZD and PF groups decreased (Halas and Sandstead 1975)
PW	Day 48 of ZD	For 1st 10 trials No. of response: ZD<PF	Reduced motivation and learning ability in ZD rats (Caldwell et al. 1970)

Passive avoidance test			
PW	Day 33 of ZD	No. of trials to reach criterion: ZD<PF	ZD rats were hyper-responsive to the mild stress (Hesse et al. 1979)
G&L	After rehab on rat chow	Pre-shock latency: CT=PF=ZD Retention latency: PF>ZD>CT	ZD and PF had enhanced reference memory (Tinius et al. 1986)
L	Four weeks after lactation	Pre-shock latency: CT=ZD Retention latency: ZD<CT	ZD rats learning ability is impaired
PW	4 or 12 weeks of ZD	Pre-shock latency: CT=ZD Retention latency: ZD<CT Zinc repletion eliminated the difference	ZD rats learning ability is impaired (Takeda et al. 2000)
G&L	79-90 day of age	Pre-shock latency: CT=PF=ZD Post-shock retention latency: CT=PF>ZD	Impaired performance in the ZD mice is associated with hippocampal damage (Golub et al. 1983)
Skinner box test			
PW	13 day of ZD	Non-performer: ZD>CT Trial to reach criteria transfer test: ZD>CT	Over-reactive to handling of ZD rats Impaired learning of ZD rats Increased motivation of ZD

		discrimination test: ZD<CT	rats (Massaro et al. 1982)
L/G	Tone-shock training at 11, 14, 17, 20 days old. Skinner box test after 42 days	Suppression ratio: Lactation groups: ZD>PF=CT in 17 and 20 days training subgroups. N/S in day 11 and 14 training groups. Gestation groups: N/S	Long term memory impaired in lactational ZD rats (Halas et al. 1979)
Lashley III water maze test			
G	Day 45 for all three progenies	ZD=CT	No comment (Caldwell et al. 1973)
PW	Day 48 of ZD	Cul-de-sac errors: ZD>PF Latency: ZD>PF	Learning impairment of ZD is confounded by lethargy (Caldwell et al. 1970)
Radial arm maze test			
L/G	100 days of age	trials to reach asymptote % correct response: Original training: ZD=PF>CT Reversal training: ZD>PF=CT	Learning impairment ZD>PF Long-term memory is not impaired Short-term memory is impaired in ZD (Halas et al. 1983, Halas et al. 1986)

		% correct response in all baited arm test (female): ZD<PF<CT	

Morris water maze test

PW & A	First week of zinc deprivation	Young rats: Retrieval swim latency : ZD>PF=CT Repletion of zinc eliminates the difference Old rats: ZD=PF=CT	ZD rats were impaired in the short-term memory (Keller et al. 2000, Keller et al. 2001)

Tolman Hozik Maze test

L	44 d of age	Running time: CT=PF=ZD Average full body error: ZD>PF=CT Average total error: ZD>PF=CT	Equal level of motivation ZD is impaired in acquiring the maze (Lokken et al. 1973)

T-water maze test

PW	ZD for 56 days and rehab for 42 days	Simple discrimination learning: N/S Simultaneous discriminate learning error: ZD>PF=CT	Learning deficit in ZD rats (Gordon 1984)

A : adult

PW : post-weanling

G : gestation

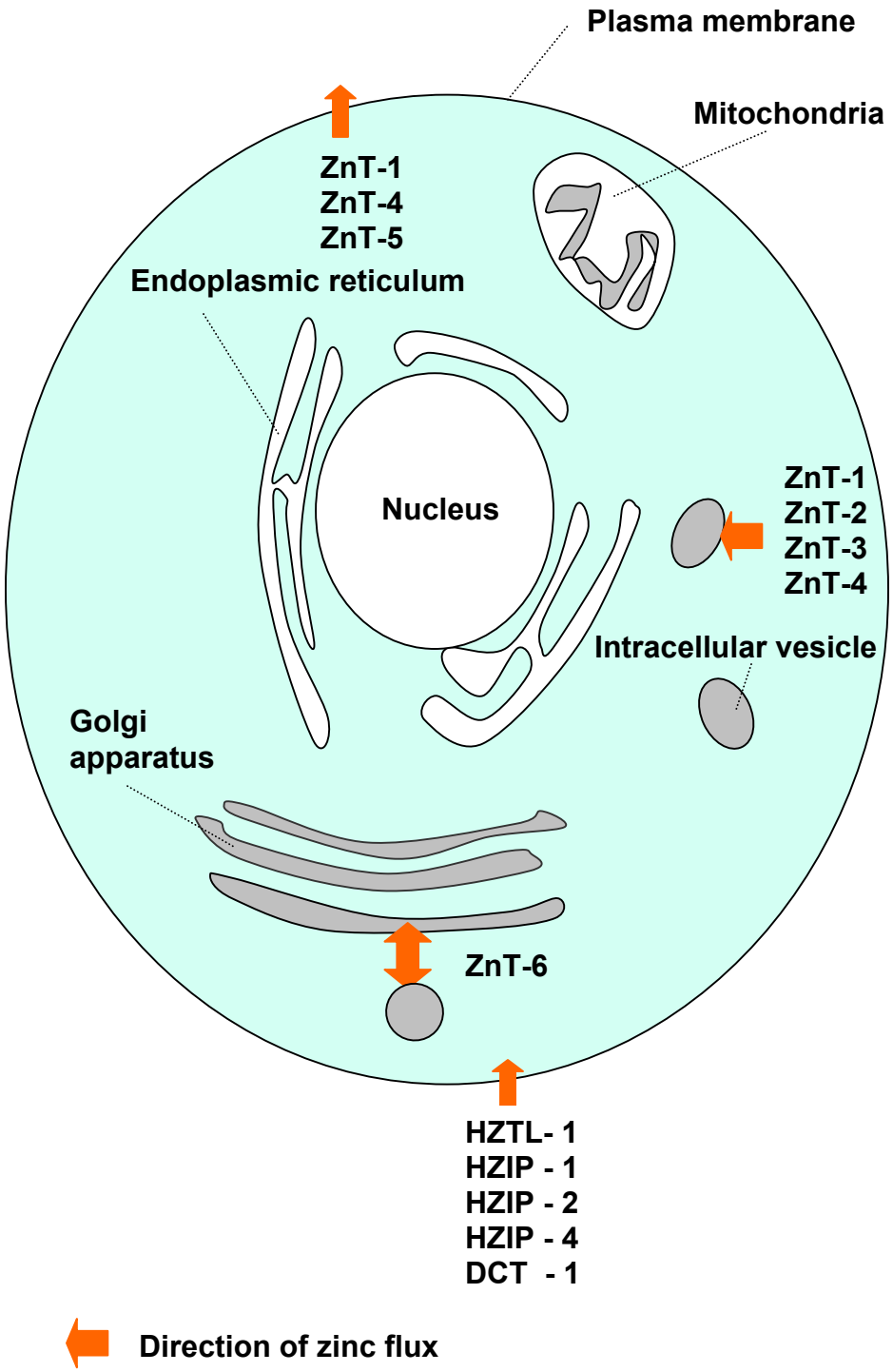
L : lactation

G/L : gestation or lactation

G&L : gestation and lactation

N/S : not significant

Fig. 2.1 Schematic illustration of zinc transporters in a cell.



CHAPTER 3

WATER MAZE PERFORMANCE AND SERUM CORTICOSTERONE IN MALNOURISHED RATS[1]

[1] Chu, Y., M.F. Mouat, R.B.S. Harris, J.A. Coffield and A. Grider. Revision to be submitted to *Physiology & Behavior*

Abstract

The aims of present study were 1) to evaluate the spatial learning, short-term memory and long-term memory of zinc-deprived and pair-fed rats, and 2) to monitor the stress response of these rats before and after swimming. Young Sprague-Dawley rats (age 27-31 d) consumed AIN93-G diet for 10 d, and then were separated into ad libitum control (CT), pair fed (PF), and zinc-deprived (ZD) groups. The zinc content of the diet was 25-30 ppm (CT and PF) or <1 ppm (ZD). After 17 days on their respective diets, a Morris water maze (MWM) was used to test spatial cognition. Delayed-matching-to-place test results indicate that both zinc deprivation and food restriction did not affect short-term memory. Learning however was significantly impaired in PF rats. Both ZD and PF groups exhibited thigmotaxic (wall-hugging) behavior during the learning and probing tests. The long-term memory test did not reveal any difference between treatments. Basal corticosterone levels before swim training were significantly elevated in the PF group. Both PF and ZD groups exhibited significantly elevated corticosterone levels after training. The data indicate that thigmotaxia and learning impairment of these rats in the water maze was associated with increased stress levels.

Introduction

Several studies have suggested a functional role for zinc in the brain. For instance, zinc supplementation improves cognitive behavior in school age children (Penland et al. 1997, Sandstead et al. 1998). Also, low-birth-weight infants exhibit improved growth rate and motor development upon zinc supplementation (Friel et al. 1993). At the molecular level, zinc is unevenly distributed in the brain with the highest concentrations in the olfactory bulb and hippocampus (Ono and Cherian 1999). In the

hippocampal region, zinc participates in neurotransmission. Glutamatergic vesicles in the mossy fiber region contain ionic zinc, which is co-released with glutamate upon stimulation (Frederickson et al. 2000). In vitro studies indicate that the $GABA_A$ (γ-amino butyric acid) and NMDA (N-methyl-D-aspartate) receptors, which participate in memory formation, are modulated by zinc (Li et al. 2001, Manzerra et al. 2001, Pang et al. 1998, Patenaude et al. 2001). However, cognitive studies using ZnT-3 knock-out mice devoid of vesicular zinc and MT-III knock-out mice with reduced zinc levels in the hippocampus (Cole et al. 2001, Erickson et al. 1997), cast doubt on the role of zinc in the memory process. Therefore, the neurophysiological function of zinc in the brain needs to be investigated further.

The cognitive behavior of young ZD rats has been evaluated by several groups and was found to be impaired using a variety of cognitive tests (Caldwell et al. 1970, Keller et al. 2000, Keller et al. 2001, Massaro et al. 1982, Takeda et al. 2000). However, factors other than hippocampal dysfunction may explain their results. The motivation, emotionality or activities of the animals potentially confound the interpretation of behavioral data. In addition, five out of twenty cognitive studies with zinc deprived rodents obviously violated the assumption of independent observation in data analysis (Halas et al. 1983, Halas et al. 1986, Halas and Sandstead 1975, Halas and Sandstead 1980, Lokken et al. 1973). Repeated measures were used in these studies but repeated trials were treated as independent measurements from different rats. This malpractice, namely 'pooling fallacy', increases the probability of falsely rejecting a true null hypothesis, a type I error (Machlis et al. 1985, Martin and Bateson 1993). Consequently, the conclusion from these studies cannot be readily accepted unless the data are re-

analyzed with appropriate repeated measure analysis. The effect of zinc deficiency on cognitive behavior, therefore, has not been addressed adequately.

Elevated physiological stress has been reported to affect the anxiety, activity, and cognitive performance of animals (Bodnoff et al. 1995, de Quervain et al. 1998, Holscher 1999, Korte 2001, McLay et al. 1998, Nishimura et al. 1999). Further, it has been shown that zinc deficiency elevates the stress response in rodents (DePasquale-Jardieu and Fraker 1979, Park et al. 1989). Zinc deficiency in young rats induces cyclic anorexia, consequently pair-feeding needs to be included as a control for the effect of reduced caloric intake. Caloric restriction, however, adversely affects cognitive function in both humans and animals (Avraham et al. 1996, Bedi 1992, Green et al. 1994, Hao et al. 2000, Polivy 1996) and involuntary food restriction increases corticosterone levels (Gallo and Weinberg 1981, Garcia-Belenguer et al. 1993). The degree of food restriction and the feeding schedule also alter the corticosterone circadian rhythm. Therefore, it is imperative to monitor the stress response of ZD and PF rats when implementing cognitive studies.

The purpose of the present study was to expand our previous investigations concerning short-term memory and zinc deprivation and to test for the effects of zinc deprivation on spatial learning and long-term memory (Keller et al. 2000). In addition, the response of the rats to swim stress was evaluated by measuring serum corticosterone levels.

Materials and Methods

Dietary schedule

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), 27-31 days old, were individually housed in stainless steel hanging cages in an AAALAC approved facility (humidity 40-60%, 12 hours light/dark cycle: 7:00 am light on, 7:00 pm light off). All rats were fed the AIN 93-G diet (either casein or egg-white based) containing 20-25 ppm zinc (Dyets, Bethlehem, PA) for the first 10 days of adaptation. Afterwards, rats were divided into three dietary groups (CT, PF and ZD). The CT and PF rats were offered the zinc-supplemented casein or egg white based diet as in the adaptation phase. Growth and behavior of these rats on different protein-based diets were compared. All ZD rats were fed AIN-93G egg-white based zinc-deficient diet with less than 1 ppm zinc (Dyets, Bethlehem, PA). The concentration of zinc levels in the diet was determined by atomic absorption (Johnson and Greger 1984). Because the anorexic eating patterns of the ZD rats were not synchronized, one-to-one pair feeding was implemented in all experiments. During the zinc depletion phase, rats were given de-ionized water to eliminate exposure to extra zinc from drinking water. Food was given at 9:00-10:00 am each day except on training and testing days when food was given at 1:00-2:00 pm after the behavioral test. All rats were tested in the water maze starting from day 17 of zinc deprivation. All procedures were approved by the Animal Care and Use Committee of the University of Georgia.

Water maze set-up

The circular pool was made of stainless steel (1.35 m diameter x 0.46 m deep). The inside was smooth and painted black with non-toxic paint to minimize intra-maze

cues. The depth of the water was 27 cm and the water temperature was maintained at 24 ± 1 °C. The water was made opaque by adding non-toxic black paint (Tempera, Heathrow, FL). A small platform of 10 cm diameter made of Plexiglas was covered with a piece of black cloth to increase the roughness on the surface, and immersed 1 cm below the water surface. The platform was invisible to the rats. In the testing room, the walls were decorated with different geometric figures, such as triangles or squares to provide extra-maze spatial cues. Behavior in the maze was recorded by a video-tracking computer system (Ethovision version 2.1, Noldus Information Technology). To disrupt any olfactory cues in the pool, the pool was wiped with 95% alcohol daily. In between each swim, the water was stirred gently to eliminate any olfactory cues arising from swimming tracks. When a rat finished its swim trials, it was dried with a towel and returned to its home cage. All swim tests were performed between 8:00 am and 1:00 pm.

Experiment 1. Delayed-matching-to-place (DMP) test

The testing procedure was adapted from Steel et al. (Steele and Morris 1999). Briefly, rats were trained in the water maze from day 2 to day 10 during the adaptation phase and tested from day 17 to day 23 of zinc deprivation. During each training day, the platform was inserted in one of six potential locations evenly distributed on two concentric circles relative to the pool (radius of 30 or 60 cm). The platform remained in a fixed location for one day of testing and moved to another location on the following day. Rats had four trials each day starting from each of four different releasing points (north, east, south, and west). They were required to locate the immersed platform as their only means of escape from the water. The inter-trial-interval (ITI) refers to the duration between the end of one trial and the initiation of the following trial. The ITI between

trials 1 and 2 was either 30 s or 2 h. These times were alternated across the training phase. The ITI between trials 2 and 3, and 3 and 4 was always 30 s. A rat was allowed 120 s to locate the platform. If it located the platform within this time, it remained on the platform for 15 s. If it was unable to find the platform, it was gently led to the platform and where it remained for 15 s before being removed from the pool.

At the beginning of the testing phase, the configuration of the platform locations, as described above, was rotated 45° clockwise from its previous configuration during training. The rats were then tested using the new platform locations. The testing procedure was the same as that for the training phase and was for four days. Mean swim distance (cm) and escape latency (s) were used to evaluate the performance of rats in this working memory test.

Experiment 2. Learning and long-term memory test

In this experiment, rats were screened for their performance on day 5 of adaptation. They swam four times to locate a fixed platform location. Rats who could not locate the platform on any of the four swims were excluded from the study (Youngblood et al. 1997). On day 15 of zinc deprivation, a 120 s probing test was given to each rat to disrupt the memory of the platform location formed during the screening test. The maze learning session started on day 17 of zinc deprivation. Rats in each dietary group were randomly assigned to four groups that searched for one of the four platform locations evenly distributed in the pool. Therefore, any quadrant bias in the water maze was minimized. The platform location was fixed for a particular rat throughout the learning phase, which lasted for three days. All rats received eight trials each day. They were released into the water from four releasing points. At each

releasing point, a rat had two trials with an ITI of 30 s. The second trial at one releasing point served as a reinforcement for spatial information acquired in the first trial. All rats searching for the same platform location were tested consecutively. The ITI between two releasing points was approximately 30 min. Two days after the learning test, rats were given the second 120 s probing test to evaluate their long-term memory of the platform location.

Learning efficiency of each day was evaluated by calculating the area under the curve of cumulative swim distance from the platform, swim distance, and swim latency, respectively. Since the reinforcement trials (even number trials) at each releasing point reflected the ability of rats to utilize the information acquired during the first trials (odd number trials), further analysis of the area under the curve of all the above parameters at both even and odd number of trials was performed. These parameters were plotted against the trials on each day and the area under the curve was determined (Youngblood et al. 1997). Swim latency reflects how fast a rat escapes from the water. Swim distance measures the length of swim path, which determines whether a rat is using the shortest route of escape from the water. The cumulative distance to the platform is the sum of distances between a swimming rat and the platform location measured every 1/6 second. The closer the rat swims to the platform, the shorter the cumulative distance. Thigmotaxia behavior was measured by calculating the thigmotaxic ratio, i.e. the time spent in the peripheral zone of the pool (10 cm from the pool edge) versus swim latency. The area under the curve of the thigmotaxic ratio for each day was calculated for statistical analysis. In the probing test, four imaginary zones (diameter 36 cm) were delineated concentric to the four platform loci. The ratios of time spent in the target and

opposite zones versus all four zones were determined to evaluate the long-term memory. The thigmotaxic ratio was also determined in this probing test.

Corticosterone measurement

To determine the corticosterone levels of rats before they were placed in their respective dietary groups, serum samples were collected on day 10 of adaptation. Rats were not disturbed for 12 h before the measurement and the experiment began at 8:00 am. Blood samples were collected by tail-bleeding (Liu et al. 1996). Time courses for the corticosterone response were measured during the two 120 s probing tests of experiment 2 starting from 8:00 am. Tail blood was taken before the probing test and 0, 30, 60, and 120 min after the test. The 0 min time point was in reality approximately 2 min after completion of swim due to the time required for drying the rat and transporting it from the swim room to the tail bleeding room. Blood samples were kept on ice until centrifugation at 3,000 x g for 10 min. Serum samples were stored at -70 ºC before measurement. Corticosterone in the serum was measured by a corticosterone ^{125}I RIA kit (ICN Biomedicals, Inc. Costa Mesa, CA) following the manufacturer's instructions.

Tissue analysis

On day 24 of zinc deprivation, rats were sacrificed by decapitation. Wet weights of the thymus and adrenal gland were measured. Cerebral cortex, hippocampus and cerebellum were dissected from the brain tissue. Zinc levels in the liver, femur, and the brain regions were determined by atomic absorption spectroscopy (AAS) (Johnson and Greger 1984).

Data analysis

Data were analyzed using one-way ANOVA or ANOVA with repeated measures (SAS version 8.2). Post-hoc analysis using least significant difference (LSD) t-test was applied when appropriate. Treatment effect with a p-value less than 0.05 was regarded as statistically significant.

Results

Effect of dietary treatment on growth

The effects of casein-based AIN-93G control diet and egg white-based AIN-93G control diet were evaluated by comparing the growth of the CT and PF rats fed on their respective diets. No significant diet effect was found in the CT ($F_{1,32}$=2.17, P=0.15), and PF ($F_{1,28}$=0.12, P=0.73) groups. In addition, the two control diets did not reveal any significant difference in the performance of rats in the water maze tests. Therefore, data collected from the casein and egg white groups were combined. The growth curve of the rats during the zinc deprivation stage is illustrated in Fig. 3.1. Two-way ANOVA with one repeated factor (day) was performed. Significant effects were detected in dietary treatment ($F_{2, 93}$=79.74, P<0.0001), day ($F_{21, 1953}$=1251.9, P<0.0001) and treatment x day ($F_{42, 1953}$=236.5, P<0.0001). The post hoc analysis showed that starting from day 5, CT rats were significantly heavier than the PF and ZD rats. Starting from day 11, PF rats were significantly heavier than the ZD rats (P<0.05). By day 22 of zinc deprivation, the body weight of the CT rats was 278.2 ± 3.9 g, PF, 210.0 ± 2.1 g, and ZD, 194.0 ± 2.5 g. The significant day effect reflected the growth of all groups of rats. The treatment x day interaction was induced by the apparent arrested growth of both PF and ZD groups compared with CT rats.

Tissue analysis

The ZD group had a significantly higher relative adrenal gland weight than that of the CT and PF groups ($F_{2, 33}$=8.44, P=0.00011) (Table 3.1). The relative weight of the thymus was significantly different across dietary groups ($F_{2,33}$=8.22, P=0.0013). The post hoc analysis showed that the thymus gland from the CT and PF groups was significantly heavier than the ZD group. No significant difference was found between the CT and PF groups. The femur zinc level of the CT group was significantly higher than that of the PF group which, in turn, was significantly higher than that of the ZD group ($F_{2,15}$=189.55, P<0.0001). Zinc levels in the liver, hippocampus, cerebellum and cerebral cortex were not different between treatment groups.

DMP test

Dietary treatment, ITI or test day had no significant effects on swim distance in the DMP test (Fig. 3.2). Swim trial effect was significant however, the swim distance in trial 1 was significantly higher than that of the trials 2, 3, and 4 ($F_{3,111}$=50.28, P<0.0001). The swim distance of trial 2 was significantly greater than trial 4 but not different from trial 3. The swim distances of trials 3 and 4 were not different from each other.

There was no effect of dietary treatment, ITI and day on swim latency in the DMP test (Fig. 3.2). However, trial 1 latency was significantly longer than that of the other trials ($F_{3,111}$=52.31, P<0.0001). Trial 2 latency was significantly higher than trials 3 and 4. But, no significant difference was found between trials 3 and 4.

Learning and reference memory test

When areas under the curve of swim distance, latency and cumulative distance for each day of training were considered, there were significant effects of dietary treatment

and day (Fig. 3.3): swim distance, $F_{2,32}$=5.2, P=0.011 and $F_{2,64}$=35.18, P<0.0001; Swim latency, $F_{2,32}$=3.49, P=0.043 and $F_{2,64}$=44.6, P<0.0001; cumulative distance, $F_{2,32}$=3.41, P=0.045, and $F_{2,64}$=55.38, P<0.0001. On day 1 of training, no group difference was found from any parameter. On day 2, the PF group displayed significantly longer swim distance and cumulative distance than either the CT or ZD groups. However, the PF group demonstrated a significantly longer latency than the control, but was not different from the ZD group. On day 3, all three parameters indicated that the PF group was significantly less effective in performance than both the CT and ZD groups. All groups, however, improved their performance across the three days of the learning test. Areas under the curves of the above parameters at both even and odd number trials were indicated in table 3.2, which suggests that the PF group performed significantly worse during even number of trials on day 2 and 3 of training. Although statically, the area under the curves of even number of trials did not reveal significant difference, the performance of the PF group appeared to be less effective than the CT and ZD groups. The day effect of all parameters was significant suggesting the improvement of performance of all groups of rats during the training.

Data analysis of the thigmotaxic ratio (diet ($F_{2,32}$=8.56, P=0.001), day ($F_{2,64}$=12.3, P<0.0001)) revealed that on day 2, PF groups spent a significantly greater proportion of time in the peripheral zone than the CT and ZD groups (Fig. 3.4). No significant difference was found between the CT and ZD groups. One day 3, both the PF and ZD groups had higher thigmotaxic ratios than the CT group. No significant difference was found between the PF and ZD groups. The day effect indicated that all three groups exhibited a reduced thigmotaxic ratio as training progressed.

Analysis of the percent of duration spent in zones during the probing test two days after the training indicated that all rats had a significant preference for the target zone, but no significant dietary effect was found in either the target or opposite zone. However, a significant dietary effect was found with the thigmotaxic ratio ($F_{2,32}$=6.32, P=0.049) (Fig. 3.5). Both the PF and ZD groups spent significantly longer than the CT group in the peripheral zone.

Serum corticosterone levels before and after water maze training

Serum corticosterone levels in rats on day 10 of adaptation did not show any differences across groups (CT=23.8 ± 13.9 ng/ml, PF=32.9 ± 19.4 ng/ml, ZD=34.1 ± 17.6 ng/ml). Before the probing swim and prior to the training session, the basal corticosterone level of the PF group was higher than that of the CT group (diet ($F_{2,32}$=7.41, P=0.0023), time ($F_{4,128}$=107.61, P<0.0001)) (Fig. 3.6). The mean corticosterone level of the ZD group was elevated; however the increment did not reach statistical significance at this stage of dietary treatment. Immediately after swimming, both the PF and ZD groups had significantly higher corticosterone levels compared to the CT group. Thirty min after swimming, only the ZD group had significantly higher corticosterone levels compared to the CT group. Sixty min after swimming, no significant differences were observed across dietary groups. The PF group had higher corticosterone levels than those of the CT and ZD groups 120 min after swimming. The corticosterone level at 30 min was the highest and at 0 and 60 min was significantly higher than basal. Corticosterone was not significantly different from basal 120 min after the swim.

After the training session, corticosterone levels of the ZD and PF groups were higher than those of the CT group both before probing and 0 min after probing (diet ($F_{2,32}$=3.49, P=0.043), time ($F_{4,128}$=126.0, P<0.0001)) (Fig. 7). At 30 and 60 min after probing, no significant dietary treatment effect was found. Corticosterone levels of the ZD group were significantly higher than those of the CT group 120 min after probing. At this time, the PF group was not significantly different from either the ZD or the CT groups. Corticosterone levels at 0, 30 and 60 min after probing were significantly higher than that of the basal levels with the difference being greatest at 30 min. The basal and 120 min corticosterone levels were not different.

Discussion

It is well established that zinc deprivation causes stunted growth of young rats (Keller et al. 2000). This phenomenon may be induced by activation of protein synthesis and an increased catabolic response to zinc deficiency (Giugliano and Millward 1987). In addition to exhibiting stunted growth, the ZD group from this study also exhibited reduced femur zinc levels. The zinc levels in the liver and hippocampus, cerebral cortex and cerebellum, however, were not affected. These results are consistent with results reported by others (Takeda et al. 2000, Wallwork et al. 1983, Wensink et al. 1987), suggesting a hierarchy of zinc conservation in the body. In Chapter 5, zinc levels in the liver and cerebral cortex were significantly affected by the dietary treatment with the ZD group exhibiting the lowest levels. Differences in dietary treatment may contribute to this inconsistency. In the current study, food pellets provided the zinc source for the PF and CT groups, whereas in Chapter 5, zinc was provided in the drinking water (25 ppm zinc). With better absorption of zinc from water, it is therefore conceivable that the PF

and CT groups accumulate more zinc in the liver and cerebral cortex with the experiments in Chapter 5 than those of this study. Tissue zinc measurements tend to bear this out.

Alterations in adrenal gland weight and thymus size indicate chronically stressed status of animals (Fukuoka and Ishida 2000). Elevation of the relative adrenal gland weight in the ZD rats is consistent with earlier reports (Quarterman and Humphries 1979, Reeves et al. 1977, Rothman et al. 1986), in which adrenal hypertrophy is accompanied by marked lipid accumulation in parenchymal cells and increased cholesterol content of this gland. In addition, the thymic involution in our ZD group is also consistent with the data of others (DePasquale-Jardieu and Fraker 1979, Quarterman and Humphries 1979, Shankar and Prasad 1998). The adrenal gland is responsible for corticosterone release and its action is regulated via the hypothalamic-pituitary-adrenal-axis (HPA axis). Enlargement of this tissue suggests the long-term dysregulation of the HPA axis (Groenink et al. 2002). The thymus is important for the maturation of B-lymphocytes and it is very sensitive to nutritional deficiency (Prentice 1999). It has been suggested that thymic involution is partly mediated by chronic corticosterone elevation in the ZD rats (Fraker et al. 1995). Therefore, by the end of the study, the ZD group exhibited signs of chronic stress. Like the CT group, the ZD group had adequate food available all day long. Others have shown that with this type of feeding schedule, the ZD animals consume food during the dark phase (Rains et al. 1998), which is consistent with our observation. Thus, the morning basal corticosterone levels in both the ZD and CT group should be at the nadir of their respective circadian rhythms (Gallo and Weinberg 1981, Garcia-Belenguer et al. 1993). A significant elevation of basal corticosterone by the end

of the water maze study may suggest a round-the-clock increment of corticosterone in the ZD group, which is consistent with the chronic stress status of the ZD animals. The non-significant elevation of basal corticosterone before the swim training session indicates that the corticosterone levels of some animals were increased at this time point. However, a synchronized elevation was not observed yet. As zinc deprivation progressed, a uniformed elevation was observed. Hence, it takes time for the effect of zinc deprivation on basal corticosterone levels to become significant.

Growth of the PF group was also significantly suppressed but the PF group eventually became significantly heavier that the ZD group despite their similar caloric intakes. Our observation is in agreement with that of the others (Keller et al. 2000). It has been suggested that efficiency of food utilization is greater in the PF group than in the ZD group (Giugliano and Millward 1984, Giugliano and Millward 1987). The PF rats exhibited a moderate but significant reduction in femur zinc as a consequence of food restriction (i.e. food is the only source of zinc for them). In contrast to the ZD rats, the relative adrenal gland and thymus weights of PF rats were not different from those of the CT group. Other reports have also shown that food restriction does not induce compromised immune function and the thymus size is normal (Weindruch et al. 1988). The adrenal gland size of food-restricted rats was not consistent in literature (Han et al. 1998, Weindruch et al. 1988), which may be related to differences in duration and degree of food restriction. Consequently, it is unlikely that the PF group was chronically stressed for the duration of the current study.

The PF group was the only group that had limited access to food. They usually consumed the daily portion of food within a couple of hours after the food was given.

The current feeding schedule was to give the food at 9:00 am on days without water maze training. Others have shown that food restricted rats with a similar feeding schedule developed a pre-prandial corticosterone peak one to two hours before the feeding time (Challet et al. 1997). The two basal corticosterone level measurements were performed at 8:00 am on different days suggesting that the corticosterone elevation can be caused by the pre-prandial effect. In addition, at both time points, corticosterone levels of the PF group were significantly greater than the CT group indicating that the duration for developing the pre-prandial effect is less than two weeks with the current feeding schedule. Therefore, it seems that, although, both the ZD and PF groups exhibited basal morning corticosterone elevation, the mechanisms underlying the elevation may not be the same.

The current study used the MWM to evaluate the effects of zinc and caloric restriction on learning, short-term memory, and long-term memory. Short-term memory or working memory is defined as the retention of information pertinent only to the current problem (Girard et al. 2000). In this study, the DMP test was used because previous investigators have shown that the DMP test is a sensitive measure of short-term memory deficits induced by hippocampal lesions (Steele and Morris 1999). Since the platform location was different for each day of testing, the rat had to acquire a new platform site in the first trial. With an intact short-term memory, the rat can apply this information to perform efficiently in the second trial. The ITI between these two trials was either 30s or 2hr since some short-term memory deficits are delay-dependent. The third and fourth swims served to stabilize the long-term memory component (Girard et al. 2000, Steele and Morris 1999). In the current study, all rats exhibited efficient retrieval of information

from the first trial by having a much shorter swim latency and distance in the second trial. No significant dietary group or ITI effect was found, which suggests that neither zinc deprivation nor pair-feeding impairs the short-term memory. These results differed from our earlier findings that the short-term memory was impaired in the ZD rats (Keller et al. 2000, Keller et al. 2001). The previous studies did not use the recently designed DMP test. In the previous testing procedure rats were tested twice a week and searched for two new platform locations in a single day. However, the DMP test requires four trials per day with a single platform location for stabilization of the long-term memory. Moreover, a 24 hr interval between blocks of trials is necessary to prevent interference between the blocks (Steele and Morris 1999). Finally, in the current study, we were able to monitor swimming behavior using a video tracking system allowing for a more comprehensive analysis of the data. In our earlier report, swim latency was the only measure of performance in the maze.

The spatial learning test was performed by training naive rats to locate a hidden platform at a fixed position in the pool. Numerous studies have used similar designs to detect learning deficits associated with brain injuries, or the pharmacological effects of drugs (Brandeis et al. 1989, D'Hooge and De Deyn 2001). Learning ability was measured as shorter swim latency, swim distance and cumulative swim distance across training days (Dalm et al. 2000). All three parameters are valid for defining the learning performance but they describe different aspects of the swimming behavior (Hamm 2001). Swim distance measures the length of the swim route that an animal takes to find the escape. This parameter indicates if a rat finds out a short route to finish the swim. Cumulative distance takes the searching pattern of the animal into account. Even if the

swim distances to reach the goal are the same for two animals, a goal-directed searching which centers around the area where the platform located gives rise to shorter cumulative distance as compared with random or concentric circling patterns. Therefore, a shorter cumulative distance suggests a better searching pattern in the maze. Swim latency describes how fast the animal escapes from the water. This measurement depends on both swim distance and speed. Since swim speed can vary between different groups, this parameter is not as sensitive as the others in determining the learning ability. Others have also found that latency is not a good parameter for the acquisition test in animals taking an indirect path to the hidden platform (Inman-Wood et al. 2000).

In the current study, all dietary groups showed improved performance in this learning test. The PF group consistently exhibited impaired learning ability but the ZD group performed normally. On day 2 of training however, the statistical difference in latency between the PF and ZD groups disappeared. This was because the PF group exhibited a transient increase in swim speed (CT: 22 ± 0.6 cm/s; PF: 25 ± 0.9 cm/s; ZD: 24 ± 0.7 cm/s) on that day. This increment shortened the time for the PF group to reach the platform. Therefore, the difference in the latency measurement between the groups was minimized. This transient elevation in swim speed may be caused by the increased activity levels of the PF group. Others have shown that the locomotor activity of the food restricted group is increased before and after the time of feeding compared with control group (Challet et al. 1997). The PF rats were fed after swimming in our study. Detailed analysis of the even and odd number of swim trials reached similar conclusion as the overall analysis of all swim trials each day. The pronounced impairment of the PF group

during even number of swim trials (reinforcement trials) suggests that the ability of PF group to utilize the acquired spatial information from the previous swim trial is impaired.

Thigmotaxia, or wall hugging, was part of the initial exploratory behavior adopted by normal rats during their search for an escape from the pool. As they learned that escape from the pool was not along the wall, they swam directly to the hidden platform via path integration or other spatial mapping processes (Whishaw et al. 1995). In our study, the thigmotaxic ratio was high in all three dietary groups on the first training day suggesting that all groups of rats exhibited a naive exploratory pattern. The CT group abandoned this ineffective searching strategy from the second training day. However, both ZD and PF were unable to completely abandon thigmotaxia throughout the training phase. The persistent thigmotaxic behavior in both PF and ZD groups was further confirmed by the subsequent 120 s probing test. Interestingly, the PF group exhibited significantly higher thigmotaxic ratio in the early phase of the probing test, whereas elevated thigmotaxic ratio in the ZD group was prominent during the later phase of the probing test (data not shown). Despite the differences in thigmotaxia, all groups showed a significant preference for the target zone. This suggests that all rats had an intact long-term memory of the platform location. Hence, performance impairment of the PF group in the water maze was related to thigmotaxia. The less severe thigmotaxia of ZD rats could have resulted in their learning curves appearing to be normal.

The impaired learning performance in the PF group and thigmotaxic behavior in both ZD and PF groups were accompanied by elevated basal serum corticosterone levels. The PF group exhibited a significantly elevated basal corticosterone level earlier than did the ZD group. The elevated basal corticosterone level of the latter was not significant

until after training. Changes in the basal corticosterone levels cannot be due to innate group differences since the corticosterone levels were not different among rats before dietary treatment. Elevated corticosterone has a negative effect on spatial learning in the water maze (de Kloet et al. 1999, Heiderstadt et al. 2000, Holscher 1999, McLay et al. 1998). Increased basal corticosterone has been shown to be associated with impaired water maze learning, and reduced corticosterone levels were associated with by normal learning in mice (Grootendorst et al. 2001). Moreover, the mice exhibited elevated basal corticosterone after they were put in the same cage with rats for two weeks. Their performance in the water maze was measured afterwards and they demonstrated a concentric searching pattern (Grootendorst et al. 2001). Although thigmotaxia was not determined in this study, the pattern was similar to what we observed with the PF group. An antidepressant that reduces plasma corticosterone levels also prevents learning impairment in the water maze (Yau et al. 1995). Middle-aged rats treated with stress level corticosterone for three months were impaired in water maze learning (Bodnoff et al. 1995). Hence, our observations are in consistent with the reports of others.

Mineralocorticoid receptors and glucocorticoid receptors are abundantly expressed in the hippocampus. The affinity of corticosterone for glucocorticoid receptor is greater than the mineralocorticoid receptor. These receptors are responsible for feedback regulation of circulating corticosterone (Korte 2001, Sousa et al. 2000). Although the mechanisms responsible for the elevation of basal corticosterone in the morning may not be the same for the PF and ZD groups, the effect of hypercorticism can induce changes in the hippocampus via these receptors. These changes can affect the water maze performance.

Persistent thigmotaxic behavior suggests an anxiety-like state of the animal which may arise from its increased stress (Ferrari et al. 1999). The stress state of malnourishment makes animals more sensitive to the stressful nature of the water maze. Other potential causes for this type of behavior may be related to the functional integrity of the cerebellum and the cholinergic system. Others have observed increased thigmotaxic behavior in animals with damaged cerebella and drug induced dysfunction of cholinergic system (Cain et al. 1996, Molinari et al. 1997, Paylor and Rudy 1990, Saucier et al. 1996).

The levels of corticosterone during the time courses were similar to reported patterns, in which the peak level of corticosterone occurred at 30 min after swimming (Szuran et al. 2000). These data indicate the stress response of all animals after a 120s swim stress. The curve reflects the overall effect of how fast corticosterone increased after the free swim, and how rapidly the elevated corticosterone was cleared out from the circulation. It appears that both the ZD and PF rats exhibited increased levels compared to the CT group at certain time points. It is unclear why the increment was limited to those time points. Increased responsiveness of malnourished rats did suggest that their HPA axis was either more responsive to swim stress or the efficacy of corticosterone clearance is less effective than that of the CT group.

In summary, our data indicate that the spatial memory of both the PF and ZD groups were not impaired. The learning impairment in the PF rats was potentially due to an increased level of stress. Thigmotaxia (ineffective searching strategy) developed in both groups of caloric restricted animals, and may be related to stress and functional integrity of the brain.

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Table 3.1 Adrenal weight, thymus weight, and zinc level in the femur

	CT	PF	ZD
Relative adrenal weight (mg/g body weight)	0.11 ± 0.01 [a]	0.10 ± 0.01 [a]	0.17 ± 0.02 [b]
Relative thymus weight (mg/g body weight)	1.96 ± 0.15 [a]	2.23 ± 0.12 [a]	1.62 ± 0.07 [b]
Femur zinc (μg/g) *	173.9 ± 2.1 [a]	159.7 ± 5.0 [b]	68.5 ± 5.8 [c]
Liver zinc (μg/g) *	27.7 ± 1.1	31.2 ± 2.8	25.9 ± 1.2
Hippocampus (μg/g) *	16.4 ± 0.3	16.9 ± 0.4	15.5 ± 0.4
Cerebral cortex (μg/g) *	16.1 ± 0.3	16.3 ± 0.2	15.4 ± 0.3
Cerebellum (μg/g) *	11.3 ± 0.3	11.4 ± 0.3	11.6 ± 0.1

Data are means ± SEM for 10 -14 rats per dietary group. * Data are means ± SEM for groups of 5-7 rats. Group means with different superscript letters indicate significant difference between each other ($P<0.05$).

Table 3.2 Area under the curve of cumulative swim distance, swim distance, and swim latency based on odd and even swim trials respectively (n=12-14 rats/dietary group).

Area under the curve of cumulative distance (odd number of swim trials)

Training day	1	2	3	Trt * P-value
CT	20923.7 ± 3482.4	10355.1 ± 2043.8	8336.2 ± 1774.5	
PF	30924.2 ± 7022.7	19677.7 ± 4198.6	12543.6 ± 1382.4	0.20
ZD	25089.6 ± 4644.7	13465.3 ± 1922.8	7047.8 ± 1273.0	

Area under the curve of cumulative distance (even number of swim trials)

Training day	1	2	3	Trt * P-value
CT	21141.3 ± 4157.1	8486.0 ± 1623.5 [a]	6470.9 ± 1252.0 [a]	
PF	25816.7 ± 4584.6	15026.4 ± 3321.3 [b]	11519.7 ± 1801.7 [b]	0.02
ZD	15038.9 ± 3225.8	8706.5 ± 1221.8 [a]	5220.8 ± 689.4 [a]	

Area under the curve of swim distance (odd number of swim trials)

Training day	1	2	3	Trt * P-value
CT	2364.9 ± 267.3	1069.1 ± 187.7	862.0 ± 152.3	
PF	2582.9 ± 556.3	1936.5 ± 428.4	1342.9 ± 128.1	0.10
ZD	2053.6 ± 335.9	1424.1 ± 188.0	815.1 ± 116.5	

Area under the curve of swim distance (even number of swim trials)

Training day	1	2	3	Trt * P-value

CT	1756.4 ± 277.6	867.3 ± 152.8 [a]	769.4 ± 135.2 [a]	0.005
PF	2288.2 ± 436.8	1553.6 ± 336.0 [b]	1296.5 ± 218.0 [b]	
ZD	1427.1 ± 292.3	929.7 ± 99.4 [a]	663.4 ± 88.7 [a]	

Area under the curve of swim latency (odd number of swim trials)

Training day	1	2	3	Trt * P-value
CT	101.9 ± 10.5	44.9 ± 7.0	38.0 ± 6.1	0.22
PF	104.8 ± 20.9	74.6 ± 14.3	53.4 ± 5.3	
ZD	88.6 ± 14.3	57.8 ± 7.7	34.4 ± 5.5	

Area under the curve of swim latency (even number of swim trials)

Training day	1	2	3	Trt * P-value
CT	79.8 ± 13.2	36.9 ± 6.1 [a]	33.7 ± 5.1 [a]	0.01
PF	97.7 ± 19.1	58.1 ± 11.0 [b]	50.8 ± 7.5 [b]	
ZD	60.1 ± 11.1	39.1 ± 4.2 [a,b]	27.7 ± 2.9 [a]	

* treatment effect

Superscripts with different letters indicate significant group difference ($P<0.05$)

Fig. 3.1 Growth curve of rats during zinc deprivation phase. Data are means ± SEM for groups of 32 to 35 rats. Repeated measure analysis indicated significant dietary treatment effect. a. The CT group was significantly heavier than the PF and ZD groups from day 5. b. The PF group was significantly heavier than the ZD group from Day 11.

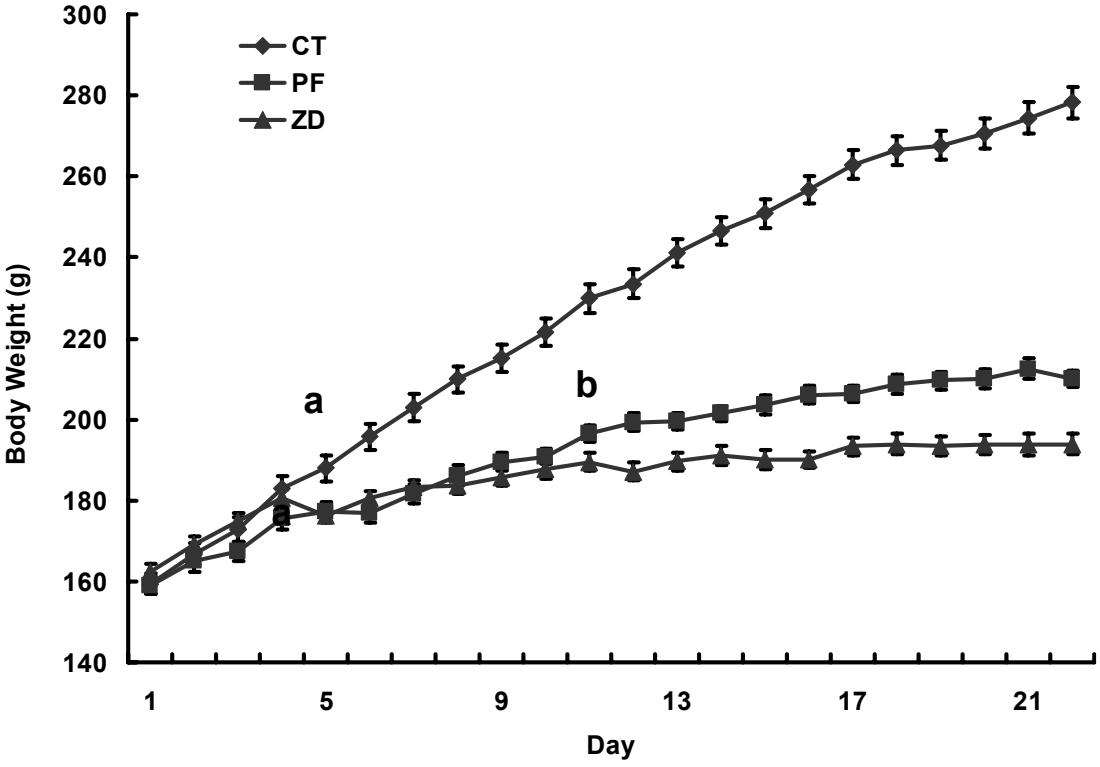

CT
PF
ZD
Body Weight (g)
300
280
260
240
220
200
180
160
140
1
5
9
13
17
21
Day
a
b

Fig. 3.2 Effect of zinc-deprivation and pair feeding on rat performance across trials on the DMP task. Data are group means of swim distance and latency to locate the platform ± SEM (n=12-14 per group). Dietary treatment and ITI effects were not significant. Data are collapsed across ITI and days. Different letters represent trial effect.

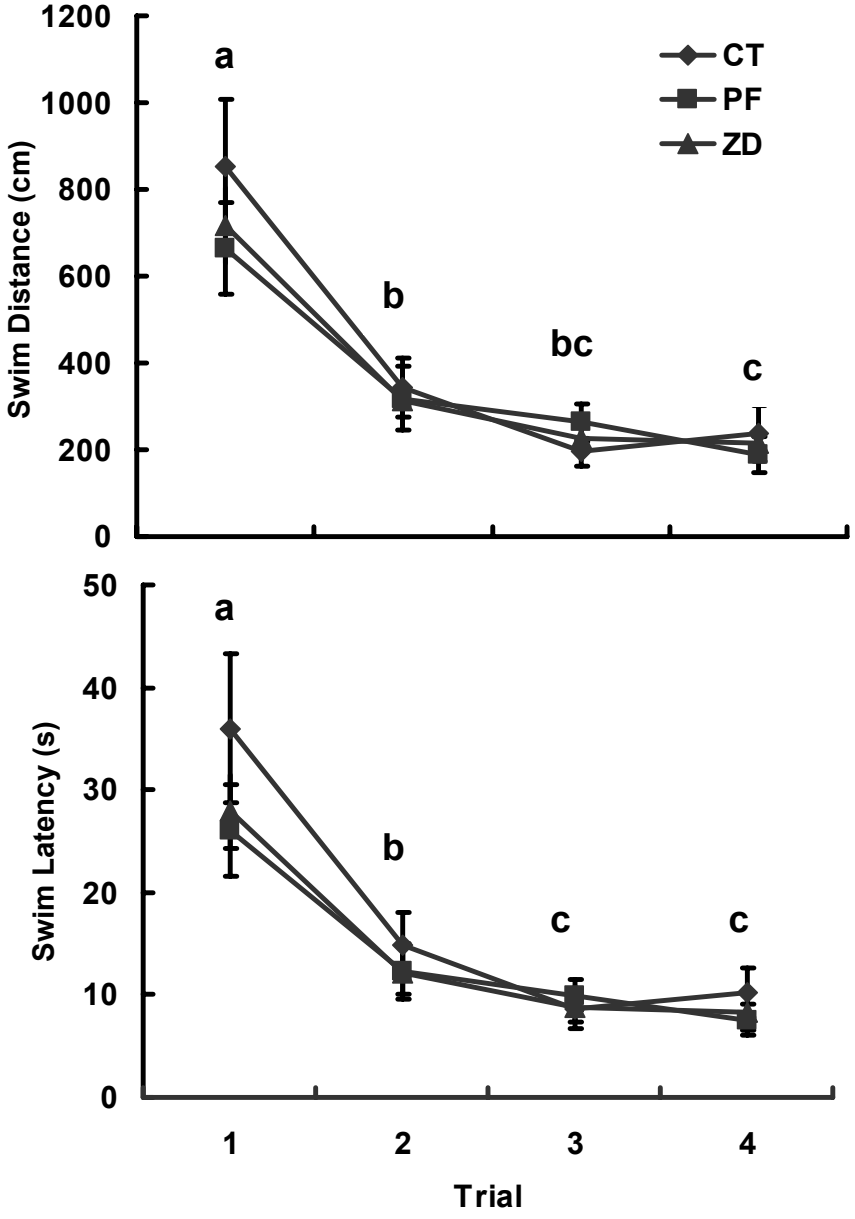

1200
1000
800
600
400
200
0
Swim Distance (cm)
a
b
bc
c
CT
PF
ZD
50
40
30
20
10
0
Swim Latency (s)
a
b
c
c
1
2
3
4
Trial

Fig. 3.3 The areas under the curves of swim distance, cumulative swim distance and swim latency were calculated for each animal during each training day of experiment 2. Data are means ± SEM for groups of 10-13 rats per group. a. Measured value of the PF group was significantly greater than both CT and ZD groups. No significant difference was found between CT and ZD groups. b. Value of the PF group was significantly greater than the CT group. At this point, the value of the ZD group was not significantly different from either the PF or CT group.

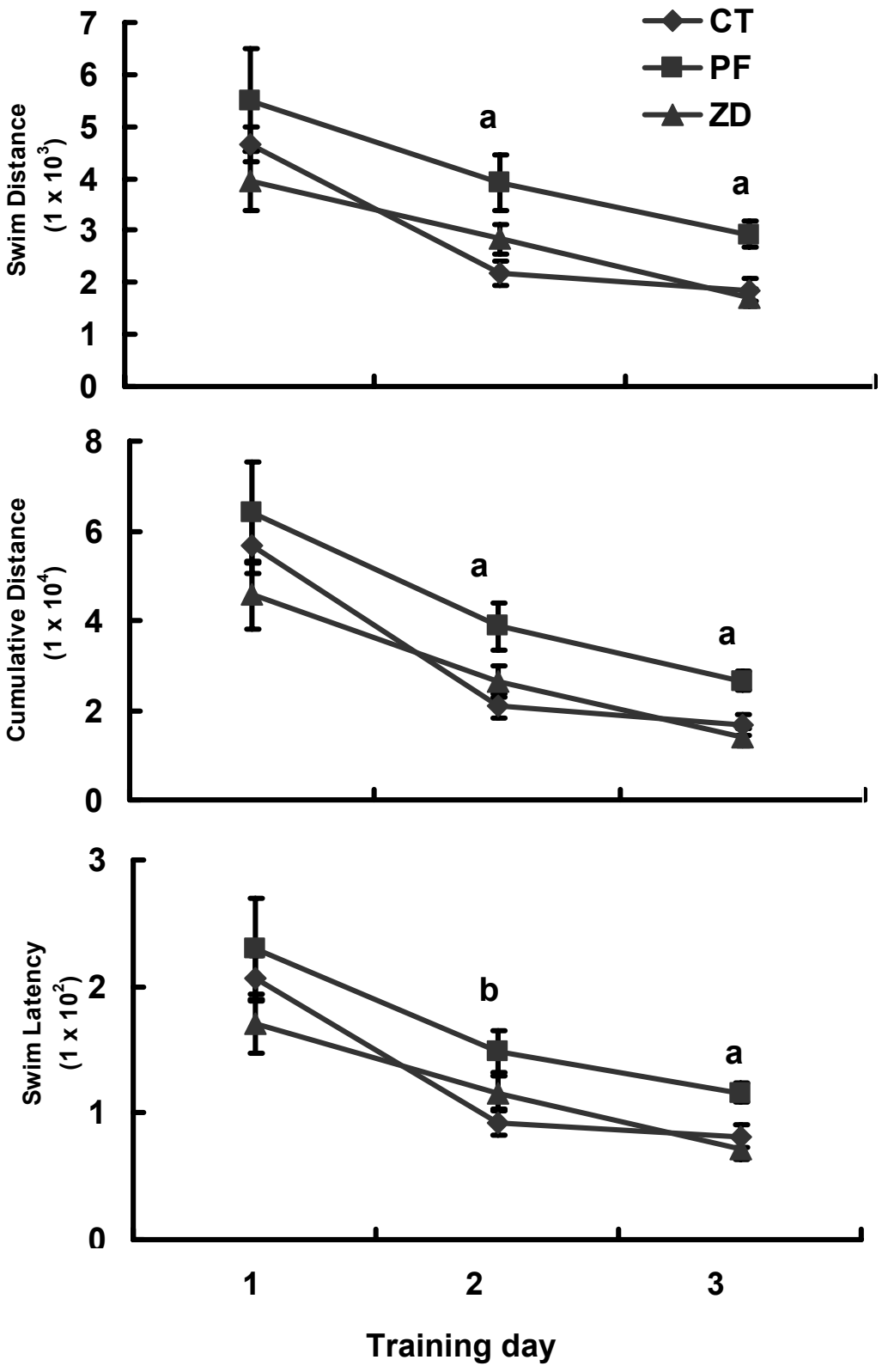

CT
PF
ZD
Swim Distance
(1 x 10^3)
Cumulative Distance
(1 x 10^4)
Swim Latency
(1 x 10^2)
a
a
a
a
b
a
Training day

Fig. 3.4 The area under the curve of thigmotaxic ratio was calculated for each animal during each training day of experiment 2. Data are means ± SEM for groups of 10-13 rats per group. a. The PF group demonstrated significantly higher thigmotaxic ratio than the CT and ZD groups on day 2. b. Both PF and ZD groups exhibited significantly higher thigmotaxic ratios than the CT group on day 3.

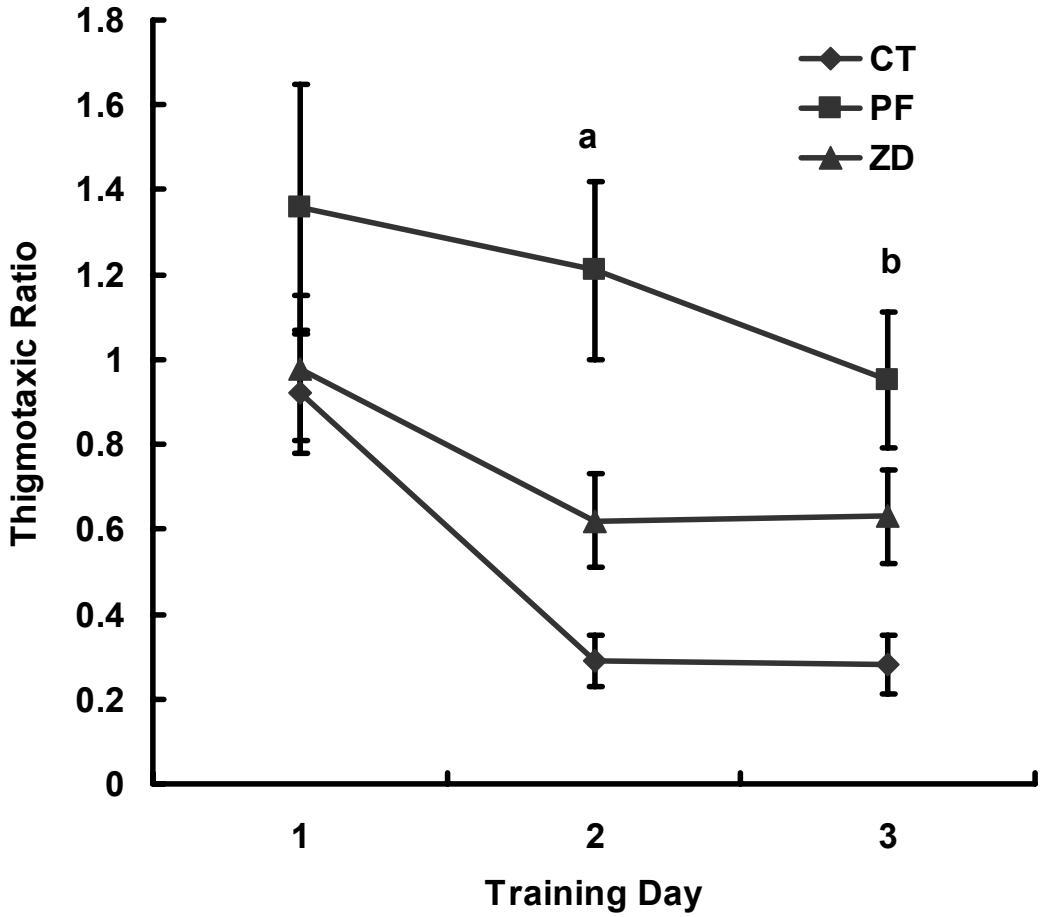

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Thigmotaxic Ratio
CT
PF
ZD
a
b
1
2
3
Training Day

Fig. 3.5 Thigmotaxic ratio and percent of duration in target and opposite zones during the 120 s of the probing test following the training session of experiment 2. Data are means ± SEM for groups of 10-13 rats per group. Superscripts with different letters indicate significant group differences ($P<0.05$). No significant dietary treatment effect was detected for the % of duration in either the target or the opposite zones.

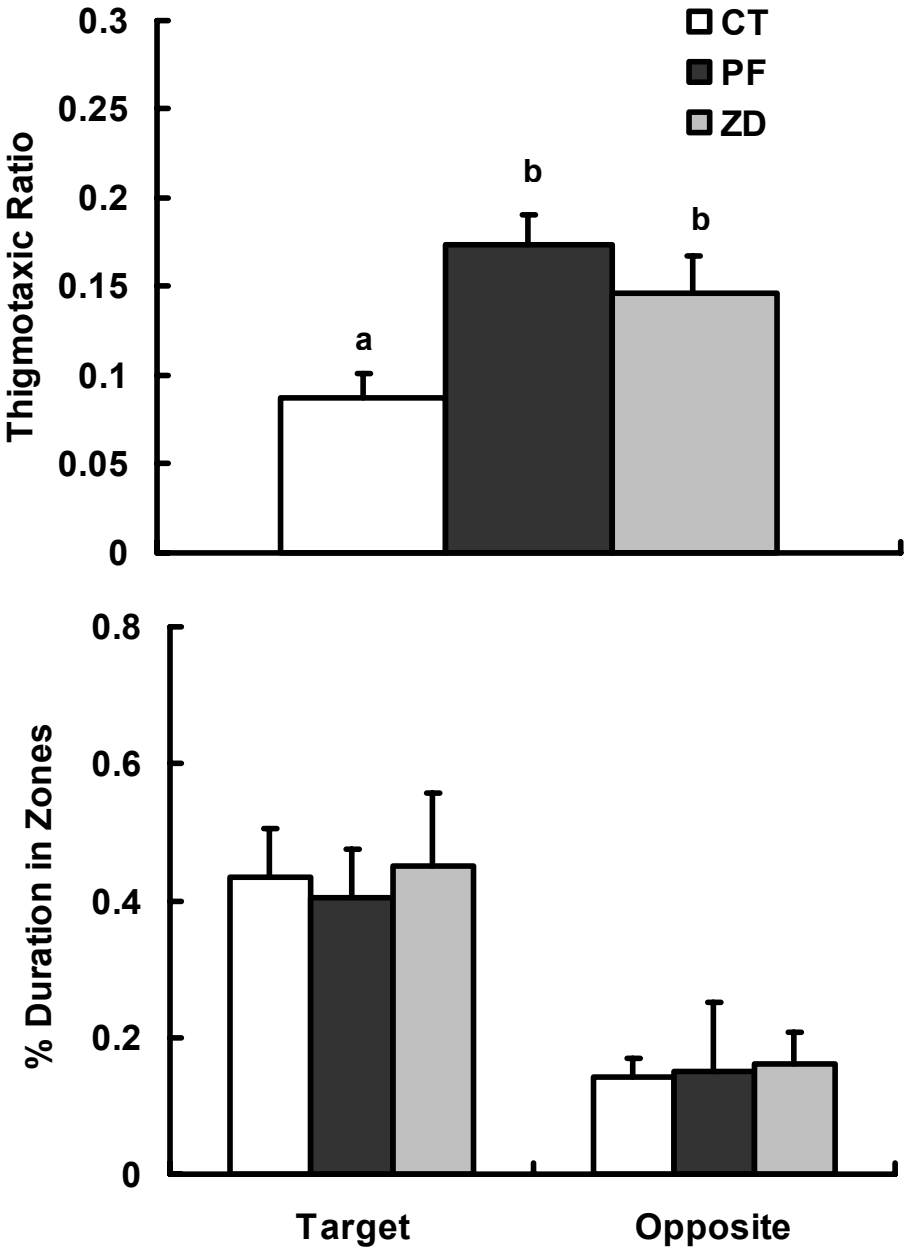
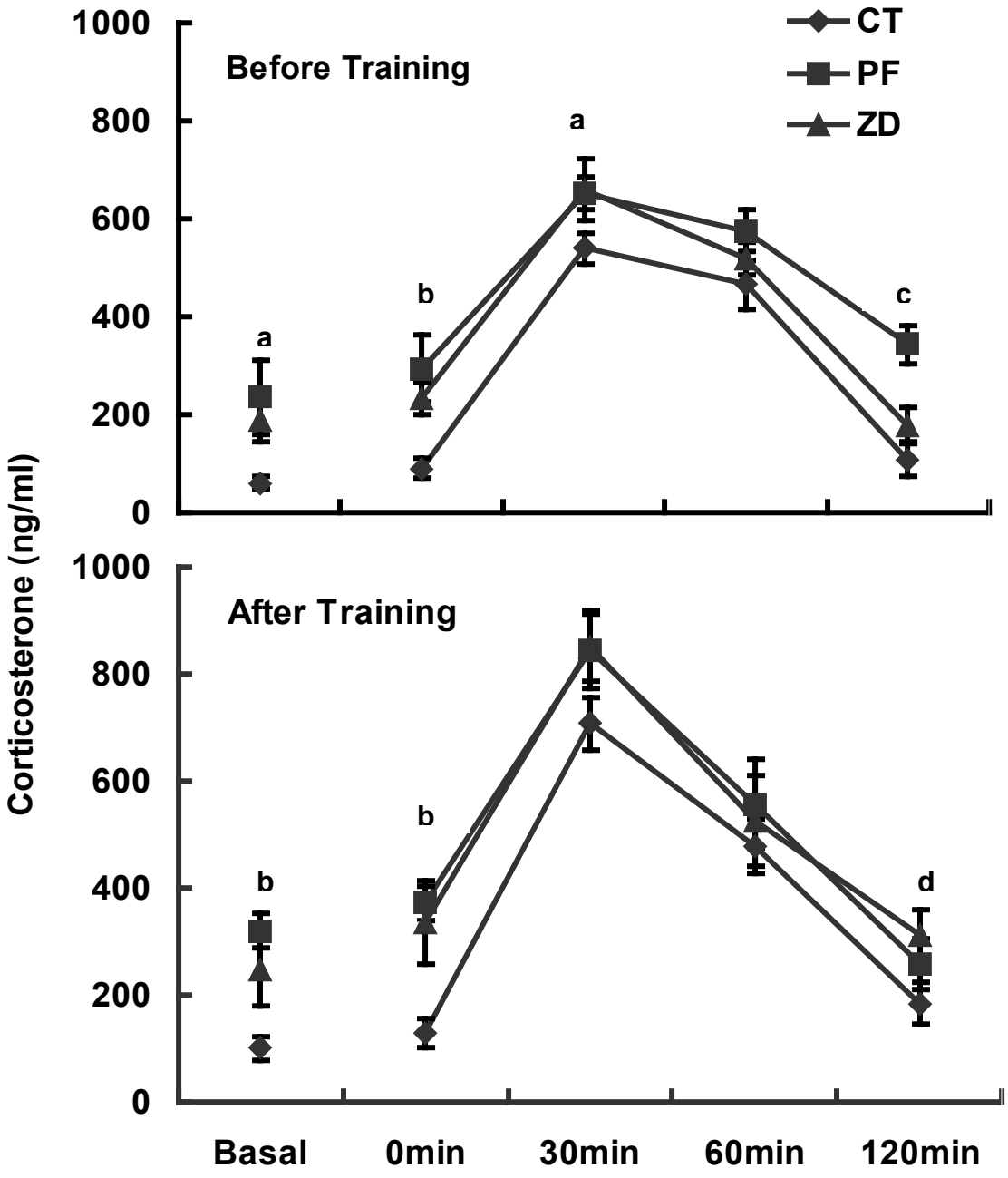

0.3
0.25
0.2
0.15
0.1
0.05
0
Thigmotaxic Ratio
CT
PF
ZD
a
b
b
0.8
0.6
0.4
0.2
0
% Duration in Zones
Target
Opposite

Fig. 3.6 Serum corticosterone levels before and after the training session in experiment 2. On day 15 of zinc deprivation, two days before the training session, basal corticosterone levels were determined from blood samples collected immediately before the pre-training probing test. 0, 30, 60, and 120 min time points represent corticosterone levels measured from samples collected 0, 30, 60, and 120 min after the probing test. In reality, 0 min time point represents 2 min after swim due to the time required to send a rat to the tail bleeding room. On day 22 of zinc deprivation, two days after the training session, corticosterone levels were measured according to the same time schedule as that of day 15. Data are means ± SEM for groups of 10-13 rats per group. a. Corticosterone levels of the PF group were significantly greater than that of the CT group. The difference between the ZD and CT was not significant. b. Both the PF and ZD groups had significantly elevated corticosterone levels than the CT group. c. The corticosterone of the PF group was significantly increased than both the ZD and CT group. d. The corticosterone level of the ZD group was significantly greater than the CT group. The difference between the PF and CT was not significant.


CT
PF
ZD
Before Training
After Training
Corticosterone (ng/ml)
1000
800
600
400
200
0
a
b
a
c
b
b
d
Basal
0min
30min
60min
120min

CHAPTER 4

PROBING INSERTIONS DURING TRAINING PHASE INTERFERE WITH RATS' PERFORMANCE IN THE LEARNING TEST

In the previous chapter, the PF group exhibited thigmotaxia and learning impairment in maze learning test and the ZD group also demonstrated thigmotaxic performance in the probing test. In order to further define the thigmotaxic behavior observed in the ZD and PF dietary groups, another learning experiment was designed that included probing tests and fewer numbers of training trials each day. No group difference was found in the learning and probing tests inserted in the learning phase. Impaired learning in the PF group was only observed in the reversal-learning phase. Data analysis indicates that proactive interference by the probing test may have contributed to the insensitivity of the learning test. Blood glucose was decreased and ACTH levels were elevated in both the PF and ZD group. Blood glucose decrement suggests an adaptive response of both malnourished groups to food restriction. The activation of the HPA axis may have affected the maze performance of these rats since stress has been shown to inhibit spatial acquisition. Blockade of the cholinergic system or cerebellum lesions also induce similar behavioral changes in rats, thus dietary treatment may also affect the normal function of these neurological systems.

Introduction

In the previous chapter, thigmotaxic behavior during the spatial learning task was observed in both PF and ZD groups. Elevated corticosterone concentrations, measured both before and after the swim trials, suggest that stress may contribute to their impaired performance during the learning test. In order to further explore this possibility, repeated probe trials were inserted between the training trials, since it has been suggested that the inserted probe trials monitor the development of the spatial preference without interrupting the training process (Gerlai 2001).

The acute energy status of the rats at the time of the behavioral test can affect the process of memory formation and glucose injection two hours before water maze training facilitates learning (Oomura et al. 1993). Although all rats were fed in the morning, their eating patterns were different. PF rats consumed the daily portion of their food in the light phase whereas the ZD and CT rats ate during the dark phase. Because swim testing was initiated in the morning, before daily feeding, the PF group was fasted before swimming whereas the CT and ZD groups were not. The blood glucose concentrations of the PF group at the time of swim test could potentially be lower that those of the CT and ZD rats, which may contribute to the greater impairment of maze performance in the PF rats compared with the ZD group.

Biosynthesis and secretion of corticosterone is, in part, activated by the hypothalamic-pituitary-adrenal axis (HPA) (Han et al. 2001). Under stressed conditions, corticotrophin-releasing factor (CRF) neuron cell bodies localized in the hypothalamus and amygdala are selectively activated (Whitnall 1989). The released CRF binds to CRF receptors in the anterior pituitary and stimulates the adrenal corticotropic hormone (ACTH) secretion (Croiset et al. 2000). ACTH in turn increases the release of corticosterone from the adrenal gland. The activity of the HPA axis exhibits a diurnal pattern with a diurnal peak being reached at the start of the dark period (Krieger 1977). Another cause of the elevation of basal corticosterone concentrations is the alteration in adrenal sensitivity to circulating ACTH. It has been shown that food restriction enhances adrenal sensitivity (Han et al. 1998). Therefore, it is unclear whether the high corticosterone concentrations in the ZD and PF groups are related to the activation of HPA axis or to adrenal hypersensitivity.

The purpose of the current study was two-fold: 1) to confirm the previous behavioral results and 2) to determine the blood glucose before the initiation of swim training and ACTH levels at the end of the swimming test, when both the ZD and PF groups exhibited increment in corticosterone concentrations. Naive rats were trained to escape from the water by finding a fixed platform as in the previous study. The number of trials for daily training was reduced from eight to four, and several probing trials were inserted in the training phase. Following the training phase, all rats were further trained to learn a new platform location opposite to the acquired platform site (reversal training). The results confirmed the thigmotaixic performance of the PF group in the reversal learning. However, proactive interference of inserted probing tests possibly prevented further definition of the effect of dietary treatment on maze performance.

Materials and Methods

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), 27-31 days old, were housed under the same condition as described in Materials and Methods in Chapter 3. During first 10 days of the adaptation phase, all rats were fed AIN-93G egg-white based diet with 20-25 ppm zinc (Dyets, Bethlehem, PA). Rats were then divided into three dietary groups (CT, PF and ZD). Diet for the CT and PF groups was the same as the diet offered during the adaptation phase whereas diet for the ZD rats was changed to modified AIN-93G egg-white based diet with less than 1 ppm zinc (Dyets, Bethlehem, PA). The feeding schedule and maze setup remained the same as described in Chapter 3. All procedures were approved by the Animal care and Use Committee of the University of Georgia.

From day 17 to day 20 of zinc deprivation, rats were trained to learn a fixed platform site within the maze. A schematic illustration of the training schedule is shown in Table. 4.1. Each day, a rat had four consecutive trials from four different releasing points. The maximum time allowed for searching during each swim was 90 s. When the rat located the platform, it remained on the platform for 15 s before it was removed from the maze. If a rat was unable to find the platform within 90 s, it was gently led to the platform and allowed to rest on it for 15 s. During the whole learning phase, the platform location was fixed for every rat. On the second and third day of training, a 45 s probing test was performed. In the probing test, the platform was removed from the water maze. As soon as a rat finished the 45 s probing test, it was picked up by hand and towel dried. The rat was rested in its home cage for half an hour before the training session started. Twenty-four hours after the last day of the training session, all rats were tested with the third probing test.

One day after the last probing test during the training phase, all rats were trained to learn a new platform location for two days. Since the platform was in a symmetrically opposite position to the previous platform site, this test has been named reversal learning. All rats were given four trials from four different releasing points each day. Twenty-four hours after the last session of reversal learning, rats were probed for 45 s.

Swimming behavior of all rats was recorded by the Ethovision video tracking system (Noldus Information Technology). Swim distance, cumulative swim distance and swim latency were calculated for the learning and reversal learning tests. The area under the curve of these parameters for each day of training was determined to evaluate the efficacy of learning for each dietary treatment group. The area under the curve of

thigmotaxia ratio of these rats during training was calculated. The swim tracks recorded from the probing tests were analyzed by calculating the percentage of testing time spent in the zone previously containing the platform and in the opposite zone. The delineation of zones was the same as described in Chapter 3.

Blood collection and measurements

On day 17 of zinc deprivation, serum samples were collected by tail bleeding from all of the rats immediately before the behavioral test. Serum glucose levels were detected colorimetrically with a glucose assay kit (Kit GAHK-20, Sigma, St. Louis, Mo).

One day after the completion of behavioral tests, all rats were sacrificed starting from 8:00 am. Trunk blood was collected into an ice-cold sterilized tube containing EDTA. The blood sample was immediately mixed with the EDTA solution and kept on ice. Plasma was collected by centrifuging the blood sample at 2,800 rpm for 30 min at 4 °C. ACTH levels in the plasma were measured by an ACTH radioimmunoassay following the manufacturer's instructions (ACTH 65T kit, San Juan Capistrano, CA).

Data analysis

Data were analyzed by either one-way ANOVA or ANOVA with repeated factors (SAS version 8.2). Post hoc analyses using least significant difference (LST) t-test was applied when appropriate. Treatment effects with a p-value less than 0.05 were regarded as statistically significant.

Results

Learning

Repeated measure analysis of the areas under the curve for swim distance, latency and cumulative distance for the first four days of learning did not reveal any significant dietary treatment effect (distance: $F_{2,36}$=1.92, P=0.16; latency: $F_{2,36}$=1.78, P=0.18; cumulative distance: $F_{2,36}$=1.55, P=0.23). The day effect, however, was significant (swim distance: $F_{3,108}$=20.19, P<0.0001; swim latency: $F_{3,108}$=23.79, P<0.0001; cumulative distance: $F_{3,108}$=29.56, P<0.0001). The day effect was caused by the daily improvement in performance of all rats, i.e. a reduction in swim time and distance. There was no significant interaction (Fig. 4.1).

No significant dietary treatment effect was found in the thigmotaxic behavior ($F_{2,36}$=1.04, P=0.36). The day effect was significant ($F_{3,108}$=3.92, P=0.01), which suggests that all rat decreased thigmotaxia as the training progressed. The treatment x day interaction was not significant (Fig. 4.2).

Reversal learning

Data analysis of reversal learning found significant dietary treatment and day effects for the three parameters of learning behavior: swim distance $F_{2,36}$=5.36, P=0.0092, $F_{1,36}$=10.81, P=0.0023; swim latency: $F_{2,36}$=6.44, P=0.0041, $F_{1,36}$=12.72, P=0.001; cumulative distance, $F_{2,36}$=6.85, P=0.003, $F_{1,36}$=11.58, P=0.002. Post hoc analysis indicated that on day 1 of reversal learning, PF group performance was significantly less efficient than that of the ZD group. Performance of the CT group was not significantly different from either PF or ZD groups. On day 2, the PF group performed significantly

worse than either the ZD or the CT groups. However, the CT and ZD rats were not significantly different from each other (day 5, 6 of Fig. 4.1).

Dietary treatment significantly influence the thigmotaxic ratio analysis ($F_{2,36}$=6.09, P=0.0053). The PF group spent significantly more portion of time in close vicinity to the wall compared to that of the ZD group on day 1 of reversal learning. Thigmotaxic ratio of the CT group was not significantly different from either the PF or the ZD group. On the second day of reversal learning, the thigmotaxic ratio of the PF group was significantly greater than those of the CT and ZD groups (day 5, 6 of Fig. 4.2).

Probing tests in the learning phase

In the probing tests, the effect of treatment on the percentage of duration in both the target and opposite zones was not significant (target: $F_{2,36}$=1.4, P=0.261; opposite: $F_{2,36}$=0.63, P=0.539). However, the test day effect was significant (target: $F_{2,72}$=16.75, P<0.0001; opposite: $F_{2,72}$=8.02, P=0.0007). Further analysis of the day effect indicated that the percent of time spent in the target zone increased and the duration spent in the opposite zone decreased across the test days (P<0.05) (Fig. 4.3).

Analysis of thigmotaxia ratio during the probing tests did not reveal any dietary treatment effect ($F_{2,36}$=2.72, P=0.079) but the test day effect was significant ($F_{2,72}$=11.23, P<0.0001). Treatment x day interaction was not significant (Fig. 4.4).

Probing test following reversal learning

Spatial preference in the probing test following the reversal learning was determined by one-way ANOVA test. No significant treatment effect was found in either the target or the opposite zones (target: $F_{2,36}$=0.5, P=0.61; opposite: $F_{2,36}$=1.26, P=0.30) (probing 4 of Fig. 4.3). The thigmotaxia ratio in this probing test however was

significantly affected by dietary treatment ($F_{2,36}$=4.95, P=0.012). The PF group spent significantly more time in the peripheral zone of the pool compared to the other two groups (probing 4 of Fig. 4.4).

Blood glucose and ACTH levels

A significant dietary treatment effect ($F_{2,12}$=4.21, P=0.041) was found in blood glucose measurement before the training phase. Both the PF and ZD groups had significantly lower blood glucose levels than the CT group, suggesting an adaptive response to food restriction in both malnourished groups (Fig. 4.5). Plasma ACTH ($F_{2,33}$=3.33, P=0.048) was significantly increased in both PF and ZD groups (Fig. 4.6). It seems that the HPA axis was activated in these animals.

Discussion

The original purpose of performing the learning test with probing insertions was to confirm the impaired maze performance in the PF and ZD groups described in the Chapter 3. Data collected from the learning experiment, however, appears to negate the previous findings because no group difference was detected in the learning trials or probing tests. The apparent discrepancy in the two experiments was carefully evaluated. In both studies, naïve rats were training to learn a fixed platform location and an optimal strategy is to swim away from the edge of the pool and escape from the water by landing onto an immersed platform (Morris 1984). All three groups of rats in the previous experiment rapidly adopted the effective searching strategy because they abandoned thigmotaxia and reached their respective asymptotic level of thigmotaxia by the second day of training (Fig. 3.4). In the current experiment, however, the decrement of thigmotaxia in the initial stage of training was not as obvious as in the previous study.

Moreover, the searching strategy of the rats was constantly improving and never reached an asymptote. This trend was observed in both the training and the probing tests (Fig. 4.2, Fig. 4.4). Therefore, in the current experiment, the establishment of an effective searching strategy was delayed in all groups of rats. One possible explanation is that the inserted probe tests disrupted the formation of the optimal strategy in all of the rats, which, in turn, obscured the difference among dietary groups. In the probing trials, the platform was removed from the water maze. Even if a rat swam to the platform location, it was not rewarded with escape from the water, and had to swim away to search for other places to escape from within the water tank. Thus, the probing trials served as extinction trials for the response-reinforcement contingency. Results from reversal learning support this explanation. After rats were training in the learning test, they started to acquire a new platform site through consecutive swimming trials that were not interrupted by probing tests. In this instance, the inferior performance of the PF group was detected. The ZD group performed as well as the CT group throughout the study, which might suggest that the subtle difference detected in the previous study was completely undetected by the current insensitive measures.

The disruptive effect of non-rewarding probing tests has also been demonstrated by others (Buresova et al. 1985, Markowska et al. 1993). In their experiments, a collapsible platform was used in the maze. At the beginning of a probing test, the platform rested at the bottom of the maze and beyond the reach of a rat. The platform was raised, and was available for escape after the rat swam for a predetermined time. The learning efficacy of the non-rewarding probing design was apparently worse than that of the collapsible platform design because the rats exhibited goal directed searching of the

platform in the collapsible platform training. In the non-rewarding tests, the searching strategy was ineffective. Concentric or random searching patterns were observed with this type of training.

In spite of the disturbance of probing trials in the learning tests, all rats demonstrated the spatial ability of recognizing the platform location. Their time in the target zone increased whereas the duration of time spent in the opposite zone decreased. Two days of reversal learning trials altered their spatial preference accordingly. Thus the chance of visiting the previous opposite zone was higher than the 25% chance level, whereas preference for the previous target zone was reduced to less than 25%. In this test, four symmetrical zones were delineated in the maze to determine the spatial memory of a rat for a specific location within one of the zones. Presumably a naïve rat does not have any spatial preference for any of the four zones and the chance of visit for each zone will be approximately 25%. If a rat is trained in the spatial task, its preference for the zone where the platform used to present should increase in the probing test. Therefore, a greater than 25% chance level of visit is deemed as spatial memory of the platform location. No group difference was detected in any of these measurements. These results conform to the observations in the previous experiment in chapter 3. Therefore, with our currently available testing methods, long-term memory for a spatial location in the water maze was not affected by either zinc deprivation or pair-feeding treatment.

The process for a naïve rat to start to learn a spatial location involves both procedure learning and declarative spatial learning (Molinari et al. 1997). Procedure learning refers to the non-spatial components of the water maze task including coordination of swimming in the water maze, learning to swim to a platform, climbing

onto it and recognizing it as an escape. Declarative learning refers to encoding the spatial information of an invisible platform site in relation to extra maze spatial cues during a swim. This learning process is believed to be associated with place cell firing and long-term potentiation (LTP) occurrence in the hippocampus (Silva et al. 1998). The hippocampal pyramidal cells fire more actively when an animal enters a specific area (Rotenberg et al. 1996). LTP refers to a stable increase in synaptic transmission in the hippocampal region. Blocking LTP by mutations or pharmacological treatments impair place learning (Bliss and Collingridge 1993). Clear dissociation of these two components in a water maze task is difficult, since both processes are modified simultaneously along with the experience of a rat in a pool (Cain 1998). In our experiment, it is likely that the impairment in both the PF and ZD rats is a procedure learning deficit rather than a-declarative learning problem. Firstly, thigmotaxia is the natural tendency for all naïve rats, i.e. instinctive behavior. Although establishment of spatial information can help with the suppression of thigmotaxia, giving up this ineffective searching strategy does not solely depend on spatial learning, it has been shown that non-spatial tasks also can eliminate this problem (Bannerman et al. 1995). Secondly, the spatial preference for the platform location can be established in all dietary groups through training, which suggests that all rats have the ability to encode the spatial information regardless of their searching strategy differences.

The beneficial effect of food intake and glucose injection on spatial learning has been shown by Oomura et al. (Oomura et al. 1993). It could be that the more severe behavioral deficits observed in the PF group compared to the ZD group is associated with a relatively lower glucose level in this group. Our results however indicate that the ZD

and PF group exhibited the same level of reduction in serum glucose before the swim trials. Therefore, reduced serum glucose may contribute in part to the thigmotaxia behavior in both the ZD and PF groups, but low serum glucose concentrations cannot explain the behavioral differences between the PF and ZD groups.

By the end of the study, plasma ACTH in both the PF and ZD rats were significantly elevated which suggests that the HPA axis was activated by the dietary treatment. Even though, we cannot rule out changes in adrenal sensitivity to ACTH and the constitutive levels of corticosterone in the blood stream, the elevated basal corticosterone levels reported in Chapter 3 were in part, the consequence of increased activity of regulatory pathway upstream of the adrenal gland. These stress hormones participate in the learning and memory processes since they can change the anxiety, fear, concentration, arousal and motivation of an animal (Croiset et al. 2000). Others have also reported a simultaneous increase in basal corticosterone levels and a spatial learning deficit in the water maze (Grootendorst et al. 2001, Yau et al. 1995).

Besides the possibility that the elevation of corticosterone and activation of the HPA axis contribute to the thigmotaxia and learning impairment in the malnourished groups, changes in other neurological systems such as the cholinergic system and the cerebellum could also induce thigmotaxic behavior and retard spatial learning in the water maze. For instance, NMDA receptor antagonists DL-2-aminophosphonovaleric acid (APV) and muscarinic antagonist scopolamine caused thigmotaxic swimming behavior (Cain et al. 1996, Paylor and Rudy 1990, Saucier et al. 1996). Hemicerebellectomized rats exhibited persistent peripheral circling in the pool (Molinari et al. 1997). Either extensive training or pre-training in a non-spatial task prevented the

impairment in spatial mapping of pharmacologically treated rats (Bannerman et al. 1995, Saucier and Cain 1995). Currently, it is unknown if zinc deprivation or pair feeding can affect the normal function of the cholinergic system and/or the cerebellum. The behavioral similarity observed here using the water maze, however, suggests that alterations in the activities of these neurological systems may occur in response to dietary treatment.

In summary, this study indicates that the learning impairment of the PF group was confined to reversal learning. Probing insertions in the learning phase, however, appeared to be a proactive interference and masked the performance differences previously observed among groups. Because both ZD and PF groups had reduced blood glucose but elevated ACTH levels, the behavioral changes in the dietary groups cannot be fully explained by blood glucose or stress. Other potential explanations, such as alterations in the cholinergic system, are discussed. Since the probing insertion test offers more information on the development of spatial memory but the application is limited by the non-rewarding nature of the test, it is necessary to acquire a collapsible platform in future studies. In addition, pre-training in a different environment may be considered and spatial ability of all dietary groups can be tested with less involvement of procedure learning.

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Table 4.1 Timetable of spatial learning test with probing insertions

Feeding schedule	Day	Experiment
Adaptation	1	
	2	
	3	
	4	
	5	Screening for swim ability
	6	
	7	
	8	
	9	
	10	
Zinc depletion	1	
	2	
	3	
	4	
	5	
	6	
	7	
	8	
	9	
	10	
	11	
	12	
	13	
	14	
	15	
	16	
	17	Blood sampling for glucose concentration 1st day of training
	18	Probing 1 2nd day of training
	19	Probing 2 3rd day of training
	20	4th day of training
	21	Probing 3
	22	
	23	1st day of reversal learning
	24	2nd day of reversal learning
	25	Probing 4
	26	
	27	Sacrifice

Fig. 4.1 Areas under the curves of swim distance, cumulative swim distance and swim latency were calculated for each animal on each training day. Data are means ± SEM (n=13 per group). a. Value for the PF group was significantly greater than that of the ZD group. b. Value for the PF group was significantly greater than both the ZD and CT groups.

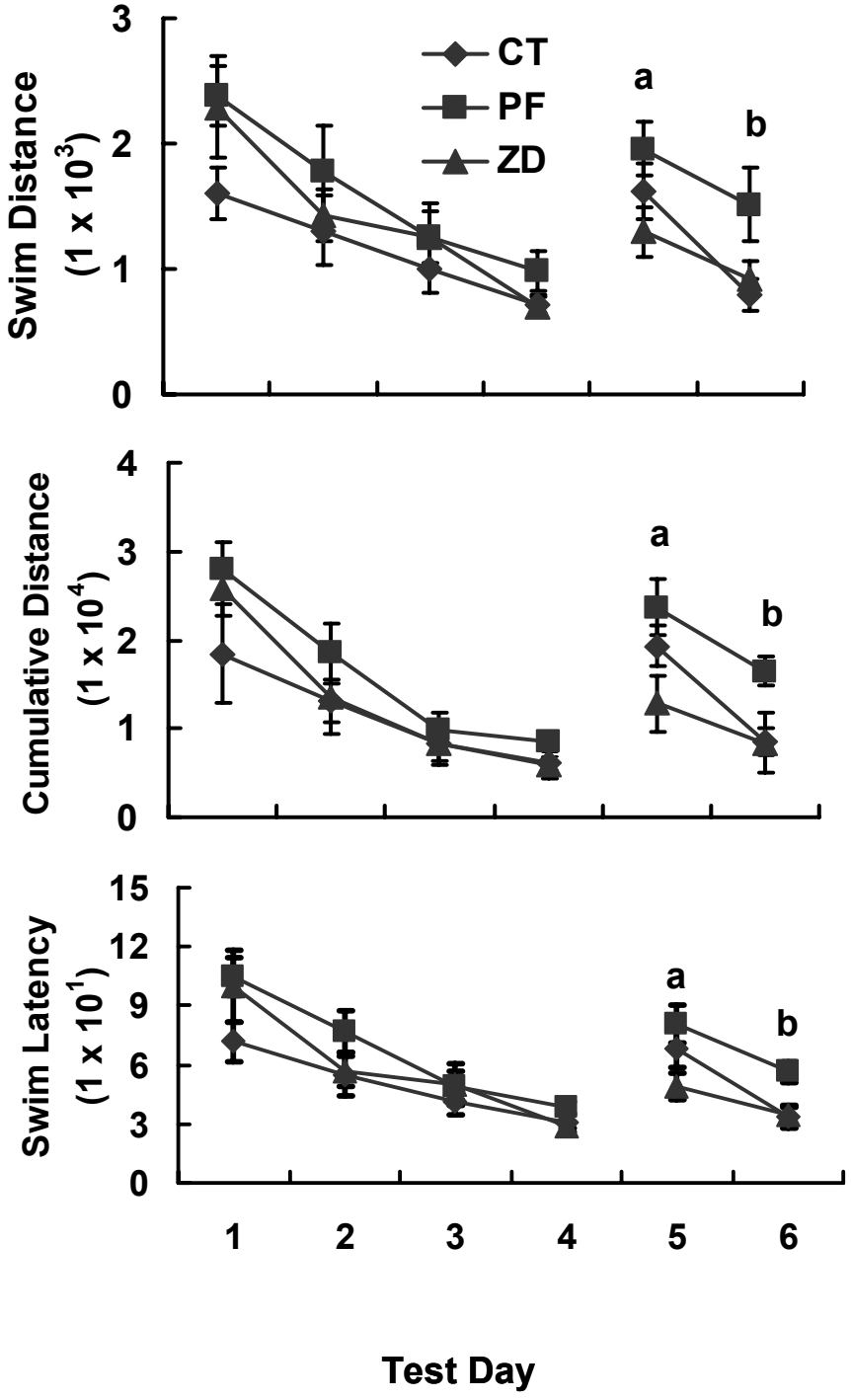

Swim Distance
(1 x 10^3)
CT
PF
ZD
a
b
Cumulative Distance
(1 x 10^4)
a
b
Swim Latency
(1 x 10^1)
a
b
0
1
2
3
4
5
6
9
12
15
Test Day

Fig. 4.2 Area under the curves of thigmotaxic ratio was calculated form each animal during each training day. Data are means ± SEM for groups of 13 rats. a. The PF group demonstrated significantly higher thigmotaxic ratio than the ZD group. b. The PF group exhibited significantly higher thigmotaxic ratios than both the ZD and CT groups.

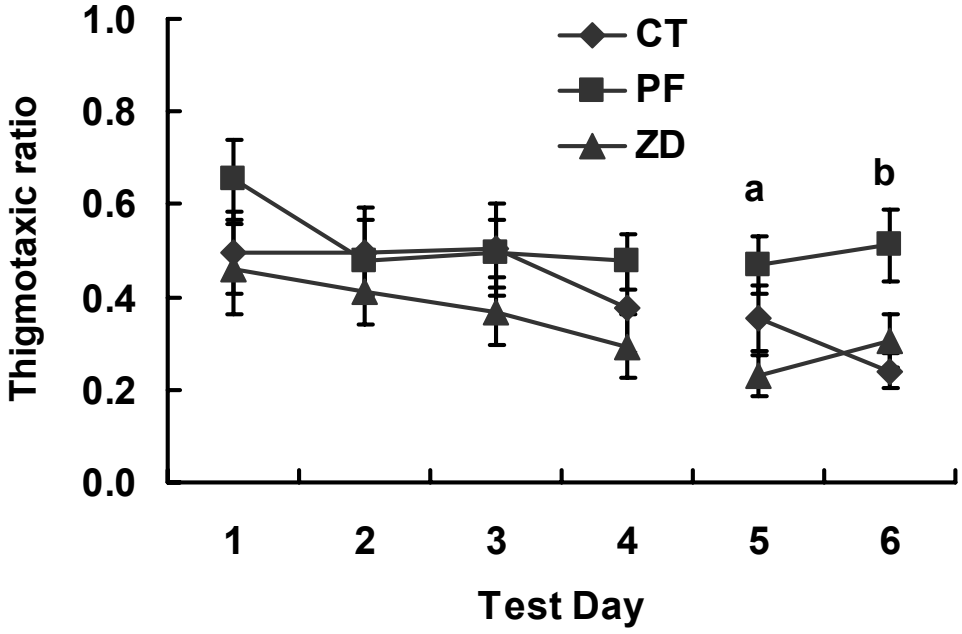

1.0
0.8
0.6
0.4
0.2
0.0
Thigmotaxic ratio
CT
PF
ZD
a
b
1
2
3
4
5
6
Test Day

Fig. 4.3 Percentage of time spent in the target zone A) and opposite zone B). n=13 in each group. Data are means ± SEM. Note: on the fourth day of probing test, the target zone and the opposite zone were in reality switched around compared with the first three probing test days because rats were subjected to reversal learning before the fourth probing test day.

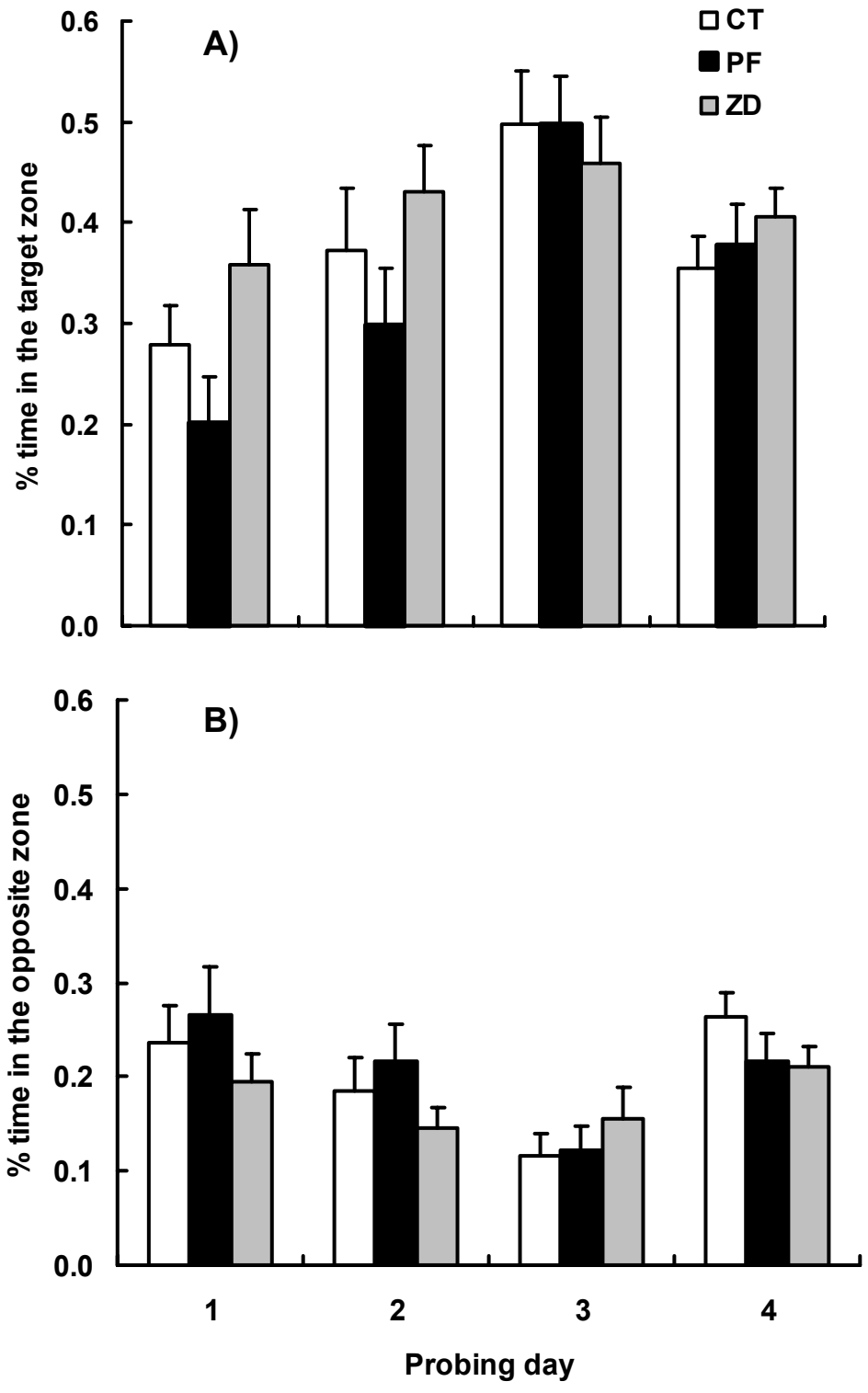

A)
CT
PF
ZD
% time in the target zone
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B)
% time in the opposite zone
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1
2
3
4
Probing day

Fig. 4.4 Area under the curves of thigmotaxic ratio during probing tests (n=13 in each group). a. PF group is significantly different from both ZD and CT group.

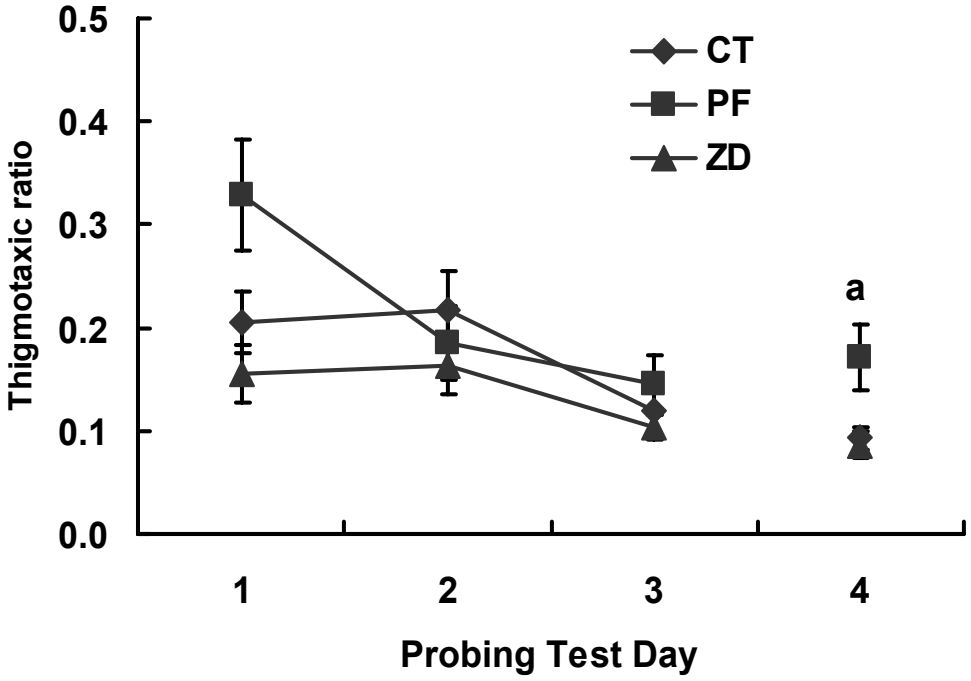

0.5
0.4
0.3
0.2
0.1
0.0
Thigmotaxic ratio
CT
PF
ZD
a
1
2
3
4
Probing Test Day

Fig. 4.5 Serum glucose levels in the CT, PF and ZD groups before swim training. Data are expressed as group means ± SEM. Superscripts with different letters indicate significant group difference (n=5 in each group).

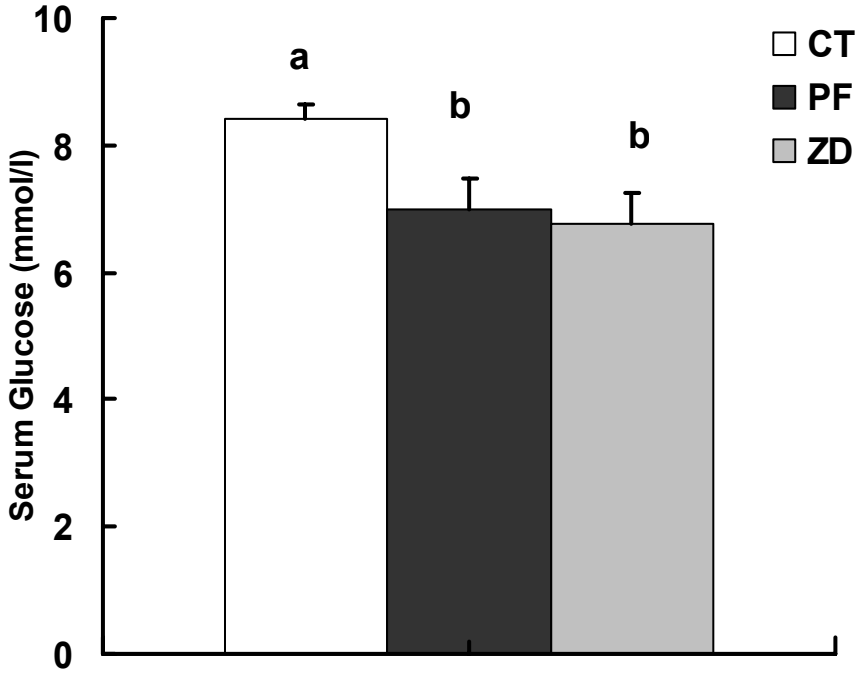
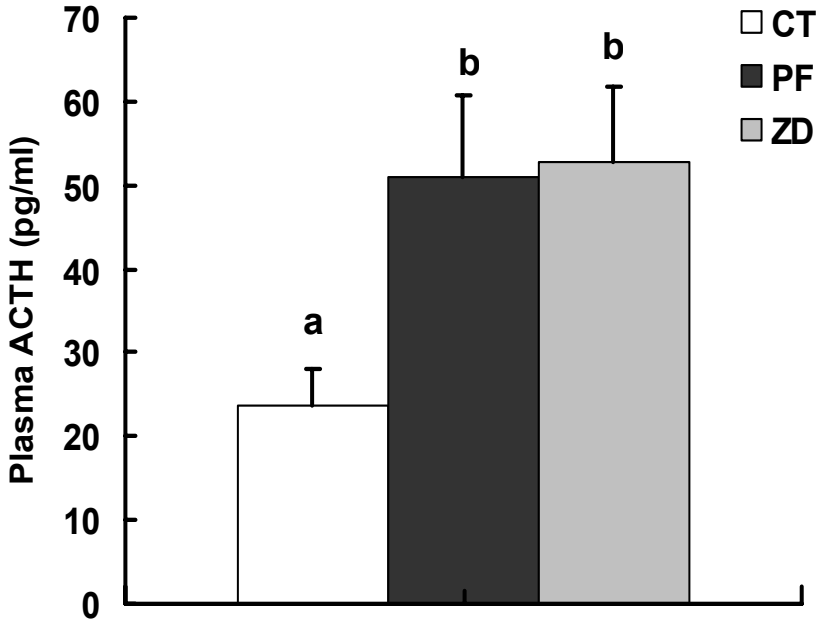

10
8
6
4
2
0
Serum Glucose (mmol/l)
a
b
b
CT
PF
ZD

Fig. 4.6 Plasma ACTH levels in the CT, PF and ZD groups measured after swim training. Data are expressed as group means ± SEM. Superscripts with different letters indicate significant group difference (n=10 in each group).


70
60
50
40
30
20
10
0
Plasma ACTH (pg/ml)
a
b
b
CT
PF
ZD

CHAPTER 5

THE EXPRESSION OF P2X6, A PURINERGIC RECEPTOR SUBUNIT, IS AFFECTED BY DIETARY ZINC DEFICIENCY IN RAT HIPPOCAMPUS[1]

[1] Chu, Y., Mouat, M.F., J.A. Coffield, R. Orlando and A. Grider. Accepted by *Biological Trace Element Research*. Reprinted here with permission of publisher, 11/05/02

Abstract

The purpose of these experiments was to determine whether dietary zinc depletion affected protein expression in the hippocampus. Eleven weanling Sprague Dawley male rats (21 d) were fed AIN-93G diet containing 1.5 ppm zinc and supplemented with 30 ppm of zinc in the drinking water. After one week, the rats were randomly divided into three groups, control (N=3), pair-fed (N=3), zinc-deprived (N=5). All groups consumed the same diet. The zinc restricted group consumed water containing no zinc. The rats were sacrificed three weeks later. Chelatable zinc levels in the hippocampus, as measured by N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) staining, were significantly reduced in the zinc-deprived group. Analysis of hippocampal protein expression by two-dimensional electrophoresis (2DE) revealed increased expression of the $P2X_6$ purinergic receptor in the zinc-deprived rats, as determined by MALDI mass spectrometry (MS) and database analysis. The data provide evidence for dual effects of dietary zinc deficiency on the hippocampus, reducing ionic zinc levels and stimulating protein expression. The role the $P2X_6$ receptor plays in the physiological response of the hippocampus to zinc depletion remains to be determined.

Introduction

Zinc is the fourth most abundant mineral in the central nervous system, exceeded only by sodium, potassium, and magnesium (Wallwork 1987, Wallwork et al. 1983). Zinc is a component of over 200 enzymes and proteins (Coleman 1992). Besides its catalytic role in numerous enzymes such as those necessary for the metabolism of GABA and catecholamines (Dreosti et al. 1981), zinc functions in gene expression through zinc finger, cluster, and twist transcription factors (Vallee et al. 1991, Vallee and Falchuk

1993). Zinc functions in a regulatory role as a modulator of GABA (zinc antagonizes (Bureau et al. 1997, Draguhn et al. 1990, Smart et al. 1991) and glutamatergic receptors (NMDA-zinc inhibits (Westbrook and Mayer 1987); AMPA-zinc potentiates (Huang 1997)). Zinc also potentiates the activity of ATP at P2X receptors (Acuna-Castillo et al. 2000, Lisman et al. 1998, Nakazawa and Ohno 1997, Seguela et al. 1996, Xiong et al. 1999). In addition, zinc has been reported to modulate homomeric α-7 receptors (Palma et al. 1998). These receptors are responsible for potentiating the excitatory action of ATP on mammalian neurons (Cloues et al. 1993, Li et al. 1993). Finally, zinc has also been found to be an endogenous ligand at σ-2 receptor sites (Connor and Chavkin 1992).

Zinc-containing neurons and nerve terminals have been identified in several regions of the brain including the hippocampus, cortex, and cerebellum (Frederickson et al. 2000). Within the neuron, zinc is found in three compartments: (1) synaptic vesicles of the nerve terminals, (2) membrane- or protein-bound available for metabolic reactions and intracellular structural support, (3) free or loosely bound within the cytoplasm (Frederickson 1989). The presence and compartmentalization of zinc within the brain suggests that it has a significant role in the normal function of the brain. The zinc-containing neurons are able to concentrate zinc within their terminals and release it in a calcium- and nerve impulse-dependent manner (Perez-Clausell and Danscher 1986).

The hippocampus participates in spatial learning and memory (Morris et al. 1982, Steele and Morris 1999) and contains the highest concentration of zinc of any brain region. It is particularly enriched in nerve terminals of the hippocampal mossy fiber system, where it may function in some aspect of synaptic transmission (Hesse 1979, Slomianka 1992, Xie and Smart 1991). Since the hippocampus contains concentrated

populations of both the NMDA and GABA receptors (Introini-Collison et al. 1994, Lisman et al. 1998, Moran et al. 1992, Paulsen and Moser 1998), hippocampal zinc may be an important modulator for their activity (Bureau et al. 1997, Draguhn et al. 1990, Huang 1997, Westbrook and Mayer 1987). For example, zinc induces pyramidal neuron hyperpolarization in organ culture by causing the release of GABA (Xie et al. 1993). The goal of the research presented here was to determine whether zinc depletion induced in rats fed a diet deficient in zinc influenced protein expression in the hippocampus of young rats. The results indicate that deficiency affected the expression of several proteins, including the $P2X_6$ purinergic receptor.

Materials and Methods

Male Sprague-Dawley rats (n=11; Harlan Sprague Dawley, Indianapolis, IN) aged 21 days were housed in 18 X 20 cm stainless steel wire-mesh cages. The room temperature of the animal facility was 22 ± 2 °C with relative humidity between 40 and 60% and a 12 h light: dark cycle (lights on 07:00 am). All rats were fed on zinc-deficient, egg-white based AIN-93G diets (Dyets Inc., Bethlehem, PA) containing 1.5 ppm zinc. The drinking water was supplemented with 30 ppm of zinc as zinc sulfate. After one week of adaptation, the animals were individually housed and randomly divided into three groups: zinc-deprived (n=3), pair-fed control (n=3), and ad libitum control (n=5). The control and pair-fed rats were fed the zinc-deprived diet and received 30 ppm zinc in their drinking water. The zinc-deprived rats were fed the zinc-depleted diet and received deionized water. The total number of days the rats were maintained on their diets was 28 days, at which time the experiment was terminated. All animals were anesthetized by halothane prior to sacrifice by decapitation. Blood was collected by heart

puncture. The blood was incubated at room temperature for 2 h and the serum separated by centrifugation at 1500 x g for 15 min at 20°C. Liver and femur samples were dissected for measurement of zinc status. The brain was dissected, half was saved for TSQ staining and the hippocampus was removed from the other half and saved.

Tissue zinc levels were measured by atomic absorption spectrophotometry (Keller et al. 2001). The brain regions, femur and liver samples were weighed in acid-washed borosilicate glass culture tubes. The samples were dried overnight in an oven set at 95°C, then ashed for 32 h in a furnace at 450°C. The ash was incubated for 45 to 60 min in 10 mol/L HCl, then diluted 10-fold with ddH_2O. The solublized samples were assayed for zinc using a flame atomic absorption spectrophotometer (Perkin-Elmer 5000; Perkin-Elmer Corp., Norwalk, CT) and compared with zinc reference solution (Fisher Scientific, St. Louis, MO). Serum samples were diluted 1:1 with ddH_2O prior to measuring the zinc concentration.

Fluorescent staining for zinc was performed as described by Frederickson et al. (Frederickson et al. 1987). Serially cryosectioned sagittal sections 20 μm thick were made through the brain using a refrigerated microtome and placed onto glass slides and air-dried. For staining with N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ; Molecular Probes; Eugene, Oregon), the slides were immersed in TSQ staining solution (140 mM sodium barbital, 140 mM sodium acetate, pH 10, 0.03% TSQ) for 90 s. and rinsed in 150 mM NaCl twice. Following the second rinse, the slides were air-dried. Fluorescence microscopy was used to visualize the zinc-containing regions within the hippocampus. An image analysis program (American Applied Biotechnology, Fullerton, CA) quantified the intensity of TSQ staining between the sections.

Hippocampus protein was isolated and the separated by two-dimensional gel electrophoresis (2DE) (Grider and Mouat 1998). The frozen hippocampus was placed in a 1.5 mL microfuge tube containing 240 µL of boiling sample buffer I (50 mM Tris-HCl, 200 mM dithiothreitol, 10.4 mM sodium dodecyl sulfate, pH 8.0) and homogenized. The homogenate was heated for 5 min at 100°C, then chilled on ice for 5 min. Sample buffer II (24 µL; 500 mM Tris-HCl, 50 mM $MgCl_2$, 1 g/L DNAse 1, 0.25 g/L RNAse A, pH 8.0) was then added to the homogenate and incubated for 8 min on ice. Proteins were precipitated by the addition of acetone for a final concentration of 13.8 M and incubation on ice for 20 min. Following centrifugation (12,000 x g for 10 min at 4°C), the supernatant was discarded and the pellet dried at room temperature for 5 min. The pellet was resuspended in 240 µL of sample buffer mix (22.4 mM Tris-HCl, 17.6 mM Tris, 7.92 M urea, 2.1 mM sodium dodecyl sulfate, 17.6 g/L ampholytes pH 3-10, 120 mM dithiothreitol, 51 mM Triton X-100). An aliquot was assayed for protein content using the Bradford method (Bradford 1976) and the remainder was stored at -80°C.

2DE was performed as previously reported (Grider and Mouat 1998). Analytical isoelectric focusing gels (180 mm x 1.2 mm i.d.) were purchased from Genomic Solutions. The gels consist of 9.5 M urea, 3.2 mM Triton X-100, 0.58 M acrylamide, 5 mM CHAPS and 0.58 g/L ampholytes pH 3-10. Sample overlay buffer (0.5 M urea, 3.2 mM Triton X-100, 1 g/L ampholytes pH 3-10, 50 mM dithiothreitol) was applied first to the tubes. The protein solution (75 µg) was then applied under the sample overlay buffer. The cathode buffer was 100 mM NaOH and the anode buffer was 10 mM phosphoric acid. The first dimension gels were run, without prefocusing, at 100 µA per gel for 17.5h.

The second dimension was run following the extrusion of the focusing gels from their tubes. The gels were incubated in the equilibration buffer (0.375 M Tris, 104 mM sodium dodecyl sulfate, 50 mM dithiothreitol, 1.4 mM bromophenol blue) for 2 min and loaded onto large format (22 cm x 22 cm x 1 mm) 12% acrylamide slab gels. The gels were run at 25 W/gel at 4°C until the dye front reached within 1 cm of the bottom of the gel. The cathode buffer contained 50 mM Tris base, 384 mM glycine and 6.9 mM sodium dodecyl sulfate. The anode buffer contained 25 mM Tris base, 192 mM glycine and 3.5 mM sodium dodecyl sulfate. The separated proteins were visualized by silver staining using a modified Rabilloud staining method (Rabilloud 1992).

Protein spots were excised from the gel and prepared for mass spectrometry as reported previously (Grider and Mouat 1998). Each gel plug was incubated with 150 µL of 50 mmol/L NH_4HCO_3 containing 12.2 mol/L acetonitrile. The gels were then dried and rehydrated with 5 µL of 50 mmol/L NH_4HCO_3 containing 0.5 µg trypsin. This solution was added in 5 µL aliquots until the gel piece regained its original size. The gel was then covered with 50 mmol/L NH_4HCO_3 and incubated overnight at 30°C. After the addition of 1.5 µL of 880 mmol/L trifluoroacetic acid the peptides were extracted with 50 mmol/L NH_4HCO_3 containing 14.6 mol/L acetonitrile. The supernatants were dried to ~10 µL. The samples were then analyzed on a Kratos SEQ MALDI-TOF (Manchester, UK) mass spectrometer. This instrument was operated in linear mode using pulsed ion extraction to detect positive ions, with an accelerating voltage of 20 kV, and a pressure below 1 x 10^{-6} Torr. The samples were desorbed/ionized from the MALDI target using a nitrogen laser source with an output wavelength of 337 nm. Calibration was performed using mixtures of peptides/proteins with known molecular masses. Alpha-cyano-4-

hydroxycinnamic acid dissolved in 70% aqueous acetonitrile with 0.01% trifluoroacetic acid was used as the MALDI matrix. Sample solutions were mixed with the matrix solution, and allowed to air dried on the MALDI target.

Results

After three weeks of zinc restriction, tissue zinc levels were analyzed by one-way ANOVA. A significant dietary effect was found in liver, femur, serum and cerebrum ($P<0.01$). Post hoc analysis indicated that the femur, liver and serum zinc levels of the pair-fed rats were the highest among the three groups ($P<0.05$). The zinc levels in these tissues from the zinc-deprived rats were all significantly lower than those of the control rats (Table 5.1). The femur zinc level in the zinc-deprived group was 50% lower than that of the control group and 54% lower than pair-fed group. The liver zinc level in the zinc-deprived group was 20% and 42% lower than the control and pair-fed groups, respectively. The serum zinc level from the zinc-deprived group was 78% and 83% lower than the control and pair-fed groups, respectively. The cerebrum zinc level of the zinc-deprived group was slightly, but significantly, less than both control groups. No significant difference was found between the control and pair-fed groups. The zinc levels in the femur, liver, and serum from the pair-fed group were significantly higher compared to the control group (increased 9%, 37%, and 33%, respectively). No significant difference in cerebellum zinc was detected between the groups.

The chelatable zinc pool in the hippocampus was detected with TSQ staining (Fig. 5.1). The hippocampus from rats fed a zinc-deficient diet exhibited reduced staining for zinc. The data indicate that the hippocampus from the zinc-deprived rats

exhibited a 32% ($P<0.05$) decrease in fluorescence compared to their pair-fed and control rats (Fig. 5.2).

Hippocampal proteins were separated by two-dimensional gel electrophoresis (Figure 5.3). The stained gels from control, pair-fed, and zinc-deprived rats were very similar in their protein expression patterns, except in the area designated by the black box. This area is reproduced for each dietary group and enlarged in Fig. 5.4. Four spots exhibited differential expression. Spots 1 and 2 were increased in the control and pair-fed groups, and disappeared in the zinc-deficient group. Spots 3 and 4 were up regulated in the zinc-deprived group. Although all of the spots were excised and analyzed by MALDI mass spectrometry, only spot 3 contained enough protein for identification despite the pooling of spots from three separate 2DE gels. This protein was identified as the $P2X_6$ purinergic receptor.

Discussion

The status of zinc deficiency was confirmed by the dramatic decrease in systemic zinc of the zinc-deprived rats. In the meantime, zinc level in the brain tissue was comparatively well conserved with only a 3% drop in cerebrum zinc concentration. These results suggest the ranking of zinc distribution upon shortage of supply in the body. An interesting observation was that pair-fed systemic zinc was even higher than that of the control. This phenomenon may have been caused by the increased water consumption in these rats. Since zinc was supplemented in the water, these rats may get more zinc than the control rats. However, the increased systemic zinc did not induce any change in the brain zinc level. Again, these observations suggest the tight regulation of zinc in the central nervous system.

Deficits in spatial memory can be caused by either the surgical lesion of the hippocampus (Morris et al. 1982) or by reducing hippocampal zinc levels (Frederickson et al. 1990, Keller et al. 2000, Keller et al. 2001). Adult rats fed a zinc-depleted diet exhibit transient impairment of learning behavior, and this was correlated with significant reduction in zinc from the hippocampal synaptosomal fraction (Takeda et al. 2000). Repletion of dietary zinc restored short-term memory in zinc-depleted young adult rats (Keller et al. 2000). Dithiozine treatment, or dietary zinc deficiency, caused a reduction in the mossy fiber zinc content of the rat hippocampus (Wensink et al. 1987). The transmission of neuronal impulses through the mossy fibers was altered with a dietary zinc deficiency (Hesse 1979). Collectively, the results of these various studies support the hypothesis that zinc is involved in memory processing (Takeda et al. 1994).

The specific roles for zinc in hippocampal function have not been fully elucidated. Since the small perturbation of zinc level in the cerebrum of zinc-deprived rats was detected, the protein expression was further analyzed in the hippocampal region beneath the cerebrum using 2D-PAGE. The results presented here indicate that dietary zinc levels also affect protein expression. Certainly one role is as a modulator of receptor function (Bureau et al. 1997, Draguhn et al. 1990, Huang 1997, Smart et al. 1991, Westbrook and Mayer 1987). The results presented here indicate that dietary zinc levels also affect protein expression. Interestingly, both increased and decreased expression was observed, and the reduction in vesicular zinc correlated with the increase in $P2X_6$ expression. The $P2X_6$ receptor is a 379 amino acid protein that acts as ATP-gated ion channel. It is widely expressed in the body, and along with the $P2X_4$ receptor is the predominate P2X receptor in the adult rat brain (Collo et al. 1996). Beside the brain, $P2X_6$ receptor is

expressed in secretory tissues such as pituitary and adrenal gland (Soto et al. 1996). Some studies show that rat $P2X_6$ receptor forms heteromeric ATP receptor complexes with $P2X_2$ or $P2X_4$ subunits (King et al. 2000, Le et al. 1998). The function of this receptor in cognitive function is not known at this time. However, P2X receptors have been shown to facilitate glutatmate release, and ATP may potentiate the NMDA receptor-mediated depolarization while adenosine may inhibit NMDA-induced current (Illes et al. 2001). Further, both P2X and NMDA receptor antagonists cause analgesic and anesthetic effects in rats (Masaki et al. 2001). Since dietary zinc-restriction induced the elevation of $P2X_6$ receptor expression, it may help modulate NMDA receptor function following the removal of the allosteric inhibitor, zinc. The $P2X_6$ receptor, the NMDA receptor, and zinc may also interact through protein kinase C (Boue-Grabot et al. 2000). The current report indicates that 2DE is useful for identifying the potential target genes whose expressions are altered by dietary zinc-restriction. Future research will focus on understanding the role(s) these zinc-responsive gene products have in hippocampal function.

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Table 5.1 Tissue Zinc Concentration Following Zinc Restriction (μg/g of wet tissue).

	Femur	Liver	Cerebrum	Cerebellum	Serum
CT	191.9 ± 2.3^{a}	32.6 ± 1.0^{a}	14.9 ± 0.1^{a}	12.5 ± 0.3^{a}	1.8 ± 0.06^{a}
PF	208.6 ± 6.7^{b}	44.8 ± 3.2^{b}	14.7 ± 0.1^{a}	12.1 ± 0.1^{a}	2.4 ± 0.08^{b}
ZD	96.1 ± 2.9^{c}	26.1 ± 0.7^{c}	14.2 ± 0.1^{b}	12.1 ± 0.1^{a}	0.4 ± 0.02^{c}

The zinc concentration was measured by atomic absorption spectrometry. The data are expressed as the mean ± pooled SEM. CT = Control group; PF = Pair-Fed group; ZD= Zinc-deprived group; N=3-5 per group of rats. Values with different superscripts are significantly different from each other ($P<0.05$). The tissues from each animal were measured in duplicate. Significance was determined using one-way ANOVA, and post-hoc analysis was performed using the T-test (LSD) (SAS version 8.2).

Fig. 5.1 Fluoresence image of zinc following TSQ staining. Histochemical staining indicates the presence of gluzinergic boutons within the hippocampus. The hilus (**H**), mossy fiber (**MF**) and the CA1 (**C**) regions are shown for the A) control, B) pair-fed, and C) zinc-deprived dietary groups.

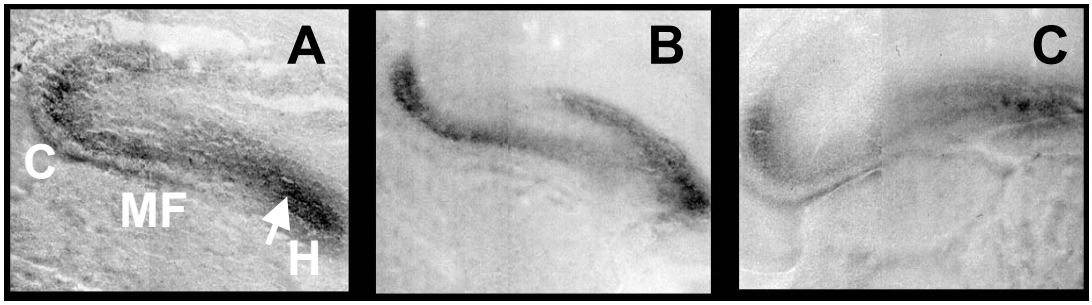

A
B
C
C
MF
H

Fig. 5.2 Hippocampus from zinc-deprived rats exhibits reduced bouton zinc. The relative density from the fluorescent staining of gluzinergic boutons (Fig. 5.1) was measured. A total of 9 images from 3 different rats were analyzed. Significance ($p<0.05$) was determined using one-way ANOVA and the T-test (LSD) post-hoc test (SAS version 8.2). The values in the graph represent the mean ± pooled SEM. The asterisk (*) indicates significant differences compared to both the control and pair-fed groups.

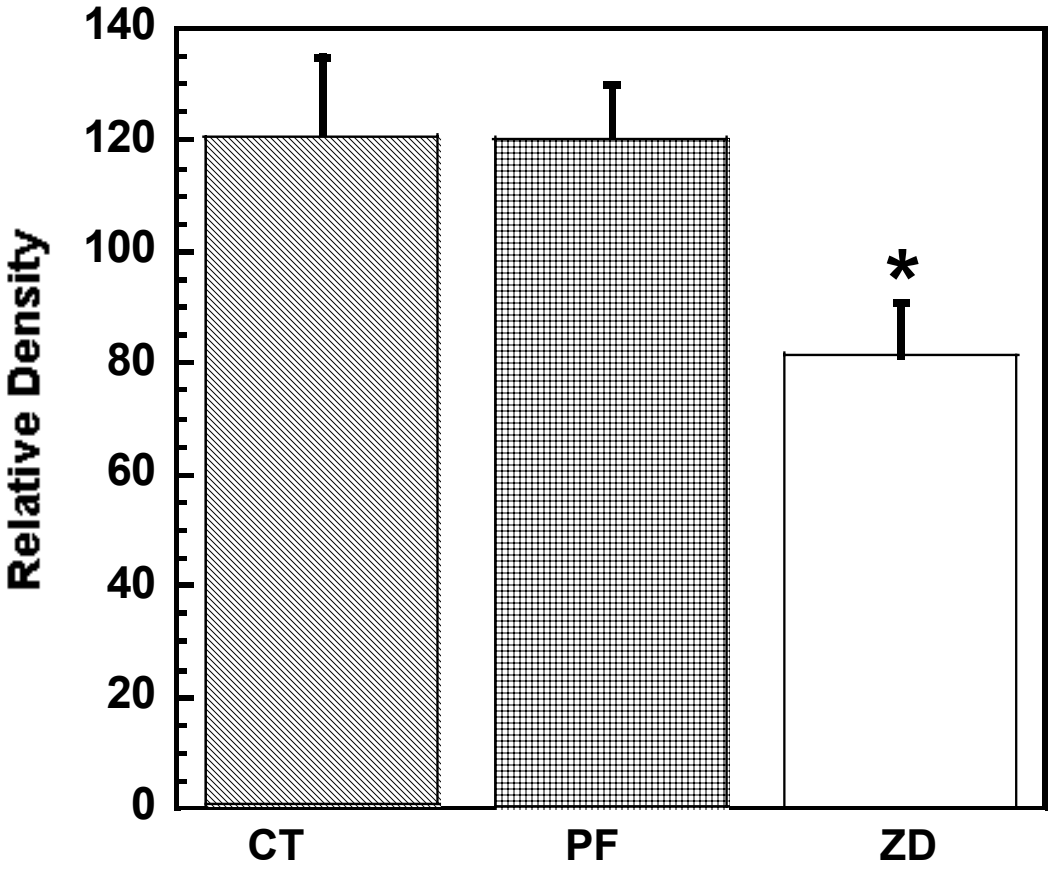

140
120
100
80
60
40
20
0
Relative Density
*
CT
PF
ZD

Fig. 5.3 Representative 2DE of total hippocampal lysate from a control rat. The sample was separated in the first dimension using carrier ampholyte isoelectric focusing, and a 12% SDS-PAGE slab gel in the second dimension. The area within the black rectangle contains the proteins that were excised and prepared for MALDI mass spectrometry.

Acidic Basic

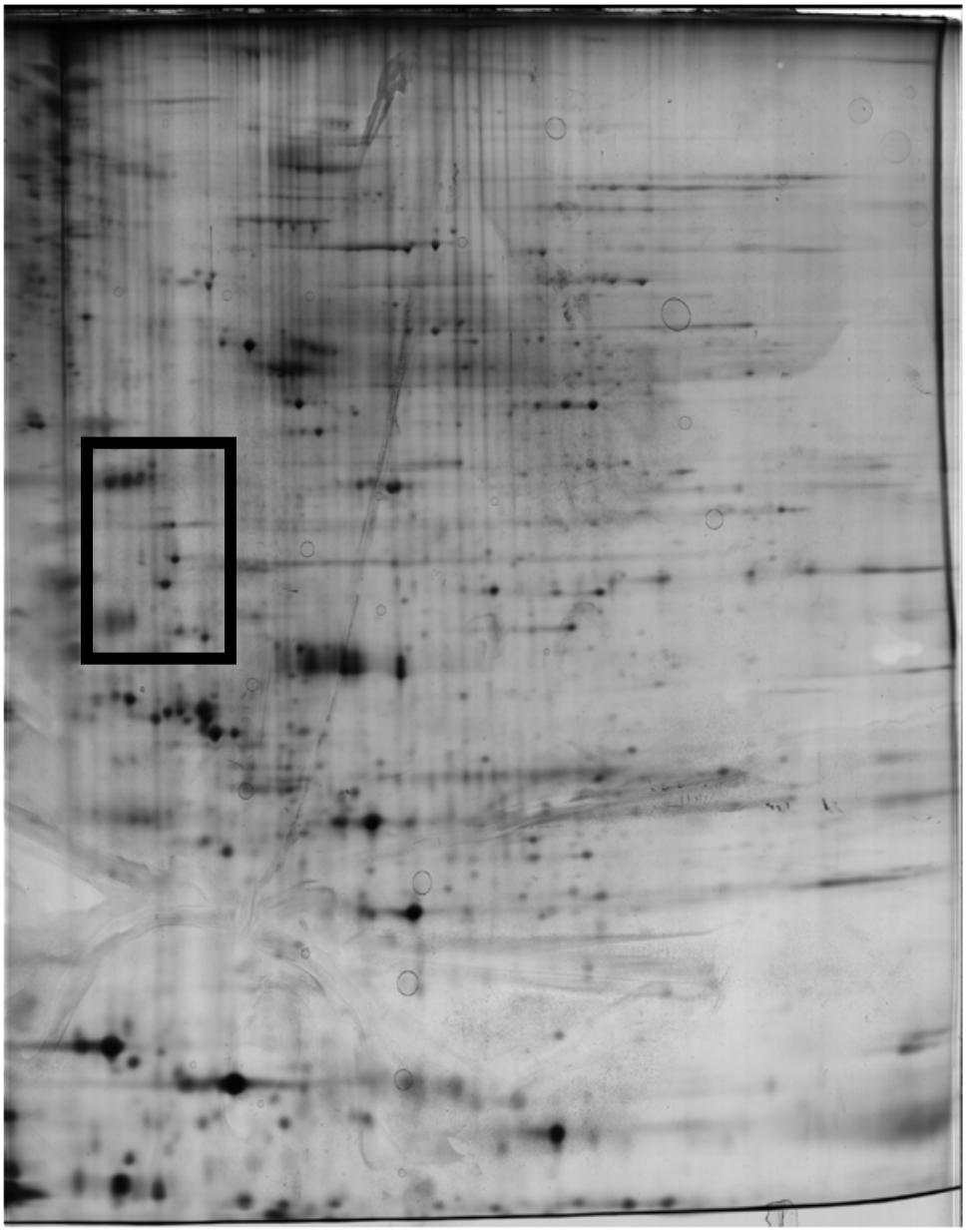
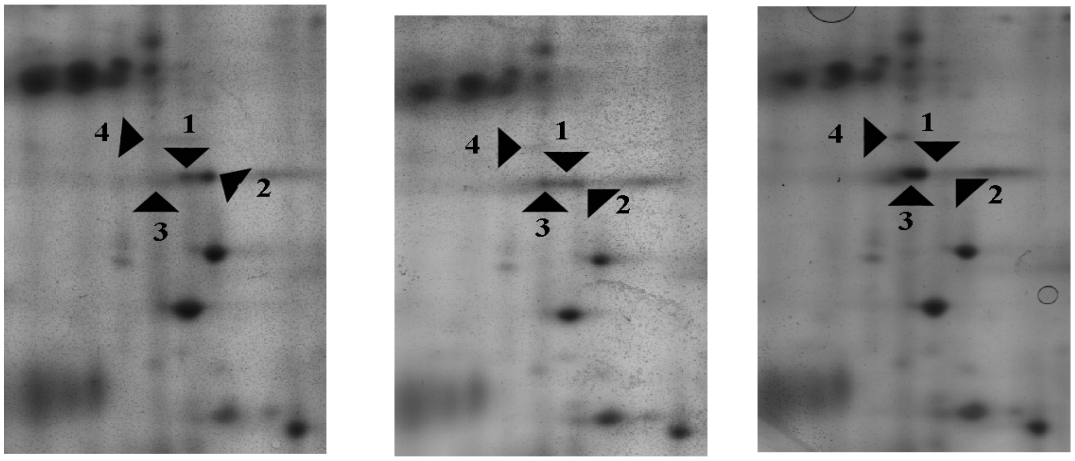


Fig. 5.4 Differential hippocampal protein expression with dietary zinc depletion. The protein spots analyzed by mass spectrometry are indicated with the arrowheads 1-4. Spots 1 and 2 were absent from the zinc deficient sample. Spots 3 increased in the pair-fed group compared to the control, and in the zinc deficient group compared to the pair-fed group. Spot 4 increased in the zinc-deprived group compared to both the control and pair-fed groups.



CT PF ZD

CHAPTER 6

PARTIAL PROTEOMIC ANALYSIS OF RAT HIPPOCAMPUS USING IMMOBILIZED PH GRADIENT GEL[1]

[1] Chu, Y., M.F. Mouat, J.A. Coffield and A. Grider. To be submitted to *Electrophoresis*

Abstract

Previously, hippocampal protein extracts from ZD, PF and ZD rats were separated by two-dimensional gel electrophoresis (2-DE). Several protein spots with differential staining intensity were submitted for sequencing. P2X6 purinergic receptor was up regulated in the ZD group. The regulatory effects of dietary treatment on hippocampal protein expression were further explored using IPG strips. Gel images were analyzed with Phoretix image analysis software. Twenty differentially expressed protein spots were tentatively identified using TagIdent of the SWISS-PROT database. Some candidate proteins play roles in stress, food intake regulation and learning processes. Definitive identification of these protein spots requires amino acid sequencing by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). The results from this study indicate that zinc deprivation and pair feeding change the expression of different proteins.

Introduction

2-DE is used to identify the differential expression patterns of gene products, and is a major tool of functional genomics (Patton 1999). The examination of protein expression at this level has been referred to as proteomics (Mahon and Dupree 2001). For instance, biological pathways that have been affected by drug treatment can be revealed by examining the effect of the drug on the regulation of protein expression. 2-DE has been used to discover early detection markers of various types of carcinomas (Brockstedt et al. 1999, Park et al. 2002, Steel et al. 2001) demonstrating the validity and reliability of this gel running technique. The effectiveness of proteomics has been

demonstrated in our previous study in which we identified the up-regulation of the $P2X_6$ receptor in zinc-deprived rats.

2-DE separates proteins by isoelectric point in the first dimension and molecular weight or size in the second dimension. In the previous study (chapter 5), carrier ampholyte IEF (isoelectric focusing) gels, which generate a pH gradient by the migration of thousands of low-molecular-weight polyamino-polycarboxylic acids, were used. Some problems with this type of IEF approach are cathodic drift and a limitation on loading capacity. Cathodic drift reduces the resolution of protein spots at the basic end of this type of gel (Bjellqvist et al. 1982). In some cases, NEPHGE (non-equilibrium pH gel electrophoresis) has been performed to resolve those basic proteins that could possibly run off the end of the gel. The loading capacity of the analytical gel is restricted to 100 μg, though one could increase the loading capacity to 300 μg with a preparative IEF gel system. Recently, immobilized pH gradient (IPG) strips were introduced whose pH gradient was covalently fixed to the polyacrylamide matrix during gel polymerization. Using IPG gels not only overcomes the cathodic drift problem inherent in carrier ampholyte gels but the loading capacity of a single gel is increased more than 50 fold (Hanash et al. 1991).

Protein identification through mass spectrometry (MS) requires trypsinizing and releasing the peptides from the gel matrix. Although silver staining is a sensitive detection method for visualizing protein spots on a gel, the compatibility of this staining method with MS is poor. Others have demonstrated low yield of peptide for sequencing following silver staining (Lauber et al. 2001). In our previous study, only one out of four submitted protein spots from silver stained gels was able to be sequenced by MALDI-

MS. Although Coomassie blue staining is less sensitive than silver staining, it is a reliable staining method and compatible with MS. More database matches are available with Coomassie blue staining (Scheler et al. 1998). In addition, this staining method is of relatively low cost. Lastly, a Phoretix 2-DE gel image analysis system was acquired, which allows for quantitiation of protein expression.

Regulatory effects of dietary treatment on hippocampal protein expression have been studied in Chapter 5. Sequencing of the proteins spots however was limited by the amount of protein in the gel. Therefore, the purpose of the current study was to increase the sample loading capacity by using IPG strips and quantifying the differentially expressed proteins after dietary treatment. Thus, 2 mg protein extracts from rat hippocampi was separated by IPG gel electrophoresis with high resolution. Protein spots with at least a 50% change in staining intensity relative to that of the control group were selected and submitted for sequencing. The results further support the regulatory effect of dietary treatment on protein expression in the hippocampus. These protein spots are awaiting MS analysis to determine their actual identities.

Materials and Methods

Sample preparation

Male Sprague-Dawley rats (Harlan, Indianapolis, IN), 27-31 days old, were housed under the same conditions as described in materials and methods in Chapter 3. During the first 10 days of the adaptation phase, all rats were fed AIN-93G egg-white based diet with 20-25 ppm zinc (Dyets, Bethlehem, PA). Then they were divided into three dietary groups (CT, PF and ZD). Diet for the CT and PF groups was the same as the diet offered during the adaptation phase whereas diet for the ZD rats was changed to

modified AIN-93G egg-white based diet with less than 1 ppm zinc (Dyets, Bethlehem, PA). After 24 days of dietary treatment, rats were sacrificed and hippocampal tissue was quickly dissected out, frozen in liquid nitrogen and stored in –80 °C until further use.

Hippocampal tissue was placed in a biopulverizer (Biospec, Bartlesville, OK) pre-chilled in liquid nitrogen. Protease inhibitor 1 and inhibitor 2 were added into the pulverizer at volumes equal to 0.08 and 0.02 times the sample tissue weight respectively. Protease inhibitor 1 contains 50 mM Tris (pH 7.0), 100 mM KCl, 20% glycerol and protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany) added at 1 tablet/2 ml of buffer. Protease inhibitor 2 contains 0.2 mM KCl, 20% glycerol, 0.1 M phosphate buffer (pH 7.1) and protease inhibitor cocktail added at 1 tablet/2 ml of buffer. Tissue and the buffer were immediately pulverized and transferred completely into a frozen eppendorf tube. Glass beads (diameter 4 mm) were added into the tube. The number of glass beads was determined by the tissue sample weight (μg) x 0.102. The sample powder was warmed up on ice, then the mixture was sonicated in ice cold water for 6 rounds. For each round, the sample was sonicated for 10s and place on ice for 50 s, meanwhile a thin wire was used to stir the sample mix to reduce overheating. Afterwards, sample buffer I was added into the sample homogenate at the volume of two times the sample weight. Sample buffer I contained 0.3% SDS, 200 mM DTT, 28 mM Tris-HCl, 22 mM Tris-base. The sample mixes were vortexed and incubated at 100°C for 5 min followed by 5 min cooling on ice. Then sample buffer II (24 mM Tris base, 476 mM Tris HCl, 50 mM $MgCl_2$, 1 mg/ml DNAase I, 0.25 mg/ml RNase A) was added and the sample mix was further incubated on ice for 10 min. Crystalline urea was added at 1:1 times the total weight of the mixture and the sample mix was stirred at room

temperature for 30 min. Subsequently, the sample mix was centrifuged at 80,000 g for 30 min. Acetone was added to the supernatant to a final concentration of 80% and the mixture was incubated at room temperature for 5 min. The protein precipitate was pelleted by centrifugation at 12,000 g for 10 min. The protein pellet was air-dried at room temperature for 5 min and re-suspended in sample loading buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% pharmalyte 3-10). All these steps were performed for the purpose of protecting the integrity of protein in the extract (Klose and Kobalz 1995). Protein concentration of the sample was measured by the Bio-Rad protein assay (Bio-Rad, Richmond, CA). The isolated protein was stored at -80°C until further use.

Two-dimensional gel electrophoresis

First dimensional isoelectric focusing was performed using immobilized pH gradient gels (IPG strip 17 cm, pH 3-10, Bio-Rad, Richmond, CA). Hippocampal protein extracts of each of three rats in each dietary group were pooled together (0.667 mg/extract). Two mg protein per dietary group was loaded onto the IPG strips. The volume of the pooled sample was increased to 300 ul by adding IPG rehydration buffer (6M urea, 2M thiourea, 2% CHAPS and 0.4% DTT). The samples were applied to dried IPG strips that were subsequently passively rehydrated for 12 hrs at room temperature. Isoelectric focusing was performed at 250 V for 15 min, followed by 10,000 V for 6 hrs at 20 ºC (Protean IEF cell, Bio-Rad, Richmond, CA). Second dimension electrophoresis was then performed (12.5% poly-acrylamide with dimensions of 22 x 22 x 0.1 cm).

Prior to running the second dimension, the IPG gel was equilibrated in 5 ml equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 0.05 M Tris, 2 mM tributylphosphine (TBP), trace amount of bromophenol blue) for 20 min. The gel was

then removed, quickly dipped into the upper running buffer, and carefully laid onto the top of the slab gel and sealed with a layer of agarose solution (1% low melting point agarose in upper running buffer). Electrophoresis was performed at 20 W/gel until the dye front migrated to 1 cm from the bottom of the gel.

Staining and image analysis

Coomassie brilliant blue (CBB) staining was used for detecting the protein spots. The gel was soaked in 200 ml staining solution (0.025% CBB, 40% methanol, 7% acetic acid) overnight and destained with destaining solution 1 (40% methanol, 7% acetic acid) for 30 min. Then the solution was changed to destaining solution II (7% acetic acid, 5% methanol) twice a day until the gel background was clear. The gel was stored in 7% acetic acid after destaining.

Protein analysis

The gel image was scanned and analyzed with Phoretix software (Nonlinear Dynamics Ltd, Newcastle, UK). The analysis included spot matching, alignment, and quantitation. To account for the variability inherent in the analysis software, a 50% up or down staining intensity was interpreted as a significant regulatory change in protein expression. Differentially expressed protein spots were determined by comparing the normalized staining volume of the spots from ZD and PF gels to those of the CT gels. Spots with staining levels of change greater than 50% were tentatively identified via the SWISS-PROT protein database (Olivieri et al. 2001).

Results

Approximately 500 spots were detected. The 2D gel image of the CT group is shown in Fig. 6.1. Twenty spots were found to exhibit differential staining intensities

between the dietary treatment and CT groups. Magnified gel regions, which demonstrate variation in protein expression levels among dietary groups, are illustrated in Fig. 6.2. The normalized volume of these spots and the level of change relative the CT gel are listed in Table 6.1. The normalized volume was calculated with consideration of both background intensity and total volume of all spots on the gel. Consequently, the comparison of the normalized volume of spots between different gels is meaningful since the variations between gels due to difference of sample loading and/or destaining efficiency were accounted for. Apparent pI and Mr of these spots, as determined by the migration of molecular weight markers and the pH gradient on the IPG strips, have also been listed in Table 6.1. Apparent pI and Mr of these spots were submitted for protein identification by the SWISS-PROT database. Candidate proteins for the spots are listed in Table 6.2. The proximity of some differentially expressed spots to one another results in an overlapping of the range of pI and Mr for protein identification. Thus the potential protein candidates for these spots have been reported together.

Among these differentially expressed protein spots, one spot (No. 16) was present in the CT gel and not detectable in the PF and ZD gels. Two spots (No. 2 and 19) were detectable only in the ZD gel but not in the PF or CT gels. Another two spots (No. 10 and 14) appeared in the PF gel but not in the ZD and CT gels. One spot (No. 1) was detectable in both the PF and ZD gels but not in the CT gel. Two spots (No. 3 and 6) in the ZD gel were down regulated compared with the CT gel whereas the level of change of those spots on the PF gel was less than 50%. Spot 13 was down regulated in both ZD and PF gels where this spot separated as doublets. The spot intensities of No. 5 and 11 increased significantly only in the ZD gel and the intensities of No. 4, 7, 8, 17 and 18

were increased only on the PF gel. However, for spots No. 9, 12, 15 and 20 the staining intensities in both the PF and ZD gels were increased more than 50% as compared with the CT gel.

Fig. 6.3 presents images at the high molecular weight region of the gel. The gel analysis program indicated several changes in spot intensity in this region. Because these spots were not as focused as the spots in the low molecular weight range, potential candidates for expression regulation were not determined.

Discussion

It has been shown that different protein expression levels between individual animals are mainly of a quantitative nature and the variations are inconsistent (Fountoulakis et al. 1999). Therefore, in the current study, pooled protein samples within each of the three dietary groups were separated on 2D gels, so that the variation of protein expression induced by individual animals was minimized. More than 90% of the spots were matched on the gels indicating the high reproducibility of IPG strips. The staining patterns of these gels were not similar to our previous silver staining. Several reasons may contribute to the difference between the results. Firstly, IPG strips were used in place of carrier ampholytes, which results in a different pH gradient in the first dimension. Secondly, the dynamic range of silver staining was nonlinear and the silver dye tends to stain differently based on protein post-translational modification and amino acid composition (Merril et al. 1984), whereas CBB staining appears to be linearly correlated with the amount of protein (Neuhoff et al. 1990). Thirdly, freeze fracturing and ultrasonic sonication in ice cold water were used for protein extraction in the current study. These procedures provide better protection against proteolysis and more complete

protein extraction, compared to homogenized tissue at room temperature (Klose and Kobalz 1995). Our Coomassie blue gel staining pattern does resemble the image of published gels from both rat brain and human hippocampus which also use IPG gels and Coomassie blue staining (Edgar et al. 1999, Fountoulakis et al. 2000). Of particular interest is that the shape and position of the strongest spots (a, b in Fig. 6.1) on our gels which were similar to spots representing tublin beta chain and actin beta chain in the neonatal brain. These two spots became much fainter in the gel of the eight-month-old rat (Fountoulakis et al. 2000). The similarity indicates that the quality of our gels is comparable to that of published results.

Twenty spots exhibited differentially protein expression among the gels from the three dietary groups. The actual identification of these proteins requires amino acid sequencing by MALDI-MS. In the absence of this information, a tentative identification was performed using the apparent Mr and pI with the TagIdent tool in the SWISS-PROT database (Olivieri et al. 2001). The limitation of this analysis is that the apparent Mr and pI of a protein spot measured on a 2D gel does not always exactly correspond to its theoretical Mr and pI calculated from the amino acid sequence, even though good agreement has been found for proteins from the hippocampus (Edgar et al. 1999). A range of observed pI $\pm$ 0.25 and Mr $\pm$ 20% was applied in the database searching to cover the deviation of apparent values. Consequently, several protein candidates were identified for each protein spot (Table. 6.2).

Among these candidates, the $P2X_6$ receptor was found to be one of the two candidate proteins for spot No. 5. This protein was up regulated in the ZD rats compared

with the CT rats. The relative level of staining intensity of this spot among dietary groups was similar to the $P2X_6$ receptor identified previously (Chapter 5).

Several candidate proteins are responsive to stress status of the animal. For instance, heat shock 70 kDa protein was one of the candidates for spot No. 2, whose expression was only detectable in the gel of the ZD group. It has been found that the expression of this protein is increased upon restraint immersion stress (Fukudo et al. 1995). Another heat shock protein with a molecular weight of 27 kDa was one of the candidates of spot No. 9. Both the ZD and PF groups exhibited elevated expression of this spot. This protein is constitutively expressed in many neurons in the brain and is strongly inducible in neurons expose to heat stress or brain injuries (Currie et al. 2000, Krueger-Naug et al. 2000). It has been suggested that these proteins play neuro-protective roles in conditions of stress. Chronic stress in the PF and ZD rats may cause the elevated expression of these candidate proteins.

Several other candidates have been implicated in appetite regulation. Selective activation of the melanocortin-4 (MC4) receptor by its agonist suppresses food intake in animals without other nonspecific effects (Benoit et al. 2000). MC4 receptor antagonist also relieves the anorexia induced by immobilization stress (Vergoni et al. 1999). In addition, MC4 receptor also participates in stress response of animals. Over expression of agouti protein, the endogenous antagonist for MC4 receptor, increases the stress responsiveness in mice (Harris et al. 2001). The melanocortin-3 (MC3) receptor in the hypothalamus may influence the release of orexigenic peptides from the arcuate nucleus (Dhillo et al. 2002). However, the direct regulatory effect of this receptor on food intake is debatable (Benoit et al. 2000, Kask et al. 2000). The melancortin-3 receptor was only

detectable in the PF group (spot No. 10). The melancortin-4 receptor was one of the candidate proteins for spot No. 11. Both the PF and ZD groups exhibited higher levels of expression of this spot. The increases observed in these spots in the PF and ZD groups might be an adaptive response to reduced food intake in both groups.

The family of 5-hydroxytryptamine (5-HT) receptors have been suggested to have a complex role in learning and memory (Meneses 1998). Studies were focused on the effects of the receptor agonists or antagonists on learning. It is not known how a change in expression in these receptors might influence memory process. 5-HT 5B receptor was identified as one of the candidate proteins that were possibly regulated by dietary treatment (Spot No. 13, 14, 15). The functional relevance of other protein candidates to dietary treatment is unknown.

Streaking was found at the 80-160 kDa molecular weight range which reduces the sharpness of spots (Fig. 6.3). Although the Phoretix image analysis system suggests that the staining intensity of some spots was different among the gels, it was difficult to determine if this truly reflected the change in protein expression levels. Due to the greater tendency of protein aggregation for those high molecular weight proteins, the migration of these proteins from the first dimensional gel into the slab gel is usually incomplete. Thus, vertical streaking is observed at the high molecular weight range (Gorg et al. 2000). In addition, the travel distance on the 12.5% constant SDS-PAGE slab gel for these high molecular weight proteins is relatively short which limits the separation of these proteins. To further identify the regulatory effect of dietary treatment on high molecular weight proteins, it will be necessary to increase the amount of reducing agents and chaotropes in the tissue lysis buffer. Using large-pore-gradient gels

could promote the migration of large proteins into the gel and give better separation of these proteins (Zuo et al. 2001).

In summary, the effect of zinc deprivation and pair feeding on hippocampal protein expression initiated in the experiment described in Chapter 5 was further explored in the current study. The identities of twenty protein spots that were regulated by the dietary treatment of pair feeding and zinc deprivation were tentatively determined. The differential regulatory patterns of protein expression between the ZD and PF groups support the hypothesis that the adaptive responses to dietary zinc and caloric restriction of both groups is unique and independent from each other and may be regulated by stress and feeding state.

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Fig. 6.1 2D images of hippocampal proteins from control rats. Equal amounts of protein extracts from three animals were combined and separated on pH 3-10 linear IPG strips. This was followed by electrophoresis on 12.5% SDS-polyacrylamide gels, as stated in Materials and Methods. The gel was stained with Coomassie blue. Differential protein expression between dietary groups was analyzed by Phoretix software. Enlarged images of the differences are presented in Fig. 6.2. a. and b. are spots exhibited similar staining intensities and locations as tublin beta chain and actin beta chain found on other 2D gels (Edgar et al. 1999, Fountoulakis et al. 2000)

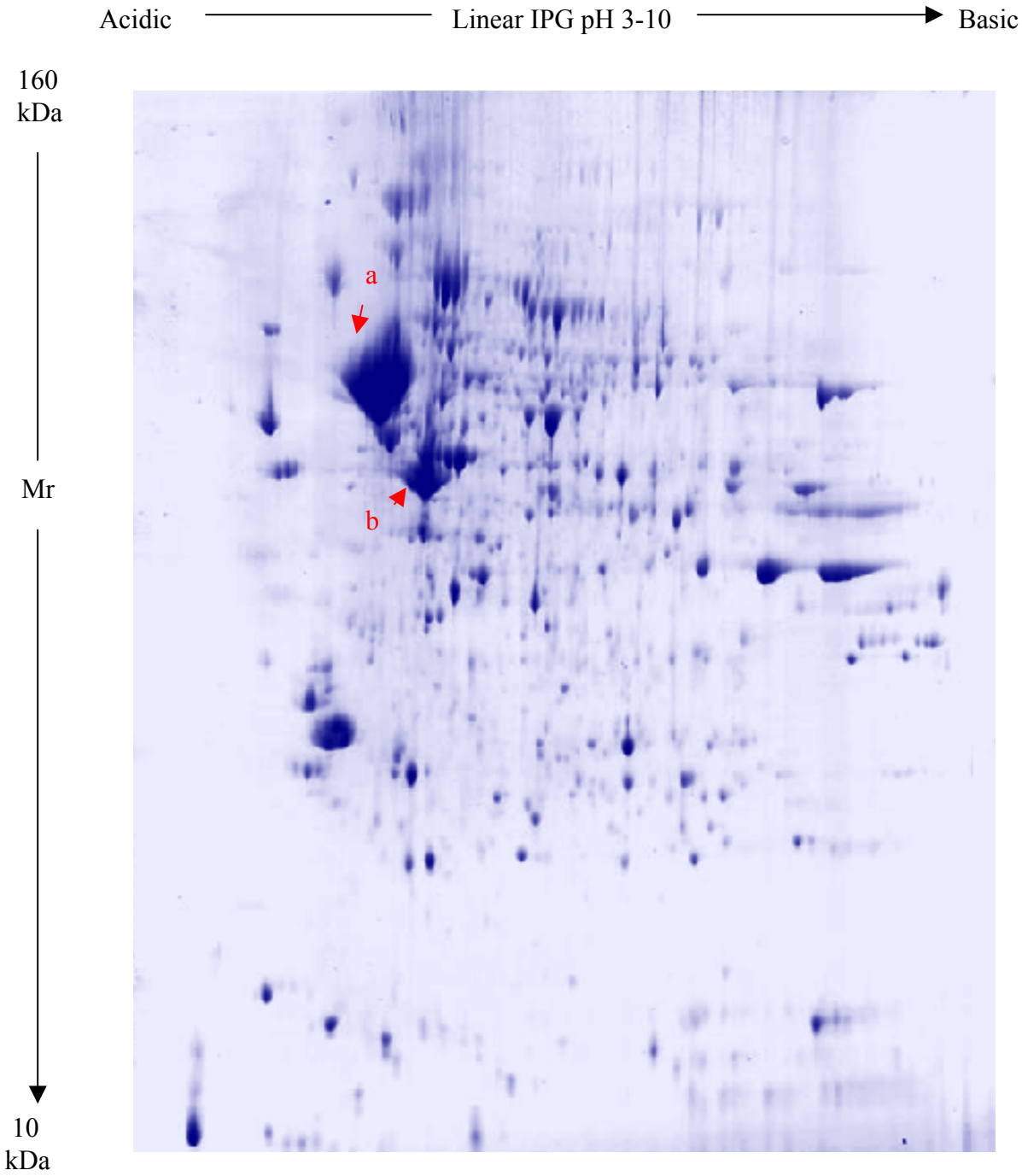
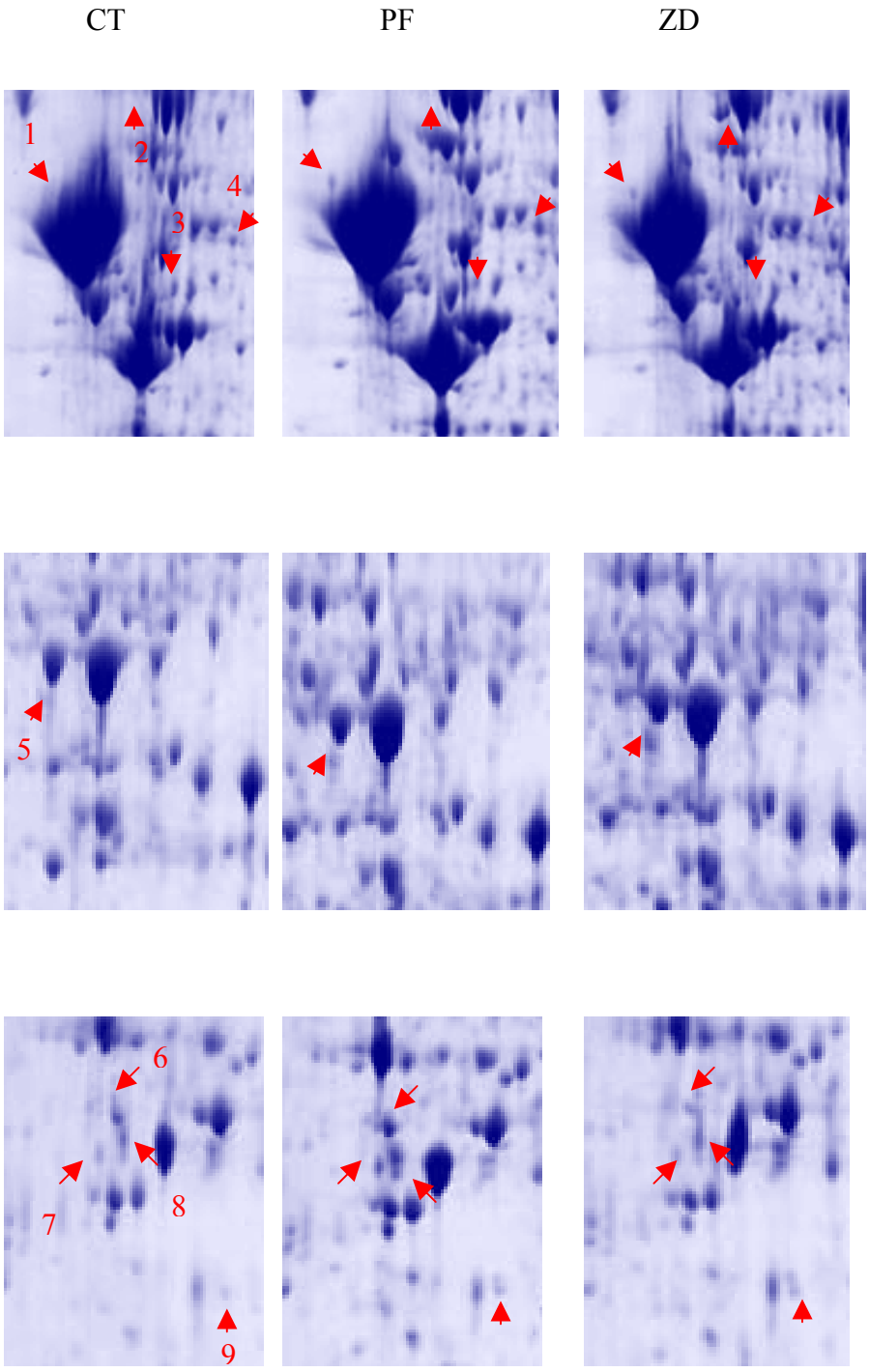

Acidic
Linear IPG pH 3-10
Basic
160
kDa
Mr
10
kDa
a
b

Fig. 6.2 Enlarged images of regions of 2D gels from all three dietary treatment groups. Protein spots in the PF and ZD gels with staining intensity greater or less than 50% of that of the CT gel are marked with arrows. The level of change and tentative identities of these spots are listed in Tables 6.1 and Table 6.2.


CT
PF
ZD
1
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6
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8
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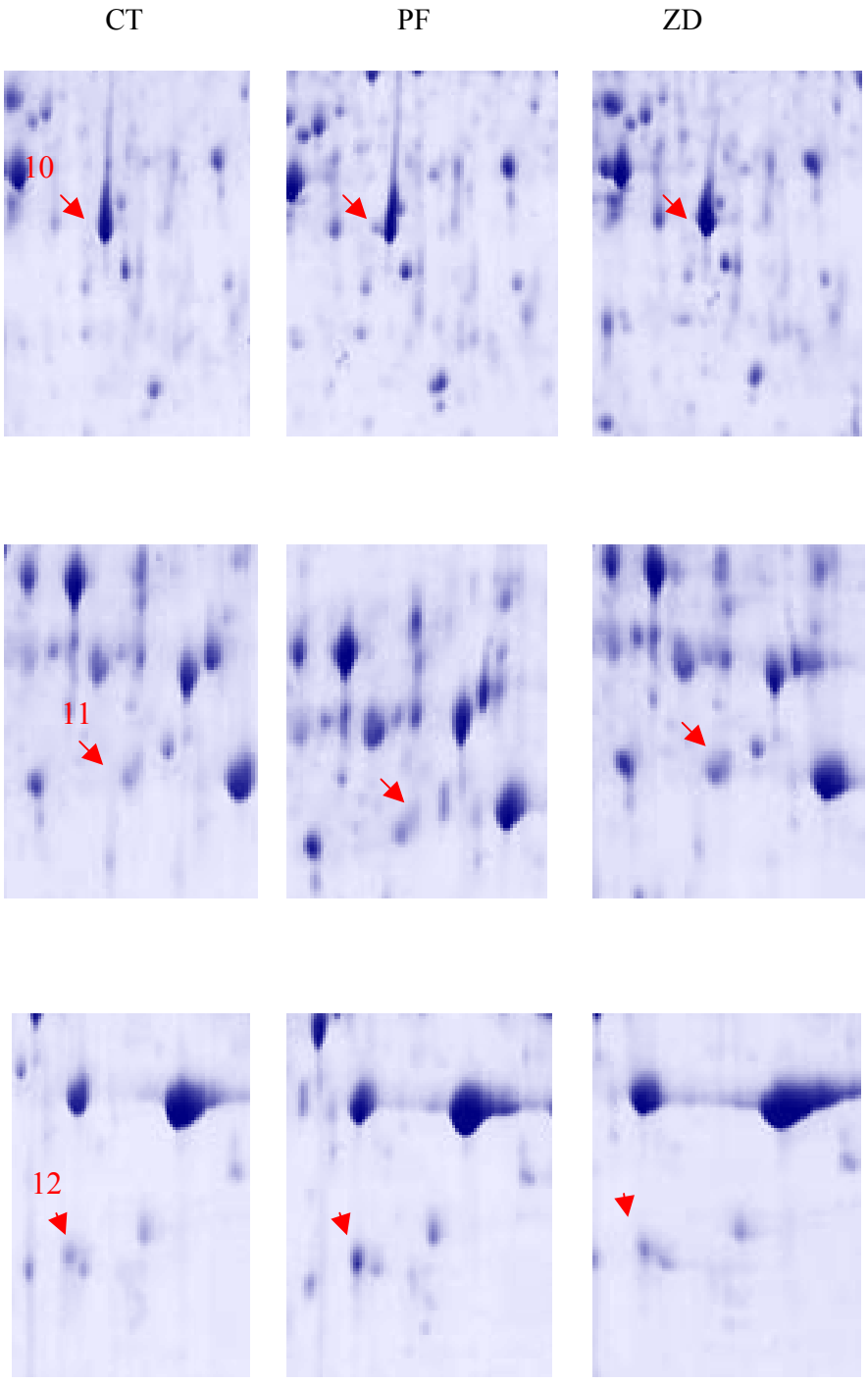
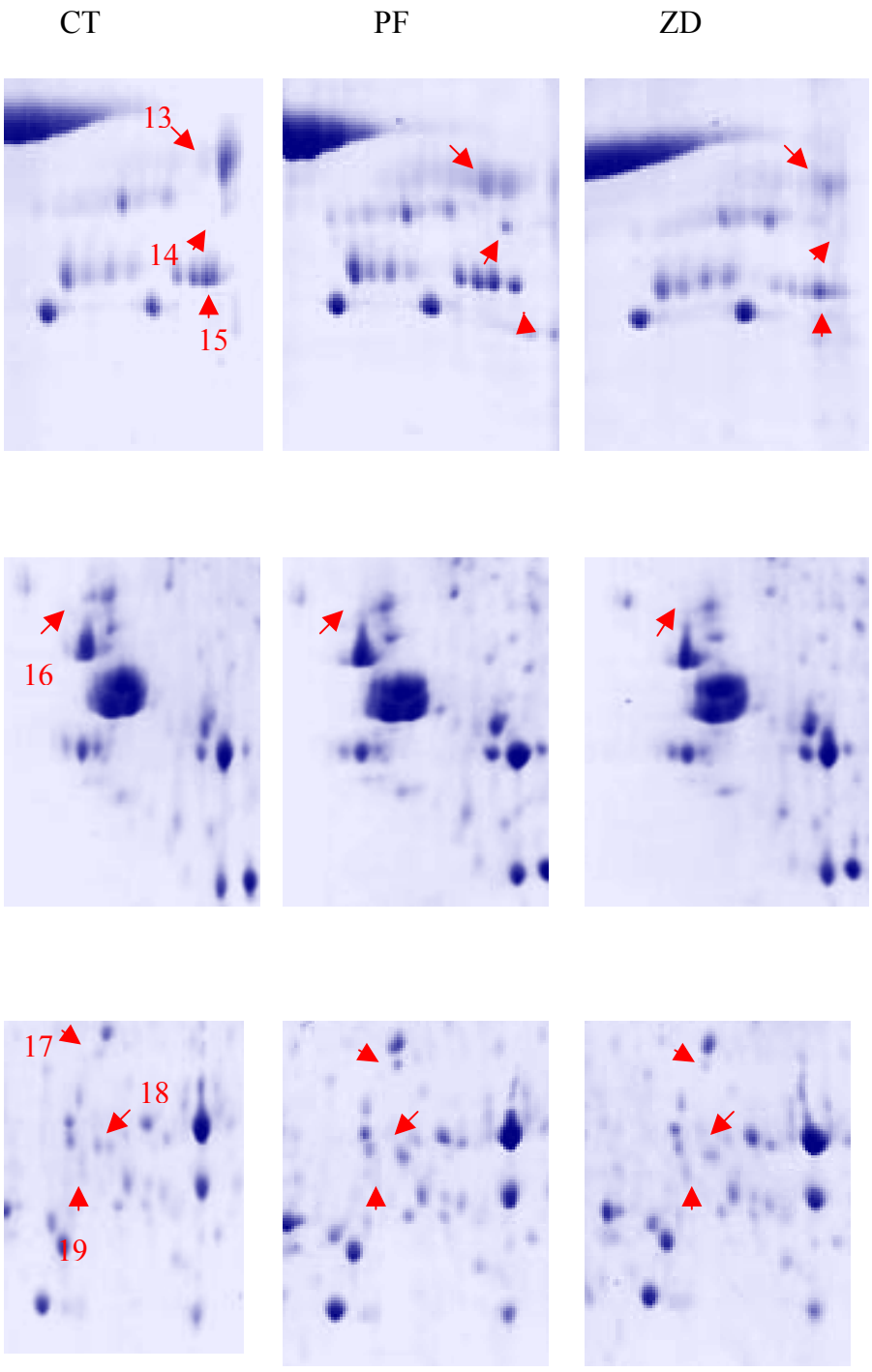

CT
PF
ZD
10
11
12


CT
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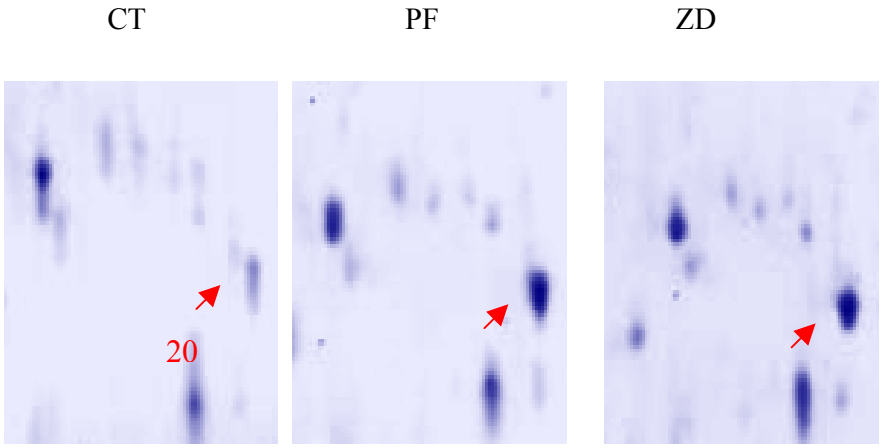

CT
PF
ZD
20

Table 6.1 Staining intensity of protein spots with 50% variation from the CT group.

Spot No.	Normalized Vol. (% of change vs. CT)			pI	Mr (kDa)
	CT	PF	ZD		
1	N.D.	0.78 /	0.67 /	4.8	59.1
2	N.D.	N.D. /	3.81 /	5.4	68.5
3	1.40	1.02 (-27%)	0.45 (-67%)	5.7	48.9
4	1.13	2.62 (131%)	1.42 (26%)	6.1	53.1
5	0.66	0.69 (5%)	1.34 (103%)	6.3	47.5
6	1.48	2.10 (42%)	0.69 (-53%)	5.4	34.1
7	0.41	0.82 (100%)	0.39 (-5%)	5.3	32.9
8	1.23	2.37 (70%)	1.15 (-7%)	5.4	33.1
9	0.21	0.55 (162%)	0.48 (129%)	6.0	28.5

10	N.D.	0.45 /	N.D. /	6.3	33.1
11	1.29	1.88 (46%)	2.38 (84%)	7.3	35.1
12	1.04	2.51 (141%)	1.74 (67%)	7.7	28.5
13	4.6	1.95 (-57%) 1.19 (-74%)	1.28 (-72%) 0.9 (-80%)	9.6 9.8	35.2 35.2
14	N.D.	0.47 /	N.D. /	9.7	33.4
15	0.24	1.19 (395%)	0.45 (88%)	9.6	29.1
16	0.81	N.D. /	N.D. /	4.6	27.3
17	0.20	0.43 (115%)	0.20 (0%)	6.6	25.2
18	0.49	0.93 (90%)	0.66 (35%)	6.6	22.2
19	N.D.	N.D. /	0.52 /	6.4	22.1

20	1.61	5.90 (266%)	6.07 (304%)	6.2	11.1

Normalized volume was determined by the percent of a spot staining volume relative to the total volume of protein spots. The values were multiplied by 1000.

The % of change vs. CT was calculated by dividing the difference of normalized staining volume by the staining volume of the control spots.

Mr was the apparent molecular weight of the protein measured by migration of molecular weight markers.

pI was determined by the pH gradient given by the manufacturer.

N.D. : not detectable

Table 6.2 Tentative protein identification by TagIdent in the SWISS-PROT database.

Spot No.	Protein Name	pI	Mr	Entry Name	Access No.
1	Calcium/calmodulin-dependent protein kinase type IV catalytic chain	4.9	53.1	KCC4_RAT	P13234
	Voltage-gated potassium channel protein Kv1.2	5.0	56.3	CIK1_RAT	P10499
	Neurosecretory protein VGF [Precursor]	4.9	68.2	VGF_RAT	P20156
2	Presynaptic density protein 95	5.6	80.4	DLG4_RAT	P31016
	Heat shock 70 kDa protein 1/2	5.5	70.1	HS71_RAT	Q07439
3	Excitatory amino acid transporter 3	6.0	56.7	EAA3_RAT	P51907
	Neuronal pentraxin I [Precursor]	5.9	45.5	NPX1_RAT	P47971
	Neuronal pentraxin II [precursor]	5.6	46.1	NPX2_RAT	P97738
4	Noelin [Precursor]	6.3	52.8	NOE1_RAT	Q62609
	Tubulin--tyrosine ligase	6.3	43.1	TTL_RAT	Q9QXJ0
5	P2X purinoceptor 6	6.5	42.4	P2X6_RAT	P51579
	NGFI-A binding protein 1	6.2	54.0	NAB1_RAT	Q62722
6/7/8	Microtubule-associated protein 1A	5.4	33.0	MAPA_RAT	P34926

	Proto-oncogene C-crk	5.4	33.8	CRK_RAT	Q63768
	Neurogenic differentiation factor 1	5.2	40.0	NDF1_RAT	Q64289
9	D1 dopamine receptor-interacting protein calcyon	5.8	24.7	D1IP_RAT	P58821
	Heat shock 27 kDa protein	6.1	22.9	HS27_RAT	P42930
10	Melanocortin-3 receptor	6.5	35.8	MC3R_RAT	P32244
	Ciliary neurotrophic factor receptor alpha [Precursor]	6.5	36.2	CNTR_RAT	Q08406
	DNA binding protein URE-B1	6.0	35.6	URB1_RAT	P51593
11	Brain-specific homeobox/POU domain protein 4	7.7	39.4	BRN4_MOUSE	P25216
	Melanocortin-4 receptor	7.1	36.9	MC4R_RAT	P70596
12	Cell division protein kinase 5	7.6	33.2	CDK5_RAT	Q03114
13/14/15	5-hydroxytryptamine 5B receptor	9.8	41.1	5H5B_RAT	P35365
	Galanin receptor type 1	9.5	38.8	GALR_RAT	Q62805
	Synaptic glycoprotein SC2	9.6	36.1	GSN2_RAT	Q64232
	Neurexophilin 4 [Precursor]	9.8	30.2	NXP4_RAT	Q9Z2N4
	Oligodendrocyte transcription factor 1	9.7	27.2	OLG1_RAT	Q9WUQ3
	Neurexophilin 4 [Precursor]	9.8	30.2	NXP4_RAT	Q9Z2N4

16	14-3-3 protein tau	4.7	27.8	143T_MOUSE	P35216
	Sodium channel beta-1 subunit [Precursor]	4.8	22.9	CIB1_RAT	Q00954
17/18/19	Neuronal protein NP25	6.5	24.7	NP25_RAT	P37805
	Stathmin 3	6.6	20.8	STN3_RAT	Q9JHU6
	ADP-ribosylation factor 1	6.4	20.5	ARF1_HUMAN	P32889
	ADP-ribosylation factor 4	6.8	20.2	ARF4_MOUSE	P36403
	ADP-ribosylation factor 5	6.4	20.4	ARF5_HUMAN	P26437
20	Vesicle-associated membrane protein 1	6.2	12.8	VAM1_RAT	Q63666
	Transforming growth factor beta 3 [Precursor]	6.1	12.7	TGF3_RAT	Q07258

PI and MW are the theoretical values calculated from the entire sequence of the protein.

Database searching parameters:

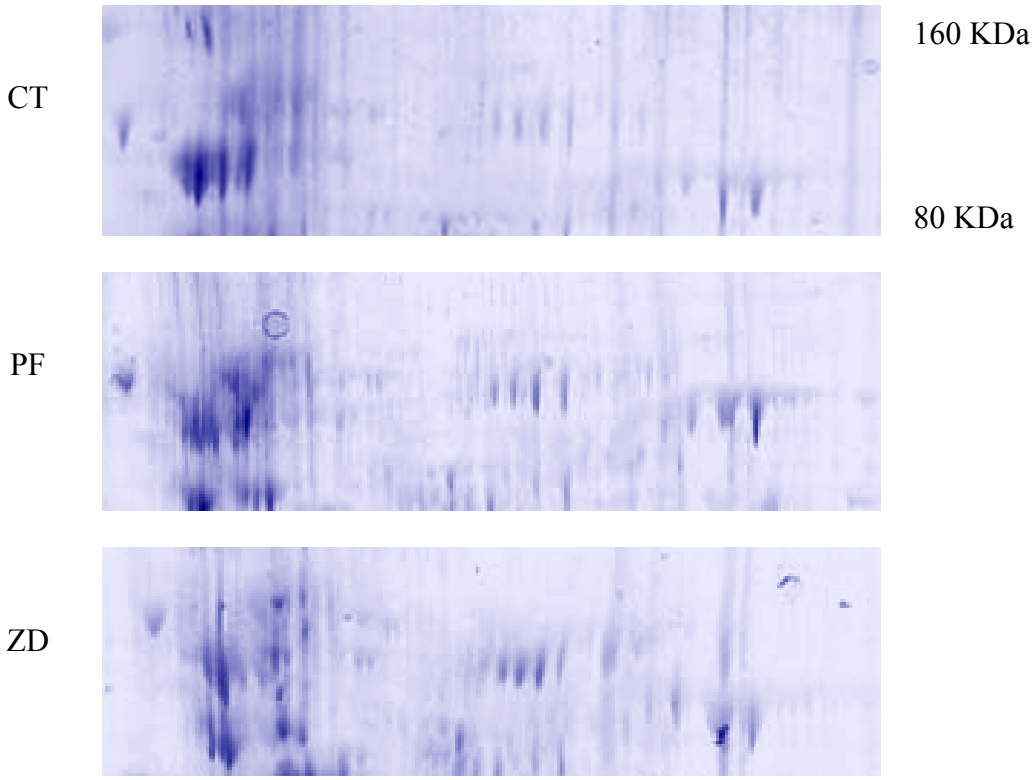
pI = apparent pI ± 0.25

Mr = apparent Mr ± 20%

Species: Rattus Norvegicus

Key word: brain

Fig. 6.3 Image of regions with high molecular weight. Spot tailing and low resolution in this region hinder the determination of variation in protein expression levels.



CHAPTER 7

CONCLUSIONS

7.1 Conclusions

The effect of zinc-deprivation and pair feeding on the performance of rats in the Morris water maze was extensively studied. Systemic zinc depletion was manifested in stunted growth, reduced femur zinc level, thymic involution and adrenal hypertrophy. Young rats fed a diet low in zinc for three weeks exhibited normal spatial cognition. Consumption of the zinc deficient diet did not significantly affect their short-term memory or long-term memory or their ability to learn. These results do not support a direct role for zinc in the process of memory formation. It is possible that under the current conditions used for zinc depletion, a sufficient amount of zinc was conserved to maintain their spatial cognition.

The hippocampal region of the brain has been implicated in spatial learning (Morris 1984) and contains a high level of chelatable zinc compared to other brain regions (Pfeiffer and Braverman 1982). We found that young weanling rats consuming a diet depleted in zinc exhibited reduced chelatable zinc in the hippocampus (as measured by TSQ staining). The total level of zinc (measured by atomic absorption) in the hippocampus however was not significantly affected. Our findings are in agreement with studies involving ZnT-3 knockout mice (Cole et al. 2001). In these studies the absence of chelatable zinc in the hippocampus did not affect the spatial cognition of these mice. The lack of a zinc effect in the rats in our study may be related to the stage of life that zinc

depletion was introduced. Others have shown behavioral impairment in animals zinc-deprived at stages of life other than young adult period (Table 2.3).

Unexpectedly, the performance of the PF group was impaired in consecutive learning tests. The learning impairment of the PF group was associated with thigmotaxic behavior, which is an anxiety-like behavior and an ineffective searching strategy (Block and Schwarz 1998). The resting corticosterone levels of these rats were elevated. The feeding schedule of these rats may have contributed to their stress since they were trained in the water maze before feeding. Their corticosterone levels before swim training could be their pre-prandial levels. Others have also reported the elevated corticosterone levels in conjunction with water maze learning impairment in rodents (Grootendorst et al. 2001, Yau et al. 1995). In the same learning test, the ZD rats performed normally. The basal corticosterone levels of the ZD group were also increased, though not statistically significant. It appears that there is a threshold of serum corticosterone level, which could affect behavioral performance in the water maze. Following a probing test, corticosterone levels of both groups were increased, and both groups exhibited thigmotaxia. The elevation of corticosterone in the ZD group could be a secondary effect of zinc deprivation. The corticosterone response curve after the probing test was elevated in both the PF and ZD group. This elevation may be the result of either an increase in sensitivity of the HPA axis to stress or a reduction in the efficiency of the corticosterone clearance. ACTH levels in both the ZD and PF group were elevated, which could partially explain the increase in corticosterone levels in these animals. Glucose levels of both groups were decreased, possibly a consequence of long-term food restriction. Impaired learning and thigmotaxic behavior of the PF group were confirmed in the

reversal-learning test. The learning test with probing insertion, however, did not reveal any group difference. The inserted probe trials in the training phase could be proactive interference, which reduces the test sensitivity.

The zinc-dependent regulation of protein expression in the hippocampus of these rats was also investigated. Zinc affects gene regulation through zinc finger proteins and metal regulatory elements in genes (Cousins 1994). Glucocorticoids and mineralocorticoid receptors are abundant in the hippocampus. Elevated corticosterone secretion in both the PF and ZD group may induce changes in protein expression via these steroid receptors. Two-dimensional gel electrophoresis was used to generate protein expression profiles of control, pair-fed, and zinc depleted dietary groups. Both carrier ampholyte gels and immobilized pH gradients (IPG) strips were used for the first dimension electric focusing. Due to the limitation of loading capacity, 100 μg of protein was loaded onto the carrier ampholyte gels and stained with silver. The $P2X_6$ purinergic receptor, identified by MALDI-MS, was up regulated in the ZD group. IPG strips allow for a higher protein load. Two mg of protein was loaded onto the IPG strips. Following separation in the second dimension, approximately 500 protein spots per gel were visualized after Coomassie blue staining. Twenty protein spots were identified as either up or down regulated in the PF and ZD gels. These were excised and submitted for sequencing by mass spectrometry. The protein spots were tentatively identified using SWISS-PROT database (Olivieri et al. 2001). The expression patterns of these gels suggest that pair feeding and zinc deprivation regulate the protein expression differently.

7.2 Future studies

Although the spatial cognition in the water maze was not affected by zinc deprivation in young adult rats, it is worthwhile to continue studying the effect of zinc depletion introduced during pregnancy, lactation or weaning on cognition. Zinc depletion is expected to induce more severe neurological damage at these stages (Golub et al. 1995, Sandstead 1995). The potential results from these studies will more fully define the role for zinc in memory formation. The importance of experimental design, particularly with the learning test, cannot be overlooked. Significantly more information could be obtained by inserting probe trials during the learning test. It will be necessary to acquire a collapsible platform for the water maze to allow for an accurate measurement of the development of place learning.

Corticosterone levels and water maze performance are correlated. To further explore this observation, the diurnal pattern of these malnourished rats needs to be studied. The effect of corticosterone on maze performance can be tested by performing the learning test when all dietary groups exhibit equal corticosterone levels. Minimizing the corticosterone effect may also correct the behavioral impairment in malnourished rats. Moreover, all dietary groups can be tested in the maze at both their nadir and peak levels of corticosterone. The comparison of performance at the two time points may also helps to resolve the role of corticosterone in water maze learning.

Two dimensional gel electrophoresis studies indicate that dietary zinc deprivation and pair feeding differentially affect protein expression in the hippocampal region. The total number of spots resolved per gel was approximately 500, which is much less than the number of expressed proteins in the tissue. Theoretically 10,000 protein spots should

be revealed on gels with mammalian tissue sample (Lopez 1999). Therefore, increasing the detection range of the current protocol will be necessary for identifying the regulation of low abundance proteins in response to dietary treatment. Several approaches may need to be adopted in the future to achieve this goal. Firstly, the sample protein extracts could be divided into several pools either by step-wise solubilization, or by isolation of the different cellular compartments (Patton 1999). Secondly, narrow pH range IPG strips could be used, which would allow for maximizing the separation of proteins with similar pI (Gorg et al. 2000). Thirdly, fluorescent dyes could be used to replace either silver staining or Coomassie blue staining (Lauber et al. 2001). These dyes improve the detection sensitivity, dynamic range and compatibility to MALDI-MS. The identified proteins that are differentially expressed in the different dietary groups will ultimately serve as the targets for investigating the pathways that are involved in the adaptive response to nutritional insults.

In summary, the current study tests the spatial ability of zinc-deprived and pair-fed animals. A potential association between the stress level and behavioral abnormality in these malnourished animals was found. The protein expression profile in the hippocampus was also determined, which provides the foundation for further identification of metabolic pathways responsive to the nutritional status of the brain.

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