UNIVERSITY OF CALIFORNIA,
IRVINE

The Role of Synaptic Zinc in Neurotransmission

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Emily Crean Vogler

Dissertation Committee:
Professor Jorge A. Busciglio, Chair
Professor Raju Metherate
Professor Andrea J. Tenner
Professor John Weiss

2014

DEDICATION

To

My dear husband Dan

who said, “You should go back to school; you would be good at it.”

and my children, D.J., Eric and Nick

who will always be in my heart

with gratitude for all the support they have given me in this and all my endeavors

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AED	antiepileptic drug
AKT	Protein kinase B (PKB)
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
apoε4	apolipoprotein ε4
APP	amyloid precursor protein
Aβ	amyloid beta
AβO	amyloid β oligomers
BDNF	brain-derived neurotrophic factor
BOLD	blood oxygen level dependent contrast imaging
CaEDTA	calcium-Ethylenediaminetetraacetic Acid
CREB	cAMP response element-binding protein
CSF	cerebral spinal fluid
D-AP5	D-(-)-2-Amino-5-phosphonopentanoic acid
DI	discrimination index
DMN	default mode network
EEG	electroencephalogram
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
EPSP	excitatory postsynaptic potential
Erk	extracellular signal-regulated kinases
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
GAP43	growth associated protein 43
GPCR	G protein-coupled receptors
i.p.	intraperitoneal injection
KCl	potassium chloride
LEV	levetiracetam
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MCI	mild cognitive impairment
MEK	mitogen-activated protein kinase kinase
mGLUR	metabotropic glutamate receptor
MTL	medial temporal lobe
NMDAR	N-methyl-D-aspartate receptor
NPY	neuropeptide Y
NT4	neurotrophin 4
OLM	object location memory
PEAQX	[(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid (NVP-AAM077)
PiB	Pittsburg compound B
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PLCγ1	phospholipase-C γ1

SSRI	selective serotonin reuptake inhibitors
TBI	traumatic brain injury
TCI	transitory cognitive impairment
TEA	transient epileptic amnesia
TLE	temporal lobe epilepsy
TrkB	tyrosine kinase B
WT	wild type
ZnT3	Zinc Transporter 3
ZnT3KO	Zinc Transporter 3 knock out transgenic mouse

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Many thanks to the members of the Busciglio lab; Pablo Helguera and Octavio Garcia, who taught me so many lab skills and techniques; Mike Hanna, Maria Torres, Silvina Catuara Solarz, Rebecca Stein and especially Ryan Bohannan, who helped tremendously. Many thanks also to Irakli Intskirveli in the lab of Raju Metherate, who taught me how to prepare acute brain slices; Malini Vashishtha in the lab of Leslie Thompson, who provided expertise and primers for BDNF mRNA qPCR; Maya Koike in the lab of Kim Green, for her surgical skills and Fluorojade experience; and Annie Vogel-Cierna in the lab of Marcelo Wood, whose assistance and guidance was invaluable for the behavioral experiments.

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CURRICULUM VITAE

Emily Vogler
20372 SW Cypress Street, Newport Beach, CA 92660
voglere@uci.edu · (949) 279-3864

EDUCATION

Ph.D	University of California, Irvine Biological Sciences; October 2014
B.S.	University of California, Irvine Biology; June 2007

RESEARCH EXPERIENCE

2008-Present GRADUATE STUDENT RESEARCHER
Ph.D. Candidate
Dr. Jorge Busciglio, Department of Neurobiology and Behavior
University of California, Irvine

Investigate molecular and cellular mechanisms of neurodegeneration. Specific responsibilities include maintaining a colony of transgenic mice, supervision of undergraduate student researchers, design and implementation of research projects, and preparation of grant proposals. Research techniques utilized include immunohistochemistry, microscopy, tissue and cell culture, and immunological protein and cell identification laboratory techniques.

2007 GRADUATE STUDENT RESEARCHER
Dr. Andrea Tenner, Department of Neurobiology and Behavior
University of California, Irvine

Conducted research on molecular basis of neurodegeneration in Alzheimer's disease. Utilized cell culture and immunological protein and cell identification laboratory techniques.

PROFESSIONAL EXPERIENCE

2011-2013 TEACHING ASSISTANT
School of Biological Sciences courses Drugs and the Brain, Brain Dysfunction, and Neurobiology & Behavior
University of California, Irvine

Write and grade exams, manage course websites, assist in utilization of smart classroom technology for large lecture hall courses.

ADMINISTRATIVE TEACHING ASSISTANT
School of Biological Sciences course DNA to Organisms Introduction to Biology Course
University of California, Irvine

Write and grade exams, manage course website, troubleshoot student concerns, assist in utilization of smart classroom technology for large lecture hall courses, including managing iClicker data for grading purposes.

2010-2013 LABORATORY MANUAL EDITOR
School of Biological Sciences Neurobiology and Behavior Laboratory Course
University of California, Irvine

Supervise laboratory manual revisions, printing and distribution.

2010-2011 TEACHING ASSISTANT
School of Biological Sciences Neurobiology and Behavior Laboratory Course
University of California, Irvine

Prepared lesson plans, lectures, and student assessments of university undergraduate students, including grading of laboratory reports and experimental designs, in biology lecture and neurobiology laboratory courses.

DISCUSSION LEADER
School of Biological Sciences DNA to Organisms Introduction to Biology Course
University of California, Irvine

Prepared lesson plans, lectures, and student assessments of university undergraduate students, including grading of assessments, for small discussion groups associated with large lecture classes.

2010 HHMI-UCI GRADUATE TEACHING FELLOW
University of California, Irvine

The HHMI-UCI Graduate Teaching Fellow Program provides teaching experience, pedagogical strategies and methods of balancing teaching and research duties for graduate students in biological sciences.

2004 TEACHING ASSISTANT
Zoology Lab
Orange Coast College

Assisted in preparation of laboratory exercises, supervising student participation and assessment of student achievement.

2003 TEACHING ASSISTANT
Honors Cell and Molecular Biology Lab
Orange Coast College

Assisted in preparation of laboratory exercises, supervising student participation and assessment of student achievement.

2002 TEACHING ASSISTANT
Honors Marine Biology Lab
Orange Coast College

Assisted in preparation of laboratory exercises, supervising student participation and assessment of student achievement.

PUBLICATIONS

2012 VOGLER, E., BUSCIGLIO, J., Disruption of Zinc Neuromodulation by Aβ Oligomers-Therapeutic Implications. *Current Pharmaceutical Design* 2014; 20(15):2520-4.

2014 VOGLER, E., BUSCIGLIO, F., BOHANNAN, R., FLYNN, D., BUSCIGLIO, J., A Novel Method of Electrode Construction and Implantation for Miniaturized EEG Recording Devices for Small Animals. In progress. *Journal of Visualized Experiments.*

2014 VOGLER, E., BOHANNAN, R., BUSCIGLIO, J., Elimination of Synaptic Zinc Induces Epileptiform Activity and Contributes to Cognitive Impairment. In progress.

2014 VOGLER, E., BUSCIGLIO, J., Elimination of Synaptic Zinc Alters Neurotrophic Signaling Pathways. In progress.

RESEARCH PRESENTATIONS

2014 SOCIETY FOR NEUROSCIENCE 2014 MEETING
Washington, D. C.
Poster (accepted)
The role of zinc in Aβ-dependent synaptotoxic mechanisms in Alzheimer's Disease

INTERNATIONAL SOCIETY FOR ZINC BIOLOGY 2014 MEETING
Asilomar Conference Grounds, Monterey, California
Poster
Genetic removal of synaptic zinc alters responses of NMDAR signaling

2013 NEUROBIOLOGY AND BEHAVIOR NEUROBLITZ GRADUATE STUDENT SEMINAR
University of California, Irvine
Presentation
Zinc and Alzheimer's disease

2012 ReMIND EMERGING SCIENTIST SYMPOSIUM ON NEUROLOGICAL DISORDERS
University of California, Irvine
Presentation
Zinc and Alzheimer's disease

ALZHEIMER'S ASSOCIATION BRAINWAVES RESEARCH SYMPOSIUM
University of California, Santa Barbara
Poster Presentation
Investigating the Role of Synaptic Zinc Deficits and Seizure Activity

ALZHEIMER'S ASSOCIATION INTERNATION CONFERENCE
Vancouver, B.C. Canada
Poster Presentation
Synaptic Zinc Deficits and Seizure Activity in Alzheimer's disease

2012 NEUROBIOLOGY AND BEHAVIOR NEUROBLITZ GRADUATE STUDENT SEMINAR
University of California, Irvine
Presentation
Title: *Zinc and Alzheimer's disease*

2011 NEUROBIOLOGY AND BEHAVIOR NEUROBLITZ GRADUATE STUDENT SEMINAR
University of California, Irvine
Presentation
Title: *Alzheimer's disease: Investigating the Role of Synaptic Zinc*

2010 NEUROBIOLOGY AND BEHAVIOR NEUROBLITZ GRADUATE STUDENT SEMINAR
University of California, Irvine
Presentation
Title: *Alzheimer's disease: From Amyloid beta to Zinc*

ACADEMIC SCHOLAR PROGRAMS

2009-Present RESEARCH AND EDUCATION IN MEMORY IMPAIRMENT DISORDERS (ReMIND)
University of California, Irvine

ReMIND is designed to encourage the next generation of scientists to study neurological disorders; to educate students and the public about neurodegenerative disorders; to support the community by helping at outreach events; and to foster a collaborative research environment by sponsoring social functions.

2004 NATIONAL SCIENCE FOUNDATION TEACHING SCHOLAR
Orange Coast College

The NSF Teaching Scholar program provides experience and mentoring to community college students exploring teaching careers.

ACADEMIC HONORS AND AWARDS

2013 EDWARD STEINHAUS TEACHING AWARD
University of California, Irvine

2005 GILBERTSON EXCELLENCE IN TEACHING ASSISTANCE AWARD
Orange Coast College

2003 COMPLETION OF HONORS PROGRAM
PRESIDENT'S LIST, DEAN'S LIST
MEMBER- ALPHA GAMMA SIGMA
Orange Coast College

2002 DEAN'S LIST
Orange Coast College

2001 PRESIDENT'S LIST
Orange Coast College

COMMUNITY SERVICE

2013-2014 UCI EXCELLENCE IN RESEARCH MENTOR
University of California Excellence in Research Program
University of California, Irvine

UCI Excellence in Research mentors directly supervise the research training program and scholarly work of an undergraduate participant in the program, including preparation of a research presentation given by the undergraduate participant at the Undergraduate Research Symposium at UCI and submission of an original research paper to the UCI Journal of Undergraduate Research.

2013 UC SURP MENTOR
University of California Summer Undergraduate Research Program (UC SURP)
University of California, Irvine

UC SURP mentors directly supervise the research and scholarly work of an undergraduate participant in the program, including preparation of a research presentation given by the undergraduate participant at state and national conferences.

2012 UC CAMP MENTOR
University of California Louis Stokes Alliance for Minority Participation (UC CAMP)
University of California, Irvine

UC CAMP mentors directly supervise the research and scholarly work of an undergraduate participant in the program, including preparation of a research presentation given by the undergraduate participant at state and national conferences.

2008-present TRUSTEE
Chapman University

Vice-Chair of Academic Committee, member of Western Association of Schools and Colleges (WASC) Interdisciplinarity Committee, and Schmid College of Science Dean Search Committee.

2006-2007 INTERNSHIP
Hoag Memorial Hospital Presbyterian
COPE Health Solutions Clinical Care Extender (CCE)

CCE interns are members of the patient care team alongside nurses, physicians and allied health professionals in clinical and administrative settings. Interns participate in basic patient care tasks as they rotate among the different departments within the hospital.

2003-2005 VOLUNTEER
Discover Science Night, Science Day at the Santa Ana Zoo
Orange Coast College Chemistry Department

Assist in preparation and presentation of scientific demonstrations to K-12 audiences to encourage the exploration of education and careers in science.

BOARD MEMBER
Children's Bureau of Orange County

The Children's Bureau coordinates social services for children identified as at risk for abuse.

2000-2003 BOARD MEMBER
YMCA Community Services

YMCA Community Services organizes services and activities for disabled adults in the Orange county community.

1994-2004 VOLUNTEER
The Prentice School, Discovery Science Center

Organize fundraising events, including preparation of printed materials and bookkeeping.

1991-1995 TREASURER
Championship Auto Racing Auxiliary (CARA)

CARA provides social services to members of the auto racing community. Duties included bookkeeping and supervising investment accounts. Recipient of the Betty Rutherford Blue Heart Award for exceptional service.

ABSTRACT OF THE DISSERTATION

The Role of Synaptic Zinc in Neurotransmission

By

Emily Crean Vogler

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Professor Jorge A. Busciglio, Chair

In recent years, soluble amyloid beta oligomers (AßO) have emerged as key elements in the cascade leading to synaptic dysfunction and neurodegeneration in Alzheimer's disease (AD). Previous work from our lab has shown that synaptic zinc released during excitatory neurotransmission increases the formation and accumulation of AβO at synaptic sites, and that the AβO-zinc interaction accelerates oligomer formation. Other recent research has shown that sequestration of synaptic zinc by AβO disrupts synaptic function, a significant finding as zinc has been demonstrated to modulate seizure activity and signaling pathways, has a high affinity for AβO and accumulates in Aβ plaques in AD brain.

We sought to investigate markers of seizure activity in zinc transporter ZnT3 knockout (ZnT3KO) mice, which lack synaptic zinc, and the effects of treatment with an anti-seizure drug on the cognitive impairment demonstrated by aged ZnT3KO mice. Hippocampus tissue collected from age-matched cohorts of wild type and ZnT3KO were assayed for markers of seizure activity, finding age-dependent alterations in levels of these markers consistent with seizure activity in ZnT3KO. To study the effects of seizure

activity on cognition, memory was assessed after acute treatment with an anti-seizure drug, finding no significant improvement in six month old ZnT3KO.

We also investigated alterations in neurotrophic signaling pathways in acute hippocampal slices from ZnT3KO mice. Neurotrophic protein expression and phosphorylation were assessed, finding reduction in basal and activity-dependent levels of Erk1/2 and p-Erk1/2, reduction in AKT, age-dependent reduction in BDNF, and reduction in activity-dependent BDNF mRNA expression. Pharmacological treatments of acute hippocampus sections were performed to investigate the effects of Zn^{2+} on activation of Erk1/2 through NMDA receptors and receptor subunits NR2A and NR2B, finding that Zn^{2+} inhibits NR2B-mediated activation of Erk1/2. Neurodegeneration was assessed through histochemistry of age-matched hippocampus sections, demonstrating an increase in neurodegeneration in aged ZnT3KO.

Collectively, the results discussed in this dissertation support the hypothesis that dysregulation of synaptic zinc results in excessive excitatory neuronal activity and neurodegenerative alterations in signaling pathways. Consequently, therapeutics targeting maintenance of zinc homeostasis and reduction of AβO interference with zinc neurotransmission may prove beneficial in managing neuronal hyperactivity and neurodegenerative changes in AD.

Chapter 1

Introduction

The characteristic hallmarks of Alzheimer's disease (AD) pathology include extracellular amyloid beta (Aβ) plaques composed of deposits of insoluble Aβ protein and deteriorating neurons, intracellular neurofibrillary tangles composed of hyper-phosphorylated tau protein (Selkoe, 1991) and neuronal loss (Thompson et al., 1998a). Recently, amyloid beta oligomers (AβO), small soluble aggregates of Aβ not associated with the Aβ deposits found in plaques, have emerged as key elements in the cascade leading to synaptic dysfunction in AD. Soluble AβO are present in AD brains and transgenic animal models of AD, induce neuronal death, reduce synaptic density (Lambert et al., 1998; Walsh et al., 2002; Lesne et al., 2006) and their levels correlate better than plaque density with cognitive impairment (Lue et al., 1999; Naslund et al., 2000). Previous research in our lab indicates that AβO form and accumulate at synaptic sites in an activity-dependent fashion, and also implicate zinc released during synaptic activity in the synaptic targeting of AβO (Deshpande et al., 2009), a significant finding as synaptic zinc has been demonstrated to modulate several signaling pathways (Westbrook and Mayer, 1987; Ruiz et al., 2004; Cohen-Kfir et al., 2005; Erreger and Traynelis, 2008a; Sindreu et al., 2011), has a high affinity for AβO (Noy et al., 2008) and accumulates in Aβ plaques in AD brain (Suh et al., 2000a), indicating that synaptic zinc/AβO interaction may contribute to AβO-induced synaptic dysfunction and neuronal death. Pharmaceuticals targeting regulation of zinc have been demonstrated to reduce memory impairment and reverse the decline in synaptic proteins in transgenic animal models of AD (Adlard et al., 2008; Grossi et al., 2009) and transgenic mice without synaptic zinc have age-dependent

cognitive impairment and biochemical changes similar to AD (Adlard et al., 2010), while recent research has linked sequestration of synaptic zinc by AβO to decreased synaptic density (Grabrucker et al., 2011a). Understanding the role of synaptic zinc in neuronal health and neurotransmission will aid in developing therapeutic treatments for AD, which presently has no cure or truly effective treatments.

Alzheimer's Disease

The U. S. National Institutes of Health Alzheimer's Disease Education and Referral Center (NIH ADEAR) states that Alzheimer's disease is the most common cause of dementia in elderly people, a condition leading to cognitive decline that interferes with the activities of daily living until the patients are no longer able to care for themselves and require substantial assistance in meeting their basic needs. The progression of the disease produces neuronal death and results in significant brain atrophy (Figure 1-1). Though a small fraction of cases, called familial AD, are caused by specific genetic mutations, the greatest risk factor associated with AD is aging and specific causes are yet undiscovered. There is presently no cure for AD and current treatments provide very modest delay in the progression of the disease. The NIH estimates that as many as 5.1 million Americans have AD and that the number of people over age 65 with AD doubles every five years as the population ages, resulting in an

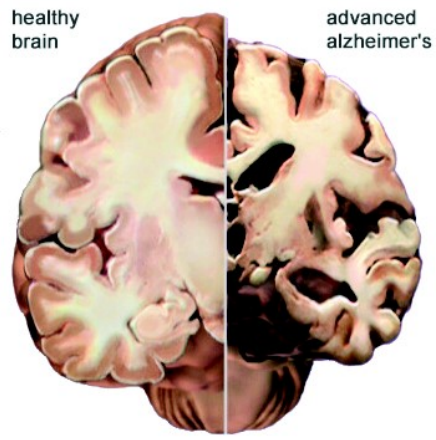

Figure 1-1. A healthy normal brain (left) and an atrophied brain of advanced Alzheimer's disease (right). Note the enlarged ventricles and shrunken gyri of the Alzheimer's diseased brain.

increasingly heavy economic burden from the costs associated with care and treatment of AD patients. The Alzheimer's Association estimates that the costs of caring for AD patients in 2013 was $214 billion in the U.S., with family and friends providing another $220 billion in unpaid care hours, and if the number of cases and associated cost of care continue to increase unabated that the American people will be facing over $1.1 trillion in costs by the year 2050. This enormous public health problem requires a multi-faceted approach involving research into the underlying causes of the disease to facilitate development of therapeutic interventions to prevent or delay the onset and progression of the disease.

Aggregation of Aβ

Alzheimer's disease is characterized by deposition of Aβ plaques and hyperphosphorylated tau neurofibrillary tangles (Selkoe, 1991) and neuronal loss (Thompson et al., 1998b). Plaque formation is associated with cholinergic abnormalities in dementia patients (Perry et al., 1978), though neurofibrillary tangles have a closer correlation with dementia than plaques (Arriagada et al., 1992). The development of transgenic mouse models expressing genetic mutations resulting in formation of plaques and tangles along with cognitive deficits has provided significant insight into the mechanisms linking AD pathology with synaptic function and cognition (Hsiao et al., 1996; Oddo et al., 2003).

Aβ is a product of sequential proteolytic cleavage of the transmembrane protein amyloid precursor protein (APP) (Selkoe, 1998). APP has been linked to maintenance of synaptic function during aging through studies utilizing transgenic mice lacking APP that develop age-dependent deficits in cognition, reduced immunoreactivity for synaptic proteins and increased reactive astrocytosis (Dawson et al., 1999). Sequential cleavage of APP by β secretase and γ secretase results in the 42 amino acid Aβ peptide. The resulting Aβ peptide is a soluble monomer that misfolds and aggregates into soluble prefibrillar oligomers (AβO) and insoluble fibrils (Figure 1-2), which are recognized by conformation-specific antibodies and ultimately form the plaques characteristic of AD (Glabe, 2008).

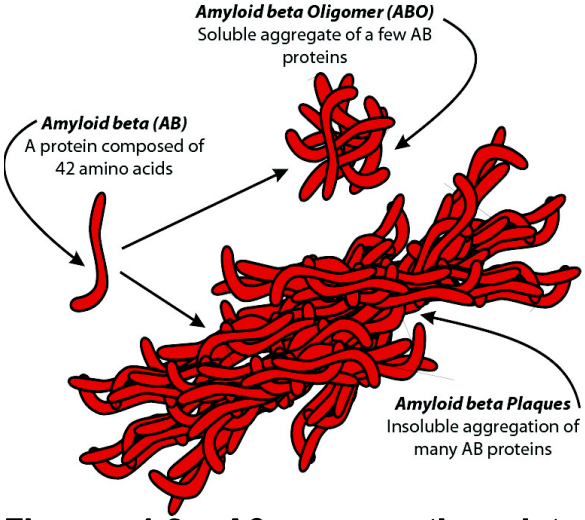

Figure 1-2. Aβ aggregation into soluble oligomers and insoluble plaques. The 42 amino acid monomer can aggregate into soluble oligomers or can form insoluble aggregations of Aβ plaques.

Amyloid Beta Oligomers

In recent years, soluble amyloid beta oligomers (AßO) have emerged as key elements in the cascade leading to synaptic dysfunction in AD. Soluble AβO are present in AD brains and transgenic models and their levels correlate better than plaque density with cognitive impairment (Lue et al., 1999; Naslund et al., 2000). In fact, Aß soluble species are toxic at low concentrations, reduce synaptic density, induce cognitive

dysfunction, and inhibit long-term potentiation (Lambert et al., 1998; Walsh et al., 2002; Lesne et al., 2006).

More recently it has been demonstrated that AβO disrupt synaptic function through various mechanisms, including increased activation of NR2B NMDARs (N-methyl-D-aspartate receptors) resulting in long-term potentiation (LTP) inhibition (Snyder et al., 2005; Rammes et al., 2011), and reduced phosphorylation of AMPAR (AMPA receptor) subunits blocking extrasynaptic delivery of AMPARs and producing memory deficits in a transgenic mouse model of Alzheimer's disease (Minano-Molina et al., 2011). NMDARs are also inhibited by zinc, with differential modulation depending on NMDAR subunit composition and zinc concentration (Legendre and Westbrook, 1990; Paoletti et al., 1997; Vogt et al., 2000; Izumi et al., 2006; Erreger and Traynelis, 2008a). Interaction between AβO and zinc may contribute to disruption of synaptic function, leading to neurodegeneration.

Synaptic Targeting of AβO and Zinc Neurotransmission

Previous results from the Busciglio lab indicate that AβO form and accumulate at synaptic sites in an activity-dependent fashion, that synaptic targeting of AβO is enhanced by NMDAR activity and that AβO colocalizes with the NR2B subunit of the NMDAR (Deshpande et al., 2009), which may then facilitate Aβ-promoted endocytosis of NMDA synaptic receptors and depressed NMDAR currents described in other research (Kharlamov et al., 2007). These previous results also implicate zinc released during synaptic activity in the synaptic targeting of AβO (Deshpande et al., 2009). Zinc is released during glutamatergic neurotransmission after having been transported into

synaptic vesicles by the zinc transporter protein ZnT3 and is most abundant in the hippocampus (Palmiter et al., 1996), a brain structure critically important in learning and memory (Milner, 1972) and a main region of neurodegeneration in AD (Bowen and Davison, 1980).

Zinc has been demonstrated to modulate several neural signaling pathways including glycine-induced inhibition (Laube et al., 2000; Miller et al., 2005; Zhang and Thio, 2007), inhibition of GABA uptake (Cohen-Kfir et al., 2005), inhibition of excitatory neurotransmission (Legendre and Westbrook, 1990; Vogt et al., 2000; Izumi et al., 2006; Erreger and Traynelis, 2008b), modulation of learning and memory and synaptic plasticity (Levine et al., 1995; Minichiello et al., 1999; Liu et al., 2008; Sindreu et al., 2011) and synaptogenesis and synapse maturity (Grabrucker et al., 2011b) (Figure 1-3).

Zinc has a high affinity for human AβO (Huang et al., 2004; Tougu et al., 2008; Talmard et al., 2009), forming aggregates in less than 5 milliseconds (Noy et al., 2008). The binding of zinc to AβO may result in reduced synaptic zinc available to modulate neural pathways.

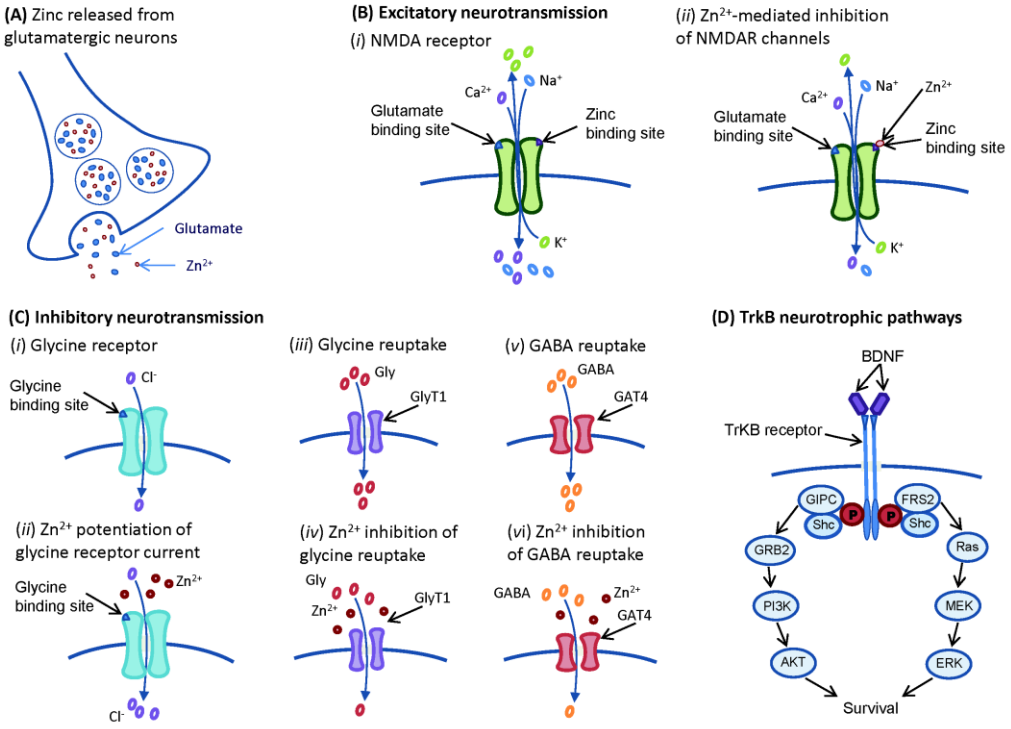

Figure 1-3. Neurotransmission pathways modulated by zinc.
A) Zn^{2+} is co-released with glutamate from glutamatergic synaptic vesicles in many regions of the brain.
B) Excitatory neurotransmission:
B*i*) Activation of the NMDAR channel by glutamate produces depolarizing current.
B*ii*) Binding of Zn^{2+} to NMDARs reduces depolarizing current.
C) Inhibitory neurotransmission:
C*i*) Activation of GlyR produces hyperpolarizing current.
C*ii*) Zn^{2+} potentiates GlyR-induced hyperpolarization.
C*iii*) The inhibitory neurotransmitter glycine is taken up by the GlyT1 glycine transporter.
C*iv*) Binding of Zn^{2+} to GlyT1 inhibits the reuptake of glycine.
C*v*) The inhibitory neurotransmitter GABA is taken up by GAT4 GABA transporter.
C*vi*) Zn^{2+} inhibits the reuptake of GABA by GAT4.
D) TrkB Neurotrophic signaling pathway:
Neuronal survival mechanisms of the AKT and ERK signaling pathways are activated by TrkB, which can be activated by exogenous Zn^{2+}.
Vogler & Busciglio, 2014, *Curr Pharm Des*.

Zinc, TrkB Receptors, NMDA Receptors and Activation of Downstream Signaling Pathways

The TrkB receptor and its endogenous ligand BDNF (brain-derived neurotrophic factor) have a significant role in neuronal survival (Ghosh et al., 1994) and learning and memory (Croll et al., 1998), with the reduction of BDNF and the catalytic form of TrkB found in the temporal lobe and hippocampus of AD patients (Phillips et al., 1991; Connor et al., 1997; Allen et al., 1999) contributing to the neurodegeneration and cognitive deficits characteristic of the disease. This is supported by research demonstrating that increasing BDNF levels improves cognition in mouse models of AD (Blurton-Jones et al., 2009).

Zinc has been demonstrated to activate the TrkB receptor (Hwang et al., 2005; Huang et al., 2008), increase pro-BDNF (Adlard et al., 2010) and increase BDNF expression in a mouse model of AD (Corona et al., 2010).

Zinc has also been shown to inhibit NMDA receptor response, though modulation is differential depending on NMDAR subunit composition (Legendre and Westbrook, 1990; Paoletti et al., 1997; Izumi et al., 2006; Erreger and Traynelis, 2008a). This is significant in that NMDAR responses are also differential depending on location and subunit type; subunits found at synapses promote cell survival and plasticity, while extrasynaptic subunits promote apoptosis and inactivation of plasticity pathways (Hardingham et al., 2002; Vanhoutte and Bading, 2003; Cull-Candy and Leszkiewicz, 2004).

Studies of the spatiotemporal dynamics of zinc released after glutamatergic mossy fiber synaptic activity show that zinc diffused through the strata radiatum and lucidum to the stratum pyramidale and did not return to baseline concentrations for ten minutes

(Ueno et al., 2002), indicating that zinc may diffuse from the synapse at concentrations sufficient to activate TrkB receptors and inhibit extrasynaptic NMDARs. The TrkB receptor has been localized with glutamatergic synapses in the hippocampus (Swanwick et al., 2004), suggesting that zinc released during glutamatergic neurotransmission may modulate TrkB receptors and both synaptic and extrasynaptic NMDARs in a differential manner, affecting the balance between activation of neurotrophic and neurodegenerative signaling pathways.

Zinc and Alzheimer's Disease

Brain regions enriched with synaptic zinc are also the regions where Aβ pathology first appears (Braak and Braak, 1991; Palmiter et al., 1996). Aβ plaques are enriched with zinc (Figure 1-4), as are the perivascular rings of amyloid angiopathy and the somata of hippocampal neurons in AD brains, which does not occur in healthy neurons (Suh et al., 2000a).

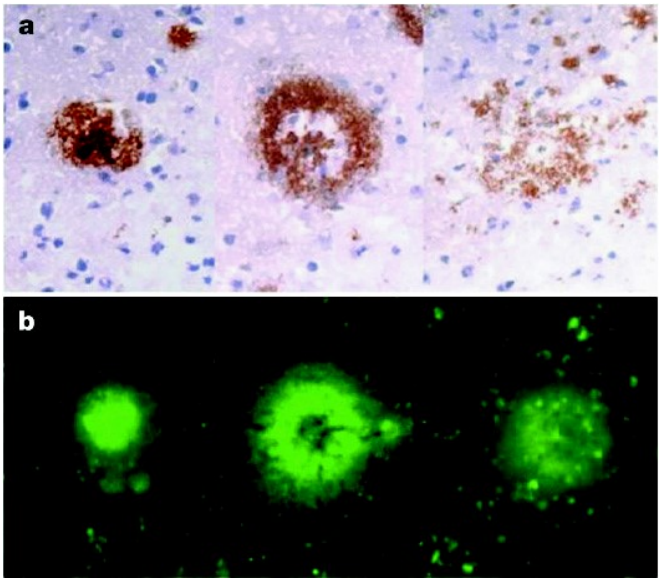

Figure 1-4. Zn^{2+} is enriched in Aβ plaques. Zinc staining of amyloid plaques in AD brain tissue. A) Anti-Aβ antibody 4G8 staining. B) TSQ fluorescent staining for Zn^{2+}.
Adapted from Fredrickson et al., 2005, *Nature Reviews Neuroscience*.

Human Aβ has a zinc-binding domain and binding of zinc induces oligomerization of Aβ that is enhanced by a post-translational modification abundant in AD brain that results in isomerization of the Asp7 residue of Aβ, changing the mechanism of Aβ-Zn^{2+} complex formation (Tsvetkov et al., 2008). However, Aβ deposition has not been found in aged rat brain tissue (Johnstone et al., 1991), which has been attributed to the observation that rat Aβ has a lower affinity for zinc and less zinc-induced aggregation (Bush et al., 1994b). There is a His13-Arg13 substitution in rat Aβ (Figure 1-5) that interferes with the Aβ-zinc binding site found in human Aβ, which has been proposed to contribute to the lack of Aβ deposition in aged rats (Huang et al., 2004) and also indicates a role for zinc in one of the main pathologies found in AD – the formation of Aβ plaques.

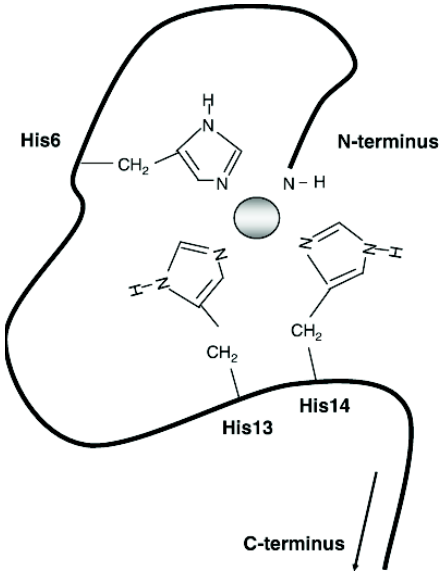

Figure 1-5. A schematic representation of the structural model of human Aβ binding zinc or copper. Rat Aβ has a His13-Arg13 substitution that interferes with binding of zinc to Aβ in rat brain. The structure was constructed using a combination of signal intensity changes, relaxation data and induced amide proton stability. Adapted from Danielsson et al., 2007, *FEBS Journal*.

Dysregulation of zinc homeostasis has been linked to the pathogenesis of AD (Bush, 2003; Sensi et al., 2008; Baum et al., 2010) (Figure 1-6) and Aβ neurotoxicity has been demonstrated to be modulated by zinc, with Zn^{2+}/Aβ aggregates blocking fast-inactivating outward K+ currents leading to a loss of Ca^{2+} homeostasis (Zhang and Yang, 2006), Zn^{2+} accelerating Aβ aggregation kinetics (Garai et al., 2007), and Zn^{2+} overload

increasing APP processing and cleavage (Wang et al., 2010a). These reports demonstrate a role for zinc in AD pathology, which is further supported by findings that clioquinol, an 8- quinolone, alters Aβ reactions to zinc, inhibits Aβ accumulation and improves cognition in transgenic mouse models of AD (Cherny et al., 2001; Adlard et al., 2008; Grossi et al., 2009). The precise mechanism of the action of clioquinol on zinc has been proposed to be as a chelator, but more recently it has been demonstrated to have a low binding affinity for Zn^{2+} and when bound to zinc clioquinol and its derivative PBT2 facilitate the transport of zinc across cell membranes, restoring zinc homeostasis and restoring cognition in aged rats (Adlard et al., 2008; Adlard et al., 2014).

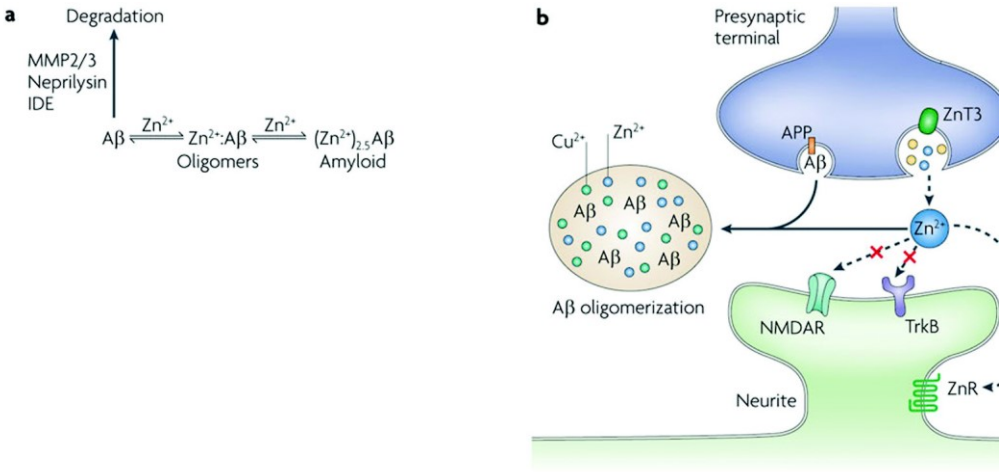

Figure 1-6. Zn^{2+}-mediated amyloid-β oligomerization and disruption of synaptic physiology by amyloid-β–Zn^{2+} complexes. A) Soluble amyloid-β (Aβ) is released upon neuronal activity. In the vicinity of the neuron, amyloid-β can encounter Zn^{2+} released through glutamatergic neurotransmission and form oligomers of increasing insolubility as the stoichiometry of Zn^{2+} binding increases. The Zn^{2+}-loaded Aβ oligomers are resistant to degradation by matrix metalloproteinases (MMPs), neprilysin and insulin degrading enzyme (IDE). B) Although there has been considerable focus on the toxic nature of Aβ and its oligomers, it is important to note that the latter act as a 'Zn^{2+} sponge,' readily binding to free Zn^{2+}. APP, Aβ precursor protein; ZnR, metabotropic Zn^{2+}-sensing receptor; ZnT3, Zn^{2+} transporter 3. Adapted from Sensi, et al., 2009, *Nature Reviews: Neuroscience*.

Zinc, Neuronal Hyperexcitability and Neurotoxicity

Zinc levels in the brain are closely regulated through activity of zinc transporters that maintain a low level of unbound "free" extracellular zinc (Sensi et al., 2009). Dysregulation of synaptic zinc has been implicated in neuronal degeneration resulting from ischemia, traumatic brain injury (TBI) and seizure activity, with both excessive and reduced levels of zinc resulting in neurodegeneration. High levels of zinc are released in ischemia and TBI, resulting in zinc translocating from pre-synaptic into post-synaptic neurons and inducing neuronal injury and death (Sensi et al., 1999; Suh et al., 2000b; Wei et al., 2004), which can prevented by zinc chelators or genetic ablation of synaptic zinc (Koh et al., 1996; Calderone et al., 2004; Doering et al., 2010; Wang et al., 2010c).

Studies of seizure activity and neuronal hyperexcitability indicate that zinc modulation generally reduces neuronal excitability. Transgenic mice that lack synaptic zinc have increased susceptibility to kainate-induced limbic seizures (Cole et al., 2000) and higher frequency of electroencephalogram (EEG) spiking after kainate treatment compared to wild-type mice (Qian et al., 2011), while infusion of zinc into the hippocampus of a rat model of epilepsy delays development of seizures (Elsas et al., 2009). Reducing zinc levels through chelation or zinc-free diet increases excitability in the hippocampus (Lavoie et al., 2007) and zinc chelation increases hippocampal cell stress markers and cell death after nonconvulsive treatment with kainic acid (Dominguez et al., 2006).

Neuronal Hyperactivity in AD

Decline in memory is associated with normal aging and has been linked to hippocampus hyperactivity. Increased CA3 activity in the hippocampus is associated with spatial memory impairment in aged rats (Wilson et al., 2005), while treating cognitively impaired aged rats with neuropeptide Y (NPY, a protein up-regulated in seizure activity thought to be neuroprotective) or antiepileptic agents improved memory performance (Koh et al., 2010). Functional magnetic resonance imaging (fMRI) studies in cognitively normal aged humans have demonstrated increased activity in the CA3/dentate gyrus during specific memory tasks, associating specific functional alterations with age-related memory difficulties (Yassa et al., 2010).

There is differential activation of brain regions in normal aging, early MCI, late MCI and AD. fMRI studies have found increased hippocampus activation in less impaired subjects with MCI compared to normal subjects and decreased hippocampus activation in MCI/AD subjects with significant impairment (Dickerson et al., 2005; Celone et al., 2006). This differential relationship was also found in PiB experiments to image Aβ deposition, with increased Aβ deposition correlating with increased brain metabolism in MCI and increased Aβ deposition correlating with decreased metabolism in AD (Cohen et al., 2009). Longitudinal studies have demonstrated that increased hippocampal activation predicts greater subsequent cognitive decline in subjects with MCI (Miller et al., 2008; O'Brien et al., 2010), linking differential activation with the different long-term outcomes observed in MCI, as not all MCI cases convert to AD. The mechanisms underlying differential activation and subsequent variations in degree of cognitive decline are not known. Though neuron loss has been thought to underlie age-related memory

impairment, it has been found that cognitive impairment is not due to loss of hippocampal neurons in aged rats with cognitive deficits (Rapp and Gallagher, 1996) and fMRI studies have revealed no decrease in hippocampal volumes in MCI, but decrease as MCI progresses to AD (Dickerson et al., 2005).

EEG, Seizure Activity and AD

EEG has been used as a tool to diagnose AD and its progression for many years as studies found hallmark EEG abnormalities in AD patients, including slowing of rhythms and decreased coherence between brain regions (Jeong, 2004), while a study of clinical changes in AD patients over time found hypersynchronization in the medial temporal lobe early in AD but becoming hyposynchronized as the disease progresses (Knyazeva et al., 2013). However, only recently has the incidence of seizures in AD patients become a focus of research (Vossel et al., 2013). It has been observed that AD patients have increased risk of unprovoked seizures (Hesdorffer et al., 1996; Amatniek et al., 2006; Lozsadi and Larner, 2006), which has been linked to Aβ-induced seizure activity and alterations in neuronal excitability and morphology (Palop et al., 2007; Minkeviciene et al., 2009). Altered levels of NPY and calbindin, common markers of seizure activity, have been also been found in human AD patients (Beal et al., 1986; Chan-Palay et al., 1986; Greene et al., 2001; Iritani et al., 2001). The link between EEG abnormalities and AD was investigated recently through generation of mice with controllable transgenic expression of amyloid precursor protein (APP), and it was found that withholding APP overexpression and subsequent overproduction of Aβ delayed development of epileptiform activity and

that the EEG abnormalities were dependent on continued overexpression of APP (Born et al., 2014).

Mouse models of AD have been demonstrated to have non-convulsive seizures, identified through simultaneous EEG and behavioral observations linking epileptiform spiking with behavioral arrest (Palop et al., 2007), and transgenic mice expressing apolipoprotein ε4 (apoε4), a gene conferring significant pre-disposition for developing AD, develop seizures as they age (Hunter et al., 2012). Studies utilizing transgenic mice have demonstrated a link between epilepsy and AD-like neuropathology (Yan et al., 2012), while reduction of tau reduces hyperexcitability in mouse models of AD (DeVos et al., 2013). Other studies have found that antiepileptic drugs reduce memory impairment (Devi and Ohno, 2013; Shi et al., 2013) and suppression of epileptiform activity (Ziyatdinova et al., 2011) in mouse models of AD. Interestingly, it has been known that the retinoid X receptor agonist bexarotene improves cognition in AD mouse models, but recently demonstrated to effect the improvement through reduction of network excitability (Bomben et al., 2014).

Epileptiform activity may go unrecognized in many AD patients. One study of AD patients found that over half of AD patients with epileptiform activity had non-convulsive or complex partial seizures, which display dyscognitive symptoms rather than myoclonic or atonic symptoms that the general public may not recognize as seizures and thus result in underreporting of seizure in AD (Vossel et al., 2013). Another recent study found that transient epileptic amnesia (TEA) has been misdiagnosed as MCI (Del Felice et al., 2014), and it has been observed that temporal lobe epilepsy (TLE) and MCI often share the similar pathology of cognitive impairment and irregular EEG and as such can be

misdiagnosed by doctors without access to neuroimaging or computational EEG analysis to distinguish between the conditions (Holler and Trinka, 2014).

However, the mechanisms by which Aβ influences seizure activity are poorly understood. Disruption by Aβ of zinc signaling may be a factor in aberrant neuronal excitability as zinc generally potentiates responses of some classes of inhibitory receptors (Bloomenthal et al., 1994; Laube et al., 2000; Cohen-Kfir et al., 2005; Miller et al., 2005) and inhibits responses of NMDA excitatory receptors (Legendre and Westbrook, 1990; Paoletti et al., 1997; Erreger and Traynelis, 2008b).

The ZnT3 Knockout Transgenic Mouse

The zinc transporter ZnT3 transports zinc from the cytosol into synaptic vesicles at glutamatergic synapses, where the zinc is then released into the synapse along with glutamate during excitatory neurotransmission. ZnT3 is expressed throughout the brain, in the retina and other parts of the nervous system (Palmiter et al., 1996; Wang et al., 2003; Wang et al., 2005; Redenti and Chappell, 2007), is concentrated in the hippocampus in mossy fibers of the dentate gyrus that project to the hilus and CA3 regions (Wenzel et al., 1997), and is most commonly imaged by a modified method of Timm's silver sulfide histochemical staining (Perez-Clausell and Danscher, 1985) (Figure 1-7). Genetic ablation of the protein in the ZnT3KO (knockout) transgenic mouse results in a lack of zinc in synaptic vesicles (Linkous et al., 2008) (Figure 1-8).

Many previous reports of zinc neurotransmission manipulated bath concentrations of organotypic brain sections, neuronal tissue cultures, cell lines and oocytes to investigate aberrant zinc signaling. There are two factors that need to be considered in these studies: binding kinetics of zinc chelators and concentration of zinc produced by

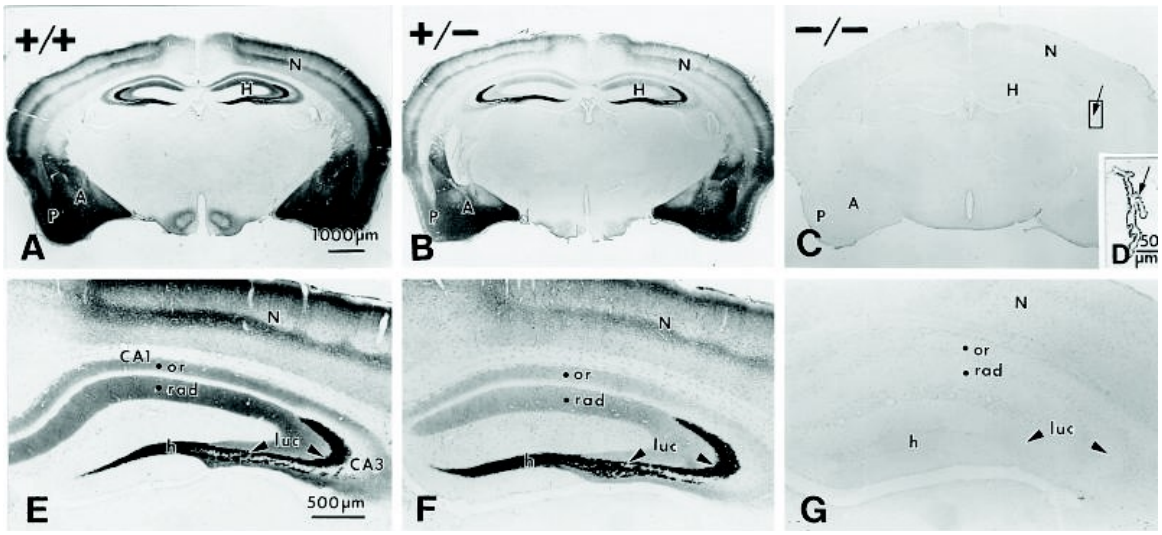

Figure1- 7. Timm stain is undetectable in the brains of ZnT3KO mice. Comparison of Timm stain between brains of ZnT3$^{+/+}$ (A and E), ZnT3$^{+/-}$ (B and F), and ZnT3KO mice (C and G). (A–C) Coronal sections through the midbrain. Timm stain in the hippocampus (H), piriform cortex (P), neocortex (N), and amygdala (A) was conspicuous in the ZnT3$^{+/+}$ brain (A), reduced in the ZnT3$^{+/-}$ brain (B), and undetectable in the brains of ZnT3KO mice (C). (E–G) Higher magnification of Timm-stained hippocampi from ZnT3$^{+/+}$ (E), ZnT3$^{+/-}$ (F), and ZnT3KO (G) mice. Timm stain was reduced (ZnT3$^{+/-}$) or absent (ZnT3KO) in the hilus (h), s lucidum (luc) of CA3, and s oriens (or) and s radiatum (rad) of CA1 and CA3. Adapted from Cole, *et al*., 1999, *PNAS*.

synaptic release. One commonly used chelator, CaEDTA, has been demonstrated to have high affinity for zinc, but slow binding kinetics that result in ineffective chelation of rapidly released zinc (Vogt et al., 2000; Kay, 2003), and the concentration of the chelator also affects efficiency of zinc chelation (Lavoie et al., 2007). Estimates of zinc concentration from synaptic release vary significantly, from as low as 100 nM (Komatsu et al., 2005) to as high as 100 μM (Vogt et al., 2000). These factors can adversely impact attempts to study zinc at physiologically relevant levels through addition of exogenous zinc and removal of zinc through addition of chelating compounds, which also eliminates both synaptic zinc and zinc from other sources. The development of the ZnT3KO mouse provides an alternative experimental method by allowing comparison of the effects of

experimental manipulations between ZnT3KO and wild-type controls to determine if the effects are from physiologically relevant synaptic zinc concentrations and also to determine if the source of zinc is from release of synaptic vesicles during excitatory neurotransmission.

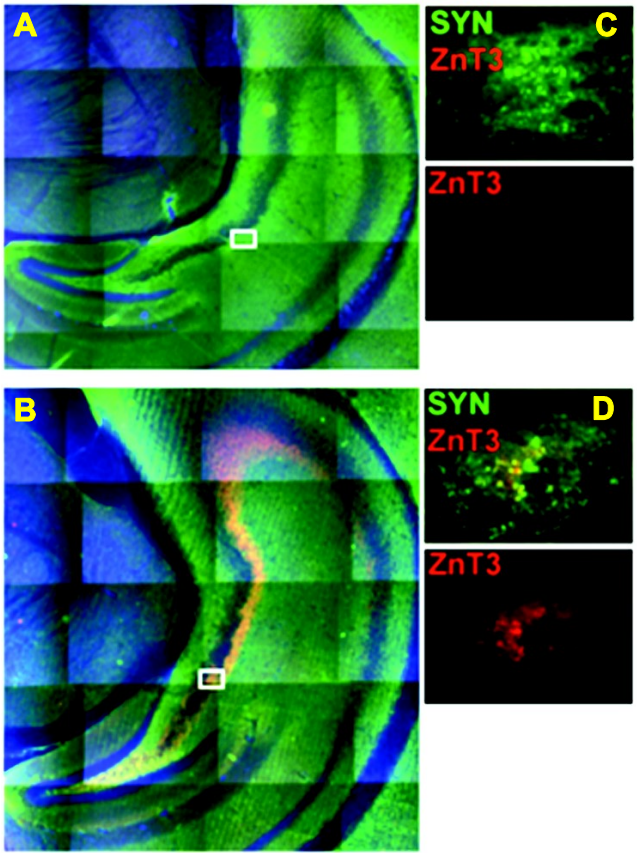

Figure 1-8. Immunolabeling of ZnT3 is undetectable in ZnT3KO hippocampus. Fluorescent labeling of paraformaldehyde-fixed hippocampus sections with:
αZnT3 (red)
αMAP2 (green)
Hoescht (blue)
A) ZnT3KO
B) Wild type
C and D) 63X magnification of regions indicated in A and B, respectively.
αZnT3 (red)
αsynaptophysin (green)

The use of transgenic mice with global ablation of a gene generates concern regarding compensatory mechanisms that may arise during development and whether the effects of the lack of the gene in organs other than the one under study may interfere with methodology and interpretation of the target effects. While ZnT3 is highly enriched in the mossy fiber tract of the hippocampus and found in many other brain regions, it is also expressed in the retina, testis, pancreas and epithelial cells (Smidt and Rungby, 2012), where Zn^{2+} has been shown to regulate light adaptation in the retina (Bai et al.,

2013), the production of insulin and viability of pancreatic β-cells (Petersen et al., 2011). Studies have demonstrated that expression of ZnT3 can be regulated by alterations in hormones (Iguchi et al., 2004; Lee et al., 2004) and environmental effects (Ackland et al., 2007). Consequently, experimental design and interpretation of results should consider these potentially confounding effects.

The Use of Mouse Models to Understand Human Disease Mechanisms

The hippocampus is an area of the brain that plays an important role in the processes of learning and memory, is enriched in synaptic zinc, and is also selectively affected in Alzheimer disease. Mice exhibit hippocampal structure similar to humans (West, 1990), making these species an optimal system allowing the extrapolation of experimental conclusions to human pathology. Recently, the development of transgenic mice has allowed targeted genetic mutations to be introduced into mice that produce pathologies similar to that produced by diseases in humans (Rosenfeld et al., 1988), providing model systems in which to investigate the effects of experimental manipulations on disease processes.

The use of transgenic animals allows investigation of physical or cognitive functions commonly impaired in diseases that can be assessed through physical or behavioral tests before and after experimental interventions to determine the effect of the intervention on the particular function and also allows investigation into the effect of physiological changes on cognition. Behavioral tasks have been developed to test many aspects of cognition, including tasks that rely on normal function of specific brain regions for normal behavioral response to the task (Morris et al., 1990).

In vitro preparations from transgenic animal tissue are widely used to study mechanisms of disease pathology. Tissue cultures of embryonic hippocampal cells provide a reduced preparation to study responses in individual neurons, while hippocampal slices are widely accepted as an excellent experimental model to study synaptic physiology and plasticity as they preserve the anatomic cytoarchitecture allowing experimental manipulations of intact neuronal signaling pathways (Lynch and Schubert, 1980).

Significance

The hallmark characteristics of Alzheimer's disease pathology are aberrant regulation of two proteins – aggregation of Aβ and hyperphosphorylation and aggregation of tau – with the symptomatic manifestation of impaired cognition. The molecular mechanisms producing aberrant regulation and the relationships between aberrant regulation and impaired cognition are not yet fully understood, though Aβ plaque load has been significantly associated with apoε4 carrier status in cognitively normal subjects, suggesting a link between ε4 gene dose and potential development of cognitive impairment (Reiman et al., 2009). This lack of understanding may also contribute to the high failure rate of clinical trials for AD therapeutics: 99.6% failure rate for the period 2002-2012 (Cummings et al., 2014). Underlying factors adding to the difficulty in developing effective therapeutics include immune response and inflammation (Fonseca et al., 2009), altered calcium signaling in cells (Chakroborty and Stutzmann, 2013), the presence of seizure activity in a minority of AD patients and mouse models (Noebels, 2011) and variability in the length of time over which the disease progresses, which hinders

determining appropriate timing for therapeutic interventions (Cummings et al., 2014). Recent demonstration that Aβ can sequester synaptic zinc (Grabrucker et al., 2011a), combined with success in treating mouse models of AD with a zinc-modulating drug (Adlard et al., 2008) that has since failed to meet target endpoints in Phase 2 clinical trials (PRANA Biotechnology press release, March 31, 2014), highlight the need for increased understanding of the role of zinc in age-related neurodegeneration.

Zinc modulation of neurotransmission is generally inhibitory and disruption of synaptic zinc contributes to neuronal hyperactivity, suggesting that lack of synaptic zinc will produce excessive excitatory activity. These experiments investigate the role of zinc in excessive neuronal hyperactivity by identifying and quantifying markers of seizure activity in the ZnT3KO hippocampus and investigating the effects of treatment with an anti-seizure drug on the cognitive impairment present in aged ZnT3KO mice in performing behavioral tasks.

Exogenous zinc has been demonstrated to modulate several cell signaling receptors and ion channels in dissociated neuronal tissue cultures, suggesting that zinc released during glutamatergic neurotransmission will modulate activation of downstream signaling molecules. The experiments in this dissertation investigate the role of endogenous zinc in acute hippocampal slices, maintaining the cytoarchitecture in which zinc is highly expressed and released during glutamatergic neurotransmission. This preparation is designed to demonstrate the effects of release of synaptic zinc on activation of downstream proteins in signaling pathways in intact hippocampus circuits and how disruption of synaptic zinc may alter the balance between neurotrophic and neurodegenerative responses.

Together, the experiments presented in this dissertation increase our understanding of the effects of synaptic zinc and may facilitate development of therapies intended to promote normal zinc function in neurodegenerative diseases.

Hypothesis and Specific Aims

The hypothesis of this dissertation is that dysregulation of synaptic zinc results in excessive excitatory neuronal activity and neurodegenerative alterations in signaling pathways. The specific aims were to investigate the effects of genetic ablation of synaptic zinc on:

- Excessive excitatory neuronal activity – Chapter 2
    - Assay age-dependent markers of seizure activity
    - Investigate the effect of acute treatment with an anti-seizure drug on impaired cognition
- Alterations in neurotrophic signaling pathways – Chapter 3
    - Assess levels of neurotrophic proteins in signaling pathways previously demonstrated to be modulated by Zn^{2+}
    - Assess alterations in activity-dependent phosphorylation of these proteins
    - Assess differential activation induced by specific receptor subunits in one of these pathways

Chapter 2

Elimination of Synaptic Zinc Increases Markers of Seizure Activity

Summary

Exogenous zinc has been demonstrated to reduce neuronal excitability (Paoletti et al., 1997) and *in vivo* reduction of zinc increases susceptibility to seizures (Cole et al., 2000), while the addition of zinc delays development of seizure in models of epilepsy (Elsas et al., 2009). Based on these results, we hypothesized that elimination of synaptic zinc promotes excessive excitatory neurotransmission, increases markers of excitotoxicity in brain regions enriched in zinc released during neurotransmission, and contributes to cognitive impairment. This hypothesis was investigated through identification and quantification of biochemical and morphological markers of seizure activity in ZnT3KO mice compared to WT from two to fifteen months of age. The role of seizure activity in cognitive impairment was investigated through behavioral testing of aged ZnT3KO mice after treatment with an anti-seizure drug. Age-dependence was considered as the ZnT3KO phenotype is cognitively and physiologically normal at two months of age (Cole et al., 2001) but develops synaptic and cognitive deficits similar to those found in AD patients by six months of age (Adlard et al., 2010). Consequently, alterations promoted by seizure activity may not be detectable in younger animals and become progressively more pronounced as the animals age.

Introduction

Types of Seizure Activity

Seizures are defined by two characteristics: observed behavior and EEG patterns. The Epilepsy Foundation of America describes the behavioral characteristics as covering a spectrum: from simple partial seizure, which typically lasts for 30-60 seconds and the subject does not lose consciousness and may even be able to converse during the episode, to primary generalized seizures featuring loss of consciousness, muscle spasms and temporary cessation of breathing. Another type of seizure, the absence seizure, is characterized by a brief period of muscle twitches and loss of consciousness with prompt recovery. The characteristic patterns seen in EEG, featuring spike and wave discharges or rapid oscillations, cover a spectrum reflected in the severity of the behavioral symptoms. Because there is such a range of seizure severity, it is possible that partial or absence seizures may not be recognized and diagnosed in AD patients with cognitive deficits that mask mild seizure symptoms.

Epileptiform Activity and Cognitive Deficits

Early EEG researchers proposed that epileptiform activity could occur without apparent behavioral effects, a phenomena considered “subclinical” or “masked” seizures. However, as the development progressed of suitable cognitive tests that could be performed during EEG recording, it was quickly demonstrated that alterations in reaction times and memory impairment frequently accompanied “subclinical” EEG discharges (Aarts et al., 1984). This condition was termed “transitory cognitive impairment (TCI) and considered a condition separate from epilepsy as it has been found in patients without a

clinical diagnosis of epilepsy (Aldenkamp, 1997; Binnie, 2003). Excessive excitatory activity in the hippocampus has been linked to cognitive impairment in both aged rats and human patients with amnestic MCI and treatment with anti-seizure drugs improved cognition in both groups (Koh et al., 2010; Bakker et al., 2012).

Cognitive deficits can also be attributed to brain injury caused by seizures. Early studies found correlation between seizures and subsequent brain damage and later animal research gave insight into specific mechanisms through which seizures impair neuronal function (Sano and Malamud, 1953; Ben-Ari, 1985). Consequently, it should be considered whether epileptiform activity is causing cognitive deficits or is causing brain damage that then results in cognitive deficits.

Anti-seizure Treatments and Their Effects on Cognition

It has been long known that first generation antiepileptic drugs (AEDs) frequently impair cognition by slowing reaction time and disrupting attention in both epileptic and non-epileptic subjects. Consequently, different classes of AEDs have been developed with fewer negative side effects, with the drug levetiracetam (LEV) having no measureable effect on cognition (Aldenkamp et al., 2003; Shannon and Love, 2005; Higgins et al., 2010). In fact, LEV has been demonstrated to improve cognition in MCI patients (Bakker et al., 2012), in mouse models of AD (Sanchez et al., 2012; Devi and Ohno, 2013) and in aged, but not young, rats (Koh et al., 2010).

The mechanism of action of LEV is not yet fully understood, but it has recently been demonstrated to reduce zinc suppression of GABA(A)-mediated pre-synaptic inhibition in a preparation of isolated synaptic boutons from the CA3 region of postnatal

rats (Wakita et al., 2014). However, this study found that LEV did not affect voltage-dependent Ca^{2+} currents, although many other studies have demonstrated that LEV inhibits Ca^{2+} channels through binding the synaptic vesicle SV2A (Vogl et al., 2012; Yan et al., 2013), for review see (Deshpande and Delorenzo, 2014).

The Hippocampus and Cognition

While ZnT3 is expressed throughout the brain, it is highly enriched in the mossy fiber tract of the hippocampus (Palmiter et al., 1996), a brain structure that has been demonstrated to be critical for spatial learning and memory through behavioral tasks in which inactivation of the hippocampus impairs performance (Parkinson et al., 1988; Bannerman et al., 1995; Steele and Morris, 1999). These tasks, including the Morris water maze and object location memory (OLM), are now commonly used to detect impairment of hippocampal function in cognition.

The hippocampal mossy fibers are the axons of the granule cells of the dentate gyrus, passing to the hilus, where they fasciculate and continue alongside the pyramidal cell layer to the CA3 region, ending at the transition to the CA1 region. Mossy fibers are distinctive in that they are highly enriched in zinc, allowing them to be visualized through sulfide silver staining. Though some axons bifurcate to form a few recurrent collaterals in the hilus, the axons primarily form synaptic contacts with dendrites of pyramidal cells of the CA3. Other mossy fiber collaterals arise just beneath the granule cell layer to connect with local interneurons, forming an inhibitory feedback circuit to the granule cells (Nicoll and Schmitz, 2005) (Figure 2-1).

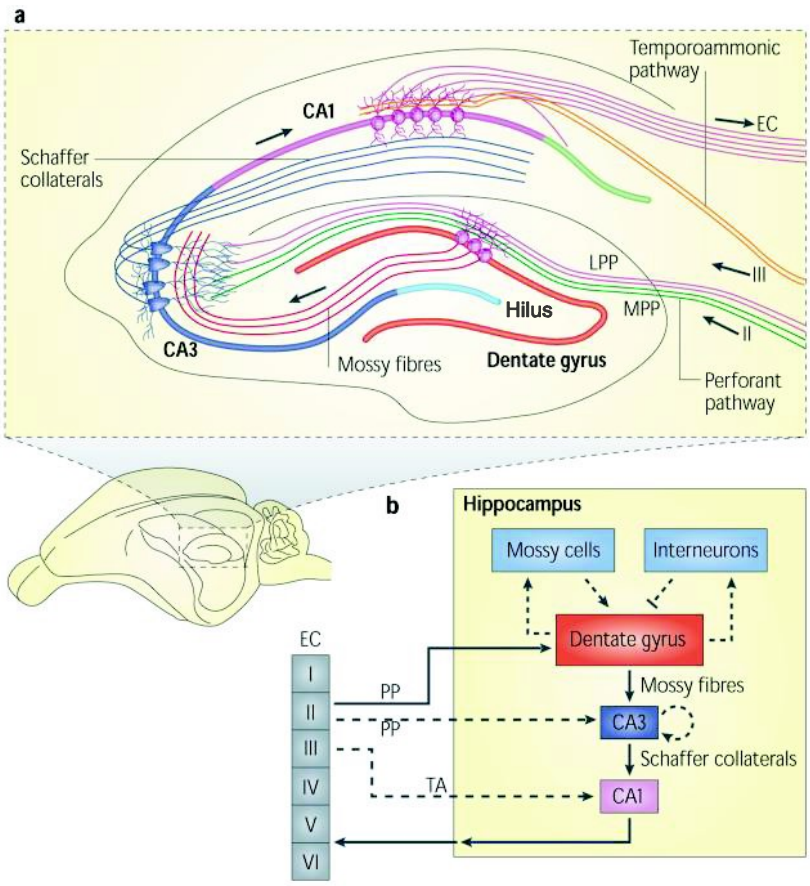

Figure 2-1. Zinc is enriched in the mossy fibers of the hippocampus.
A) An illustration of the hippocampal circuitry.
B) Diagram of the hippocampal neural network.
The traditional excitatory trisynaptic pathway (EC–dentate gyrus–CA3–CA1–EC) is depicted by solid arrows:

- •The dentate gyrus (red) sends projections to the pyramidal cells in CA3 (dark blue) through mossy fibers.
- •CA3 pyramidal neurons relay the information to CA1 (pink) pyramidal neurons through Schaffer collaterals.
- •CA1 pyramidal neurons send back-projections into deep-layer neurons of the EC (gray).

Other pathways are depicted by dotted arrows:

- •CA3 also receives direct projections from EC layer II neurons through the PP.
- •CA1 receives direct input from EC layer III neurons through the TA.
- •The dentate granule cells also project to the mossy cells in the hilus and hilar interneurons, which send excitatory and inhibitory projections, respectively, back to the granule cells.

EC, entorhinal cortex; PP, perforant pathway; LPP, lateral perforant pathway; MPP, medial perforant pathway; TA, temporoammonic pathway.
Adapted from Deng et al., 2010, *Nature Reviews Neuroscience*.

Markers of seizure activity

Seizure-related activity increases expression of neuropeptide Y (NPY) (Gruber et al., 1994; Vezzani et al., 1999) and decreases calbindin expression in the hippocampus (Tonder et al., 1994; Yang et al., 1997). Altered levels of NPY and calbindin have also been found in human AD patients (Beal et al., 1986; Chan-Palay et al., 1986; Greene et al., 2001; Iritani et al., 2001; Krezymon et al., 2013). It has been proposed that these measures can serve as surrogate biomarkers to supplement behavioral tests of brain function in mouse models of AD as they correlate better with memory deficits than does plaque density in both mouse models of AD and AD patients (Palop et al., 2011).

Kainate-induced seizures result in extensive hippocampal neuronal loss and aberrant sprouting of mossy fibers of the dentate granule cells across the granule cell layer into the inner molecular layer (Nadler et al., 1980; Cronin and Dudek, 1988). Induction of less severe seizures provoked through kindling (a model of epilepsy induced by repeated electrical or chemical stimulation to a brain region) or hyperthermia produces similar mossy fiber sprouting without neuronal loss (Sutula et al., 1988; Bender et al., 2003), indicating that mossy fiber sprouting can be produced from brief epileptiform activity without convulsive seizure and also serves as a marker for seizure activity.

Calbindin D28K

Calbindin D28K is a calcium-binding protein found throughout the nervous system (Baimbridge et al., 1992), where it contributes to Ca^{2+} buffering in neurons (Kohr et al., 1991). Null mutation of the calbindin gene results in severe impairment in motor coordination, highlighting its importance in the cerebellar function (Airaksinen et al.,

1997), while an antisense mutation targeting expression in forebrain and midbrain result in deficits in memory and hippocampal long-term potentiation (Molinari et al., 1996). In the hippocampus, calbindin is found in the dentate gyrus in granule cells, CA1 pyramidal cells and CA3 GABAergic cells (Baimbridge and Miller, 1982; Toth and Freund, 1992).

Neurons in the hippocampus expressing calbindin are more resistant to prolonged stimulation and calcium or glutamate toxicity compared to neurons lacking the protein (Scharfman and Schwartzkroin, 1989; Mattson et al., 1991) and it is reduced in the dentate gyrus by seizure activity (Kohr et al., 1991), with significant reduction after kindling (Sonnenberg et al., 1991).

Calbindin is also reduced in normal aging and in discrete areas in neurodegenerative diseases, including AD hippocampus and cholinergic neurons (Iacopino and Christakos, 1990; Riascos et al., 2014) and is lost at a significantly higher ratio in the dentate gyrus of AD patients compared to other neurodegenerative diseases (Stefanits et al., 2014). It has recently been demonstrated that targeted elimination of calbindin in a mouse model of AD results in neuronal loss, reduced levels of phosphorylated Erk1/2 and CREB and reduction of synaptic proteins, contributing to AD pathogenesis (Kook et al., 2014).

Neuropeptide Y

NPY is a 36 amino acid protein found abundantly in neural tissue (Allen et al., 1983; Hendry et al., 1984a) and is present in interneurons of the hippocampus (Hendry et al., 1984b; Jinno and Kosaka, 2003) that form symmetric synaptic contacts on granule cell bodies and dendrites in the hilus, granule cell layer, molecular layer and CA3 (Deller

and Leranth, 1990). Seizure-induced alteration of NPY expression and remodeling of hippocampal circuitry have been proposed to function in seizure modulation (Redrobe et al., 1999; Vezzani et al., 1999; Nadler et al., 2007). NPY reduces the amplitude of excitation through hippocampal circuits (Haas et al., 1987; Klapstein and Colmers, 1993) (Figure 2-2), inhibits potassium-stimulated release of glutamate in hippocampal slices (Greber et al., 1994), and activation of NPY receptors is neuroprotective against AMPA-induced excitoxicity in CA1 and dentate granule cells hippocampal slices (Silva et al., 2003).

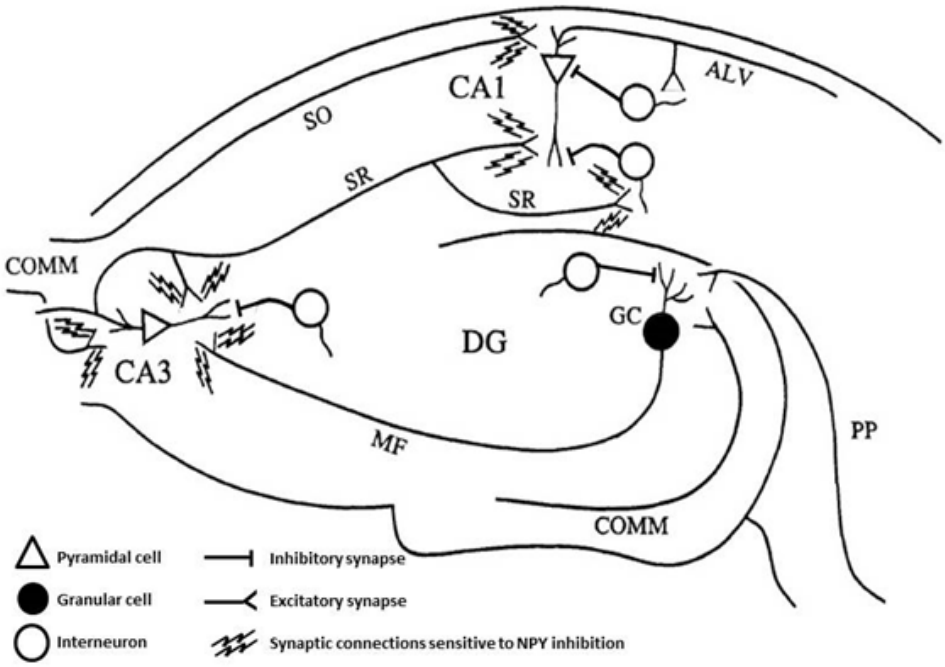

Figure 2-2. NPY is present in interneurons of the hippocampus. A schematic diagram of a transverse hippocampal slice, showing sites of NPY presynaptic inhibition in the rodent hippocampus. ALV, alveus; CA1, CA3, pyramidal cells of areas CA1 and CA3; COMM, commissural fibers entering dentate or CA3 from contralateral hippocampus; DG, dentate gyrus; GC, dentate granule cell; MF, mossy fibers; PP, performant path; SO, stratum oriens; SR, stratum radiatum. Adapted from Klapstein and Colmers, 1993, *Hippocampus.*

NPY has a protective role in seizure activity, reducing spontaneous and evoked excitability (Klapstein and Colmers, 1997), and reducing mortality in kainate-induced seizures (Baraban et al., 1997). Hippocampal kindling increases expression of NPY in CA1, CA3 and dentate gyrus (Bendotti et al., 1993; Schwarzer et al., 1995). Increased NPY protein and mRNA expression persists for many months following seizure activity (Sperk et al., 1992; Gruber et al., 1994; Kharlamov et al., 2007). NPY receptor binding sites are reduced in AD brain compared to age-matched controls (Martel et al., 1990) and NPY is reduced in post-mortem AD brain though considerable networks of NPY cells survive in advanced AD brain compared to age-matched control brain (Chan-Palay et al., 1985).

Initial attempts to quantify the 11kDa NPY protein by western blot found that multiple bands formed during gel electrophoresis, demonstrating aggregation of NPY. Therefore, dot blot analysis of NPY protein levels was performed using known concentrations of NPY protein and a standard curve derived from serial dilutions to validate the dot blot method for quantification purposes (Figure 2-4).

Mossy fiber sprouting

Kainic acid is an agonist of the kainate receptor, an ionotropic glutamate receptor, and a potent convulsant used in studies of seizures and epilepsy. Synapses of the mossy fiber-CA3 region have a high density of kainic acid binding sites and intraventricular injection of kainic acid results in seizures and pattern of brain damage similar to that found in temporal lobe epilepsy (Nadler, 1981). Studies have also demonstrated correlation between kainic acid-induced seizures and sprouting of mossy fibers (Cronin and Dudek,

1988) (Figure 2-3). Even brief seizure episodes can induce mossy fiber sprouting (Ben-Ari and Represa, 1990).

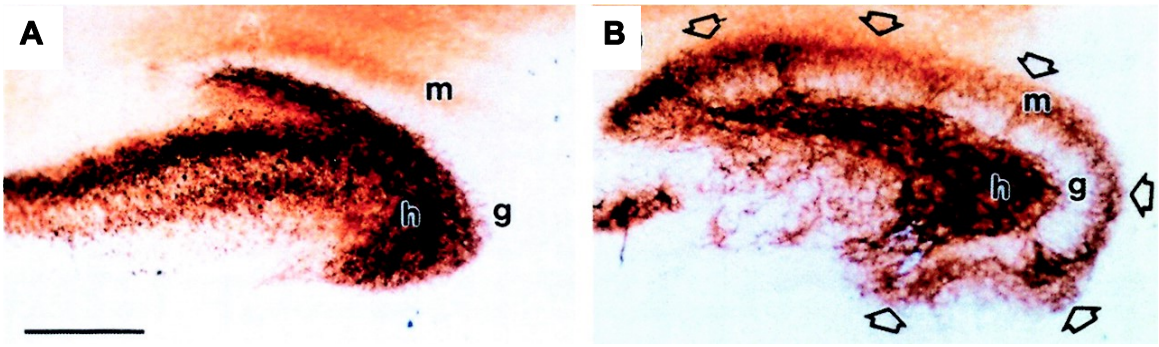

Figure 2-3. Kainic acid (KA) treatment *in vitro* causes mossy fiber sprouting. Vehicle-treated (A) and KA-treated (B) hippocampal slice cultures were stained with Timm's stain (black). A) Timm's staining revealed a relative paucity of zinc-positive mossy fibers in the inner molecular of vehicle-treated cultures. B) Timm's staining revealed mossy fiber sprouting into the inner molecular layer (arrows) was increased after KA treatment. Scale bar is 100μm. DG, dentate gyrus; g, dentate granule cell layer; h, hilus; m, molecular layer. Adapted from Bausch & McNamara, 2004, *J Neurophys*.

Early studies found that various experimental manipulations resulted in mossy fiber afferents rerouted to abnormal regions, forming aberrant connections. Kindling results in mossy fiber afferents sprouting into the molecular layer (Sutula et al., 1988), as do lesion studies removing major afferents to the dentate molecular layer (Laurberg and Zimmer, 1981), while destruction of CA3 afferents with kainic acid produce similar results (Nadler et al., 1980). These studies found that many of the aberrantly sprouting mossy fibers made contact with granule cell dendrites in the molecular layer. A subsequent study demonstrated that sprouting fibers induced by kainic acid lesion form functional synapses and a recurrent excitatory circuit in the molecular layer of hippocampal slice preparations (Tauck and Nadler, 1985).

Mossy fiber synapses are altered in AD hippocampus (Kiktenko et al., 1995), a mouse model of familial AD has reduced mossy fiber synaptic complexity and contacts

(Wilke et al., 2014), transgenic mice lacking Apolipoprotein ε (Apoε), which is a risk factor for AD, have limited mossy fiber sprouting after neural injury (Teter et al., 1999), and mouse models of AD with neuronal hyperexcitability demonstrate mossy fiber sprouting (Palop et al., 2007), indicating that AD pathology disrupts mossy fiber structure and function. Though mossy fiber sprouting specifically is not commonly reported in AD brain, AD pathology includes significant aberrant neuronal sprouting throughout the brain (Teter and Ashford, 2002).

Visualizing mossy fiber sprouting

Mossy fiber sprouting has been most commonly visualized and studied through use of the Timm's method of silver sulfide staining that labels unbound or "free" Zn^{2+}, the zinc that is highly enriched in synaptic vesicles of mossy fibers. Consequently, studies utilizing transgenic mice lacking synaptic zinc require other methods of identifying mossy fibers. One method uses a protein associated with growth cones of axonal projections, GAP-43 and the other uses a synaptic protein enriched in mossy fibers, synaptoporin.

GAP-43

GAP-43 is a protein linked to axonal growth and plasticity that is normally found in the molecular layer of the stratum lacunosum of the hippocampus (Benowitz et al., 1989), and in growth cones and immature synapses during establishment of neuronal connections (Benowitz and Routtenberg, 1997). Expression of GAP-43 is significantly reduced in mature synapses, but expression persists in mature hippocampus, where changes in GAP-43 phosphorylation has been found to correlate with induction of long-

term potentiation (Benowitz et al., 1989). GAP-43 up-regulation in sprouting mossy fibers is also associated with hippocampal kindling (Bendotti et al., 1993), epileptogenesis (Tolner et al., 2003), and induced seizures (Cantallops and Routtenberg, 1996; Bendotti et al., 1997; Naffah-Mazzacoratti et al., 1999). GAP-43 levels are increased in AD hippocampus (Rekart et al., 2004) and the protein is also found associated with APP immuno-labeling in aberrant sprouting neurites in AD brain (Masliah et al., 1992).

Synaptoporin

Synaptoporin is a synaptic vesicle membrane protein that shares sequence homology with synaptophysin (Knaus et al., 1990) but is differentially expressed with high abundance in the mossy fibers of the hippocampus (Singec et al., 2002). Recently, synaptoporin was shown to be abundant in sprouting mossy fibers after induced seizures (Ito et al., 2012).

Significance

Quantification of age-dependent alterations in levels of these biochemical and morphological features in ZnT3KO mice compared to age-matched WT mice provides significant evidence of excessive excitatory or seizure activity in aging ZnT3KO hippocampus.

Both seizure activity and cumulative brain damage from seizure activity have been demonstrated to impair cognition. This research attempted to differentiate between the two possibilities through acute treatment with an anti-epileptic drug to suppress seizure activity during the training and testing periods in a hippocampus-dependent behavioral

memory task to determine if seizure activity was the primary contributor to cognitive deficits.

Another approach would be long-term administration of an anti-epileptic drug beginning at an age when the animals do not demonstrate impairment until the age where impairment has been observed, then performing the same behavioral test for performance in a memory task combined with biochemical and morphological analysis of the treated subjects to determine if suppression of seizure activity prevented development of the cognitive deficits and alterations in biochemical and morphological characteristics.

Together, these experiments provide more understanding of how interference with zinc neurotransmission can contribute to aberrant neuronal activity, age-dependent cognitive impairments and neurodegeneration and assist in development of therapeutics to delay or prevent the pathology.

Materials and Methods

All animal experiments were approved by the University of California, Irvine's Institutional Animal Care and Use Committee and conformed to all University and USDA animal care regulations. 129Sv wild-type (WT) or ZnT3KO mice (from Richard Palmiter, University of Washington) were housed in the animal facility at the University of California, Irvine, maintained on a 12 hour day/night cycle and provided food *ad libitum*.

Preparation of animal tissues. Mice were deeply anesthetized and perfused with PBS, the brains removed and hemispheres separated. One hemisphere was placed in ice cold 4% paraformaldehyde (PFA) and fixed for 48 hours at 4°C, then transferred to PBS until

sliced into 40μM sections bathed in ice cold PBS with a Vibratome 600 and stored in PBS/0.03% sodium azide for immunohistochemistry. The other hemisphere was quickly dissected in ice cold PBS to remove the hippocampus, which was transferred to a microfuge tube and flash frozen in ethanol with dry ice for immunoblotting.

Biochemical analyses. Immunoblotting was used for quantification of protein levels. Frozen hippocampi were transferred to RIPA buffer containing 10μL/mL Halt protease and phosphatase inhibitor cocktail (ThermoScientific), homogenized for 20 seconds with an IKA Ultra-Turrax T8 homogenizer, centrifuged at 44,000 x *g* for one hour, the supernatant removed and stored at -80°C until immunoblotting. Protein concentrations were estimated using the BioRad DC Lowry-based protein assay.

Western blot. Proteins were prepared for SDS-PAGE using the BioRad Criterion electrophoresis system. Protein concentrations were equalized with RIPA buffer, diluted 1:1 with Laemmli sample buffer (BioRad)/β-Mercaptoethanol prepared at a ratio of 95:5, heated to 100°C for 5 minutes, loaded into wells of Criterion 4-20% Tris/HCl, 1.0mm precast gels and separated by gel electrophoresis for 2 hours at 125mV in BioRad tris/glycine/SDS running buffer. Proteins were then transferred onto PVDF membrane (Immobilon) by western blot at 100mV for 3 hours at 4°C in Towbin buffer (BioRad) with Precision Plus Protein All Blue Standards (BioRad) to identify molecular weights. Membranes were blocked overnight at 4°C in 5% nonfat dried milk with 0.1% Tween20 in TBS and probed with primary antibodies overnight at 4°C in 2.5% nonfat dried milk with 0.05% Tween20 in TBS. Primary antibodies used were rabbit anti-GAPDH (abcam 37168) and mouse anti-calbindin (Swant 300). Membranes were then washed three times

for five minutes each with 0.1% Tween20 in TBS and incubated in secondary antibodies goat anti-mouse and goat anti-rabbit IgG (H+L) HRP conjugated (ThermoScientific) in 2.5% nonfat dried milk with 0.05% Tween20 in TBS for two hours at room temperature. Membranes were washed three times for five minutes each with 0.1% Tween20 and incubated 5 minutes in SuperSignal West Pico chemiluminescent substrate and imaged on a BioRad ChemiDoc XRS+ imaging system and analyzed for quantification with ImageLab software (BioRad). All data were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a loading control.

Dot blot. To determine accuracy of relative protein level quantification by dot blot, neuropeptide Y (NPY) protein (American Peptide Company 60-1-33) was used to derive a standard curve of serial dilutions by dot blot (Figure 2-4). Neuropeptide Y (scrambled) (Tocris 3903) was used as a control for non-specific binding of antibody. To determine protein levels of NPY in hippocampal tissue, protein concentrations were equalized with RIPA buffer and the prepared samples loaded into two adjacent wells of an S&S manifold-I dot-blot apparatus housing a nitrocellulose membrane. The samples were drawn through the apparatus and into the membrane by vacuum and blocked overnight at 4°C in 5% nonfat dried milk 0.1% Tween20 in TBS and probed with primary antibodies overnight at 4°C in 2.5% nonfat dried milk 0.1% Tween20 in TBS. Antibodies used were rabbit anti-NPY (abcam 10980) and mouse anti-MAP2 (Millipore MAB4318). Membranes were washed three times for five minutes each with 0.1% Tween20 in TBS and incubated in secondary antibodies-goat anti-mouse and goat anti-rabbit IgG (H+L) HRP conjugated (ThermoScientific) for two hours at room temperature. Membranes were washed three times for five minutes each with 0.1% Tween20 and incubated 5 minutes in SuperSignal

West Dura chemiluminescent substrate and imaged on a BioRad ChemiDoc XRS+ imaging system and analyzed for quantification with QuantityOne 1-D analysis software (BioRad). All data were normalized to MAP2 adjacent samples.

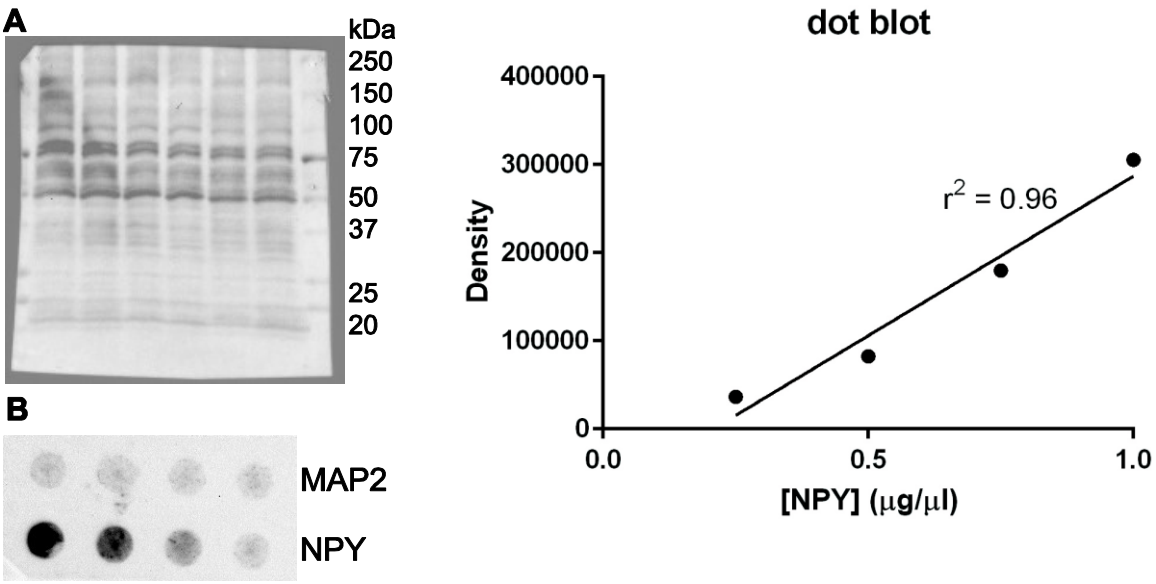

Figure 2-4. NPY protein forms aggregates during gel electrophoresis.
A) Western blot of 11kDa NPY protein.
B) Dot blot of known concentrations of NPY protein. Concentration left to right: 1.0 ug/ul, 0.75 ug/ul, 0.5 ug/ul, 0.25 ug/ul.
C) Standard curve of NPY dot blot in B.

Immunohistochemistry. Free-floating 40μm-thick sections of PFA-fixed mouse brain sections were permeablized and blocked with 5% BSA/0.3% Triton-X/PBS for 1-2 hours and incubated overnight at 4°C with primary antibodies in 2.5% BSA/0.015% Triton-X/PBS. Primary antibodies used were rabbit anti-ZnT3 (Synaptic Systems 197002 and a gift from R. Palmiter), mouse anti-calbindin (Swant 300), rabbit anti-NPY (abcam 10980), mouse anti-GAP-43 (Calbiochem CP09L), rabbit anti-synaptoporin (Synaptic Systems 102002), rabbit anti-MAP2 (abcam 32454), and mouse anti-MAP2 (Millipore MAB4318). The sections were washed three times for five minutes each with PBS and incubated one hour at room temperature with secondary antibody in 2.5% BSA/0.015% Triton-X/PBS.

Secondary antibodies were goat anti-rabbit and goat anti-mouse fluorescent conjugated Alexa 488 and Alexa 594 (Invitrogen). The sections were washed again three times for five minutes each with PBS and incubated in Hoescht 33342 solution (Invitrogen) 1:10,000 in PBS for five minutes, washed again as before and mounted on a microscope slide with Vectashield mounting media (Vector Laboratories) with a microscope cover glass. Slides were allowed to dry for one hour at 37°C and stored at 4°C.

Microscopy. Images were captured with an Axiovert 200 inverted microscope (Zeiss) and processed using AxioVision software (Zeiss) for visualization of calbindin and NPY immunolabeling. An Apotome device (Zeiss) was used for optical "Z" sectioning of multifluorescence signals, then a maximum intensity projection of all z planes was created for analysis of synaptoporin and GAP-43 immunolabeling. This allowed visualization of individual processes through multiple planes of focus.

Image analysis. Calbindin and NPY immunolabeling in specific hippocampal regions was quantified as percent area in two fields from two samples for each condition. Background intensity was normalized between images. Synaptoporin puncta were counted at 63X magnification with three fields imaged for each subject in the molecular layer of the dentate gyrus of ZnT3KO and values expressed as fold change relative to WT puncta for each subject. Puncta were counted if the size and intensity were above a threshold that was determined to be background fluorescence. GAP-43 levels were analyzed in ten fields from each subject imaged at 63X magnification and the percent area of GAP-43 immunofluorescent labeling in each field was quantified using the

Axiovision (Zeiss) automated measurement program and the average percent area calculated.

Behavioral Paradigms

Elevated plus maze. The elevated plus maze consisted of a platform with two open arms and two closed arms, each arm 30cm long and 5 cm in width. The enclosed arms contained walls that were 15 cm in height. The platform was elevated 40 cm above the floor and light in the test room was adjusted to 15 lux. Testing consisted of the animal being placed in the center of the platform and allowed to explore the maze arms for 5 minutes. The testing session was recorded and analyzed using Noldus Ethovision. Total distance traveled and percent of time spent in each arm was analyzed. The maze was cleaned with 70% ethanol in between subjects.

This task is an ethological animal model of anxiety with the aversion to the open arms demonstrated in the task having ecological meaning to the animal in that while rodents spontaneously explore the environment, they also have an aversion to open spaces to reduce risk of predation. Normal behavior results in the animal spending more time in the closed arms than in the open arms, while an animal with heightened anxiety will spend very little time in the open arms. The test is used to assess anxiety in rodents and to test discrimination of anxioselective drugs as a subject with heightened anxiety will enter the open space with much less frequency than the normal counterpart (Wall and Messier, 2001; Carobrez and Bertoglio, 2005). Performance in the EPM task is an important control measure in behavioral tests based on spontaneous exploration patterns of normal subjects, such as object location memory (OLM), as heightened anxiety can

interfere with performance if the subjects have heightened aversion to open spaces, reducing exploration of objects.

Habituation. Habituation is a form of learning defined as a decreased response to repeated stimulation (Thompson and Spencer, 1966). In this case, the stimulation is a novel context, the context used for training and testing in OLM, and the protocol consists of placing the subjects individually into the context for 5 minutes each day for 6 consecutive days and recording the distance traveled for each session. As the subjects become familiar with the new context they will explore it less during subsequent introductions until reaching an asymptotic level (Rankin et al., 2009).

Object location memory. OLM, also referred to as "novel place learning" or "novel object location," is a memory task that assesses rodents' preference for novel objects, in this case the novelty of the object is placement in a different location. Mice were handled for 2 minutes for 5 consecutive days and habituated to the testing arena for 5 minutes for 6 consecutive days, with days 4 and 5 of handling overlapping with days 1 and 2 of habituation. On training day, two identical objects were introduced into the training area, spaced equally apart. Animals were allowed to explore the objects for 10 minutes to become familiarized with the objects and their location. After 24 hours the mice were tested for long-term object location memory by returning the mice to the testing arena, with one of the previous objects placed in the same location and the other object placed in a novel location, allowing the mice to explore the objects. All extra-maze cues in the room were kept constant throughout training and testing. Habituation, training, and testing

were all filmed and analyzed in EthoVision software. Exploration of the objects was scored by two investigators blind to subjects' condition. Time of exploration was determined by the subject being within 1 cm of the object and the elongated line from eyes to nose would intersect the object. The discrimination index was determined by: (DI = ($t$novel – $t$familiar) / ($t$novel + $t$familiar) × 100%). Mice that explored less than 3 seconds during training or testing were excluded from analysis.

Statistical analysis

Data were analyzed by two-tailed unpaired t test with the results expressed as the mean ±SEM, via Graphpad Prism software. Significance was defined at $p<0.05$. Representative immunoblots correspond to individual experiments.

Results

ZnT3KO hippocampus have age-dependent alterations in markers of seizure activity. We assessed biochemical markers of seizure activity using cellular homogenates of hippocampus tissue collected from 2, 3, 6, 12, 13, and 15 month old ZnT3KO and age-matched WT mice and found age-dependent alterations of calbindin and NPY protein levels in ZnT3KO. Calbindin levels were significantly reduced in 12 and 15 month old (Figure 2-5), with decrease in the dentate gyrus, but not in the CA1 regions of the hippocampus (Figure 2-6). NPY was significantly increased in 12 and 15 month old in ZnT3KO hippocampus compared to WT, with abundant immunolabeling in the CA3 region of the hippocampus (F

igure 2-7).

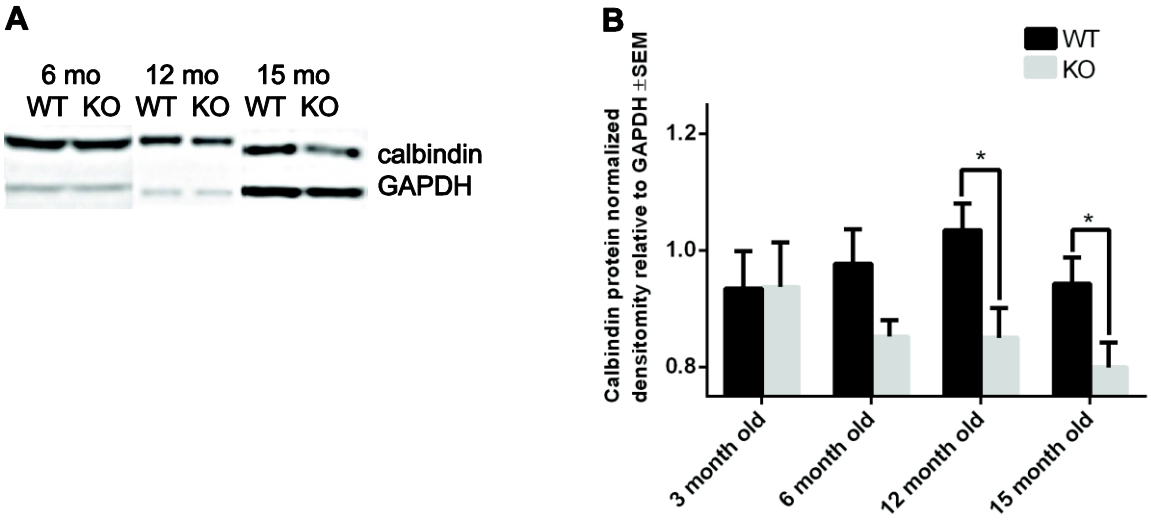

Figure 2-5. ZnT3KO hippocampus have age-dependent reduction in calbindin protein levels.

A) Representative immunoblot analysis of cellular homogenates of hippocampus tissue of WT and ZnT3KO hippocampus tissue prepared as described in Materials and Methods.

B) Calbindin protein levels in WT and ZnT3KO hippocampal tissue.

3 mo. WT 0.93 ± 0.06, n = 5; KO 0.94 ± 0.08, n = 5

6 mo. WT 0.98 ± 0.06, n = 5; KO 0.85 ± 0.03, n = 5

12 mo. WT 1.03 ± 0.05, n = 8; KO 0.85 ± 0.05, n = 8

15 mo. WT 0.94 ± 0.04, n = 7; KO 0.79 ± 0.04, n = 7

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM

*p<0.05. WT, wild type; KO, ZnT3KO.

We assessed a morphological marker of seizure activity, mossy fiber sprouting in the molecular layer of the dentate gyrus, using two different markers and methods of quantification. The percent area of immunolabeling for GAP-43 in ten fields was quantified in 3 and 12 month old (figure 2-8), while synaptoporin-immunolabeled puncta were counted in three fields in 2, 6, 13 and 15 month old (figure 2-9) age-matched WT and ZnT3KO at 63X magnification for both methods, finding that both markers indicated increased sprouting of mossy fibers into the molecular layer in the aged ZnT3KO.

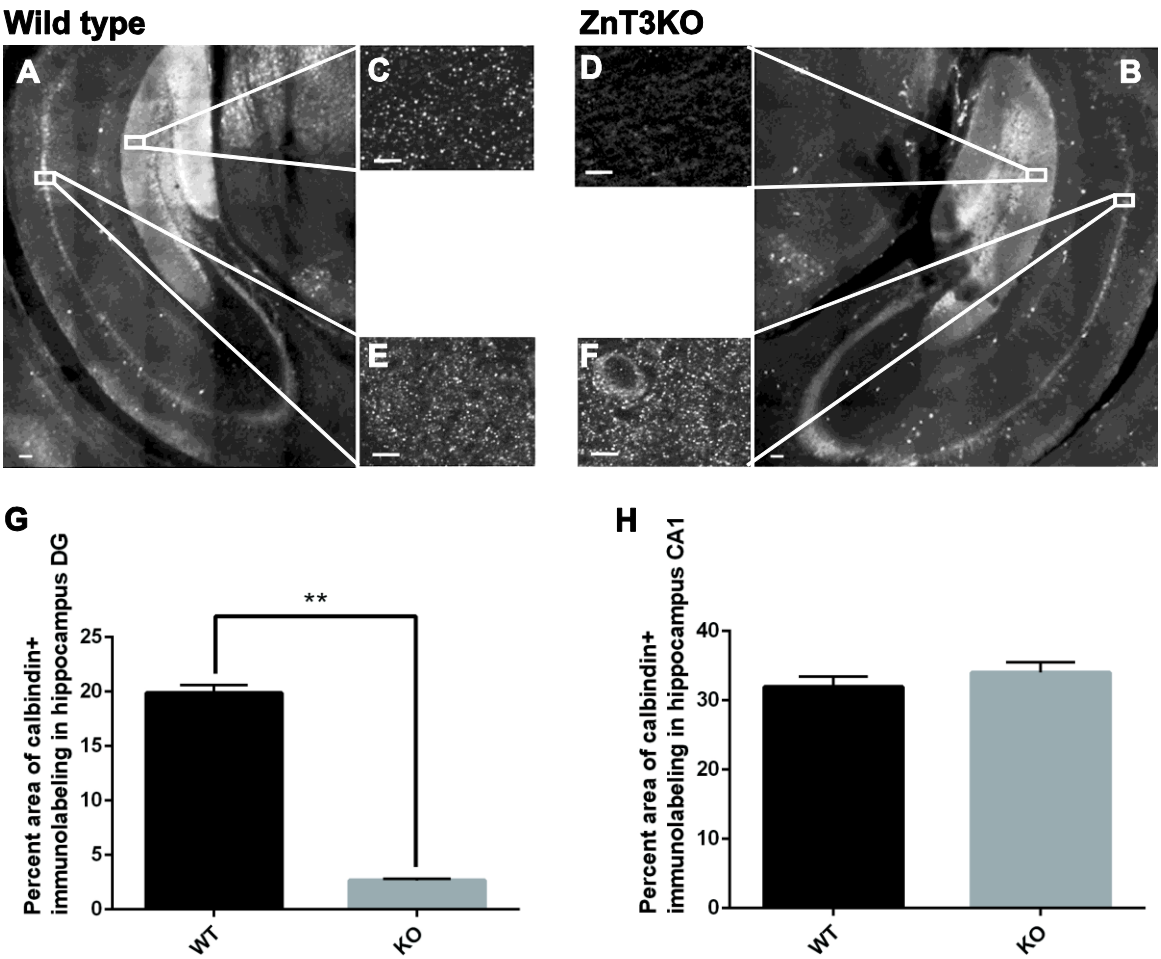

Figure 2-6. Calbindin expression is decreased in the dentate gyrus but not in the CA1 of the hippocampus in ZnT3KO mice.

A and B) Immunostaining of hippocampus sections showing anti-calbindin labeling in 12 month old WT and ZnT3KO, respectively.

C and D) High magnification of the dentate gyrus region indicated in A and B.

E and F) High magnification of the CA1 region indicated in areas in A and B.

G) Percent area of calbindin immunolabeling in dentate gyrus.

WT 19.85 ± 0.75, n = 2; KO 2.62 ± 0.156, n = 2.

H) Percent area of calbindin immunolabeling in CA1.

WT 31.90 ± 1.50, n = 2; KO 33.98 ± 1.49, n = 2.

A and B - scale bars 100μm, C, D, E and F scale bars 10μm. Data were analyzed by two-tailed t test. Error bars indicate the mean ±SEM. **p<0.001.

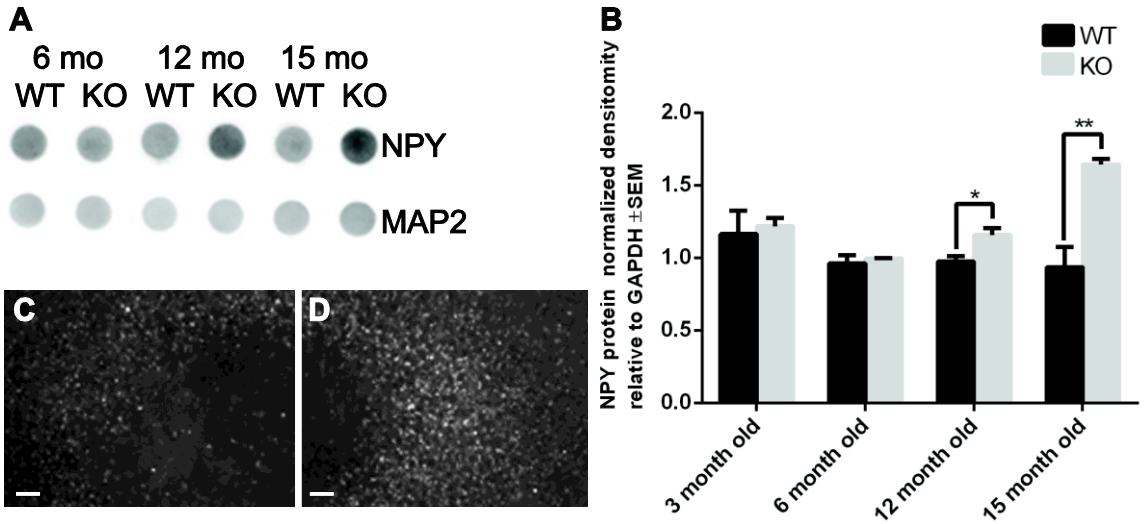

Figure 2-7. ZnT3KO have age-dependent increase in Neuropeptide Y protein levels in the hippocampus.

A) Representative immunoblot analysis of cellular homogenates of hippocampus tissue of WT and KO hippocampus tissue prepared as described in Materials and Methods.

B) NPY protein levels in WT and ZnT3KO hippocampal tissue.

3 mo. WT 1.16 ± 0.16, n = 4; KO 1.21 ± 0.06, n = 4
6 mo. WT 0.96 ± 0.05, n = 3; KO 0.99 ± 0.03, n = 3
12 mo. WT 0.97 ± 0.04, n = 5; KO 1.16 ± 0.06, n = 5
15 mo. WT 0.94 ± 0.1, n = 4; KO 1.64 ± 0.04, n = 4

C and D) Immunostaining of the CA3 region of the hippocampus in 12 mo. WT and KO, respectively, shows abundant anti-NPY labeling in the KO, but not in the WT. Scale bars 10μm.

Values expressed as normalized densitometry relative to MAP2. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. *p<0.05, **p<0.001. WT, wild type; KO, ZnT3KO; NPY, Neuropeptide Y.

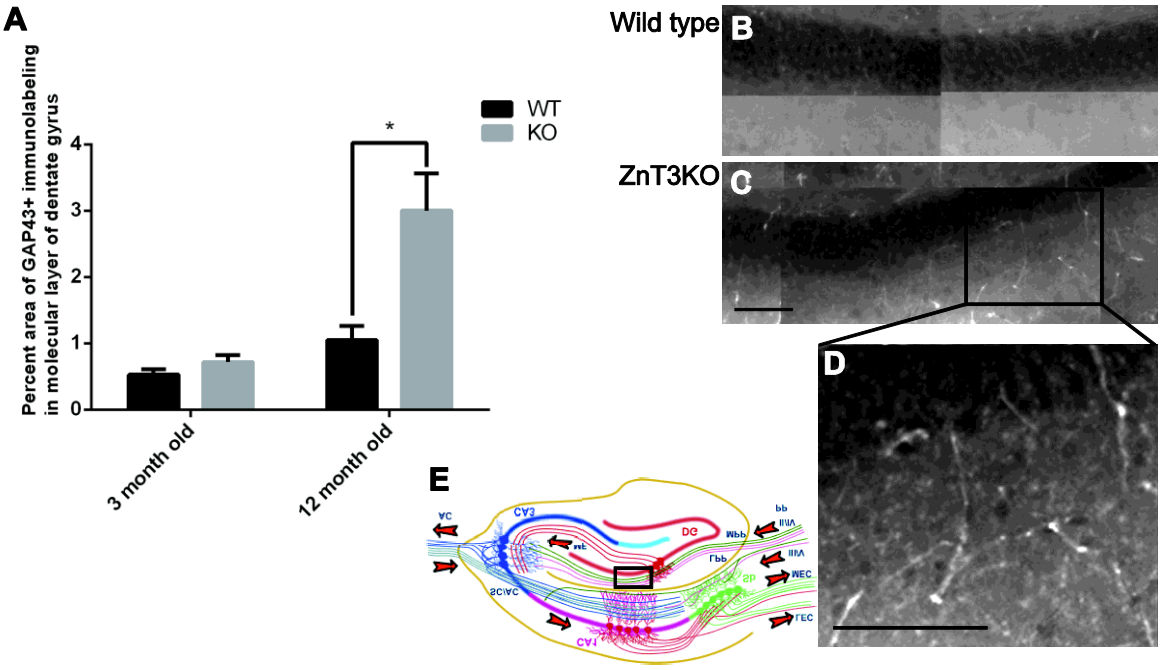

Figure 2-8. GAP43 immunolabeling is increased in aged ZnT3KO hippocampus.

A) Percent area of GAP43 immunolabeling in the molecular layer of the dentate gyrus of the hippocampus.

3 mo. WT 0.6 ± 0.08, n = 5; KO 1.0 ± 0.3, n = 5

12 mo. WT 1.1 ± 0.2, n = 5; KO 3.0 ± 0.5, n = 5

B and C) Immunostaining of molecular layer of dentate gyrus showing anti-GAP43 labeling in 12 month old WT and KO, respectively.

D) Magnification of region indicated in C.

E) Inset box indicated region imaged in B and C.

Scale bars 100µm. Ten fields from each subject was imaged at 63X magnification and the percent area of GAP43 immunofluorescent labeling in each field was quantified using the Axiovision (Zeiss) automated measurement program and the average percent area calculated. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM Error bars indicate the mean ±SEM. *p<0.05. WT, wild type; KO, ZnT3KO.

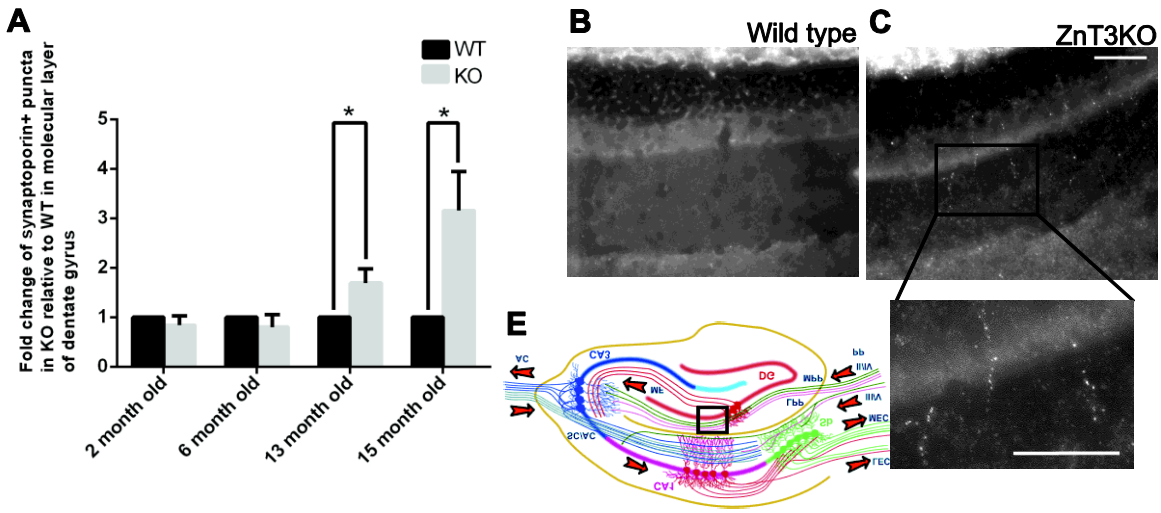

Figure 2-9. Synaptoporin puncta are increased in aged ZnT3KO hippocampus.

A) Quantification of synaptoporin puncta counted in the molecular layer of the dentate gyrus.
- 2 mo. WT n = 6; KO 0.8 ± 0.2, n = 6
- 6 mo. WT n = 6; KO 0.8 ± 0.2, n = 8
- 13 mo. WT n = 6, KO 1.7 ± 0.3, n = 7
- 15 mo. WT n = 9, KO 3.1 ± 0.8, n = 8

B and C) Immunostaining of molecular layer of dentate gyrus showing anti-synaptoporin labeling in 13 month old WT and KO, respectively.
D) Magnification of region indicated in C.
E) Inset box indicated region imaged in B and C.
Scale bars 100µm. Three fields were imaged at 63X and values expressed as fold change relative to wild type puncta for each subject. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. *p<0.05,. WT, wild type; KO, ZnT3KO.

Aged ZnT3KO mice have normal anxiety response. We assessed anxiety response to the elevated plus maze (EPM) in aged ZnT3KO mice. As with previous reports that found no age-dependent impairment in motor function or open field anxiety (Cole et al., 2001; Martel et al., 2011), we found no reports of anxiety response in this task, which can affect performance in behavior tasks based on rodents' exploration patterns. Ratio of time spent in open arm of the EPM versus the time spent in the closed arm in 6 month old subjects shows that aged ZnT3KO have anxiety response comparable to WT (Figure 2-10).

Distance traveled also was similar for both genotypes (data not shown). Consequently, variation in anxiety response would not act as a confound in subsequent cognitive behavioral testing of ZnT3KO.

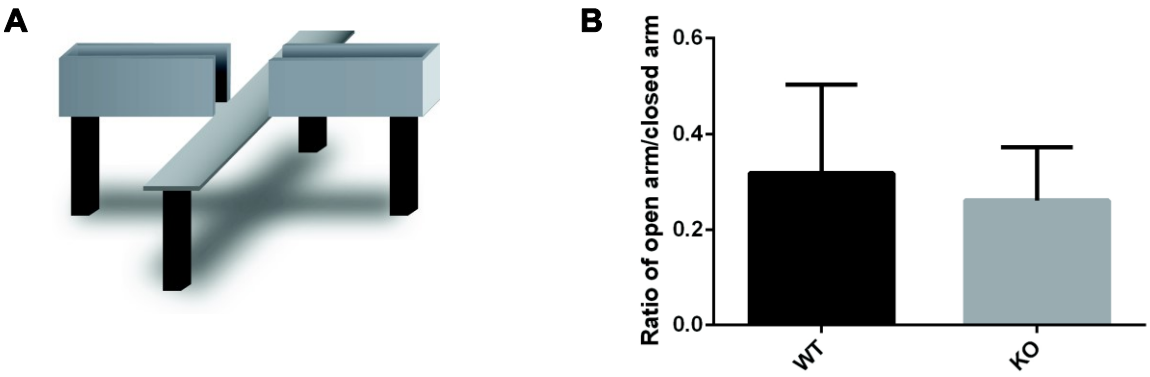

Figure 2-10. Aged ZnT3KO mice have normal anxiety response.
A) The EPM apparatus is used to assess anxiety response in rodents.
B) Ratio of time spent in open arm of the EPM versus the time spent in the closed arm in 6 month old subjects.
WT 0.3 ± 0.1, n = 5; KO 0.3 ± 0.1, n = 10
Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. WT, wild type; KO, ZnT3KO; EPM, elevated plus maze.

Habituation to a novel context is reduced in 6 month old ZnT3KO mice. Habituation to context is part of the OLM protocol to introduce the subjects to the experimental context until they have habituated so that when the novel objects are introduced the subject's response is to the object and not to the context. Habituation measured as distance traveled in each 5 minute session for 6 days of habituation to context (Figure 2-11A) shows that while 6 week old ZnT3KO habituate similarly to age-matched WT (Figure 2-11B), 6 month old ZnT3KO habituate less than age-matched WT (Figure 2-11C), as indicated by the greater distance traveled on day six. Three month old ZnT3KO also habituated similarly to age-matched WT (data not shown). The difference between aged ZnT3KO and WT habituation is most pronounce at the second day of habituation, where distance moved is reduced significantly in WT, but not in ZnT3KO (Figure 2-11D). This

reduced habituation response is consistent with memory impairment as habituation occurs due to the subject demonstrating less exploratory activity as a context becomes familiar.

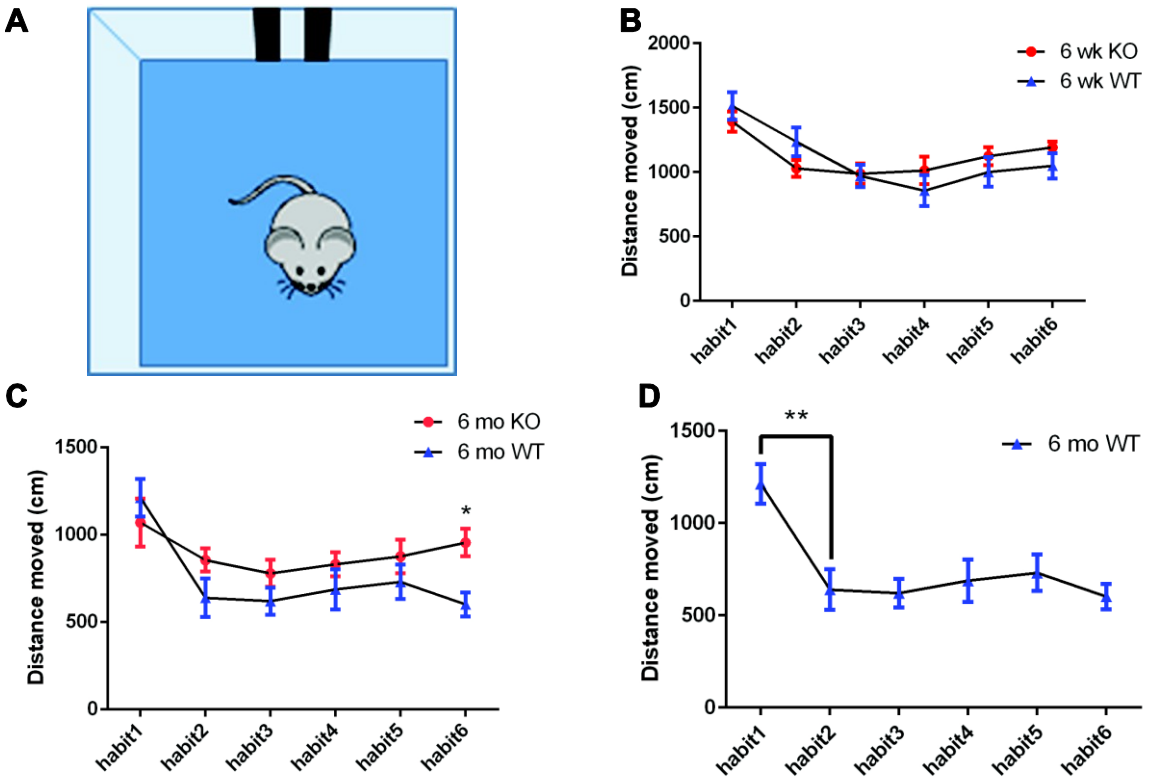

Figure 2-11. Habituation to a novel context is reduced in 6 month old ZnT3KO mice. Habituation measured as distance traveled in each 5 min session for 6 days of habituation to context.
A) Habituation context design.
B) 6 wk old KO habituate similar to WT.
C) 6 mo old KO habituate less than WT.
Habit 6 WT 601.5 ± 69.7, n = 8; KO 955.5 ± 79.0, n = 10)
D) Distance moved is reduced after the first day of habituation in WT
Habit 1 1213 ± 107.5, n = 8, Habit 2 639.6 ± 110.6, n = 8
Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. *p<0.05, **p<0.001. WT, wild type; KO, ZnT3KO.

ZnT3KO mice develop age-dependent cognitive impairment in an object location memory (OLM) task. We assessed age-dependent cognitive impairment in ZnT3KO through a hippocampal-dependent behavioral task. OLM discrimination index shows age-

dependent cognitive impairment of ZnT3KO mice, with 6 week old ZnT3KO performing similar to WT; however, the 3 month old ZnT3KO show impairment compared to age-matched WT, and by 6 months old ZnT3KO perform at chance showing no recognition of the novel object location (Figure 2-12). The lack of preference for investigating the object that was placed in a new location indicates that the aged ZnT3KO have impaired memory for the previous location of the object.

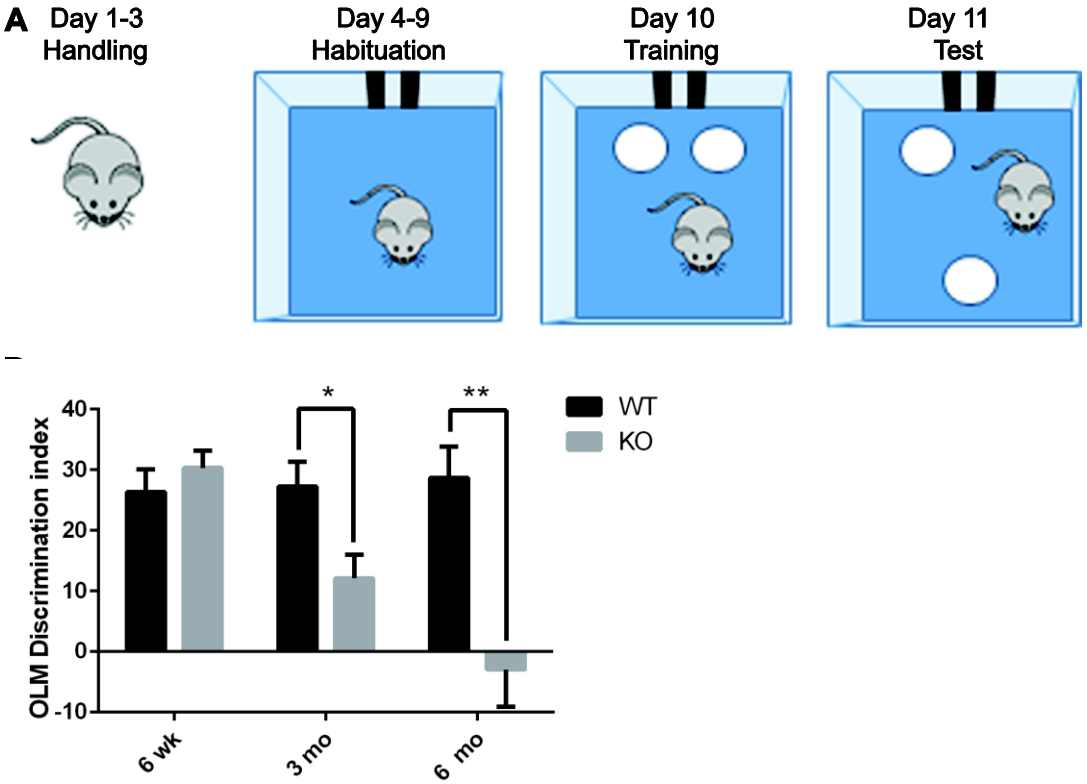

Figure 2-12. ZnT3KO mice develop age-dependent cognitive impairment in an OLM task.

A) Study design of OLM.

B) OLM discrimination index shows age-dependent cognitive impairment of ZnT3KO mice

6 wk. WT 26.3 ± 3.7, n = 13, KO 30.2 ± 2.8, n = 16

3 mo. WT 27.2 ± 4.1, n = 9, KO 12.07 ± 3.9, n = 14

6 mo. WT 28.6 ± 5.1, n = 8, KO -2.9 ± 6.1, n = 14

Values expressed as discrimination index, the time of the (novel - familiar) / (novel + familiar) * 100. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ± SEM. ** $p<0.001$, * $p<0.05$. OLM, object location memory; WT, wild type; KO, ZnT3KO; wk, week; mo, month.

Treatment with an anti-epileptic drug does not reverse cognitive impairment in aged ZnT3KO mice. We explored the effect of an anti-seizure drug, levetiracetam, in the cognitive impairment demonstrated in aged ZnT3KO to determine if seizure activity contributes to the impairment. OLM discrimination index of six month old mice after acute treatment with anti-epileptic drug levetiracetam shows that the treatment did not significantly improve cognition in wild type or ZnT3KO (Figure 2-13).

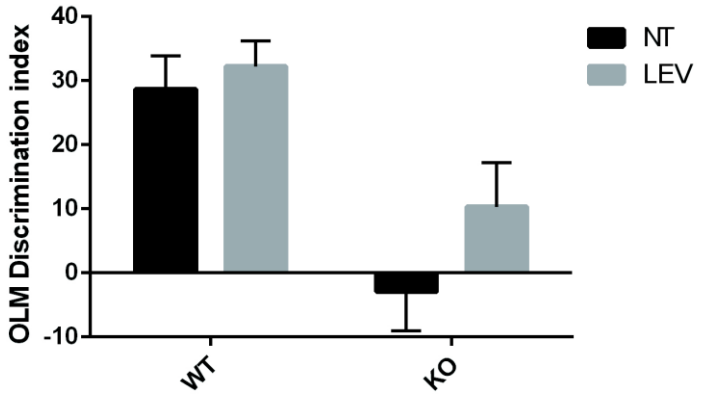

Figure 2-13. Treatment with an anti-epileptic drug does not reverse cognitive impairment in 6 mo. ZnT3KO mice.
A) OLM discrimination index of six month old mice after acute treatment with anti-epileptic drug levetiracetam.
WT: NT 28.6 ± 5.1, n = 8
LEV 32.2 ± 3.9, n = 10
KO: NT -2.9 ± 6.1, n = 14
LEV 10.3 ± 6.8, n = 14
Values expressed as discrimination index, the time of the (novel - familiar) / (novel + familiar) * 100. Data were analyzed by two-tailed unpaired t test. WT, wild type; KO, ZnT3KO; NT, no treatment; LEV, acute injection of 75mg/kg levetiracetam 3.5 hr before OLM training and testing.

Discussion

Previous research found a lower threshold for seizure and EEG spiking after treatment with kainate in ZnT3KO mice, though epileptiform EEG activity was not detected during hour long recordings of young subjects taken days after kainate treatment (Cole et al., 2000), indicating that while spontaneous seizures are not observed in young Znt3KO mice, that either their basal neuronal activity is elevated and close to threshold for seizure or that they have impaired inhibitory response when challenged by excitatory agents. But whichever is the case, the ablation of synaptic zinc results in alterations in hippocampal morphology and biochemical profiles consistent with excessive excitatory activity as the animals age.

We found alterations in protein levels consistent with previous reports of the effects of seizure activity, including reduced calbindin in ZnT3KO hippocampus localized to the dentate gyrus, but not reduced in the CA1 region (Miller and Baimbridge, 1983) (Figure 2-6), increased NPY expression in the hippocampus (Figure 2-7), and mossy fiber sprouting identified through two immunohistochemical labelings, GAP-43 (Figure 2-8) and synaptoporin (Figure 2-9). These alterations may contribute to age-dependent cognitive impairment as the observation that young ZnT3KO mice are phenotypically normal implies that it is not the lack of synaptic zinc that is the direct cause of the impairment but instead contributes indirectly. Both cumulative insults result in neurodegeneration and compensatory mechanisms in young ZnT3KO that become less effective as the animals age, likely contributing to impairment.

Cognition in ZnT3KO has been investigated through several behavioral tasks, with mixed results. Initial phenotyping of 6 week old ZnT3KO found no deficits (Cole et al.,

2001). Adlard and colleagues found no impairment in 3 month old ZnT3KO in the Morris water maze, but significant impairment in 6 month old (Adlard et al., 2010). Martel et al. tested 3-4 month old ZnT3KO, finding normal cognition in a water maze task, but impaired performance in contextual fear conditioning and object recognition (Martel et al., 2011). Impairment in contextual fear conditioning in the same age cohort was also reported in other research (Sindreu et al., 2011). These mixed results indicate that young ZnT3KO have normal cognition but at three months of age their performance begins to decline and becomes sensitive to task type and variations in testing methodology, and by 6 months of age ZnT3KO are profoundly impaired in cognition.

Our investigation into markers of seizure activity did not find significant alterations in ZnT3KO until 12 months of age, though some markers approached significance at 6 months. This strongly suggests that pathological changes are beginning by 6 months, reflected by the impaired cognition demonstrated in both habituation and OLM performance and that excessive excitatory activity may be contributing to the impairment. This research investigated the possibility that excessive excitatory activity contributes to impaired cognition through observing the effects of acute treatment with an anti-seizure drug on performance in a memory task. Improved performance with acute treatment during training and testing would demonstrate that reduction of excessive excitatory activity rescues impaired cognition and that it is the activity contributing to the impairment, while improved performance with long-term treatment would demonstrate that neurodegeneration induced by excessive activity is contributing to the impairment. Acute treatment of six month old ZnT3KO did not result in improvement in cognition. It may be that while the pharmaceutical intervention provided some benefit, the cumulative damage

cannot be overcome to restore normal cognition. Consequently, three month old ZnT3KO will be investigated to determine if their modest impairment can be rescued by acute treatment with an anti-seizure drug.

Investigating the hypothesis that lack of synaptic zinc contributes to seizure activity and cognitive impairment in a transgenic mouse with global ablation of a gene requires consideration of other potential effects of the lack of that gene. ZnT3 is expressed primarily in the brain, but is also found to have roles in retinal and pancreatic function (Petersen et al., 2011; Bai et al., 2013). These functions do not have a direct effect on neuronal activity; however, impaired pancreatic function can contribute to type 2 diabetes-like pathology which may have an age-dependent effect on cognition. Consequently, research into age-dependent alterations of glucose tolerance of ZnT3KO would provide insight into diabetic-induced impairment of cognition and further link disruption in zinc homeostasis to AD pathology as type 2 diabetes is one of the significant risk factors in developing AD (Arvanitakis et al., 2004).

ZnT3 is also expressed in the cerebellum (Wang et al., 2005), and so global ablation could contribute to motor deficits; however, young ZnT3KO demonstrate normal motor function and aged ZnT3KO initially travel the same distance during habituation so it can be inferred that the lack of ZnT3 does not directly contribute to motor deficits that may impair performance in behavioral tasks. ZnT3 and Zn^{2+} are critical for light adaptation in the retina. ZnT3KO mice are more susceptible to retinal damage after exposure to extreme light/dark conditions than WT mice (Bai et al., 2013), which would result in vision impairment likely to interfere with OLM performance. The retinal damage seen by Bai et

al. was light exposure-dependent, not age dependent, and so would not be expected to be present in ZnT3KO housed under normal conditions and light exposure.

In summary, our results demonstrate that interference with zinc neurotransmission through the genetic ablation of synaptic zinc contributes to excessive excitatory neurotransmission. The link between the excessive activity and impaired cognition is yet to be determined.

Chapter 3

Elimination of Synaptic Zinc Alters Neurotrophic Signaling Pathways

Summary

Exogenous zinc has been demonstrated to activate TrkB receptors (Hwang et al., 2005; Huang et al., 2008) and to inhibit NMDA receptors (Legendre and Westbrook, 1990; Erreger and Traynelis, 2008a), both receptors that initiate signaling pathways activating Erk1/2 and AKT (Mao et al., 2004; Minichiello, 2009) and regulate expression of BDNF, an endogenous ligand for the TrkB receptor (Hardingham et al., 2002; Chen et al., 2007; Yasuda et al., 2007). These findings suggest a hypothesis that elimination of synaptic zinc will alter activity-dependent phosphorylation of AKT and Erk1/2 and expression of BDNF, which are critical for neuronal health, synaptic plasticity and cognition (Ghosh et al., 1994; Croll et al., 1998; Hardingham et al., 2002; Gomez-Palacio-Schjetnan and Escobar, 2008). This hypothesis was investigated through chemical stimulation of neurotransmission in acute hippocampus slices and assaying levels of phosphorylated signaling proteins and biochemical analysis of BDNF protein and mRNA expression from ZnT3KO compared to WT mice. The hippocampus was targeted because the mossy fiber tract of the hippocampus is enriched with synaptic zinc (Palmiter et al., 1996), is a brain region where AD pathology first appears (Braak and Braak, 1991) and is critical for memory (Milner, 1972).

Introduction

Investigating the role of zinc in neurotransmission

The effects of exogenous zinc on transporters and postsynaptic responses elicited by many receptors have been studied through the addition of Zn^{2+} or zinc chelators to the incubation media. These studies typically utilized oocytes, cell lines or tissue culture, usually of primary neurons, and focused on response of one transporter or receptor type (Westbrook and Mayer, 1987; Smart and Constanti, 1990; Laube et al., 2000; Cohen-Kfir et al., 2005; Hwang et al., 2005; Erreger and Traynelis, 2008a; Huang et al., 2008). These studies demonstrated that zinc does bind and alter responses to various receptors and transporters, but the effects of endogenously available zinc in neurotransmission in the hippocampus are not well understood.

NMDA receptors

The NMDA receptor is a heteromeric assembly of two NR1 and two NR2 or NR3 subunits, with four variants of NR2 subunits, NR2A, NR2B, NR2C and NR2D, widely expressed in adult brain both at synapses and extrasynaptic sites (Figure 3-1). Synaptic NMDARs are primarily comprised of two NR2A or NR2A/NR2B subunits, while extrasynaptic NMDARs primarily contain NR2B subunits. (Cull-Candy and Leszkiewicz, 2004). NMDA receptor localization determines the route of Ca^{2+} entry and subsequent influences on differential activation of downstream signaling pathways (Cull-Candy and Leszkiewicz, 2004; Kohr, 2006), with synaptic and extrasynaptic NMDARs demonstrating opposing effects; synaptic NMDARS promote cell survival and plasticity through stimulating CREB, BDNF and Erk expression and pathways and also promoting LTP, while extrasynaptic NMDARs

promote apoptosis and inactivate pathways associated with plasticity (Figure 3-2), (Hardingham et al., 2002; Vanhoutte and Bading, 2003; Ivanov et al., 2006; Mulholland et al., 2008) and also trigger long-term depression (LTD) (Massey et al., 2004).

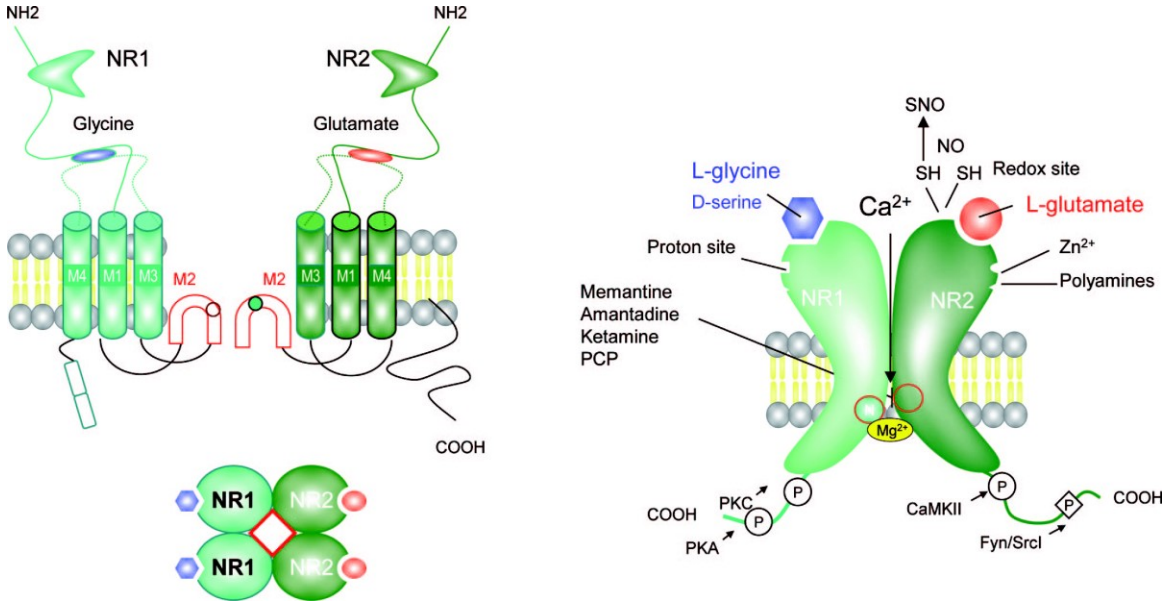

Figure 3-1. Structure, gating, and allosteric modulation of NMDA receptors (NMDARs). The NMDARs are heteromeric complexes composed of 4 subunits derived from 3 related families: NR1, NR2, and NR3. The typical NMDAR requires 2 NR1 subunits, which bind glycine, and 2 NR2 subunits, which bind glutamate. The typical NR1/NR2 receptors are permeable to Ca^{2+} and their activation requires binding of 2 molecules of glutamate to the NR2 and 2 molecules of glycine (or d-serine) to the NR1 subunit. Several modulatory sites affect the function of the NMDAR channels. These include the polyamine site, Zn^{2+} site, proton-sensitive site, and a redox modulatory site. Adapted from Benarroch, E., 2011, *Neurology*.

Zinc inhibits NMDARs, decreasing mean single-channel open duration, open probability and conductance (Legendre and Westbrook, 1990; Erreger and Traynelis, 2008b) and also inhibits NMDAR excitatory postsynaptic potentials (EPSPs) in CA1 and CA3 (Vogt et al., 2000; Izumi et al., 2006). Zinc has differential modulation of NMDARs depending on subunit composition and zinc concentration. Micromolar concentrations of

zinc inhibit LTD through inhibition of NR2B receptors, while higher concentrations inhibit LTP through inhibition of both NR2A and NR2B, and nanomolar concentrations of zinc promote LTP by inhibiting untimely NR2A activation (Izumi et al., 2006). Voltage-dependent inhibition by zinc is similar in both NR2A and NR2B subunits, while voltage-independent inhibition of the NR2B subunit requires higher zinc concentrations than does inhibition of the NR2A subunit (Paoletti et al., 1997), indicating differential inhibitory levels as a mechanism for regulation of activity.

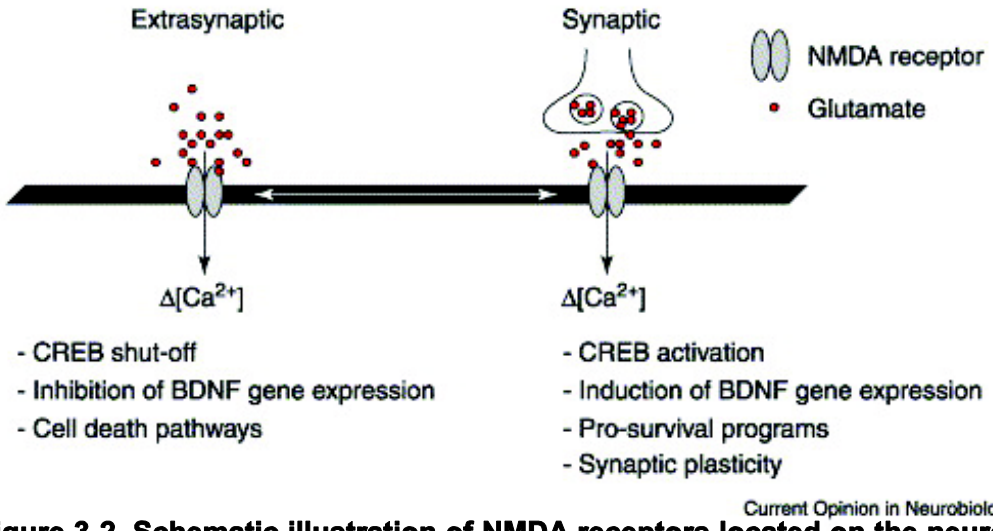

Figure 3-2. Schematic illustration of NMDA receptors located on the neuron surface at synaptic and extrasynaptic locations. Lateral receptor mobility is indicated by an arrow. NMDA receptors are glutamate and voltage-gated calcium permeable ion channels; activation of either synaptic or extrasynaptic receptors causes changes in the intracellular calcium concentration (indicated as Δ[Ca2+]). The biological responses of NMDA receptor-induced calcium signals are specified by the location of the receptor activated, with synaptic and extrasynaptic NMDA receptors having directly opposing actions on CREB function, BDNF gene regulation and neuronal survival. Adapted from Vanhoutte and Bading, 2003,Curr. Opin. Neurobiol.

The TrkB receptor

The TrkB receptor has a role in the survival of cortical neurons (Ghosh et al., 1994), hippocampus-mediated learning and memory and synaptic plasticity (Levine et al., 1995; Minichiello et al., 1999; Liu et al., 2008) (Figure 3-3). TrkB is up-regulated and targeted to dendrites by neuronal activity (Tongiorgi et al., 1997; Righi et al., 2000), has been localized with glutamatergic and GABAergic synapses in the hippocampus (Swanwick et al., 2004) and is activated by BDNF and neurotrophin 4 (NT4) or through indirect activation by G protein-coupled receptors (GPCRs) in the hippocampus (Lee et al., 2002).

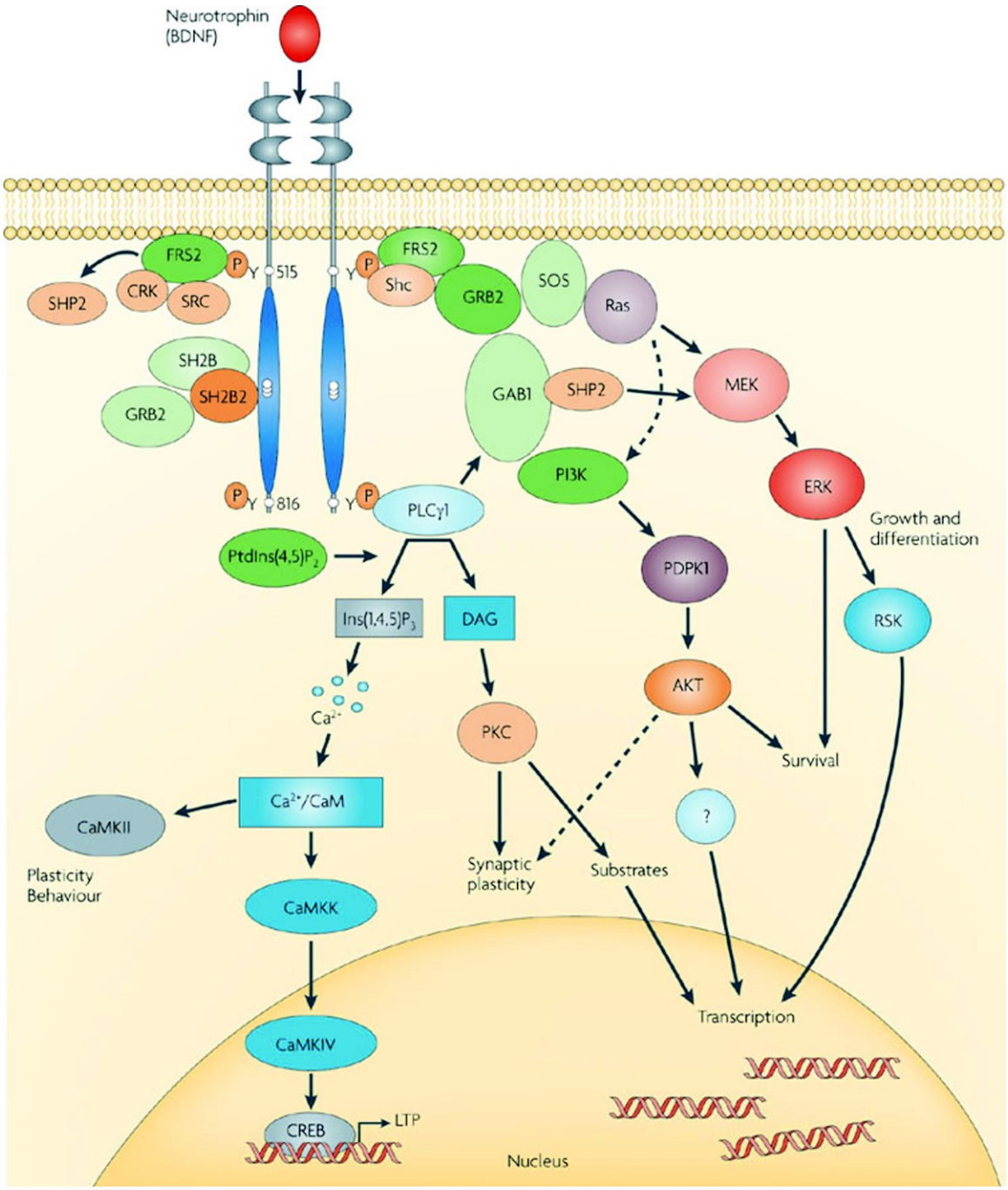

Figure 3-3. The interaction between the receptor tyrosine kinase TrkB and neurotrophins activates three main intracellular signaling pathways. Phosphorylation and recruitment of adaptors to Y515 leads to activation of the Ras-mitogen-activated protein kinase (MAPK) signaling cascade, which promotes neuronal differentiation and growth through MAPK/Erk kinase (MEK) and extracellular signal-regulated kinase (Erk), and leads to activation of the phosphatidylinositol 3-kinase (PI3K) cascade, which promotes survival and growth of neurons and other cells through Ras or GRB-associated binder1 (GAB1). Adapted from Minichiello 2009 *Nature Reviews Neuroscience*.

More recently it has been shown that TrkB can be activated by exogenous zinc (Hwang et al., 2005; Huang et al., 2008), subsequently activating downstream TrkB signaling cascades, including Erk and AKT (Huang et al., 2008). TrkB receptors are activated in neuronal cultures incubated with exogenous zinc; however, there are conflicting reports on the role of BDNF in the process. Pre-incubation with a function-blocking BDNF antibody prevented zinc-mediated phosphorylation of TrkB while incubation with exogenous zinc increased pro-BDNF levels and TrkB activation (Hwang et al., 2005); however, scavenging BDNF with TrkB-Ig had no effect on phospho-TrkB levels after zinc incubation (Huang et al., 2008). Significantly, the latter study also found that stimulation with KCl and blockade of $GABA_A$ receptors enhanced zinc-mediated phosphorylation of TrkB, suggesting zinc-induced activation of TrkB may be regulated by synaptic activity.

BDNF

BDNF is critical for neuronal survival (Ghosh et al., 1994) and mediates many synaptic functions, including depression of hippocampus mossy fiber-induced NMDAR-mediated excitatory post-synaptic currents (Li et al., 2010) and synaptic plasticity in the hippocampus (Gomez-Palacio-Schjetnan and Escobar, 2008).

There is significant reduction of BDNF in the brain and hippocampus of AD patients (Phillips et al., 1991; Connor et al., 1997), with the amount of AβO in the brain of mouse models of AD having a negative correlation with BDNF expression (Peng et al., 2009). It has also been demonstrated that AβO impair BDNF trafficking (Poon et al., 2011) and

increasing BDNF levels improves cognition in mouse models of AD (Blurton-Jones et al., 2009; Caccamo et al., 2010).

There is also a link between zinc, reduced BDNF and AD symptoms. Reduction of BDNF has been proposed as a contributing factor to the depression commonly found in AD patients (Tsai, 2003), demonstrated by experiments treating rats with soluble Aβ and finding the treatment induces a depressive state (Colaianna et al., 2010). Dysregulation of zinc has long been implicated in mood disorders, primarily depression (Levenson, 2006), with dietary zinc supplementation of SSRI therapies improving outcomes in clinical trials of patients with depression (Nowak et al., 2003; Ranjbar et al., 2013), with the anti-depressive effects of zinc including increased expression of BDNF and phosphorylation of Erk (Nowak et al., 2004; Franco et al., 2008).

Zinc promotes BDNF production as incubation of neuronal cultures with exogenous zinc increases release of pro-BDNF and conversion to mature BDNF (Hwang et al., 2005), ZnT3KO mice have decreased hippocampal pro-BDNF (Adlard et al., 2010) and dietary supplementation of zinc in transgenic mouse models of AD increases BDNF levels (Corona et al., 2010). Adlard et al. found mixed results, with decreased pro-BDNF and no difference in BDNF levels in three month old ZnT3KO compared to wild-type mice; however, at six month old the difference in BDNF levels approached significance, indicating a possible age-dependent alterations of BDNF levels. Older mice were not included in the Adlard study.

Erk1/2

The Ras/Raf/MEK/Erk signaling pathway has been demonstrated to regulate many different cellular functions, depending on the strength and timing of activation by effector molecules, including neurotrophins and Ca^{2+} entry into cells (Grewal et al., 1999). Erk (extracellular signal-regulated kinase) has two primary isoforms in neurons, p44 MAPK and p42 MAPK (mitogen-activated protein kinase), which are nearly 85% identical with the same phosphorylation site motif and are commonly referred to as Erk1/2. MAP kinase signaling cascades have overlapping substrate specificities and can form complexes that impact targets and localization to integrate responses and modulate outputs (Figure 3-4) (Pearson et al., 2001).

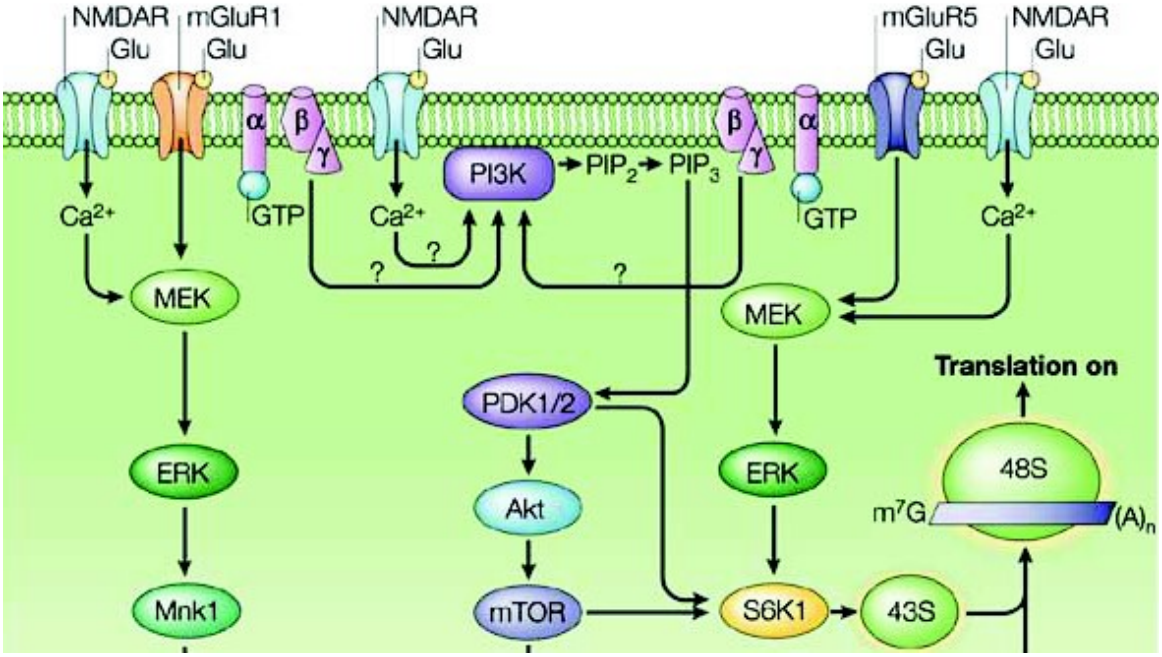

Figure 3-4. Activation of group I metabotropic glutamate receptors (mGluRs) and N-methyl-D-asparate receptors (NMDARs) activate the mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways. Sequential activation of PI3K, phosphoinositide-dependent kinase 1 or 2 (PDK1/2), Akt and mammalian target of rapamycin (mTOR) results in activation of S6 kinase 1 (S6K1). Adapted from Klann & Dever, 2004, *Nature Reviews Neuroscience*.

AKT

The PI3K/AKT (PI3K/PKB) signaling pathway is a regulator of many cellular processes, including metabolism, cell cycle and apoptosis, and is activated by growth factors through receptor tyrosine kinases (Kandel and Hay, 1999). This pathway is highly conserved from *drosophila* to human and is activated through a multistep process with transient regulation by AKT-binding proteins (Fayard et al., 2005). Among more than fifty other substrates, AKT activates GS3K, BAD and FoxO, which are regulators of glucose metabolism, apoptosis, and transcription, respectively (Hanada et al., 2004).

Fluorojade labeling

The identification of degenerating neurons in tissue samples is critical in the study of neurodegenerative diseases and insults, consequently several methods have been developed with varying degrees of success due to equivocal labeling results or difficulty in processing. One method is fluorojade labeling, a derivative of anionic fluorescein, with which experiments using various methods of known neurodegenerative characteristics and co-labeling with other markers of neurodegeneration have demonstrated a high degree of specificity (Schmued and Hopkins, 2000; Zuch et al., 2000).

Significance

These experiments were designed to study the effects of endogenous zinc released at the synapse during neurotransmission through stimulation of vesicle release by the addition of potassium chloride (KCl) to the incubation medium, a method that depolarizes the tissue, inducing Ca^{2+} entry into the axon terminal and triggering release of neurotransmitters (Dudel et al., 1983). This method was chosen because it stimulates vesicle release producing excitatory postsynaptic potentials similar to that produced by electrical stimulation (Baker et al., 1973). The tissues used in this study were acute hippocampus slices as this method preserves the cytoarchitecture of a brain region enhanced with synaptic zinc and preliminary research found that ZnT3 protein expression is sparse in synapses of mature cultures of dissociated hippocampal neurons (results not shown), and thus would have minimal quantities of zinc transported into synaptic vesicles in WT control samples. It is intended that the results of these experiments will provide more understanding of the neuronal signaling effects elicited by removal of synaptic zinc

in a systemic manner to demonstrate the physiological relevance of zinc neurotransmission in brain function.

As previously noted in Chapter 2, young ZnT3KO mice are phenotypically normal, indicating that the lack of synaptic zinc is not the direct cause of the cognitive impairment in ZnT3KO. This suggests the possibility that either the neurodegenerative effects of the lack of synaptic zinc are cumulative or there may be compensatory mechanisms in young ZnT3KO that become less effective as the animals age.

Finding altered NMDAR or TrkB responses and reduced BDNF expression in hippocampus slices from 3-4 week old ZnT3KO, before cognition becomes impaired, indicates that the altered responses do not directly cause impairment of cognition. Instead, such findings would suggest that it is the cumulative effects of these altered responses, in combination with effects from excessive excitatory activity, that contribute to neurodegeneration, resulting in impaired synaptic function, neuronal health and cognition.

These possible effects of AβO interference with zinc neurotransmission resulting in reduced capacity to survive later insults, combined with observations that Zn^{2+} induces aggregation of Aβ (Bush et al., 1994b) and binding of Zn^{2+} to Aβ reduces clearance of Aβ (Bush et al., 1994a) and increases Aβ load, indicate that Zn^{2+} has a significant role in the pathological progression of AD. Identifying specific effects of interference with Zn^{2+} neurotransmission will contribute to a broader understanding of the mechanisms responsible for the neurodegeneration that is characteristic of AD.

Methods

Preparation of hippocampal slices. Three to four week old 129Sv WT or ZnT3KO mice were used to prepare acute hippocampal slices. Animals were anesthetized with 5% isoflurane and decapitated, their brains rapidly removed and rinsed with ice-cold artificial CSF (ACSF). The hippocampus was dissected out and sliced into 350μm thick sections with a chilled Stoelting tissue chopper and transferred with a glass pipet to individual wells in a 48-well plate containing cold ACSF. The ACSF was replaced with 500μL 37°C Neurobasal medium and stabilized at 37°C with 5% CO_2 for one hour before experimental procedures. Slices from two subjects were pooled for each experiment to result in an equal number of slices for each condition in the experiment. Conditions were vehicle (no treatment) and incubation with KCl or with KCl and antagonist.

Chemical modulation of neuronal activity. Hippocampal slices were incubated with KCl (20mM, Sigma). The following drugs were used to modulate NMDA receptor activity:

Table 3-1. Receptor antagonists.

Function	Chemical name	Name	[μM]	Supplier
NMDAR antagonist	D-(-)-2-Amino-5-phosphonopentanoic acid	D-AP5	50	Tocris
NR2A antagonist	[(R)-[(S)-1-(4-bromo-phenyl)-ethylamino]-(2,3-dioxo-1,2,3,4-tetrahydroquinoxalin-5-yl)-methyl]-phosphonic acid	PEAQX (NVP-AAM077)	10	Sigma
NR2B antagonist	4-[2-[4-(cyclohexylmethyl)-1-piperidinyl]-1-hydroxypropyl]phenol	ifenprodil	50	Sigma

Drugs were reconstituted with sterile H_2O and diluted in Neurobasal.

Quantification of protein levels after chemical modulation. Hippocampal slices were transferred to RIPA buffer containing 10μL/mL Halt protease and phosphatase inhibitor cocktail (ThermoScientific), homogenized for 20 seconds with an IKA Ultra-Turrax T8 homogenizer, centrifuged at 14,000 x *g* for one hour, the supernatant removed and stored at -80°C until immunoblotting.

Western blot. Proteins were prepared for SDS-PAGE using the BioRad Criterion electrophoresis system. Protein concentrations were equalized with RIPA buffer, diluted 1:1 with Laemmli sample buffer (BioRad)/β-Mercaptoethanol prepared at a ratio of 95:5, heated to 100°C for 5 minutes, loaded into wells of Criterion 4-20% Tris/HCl, 1.0mm precast gels and separated by gel electrophoresis for 2 hours at 125mV in BioRad tris/glycine/SDS running buffer. Proteins were then transferred onto PVDF membrane (Immobilon) by western blot at 100mV for 3 hours at 4°C in Towbin buffer (BioRad) with Precision Plus Protein All Blue Standards (BioRad) to identify molecular weights. Membranes were blocked overnight at 4°C in 5% nonfat dried milk or 5% BSA, both with 0.1% Tween20, in TBS and probed with primary antibodies overnight at 4°C in 2.5% nonfat dried milk or 2.5% BSA, both with 0.05% Tween20, in TBS. Primary antibodies used were mouse anti-GAPDH (abcam 125247), rabbit anti-GAPDH (abcam 37168), rabbit anti-BDNF (Santa Cruz Biotechnology sc-546), rabbit anti-MAPK (Erk1/2) (Cell Signaling Technology 4695), mouse anti-phospho-p44/42 MAPK (Erk1/2) (Cell Signaling Technology 9106), rabbit anti AKT (pan) (Cell Signaling Technology 4691), and rabbit anti-phospho-AKT (Cell Signaling Technology 2965). Membranes were then washed three times for five minutes each with 0.1% Tween20 in TBS and incubated in secondary antibodies goat anti-mouse and goat anti-rabbit IgG (H+L) HRP conjugated (ThermoScientific) in 2.5% nonfat dried milk or 2.5% BSA, both with 0.05% Tween20, in TBS for two hours at room temperature. Membranes were washed three times for five minutes each with 0.1% Tween20 in TBS and incubated 5 minutes in SuperSignal West Femto, Dura or Pico chemiluminescent substrate, imaged on a BioRad ChemiDoc XRS+ imaging system and analyzed for quantification with ImageLab software (BioRad). All data

were normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a loading control.

BDNF mRNA levels after depolarization

Extraction of RNA from tissue and purification. Hippocampal slices were prepared as previously described for chemical modulation and incubated in 20mM KCl or vehicle for 3.5 hours at 37°C and 5% CO_2, transferred to 1 mL of Trizol (Invitrogen 15596-026) and homogenized for 20 seconds with an IKA Ultra-Turrax T8 homogenizer. RNA was isolated using the Trizol method (Invitrogen) and stored at -80°C until purification using a Qiagen RNeasy mini-kit (Qiagen 74104) with on-column DNase I digestion according to manufacturer's protocol. RNA concentrations and purity were measured using a NanoDrop 2000 spectrophotometer (ThermoScientific) and analyzed using NanoDrop software (ThermoScientific).

RT-PCR and qPCR. cDNA was prepared from up to 1.0 µg RNA using the BioRad iScript Reverse Transcription (RT) Supermix system (BioRad 170-8840) and RT-PCR was performed with a PCR Sprint thermalcycler using the BioRad iScript RT protocol. The resulting cDNA was diluted 1:5 in DNAse/RNAse free H_20 and used for qPCR by the SYBR green method (BioRad 170-8880) with a BioRad DNAengine Peltier thermalcycler using the iQ SYBR green protocol. Primer sequences (Eurofins MWG Operon) used were:

TCGTTCCTTTCGAGTTAGCC (mBDNFexS)

TTGGTAAACGGCACAAAAC (mBDNFexAS)

AGGTATCCTGACCCTGAAG (mActin S)

GCTCATTGTAGAAGGTGTGG (mActin AS)

The resulting data were analyzed using MJ Opticon Monitor (BioRad) and mRNA expression was measured with satisfactory reproducibility between triplicates, and fold change relative to average ΔC_t was calculated as $2^{-\Delta\Delta Ct}$ and normalized to actin.

Fluorojade Labeling. 40µm PFA-fixed mouse brain sections were mounted on microscope slides with 0.5% gelatin (Sigma) mounting solution and dried for 5 minutes at 45°C. The slides were then immersed in 100% ethanol for 5 minutes, 70% ethanol for 2 minutes and then rinsed in deionized water for 2 minutes. Slides were then immersed in a solution of 0.06% $KMnO_4$ (Sigma) for 10 minutes with gentle shaking and rinsed in deionized water for two minutes. Slides were then immersed in a solution of 0.0004% Fluorojade B (Millipore) in 0.1% acetic acid for 20 minutes with gentle shaking in the dark, rinsed in deionized water three times for one minute each time and dried at 45°C for 10 minutes. Slides were then immersed in xylene for three changes of one minute each and coverslipped with DPX mountant (Sigma).

Microscopy. Mosiac (tiled) images of the CA3 region of the hippocampus were captured at 20X with an Axiovert 200 inverted microscope (Zeiss) and processed using AxioVision software (Zeiss).

Image analysis. Background intensity was normalized between samples and Fluorojade puncta were marked if the size and intensity were above a threshold that was determined

to be background fluorescence and counted as positive labeling if the Fluorojade puncta was co-localized with Hoescht staining to eliminate non-specific Fluorojade label.

Statistical analysis

Data were analyzed by two-tailed unpaired t test with the results expressed as the mean ±SEM with Graphpad Prism software. Significance was assessed at $p<0.05$. Representative immunoblots correspond to individual experiments.

Results

To determine if the release of synaptic zinc has a role in activation of proteins in the NMDAR and TrkB signaling pathways, acute hippocampal slices were incubated in 20mM KCl to stimulate vesicle release (as described under Methods) and the changes in levels of two phosphorylated proteins, AKT and Erk1/2, found in both signaling pathways were compared to vehicle-treated slices.

AKT and p-AKT levels are reduced in ZnT3KO hippocampus at basal conditions; however, depolarization with KCl did not increase p-AKT significantly in either WT or ZnT3KO. Western blot analysis of cellular homogenates of the slices show reduced AKT and p-AKT at basal conditions in ZnT3KO compared to WT (Figure 3-5A). While AKT protein levels are not altered by 10 minute incubation with 20mM KCl in ZnT3KO or WT (Figure 3-5B), p-AKT protein levels increased, but not significantly (Figure 3-5C). The percent change in p-AKT after depolarization with 20mM KCl in ZnTKO compared to WT is not significant (Figure 3-5D). These results indicate that while release of synaptic zinc does not have a significant role in activity-dependent phosphorylation of AKT, the lack of synaptic zinc reduces expression of AKT.

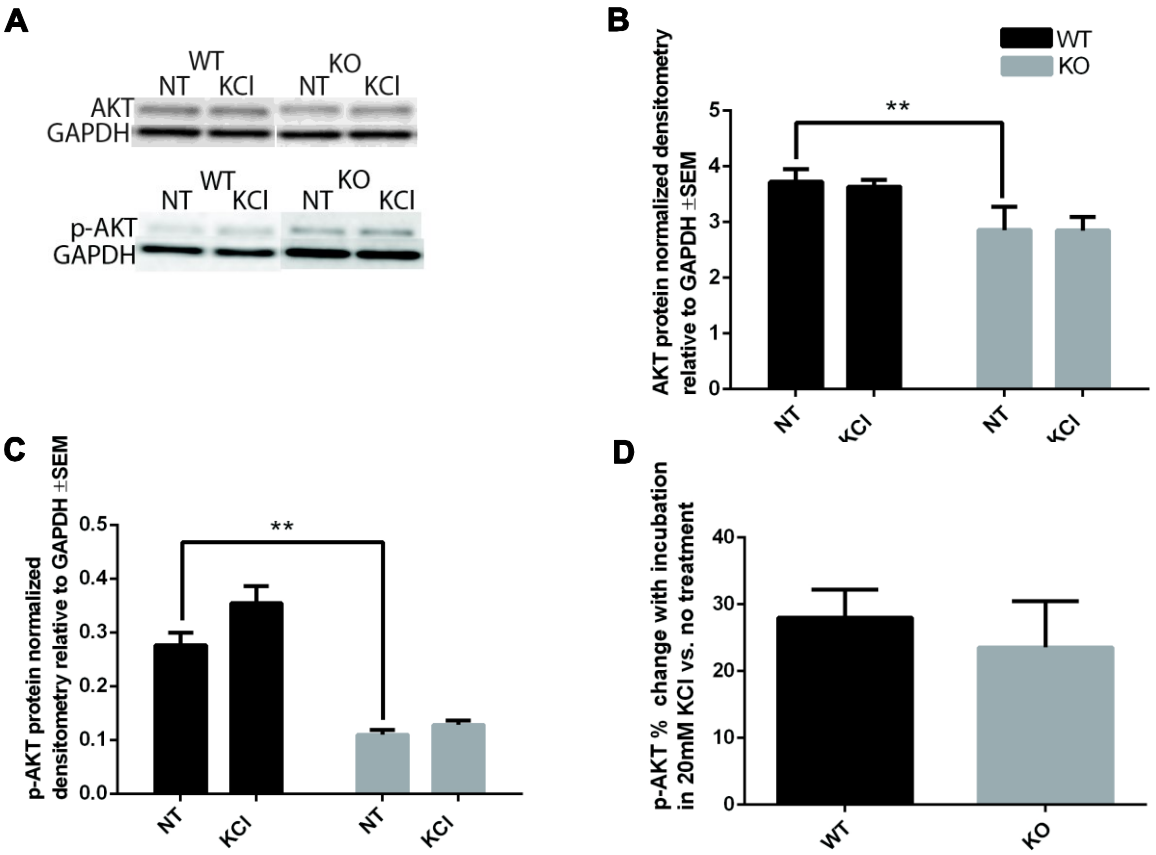

Figure 3-5. AKT and p-AKT levels are reduced in ZnT3KO hippocampus.

A) Representative image of immunoblot analysis of cellular homogenates of acute hippocampus slices with no treatment or with 10 minute incubation in 20mM KCl.

B) AKT protein levels are reduced in KO, but AKT protein levels are not altered by depolarization with 20mM KCl in WT or KO.
WT 3.7 ± 0.2, n = 6 (NT); 3.6 ± 0.1, n = 5 (KCl)
KO 2.7 ± 0.1 (NT), 2.8 ± 0.1 (KCl), n = 6

C) p-AKT protein levels are reduced in KO, but not altered significantly by depolarization with 20mM KCl in WT or KO.
WT 0.27 ± 0.02, n = 5 (NT), 0.35 ± 0.03, n = 5 (KCl)
KO 0.11 ± 0.008, n = 5 (NT), 0.12 ± 0.008, n = 5 (KCl)

D) The percent change in p-AKT after depolarization with 20mM KCl in KO compared to WT is not significant.
WT 27.97% ± 4.23, n = 5,
KO 23.53% ± 6.93, n = 5, p=0.6

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. ** p<0.01. WT, wild type; KO, ZnT3KO; NT, no treatment; KCl, 10 min incubation in 20mM KCl.

Erk1/2 and p-Erk1/2 levels under basal conditions and the increase in p-Erk1/2 after depolarization with KCl are reduced in ZnT3KO hippocampus. Western blot analysis of cellular homogenates of acute hippocampal slices show Erk1/2 protein levels are not altered by depolarization with 20mM KCl in WT or ZnT3KO, (Figure 3-6B); however Erk1/2 protein levels are reduced in ZnT3KO compared to WT under basal conditions (Figure 3-6B). While p-Erk1/2 protein levels after 10 minute incubation with 20mM KCl increased in ZnT3KO and WT (Figure 3-6C), the percent increase in p-Erk1/2 is less in ZnT3KO (Figure 3-6D). These results indicate that the release of synaptic zinc has a role in regulation of Erk1/2 expression and activation.

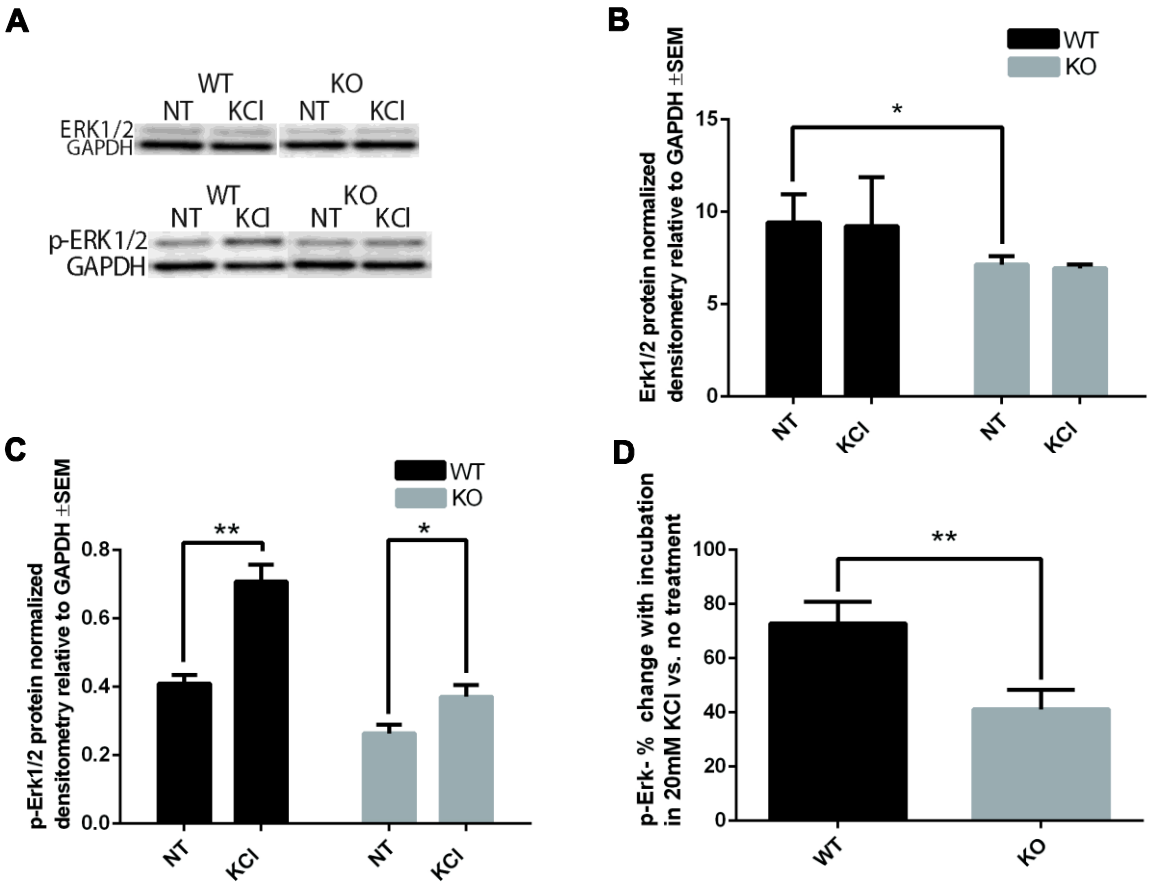

Figure 3-6. Erk1/2 and p-Erk1/2 levels are reduced in ZnT3KO hippocampus.

A) Representative image of immunoblot analysis of cellular homogenates of acute hippocampus slices with no treatment or with 10 minute incubation in 20mM KCl.

B) Erk1/2 protein levels are reduced in KO, but Erk1/2 protein levels are not altered by depolarization with 20mM KCl in WT or KO.

WT 9.4 ± 0.6, n = 6 (NT), 9.2 ± 1.1, n = 5 (KCl)

KO 7.1 ± 0.4 (NT), 6.9 ± 0.2 (KCl), n = 6

C) p-Erk1/2 protein levels are increased by depolarization with 20mM KCl in WT and KO.

WT 0.41 ± 0.02, n = 4 (NT), 0.71 ± 0.02, n = 4 (KCl)

KO 0.26 ± 0.02, n = 5 (NT), 0.37 ± 0.03, n = 5 (KCl)

C) The percent increase in p-Erk1/2 with NT vs. KCl is less in KO than in WT

WT 72.82% ± 3.94, n = 4

KO 41.05% ± 3.19, n = 5

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. * $p<0.05$, ** $p<0.001$. WT, wild type; KO, ZnT3KO; NT, no treatment; KCl, 10 min incubation in 20mM KCl.

Erk1/2 phosphorylation is not significantly reduced by blockade of NMDA receptors in ZnT3KO hippocampus during depolarization with KCl. To determine if zinc-mediated activation of Erk1/2 is through NMDARs, we treated samples with the NMDAR antagonist D-AP5. Western blot analysis of acute hippocampus slices after 30 minute pre-incubation with D-AP5 (50μM) followed by 10 minute co-incubation with 50μM D-AP5 and 20mM KCl show reduced p-Erk1/2 protein levels after incubation with D-AP5/KCl compared to incubation with KCl in WT, but not ZnT3KO (Figure 3-7B). The decrease in p-Erk1/2 after incubation with D-AP5/KCl in WT demonstrates that Erk1/2 is activated through NMDAR receptors. Calculation of the percent decrease in p-Erk1/2 after incubation D-AP5/KCl compared to KCl shows that the decrease is significantly greater in WT than in ZnT3KO (Figure 3-7C), suggesting that synaptic Zn^{2+} has a role in NMDAR activation of Erk1/2.

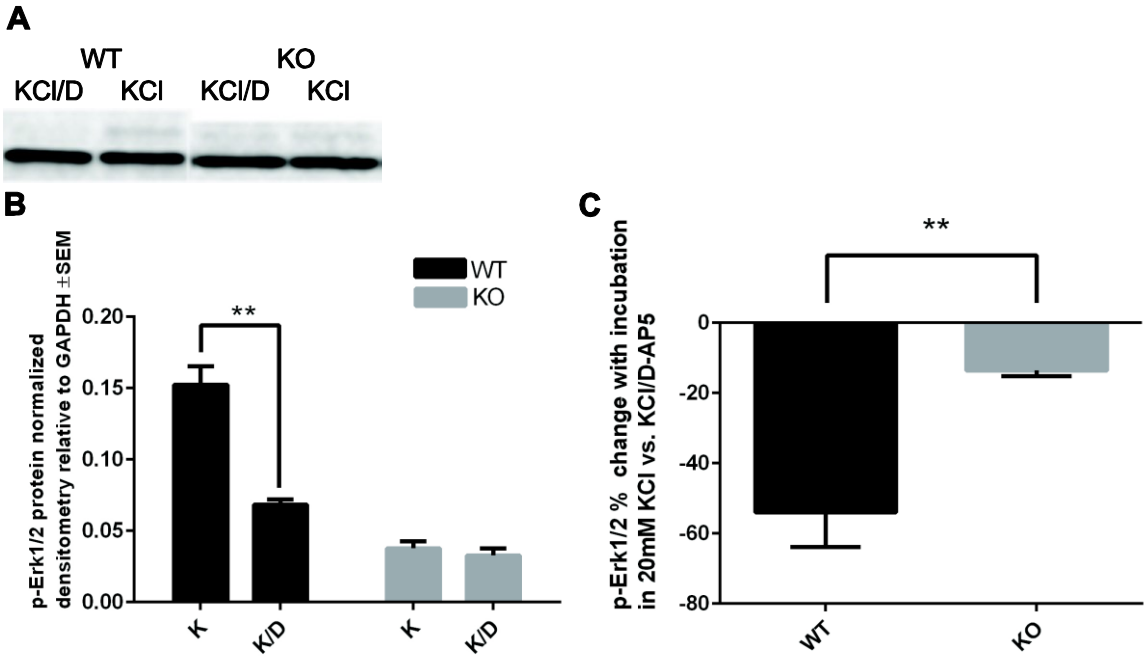

Figure 3-7. Erk1/2 phosphorylation is not significantly reduced by blockade of NMDA receptors in ZnT3KO hippocampus during depolarization with KCl.

A) Immunoblot analysis of acute hippocampus slices after 10 minute incubation with 20mM KCl or 30 minute pre-incubation with NMDAR antagonist D-AP5 (50μM) followed by 10 minute co-incubation with 50μM D-AP5 and 20mM KCl

B) p-Erk1/2 protein levels are reduced in WT after incubation in D-AP5/KCl, but not in KO.

WT 0.152 ± 0.013, (KCl), 0.068 ± 0.003 (KCl/D-AP5), n = 5

KO 0.037 ± 0.002 (KCl), 0.032 ± 0.002 (KCl/D-AP5), n = 5

C) Percent decrease in p-Erk1/2 after incubation in D-AP5/KCl compared to KCl is greater in WT than in KO.

WT -53.94 ± 4.46, n = 5

KO -13.58 ± 1.60, n = 5

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. ** $p<0.001$. WT, wild type; KO, ZnT3KO; K, 10 min incubation in 20mM KCl; K/D, 30 minute pre-incubation with 50μM D-AP5 followed by 10 minute co-incubation with 50μM D-AP5/20mM KCl.

Zn^{2+} inhibits NR2B-mediated activation of Erk1/2. Zn^{2+} has been shown to inhibit current flow and EPSPs through both NR2A and NR2B NMDAR subunits depending on methods used to chelate Zn^{2+}, voltage-dependence of response, and the concentration and timing of application of exogenous Zn^{2+} (Legendre and Westbrook, 1990; Paoletti et

al., 1997; Izumi et al., 2006; Erreger and Traynelis, 2008a). To determine if there is NMDAR subunit specificity of synaptic zinc modulation of Erk1/2 activation, we treated acute hippocampal slices with antagonists of the NMDAR subunits NR2A and NR2B.

Western blot analysis of acute hippocampus slices after 10 minute incubation in 20mM KCl or 10 minute co-incubation in NR2B antagonist ifenprodil (50μM) and 20mM KCl show decreased p-Erk1/2 protein levels after incubation with ifenprodil/KCl compared to KCl in both WT and ZnT3KO (Figure 3-8B), with a similar percent decrease in p-Erk1/2 (Figure 3-8C).

Western blot analysis of cellular homogenates of slices after 10 minute incubation with 20mM KCl or 30 minute pre-incubation in NR2A antagonist PEAQX (NVP-AAM077) (0.5μM), followed by 10 minute co-incubation in 0.5μM PEAQX and 20mM KCl show increased p-Erk1/2 protein levels after incubation with PEAQX/KCl compared to KCl in ZnT3KO, but not in WT (Figure 3-9B), with a significantly greater percent increase in ZnT3KO compared to WT (Figure 3-9C).

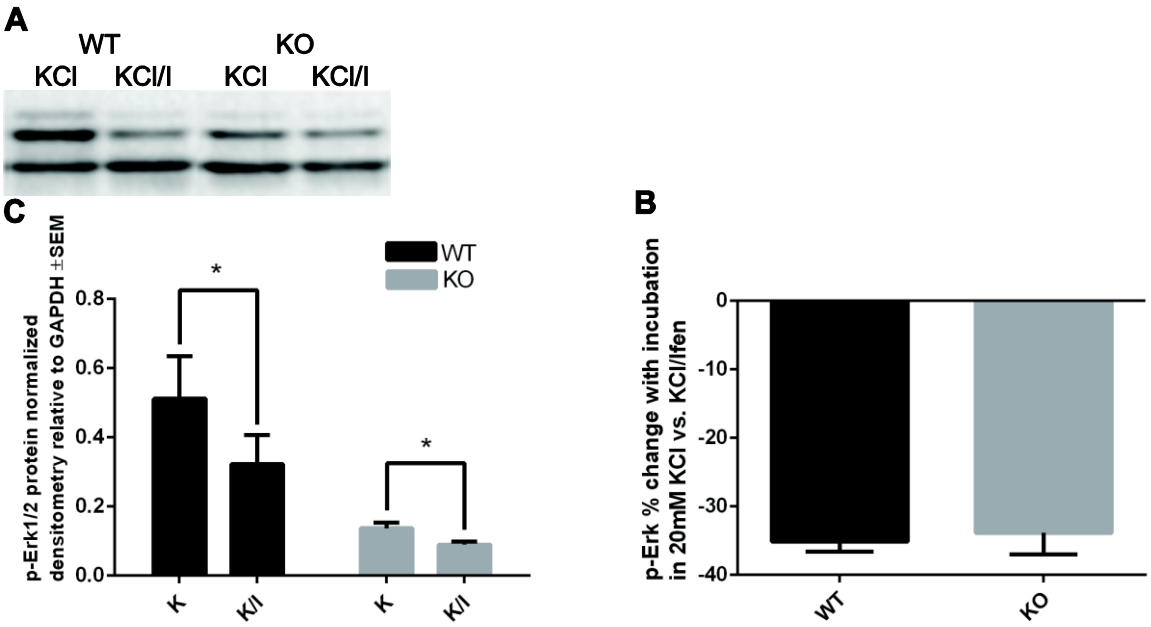

Figure 3-8. Decrease in Erk1/2 phosphorylation is not altered with blockade of NR2B subunits of NMDA receptors in ZnT3KO hippocampus during depolarization with KCl.

A) Immunoblot analysis of acute hippocampus slices with 10 minute incubation in 20mM KCl or 10 minute co-incubation in NR2B antagonist ifenprodil (50μM) and 20mM KCl.

B) p-Erk1/2 protein levels are decreased after incubation with ifenprodil/KCl compared to KCl in both WT and KO.

WT 0.7 ± 0.06 (KCl), 0.4 ± 0.04 (KCl/ifenprodil), n = 4

KO 0.1 ± 0.01 (KCl), 0.09 ± 0.009 (KCl/ifenprodil), n = 6

C) Percent decrease in p-Erk1/2 after incubation with ifenprodil/KCl compared to KCl does not differ between WT and KO.

WT -35.19 ± 1.42, n = 4

KO -33.87 ± 3.13, n = 6

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. * $p<0.05$. WT, wild type; KO, ZnT3KO; K, 10 min incubation in 20mM KCl; K/I, 10 minute co-incubation with 50μM ifenprodil/20mM KCl.

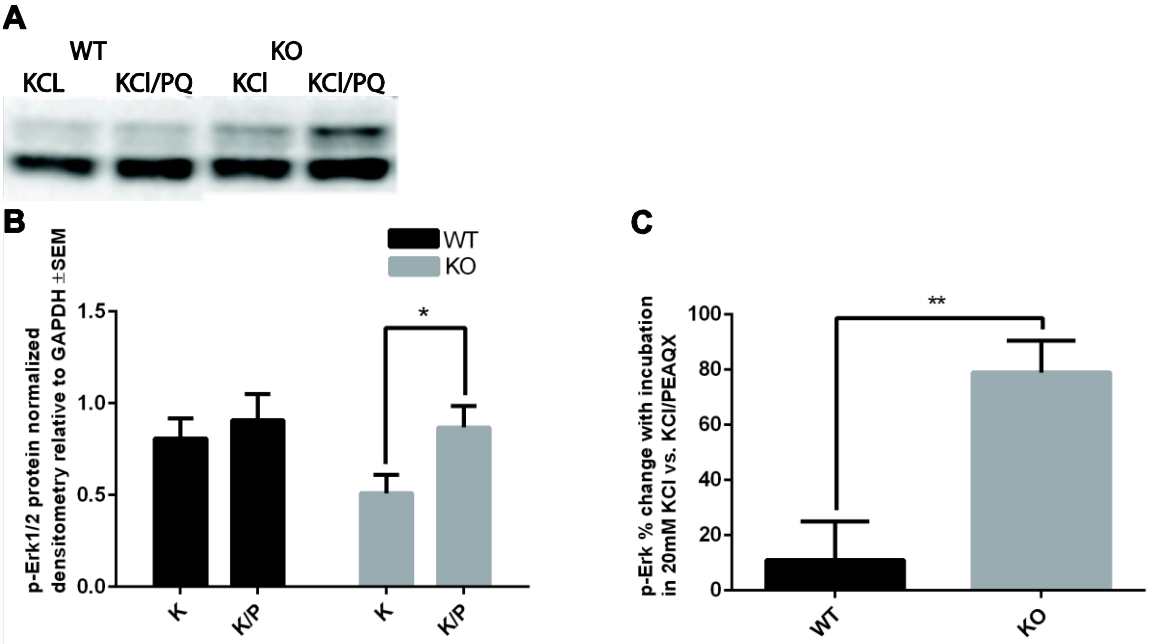

Figure 3-9. Erk1/2 phosphorylation is significantly increased with blockade of NR2A subunits of NMDA receptors in ZnT3KO hippocampus during depolarization with KCl.

A) Immunoblot analysis of acute hippocampus slices after 10 minute incubation with 20mM KCl or 30 minute pre-incubation in NR2A antagonist PEAQX (NVP-AAM077) (0.5μM), followed by 10 minute co-incubation in 0.5μM PEAQX and 20mM KCl.

B) p-Erk1/2 protein levels are increased in KO after incubation with PEAQX/KCl compared to KCl.

WT 0.8 ± 0.1 (KCl), 0.9 ± 0.1 (KCl/PEAQX), n = 5

KO 0.5 ± 0.1 (KCl), 0.9 ± 0.1 (PEAQXKCl), n = 5

C) Percent increase in p-Erk1/2 after incubation with PEAQX/KCl compared to KCl is greater in KO.

WT 10.95 ± 6.21, n = 5

KO 78.90 ± 11.55, n = 5

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. * $p<0.05$, ** $p<0.001$. WT, wild type; KO, ZnT3KO; K, 10 min incubation in 20mM KCl; K/P, 30 minute incubation with PEAQX followed by 10 minute co-incubation with 0.5μM PEAQX/20mM KCl.

Blockade of NR2B subunits uncovers in any alterations in p-Erk1/2 being facilitated through NR2A subunits. The percent decrease of p-Erk1/2 in the presence of a NR2B antagonist was significant and similar in WT and ZnT3KO, indicating that Erk1/2 is activated through NR2B-containing NMDARs as blockade of those subunits reduced activation and that the removal of synaptic Zn^{2+} does not alter activation of Erk1/2 through NR2A subunits.

Blockade of NR2A subunits permits analysis of any alterations in p-Erk1/2 being facilitated through NR2B subunits. Observation that the removal of synaptic Zn2+ with the blockade of NR2A subunits results in no change in WT but increased p-Erk1/2 in ZnT3KO suggests that in the WT the presence of Zn^{2+} inhibits NR2B-mediated activation of Erk1/2 and that removal of synaptic Zn^{2+} in the ZnT3KO removes Zn^{2+} inhibition of NR2B-mediated activation of Erk1/2, thus increasing p-Erk1/2.

BDNF protein levels are reduced in an age-dependent manner in ZnT3KO hippocampus. Activation of synaptic NMDARs, composed primarily of NR2A subunits, stimulates expression of BDNF, while activation of extrasynaptic NMDARs, composed primarily of NR2B subunits, inhibits expression of BDNF (Figure 3-2), so to further investigate the role of Zn^{2+} in the differential activation of NMDAR subunits, we assessed age-dependent expression of BDNF. Western blot analysis of cellular homogenates of hippocampus tissue of WT and ZnT3KO hippocampus tissue show an age-dependent decrease in BDNF protein levels in ZnT3KO compared to age-matched WT, reaching significance at 15 months of age (Figure 3-10). This indicates that while synaptic zinc

does not directly influence BDNF protein levels, there are age-dependent neurodegenerative alterations in ZnT3KO reducing BDNF levels.

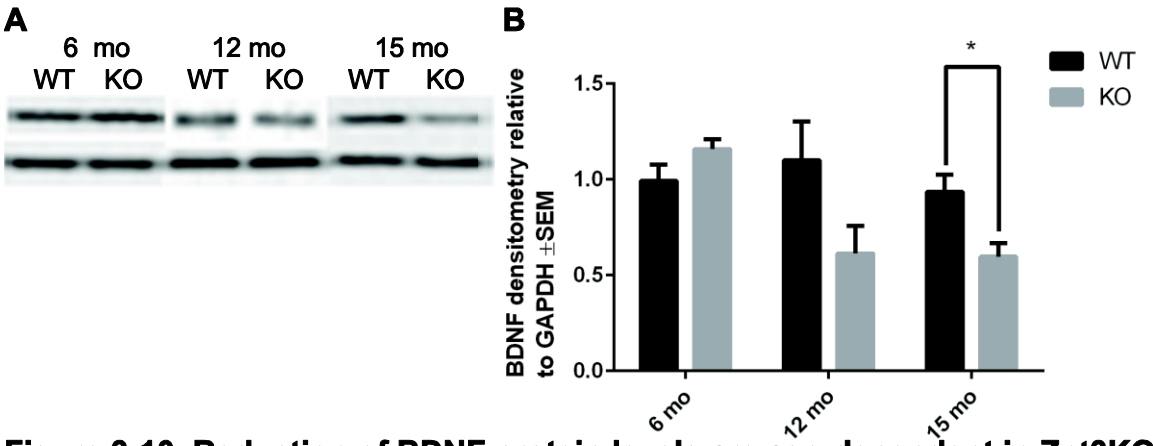

Figure 3-10. Reduction of BDNF protein levels are age-dependent in Znt3KO hippocampus.

A) Immunoblot analysis of cellular homogenates of hippocampus tissue of wild type and ZnT3KO hippocampus tissue prepared as described in Materials and Methods.

B) BDNF protein levels are reduced in 15 month old ZnT3KO hippocampal tissue.
6 mo. WT 1.0 ± 0.08, n = 6; KO 1.1 ± 0.02, n = 5
12 mo. WT 1.1 ± 0.2, KO 0.6 ± 0.1, n = 5
15 mo. WT 0.9 ± 0.09, n = 6, KO 0.6 ± 0.07, n = 6

Values expressed as normalized densitometry relative to GAPDH. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM *p<0.05. WT, wild type; KO, ZnT3KO.

***BDNF* mRNA expression after 3.5 hour incubation in 20mM KCl is reduced in ZnT3KO hippocampus slices.** BDNF protein levels in young ZnT3KO are not altered; however, previous research found reduced pro-BDNF in three and six month old ZnT3KO (Adlard et al., 2010), indicating that synaptic zinc increases pro-BDNF expression. We investigated the role of synaptic zinc on activity-dependent *BDNF* mRNA expression through depolarization with KCl, which has been demonstrated to upregulate expression of BDNF (Cosi et al., 1993). RT-qPCR analysis of acute hippocampal slices incubated 3.5 hr in Neurobasal medium shows that basal *BDNF* mRNA expression fold change relative to *actin* mRNA is not altered in ZnT3KO compared to WT (Figure 3-11A), while

analysis after 3.5 hr incubation in Neurobasal medium with 20mM KCl shows reduced *BDNF* mRNA expression fold change relative to *actin* mRNA in ZnT3KO (Figure 3-11B). These results demonstrate that while basal *BDNF* mRNA expression is not altered in ZnT3KO, activity-dependent expression is reduced, suggesting that while synaptic zinc can stimulate BDNF expression, there are compensatory mechanisms at work to maintain BDNF homeostasis that become less effective as the animals age.

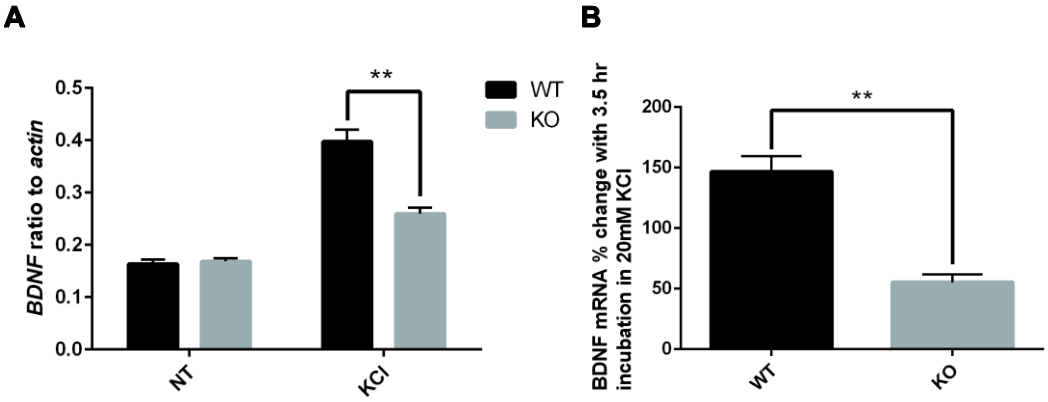

Figure 3-11. Increase in *BDNF* mRNA expression after 3.5 hour incubation in 20mM KCl is reduced in ZnT3KO hippocampus slices.

A) Basal *BDNF* mRNA expression in acute hippocampal slices is not altered in KO; however, the increase after 3.5hr incubation in Neurobasal medium with 20mM KCl is reduced in KO.
WT 0.17 ± 0.01 (NT), 0.40 ± 0.02 (KCl), n = 4
KO 0.16 ± 0.01, (NT), 0.26 ± 0.01 (KCl), n = 4

B) Percent increase in *BDNF* mRNA is significantly less in KO after 3.5hr incubation in Neurobasal medium with 20mM KCl compared to WT.
WT 146.5 ± 12.4, n = 4
KO 55.1 ± 6.6, n = 4

Values expressed as *BDNF* mRNA expression fold change relative to *actin* mRNA. Error bars indicate the mean ±SEM. **p<0.001, WT, wild type; KO, ZnT3KO.

Fluorojade+ cells are increased in aged ZnT3KO mice. Our results demonstrate that genetic ablation of synaptic zinc results in several neurodegenerative processes, including excessive excitatory neuronal activity and altered neurotrophic signaling. Consequently, we investigated age-dependent levels of degenerating neurons using the compound Fluorojade B. While the specific mechanism of the fluorojade reaction with cells is not known, it has been widely demonstrated through co-labeling experiments with other markers of neurodegeneration that fluorojade labeling effectively identifies neurons degenerating due to various insults (Poirier et al., 2000; Schmued et al., 2005) and consequently is widely used for this purpose. Fluorojade labeling was quantified in the CA3 region as that was the area with the most pronounced labeling of individual cells. Quantification of Fluorojade+ cells in the CA3 region of the hippocampus found a 25.4% increase in aged ZnT3KO compared to age-matched WT (Figure 3-12).

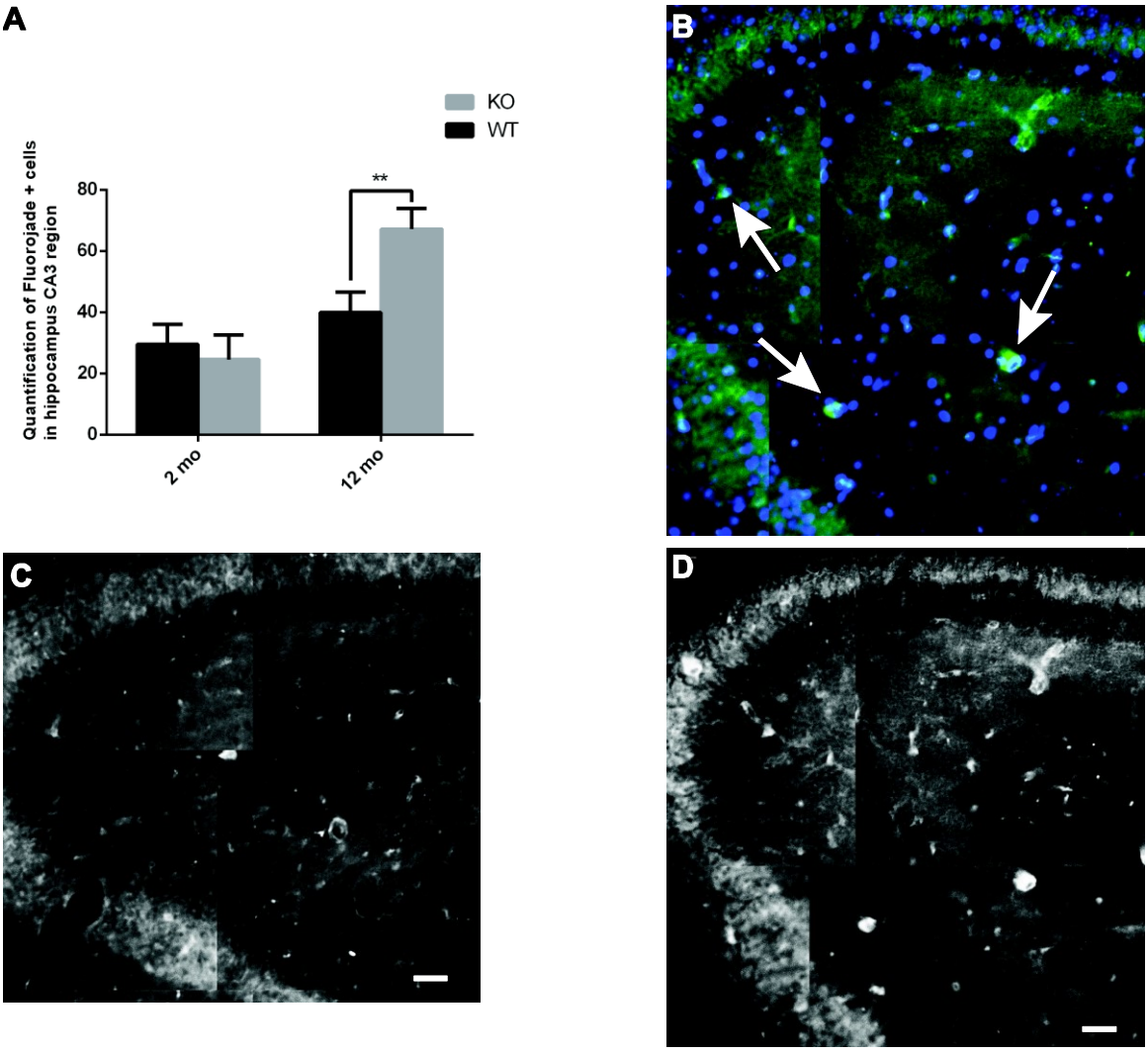

Figure 3-12. Fluorojade+ cells are increased in aged ZnT3KO hippocampus.

A) Quantification of Fluorojade+ cells counted in the CA3 region of the hippocampus show that while there is no difference in 2 month old KO, there is an increased number in 12 month old KO.

2 mo: WT 29.5 ± 6.5, n = 8
KO 24.6 ± 8.0, n = 9

12 mo: WT 39.9 ± 6.6, n = 11
KO 67.1 ± 6.7, n = 11

B) Co-localization of Fluorojade (green) and Hoescht (blue) stains indicate degenerating neurons in 12 month old KO CA3 region of the hippocampus (arrows).

C) Fluorojade labeling in 12 month old WT hippocampus.

D) Fluorojade labeling of image seen in B.

The CA3 region was imaged at 20X magnification using the Mosaic tool in Axiovision to image and stitch together adjacent fields. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM. **p<0.001, WT, wild type; KO, ZnT3KO. Scale bars 50μm.

Discussion

The observation that basal levels of phosphorylated Erk1/2 are reduced in ZnT3KO compared to WT is consistent with research by Sindreu and colleagues (Sindreu et al., 2011), which also found a 30% reduction in p-Erk in ZnT3KO compared to WT at baseline conditions, compared to the 55% reduction observed in this study. Also consistent with the Sindreu study, activity-dependent phosphorylation of AKT was not altered in ZnT3KO. Sindreu *et al*. also found that activity-dependent increase in p-Erk was suppressed in ZnT3KO, proposing a mechanism of increased MAPK tyrosine phosphatase activity in the reduction of p-Erk1/2 in ZnT3KO. Those experiments were performed in tissue collected and assayed 90 minutes after i.p. injection of a sub-convulsive dose of kainate and phosphatases. The present study assayed tissue after 10 minute incubation in KCl, consequently the time course of activity of kinases and phosphatases and phosphorylation state of Erk1/2 differed from the conditions in the Sindreu experiments. However, our results demonstrating suppression of activity-dependent increase of p-Erk1/2 in ZnT3KO are consistent with those of Sindreu and colleagues, supporting a role for synaptic zinc in regulation of Erk1/2 phosphorylation.

Sindreu and colleagues associated altered zinc signaling to impaired cognition, investigating the role of synaptic zinc in the performance of a hippocampal-dependent memory task. Infusion of a Zn^{2+} chelator, TPEN, into the CA3 region of the hippocampus in WT mice resulted in impaired contextual discrimination in a foot shock-mediated memory test similar to the impairment shown by 3-4 month old ZnT3KO. These results indicate that interference with synaptic zinc can result in cognitive impairment, which brings into question why young ZnT3KO, with the lack of synaptic zinc, perform normally

in behavior tasks. One hypothesis is that young ZnT3KO may have developed compensatory mechanisms that wild type would not have when Zn^{2+} is abruptly rendered inactive with a chelator, possibly up-regulation of receptors activated or suppressed by Zn^{2+}.

The effect of altered Erk1/2 activation on cognition was also investigated by Sindreu *et al*. through infusion of a MEK1/2 inhibitor into the CA3 region of the hippocampus in WT to reduce p-Erk1/2 levels to those found in ZnT3KO. The results demonstrated cognitive impairment similar to those found in ZnT3KO and with zinc chelators in WT. These results demonstrate that Zn^{2+} and normal Erk1/2 activation in the hippocampus are necessary for normal performance in a hippocampus-dependent memory task. However, our results show altered basal and activity-dependent p-Erk1/2 levels and activity-dependent expression of *BDNF* mRNA in acute slices from 3-4 week old ZnT3KO, which is before cognition becomes impaired, demonstrating that these alterations are present in young ZnT3KO with normal cognition and do not develop during maturity. The mechanisms that result in normal cognition in young ZnT3KO remain to be discovered and the present research is discovering age-dependent alterations that may contribute to impaired cognition in aged ZnT3KO, specifically increased neurodegeneration and seizure activity and reduced BDNF.

Research reporting on the role of NMDARs on phosphorylation of Erk agree that differential activation is mediated through NR2A and NR2B subunits of NMDARs but report conflicting results, attributing the differences to tissue substrate type, experimental methods used, time course recorded and other variations in methodology. Previous results have demonstrated that activation of NR2A-containing NMDARs activates Erk

(Choo et al., 2012), activation of NR2B-containing NMDARs activates Erk (Krapivinsky et al., 2003; Chen et al., 2007), and that activation of NR2B-containing NMDARs inhibit Erk activation (Kim et al., 2005; Choo et al., 2012). The results reported here support a minimal role for NR2A subunits in activation of Erk1/2 as blockade of NR2B subunits results in significantly greater decrease of p-Erk1/2 in WT than found with blockade of NR2A subunits (Figures 3-13A and B). These results also support a role for NR2B-containing NMDARs in activation of Erk1/2 in the experimental design used, with synaptic zinc suppressing NR2B-mediated response (Figure 3-13C).

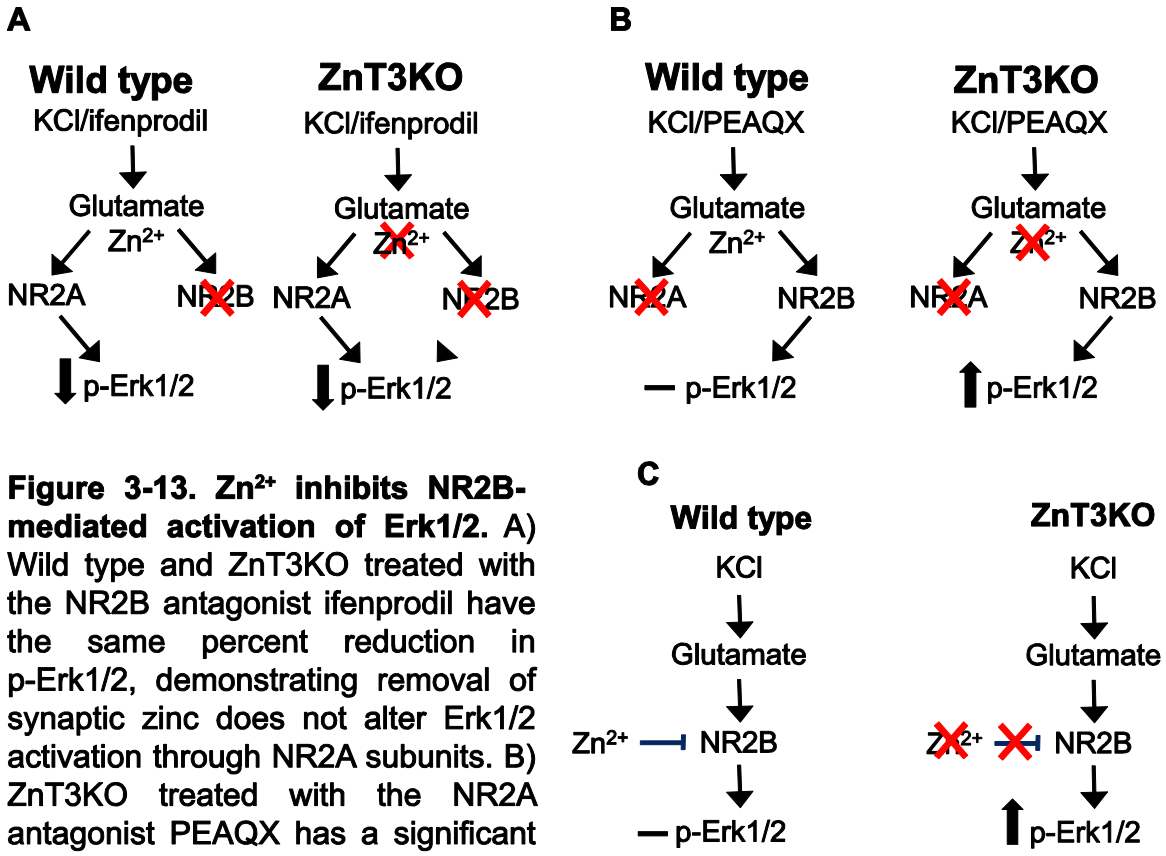

Figure 3-13. Zn^{2+} inhibits NR2B-mediated activation of Erk1/2. A) Wild type and ZnT3KO treated with the NR2B antagonist ifenprodil have the same percent reduction in p-Erk1/2, demonstrating removal of synaptic zinc does not alter Erk1/2 activation through NR2A subunits. B) ZnT3KO treated with the NR2A antagonist PEAQX has a significant increase in p-Erk1/2, while wild type does not, suggesting that Zn^{2+} inhibits activation of Erk1/2 through NR2B subunits. C) Proposed model of the role of Zn^{2+} in NMDAR activation of Erk1/2. Removal of synaptic zinc results in increased activation of Erk1/2 by removing Zn^{2+} inhibition of NR2B subunits.

Investigation of BDNF expression found an age-dependent and activity-dependent decrease in ZnT3KO hippocampus tissue, indicating that removal of synaptic zinc has a role in BDNF synthesis or metabolism. While basal *BDNF* mRNA expression was similar in WT and ZnT3KO, the fold change increase after stimulation was reduced by more than 50% in ZnT3KO, suggesting that synaptic zinc enhances signaling pathway(s) that stimulate activity-dependent BDNF expression. These results are consistent with studies finding that though basal BDNF is not altered in young ZnT3KO, pro-BDNF levels are reduced (Adlard et al., 2010) and that that Zn^{2+} increases secretion of pro-BDNF in tissue culture (Hwang et al., 2005).

Along with the other experiments performed for this dissertation, the effects of genetic ablation of ZnT3 need to be considered. Eight week old ZnT3KO are phenotypically normal in a battery of motor and cognitive tests, indicating that the genetic removal of synaptic zinc does not directly result in significant alterations in functions that are detectable by such tests. Impaired ZnT3 function in the retina is not likely to affect neuronal signaling, but impaired insulin secretion and potential hyperglycemia may influence development. Children with early onset type 1 diabetes have impaired cognition (Ferguson et al., 2005); however, 6-8 week old ZnT3KO do not demonstrate cognitive impairment, indicating that any impairment in insulin regulation or hyperglycemia is not significant in young ZnT3KO. These experiments focused on alterations in signaling pathways in hippocampal slices from 3-4 week old mice, well before detection of any cognitive impairment or development of markers of seizure activity, and likely reflect changes in signaling directly mediated by synaptic zinc before any age-related deficits develop.

Chapter 2 found markers of seizure activity, but acute treatment with an anti-seizure drug did not result in improved cognition in 6 month old ZnT3KO, indicating that seizure activity does not directly contribute to cognitive deficits in aged ZnT3KO. In these experiments we found evidence that removal of synaptic zinc tips the balance from neurotrophic synaptic to neurodegenerative extrasynaptic NMDAR response, reduces expression of Erk1/2 and AKT, and reduces BDNF expression. These alterations, combined with effects of excessive excitatory neuronal activity and potential impairment of ZnT3 regulation of insulin secretion, likely contribute to the age-dependent neurodegeneration found in aged ZnT3KO, resulting in impaired cognition.

In summary, our results demonstrate that synaptic zinc has a role in the activation of Erk1/2 through inhibition of the NR2B-containing NMDA receptor. Synaptic zinc also has a role in maintaining Erk1/2 and AKT protein levels in the hippocampus, in activity-regulated expression of BDNF and in maintaining neuronal health.

Chapter 4

Summary and Concluding Remarks

The Alzheimer's Association notes that progression through stages of cognitive impairment and ultimately death in AD varies significantly from patient to patient, with some patients surviving as few as four years and others as long as twenty years before succumbing to the effects of the disease. These varied time courses indicate that the pathologies leading to AD may be due to a variety of factors, with no one single cause, and consequently no one single prevention or cure, highlighting the importance of understanding the underlying molecular mechanisms of synaptic loss and neurodegeneration characteristic of the disease and recognizing that there may be interplay between myriad mechanisms at work. It is also critical to identify mechanisms at work early in the disease, before the neuronal damage is catastrophic.

This study aimed to elucidate specific targets of AβO early in the process of disruption of synaptic neurotransmission that can lead to synapse loss and neuronal death, looking at brain regions impacted in AD and specific neuronal signaling pathways impaired by AβO, and more specifically, zinc released during neurotransmission. It has been demonstrated that therapeutics modulating zinc homeostasis, clioquinol and its derivative PBT2, improve cognition in transgenic mice models of AD (Adlard et al., 2008; Grossi et al., 2009) and AD patients (Lannfelt et al., 2008), with recent research focusing on the mechanisms of action of those therapeutics on Aβ processing and associated pathways (Adlard et al., 2008; Crouch et al., 2011) and synaptic health (Adlard et al., 2011).

A Phase 2 clinical trial completed in 2007 found that 12 weeks of treatment with PBT2 in a cohort of mild AD subjects significantly reduced cerebral spinal fluid soluble Aβ and improved cognition (Lannfelt et al., 2008; Faux et al., 2010); however, results of a recent Phase 2 clinical trial treating prodromal and mild AD patients with PBT2 for 12 months failed to meet target endpoints of reduced Aβ plaque load as detected by PiB imaging and improved cognition (PRANA Biotechnology press release, March 31, 2014). In fact, the study found an atypical reduction of PiB signal in both the treatment and control group, which confounded interpretation of the results.

This is not the end of the story for PBT2. The 2014 study did not look at soluble AβO levels, which is the more toxic species of Aβ (Lambert et al., 1998). Instead, the target endpoint was PiB imaging, which does not detect soluble AβO, only insoluble Aβ plaques. Clioquinol and PBT2 have a low affinity for Zn^{2+} and may not be able to disrupt binding in insoluble Aβ plaques, but have been shown to reduce soluble AβO (Cherny et al., 2001; Adlard et al., 2008). The 2014 study also required that participants have Aβ plaque load detectable by PiB imaging before initiating treatment, while the 2007 study did not. It is likely that the 2007 study had a significant number of participants without significant Aβ plaque load, as plaque load does not correlate well with cognitive impairment (Lue et al., 1999; Naslund et al., 2000). This would skew the results as the second study had subjects with insoluble Aβ aggregates resistant to treatment while the first study likely had both insoluble and soluble forms of Aβ aggregation so was more amenable to successful treatment. Significant Aβ plaque load may also indicate a more advanced stage of AD with increased neurodegeneration compared to subjects without detectable plaques, in which treatment may be too late in the process to reverse cognitive

impairment. Our results support further research into treatment with PBT2, such as an end target of reduction of AβO determined by Bis-ANS imaging, as AβO have been shown to sequester synaptic zinc (Grabrucker et al., 2011a) and we have shown that impairment of zinc neurotransmission increases neurodegeneration and results in cognitive impairment.

The potential effects of interference with zinc signaling are not well known. The discovery of significant alterations in neuronal signaling and degradation in synaptic and neuronal health caused by zinc dysregulation may provide additional therapeutic targets, such as hippocampal-targeted up-regulation of BDNF or suppression of neuronal hyperactivity, to prevent or ameliorate the neurodegeneration characteristic of AD.

Mechanisms for age-dependent cognitive impairment of ZnT3KO mice

The initial phenotyping of the ZnT3KO transgenic mouse found no cognitive, motor or sensory deficits in six to ten week old subjects and concluded that zinc modulation of neurotransmission was not relevant in the battery of tasks tested or that other mechanisms compensated for the lack of synaptic zinc (Cole et al., 2001). More recent phenotyping tested older ZnT3KO subjects and found age-dependent cognitive deficits and reduction in synaptic and neurotrophic proteins (Adlard et al., 2010). The observation that young ZnT3KO mice are phenotypically normal, but develop cognitive deficits and neurodegenerative alterations with age indicates that the lack of synaptic Zn^{2+} does not necessarily directly cause cognitive deficits, it simply contributes to neurodegenerative alterations that result in impaired cognition, and the contributions are subtle and cumulative. The results presented here support the hypothesis of age-dependent

alterations leading to neurodegeneration and cognitive impairment and propose mechanisms of impaired Zn^{2+} enhancement of neurotrophic signaling pathways and suppression of excessive excitatory neurotransmission as contributors to these alterations.

Aβ pathology, neuronal hyperactivity and AD

The incidence of seizure activity in AD patients had not been considered significant until recently, with a broad array of findings linking AD pathology, excessive neuronal hyperactivity, and impaired network connectivity and cognition.

Recent research using *in vivo* two-photon Ca^{2+} imaging in a mouse model of AD demonstrated increased hippocampus Ca^{2+} transients triggered by action potentials in young transgenic mice before the deposition of amyloid plaques and that the transients were suppressed by treatment with γ-secretase inhibitors, demonstrating that reducing the production of soluble Aβ is linked to hippocampal hyperactivity early in AD pathology (Busche et al., 2012). Subsequently, overproduction of Aβ has been demonstrated to produce aberrant EEG and epileptiform activity in a transgenic mouse with controllable expression of APP in which abnormal EEG discharges were dependent on overexpression of APP, showing a direct link between Aβ and neuronal hyperexcitability (Born et al., 2014).

Differential hippocampal activation has been linked to cognition in normal, MCI and AD subjects, with increased activation in less impaired MCI and decreased activation in more impaired MCI and AD patients compared to normal subjects (Celone et al., 2006) and increased cognitive decline in MCI patients with increased hippocampal activation

compared to MCI patients with normal hippocampus activity (Miller et al., 2008; O'Brien et al., 2010). AD pathology is associated with these observations through PiB imaging showing Aβ deposition correlates with increased metabolism in MCI and decreased metabolism in AD (Cohen et al., 2009). These findings demonstrate that hippocampal hyperactivity early in the progression to AD converts to reduction of hippocampal activity as cognition declines, suggesting that neuronal hyperactivity may contribute to neurodegeneration and cognitive impairment in AD.

Hippocampal hyperexcitability has recently been linked to impaired connectivity of the default mode network (DMN) in AD through an fMRI study of hippocampus and network activity in normal, MCI and AD subjects that found increasing activity in the hippocampus was associated with decreasing connectivity of the DMN in MCI and mild AD (Pasquini et al., 2014). Increased hippocampal activity is also associated with decreased DMN activity in temporal lobe epilepsy (Zeng et al., 2013), suggesting that hippocampal hyperactivity and seizures contribute to impaired DMN function.

The DMN and other networks of functional connectivity in the brain have been identified through brain imaging research utilizing fMRI and PET scans that use changes in blood oxygen levels or blood flow to determine which brain regions are active while a subject is performing specific tasks. Research to determine brain activity during a control condition while a subject is not engaged in any specific task found that a group of brain regions consistently demonstrated activity when the subject was not engaged with the external environment or in any goal-directed behavior and became inactivated when the subject initiated a task. Subsequently, these collective brain regions and structures became known as the DMN (Raichle et al., 2001). These regions are also sites of Aβ

deposition, atrophy and metabolism reduction (Buckner et al., 2005) and reduced normal connectivity (Agosta et al., 2011; Binnewijzend et al., 2011; Soldner et al., 2011) in brains of Alzheimer's patients and aMCI patients (Sorg et al., 2007), who are at high risk of developing AD.

AD pathology has been linked to reduced DMN connectivity and impaired cognition. PiB imaging, a marker for Aβ plaques, in cognitively normal elder subjects found aberrant connectivity in regions of the DMN correlated with Aß deposition, suggesting that altered DMN connectivity is a sign of Aβ toxicity early in the development of AD before cognition becomes impaired (Sperling et al., 2009; Sheline et al., 2010) Sperling et al, 2009). DMN connectivity has been linked to performance in memory tasks through a study of cognitively intact older individuals, finding reduced connectivity correlated with reduction in performance (Wang et al., 2010b), while recent research found that increased PiB uptake correlated with reduced DMN connectivity and that the reduced DMN activity correlated with increased cognitive impairment in patients with prodromal AD (Koch et al., 2014). How Aβ contributes to reduced DMN connectivity is not known, but we propose that Aβ-induced increase in neuronal activity through the disruption of Zn^{2+} signaling in the hippocampus may play a role in aberrant DMN function in AD.

The link between Aβ and neuronal hyperactivity in MCI and early AD suggests that reduction of excessive excitatory neuronal activity may be a potential target for therapeutic treatment to prevent or slow the decline in cognition as AD progresses; however, only a subset of AD patients suffers from seizures, indicating that early identification and treatment may benefit this population. This still leaves unanswered the

question of how Aβ influences hyperactivity. Our results suggest that disruption of zinc modulation of neurotransmission is one target of Aβ pathology and that potential therapies targeting zinc homeostasis and neuronal hyperactivity, along with reducing Aβ, may mirror the successful treatment regimens found in drug combination therapies used to treat other diseases such as cancer and HIV.

The role of Zn^{2+} in neurotrophic pathways

We have demonstrated that Erk1/2 and AKT levels, and activity-dependent expression of *BDNF* mRNA, are reduced in ZnT3KO, indicating a role for Zn^{2+} in regulation of these proteins. Zn^{2+} inhibits NR2B-mediated activation of Erk1/2 and enhances Erk1/2 activation through other signaling pathways in addition to NMDARs as activation of Erk1/2 is reduced in ZnT3KO compared to WT with blockade of NMDARs (Figure 3-7). The observation that Zn^{2+} suppresses NR2B-containing NMDARs, which are predominantly extrasynaptic (Cull-Candy and Leszkiewicz, 2004), supports a neurotrophic role for synaptic zinc in neurotransmission, as it has been demonstrated that CREB (cAMP response element binding protein)-mediated neurotrophic pathways are activated through synaptic NMDARs and suppressed through extrasynaptic NMDARs (Hardingham et al., 2002). This result also suggests that interference with Zn^{2+} suppression of NR2B subunits can be a mechanism by which AβO disrupt synaptic function through increased activation of NR2B-containing NMDARs, resulting in inhibition of LTP (Snyder et al., 2005; Rammes et al., 2011).

Our observations that basal BDNF and *BDNF* mRNA are not altered in young ZnT3KO, but activity-dependent expression of *BDNF* mRNA is reduced in ZnT3KO acute

hippocampus slices, are consistent with previous reports finding (1) reduction in pro-BDNF, but not BDNF in ZnT3KO (Adlard et al., 2010) and (2) Zn^{2+} increases secretion of pro-BDNF (Hwang et al., 2005). The release of synaptic zinc triggering an increase in secretion of pro-BDNF should increase post-translational processing into mature BDNF, resulting in activity-dependent increase of BDNF. Conversely, recent research found a 51% increase of BDNF protein in ZnT3KO hippocampal lysates by ELISA (Helgager et al., 2014), a significant difference from Adlard *et al*. and our results, which found no alteration at basal levels. At this point these differences are difficult to reconcile other than through methods and materials used. Both Adlard's and our studies analyzed BDNF levels by western blot and Helgager's study used ELISA; both Helgager's and our study sampled hippocampus tissue. It is unclear whether Adlard's study sampled cortical or hippocampal tissue in 3 month old ZnT3KO.

Helgager *et al*. also investigated TrkB activation by endogenous zinc using ZnT3KO, finding that removal of synaptic zinc does not alter phosphorylation of TrkB at Y816, which activates the phospholipase-C γ1 (PLCγ1) signaling pathway. However, that study did not investigate phosphorylation of TrkB at Y515, which activates the MAPK signaling pathway (Minichiello, 2009) so did not address activation of Erk1/2 by synaptic zinc.

Future Directions

Aberrant neuronal activity

Previous research investigated the threshold for kainate-induced seizures in four to twelve week old ZnT3KO mice, finding a reduced threshold for seizures, but did not detect epileptiform EEG activity before or after kainate treatment (Cole et al., 2000). Cole

et al., however, did not investigate alterations in EEG in older subjects where the difference between wild type and ZnT3KO may be more pronounced or discuss the criteria considered for determining aberrant EEG activity. Consequently, analysis of EEG to detect aberrant but not necessarily epileptiform activity may uncover alterations in neuronal activity in young ZnT3KO mice. Alternatively, analysis of EEG in aged ZnT3KO may reveal age-dependent development of epileptiform activity.

The effect of anti-seizure drug on cognition

While we found an age-dependent increase in markers of excessive excitatory activity, we did not find that acute treatment with an anti-seizure drug during training and testing resulted in improved cognition in 6 month old ZnT3KO. Markers of seizure activity are not found in three month old ZnT3KO, suggesting that EEG of subjects in this age group would not show epileptiform activity, which was reflected in the normal habituation profile (data not shown). Though they show impaired cognition in the OLM task, they do not perform at chance as do six month old ZnT3KO subjects. This suggests that acute treatment of 3 month old ZnT3KO with an anti-seizure drug may improve cognition and that chronic treatment with an anti-seizure drug during the time course when cognitive impairment becomes profound between the ages of three and six months may reduce the progressive cognitive impairment and consequently indicate that the impairment is due to neurodegeneration induced by seizure activity.

The role of synaptic zinc in neurotransmission

Activation of Erk1/2 is significantly reduced in ZnT3KO, though the decrease in Erk1/2 activation is significantly less in ZnT3KO in the presence of an NMDAR antagonist (Figure 3-7C), suggesting that synaptic Zn^{2+} has a role in Erk1/2 activation independent of NMDARs. The most likely candidate is the TrkB receptor, which is activated by exogenous Zn^{2+}, affecting the downstream proteins Erk1/2, AKT and CREB; however, we found no difference in AKT phosphorylation in ZnT3KO which would not be expected if synaptic zinc activates TrkB receptors that then subsequently activate downstream proteins. However, activation of a protein can be initiated or inhibited by several pathways so determining if synaptic zinc activates a specific pathway would need evidence from more than activation of one downstream protein, or differential activation, as demonstrated by NMDAR subunit antagonists. This suggests future experiments comparing activation of the TrkB receptor with BDNF with and without KCl in WT and ZnT3KO, and also comparing phosphorylation of Erk1/2, AKT and CREB before and after these treatments.

Other neurodegenerative effects of disruption of zinc function

One observation from working with aging ZnT3KO is that they become significantly obese by 12 months of age compared to non-transgenic mice of the same 128Sv strain, suggesting that genetic ablation of ZnT3 has effects other than those found in neuronal activity and signaling.

ZnT3 is also found in the retina and pancreatic β-cells, so ablation of the gene would be expected to impair Zn^{2+} homeostasis in those tissues. The age-dependent

obesity in ZnT3KO suggests that impaired Zn^{2+} function may lead to a diabetic condition. Zinc deficiency has been linked to diabetes, and a diet deficient in zinc results in impaired insulin secretion and decreased glucose tolerance (Quarterman et al., 1966; Boquist et al., 1968). In addition, diabetic patients have been found to have significant decrease in serum Zn levels and increased Zn secretion in urine (el-Yazigi et al., 1993; Garg et al., 1994). While islet amyloid polypeptide (IAPP) is the primary component of the amyloid plaques found in the pancreas of type 2 diabetics, Aβ has also been found in the pancreas of type 2 diabetic patients (Miklossy et al., 2010) and of transgenic mice overexpressing APP (Kawarabayashi et al., 1996), suggesting that Aβ may contribute to dysregulation of Zn^{2+} homeostasis and function in the pancreas and may be a link between AD and type 2 diabetes.

Zinc is found in abundance in pancreatic β-cells where zinc binds to insulin, regulating insulin storage and release, and is regulated by zinc transporters, including ZnT8 and ZnT3. While genetic deletion of ZnT8 has been found to produce modest effects (Davidson et al., 2014), deletion of ZnT3 has been found to impair insulin metabolism. Recent research found that treatment with streptozotocin, a drug toxic to pancreatic β-cells, resulted in significantly impaired glucose metabolism in ZnT3KO mice (Smidt et al., 2009), while another study demonstrated that knock down of ZnT3 in INS-1E cells (derived from pancreatic β-cells) increases apoptosis and reduces insulin secretion (Petersen et al., 2011). The results of these studies indicate that disruption of Zn^{2+} function may contribute to development of diabetes. Smidt and colleagues used 3-month old male ZnT3KO to investigate glucose tolerance and metabolism, finding no difference

in body weight between WT and ZnT3KO, and that low dose streptozotocin did not induce a diabetic condition in ZnT3KO.

We suggest that examination of glucose tolerance in 12 month old ZnT3KO may reveal an age-dependent onset of diabetes induced by loss of zinc homeostasis which may contribute to cognitive impairment and neurodegeneration. Furthermore, it would be worthwhile to investigate potential rescue of cognition in aged ZnT3KO by administration of treatments for diabetes that stimulate the release of insulin and possibly restore normal glucose metabolism.

Conclusion

Together, these experiments demonstrate that disruption of zinc-dependent signaling during neurotransmission results in neurodegeneration. While current efforts to treat AD patients with drugs targeting zinc homeostasis have not yet been successful, continuing investigation into therapeutics targeting Aβ/Zn^{2+} interaction, reduction of Aβ to preserve synaptic zinc and treatments reducing neuronal hyperexcitability should be pursued as the multifactorial pathology of AD will likely require complex treatments to prevent the devastating symptoms of the disease and its burden on families and society.

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