UNIVERSITY OF CALIFORNIA,
IRVINE

Levetiracetam prevents age-related cognitive impairment in a sex-specific manner in mice lacking synaptic zinc

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Matthew Mahavongtrakul

Dissertation Committee:
Professor Jorge A. Busciglio, Chair
Professor Karina Cramer
Professor Raju Metherate
Associate Professor Michael Yassa

2018

DEDICATION

To

My wife Minhan,

who never gave up on me.

#pipsyandpoppymusings

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First and foremost, I would like to thank my advisor, Dr. Jorge Busciglio, for giving me the opportunities to be successful not only as a neuroscientist, but as a well-rounded person. Words cannot express how grateful I am for his trust in my abilities to formulate hypotheses, design experiments, mentor undergraduates, and pursue interests outside of the laboratory.

Thanks to Dr. Emily Vogler, who mentored me when I started in the laboratory. She laid the groundwork for my success and continued to be intimately involved in the experiments.

This work would not be possible with the amazingly talented undergraduate researchers with whom I have had the pleasure of working. First to Alison Tran, who showed her ability to perform surgical techniques with finesse and had unparalleled enthusiasm and dedication. Best of luck in her endeavors as a veterinarian at the UC Davis School of Veterinary Medicine. Next to Robbin Nameki, who not only managed to complete a project in eight weeks during a summer fellowship, but also for continuing to develop scientifically throughout her stay in the laboratory. Best of luck as a doctoral student in the Cedars-Sinai Biomedical Science and Translational Medicine program. Lastly to Carlene Chinn, who was brave enough to extend the projects from this dissertation and cultivate collaborations with other labs to push additional experiments forward.

Thanks to the rest of the Busciglio laboratory for their support, especially to Dr. Maria Torres and Dr. Pablo Helguera, who kept the atmosphere light and were willing to talk about anything from experiments to culture. I will always cherish maté time! Thanks also to Jessica Noche, whose perseverance and upbeat attitude helped immensely during the latter stages of the project.

Special thanks to Dr. Michael Phelan for his statistical consulting, and to Dr. Yama Akbari and Dr. Beth Lopour for their guidance in electroencephalogram analysis. Also to Dr. Conor Cox, whose programming expertise is second to none.

I would also like to thank the Division of Teaching Excellence and Innovation here at UC Irvine. Being a part of the Pedagogical Fellowship has been one of the best experiences and continuing to be a part of the team has been integral in my development both as a scientist and as an educator.

Lastly, thank you to my wife, Minhan, who has been my pillar of support for the past 12 years. She always believed in me, even when I doubted myself. This marathon would not be possible without her.

CURRICULUM VITAE

Matthew Mahavongtrakul
mmahavon@uci.edu

EDUCATION

University of California, Irvine

Doctor of Philosophy, Neurobiology and Behavior 2018

Dissertation: "Levetiracetam prevents age-related cognitive impairment in a sex-specific manner in mice lacking synaptic zinc"

Advisor: Jorge Busciglio, Ph.D

Master of Science, Biological Sciences 2013 – 2016

California State University, Long Beach

Master of Science, Biology 2010 – 2013

Thesis: "Estradiol dose dependently regulates membrane estrogen receptor-α and metabotropic glutamate receptor-1a complexes in the arcuate nucleus of the hypothalamus"

Advisor: Kevin Sinchak, Ph.D

University of California, Davis

Bachelor of Science, Neurobiology, Physiology, and Behavior 2005 – 2009

QUALIFICATION SUMMARY

- Four years laboratory research experience for the Department of Neurobiology and Behavior at UC Irvine
- Three years laboratory research experience for the Department of Biological Sciences at CSU Long Beach
- Three years laboratory research experience for the Department of Cell Biology and Human Anatomy at the UC Davis School of Medicine
- One year laboratory research experience for the Department of Human Physiology: Membrane Biology at the UC Davis School of Medicine
- One year clinical research experience for the Emergency Medical Research Associate Program at the UC Davis Medical Center
- One year Teaching Associate experience at CSU Long Beach
- Two years Teaching Associate experience at UC Irvine
- Two years instructor of record experience at Santa Ana College
- Experience in biostatistics, preparing poster presentations, and writing manuscripts, protocols, and grants

RESEARCH EXPERIENCE

University of California, Irvine

Doctoral Student Researcher April 2015 – January 2018

Investigate the role of synaptic zinc deficiency in age-related cognitive impairment, chronic hyperexcitability, and neurogenesis in the zinc T3 knockout (*ZnT3*$^{-/-}$) model of Alzheimer's Disease. Utilize a novel method for recording electroencephalogram (EEG) activity and analyze EEG data using Python. Chronically treat *ZnT3*$^{-/-}$ mice with the anti-epileptic drug Levetiracetam characterize to gene networks involved in the physiological effects that lead to improved cognitive function. Dr. Jorge Busciglio, Department of Neurobiology and Behavior.

Doctoral Student Researcher March 2015 – April 2015

Studied the activity-dependent regulation of long non-coding RNAs (lncRNAs) that regulate proximally-located brain-specific genes. Used *in vitro* primary cortical neuron depolarization and *in vivo* mouse fear extinction training to study activity-dependent lncRNA expression. Began to develop a fluorescent-activated cell sorting (FACS) protocol that would allow for sorting only those neurons activated by learning while maintaining RNA integrity. Maintained primary cortical cultures, N2A, and HEK293T cell lines. Dr. Timothy Bredy, Department of Neurobiology and Behavior.

Rotating Doctoral Student Researcher December 2013 – March 2014

Stereotactic surgery for injection of morpholino and siRNA to knockdown expression of the calcium-responsive transactivator (CREST) in the dorsal hippocampus to study its effects on learning and memory. Ran object location memory behavior on mice and scored behavior. Generated PCR primers for both subcloning CREST into a TA vector and for quantifying CREST mRNA with qRT-PCR. Cryosection, immunohistochemistry, and qRT-PCR to analyze CREST knockdown efficiency. Dr. Marcelo Wood, Department of Neurobiology and Behavior.

Rotating Doctoral Student Researcher September 2013 – December 2013

Studied the role of Apolipoprotein E (ApoE) in induced pluripotent stem (iPS) cells and ENStem-A human neural progenitor cells. Generated ApoE shRNA lentiviral constructs for transduction in iPS cells and ENStem-A cells. Ran Western blots, immunocytochemistry, and scratch assays on cultured cells to study the effects of knocking down ApoE. Dr. Mathew Blurton-Jones, Department of Neurobiology and Behavior.

California State University, Long Beach

Graduate Student Researcher December 2010 – September 2013

Western blot and co-immunoprecipitation analysis to study behaviorally relevant neuroendocrine control by estrogen and progesterone in the arcuate nucleus of the hypothalamus. Maintain laboratory. Train undergraduates in molecular biology techniques and manuscript and poster preparation. Prepare posters for presenting at international conferences such as the Society for Neuroscience. Prepare manuscripts for publication. Prepared figures and experiments for an R01 grant. Assist in creating material for an upper division Principles in Neurobiology undergraduate course. Dr. Kevin Sinchak, Department of Biological Sciences.

Thesis: Estradiol dose dependently regulates membrane estrogen receptor-α and metabotropic glutamate receptor-1a complexes in the arcuate nucleus of the hypothalamus

University of California, Davis

Junior Specialist June 2007 – September 2010

Maintained and genotyped multiple mouse colonies, and conditionally knocked-out *Rac1* and *MeCP2* mouse strains using the Cre-lox system. Golgi stained mouse olfactory bulb sections to study dose-dependent *Rac1* dendritic morphology. Paraffin sectioned and cryosectioned brain sections for immunohistochemistry. Maintained 293T and 3T3 cell culture lines for dominant-negative and constitutively active *Rac1* studies. RNA extracted olfactory tissue and RT-PCR to isolate synaptophysin and ligate into lentiviral vectors. Prepared plasmids for cloning. Produced riboprobes for use in *in situ* hybridization. Maintained laboratory. Prepared a pWPI-synaptophysin lentiviral vector construct to visualize the effects of different knockout models on the mouse olfactory system. Prepared posters for presenting at local research conferences such as the Undergraduate Research Conference. Dr. Qizhi Gong, Department of Cell Biology and Human Anatomy.

Senior Research Associate April 2007 – June 2008

Screened and enrolled patients in various clinical studies associated with the emergency room. Interviewed and trained prospective Research Associates. Helped lead a study concerning pulmonary emboli. Abhi Gorhi MBBS, CCRP, Department of Emergency Medicine.

PUBLICATIONS

Vogler, E., Flynn, D., Busciglio, F., Bohannan, RC, Tran, A., **Mahavongtrakul, M.**, Busciglio, J. 2017. Low cost electrode assembly for EEG recordings in mice. *Front Neurosci*. E-pub ahead of print.

Vogler, E., **Mahavongtrakul, M.**, Busciglio, J. 2017. Oligomers at the Synapse: Synaptic Dysfunction and Neuro-Degeneration. In: Protein Folding Disorders of the Central Nervous System (Ghiso, J. and Rostagno A., ed.), *ahead of print*.

Sanathara, N.M., Moreas, J., **Mahavongtrakul, M.,** Sinchak, K. 2014. Estradiol upregulates progesterone receptor and orphanin FQ colocalization in arcuate nucleus neurons and opioid receptor-like receptor-1 expression in proopiomelanocortin neurons that project to the medial preoptic nucleus in the female rat. *Neuroendocrinology* 100(2-3):103-18. **Featured on journal cover.**

Mahavongtrakul, M., Kanjiya, M.P., Maciel, M., Kanjiya, S., Sinchak, K. 2013. Estradiol dose-dependent regulation of membrane estrogen receptor-α, metabotropic glutamate receptor-1a, and their complexes in the arcuate nucleus of the hypothalamus in female rats. *Endocrinology* 154(9):3251-3260.

ABSTRACTS AND PRESENTATIONS

Mahavongtrakul, M., Vogler, E., Tran, A., Nameki, R., Chinn, C., Cox, C., Busciglio, J. Levetiracetam prevents age-related cognitive impairment in a sex-specific manner in mice lacking synaptic zinc. Presented at the 47th Annual Society for Neuroscience Conference

Mahavongtrakul, M., Vogler, E., Yao, J., Tran, A., Stevenson, R., Tran, D., Busciglio, J. Abnormal increased basal EEG activity in the ZnT3KO mouse model of Alzheimer's Disease. Presented at the 46th Annual Society for Neuroscience Conference.

Vogler, E., Wang, X., Michalski, S., Gao, X., **Mahavongtrakul, M.**, Bohannan, R., Chen, J., Busciglio, J. Synaptic zinc deficiency induces neuronal hyperexcitability and impairs adult neurogenesis in the hippocampus of ZnT3KO transgenic mice. Presented at the 46th Annual Society for Neuroscience Conference.

Sinchak, K., Maciel, M., Thach, V., Tea, P., Seng, H., Serey, C., **Mahavongtrakul, M.**, Phan, J. Estradiol increases colocalization of D1 Dopamine receptors and progesterone receptor in the arcuate nucleus of the hypothalamus associated with facilitation of lordosis. Presented at the 44th Annual Society for Neuroscience Conference.

Serey, C.S., Huss, B., Chuon, T., **Mahavongtrakul, M.**, Sinchak, K. The signaling pathways of progesterone receptor, Src kinase, and dopamine D1 receptor converge in the arcuate nucleus of the hypothalamus to facilitate lordosis. Presented at the 44th Annual Society for Neuroscience Conference.

Phan, J., **Mahavongtrakul, M.**, Sinchak, K. Progesterone receptor-B and Src kinase complex in the plasma membrane of the arcuate nucleus of the hypothalamus of female rats. Presented at the 44th Annual Society for Neuroscience Conference.

Phan, J., **Mahavongtrakul, M.**, Sinchak, K. Plasma membrane progesterone receptors complexes with Src but not dopamine D1 receptor in the arcuate nucleus of the hypothalamus. Presented at the 45th Annual American Society for Neurochemistry Conference.

Serey, C.S., **Mahavongtrakul, M.**, Huss, B., Chuon, T., Welborn, A., Ponce, L., Sinchak, K. Progesterone receptor, Src kinase, and dopamine D1 receptor signaling pathways in arcuate nucleus converge to facilitate lordosis. Presented at the 45th Annual American Society for Neurochemistry Conference.

Mahavongtrakul, M., Phan, J., Sinchak, K. Plasma membrane progesterone receptors A and B do not complex with dopamine D1 receptor in the arcuate nucleus of the hypothalamus. Presented at the 43rd Annual Society for Neuroscience Conference.

Mahavongtrakul, M., Maciel, M., Garcia, M.P., Sinchak, K. Estradiol dose dependently downregulates estrogen receptor-α in the mediobasal hypothalamus, but not in the amygdala. Presented at the 42nd Annual Society for Neuroscience Conference.

Estep, J., **Mahavongtrakul, M.**, Chaimanont, C., Gong, Q. Postnatal requirement of Rac1 for mitral cell dendritic development. Presented at the 2012 Annual Association for Chemoreception Sciences Meeting.

Mahavongtrakul, M., Kanjiya, S.M., Garcia, M.P., Charukulvanich, P., Sinchak, K. Estradiol down regulates estrogen receptor-α in a behaviorally relevant and dose dependent manner in the arcuate nucleus. Presented at the 41st Annual Society for Neuroscience Conference.

Mahavongtrakul, M., Gong, Q. Mitral cell dendritic remodeling and the role of Rac1 in the mouse olfactory system. Presented at the 20th Annual Undergraduate Research Conference.

Chaimanont, C., **Mahavongtrakul, M**., Gong, Q. Characterization of olfactory mitral cell specific Cre expressing transgenic mouse strains. Presented at the 20th Annual Undergraduate Research Conference.

Chaimanont, C., **Mahavongtrakul, M**., Gong, Q. Generation of a transgenic mouse model to study the development of the olfactory neuronal connections. Presented at the 19th Annual Undergraduate Research Conference.

GRANTS, AWARDS, CERTIFICATIONS, AND HONORS

University of California, Irvine

UCI MIND T32 AG000096-33 Aging Fellowship 2016 – 2018
Competitive with a monetary award: $1910/month stipend + $500 for travel + $3000/quarter tuition.

GPS-BIOMED Professional Development Certificate (NIH BEST Initiative) 2017

Trainee Professional Development Award (Society for Neuroscience) 2017
Competitive with a monetary award: $1000

Renee Harwick Advanced Graduate Student Award 2017
Competitive with a monetary award: $1000

Center for the Integration of Research, Teaching and Learning Associate 2017

Certification in Course Design 2017

Outstanding Predoctoral Scholar 2017
Competitive with a monetary award: $100

Faculty Research and Travel Fund 2015 – 2016
Competitive grant: $7,797 (co-authored with Jorge Busciglio)

Institute for Clinical and Translational Science 2016
Competitive grant (UL1 TR001414): $4,985 (co-authored with Jorge Busciglio)

Edward A. Steinhaus Teaching Award 2016
Competitive with a monetary award: $750

Pedagogical Fellowship 2016
Competitive with a monetary award: $2000

Certification in Teaching Excellence 2016

Graduate Assistance in Areas of National Need Fellowship 2014 – 2015
Competitive with a monetary award: $3000/quarter tuition

Federal Work Study 2015

Monetary award: $7500 for tuition and fees

Certification in Mentoring Excellence 2014

PEDAGOGY, CAREER, AND EDUCATIONAL DEVELOPMENT

University of California, Irvine

Division of Teaching Excellence and Innovation

Instructional Development Associate June 2017 – January 2018

Work in a team to design new programs and refine existing ones to support graduate students, post-doctoral fellows, and faculty in their teaching and pedagogical development. Programs include the Instructor of Record Training Program, the Developing Teaching Excellence course (UNI STU 390X), the Pedagogical Fellows Program, the Certificate in Teaching Excellence, and the Course Design Certification Program. Facilitate multi-day workshops in Course Design Certification and New Faculty Training Academy. Assist with refining TA orientation programs. Develop program-level assessments and draft a proposal for external funding.

Senior Pedagogical Fellow January 2017 – June 2017

Developed and facilitated workshops in Course Design Certification and Time Management, which was open to graduate students, post-doctoral fellows, and faculty. Held one-on-one teaching consultations.

Pedagogical Fellow January 2016 – December 2017

Prepared for and lead a 12-hour TA Professional Development Program to train TAs in preparing for teaching. Consulted TAs on their teaching. Worked in a team to interview and recruit graduate students into the Pedagogical Fellows Program.

Graduate Professional Success for PhD Students and Postdocs in the Biomedical Sciences

Trainee 2015 – 2018

An NIH-funded Broadening Experiences for Scientific Training (BEST) program focused on professional development and preparing for a variety of careers both inside and outside of academia.

Trainee 2016 – 2017

A competitive teaching apprenticeship in STEM (TAP-STEM) program for formal training in teaching in STEM fields. On a quarterly basis, met with Dr. Brian Sato, a faculty mentor, to discuss pedagogy, and with Bri McWhorter, a communications specialist, to discuss strategies to effectively communicate. Taught Molecular Biology as Instructor of Record during Summer 2017. Involved monetary compensation for teaching.

Member, STEM Ed 2014 – 2016

A monthly seminar series focused on educational and professional issues in STEM fields including implementing active learning, teaching to different class sizes, and different teaching positions at the various levels of higher education.

RELEVANT TEACHING EXPERIENCE

Santa Ana College

Instructor of Record, Fundamentals of Biology Fall 2016-2017, Spring 2016
Instructor of Record, Fundamentals of Biology Lab Fall 2015, Summer 2016
Instructor of Record, Microbiology Laboratory Spring 2016

University of California, Irvine

Instructor of Record, Molecular Biology Summer 2016-2017
Teaching Assistant, Neurobiology Lab Winter 2015-2016, Spring 2015-2016

California State University, Long Beach

Teaching Associate, Principles of Neurobiology Fall 2012
Teaching Associate, Biostatistics Spring 2011, Summer 2011, Fall 2011

INVITED TALKS

University of California, Irvine

Enhancing Your Professional Presence & Career Prep 2017
Hosted by GPS-BIOMED as part of the Peer Seminar series

TA Professional Development Program Prep Panel 2017
Hosted by the Division of Teaching Excellence and Innovation as part of the Pedagogical Fellows Program

Teaching Associate Panel 2015
Hosted by the Division of Teaching Excellence and Innovation as part of the TA Professional Development Program

California State University, Long Beach

History of Endocrinology 2013
Part of Dr. Kevin Sinchak's Endocrinology undergraduate and graduate course

SERVICE

University of California, Irvine

Event Supervisor, Science Olympiad 2016

Judge, Intel International Science and Engineering Fair 2014 – 2015

Volunteer, Los Angeles Brain Bee 2015

Volunteer, Steve Luther Elementary Science Night 2015

University of California, Davis
Science Outreach Instructor, Adventures in Science 2009

RELEVANT SKILLS

Laboratory Techniques: Bacterial cloning, cell culture, stereotactic surgery, Western blots, sectioning, immunostaining, Golgi staining, lentivirus packaging, microscopy, PCR, *in situ* hybridization, co-immunoprecipitation, chromatin immunoprecipitation, calcium imaging, qRT-PCR, behavioral training.

Communication: Active listener, clear speaker, and comfortable speaking in front of a group. Able to write clearly, concisely, and logically. Experience in grant writing and manuscript preparation.

Quantitative and Analytical Reasoning: Able to perform and analyze. Able to develop and troubleshoot protocols for scientific research.

Languages: Read and speak Thai, conversational Spanish.

Computer: Experience in Microsoft Office, Adobe, MiniTab, SigmaStat, Prism, SPSS, and Python.

Work Style: Quick learner, organized, independent, attentive to detail. Self-motivated, team-spirited, cooperative, leader.

RELEVANT COURSEWORK

Neurobiology, Neuroscience, Systems Neuroscience, Molecular Neuroscience, Cellular Neuroscience, Foundations of Neuroscience, Behavioral Neuroscience, Neuroepigenetics, Epigenetics, Genetics, Regulation of Gene Function, Biostatistics, Statistics for Neurobiologists, Experimental Design, Scientific Communication, Systemic Physiology, Advanced Systemic Physiology, Cardiovascular Physiology, Gastrointestinal Physiology, Renal Physiology, Respiratory Physiology, General Chemistry, Organic Chemistry, General Biology, Biochemistry, Bioenergetics, Engineering Calculus, Physics, Endocrinology, and associated laboratories.

PROFESSIONAL MEMBERSHIPS

2015-2018: University of California Graduate Professional Success (UCI GPS)-BIOMED

2010-2018: Society for Neuroscience

ABSTRACT OF THE DISSERTATION

Levetiracetam prevents age-related cognitive impairment in a sex-specific manner in mice lacking synaptic zinc

By

Matthew Mahavongtrakul

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2018

Professor Jorge A. Busciglio, Chair

While it has been known for a while that patients with Alzheimer's Disease (AD) have increased risk of unprovoked seizures, only recently has hyperexcitability become a major focus of AD research. Increased hippocampal activity in patients with mild cognitive impairment (MCI) was initially thought to be a beneficial compensatory mechanism; however, recent research has shown that patients with MCI treated with antiepileptic drugs have improved cognition. Synaptic zinc, co-released with amyloid-β (Aβ) during neurotransmission, is implicated in both oligomer formation and modulation of excitatory neurotransmission. Synaptic zinc is packaged into vesicles by ZnT3; thus, *ZnT3*$^{-/-}$ mice lack synaptically-released zinc. These mice exhibit increased seizure susceptibility, synaptic dysfunction, and age-dependent increases in markers of seizure activity, synaptic loss, and neurodegeneration, strikingly similar to AD mouse models. Although *ZnT3*$^{-/-}$ mice do not exhibit spontaneous convulsive seizures, our data suggest that these mice exhibit epileptiform activity. In addition, our data demonstrate that chronic, but not acute, treatment with the antiepileptic drug Levetiracetam (LEV) prevents age-

related cognitive decline in these mice, suggesting a mechanism of action independent of antiseizure activity. In this regard, the mechanism by which LEV prevents cognitive decline is not understood.

The goal of this project was to begin characterizing the mechanism of action of LEV leading to the prevention of cognitive decline in aged *ZnT3*$^{-/-}$ mice. mRNA was extracted from 6-month-old *ZnT3*$^{-/-}$ and wildtype (WT) sex-matched mice and analyzed using NanoString. Gene analysis indicated sex-specific changes genes involved in epigenetics, neurogenesis, and hyperexcitability following LEV treatment. To investigate the effects of chronic LEV on neurogenesis important for learning and memory, *ZnT3*$^{-/-}$ mice were fed bromodeoxyuridine and immunohistochemistry was performed on brain sections. Although there was an increase in neurogenesis in LEV-treated animals, this increase was restricted to females, further suggesting a sex-specific effect of LEV. Lastly, *ZnT3*$^{-/-}$ mice were implanted with surface electroencephalogram (EEG) electrodes and the results suggest abnormal EEG activity in aged *ZnT3*$^{-/-}$ mice. Taken together, these results suggest that LEV prevents cognitive impairment in *ZnT3*$^{-/-}$ mice in a sex-specific manner at multiple layers of regulation, including epigenetics, neurogenesis, and hyperexcitability.

Chapter 1

Overview

This dissertation seeks to explore the mechanisms by which the antiepileptic drug Levetiracetam (LEV) prevents cognitive impairment. Alzheimer's Disease is the most common form of dementia and is an age-related neurodegenerative disorder that destroys memory and impairs the ability to perform basic tasks. It is currently the 3rd leading cause of death in the United States. Increased hippocampal excitability precedes cognitive impairment, and antiepileptic drugs have been shown to enhance memory in patients with mild cognitive impairment. LEV was originally discovered to be a nootropic drug, but is currently being used as an acute dose for treating seizures. Other laboratories have shown that a chronic low dose of LEV (75mg/kg/day), can prevent cognitive decline in the hAPP mouse model. On the other hand, an acute high dose of LEV (200mg/kg) is typically used to treat seizures. Based on these experimental results, we sought to explore the mechanisms by which the chronic low dose of LEV act on synaptic plasticity to prevent cognitive decline. In our laboratory, we use the *ZnT3*$^{-/-}$ mouse model because these mice lack synaptic zinc and have decreased synaptic function, cognitive impairment, and increased excitability, similar to what is seen in Alzheimer's mouse models. Although it is known that LEV interacts with the synaptic vesicular protein Sv2a, the observation that only the chronic dose of LEV prevents cognitive impairment suggests that chronic LEV may be acting through other mechanisms as well. To understand how chronic LEV prevents cognitive impairment, this dissertation addresses the following three aims:

Aim 1: Investigate the effect of chronic LEV treatment on age-related changes in EEG activity. This was done by developing a novel electroencephalogram electrode and implantation method, followed by spiking and power analysis using Python.

Aim 2: Characterize gene and signaling pathway networks involved in the mechanism of action of chronic LEV in preventing age-related cognitive decline. This was done using NanoString on hippocampal tissue followed by cluster analysis and statistical modeling.

Aim 3: Characterize age-related impairments in neurogenesis in *ZnT3*$^{-/-}$ mice and investigate the effect of LEV on neurogenesis. This was done by orally administrating 5-bromo-2'-deoxyuridine (BrdU) over the course of a week, followed by immunohistochemistry analysis.

Together, the results from these three aims indicate that aged *ZnT3*$^{-/-}$ mice exhibit increased neural excitability and that a low chronic dose of LEV, but not a high acute dose typically used to treat seizures, prevents cognitive impairment in the *ZnT3*$^{-/-}$ mouse model. Chronic LEV enhances neurogenesis only in aged female *ZnT3*$^{-/-}$ mice and modifies gene expression in a sex-specific manner. Future studies will further define the mechanisms underlying the therapeutic effect of chronic LEV treatment on cognition.

General Introduction

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is an age-related, progressive neurodegenerative disorder that is characterized by synaptic and neuronal loss and accumulation of neurofibrillary tangles and senile plaques which are primarily composed of fibrillary amyloid beta (Aβ), produced by proteolytic cleavage of amyloid precursor protein (APP) and triggers pathological changes such as hyperphosphorylation of tau leading to neuronal dysfunction and neurodegeneration (Busciglio et al., 1992, Pike et al., 1992, Busciglio et al., 1995, Geula et al., 1998, Selkoe, 2001). Fibrillization of Aβ is preceded by multiple conformation changes including high molecular weight complex formation of Aβ-derived diffusible ligands (ADDL; Lambert et al., 1998), protofibrils (Nguyen and Hall, 2004), and Aβ oligomers (AβO; Kayed et al., 2003). These intermediate Aβ species are soluble in aqueous buffer and remain in solution after high-speed centrifugation, which is why they are known as "soluble Aβ" (Glabe, 2004, Sakono and Zako, 2010). Soluble Aβ are found in the cerebrospinal fluid (CSF) of AD patients (Kuo et al., 1996, Georganopoulou et al., 2005), can be neurotoxic at low concentrations, and can induce inhibition of long-term potentiation (LTP) and lead to cognitive dysfunction in rodents (Lambert et al., 1998, Hartley et al., 1999, Dahlgren et al., 2002, Walsh et al., 2002, Lesne et al., 2006). In fact, soluble AβO levels are more cytotoxic and correlate much better than the location and number of senile plaques with local neuronal cell death, synaptic loss, LTP inhibition, and cognitive impairment (Terry, 1996, Lambert et al., 1998, Lue et al., 1999, McLean et al., 1999, Naslund et al., 2000, Klein et al., 2001, Walsh et al., 2002, Caughey and Lansbury, 2003, Chiti and Dobson, 2006, Lesne et al., 2006, Ferreira et al.,

2007, Haass and Selkoe, 2007, LaFerla et al., 2007; Figure 1-1). These studies indicate that soluble AβO are likely to play a key role in AD pathogenesis.

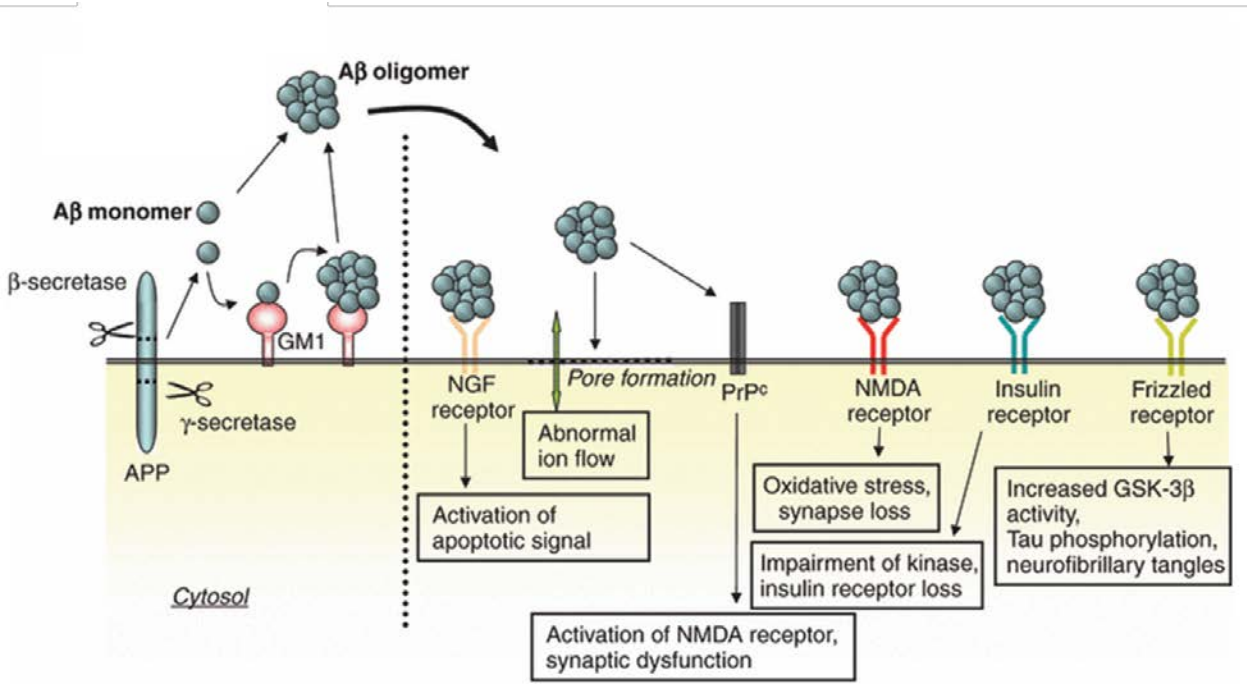


Figure 1-1. **Effects of soluble AβO**. Aβ monomers oligomerize following proteolytic cleavage and can affect many different receptors and physiological processes including apoptosis, ion flow, synaptic dysfunction, oxidative stress, insulin dysregulation, and tau phosphorylation. Adapted from Sakono and Zako, 2010.

1.2 Amyloid Beta Oligomers

AβO range from basic dimers to multimers of 24-mers or even higher molecular weight (Haass and Selkoe, 2007, Glabe, 2008, Roychaudhuri et al., 2009). Although the exact mechanisms by which soluble AβO are formed *in vivo* have not been fully elucidated, sequential proteolytic cleavage of APP by β-secretase (BACE) and γ-secretase results in the production of the 42 amino acid Aβ peptide, which is a soluble monomer that, under pathological conditions, misfolds and aggregates into soluble

prefibrillar AβO and insoluble fibrils. These structures can be recognized by different anti-AβO or anti-fibrillar conformation-specific antibodies (Glabe, 2008).

Aβ can be localized intracellularly by the cleavage of APP by β-secretase and γ-secretase in endosomes generated from the endoplasmic reticulum (ER) or the Golgi apparatus, or by the uptake of extracellular Aβ (Kinoshita et al., 2003, LaFerla et al., 2007). The uptake of extracellular Aβ may occur via various receptors and transporters, such as FPRL1 or RAGE. These complexes are internalized into early endosomes, where Aβ can be degraded. However, Aβ in the lysosome can leak into the cytosol by lysosomal membrane destabilization. Although cytosolic Aβ can be degraded by the proteasomal degradation system, inhibition of the proteasome function by AβO causes cell death (Sakono and Zako, 2010). In addition, AβO can bind rapidly and with very high affinity to synaptic contacts and cellular membranes, inducing rapid and massive neuronal death which may contribute to memory deficits (Deshpande et al., 2006). For example, reduced phosphorylation of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor subunits by AβO blocks extrasynaptic delivery of AMPA receptors and produces memory deficits in the $APP_{Sw,Ind}$ mouse model of AD, which develops age-dependent amyloid pathology and memory deficits (Mucke et al., 2000, Espana et al., 2010, Minano-Molina et al., 2011). In addition, AβO accumulate at synaptic sites in an activity-dependent manner and synaptic targeting of AβO is enhanced by N-methyl-D-aspartate (NMDA) receptor activity (Deshpande et al., 2009), causing abnormal calcium homeostasis leading to increased oxidative stress and synaptic loss (De Felice et al., 2007, Shankar et al., 2007). AβO can impair presynaptic calcium currents at both glutamatergic and γ-aminobutyric acid (GABA)-ergic synapses (Nimmrich et al., 2008). This impairment may

occur through AβO-mediated destabilization of the cell membrane, leading to the abnormal flow of ions (Demuro et al., 2005, Deshpande et al., 2006, Valincius et al., 2008). AβO can also increase calcium influx and elicit cellular changes that are consistent with the activation of a mitochondrial death pathway (Deshpande et al., 2006).

In addition to having effects on NMDA receptors themselves, AβO also colocalize with, and increase the activation of, the NMDA receptor subunit NR2B, induce insulin receptor loss from the neuronal surface, and can impair kinase activity, resulting in LTP inhibition (Snyder et al., 2005, Townsend et al., 2007, Zhao et al., 2008, De Felice et al., 2009, Deshpande et al., 2009, Rammes et al., 2011). Colocalization with NR2B may also facilitate Aβ-promoted endocytosis of NMDA receptors and depress NMDA receptor currents at the synapse (Deshpande et al., 2009). The zinc chelator clioquinol reduces synaptic targeting of AβO, a result that mimics what is seen in mice lacking vesicular zinc (*ZnT3*$^{-/-}$), indicating that vesicular zinc released during excitatory neurotransmission is essential for synaptic targeting of AβO (Deshpande et al., 2009).

1.3 Synaptic Zinc

Zinc is essential for the function of over 1000 proteins, is critical for brain function, and is transported into the brain via the blood-brain barrier and the blood-CSF barrier (Takeda, 2000; Sandstead, 2012). Zinc is released during glutamatergic neurotransmission in an activity- and calcium-dependent manner and can modulate both excitatory and inhibitory neurotransmission (Assaf and Chung, 1984, Deshpande et al., 2009, Vogler and Busciglio, 2014; Figure 1-2), after having been transported into synaptic

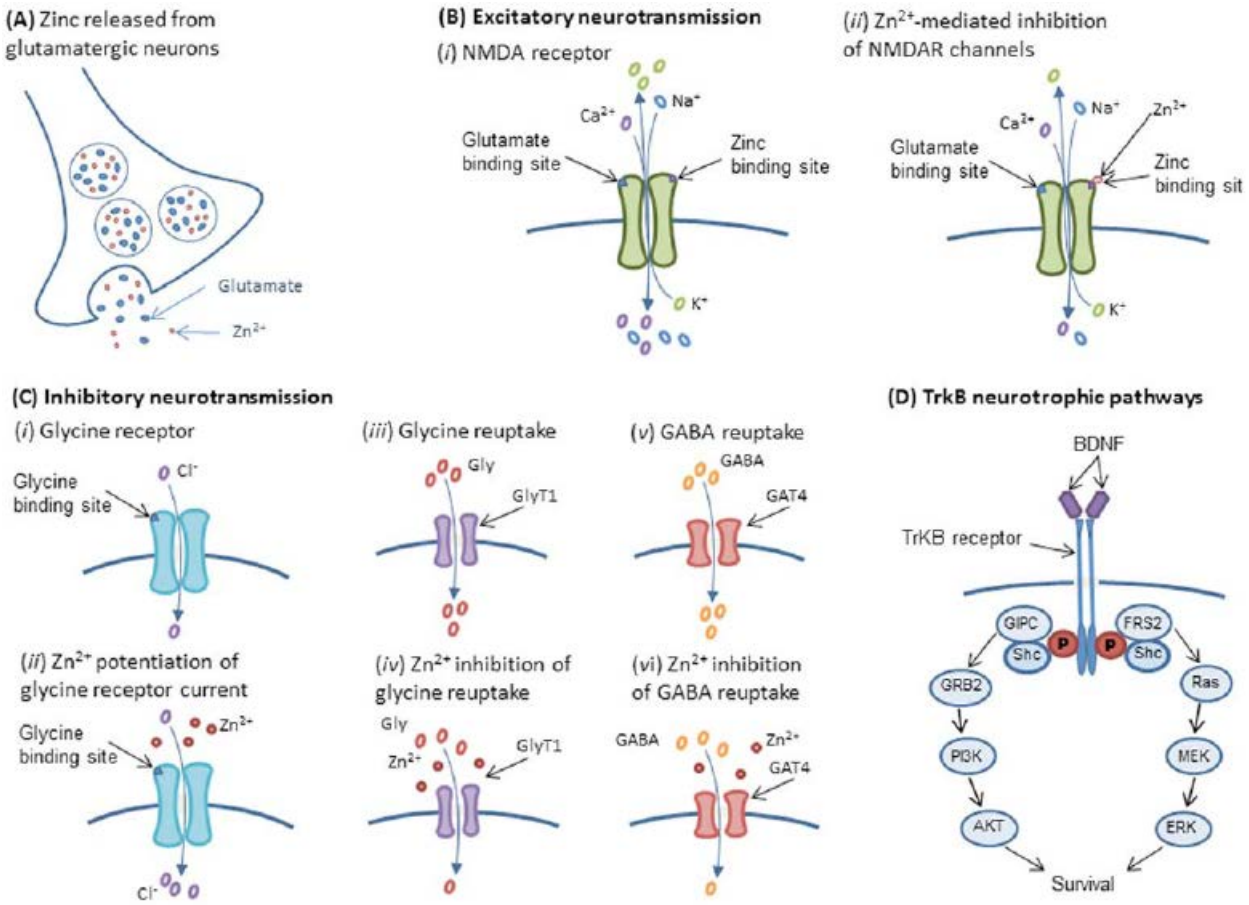


Figure 1-2. **Effects of zinc on both excitatory and inhibitory neurotransmission**. **A)** Zinc is packaged into synaptic vesicles by the ZnT3 protein, where it is released into excitatory synapses. There, it can affect **B)** excitatory neurotransmission through NMDA receptors, or it can affect **C)** inhibitory neurotransmission through glycine and GABA receptors by affecting reuptake of neurotransmitters or potentiating inhibitory current. **D)** Zinc can also modulate learning and memory and synaptic plasticity through activation of the TrkB receptor. Taken from Vogler and Busciglio, 2014.

vesicles by the zinc T3 transporter (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 region where neurodegeneration first appears in AD (Bowen and Davison, 1980, Braak and Braak, 1991, Palmiter et al., 1996). Although vesicular zinc is found throughout the hippocampus, neocortex, amygdala, striatum, and olfactory bulb, zinc expression is most abundant in the mossy fiber terminals of dentate gyrus (DG) cells (Frederickson, 1989, Slomianka, 1992). In fact, Cornu Ammonis region 3 (CA3) neurons,

which are innervated by the mossy fiber tract, contain the highest concentration of zinc (Frederickson et al., 1983), and are the first to be destroyed by kainic acid (KA) administration (Nadler et al., 1978). The distribution of the ZnT3 protein matches what is seen using the Timm stain, and is completely abolished after knocking out the *ZnT3* gene (Palmiter et al., 1996, Wenzel et al., 1997, Cole et al., 1999). Additionally, age-related decreases in ZnT3 protein expression may be involved in cognitive decline (Adlard et al., 2010, Adlard et al., 2015, Tamano et al., 2016). In fact, previous research has shown that: 1) Aβ monomers are present in synaptic vesicles and are released in an activity-dependent fashion at excitatory synapses (Abramov et al., 2009, Sensi et al., 2009, Palop and Mucke, 2010); and 2) Aβ monomers can rapidly form soluble amyloid beta oligomers (AβO) which accumulate at excitatory synapses (Deshpande et al., 2009). Synaptic zinc is implicated in this process because it is also present in synaptic vesicles in excitatory synapses, albeit in different vesicles than Aβ (Sensi et al., 2009; Figure 1-3), and is co-released into the cleft along with Aβ during synaptic transmission. This observation is significant because synaptic zinc modulates several signaling pathways that reduce neuronal excitability (Westbrook and Mayer, 1987, Cohen-Kfir et al., 2005, Erreger and Traynelis, 2008, Vogler

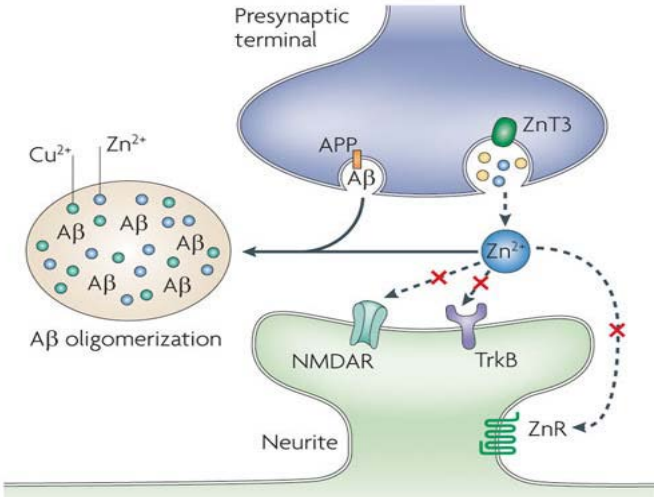


Figure 1-3. **A role for zinc at the synapse**. Zinc is packaged into synaptic vesicles by the ZnT3 transporter and is released into excitatory synapses. Synaptically released Aβ, co-released with zinc during synaptic transmission, can rapidly bind and sequester zinc, leading to a lack of postsynaptic neuromodulation by zinc. Taken from Sensi et al., 2009

and Busciglio, 2014). Thus, the *ZnT3*$^{-/-}$ mouse model recapitulates one key aspect of AD pathology: the lack of neuromodulation by synaptic zinc.

Dysregulation of synaptic zinc has been implicated in neurodegeneration resulting from ischemia, traumatic brain injury (TBI), and seizure activity, with both excessive and reduced levels of zinc resulting in neurodegeneration. Toxicity arising from high concentrations of zinc may occur through intracellular accumulation of zinc after uptake through NMDA receptors, voltage-gated calcium channels, AMPA receptors, kainite receptors, or through transporter-mediated intracellular sodium exchange (Yokoyama et al., 1986, Lees et al., 1990, Freund and Reddig, 1994, Koh and Choi, 1994, Yin and Weiss, 1995, Sensi et al., 1997, Choi and Koh, 1998, Yin et al., 1998). High levels of zinc are released in ischemia and TBI, resulting in the translocation of zinc from presynaptic into postsynaptic neurons, inducing neuronal injury, degeneration arising after prolonged seizures, and death (Sloviter, 1985, Frederickson, 1989, Weiss et al., 1993, Sensi et al., 1999, Suh et al., 2000), which may be prevented by zinc chelators or genetic removal of synaptic zinc (Koh et al., 1996, Calderone et al., 2004, Doering et al., 2010). Interestingly, zinc chelators and zinc deficiency are also associated with increased seizure susceptibility (Noebels and Sidman, 1989, Fukahori and Itoh, 1990, Feller et al., 1991, Mitchell and Barnes, 1993, Kantheti et al., 1998). In addition, *ZnT3*$^{-/-}$ mice have increased sensitivity to KA-induced seizures, suggesting that the overall effect of synaptic zinc is to dampen excitability. Zinc has been found to block augmentation of GABAergic inhibition in DG cells, which may lead to hyperexcitability in the epileptic hippocampus (Buhl et al., 1996). Other studies have shown that *ZnT3*$^{-/-}$ mice have higher incidences of seizures in response to KA, and that the seizures are more severe. The researchers also found that

epileptiform activity persists for more than an hour in *ZnT3*$^{-/-}$ mice, with only occasional behavioral indications that an electrical seizure was actually occurring, suggesting that explicit behavioral seizure severity scores are imprecise at measuring intensity of electrographic seizures (Cole et al., 1999, Qian et al., 2011). Other studies have found that infusion of zinc into the hippocampus of a rat model of epilepsy delays the development of seizures (Elsas et al., 2009), and reducing zinc levels through chelation or through the diet increases excitability in the hippocampus (Lavoie et al., 2007). Zinc chelation also increases hippocampal cell stress markers and cell death after nonconvulsive treatment with KA (Dominguez et al., 2006). KA-infused rat hippocampi are depleted of zinc compared to saline-infused rats, and the serum of KA-infused rats have higher concentrations of zinc compared with saline-infused rats, indicating that during epileptic episodes, hippocampal zinc is depleted (Assaf and Chung, 1984).

Dysregulation of zinc homeostasis has also been linked to the pathogenesis of AD (Bush, 2003, Sensi et al., 2008, Baum et al., 2010), and Aβ neurotoxicity is modulated by zinc, with Aβ-zinc aggregates blocking fast-inactivating outward potassium currents in CA1 hippocampal pyramidal neurons (Zhang and Yang, 2006). Zinc also accelerates Aβ aggregation kinetics (Garai et al., 2007), and zinc overload increases APP processing and cleavage (Wang et al., 2010), which may give rise to higher concentrations of AβO. The link between AD and zinc may be due to the fact that human Aβ has a zinc-binding domain and binding of zinc has been found to induce oligomerization of Aβ that is enhanced by the isomerization of the Asp7 residue of Aβ (a post-translational modification found abundantly in the AD brain), changing the mechanism of Aβ-zinc complex formation (Tsvetkov et al., 2008).

These studies, among others, have implicated synaptic zinc in a variety of physiological processes. Zinc has been shown to differentially modulate NMDA receptors depending on the subunit composition and zinc concentration (Peters et al., 1987, Westbrook and Mayer, 1987, Christine and Choi, 1990, Legendre and Westbrook, 1990, Paoletti et al., 1997, Vogt et al., 2000, Izumi et al., 2006, Erreger and Traynelis, 2008), potentiate AMPA receptor responses via co-release with glutamate (Rassendren et al., 1990, Kalappa et al., 2015), inhibit GABA uptake and GABA-A receptors that lack gamma subunits (Westbrook and Mayer, 1987, Draguhn et al., 1990, Legendre and Westbrook, 1990, Celentano et al., 1991, Smart et al., 1991, Cohen-Kfir et al., 2005), inhibit glycine (Laube et al., 2000, Miller et al., 2005, Zhang and Thio, 2007), inhibit glutamate uptake through the glutamate transporter EAAT-1 (Spiridon et al., 1998, Vandenberg et al., 1998), modulate learning and memory and synaptic plasticity (Sindreu et al., 2011, Takeda et al., 2014), modulate synaptogenesis and synapse maturity (Grabrucker et al., 2011), and antagonize voltage-gated calcium channels (Winegar and Lansman, 1990). In addition, zinc has a high affinity for AβO (Huang et al., 2004, Tougu et al., 2008, Talmard et al., 2009), forming aggregates in less than five milliseconds (Noy et al., 2008).

The link between zinc and AD is further evidenced by the observation that ZnT3 protein levels decline with age and in AD and is crucially involved in learning and memory (Lee et al., 2002, Frederickson et al., 2005, Adlard et al., 2010, Takeda et al., 2014, Takeda and Tamano, 2014), through its interactions with GPR39 (Besser et al., 2009), TrkB (Huang et al., 2008), glutamate receptors (Paoletti et al., 2009), and the p75 pathway involved in caspase-3-dependent apoptosis (Park et al., 2000, Lee et al., 2008). *ZnT3*$^{-/-}$ mice also display cognitive impairment with affected proteins paralleling those involved

with synaptic plasticity, such as SNAP25, PSD95, AMPA receptors, NR2A, NR2B, and doublecortin, and these mice have a decrease in spine density as well (Adlard et al., 2010). Pharmacological treatment with clioquinol, a metal chaperone, restores levels of some of these affected proteins, possibly due to a selective increase in synaptic zinc in the hippocampus (Adlard et al., 2015). *ZnT3*$^{-/-}$ mice treated with clioquinol also have improved cognitive performance and restored LTP compared with untreated controls, indicating that restoration of zinc homeostasis can prevent cognitive impairment (Takeda et al., 2014, Adlard et al., 2015).

1.4 Neurogenesis

The hippocampus is one of the few brain regions where neurogenesis continues to occur in adults (Altman, 1962). Hippocampal neurogenesis structurally and functionally modifies neuronal circuitry that is relevant for formation of memory in adults (Leuner et al., 2006, Deng et al., 2010, Aimone et al., 2014, Yu et al., 2014, Lopez-Virgen et al., 2015). Stem cells located in the subgranular zone (SGZ) of the adult dentate proliferate extensively before migrating to the granular cell layer, where they mature and are incorporated into the existing hippocampal circuitry by sending dendrites to the molecular layer and axons to CA3 (Kuhn et al., 1996, Eriksson et al., 1998, Hastings and Gould, 1999, Markakis and Gage, 1999, Zhao et al., 2006, Suh et al., 2009; Figure 1-4). This occurs through various molecular (Wnt, Notch, Shh, Eph:ephrin signaling, etc.) and epigenetic (DNA methylation, histone modifications, and microRNA, etc.) factors that are known to play a role in AD pathology (Mu and Gage, 2011, Schouten et al., 2012, Faigle and Song, 2013, Fitzsimons et al., 2014, Hussaini et al., 2014, Laussu et al., 2014). For

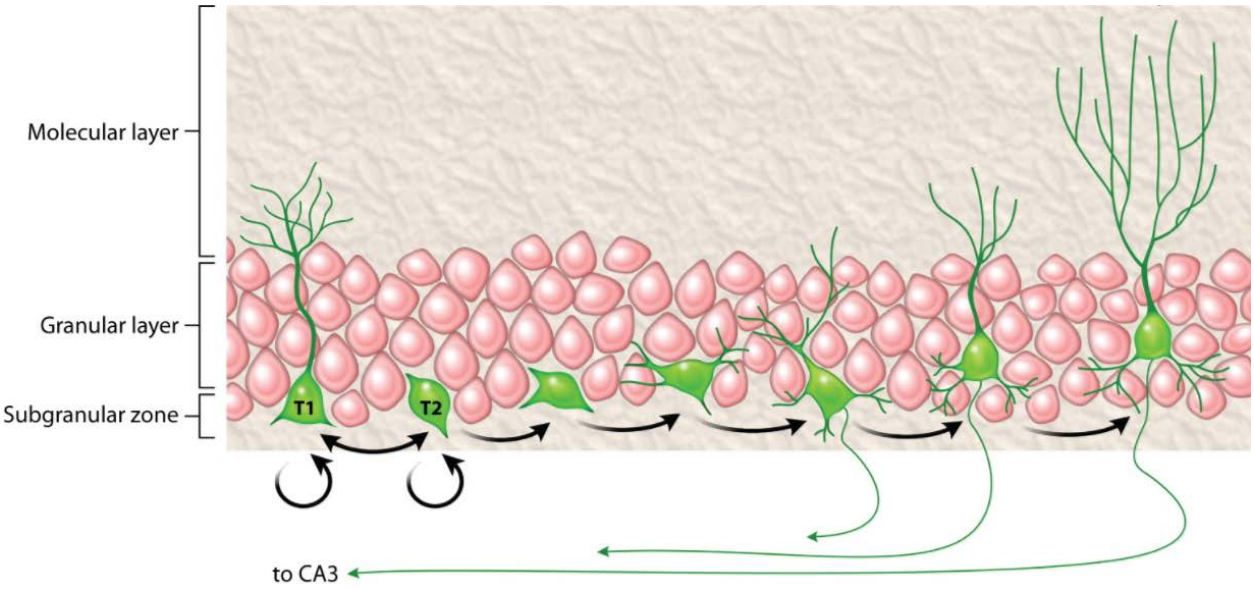


Figure 1-4. **Hippocampal neurogenesis**. Cells in the subgranular zone of the adult dentate gyrus proliferate before migrating to the granular layer, where they begin sending their dendrites into the molecular layer and their axons to CA3 to be integrated into the hippocampal network. Adapted from Suh et al., 2009.

example, DG neural stem cells express β-catenin, which affects proliferation of stem cells, as well as dendritic and axonal development and Wnt signaling protects neurons from neurotoxins such as Aβ through activation of Protein Kinase C (PKC), which phosphorylates and degrades β-catenin and inhibits apoptosis of progenitor cells (Gabuzda et al., 1993, Garrido et al., 2002, Toledo et al., 2008). Another example is the observation that EphB1 is expressed in neural stem cells (NSCs) in the SGZ and controls proliferation, migration, and the polarity of progenitor cells during hippocampal neurogenesis (Chumley et al., 2007). Epigenetics also plays very important roles in neurogenesis. The methyl-CpG-binding domain MBD-1 plays a crucial role in the differentiation of NSCs by binding to, and inhibiting the expression of, FGF2 to inhibit differentiation during adult neurogenesis (Zheng et al., 2004, Singh et al., 2009). Other epigenetic factors which contribute to neurogenesis include histone acetylation (Sun et al., 2007), as well as noncoding RNAs and microRNAs (Horgusluoglu et al., 2016). The

emergence of epigenetics in neuroscience in general has brought to light the importance of looking beyond the genome for therapeutic targets. This emergence is especially important because numerous studies have shown that adult neurogenesis decreases with age in rodents, non-human primates, and humans (Kuhn et al., 1996, Eriksson et al., 1998, Gould et al., 1999, Kornack and Rakic, 1999, Bondolfi et al., 2004, Heine et al., 2004, Shetty et al., 2005, Kronenberg et al., 2006, Montaron et al., 2006, Manganas et al., 2007, Mirochnic et al., 2009, Knoth et al., 2010, Curtis et al., 2011, Jinno, 2011, Couillard-Despres, 2013, Spalding et al., 2013).

While it is not entirely understood how hippocampal neurogenesis affects AD pathology, there have been many studies looking at alterations of neurogenesis and how it relates to AD. Presenilin-1 modulates NSC differentiation in the adult brain, and sAPPα, a product of APP cleavage, regulates proliferation of NSCs (Gakhar-Koppole et al., 2008, Gadadhar et al., 2011). Increased Aβ deposition inhibits hippocampal neurogenesis and impairs maturation of newborn neurons (Mu and Gage, 2011). AD patients have elevated levels of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6, which may reflect NSC proliferation as a compensatory mechanism during neurodegeneration (Swardfager et al., 2010), inhibiting NSC survival, proliferation, and neurogenesis.

Increased hippocampal neurogenesis follows brain insults such as TBI and seizures, and is impaired by injections of the zinc chelator clioquinol or zinc-deficient diets, which can be rescued by zinc supplementation (Corniola et al., 2008, Gao et al., 2009, Suh et al., 2009, Kim et al., 2012, Choi et al., 2014, Han et al., 2015, Cope et al., 2016). Specifically, genetic removal of synaptic zinc using the *ZnT3*$^{-/-}$ mouse model also decreases the number of proliferating progenitor cells and immature neurons in the

hippocampus following hypoglycemia (Suh et al., 2009). Of the progenitors that persist following zinc deficiency, a reduced proportion of those cells actually survive (Corniola et al., 2008), likely due to increased p53-mediated caspase-3-mediated apoptosis and increased production of reactive oxygen species (Gao et al., 2009, Adamo et al., 2010, Seth et al., 2015). The cells that actually survive also do not readily differentiate into hippocampal neurons following zinc-deficiency, as indicated by a decrease in double-labeling of bromodeoxyuridine (BrdU) and doublecortin (DCX)-positive cells (Gao et al., 2009, Suh et al., 2009). The mechanism by which zinc impairs hippocampal neurogenesis involves FOXO3a, a transcription factor involved in metabolism, differentiation, proliferation, survival, stress resistance, and aging (Accili and Arden, 2004, Barthelemy et al., 2004, Han et al., 2015). Our laboratory has recently shown that adult *ZnT3*$^{-/-}$ mice exhibit impaired neurogenesis in an age-dependent manner, as evidenced by decreased BrdU labeling and reduced markers of neuronal-specific precursor cells, in addition to reductions in NSC proliferation and production (Wang et al., *submitted*). This result is important because the present project characterized neurogenesis involved in age-related cognitive impairment and the modulation by chronic treatment of the antiepileptic drug Levetiracetam (LEV), which has been shown to improve cognition.

1.5 Hyperexcitability

Hippocampal hyperactivity is associated with impaired cognition in patients with mild cognitive impairment (MCI), as evidenced by hyperactive blood-oxygen-level dependent (BOLD) activity in the DG/CA3 region of the hippocampus (Yassa et al., 2010). In nondemented older adults, there is increased hyperactivity in the DG/CA3 region

(Putcha et al., 2011), which may precede and predict subsequent cognitive decline (Miller et al., 2008). This hyperactivity is also associated with impairments in pattern separation, which is the ability to differentiate similar but novel information (Yassa et al., 2011). Perturbation of neuronal network activity is an early major contributor to AD pathogenesis and AD patients have increased frequency of epileptic, as well as nonconvulsive, seizures (Dickerson et al., 2005, Celone et al., 2006, Palop et al., 2007, Palop and Mucke, 2009, Sperling et al., 2010, Putcha et al., 2011). In addition, AD patients exhibit cortical hyperexcitability following magnetic stimulation of the cortex (Di Lazzaro et al., 2004). AD patients also have increased inactivity in the prefrontal cortex when performing memory tasks (Grady et al., 2003). As a consequence, these patients are often more susceptible to developing seizures, and seizure susceptibility appears to increase with disease progression, which may contribute to dementia symptoms (Romanelli et al., 1990, Rabinowicz et al., 2000, Mendez and Lim, 2003, Amatniek et al., 2006, Larner, 2010, Nimmrich et al., 2015). Several transgenic AD models of mice also exhibit altered electroencephalographic (EEG) activity, such as altered hippocampal theta oscillations and delta frequency, which may precede cognitive impairment (Palop et al., 2007, Minkeviciene et al., 2009, Harris et al., 2010, Jyoti et al., 2010, Platt et al., 2011, Roberson et al., 2011, Vogt et al., 2011, Scott et al., 2012).

Pathological accumulation of AβO suppresses glutamatergic excitatory synaptic transmission and triggers abnormal neuronal circuit activity and epileptiform activity (Chapman et al., 1999, Hsia et al., 1999, Walsh et al., 2002, Palop and Mucke, 2010). AβO interact with synaptic zinc to modulate many neurotransmitter signaling pathways by being released at excitatory synapses, especially in the hippocampal mossy fiber tract,

which has projections that target GABAergic interneurons in CA3 (Palmiter et al., 1996, Valente and Auladell, 2002, Shen et al., 2007), leading to hyperexcitability and increased hippocampal activity, providing evidence that modulation of synaptic zinc may be able to ameliorate certain aspects related to AD (Vogler and Busciglio, 2014). Zinc reduction increases susceptibility to seizures (Cole et al., 2000), whereas exogenous application of zinc reduces neuronal excitability and delays seizure development in models of epilepsy (Paoletti et al., 1997, Elsas et al., 2009).

In vivo whole-cell patch-clamp recordings and high resolution stimulated emission depletion microscopy have demonstrated that dendritic structure and hyperexcitability are crucially linked (Siskova et al., 2014), probably through alterations in cable properties leading to impaired action potential propagation (Hausser et al., 2000, Magee, 2000, Spruston, 2008). This impairment is especially important because reduced dendritic branching and length are consistently found in the hippocampus and cortex of AD patients (Geula et al., 1998, Adlard and Vickers, 2002, Falke et al., 2003, Spires and Hyman, 2004, Grutzendler et al., 2007), as well as in animal models (Moolman et al., 2004, Grutzendler et al., 2007). The consequences of impaired dendritic structure include not only hyperexcitability, but also abnormal circuit synchronization, which may contribute to cognitive impairment (Palop et al., 2007, Busche et al., 2008, Minkeviciene et al., 2009, Busche et al., 2012, Vossel et al., 2013, Busche and Konnerth, 2015).

3xTg-AD mice, an extensively used preclinical triple transgenic mouse model of AD (Oddo et al., 2003), have enhanced spike frequency and decreased intensity compared with wild-type mice, which may be due to reduced hippocampal expression and functionality of Kv2.1 channels, the most widely expressed Kv channel in the brain

(Murakoshi and Trimmer, 1999, Mohapatra et al., 2009, Frazzini et al., 2016). The mechanism by which this occurs could be due to increased production of reactive oxygen species (ROS), a negative regulator of Kv2.1 from glutamate receptor activation, which can be rescued with antioxidant treatment to reduce hyperexcitability, recapitulating the crucial role of oxidative stress in early stages of AD (Good et al., 1996, Resende et al., 2008, Torres et al., 2011, Cotella et al., 2012, Isopi et al., 2015, Frazzini et al., 2016). This result is an important finding because Kv2.1 channels play a role in neuronal apoptosis and control excitability because they have very slow kinetics, which prevent complete channel deactivation upon sustained neuronal activation (Pal et al., 2003, Shah et al., 2014). In addition, chronic administration of pyruvate, an energy substrate shown to have neuroprotective properties, has been shown to inhibit both short-term and long-term memory deficits in 3xTg-AD mice and reduce hyperexcitability and oxidative stress (Isopi et al., 2015).

The Tg2576 mouse model of AD displays early expression of seizure markers, increased susceptibility to kindling (Chan et al., 2015), and hypersynchronous network activity, which may contribute to memory deficits (Bezzina et al., 2015). These mice also exhibit increased seizure susceptibility following GABA receptor antagonism and hypersynchrony at 1.5 months of age, which precedes behavioral memory impairment (Bezzina et al., 2015). In addition, the lateral entorhinal cortex, one of the first brain regions to exhibit AD pathology (Braak and Braak, 1996, Braak et al., 2011), in these mice exhibit early hyperexcitability in 3- and 6-month old mice, as shown by local field potentials and single-unit spontaneous activity (Xu et al., 2015). The mounting evidence of hyperexcitability preceding cognitive impairment raises the tantalizing possibility of pre-

emptively treating hyperexcitability, which may prevent cognitive impairment. The present project examined the effects of chronic LEV treatment on excitability in the *ZnT3*$^{-/-}$ mouse model of AD, which may lead to prevention of cognitive decline.

1.6 Anti-Epileptics, Selective Serotonin Re-uptake Inhibitors, and Levetiracetam

Therapies for AD targeting Aβ are being tested in clinical trials (Mucke, 2009, Palop and Mucke, 2010, Golde et al., 2011, Huang and Mucke, 2012); however, strategies to enhance clearance of Aβ have been associated with serious side effects that may be unrelated to the reduction of Aβ (Golde et al., 2010, Golde et al., 2011). In addition, current symptomatic therapeutics, such as acetylcholinesterase inhibitors and memantine (an NMDA receptor antagonist) only modestly improve symptoms, and the improvements are only temporary and do not significantly alter disease progression (Schneider et al., 2011). Dimebon, an antihistamine that also has been thought to have neuroprotective properties, showed promising phase 2 clinical trial results, but failed to show efficacy during phase 3 clinical trials (Doody et al., 2008, Jones, 2010). Another target for therapeutics is tau, but the desired effect (decreasing hyperphosphorylation, preventing aggregation, blocking proteolysis) has yet to be defined (Salloway et al., 2008, Golde et al., 2010). In addition, the mechanistic link between Aβ and tau pathology has yet to be established, which not only makes creating animal models difficult, but also makes understanding the biological connection between Aβ and tau pathology challenging (Golde et al., 2011).

Other approaches for therapeutic intervention of AD include targeting BACE1, the enzyme responsible for Aβ generation. However, reports have been inconsistent with

correlating the level of BACE1 with AD progression, with some studies indicating higher BACE1 in AD (Yang et al., 2003, Verheijen et al., 2006, Zhong et al., 2007, Ewers et al., 2008, Zetterberg et al., 2008), or lower BACE1 in AD (Wu et al., 2008). Nonetheless, BACE1 inhibitors have entered clinical trials, but have had limited success and/or severe side effects such as retinal, neuronal, and glial cell degeneration, and liver toxicity (May et al., 2011, May et al., 2015). Other BACE1 inhibitors have had more success and have advanced to phase 3 clinical trials that may be completed in 2019. These advanced phase trials seek to understand the dosage and effect of the inhibitor at different stages of AD progression (Forman et al., 2012, Forman et al., 2013).

The FDA-approved antiepileptic drugs Ethosuximide, Gabapentin, Phenytoin, Pregabalin, Valproic Acid, and Vigabatrin have previously been shown to be effective in humans and animal models with epilepsy (Bialer and White, 2010). However, these drugs do not reduce spiking activity in hAPP mice (Sanchez et al., 2012). In fact, Phenytoin and Pregbalin exacerbates spiking in hAPP mice (Sanchez et al., 2012). In contrast to these drugs, Levetiracetam (LEV) decreases spiking activity in the cortex and hippocampus in a dose-dependent manner and rescues LTP deficits (Sanchez et al., 2012). In addition, chronic, but not acute, treatment with LEV prevents age-related cognitive decline in hAPP mice (Sanchez et al., 2012). The differences in mechanism of action of LEV compared with these other drugs is unclear. The effects of chronic versus acute doses of drugs has been well-studied in depression, with selective serotonin reuptake inhibitors (SSRIs) requiring at least 2-4 weeks of administration before therapeutic effects are achieved, despite the fact that serotonin levels rapidly rise after acute treatment with SSRIs (Rutter et al., 1994, Kreiss and Lucki, 1995, Wong and Licinio, 2001, Anderson et al., 2005, Wang

et al., 2008). Chronic, but not acute, treatment with the SSRI fluoxetine reduces anxiety and depressive symptoms in mice (Dulawa et al., 2004), stimulates hippocampal neurogenesis (Mendez-David et al., 2014), and enhances dendritic maturation of adult neurogenic granule cells (Wang et al., 2008). These results indicate that stable changes in neuroplasticity can be achieved through chronic effects of drugs, which was explored in this dissertation.

The anti-epileptic drug Levetiracetam (LEV, also known as Keppra) has been approved in the United States to treat certain types of seizures with relatively mild side effects, which are often reversed upon discontinuation (Gambardella et al., 2008). It is an enantiomer of piracetam, which was initially investigated for its nootropic effects, which are cognition-enhancing effects for which no commonly accepted mechanism of action has been established (Gouliaev and Senning, 1994). Around the same time, LEV was found to be an atypical anticonvulsant in that there were no obvious mechanisms for which it could suppress seizures (Gower et al., 1992), affecting cholinergic function (Wulfert et al., 1989), GABA function (Margineanu and Wulfert, 1995, Loscher et al., 1996), and calcium channel function (Niespodziany et al., 2001, Zona et al., 2001, Lukyanetz et al., 2002).

Further research has shown that LEV also has beneficial effects for other syndromes including Tourette's Syndrome (Martinez-Granero et al., 2010), anxiety (Farooq et al., 2009), depression (Husum et al., 2004, Ookubo et al., 2013), and AD (Sanchez et al., 2012). In addition, LEV has anti-inflammatory properties through normalization of astrocytic properties by restoring membrane current kinetics to normal and promoting TGFβ1 activation, a cytokine often overproduced during inflammation, and

inhibiting IL-1β, a proinflammatory cytokine that is upregulated following epilepsy, in the hippocampus (Wan and Flavell, 2007, Haghikia et al., 2008, Zou et al., 2013). However, whether or not the alterations in gene expression are enough to alter disease progression has not been consistent (Thone et al., 2012).

Although the main use of LEV is to treat certain types of epilepsy, the exact mechanisms by which LEV acts are not fully characterized. LEV has been shown to interact with synaptic vesicle glycoprotein 2A (SV2A) and reduces neurotransmitter release by inhibiting presynaptic calcium channels to block action potential propagation (Lukyanetz et al., 2002, Lynch et al., 2004, Rogawski, 2006, Vogl et al., 2012). For example, LEV modifies different aspects of neurotransmission, including GABA synthesis, degradation, and turnover in the striatum, which alters neuronal activation of projections to the substantia nigra pars reticulata, a critical structure that protects against certain types of seizures (Loscher et al., 1996). In addition, LEV partially reduces glutamate-induced excitotoxicity and increases GABAergic inhibition in a chronic rat seizure model (Ueda et al., 2007). Microarray analysis on temporal lobe tissue from a rat kindling model treated chronically with daily LEV has revealed that epilepsy-related genes such as NPY, TRH, and GFAP are partially normalized following treatment (Gu et al., 2004). LEV also has neuroprotective properties, protecting brain tissue following middle cerebral artery occlusion, a model of cerebral ischemia (Hanon and Klitgaard, 2001).

When given orally twice daily over the course of two weeks, patients with amnestic mild cognitive impairment exhibit decreased DG/CA3 hyperactivity as analyzed with BOLD imaging compared with the placebo-treated group, and have improved performance on a memory scanning task, which is designed to test pattern separation

and completion (Bakker et al., 2012, Bakker et al., 2015). In rodents, LEV treatment suppresses neuronal network dysfunction and improves cognitive impairment in a dose-dependent manner (Koh et al., 2010, Sanchez et al., 2012), possibly through normalizing the amount of DNA double-strand breaks (Suberbielle et al., 2013), or through decreased Aβ production and increased Aβ clearance by inhibiting GSK-3β activation and enhancing AMPK/Akt signaling (Shi et al., 2013). LEV also increases acetylation of histone H4 (Eyal et al., 2004, Shi et al., 2013), which is important because increasing acetylation by inhibiting histone deacetylase (HDAC) activity is known to facilitate the formation of long-term memory (Stefanko et al., 2009). In astrocyte cultures, LEV increases the expression of brain-derived neurotrophic factor (BDNF) in a dose-dependent manner (Cardile et al., 2003). *In vivo*, LEV prevents changes in BDNF and NPY levels in the hippocampus of a rat model of amygdala kindling, providing further evidence for a potential mechanism by which LEV prevents cognitive decline and prevents seizure (Husum et al., 2004). In our laboratory, chronic, but not acute, treatment with LEV prevents cognitive impairment in *ZnT3*$^{-/-}$ mice, which also exhibit age-dependent decreases in BDNF and markers of seizure activity (Vogler et al., *submitted*). This suggests that LEV may be acting through an SV2a-independent mechanism that is not necessarily related to its antiepileptic properties to improve cognition, which was explored in this dissertation.

Hypothesis and Aims

Hippocampal hyperexcitability precedes cognitive impairment in Alzheimer's Disease (AD) and antiepileptic drugs can enhance memory. In the clinic, a high acute dose of the antiepileptic drug Levetiracetam (LEV) is used to treat seizures in patients. However, other laboratories have shown that in hAPP mice it is the chronic low dose of LEV that prevents cognitive impairment. Our laboratory utilizes the *ZnT3*$^{-/-}$ mouse model which lacks synaptic zinc. These mice exhibit increased age-related hippocampal excitability and are more susceptible to seizures. They also exhibit synaptic loss, neurodegeneration, increased markers of seizure activity, and impaired cognition, similar to what is seen in AD patients. Since chronic LEV prevents cognitive impairment in hAPP mice and *ZnT3*$^{-/-}$ mice exhibit increased hippocampal excitability, the projects in this dissertation tested the hypothesis that chronic LEV prevents cognitive impairment in *ZnT3*$^{-/-}$ mice through changes in gene expression and enhancing neurogenesis. The specific aims were to assess chronic LEV on electroencephalogram activity, neurogenesis, and gene expression:

Chapter 2 describes a novel electroencephalogram (EEG) electrode and implantation method to obtain robust EEG data in mice. This method is an alternative to using wired devices which could have a lot more bulk, screw implantation methods which could interfere with signal, and smaller implantation devices which may not be able to record for longer periods of time accurately.

Chapter 3 demonstrates the effects of chronic LEV on hippocampal-dependent behavior, excitability, neurogenesis, and gene expression. This was done using the object location memory task to assess memory, EEG electrode implantation

and custom Python scripts to analyze spiking and power, BrdU administration to assess neurogenesis, and NanoString to characterize gene expression.

Chapter 2

Low Cost Electrode Assembly for EEG Recordings in Mice

This work has been published in Frontiers in Neuroscience 10.3389/fnins.2017.00629

October 30th, 2017

2.1 Abstract

Wireless electroencephalography (EEG) of small animal subjects typically utilizes miniaturized EEG devices which require a robust recording and electrode assembly that remains in place while also being well-tolerated by the animal so as not to impair the ability of the animal to perform normal living activities or experimental tasks. We developed simple and fast electrode assembly and method of electrode implantation using electrode wires and wire-wrap technology that provides both higher survival and success rates in obtaining recordings from the electrodes than methods using screws as electrodes. The new wire method results in a 51% improvement in the number of electrodes that successfully record EEG signal. Also, the electrode assembly remains affixed and provides EEG signal for at least a month after implantation. Screws often serve as recording electrodes, which require either drilling holes into the skull to insert screws or affixing screws to the surface of the skull with adhesive. Drilling holes large enough to insert screws can be invasive and damaging to brain tissue, using adhesives may interfere with conductance and result in a poor signal, and soldering screws to wire leads results in fragile connections. The methods presented in this article provide a robust implant that is minimally invasive and has a significantly higher success rate of electrode implantation. In addition, the implant remains affixed and produces good recordings for

over a month, while using economical, easily obtained materials and skills readily available in most animal research laboratories.

2.2 Introduction

The use of animals to model human disease pathology has required the development of technology to investigate the effects of experimental interventions in subjects for which existing equipment designed for imaging, recording, or measuring physiology of humans are inappropriate due to the differences between humans and animals in size, other physical attributes, and compliance with equipment requirements. One such physiological recording is electroencephalography (EEG), the recording of changes in electrical potentials at the surface of the brain through scalp or skull, which are a result of ion flow across neural membranes and a measure of neuronal activity (Petsche et al., 1984). EEG is commonly used in the clinical study and diagnosis of medical disorders, and with the advent of computer quantitative analysis, is also used to quantify the effects of pharmacological, dietary, or genetic alterations in research studies (Shipton, 1975, Bronzino, 1984).

The process for EEG in humans involves attaching electrodes directly to the scalp with an adhesive or wearing a cap with the electrodes attached, with wires connecting the electrodes to the recording equipment. This process benefits from a compliant subject and limits mobility and so is difficult to use in experiments requiring awake and mobile animal subjects. Advances in miniaturization of recording equipment have resulted in wireless EEG recording devices that can be implanted in the animal or mounted on the animal's head, providing mobility for the animal during recording and allowing recording

for hours and even days (Higashi et al., 1979). Such devices incorporate either telemetry or data saved to a microchip and typically require inserting screws into holes drilled into the skull, affixing screws with cyanoacrylate glue to the surface of the skull, or attaching a pre-fabricated headmount with screws and dental cement, which require lengthy and invasive surgical procedures and costly materials. The screws or headmounts are frequently connected to wires by soldering or glue, which can form fragile connections or interfere with conductance of the electrical signal.

In our initial experiments using the Neurologger device (TSE Systems) to record EEG in mice, we used the protocol and electrode material provided by TSE Systems, which calls for soldering and screws, either inserted in drilled holes or glued on the surface of the skull. The TSE protocol also calls for removal and re-insertion of the pins of the screw leads into the connecting block while the animal is under anesthesia, which increases the risk of damage to the soldered connections and results in a lengthy surgical procedure. Using this protocol, we experienced a significant failure rate, typically failed connectivity between neural tissue and recording device with one or more electrodes, and decreased tolerability of the lengthy surgery in aged mice.

Consequently, we developed a new method that combines fabrication of a simple wiring harness with insulated silver-plated copper wire electrodes, which eliminated the screws, solder, and glue in the electrode assembly and eliminated the process of re-inserting the pins into the connector during surgery. This method results in electrodes with direct contact of brain tissue and minimal headmount apparatus. The headmount is well-tolerated and will remain on the animal for a month or more and, most importantly, these procedures simplify the implantation process, resulting in a quick and efficient

surgery that minimizes discomfort to the animal and promotes swift recovery. The procedures were designed to utilize laboratory and surgical equipment customarily found in animal research facilities and readily available tools and materials. It greatly facilitates implementation of wireless EEG recording devices in animal research.

2.3 Materials and Methods

All procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Materials for constructing and implanting the electrode assembly are readily available from electronic supply stores or online.

2.3.1 Preparing the components for the electrode connector assembly

The electrode assembly has three main components: the 6-pin connector block, the posts that connect the block to the leads, and the leads with the recording electrodes at their tips (Figure 2-1). The first step is to make the 6-pin connector block from the supplied 25-pin connector block. To do this, count out 7 pins, giving you one sacrificial pin, and then cut between the 7th and

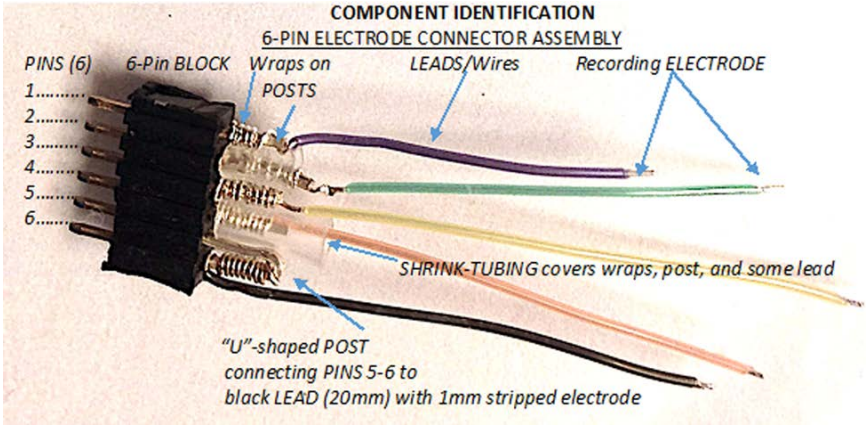


Figure 2-1. The 3 main components of the 6-pin electrode connector assembly. The 6-pin electrode connector assembly is comprised of the 6-pin block, the posts, and the recording leads/wires, 1 of which serves as the ground-reference electrode and 4 that serve as the ADC electrodes.

8th pin of the larger 25-pin connector block. Trim the excess pin and material so you have 6 pins comprising one 6-pin block.

Then prepare posts, which are created from the legs of standard 3mm LEDs. The diameter of each LED leg is 0.5mm and fits securely in the holes of the pin block. Use sand paper or an abrasive sponge to clean the oxidation from the LED's legs. An electronic designer breadboard holds the LEDs and components for the construction process. Cut the LED component off as scrap, leaving two 20mm-long LED legs to be used as posts to connect the leads into the 6-pin connector block. After creating the posts, the next step is to secure the lead-to-post connection using the "wire-wrap" process. There are 2 separate types of leads. One lead will serve as the ground and reference lead and will connect to 2 pins in the connector block. The remaining 4 leads are ADC or signal leads and each connect individually to a single post and single pin in the connector block.

2.3.2 Building the ground-reference lead

Making the ground and reference lead, which has 1 lead for 2 pin connections, requires only 1 post made from 1 LED leg. Fully insert 1 post into the designer's breadboard. To wire-wrap the lead wire to the post, strip a 25mm length of black insulated wire and insert the stripped section into the small hole at the outer-edge of the wire wrap tool. With the stripped section of the wire fully inserted into the edge hole of the tool, place the center hole of the wire wrap tool completely over the post held in the breadboard until the tool rests on the breadboard. Holding the insulated section of the wire onto the breadboard, slowly twist the tool to wrap the stripped section of wire securely around the

post. When done accurately, the wraps will be neat and close together and there will be no insulated wire wrapped around the post.

Unique to the ground and reference lead, the connection is to 2 pins of the 6-pin block. Thus, the single post is bent into a tight “U” with the wrap at the apex of the curve. To accomplish this, hold the post with needle-nose pliers at the wraps. Then bend the post into a tight “U”. After bending into the tight “U”, the black lead is now functionally connected to two parallel lengths of post which, when trimmed to “pin-length,” will be inserted into holes 5 and 6 in the pin connector. Pinch the “U” close together so it matches the width of the receiving holes in the 6-pin block. For the pins and block to connect properly, approximately 3mm of post is exposed beyond the wraps. This is approximately the same length as the pins that are extending from the opposite side of pin block. Insert the 2 posts of the “U” into the last 2 holes in the 6-pin block corresponding to pins 5 and 6. Make sure to insert the posts solidly into the pin block. Check for continuity between pins 5 and 6 using a digital multi-meter to insure a mechanical connection between the lead wire and both pins after insertion.

Shrink tubing on the exposed lead at the wire wrap provides 2 things: (1) Electrical insulation from neighboring wrapped posts, and (2) Strain-relief for the wire lead. Cut a small (approximately 5mm) piece of shrink tubing - just enough to cover the exposed wraps and a small section of insulated wire. Slide the shrink tubing over the exposed lead and wrapped section of the post going into the pin block. Use a micro-torch to shrink the tubing onto the inserted post’s wrap and lead. Strip the insulation from the lead to leave approximately 10mm of insulated wire beyond the wrap. Just beyond the insulation at the opposite end of the lead, trim to approximately 0.5mm of uninsulated lead. This will be

the recording electrode that will be gently implanted into the hole drilled into the mouse's skull. Be careful to firmly hold only the lead when stripping insulation.

2.3.3 Building the analog-digital channel (ADC) leads

The next step is to make the 4 ADC leads. Since 4 leads are required for each assembly, prepare 4 posts from the legs of 2 LEDs. Each ADC lead will each have only 1 lead connected to 1 post to be inserted into 1 hole in the 6-pin block. It is helpful during the surgical process to differentiate ADC leads by differing insulation color. Follow the same wire wrapping procedure as before for each ADC lead. Wire wraps should be tight and evenly spaced. For ADC leads, trim and remove the post above the wire wrap, where the insulated section of wire begins, so the wraps are on the post where the wire lead's insulation begins. Trim the post below the wire wraps to create approximately 3mm of exposed post below the wraps. Insert the 3mm of exposed post into hole 4 in the pin block adjacent to the ground-reference lead in holes 5 and 6 until it is completely and securely inserted. Strip the trailing end of the lead to have approximately 10mm of insulated wire extending from the wrapped post. Trim the stripped section of lead to leave only 0.5mm of uninsulated wire at the end of the lead to serve as the recording electrode. Repeat that process for the remaining 3 ADC leads, placing each new lead into the next adjacent hole. Putting shrink tubing on every post and wrap is not possible due to size constraints. Insulating alternating post-wraps can ensure insulation and strain relief between posts. Check continuity to make sure that electrical connections between each pin of the block and the end of each lead are intact. Confirm that only the pins that are connected to the leads have continuity and that there are no shorts or connections between adjacent pins,

wraps, or electrodes. Sterilize the completed assembly with a hard surface disinfectant such as Cetylcide-II per manufacturer's directions. Do not autoclave the assembly.

2.3.4 Preparing for surgical implantation of electrodes

Prepare and anesthetize the mouse for surgery, then place the mouse into a stereotaxic device. Lift a section of the scalp with forceps to make an incision through the raised area. Then cut a teardrop-shaped piece of scalp encompassing the electrode implantation sites, removing the scalp and any underlying tissue to expose bare skull. Swab the exposed skull with 70% ethanol to remove any remaining tissue and clean the skull. Locate bregma or any other reference point you will use to determine the stereotaxic locations. Insert a marking pen into the stereotaxic device, center the pen on bregma (or your point of choice), and use the micrometer function of the stereotaxic device and pen to locate and mark the electrode implantation sites relative to your reference point. Using a sterilized drill bit of the same diameter as the electrodes, drill holes just penetrating the skull at each marked electrode location, careful not to pass into the brain. The skull will discolor as the drill penetrates. Remove the drill if any blood appears or when there is a sudden drop in resistance as this indicates penetration of the skull. Swab skull material off the drill bit with a sterile tissue or gauze with 70% ethanol after drilling each hole. Swab off any blood so it does not coagulate and plug the hole.

2.3.5 Implanting the electrodes

Place the electrode assembly in the desired location over the back of the mouse, keeping the pin block parallel to the mouse's back, and use forceps to bend the electrode

wires to align with the drilled holes. Load a gel-loading tip on a 20µl pipettor with Vetbond by depressing the pipettor plunger, inserting the gel tip into the Vetbond bottle, inverting the bottle and flicking it gently to get the adhesive to settle into the tip of the bottle and then drawing the Vetbond into the gel tip with the pipettor plunger. The Vetbond will stay fluid in the tip for at least 10-15 minutes. Use bent toothed forceps to insert the electrode wires into the drilled holes, then secure each wire to the skull with 2-3µl of Vetbond. Take care not to allow Vetbond to get into the mouse's eyes or flow into empty drilled holes. Keep a sterile cotton swab handy to wipe up any excess Vetbond. Repeat the insertion and securing process for the remaining electrodes.

2.3.6 Affixing the electrode assembly to the mouse head

Use dental cement to further secure the electrodes in the skull and to build a pedestal to support the recording device. Mix resin into the cement powder and apply the cement around all the electrodes, covering the entire exposed skull and taking care not to allow the cement to get in the subject's eyes. Keep a sterile cotton swab handy to catch any dripping cement. Position the pin connector block in the desired location and completely cover all leads between the skull and the connector block by applying subsequent layers of dental cement, creating a secure pedestal of dental cement to support and secure the pin connector. Allow the cement to set up between layers and to prevent the formation of holes or gaps which could allow the mouse to snag and pull off the assembly. It is critical that the supporting cement places the pin connector centered on the mouse's midline and high enough that the recording apparatus will not rub the animal's back or become dislodged if the animal has a seizure as a result of anesthesia

administration. Check that the dental cement has set, then secure the seam between the cement and the scalp with a thin line of Vetbond. Check that the cement and Vetbond forms a seal all around the scalp incision. Test the continuity between the reference and ground electrodes to ensure that the connections of the electrode assembly were not compromised during the implantation process. This completes the surgical process.

2.3.7 Attaching the recording device

The recording device can be attached to and removed from the implanted electrode assembly without anesthesia on animals that can be held securely. Aggressive animals may need to be briefly anesthetized for attachment and removal. Subsequent recordings can be made during the course of a month or more.

2.4 Results

Twenty-four mice were implanted with screw electrodes and 24 mice were implanted with wire electrodes. Each animal was implanted with 4 recording electrodes placed at -1.34 A/P, +1.50 L; -1.34 A/P, -1.50 L; -3.5 A/P. +3.00 L; -3.50 A/P. -3.00 L and one ground/reference electrode place at -6.00 A/P, +0.50 L relative to bregma. A recording electrode implantation was considered successful if the electrode recorded data consistent with the other electrodes and was considered unsuccessful if it showed periods with clipped signal both at minimum and maximum voltage, no change in potential and/or a 60 Hz mains hum.

The new wire method of electrode assembly and implantation resulted in a 51% increase in the number of successfully implanted electrodes, from an average of 2.5 of

the 4 recording electrodes per mouse providing signal with the screw method to an average of 3.8 of the 4 recording electrodes providing signal with the wire method (Figure 2-2). An unsuccessful implantation typically results in a clipped signal (Figure 2-3A) with a 50 or 60 Hz mains hum from background electromagnetic emissions (Figure 2-3B).

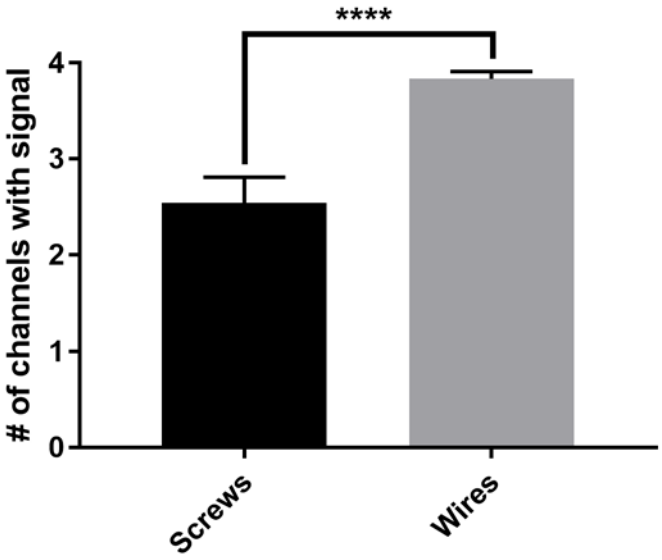


Figure 2-2. The average number of successful electrode implantations increases 51% with the wire method. The new wire electrode method resulted in an average of 3.8±0.08 of the 4 recording electrodes implanted per mouse returning analyzable signal, compared to an average of 2.5±0.27 of the 4 recording electrodes implanted per mouse with the screw electrode method, an increase of 51% in successful implantations as measured by individual implanted electrodes. Screws, n=24 mice; wires, n=24 mice, ****p<0.0001. Data were analyzed by two-tailed unpaired t test. Error bars indicate the mean ±SEM.

This method also results in an implantation that is well-tolerated by the animal for weeks and provides the opportunity for repeated recordings after experimental interventions. EEG traces recorded from the same animal 4 days after electrode implantation (Figure 2-4) and 1 month after the first recording (Figure 2-5) indicate no degradation of the signal over that time period, demonstrating the robustness of the electrode implantation process including the dental cement headmount. There were 4 separate 2-day recording sessions, 1 each week during the month, for a total recording time of over 200 hours. The subject mouse ate, nested, and groomed normally during the course of the month and was caged individually to prevent cage mates from chewing on the assembly.

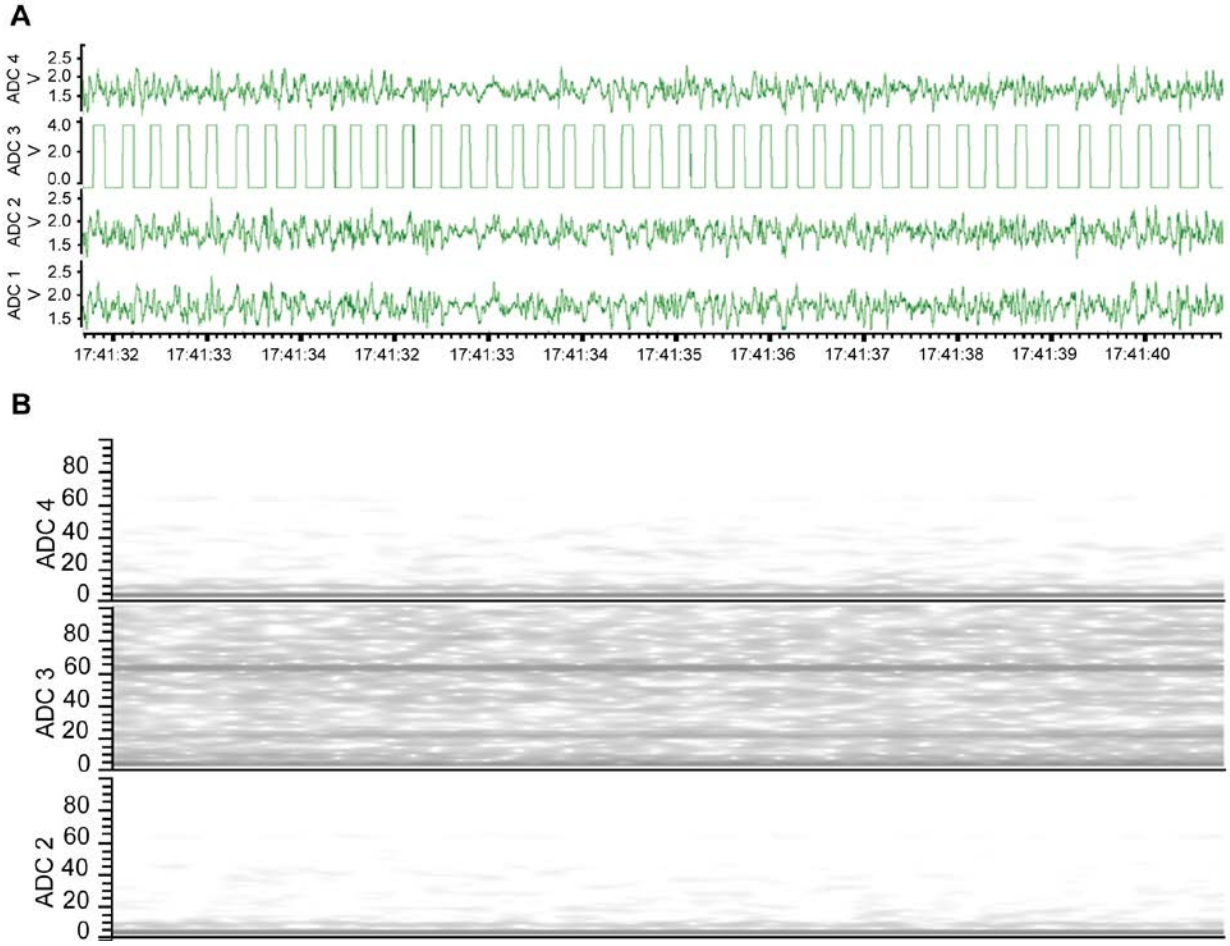


Figure 2-3. EEG of electrode implantation failure. (A) Waveform representation of the analog signal illustrates a clipped signal from the improperly implanted electrode of ADC2 as the recorded signal exceeded the Neurologger's EEG-specific signal magnitude capability. **(B)** Time-frequency representation of the analog signal indicates the continuity between the electrode and recording device failed with electrode of ADC2, resulting in no EEG signal recorded. Consequently, the graph has a band at 60 Hz recorded from other electrical equipment present.

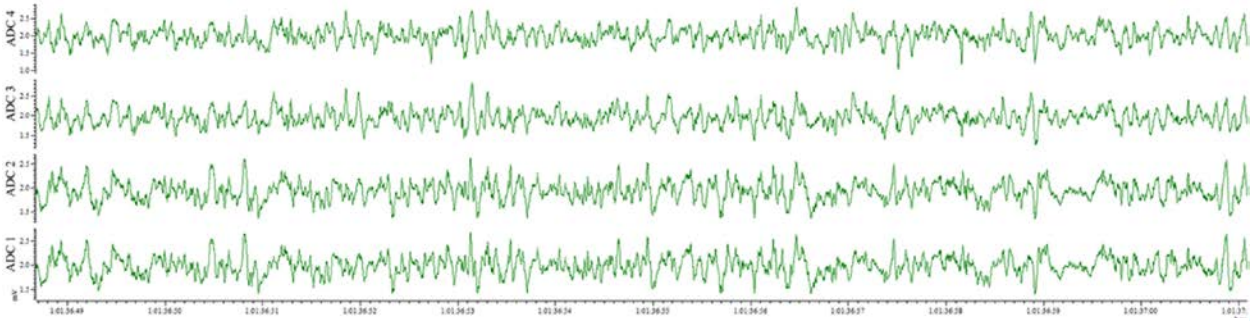


Figure 2-4. EEG recorded 4 days post-surgery utilizing the wire method. All 4 electrodes record analyzable signal.

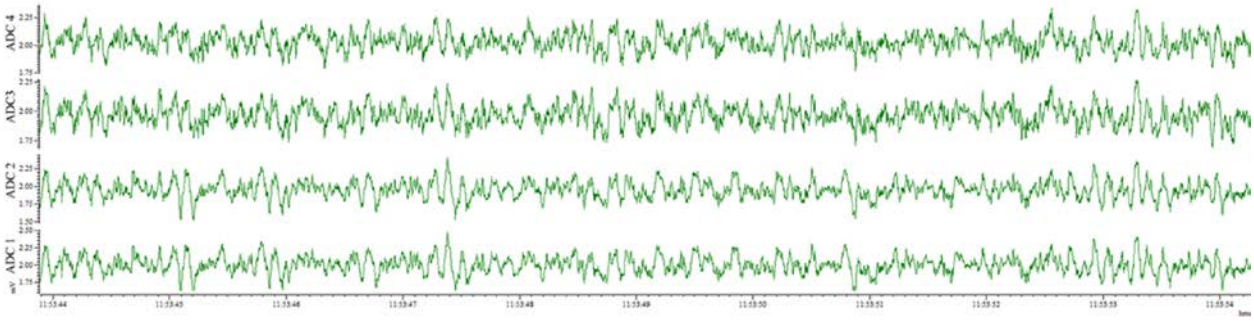


Figure 2-5. EEG recorded 28 days post-surgery utilizing the wire method. All 4 electrodes record analyzable signal, with no deterioration compared to 4 days post-surgery.

2.5 Discussion

The use of animals in research was for many years limited to studies of diseases and pathologies of the particular species of animal or testing of pharmaceuticals for lethality or adverse effects, which has limited translation to effects on humans. However, use of animals to study disease has increased significantly in the last 30 years due to the creation of genetically modified (transgenic) animals to study gene function and to model human diseases. While many animal species have been successfully genetically modified, mice are the most commonly used species because more than 80% of human genes have corresponding counterparts in the mouse genome (Emes et al., 2003), their short life span reduces the time needed to complete age-dependent studies, and colonies are easily maintained. It is now possible to mimic pathology characteristic of a human disease to which mice are not normally susceptible, such as the neurodegenerative conditions of Alzheimer's and Parkinson's. The pathologies can then be studied both in vivo and in vitro and potential therapeutics can be administered to the animal subjects to find candidate treatments for human clinical trials. These genetic advances in research technology require the adaptation of equipment designed to quantify changes in physiology of human subjects to suit the physical and behavioral characteristics of mice,

which in general requires miniaturizing while maintaining robustness of the equipment so it is not overly obstructive to the mouse's activities but can withstand damage from normal grooming activities.

The ability to record EEG in mice has led to significant research findings, including age-related sleep disturbances and changes in EEG profile (Jyoti et al., 2015) and the presence of seizure activity in mouse models of Alzheimer's Disease (AD) (Palop et al., 2007), a condition previously not often recognized in AD patients due to their cognitive deficits (Vossel et al., 2013). Just a few years ago, EEG studies of AD patients focused on sleep abnormalities (Jeong, 2004), altered regional connectivity, and rhythms (Knyazeva et al., 2013), while more recent research is also utilizing mouse models of AD to discover the underlying causes of seizure activity in AD pathology and investigate therapeutic treatments (Sanchez et al., 2012, DeVos et al., 2013, Bomben et al., 2014, Born et al., 2014).

Introducing the ability to record EEG on freely moving mice performing behavioral tasks makes it possible to observe and test interventions in mice designed to affect learning and memory that would be hampered by tethering or other invasive equipment. Typically, screws are used as electrodes or anchors for wires serving as electrodes and there are 2 different approaches for affixing the screws. One features drilling holes in the skull and inserting screws, which requires careful precision to avoid drilling or inserting the screws too deep and causing brain damage (Lapray et al., 2008, Armstrong et al., 2013), while the other avoids drilling and instead affixes the screws to the surface of the skull with cyanoacrylate glue (Etholm et al., 2010), which can result in poor contact if there is excessive glue between the skull and screw. After utilizing both approaches to record

EEG on mice with a wireless recording system, with a lack of consistent successful implantation and adequate signal conduction, our laboratory decided to investigate alternative approaches and have developed procedures that result in consistent successful surgeries and recordings, with a robust implant that is minimally invasive and produces recordings for a month or more after implantation. The use of screws appeared to be the main impediment to consistent results due to the large size relative to a mouse. A flat screw base does not provide a secure conductance area on a curved mouse skull, while drilling several holes large enough for screws in the small and thin mouse skull resulted in a fragile setup that failed to maintain the apparatus for long periods of time. We also found that tissue growing around the wound with screw implants would degrade the signal within 2 weeks of electrode attachment. The other weak link in the process is the attachment of wires to the screws and pin connectors with the use of solder or conductive adhesive. These connections are difficult to manufacture and fragile in use.

Our electronic technician suggested inserting just a small length of highly conductive stripped wire through a small hole drilled in the skull and affixing the wire jacket insulation to the skull with adhesive, with the wire wrapped around the pin connector instead of soldered to it, and proposed to make a pre-assembled wiring harness that would be strong and simple to affix. A similar process without screws has been described for telemetry recording of EEG (Weiergraber et al., 2005), but specific details were not provided. Consequently, we have completely revamped our electrodes and implantation procedures, finding that the percentage of successful recordings from all electrodes in an implanted electrode assembly increased from 29% with the TSE screw method to 83% with the new wire method (Figure 2-6). The success rate is calculated

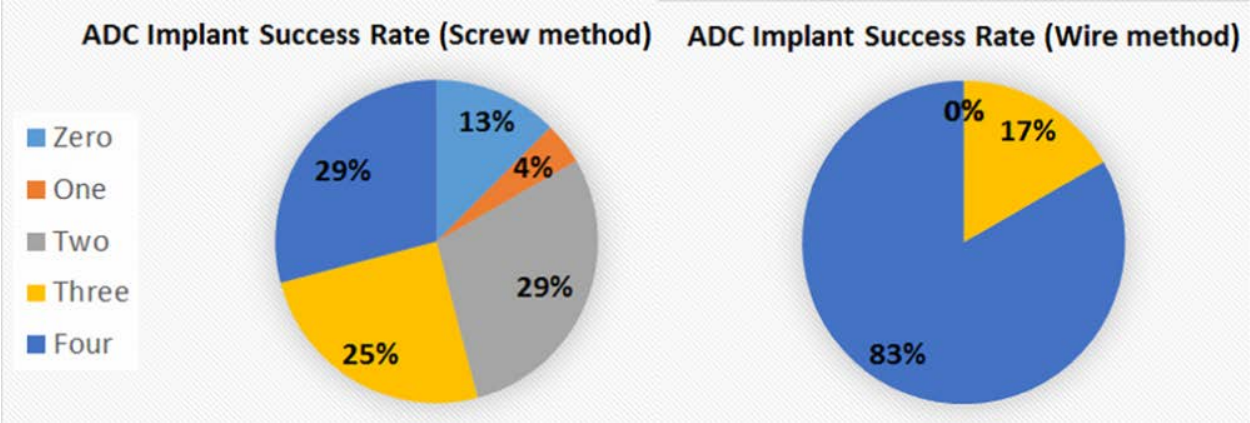


Figure 2-6. Percentage of electrodes successfully implanted. The percentage of fully successful electrode implantation, as measured by implant recording signal on all 4 ADC channels, increased from 29% with the screw method to 83% with the wire method. The different areas represent the number of electrodes recording analyzable signal after implantation for the 2 methods.

based on signal return from 0 through 4 electrodes as failure of the ground/reference electrode typically results in loss of signal from all recording electrodes even if the recording electrodes are all properly implanted. Other benefits of the wire method are that the implants remain in place for extended periods with no adverse effects or reactions and the assembly can be constructed with equipment readily available from typical electronic stores. On the other hand, some limitations of this method include not being well-suited for studies where more electrodes are needed to resolve different frequencies during sleep stages or for studies targeting deep brain structures which require implantation of depth electrodes.

Advances in science and technology over the past 30 years have made possible the development of transgenic animals to model human diseases and electronic equipment that can wirelessly record 72 hours of EEG data on a device weighing slightly more than the U.S. Mint specifications for a dime (www.USMint.gov). However, it is still important to think “keep it simple” when using such equipment to reduce the opportunities for failure of components or steps in the process. Simplifying the electrodes and

implantation process is also in accordance with the animal research principles of "replacement, reduction and refinement (Russell et al., 1959)," in that this is a refinement that minimizes the invasiveness of the experimental intervention and reduces the number of animal subjects needed to complete a study by increasing the reliability of data generation from each animal subject. Technology advances by both grand leaps and small adjustments; ironically, the wire-wrapping technology is well over a half century old and relatively novel to the current generation of scientists and has been documented to have the greatest reliability among various methods of electronic connections (Wagner, 1999). We have presented a protocol for improvements in the implementation of miniaturized EEG equipment that will facilitate successful use of the equipment in animal research.

Chapter 3

Levetiracetam Prevents Cognitive Impairment in a Sex-Specific Manner in Mice Lacking Synaptic Zinc

3.1 Abstract

While it has been known for a while that patients with Alzheimer's Disease (AD) have increased risk of unprovoked seizures, only recently has hyperexcitability become a major focus of AD research. Increased hippocampal activity in patients with mild cognitive impairment (MCI) was initially thought to be a beneficial compensatory mechanism; however, recent research has shown that patients with MCI who are treated with antiepileptic drugs improved their cognition. Synaptic zinc, co-released with amyloid-β (Aβ) during neurotransmission, is implicated in both oligomer formation and modulation of excitatory neurotransmission. Synaptic zinc is packaged into synaptic vesicles by ZnT3; thus, *ZnT3*$^{-/-}$ mice lack synaptically-released zinc. These mice exhibit increased susceptibility to seizures and synaptic dysfunction, consistent with the finding that synaptic zinc is sequestered by Aβ oligomers. In fact, *ZnT3*$^{-/-}$ mice exhibit age-dependent increases in markers of seizure activity, synaptic loss, neurodegeneration, strikingly similar to that in AD mouse models. Although *ZnT3*$^{-/-}$ mice do not exhibit spontaneous convulsive seizures, our data suggest that these mice exhibit epileptiform activity. In the hAPP mouse model, treatment with an acute high dose (200mg/kg) of the antiepileptic drug Levetiracetam (LEV) reduces spiking, and treatment with a chronic low dose (50mg/kg/day) of LEV enhances memory. Importantly, this low dose is not sufficient to treat seizures. In this regard, the mechanism by which chronic LEV prevents cognitive decline is not understood. The goal of this project was to begin characterizing gene

networks involved in the mechanism of action of chronic LEV leading to the prevention of cognitive decline in the *ZnT3*$^{-/-}$ mouse model. First, the hippocampal-dependent object-location memory task was used to assess memory in aged *ZnT3*$^{-/-}$ mice treated with chronic LEV. Next, we implanted mice with an electrode assembly to characterize electroencephalogram activity. mRNA was then extracted from aged *ZnT3*$^{-/-}$ mice and analyzed using NanoString. To investigate the effects of chronic LEV on neurogenesis important for learning and memory, *ZnT3*$^{-/-}$ mice were fed 5-bromo-2'-deoxyuridine and immunohistochemistry was performed. Our results indicate that aged *ZnT3*$^{-/-}$ mice have impaired hippocampal cognition. Chronic, but not acute, treatment with LEV completely prevented this cognitive impairment. Although chronic LEV had a more profound effect on spiking in aged female *ZnT3*$^{-/-}$ mice, it did not modify the power characteristics of the different treatment groups, indicating that the low chronic dose of LEV may not be sufficient to treat seizure activity. Gene analysis indicated sex-specific changes in genes involved in epigenetics, neurogenesis, and excitability. Although there was an increase in neurogenesis in LEV-treated animals, this increase was restricted to females, further suggesting a sex-specific effect of chronic LEV. Taken together, these results suggest that a low chronic dose of LEV prevents cognitive impairment in *ZnT3*$^{-/-}$ mice in a sex-specific manner acting at multiple layers of regulation, including epigenetics, adult neurogenesis, and gene expression.

3.2 Introduction

Alzheimer's Disease (AD) is the leading cause of dementia worldwide and is ranked as the third leading cause of death in the United States (reviewed in Whittington

et al., 2017). It is irreversible and progressive, destroying memory, thinking, and ability to perform basic tasks. The number of people with AD is projected to rise exponentially, and some estimates indicate 13 million Americans will be affected by 2050 (Hebert et al., 2003). The cost of care is similarly expected to rise, with estimates at $1.1 trillion by the year 2050 (Alzheimer's Study Group; Alzheimer's Association; National Institute of Health Office of the Budget). Although there has been therapeutic promise in pre-clinical AD studies, over 99% of clinical trials have failed during 2002-2012, with 14 drugs in Phase 3 clinical trials and only one (memantine, an NMDA receptor antagonist) approved for marketing as of 2012 (Cummings et al., 2014). Thus, there is an urgent need to develop new strategies for tackling this devastating disease.

Although the major focus of AD research has been on amyloid plaques and neurofibrillary tangles, a better correlation with AD pathology is soluble amyloid beta oligomers (AβO) (Terry, 1996, Lambert et al., 1998, Lue et al., 1999, McLean et al., 1999, Naslund et al., 2000, Klein et al., 2001, Walsh et al., 2002, Caughey and Lansbury, 2003, Chiti and Dobson, 2006, Lesne et al., 2006, Ferreira et al., 2007, Haass and Selkoe, 2007, LaFerla et al., 2007). Previous research has shown that AβO form and accumulate at the synapse, and are released into the cleft in response to neuronal stimulation (Deshpande et al., 2009). Synaptic zinc is implicated in this process, which is significant because synaptic zinc modulates several signaling pathways that reduce neuronal excitability (Westbrook and Mayer, 1987, Cohen-Kfir et al., 2005, Erreger and Traynelis, 2008, Vogler, 2014). Synaptic zinc is packaged into synaptic vesicles by the zinc T3 transporter (ZnT3); thus, *ZnT3*$^{-/-}$ mice lack synaptic zinc (Cole et al., 1999). These mice exhibit increased susceptibility for seizures and synaptic dysfunction and loss, consistent

with the finding that synaptic zinc is sequestered by AβO (Cole et al., 2000, Grabrucker et al., 2011, Qian et al., 2011). In fact, *ZnT3*$^{-/-}$ mice display age-related cognitive impairment strikingly similar to that in AD mouse models (Adlard et al., 2010). This similarity could be due to the fact that *ZnT3*$^{-/-}$ mice recapitulate a key aspect of AD: the lack of neuromodulation of synaptic zinc.

Recently, our laboratory has shown that *ZnT3*$^{-/-}$ mice exhibit age-dependent increases in synaptic loss and markers of seizure activity (Vogler et al., *submitted*). Although these mice do not exhibit convulsive seizures, our data show that these mice display increased spiking activity, consistent with observations in the clinic in AD patients and several AD transgenic animal models (Palop et al., 2007, Minkeviciene et al., 2009, Gurevicius et al., 2013). In addition, while it has been known that patients with AD have increased risk of unprovoked seizures (Hesdorffer et al., 1996, Amatniek et al., 2006, Lozsadi and Larner, 2006), seizure activity in these patients has only recently become a major area of research. Increased hippocampal activity in patients with mild cognitive impairment (MCI) was initially thought to be a beneficial mechanism to compensate for cognitive impairment; however, patients with MCI treated with the antiepileptic drug Levetiracetam (LEV) have improved cognition (Miller et al., 2008, Bakker et al., 2012). Moreover, hippocampal hyperexcitability is an upstream predictor of AD (reviewed in Palop and Mucke, 2009), and neuronal pathology is linked to increased hyperexcitability in both *in vitro* induced pluripotent stem cell models and *in vivo* mouse models of AD (Siskova et al., 2014, Balez et al., 2016, Kazim et al., 2017, Lam et al., 2017).

Acute treatment with LEV reduces spiking activity in the hAPP mouse model of AD and chronic treatment with LEV improves spatial memory (Sanchez et al., 2012).

Similarly, acute treatment of LEV modifies neuronal oscillations in AD patients, but does not improve cognition (Musaeus et al., 2017). In contrast, chronic LEV treatment in patients without epilepsy results in improved memory; although in this study there were some side effects such as irritability and fatigue, likely due to the high dose given to patients (Schoenberg et al., 2017). These results suggest that temporal dynamics play a role in the mechanism of action of LEV, and that the differential effects of acute and chronic LEV on spiking and cognition may have diverging pathways. Although a binding site for LEV is the synaptic protein SV2a (Lynch et al., 2004, Stockburger et al., 2016), the exact downstream mechanisms by which LEV acts to improve cognition is not completely clear. Since chronic LEV improves cognition, we hypothesized that chronic treatment with LEV prevents cognitive impairment in aged *ZnT3*$^{-/-}$ mice by reducing excitability and increasing neurogenesis important for the formation of new memories. Because there are sex-specific effects of knocking out *ZnT3*$^{-/-}$ (Thackray et al., 2017), we also sought to investigate genetic differences between male and female *ZnT3*$^{-/-}$ mice and the effects of LEV on each sex. Our data indicate that treatment in aged *ZnT3*$^{-/-}$ mice with a chronic, but not acute, low dose of LEV prevents cognitive impairment, suggesting that chronic LEV acts through mechanisms distinct from those that ameliorate epilepsy. Analysis of mRNA using NanoString suggests that chronic LEV modifies gene expression in a sex-specific manner, and suggests a significant role for epigenetic regulation of genes involved in cognition and neurogenesis. In addition, chronic LEV restores neurogenesis in aged female *ZnT3*$^{-/-}$ mice, but not males, further suggesting sex-specific actions of LEV on cognition. Taken together, these results indicate that unlike a high acute dose of LEV typically used to treat seizures, a low chronic dose of LEV prevents cognitive impairment

by altering neurogenesis and gene expression involved in synaptic plasticity and the epigenetic regulation of gene expression.

3.3 Materials and Methods

All animal experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee. Male and female 129Sv wild-type (WT) or *ZnT3*$^{-/-}$ 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 and water *ad libitum*.

3.3.1 Levetiracetam treatment

Levetiracetam (LEV; Sequoia Research Products) was dissolved in saline to a final concentration of either 200mg/kg (acute; aLEV) or 50mg/kg/day (chronic; cLEV). For the acute treatment, mice were injected interperitoneally 3.5 hours before training. For the

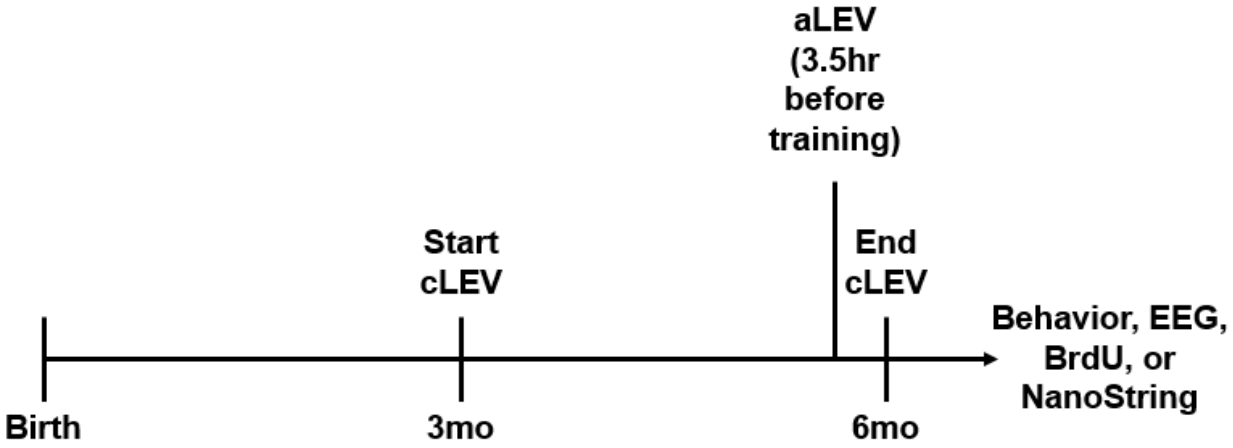


Figure 3-1. Timeline of experiments. For the chronic Levetiracetam (cLEV, 50mg/kg/day) treatment, mice were implanted with osmotic minipumps starting at 3 months of age. After the 10-week treatment, these mice underwent behavioral testing, EEG electrode implantation, BrdU administration, or hippocampal mRNA extraction followed by NanoString analysis. For the acute LEV (aLEV, 200mg/kg) treatment, mice were injected interperitoneally 3.5 hours before behavioral training.

chronic treatment, mice were implanted with Alzet osmotic minipumps (Durect Corp.) for 10 weeks before training (minipumps were switched out halfway through the treatment). After 24 hours of priming LEV in saline at 37°C, the minipumps were surgically implanted subcutaneously into the upper back of the mice anesthetized with 3% isoflurane. Minipumps delivered LEV at a rate of 0.11µL/hr and were replaced halfway through the cLEV treatment (Figure 3-1).

3.3.2 Object location memory test

The hippocampus-dependent Object Location Memory (OLM) task was performed as previously described in Vogel-Ciernia and Wood, 2014. Briefly, mice were handled for two minutes for five consecutive days followed by habituation to the testing arena for five minutes for six consecutive days, with Days 4 and 5 of handling overlapping with Days 1 and 2 of habituation. During training, two identical objects were placed in the training arena and individual mice were allowed to explore the objects for 10 minutes. Twenty-four hours after the conclusion of training, the mice were returned to the arena with one object moved to a novel location, and the mice were allowed to explore the objects for five minutes (Figure 3-2). All portions of the behavior training were video monitored using EthoVision and the time (t) spent exploring each object was used to calculate the discrimination index (DI) using $DI = (t_{novel} - t_{familiar})/(t_{novel} + t_{familiar}) \times 100\%$.

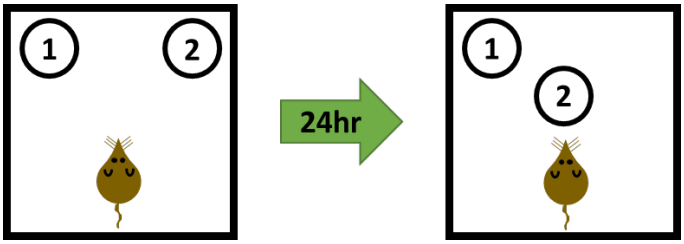


Figure 3-2. Object location memory behavioral task. Individual mice received a 10-minute training in an arena with two identical objects. Twenty-four hours later, one of the objects was moved to a new location and the mice explored the arena for 5 minutes to study retention. The discrimination index was used to assess long-term memory.

Data were analyzed using unpaired, two-tailed t-tests with a significance threshold of $p<0.05$.

3.3.3 Electrode implantation

Electroencephalogram (EEG) electrode creation and implantation were done as previously described in Vogler et al., 2017. Briefly, four analog-digital channel (ADC) leads and a ground/reference lead were made by connecting five leads to a six-pin connector block. At the end of each lead, the insulation was stripped to expose a portion of the wire that would be inserted into the holes drilled into the skull. A stereotaxic apparatus was used to mark the coordinates: two recording electrodes were placed at -1.34mm A/P, ±1.5mm M/L and two at -5.34mm A/P, ± 3.00mm M/L relative to bregma and the reference electrode was placed at -7.00mm A/P, 0.50mm L relative to bregma. Direct contact between the leads and the brain allowed for signals in the brain to be received and recorded by a Neurologger device (TSE Systems).

EEG data collection was performed by connecting the Neurologger to the EEG assembly by inserting the pins on the connector block of the electrode into the Neurologger. A grounding wire was used while handling the Neurologger to prevent electrical faults from damaging the device. Two p312 batteries were used to power the neurologger. A slow, blinking red light on the Neurologger indicated whether or not the device was recording. After ensuring that the device was functioning properly, the Neurologger was then wrapped in parafilm for protection and connected to the electrode assembly on the subject's head. The mouse cage was placed in a grounded Faraday cage to prevent 60Hz interference from electrical currents in the environment during the

24-hour recording period. The electroencephalogram data were then downloaded from the Neurologger onto Spike2 files for analysis.

3.3.4 Electroencephalogram analysis

Analysis was done using the modified EEGrunt Python script as well as custom Python scripts to analyze neuronal spiking, coherence, and power. The Neurologger sampled data at a frequency of 163Hz; thus, the maximum frequency analyzed was the Nyquist frequency of roughly 80Hz. One hour into the recording, a 30-minute window was extracted and analyzed. A 1-80Hz broadband filter, 1.0Hz highpass filter, and a 57.0-63.0Hz notch filter was applied to the data. Rate of spiking, defined as a deflection greater than two standard deviations above the mean, was calculated for each recording site. After obtaining the spiking counts using our script, we manually scanned the traces and subtracted waveforms not indicative of true spiking (for example, double spikes were removed). Power was analyzed for the delta (1-4Hz), theta (4-8Hz), alpha/beta (8-35Hz), low gamma (35-50Hz), and high gamma (50-80Hz) bands (Ehlers and Criado, 2009). Initial analysis was performed on each electrode separately by using a General Linear Model with a significance threshold of $p<0.05$. To increase statistical power and determine whether additional differences could be uncovered, the data for these were collapsed and analyzed together. All spiking data were normalized with respect to wildtype male levels. Data were analyzed using a one-way ANOVA with Tukey's *post hoc* test in SPSS v24 (IBM) with a significance threshold of $p<0.05$.

3.3.5 Neurogenesis

The thymidine analog 5-bromo-2'-deoxyuridine (BrdU, 1mg/kg, Sigma Aldrich B5002), supplemented with 1% glucose, was dissolved in drinking water and administered orally for seven days to both WT and ZnT3KO mice after completion of chronic LEV treatment. The BrdU water solution was prepared in low-light conditions and stored in black water bottles since it is light-sensitive. After three days, the BrdU was replaced with a fresh solution for the remaining days to prevent degradation and preserve efficiency of the compound. After one week treated mice were perfused and brains extracted and stored for RNA extraction and immunostaining.

3.3.6 Perfusions

An intraperitoneal injection of Ketamine/Xylazine (67mg/kg of Ketamine and 27mg/kg of Xylazine) was given to anesthetize mice three to five minutes before performing perfusions. A buffer solution of 1x phosphate-buffered saline (PBS) was placed on ice, with the input end of the pump partially submerged in the solution and the output connected to a 25G needle. Once mice were sedated and paralyzed but still breathing, the thoracic cavity and diaphragm of the mice were opened to allow access to the heart. The right atrium was cut to create an exit hole for the PBS while the left ventricle was punctured with the 25G needle. Perfusions continued for up to 30 seconds or until the liver and lungs were visibly white to ensure completion. Mice were then decapitated and the exposed brain was carefully extracted.

Upon completion of extraction, a cold blade was used to separate the right and left hemispheres. The left hemisphere was stored immediately in 4% paraformaldehyde for

two days before being washed three times with 1x PBS and incubated in 1x PBS and 0.02% sodium azide and stored at 4°C until use. The right hemisphere was flash frozen in a solution of 2-methylbutane in dry ice and subsequently placed in a cryotube and stored at -80ºC.

3.3.7 Immunohistochemistry

Extracted brains were fixed in a 4% paraformaldehyde solution at 4°C overnight, then stored in a 0.02% sodium azide solution in PBS. Samples were soaked in a sucrose gradient of 10%, 20%, and 30% over three days and stored in 30% sucrose for up to 72 hours and sectioned coronally into 40µm sections with a vibrotome (Vibrotome Series 100). Collected sections were then stored in a 0.02% sodium azide solution in PBS and stored at 4°C. Equivalent sections within the dentate gyrus of the hippocampus were selected using a mouse brain atlas, then stained with primary antibodies: mouse anti-NeuN (1:1000, EMD Millipore) and rat anti-BrdU (1:500, Accurate Chemical & Scientific Corporation). Immunohistochemistry was performed by washing free-floating sections in PBS for five minutes, followed by permeabilization in 0.2% Triton X-100 in PBS for five minutes and denaturation in 2N HCl for 30 minutes at 37°C. Samples were then neutralized in 0.1M borate buffer twice at 15 minutes each. Sections were washed in PBS three times for five minutes each, and blocked in 0.3% Triton X-100 + 10% Normal Goat Serum (NGS) + PBS for one hour. The sections were then incubated in primary antibodies diluted in a 0.15% Triton X-100 + 5% normal goat serum + PBS and left on a rocker at 4°C overnight. Sections were then labeled with the fluorescent secondary antibodies goat anti-rat (1:1000, Alexa Fluor 488) and goat anti-mouse (1:1000, Alexa Flour 555) for one

hour, and labeled with Hoescht's solution for five minutes. Sections were then mounted on Superfrost+ slides using Aquamount, dried at 37°C for 30 minutes and sealed and stored at 4°C.

3.3.8 Microscopy and image analysis

Images were captured using an Axiovert 200 inverted microscope (Zeiss) and processed using AxioVision to visualize BrdU-positive immunolabeling at 200x total magnification as previously described (Vogler et al., *submitted*). The entirety of the dentate gyrus was visualized by stitching together individual 20x Mosaix images into a streamlined image. Briefly, immunolabeled cells were counted with three fields imaged for each subject in the dentate gyrus of mice and values were expressed as a fold change relative to wild-type (WT) puncta. Background intensity was normalized between images and immunopositive cells will be counted using ImageJ using the Cell Counter plugin (National Institutes of Health) and only puncta that are above a threshold determined by subtracting background will be included for the analysis. Quantification of BrdU and BrdU+NeuN labeling in the dentate gyrus of the hippocampus was done by cell counting immunopositive labels that co-localized with Hoescht. The data for the three-month-old mice were analyzed using a two-tailed t-test, and the data for the six-month-old mice with or without LEV were analyzed using a one-way ANOVA with a significance threshold of $p<0.05$.

3.3.9 RNA extraction and NanoString

Hippocampal tissue was freshly extracted following cervical dislocation and RNA was extracted using the RNeasy Micro Kit (Qiagen) and analyzed using NanoString, a high-throughput, amplification-free method that allows for accurate quantification of differences in RNA transcript expression. RNA quality was assessed using an Agilent Bioanalyzer 2100 (UCI Genomics High-Throughput Facility) and only RNA with an A260/280 ratio of at least 1.9, an A260/230 ratio of at least 1.8, and an RNA Integrity Number score of at least 9.0 was used, per NanoString recommendation, indicating relatively pure RNA preparation with minimal fragmentation. The RNA was hybridized to a custom gene CodeSet containing 150 different probes targeting genes involved in synaptic plasticity, Alzheimer's Disease, neurogenesis, learning and memory, hyperexcitability, neuroinflammation, epigenetics, gene expression, and cellular signaling (Appendix 1). Thus, instead of an unbiased screen, we utilized a broad candidate gene approach which allowed for the identification of changes in gene expression even in low-expressed transcripts, as opposed to using RNA-seq where amplification bias might wash out signal from any targets not expressed at a high level (Geiss et al., 2008). The hybridized samples were purified, immobilized, and counted using the automated nCounter Prep Station. Data were be analyzed using both the proprietary nSolver Analysis Software with guidance from the NanoString bioinformatics team and the programming environment R in collaboration with Dr. Michael Phelan from the Center for Statistical Consulting at the University of California, Irvine.

Data obtained from the nCounter platform were imported into the proprietary nSolver Analysis Software. Quality control was done by setting parameters for

background imaging and binding density, which analyzed the fields of view to ensure that the instrumentation worked as expected. The average binding density (spots/μm^2) was calculated to ensure that the amount of binding of the probes for each lane fell within the acceptable range (0.005-2.25 spots/ μm^2). The housekeeping genes *Gapdh*, *Hprt*, *Polr1b*, *Polr2a*, *Rpl19*, and *Tbp* were analyzed using linear correlation to ensure proper hybridization reactions, with only a correlation coefficient >0.95 being acceptable. After quality control, the data were organized in the nSolver Analysis Software and annotated according to the custom CodeSet. Background was subtracted before normalization of the data to the mean of the housekeeping probes. Fold changes were calculated by taking the average of normalized lanes associated with the same treatment group, running in triplicate, and dividing by the average of controls. Agglomerative clustering was done based on the similarity in transcript counts to visualize potential relationships between all of the samples and treatment groups, with the output being a heat map with a dendrogram tree. All statistical tests were be done with a significance threshold of $p<0.05$.

The following approach was used to screen for active genes. For each gene in the probe set, a linear model was used for the regression of the mean expression level of the gene based on an indicator of the drug, genetic type, and sex. To account for potential modifications of drug effects by mouse type and sex, the model included terms for all two-factor interactions and the three-factor interaction. Expression values were log transformed (base 2) prior to the analysis. The models were subject to a residual diagnostics as a check on the assumption of normally distributed measurement errors. Each gene-comparison produced a t-value for the test statistic of a two-sample t-test of the equality of means. The t-values were then transformed to z-values, using a standard

approach based on the t-distribution and the inverse standard normal distribution function (Efron, 2010). Under the null hypotheses these z-values will follow the theoretical null, namely N(0,1), for the normal distribution with mean 0 and standard deviation 1. Thus, z-values that pile up around zero were indicative of `true' null hypotheses. Those that pile up in the tails (large negative or large positive z-values) were indicative of findings of mean differences in gene expression. Naive P-values were then calculated and reported for each gene-comparison based on a two-sided test of the comparison for the particular gene with a false-discovery rate (FDR) of 0.10.

3.4 Results

3.4.1 Chronic LEV prevents age-related cognitive decline in *ZnT3*$^{-/-}$ mice

Although *ZnT3*$^{-/-}$ mice were first assessed to have normal cognition, subsequent studies found age-dependent cognitive impairment (Cole et al., 2000, Adlard et al., 2010). However, the mechanisms by which the lack of synaptic zinc contribute to cognitive impairment are not well understood. Since chronic LEV has previously been shown to prevent cognitive impairment in the human APP mouse model of AD (Sanchez et al., 2012), we sought to investigate the effect of chronic LEV on cognitive impairment in *ZnT3*$^{-/-}$ mice. Previous research in our laboratory has indicated that using the hippocampal-dependent OLM task, *ZnT3*$^{-/-}$ mice demonstrate normal performance at six weeks of age, decreased performance at three months of age, and complete impairment at six months of age (Vogler, 2014). An acute high-dose treatment with LEV had no effect on performance in *ZnT3*$^{-/-}$ mice, whereas a chronic low-dose treatment with LEV completely

prevented cognitive impairment in aged *ZnT3*$^{-/-}$ mice (Figure 3-3). These results suggest that the mechanism by which chronic LEV acts to prevent cognitive impairment in aged *ZnT3*$^{-/-}$ mice.

3.4.2 Chronic LEV reduces spiking in aged *ZnT3*$^{-/-}$ female mice

Previous research in our laboratory indicates that *ZnT3*$^{-/-}$ mice exhibit age-related increases in markers of seizure activity such as decreased calbindin expression in the dentate gyrus, increased neuropeptide Y expression in CA3, and aberrant mossy fiber sprouting in the molecular layer of the dentate gyrus (Vogler, 2014). These results expand

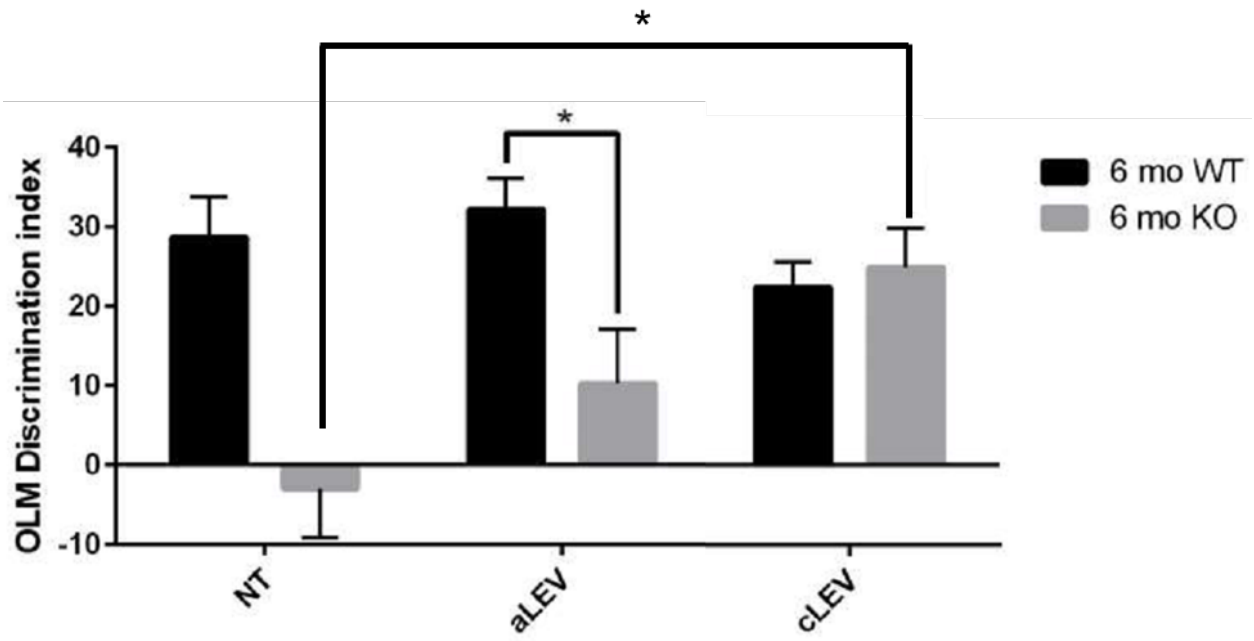


Figure 3-3. Chronic, but not acute, treatment with LEV prevents cognitive impairment in *ZnT3*$^{-/-}$ mice. The hippocampal-dependent object-location memory (OLM) task was used to assess cognition in *ZnT3*$^{-/-}$ mice. Mice were habituated to the testing arena followed by a 10-minute training period with two identical objects. Twenty-four hours later, one of the objects was moved to a novel location, the mice explored the arena for a 5-minute testing period, and the discrimination index was calculated. Nontreated *ZnT3*$^{-/-}$ mice (NT) were unable to discriminate the object placed in the novel location. Acute treatment with LEV (aLEV, 200mg/kg) 3.5 hours before training did not significantly improve performance in *ZnT3*$^{-/-}$ mice at six months of age. Chronic treatment with LEV (cLEV, 50mg/kg/day for 10 weeks) prevented cognitive impairment in *ZnT3*$^{-/-}$ mice. Two-tailed unpaired t-test, *p<0.05, **p<0.01, n=5/group. OLM = Object location memory, NT = nontreated, aLEV = acute Levetiracetam, cLEV = chronic Levetiracetam, WT = wildtype, KO = *ZnT3*$^{-/-}$.

upon other studies indicating that *ZnT3*$^{-/-}$ mice have increased susceptibility to induced seizures (Cole et al., 2000; Qian et al., 2011). Since LEV is currently on the market as an antiepileptic drug, we investigated its effects on spiking activity in aged *ZnT3*$^{-/-}$ mice using EEG and custom Python scripts. Since a high acute dose of LEV (200mg/kg) reduces spiking in hAPP mice, and a low chronic dose of LEV (75mg/kg/day for 4 weeks) reduces spiking that returns back to baseline 12 days after the end of treatment (Sanchez et al., 2012), we hypothesized that an even lower chronic dose of LEV (50mg/kg/day for 10 weeks) would similarly reduce spiking in aged *ZnT3*$^{-/-}$ mice. We chose this dose because

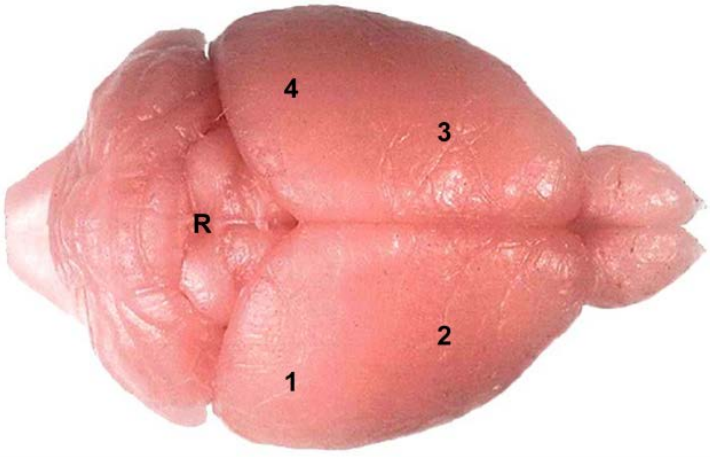


Figure 3-4. Placement of electrodes for EEG recording. To investigate EEG activity, four recording electrodes (1-4) and one reference/ground electrode (R) were implanted. The implantation locations are shown in the figure. 1 = right posterior, 2 = right anterior, 3 = left anterior, 4 = left posterior.

higher doses of LEV are associated with side effects (Schoenberg et al., 2017). In addition, our *ZnT3*$^{-/-}$ mice begin exhibiting cognitive impairment at 3 months of age and are completely impaired by 6 months of age (roughly 10 weeks); thus, we decided to treat these mice for 10 weeks. Four recording electrodes were implanted and referenced/grounded to an electrode placed at the cerebellum (Figure 3-4). Representative traces between 6-month-old WT and *ZnT3*$^{-/-}$ male mice as well as an example spike are shown in Figure 3-5A.

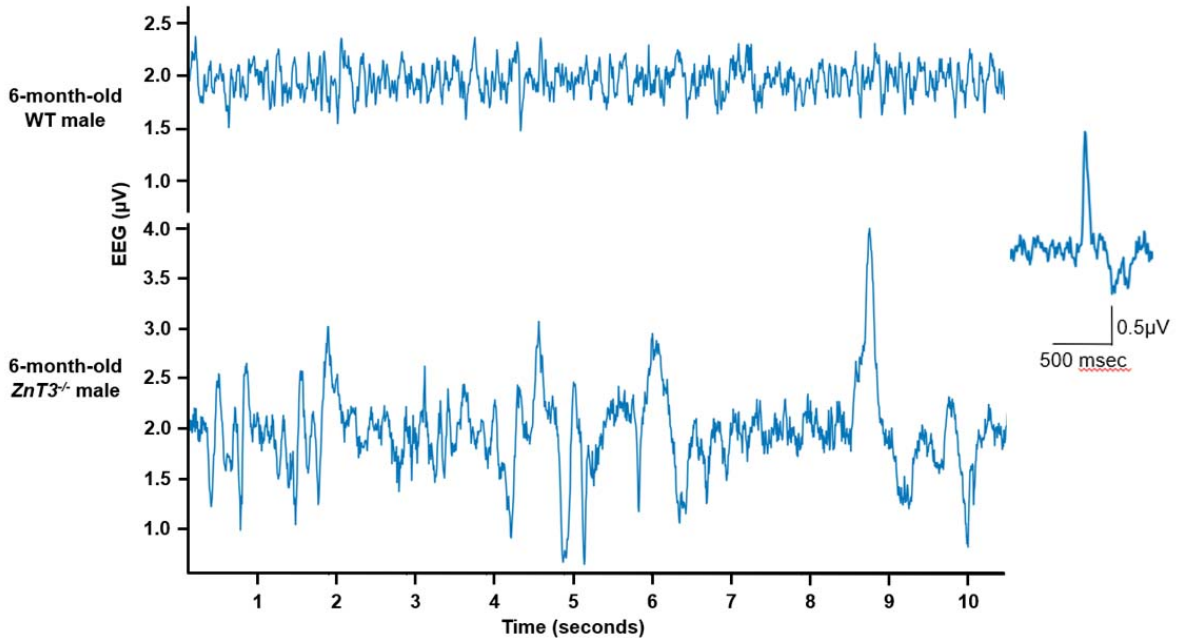
A summary of the spiking data is shown in Table 3-1. We first compared WT to *ZnT3*$^{-/-}$ mice and found increased spiking in the right posterior electrode (ADC1) for *ZnT3*$^{-}$

$^{-/-}$ males. Next, we investigated sex differences and found increased spiking in WT males compared to WT females in the left side of the brain (ADC3 and ADC4). In addition, male *ZnT3*$^{-/-}$ mice had more spiking compared to female *ZnT3*$^{-/-}$ mice in the right posterior electrode (ADC1). Although chronic LEV had no effect on spiking within the same sex, the overall effect of chronic LEV resulted in reduced spiking in *ZnT3*$^{-/-}$ female mice when compared to male *ZnT3*$^{-/-}$ mice in all four recording sites (ADC1-4; Figure 3-5B). To increase statistical and potentially uncover additional differences, we also collapsed the data from the electrodes and normalized the spiking data to WT males (Figure 3-6). Both WT and *ZnT3*$^{-/-}$ males had higher spiking compared to their female counterparts, and male *ZnT3*$^{-/-}$ mice had higher spiking compared to WT males. In addition, similar to the uncollapsed data, chronic LEV did not have an effect on spiking within the same sex, but did result in a profound reduction in spiking when comparing female *ZnT3*$^{-/-}$ mice to male *ZnT3*$^{-/-}$ mice. These results suggest that male *ZnT3*$^{-/-}$ mice have localized increases in spiking compared to their WT counterparts. In addition, the overall effect of chronic LEV is more profound in female *ZnT3*$^{-/-}$ mice compared with male *ZnT3*$^{-/-}$ mice. Interestingly, the fact that we observed these abnormalities in spiking in male but not female *ZnT3*$^{-/-}$ mice, but we see amelioration in both sexes, is consistent with the possibility that the effect of LEV on neuronal activity is not the main driver the improvement in cognition.

EEG activity can be analyzed using the different frequency bands: delta (1-4Hz), theta (4-8Hz), alpha/beta (8-35Hz), low gamma (35-50Hz), and high gamma (50-80Hz; Ehlers and Criado, 2009). In particular, previous research on various transgenic AD mouse models have demonstrated altered hippocampal theta oscillations and delta frequency, which may precede cognitive impairment, although there is no consensus as

to the direction of change of the various frequency bands (Palop et al., 2007, Minkeviciene et al., 2009, Harris et al., 2010, Jyoti et al., 2010, Platt et al., 2011, Roberson et al., 2011, Vogt et al., 2011, Scott et al., 2012). Thus, we sought to investigate the effects of chronic LEV on the power profiles of *ZnT3*$^{-/-}$ mice (Figure 3-7). Although there were slight changes in the power composition, chronic LEV did not change the overall power characteristics of *ZnT3*$^{-/-}$ mice when compared to untreated controls. These results suggest that chronic LEV does not significantly modify oscillatory network characteristics in *ZnT3*$^{-/-}$ mice. Because the low chronic dose of LEV might be too low to act through its antiepileptic properties, we explored its effects on gene expression to further explore the mechanism by which this dose prevents cognitive impairment.

A



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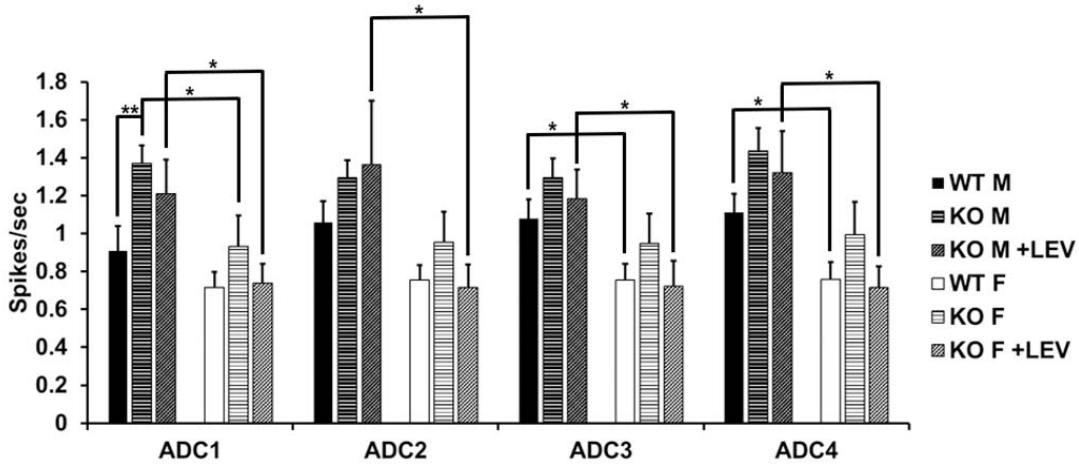

Figure 3-5. EEG spiking activity in sex-matched *ZnT3*$^{-/-}$ and WT mice. Four recording EEG electrodes were implanted and referenced to a ground electrode placed at the cerebellum. Spiking was determined as any deflection greater than two standard deviations above the mean. **(A)** Example 10-second window of a 30-minute EEG recording for a 6-month-old male WT mouse (top) and a male *ZnT3*$^{-/-}$ mouse (bottom). Right panel shows details of a spike. **(B)** Male *ZnT3*$^{-/-}$ mice had more spiking than WT males in the right posterior electrode (ADC1), and there were sex differences in *ZnT3*$^{-/-}$ mice in the left side of the brain (ADC3-4), with males having more spiking. Although chronic LEV did not alter spiking in *ZnT3*$^{-/-}$ mice relative to their untreated counterparts, it had an overall effect of lowering spiking in female *ZnT3*$^{-/-}$ mice compared to male *ZnT3*$^{-/-}$ mice. General linear model, *p<0.05, **p<0.01, n=7-15/group.

Table 3-1. Summary of spike counts (spikes/sec±SEM) for sex-matched WT and *ZnT3*$^{-/-}$ mice at each recording site, as well as the collapsed ADC normalized average counts used in the analysis.

	Male WT	Male KO	Male KO +LEV	Female WT	Female KO	Female KO +LEV
ADC1	0.91±0.13	1.36±0.09	1.21±0.18	0.71±0.08	0.93±0.16	0.74±0.10
ADC2	1.06±0.11	1.30±0.09	1.36±0.34	0.76±0.08	0.95±0.16	0.72±0.12
ADC3	1.08±0.10	1.29±0.10	1.18±0.15	0.75±0.09	0.94±0.16	0.72±0.13
ADC4	1.11±0.10	1.43±0.12	1.32±0.22	0.76±0.09	0.99±0.17	0.72±0.11
Normalized Average (ADC1-4 collapsed)	1.00±0.05	1.31±0.05	1.23±0.11	0.72±0.04	0.93±0.08	0.70±0.05

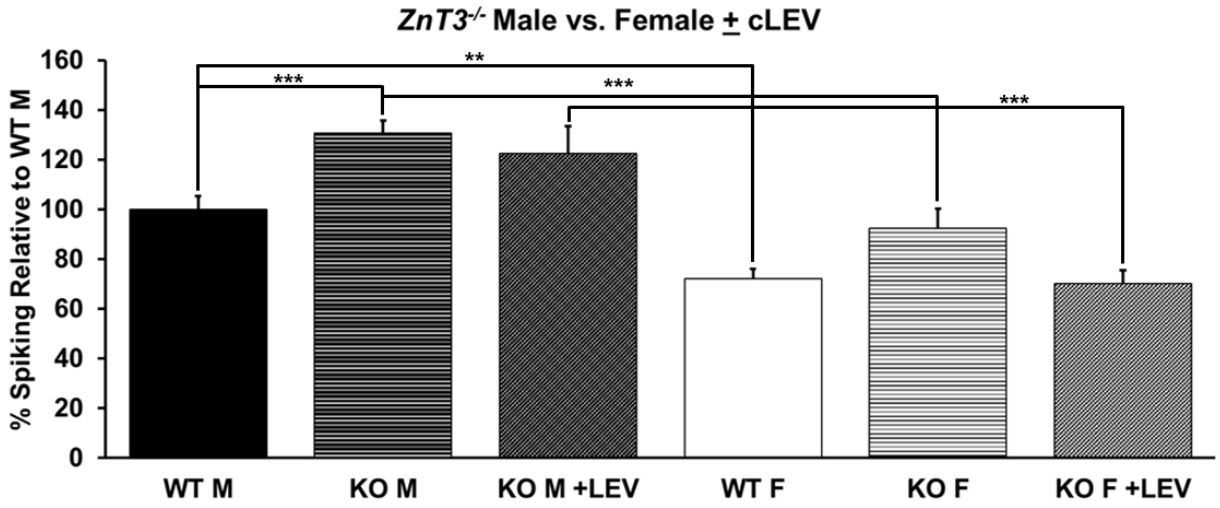

Figure 3-6. Collapsed EEG spiking activity normalized to WT males. Male *ZnT3*$^{-/-}$ mice had higher spiking, whereas female WT mice had lower spiking, when compared to male WT mice. In addition, female *ZnT3*$^{-/-}$ mice had lower spiking compared to their male counterparts, and chronic LEV treatment decreased spiking in female *ZnT3*$^{-/-}$ mice compared to chronic LEV in male *ZnT3*$^{-/-}$ mice. One-way ANOVA with Tukey's *post-hoc* test, **p<0.01, ***p<0.001.

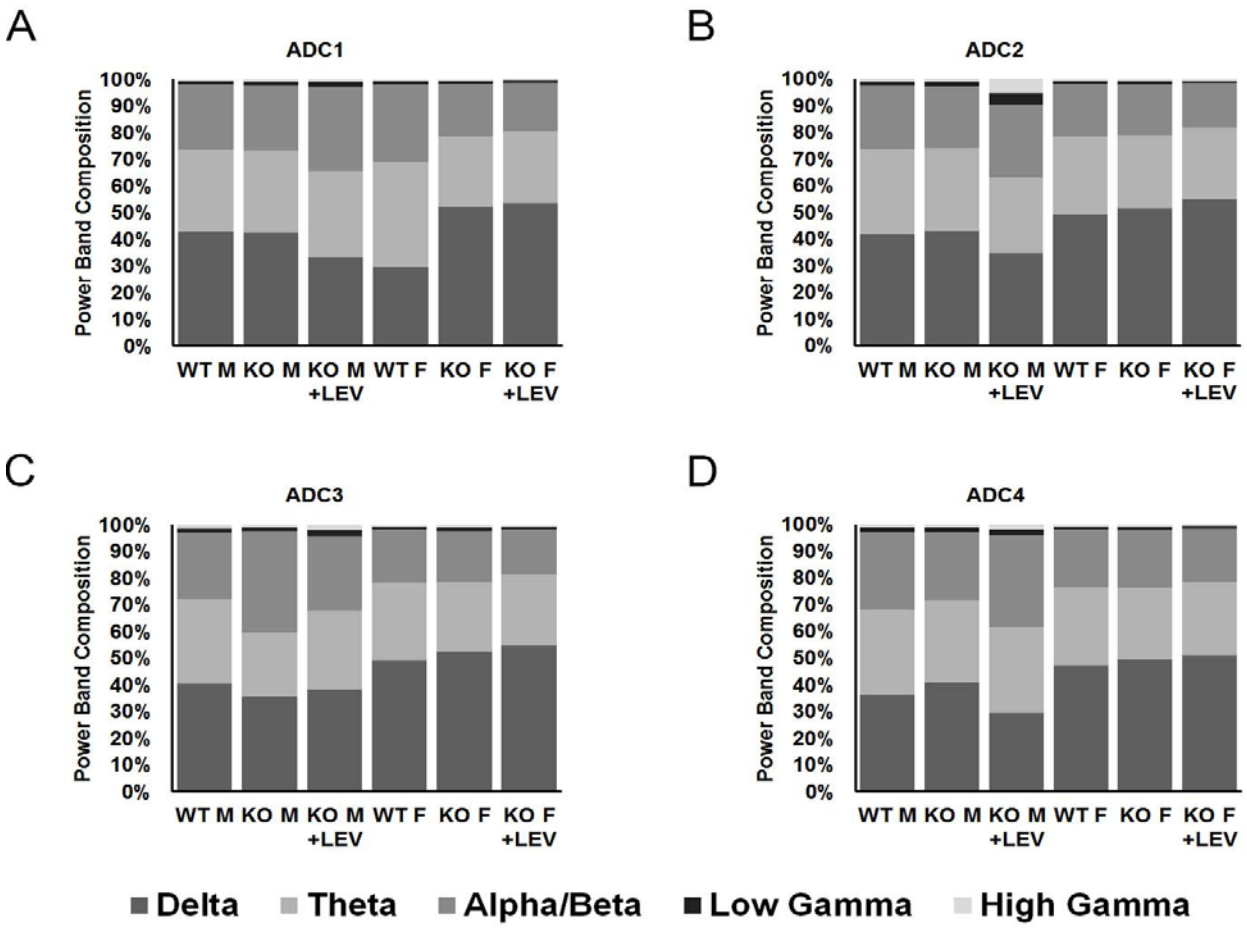

Figure 3-7. Chronic LEV does not alter overall power characteristics. Four recording electrodes were implanted as previously described and custom Python scripts were used to analyze power characteristics at **(A)** ADC1 (right posterior electrode), **(B)** ADC2 (right anterior electrode), **(C)** ADC3 (left anterior electrode), and **(D)** ADC4 (left posterior electrode). Although there were slight changes in individual band frequencies, chronic LEV did not alter the overall power characteristics when compared to untreated controls. n=7-15/group.

3.4.3 LEV modifies gene expression in a sex-specific manner

Because chronic LEV prevents cognitive impairment in *ZnT3*$^{-/-}$ mice and its effects on hippocampal gene expression in mice have been sparsely studied, we next sought to explore the genetic changes induced by chronic LEV treatment in *ZnT3*$^{-/-}$ mice. We created a 150 custom gene panel for analysis using the NanoString nCounter platform on mRNA extracted from hippocampal tissue. We chose to use this system because it is a

high-throughput, sensitive, no amplification method of studying gene expression. After receiving the transcript counts from the experiment, we used a combination of the nSolver software and a linear model to analyze the data. A normal distribution did not fit well with the data (Figure 3-8A-C). Z-values close to zero were indicative of a null distribution, whereas Z-values that piled up near the tails (large negative or large positive values) were indicative of differences in gene expression and could be subject to further investigation. There were gene expression differences between *ZnT3*$^{-/-}$ mice and WT mice (Figure 3-8A), which were sex-specific. For example, male *ZnT3*$^{-/-}$ mice had lower expression in genes involved in the epigenetic regulation of gene expression (ex. *Hdac3* and *Ncor1*) and synaptic plasticity (ex. *Elk1* and *Cdk5*) when compared to male WT mice. Female *ZnT3*$^{-/-}$ mice had higher expression in genes involved in the epigenetic regulation of gene expression (ex. *MeCP2* and *Pcaf*) and apoptosis (ex. *Sod1* and *Fas*). In addition, cluster analysis indicated that chronic LEV treatment modified gene expression in both WT and *ZnT3*$^{-/-}$ mice (Figure 3-8B-D). LEV modified the expression of 50 genes from our CodeSet in female *ZnT3*$^{-/-}$ mice, including genes involved in the epigenetic regulation of gene expression (ex. *Hdac3* and *Dnmt1*), excitability (ex. *Kcnq2* and *Gabrg2*), and neurogenesis (ex. *Ki67* and *Dcx*), among many others. In contrast, only 11 genes from our CodeSet were changed following LEV treatment in male *ZnT3*$^{-/-}$ mice. A summary of the gene differences is shown in Tables 3-2 through 3-9. These results indicate that there are sex-specific differences in hippocampal gene expression in both WT and *ZnT3*$^{-/-}$ mice, and that chronic LEV exerts its effects in a sex-specific manner.

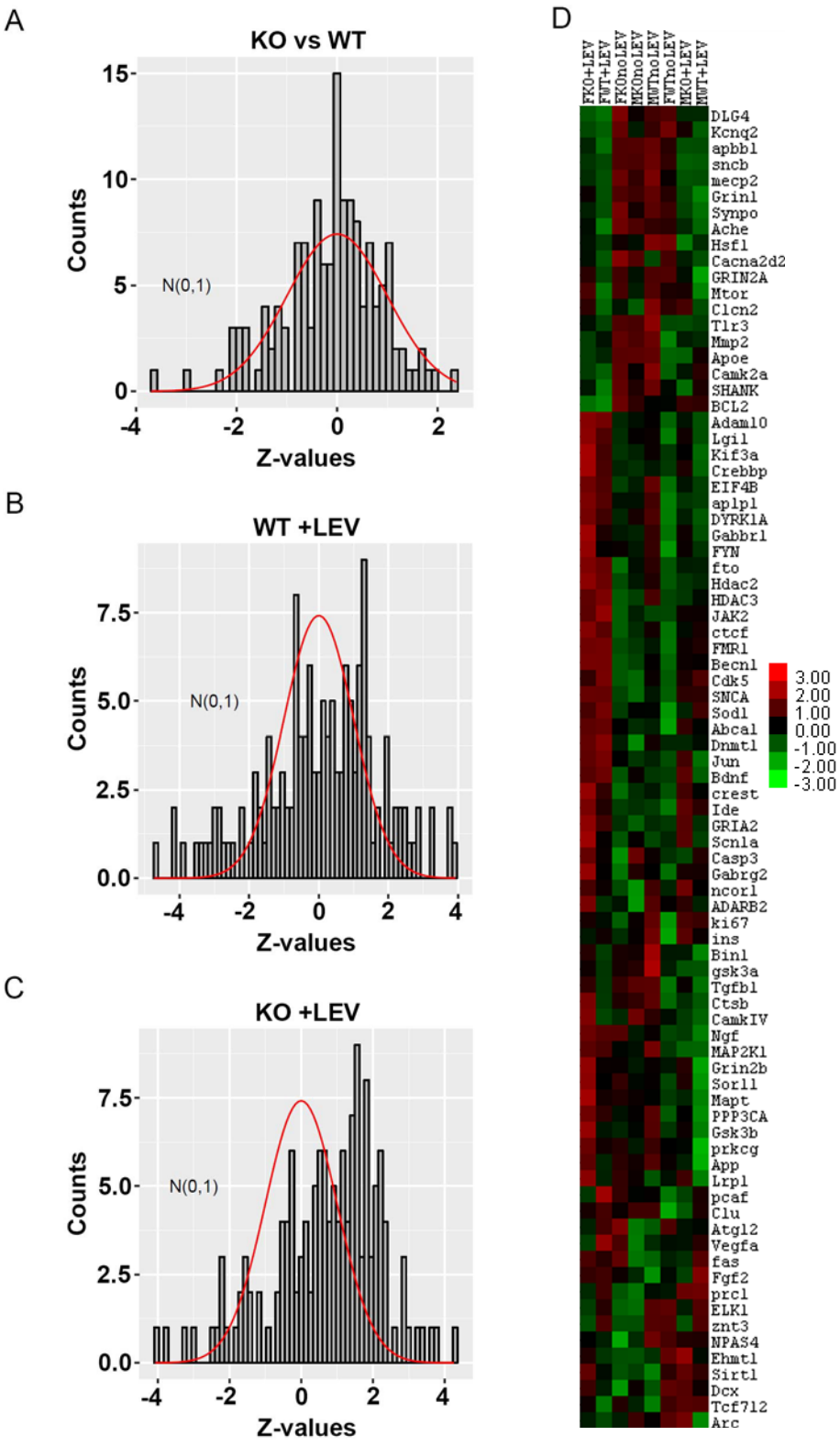

Figure 3-8. Histograms of Z-values for the comparisons on all genes. Hippocampal mRNA was extracted and analyzed using the NanoString nCounter platform. A linear model was created and fit to the data. The theoretical null (N(0,1)) was superimposed and scaled to have the same area for all three comparisons: **(A)** *ZnT3*$^{-/-}$ vs WT, **(B)** LEV in WT, and **(C)** LEV in *ZnT3*$^{-/-}$. The normal distribution did not fit well, indicating that there is a clear shift in gene expression. **(D)** nCounter gene cluster analysis indicates sex-specific effects of chronic LEV on gene expression. Red shades indicate upregulated genes, and green squares indicate downregulated genes. Significant gene differences are shown in Tables 3-2 through 3-9. n=3/group.

Table 3-2. Significant gene expression differences between male and female WT mice. p<0.05

Male WT vs. Female WT	
Gene Name	**Higher in:**
MeCP2	Male
Ins	Male
Sod1	Male
Abca1	Male
Mmp2	Male
Snca	Male
Gsk3a	Male
Clu	Male
Cdk5	Male

Table 3-3. Significant gene expression differences between male and female *ZnT3*$^{-/-}$ mice. p<0.05

Male *ZnT3*$^{-/-}$ vs. Female *ZnT3*$^{-/-}$	
Gene Name	**Higher in:**
Grin2a	Female
Prkcg	Female
Mtor	Female
Abca1	Female
Atg12	Female
Gapdh	Female
Kcnq2	Female
Sorl1	Female
Fto	Male
Sod1	Male
Casp3	Male
Htr1a	Male
Dcx	Male
Gabrg2	Male
Bdnf	Male
Tgfb1	Male

Table 3-4. Significant gene expression differences between male *ZnT3*$^{-/-}$ and male WT mice. p<0.05

Male *ZnT3*$^{-/-}$ vs. Male WT	
Gene Name	**Higher in:**
Lrp1	*ZnT3*$^{-/-}$
Hsf1	WT
Elk1	WT
Ncor1	WT
Clcn2	WT
Gsk3a	WT
Becn1	WT
Bin1	WT
Npas4	WT
Cdk5	WT
Adarb2	WT
Hdac3	WT

Table 3-5. Significant gene expression differences between female *ZnT3*$^{-/-}$ and female WT mice. p<0.05

Female *ZnT3*$^{-/-}$ vs. Female WT	
Gene Name	**Higher in:**
MeCP2	*ZnT3*$^{-/-}$
Mmp2	*ZnT3*$^{-/-}$
Abca1	*ZnT3*$^{-/-}$
Sod1	*ZnT3*$^{-/-}$
Pcaf	*ZnT3*$^{-/-}$
Ngf	*ZnT3*$^{-/-}$
Fas	*ZnT3*$^{-/-}$
Ehmt1	WT
Elk1	WT
Npas4	WT

Table 3-6. Significant gene expression differences between LEV-treated male WT mice and untreated controls. p<0.05

Male WT ± LEV	
Gene Name	**Higher in:**
Prc1	+LEV
Gsk3a	-LEV
Apbb1	-LEV
Ppp3ca	-LEV
Grin2a	-LEV
Kcnq2	-LEV
MeCP2	-LEV

Table 3-7. Significant gene expression differences between LEV-treated female WT mice and untreated controls. p<0.05

Female WT ± LEV	
Gene Name	**Higher in:**
Pcaf	+LEV
Abca1	+LEV
Cdk5	+LEV
Rps6	+LEV
Snca	+LEV
Vegfa	+LEV
Becn1	+LEV
Jun	+LEV
Htr1a	+LEV
Ngf	+LEV
Bdnf	+LEV
Jak2	+LEV
Hsd17b10	+LEV
Gal	+LEV
Kcnq2	-LEV
Apbb1	-LEV
Hsf1	-LEV

Table 3-8. Significant gene expression differences between LEV-treated male *ZnT3*$^{-/-}$ mice and untreated controls. p<0.05

Male *ZnT3*$^{-/-}$ ± LEV	
Gene Name	**Higher in:**
Ide	+LEV
Abca1	+LEV
Tcf7l2	+LEV
Prc1	+LEV
Ncor1	+LEV
Snca	+LEV
Clcn2	+LEV
Ehmt1	+LEV
Tlr3	-LEV
Clu	-LEV
MeCP2	-LEV

Table 3-9. Significant gene expression differences between LEV-treated female *ZnT3*$^{-/-}$ mice and untreated controls. p<0.05

Female *ZnT3*$^{-/-}$ ± LEV			
Gene Name	**Higher in:**	**Gene Name**	**Higher in:**
Fmr1	+LEV	Dyrk1a	+LEV
Aplp1	+LEV	Eif4b	+LEV
CamKIV	+LEV	Ctcf	+LEV
Kif3a	+LEV	Adarb2	+LEV
Lgi1	+LEV	Grin2b	+LEV
Snca	+LEV	Dcx	+LEV
Sod1	+LEV	Map2k1	+LEV
Becn1	+LEV	Mapt	+LEV
Fto	+LEV	Casp3	+LEV
Gsk3b	+LEV	App	+LEV
Hdac3	+LEV	Gria2	+LEV
Fyn	+LEV	Kcnq2	-LEV
Scn1a	+LEV	Sncb	-LEV
Ctsb	+LEV	Ache	-LEV
Gabbr1	+LEV	Vegfa	-LEV
Jak2	+LEV	Grin1	-LEV
Dnmt1	+LEV	Dlg4	-LEV
Cdk5	+LEV	MeCP2	-LEV
Crest	+LEV	Cacna2d2	-LEV
Crebbp	+LEV	Apoe	-LEV
Ide	+LEV	Shank	-LEV
Gabrg2	+LEV	CamK2a	-LEV
Hdac2	+LEV	Synpo	-LEV
Polr2a	+LEV	Bcl2	-LEV

3.4.4 LEV restores neurogenesis only in female *ZnT3*$^{-/-}$ mice

Adult hippocampal neurogenesis, particularly in the dentate gyrus, is required for formation of new memories (Leuner et al., 2006; Deng et al., 2010; Aimone et al., 2014; Yu et al., 2014; Lopez-Virgen et al., 2015). While it is well-known that neurogenesis declines naturally with age (Kuhn et al., 1996; Eriksson et al., 1998; Gould et al., 1999; Kornack and Rakic, 1999; Bondolfi et al., 2004; Heine et al., 2004; Shetty et al., 2005; Kronenberg et al., 2006; Montaron et al., 2006; Manganas et al., 2007; Mirochnic et al., 2009; Knoth et al., 2010; Curtis et al., 2011; Jinno, 2011; Couillard-Despres, 2013; Spalding et al., 2013), it can also be inhibited by Aβ (Mu and Gage, 2011), and is impaired by zinc deficiency (Corniola et al., 2008; Gao et al., 2009; Suh et al., 2009; Kim et al., 2012; Choi et al., 2014; Han et al., 2015). Because chronic LEV prevented age-related cognitive impairment, we sought to investigate the effects of LEV on neurogenesis in *ZnT3*$^{-/-}$ mice. Following chronic LEV treatment, mice were fed the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) in the drinking water and immunohistochemistry was performed on coronal brain sections from 3-month- and 6-month-old WT and *ZnT3*$^{-/-}$ mice, investigating both the number of neurogenic cells and the number of neurogenic cells that express the neuronal marker NeuN (Figure 3-9A and Figure 3-10A). A summary of the immunopositive cells is shown in Table 3-10. Three-month-old *ZnT3*$^{-/-}$ mice had lower BrdU immunolabeled cells and BrdU+NeuN co-labeled cells (Figure 3-9B). There were no sex differences at three months of age (data not shown). Both male and female six-month-old *ZnT3*$^{-/-}$ mice had lower numbers of BrdU immunolabeled cells and BrdU+NeuN co-labeled cells compared to their WT counterparts (Figure 3-10A-B), indicating reduced neurogenesis. Treatment with chronic LEV restored the number of BrdU immunolabeled

cells and BrdU+NeuN co-labeled cells only in female *ZnT3*$^{-/-}$ mice (Figure 3-10B). These results indicate the chronic LEV completely rescues adult hippocampal neurogenesis in a sex-specific manner.

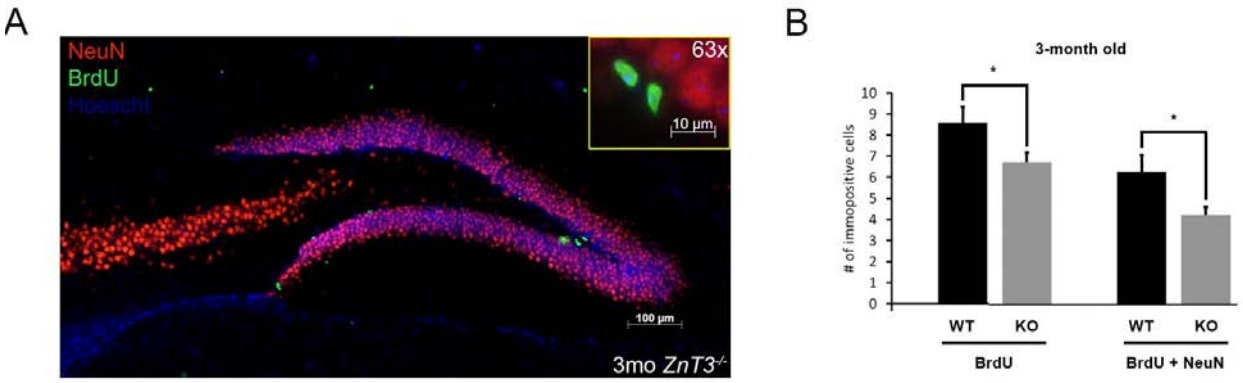

Figure 3-9. Three-month-old *ZnT3*$^{-/-}$ mice have impaired neurogenesis. Three-month old WT and *ZnT3*$^{-/-}$ mice were given oral BrdU for seven days. Afterwards, brains were extracted and coronally sectioned, followed by immunohistochemistry. **(A)** Immunolabeling of the hippocampus showing BrdU (green), NeuN (red), and Hoescht (blue). Note the labeling of BrdU in the hilus of the hippocampus. Inset=63x. Quantification of image analysis indicates **(B)** three-month-old *ZnT3*$^{-/-}$ mice have impaired neurogenesis. Two-tailed t-test, *p<0.05, n=4-6/group.

3.5 Discussion

The purpose of this study was to investigate the effects of chronic treatment with a low dose of the antiepileptic drug Levetiracetam (LEV) on age-related cognitive impairment in the *ZnT3*$^{-/-}$ mouse model. Previous research has shown that three-month-old *ZnT3*$^{-/-}$ mice have normal performance on the Morris Water Maze memory task, but that these mice become impaired at six months of age (Adlard et al., 2010). We utilized the hippocampal-dependent object location memory which has the advantage of not having a motivational component for the mice. Previous work from our laboratory demonstrates that *ZnT3*$^{-/-}$ mice become cognitively impaired starting at three months, and become progressively worse by six months of age (Vogler, 2014). Because synaptic zinc is concentrated at excitatory synapses in the mossy fiber tract, genetic removal of

synaptic zinc results in lack of zinc neuromodulation on postsynaptic receptors. Since zinc has a very high affinity for amyloid β (Aβ) and rapidly forms Zn-Aβ complexes leading to increased oligomerization of Aβ and thus reduced zinc availability in the synapse (reviewed in Sensi et al., 2009), our data showing age-dependent cognitive impairment suggests that the *ZnT3*$^{-/-}$ mouse model approximates neurodegeneration and cognitive impairment related to Alzheimer's Disease (AD).

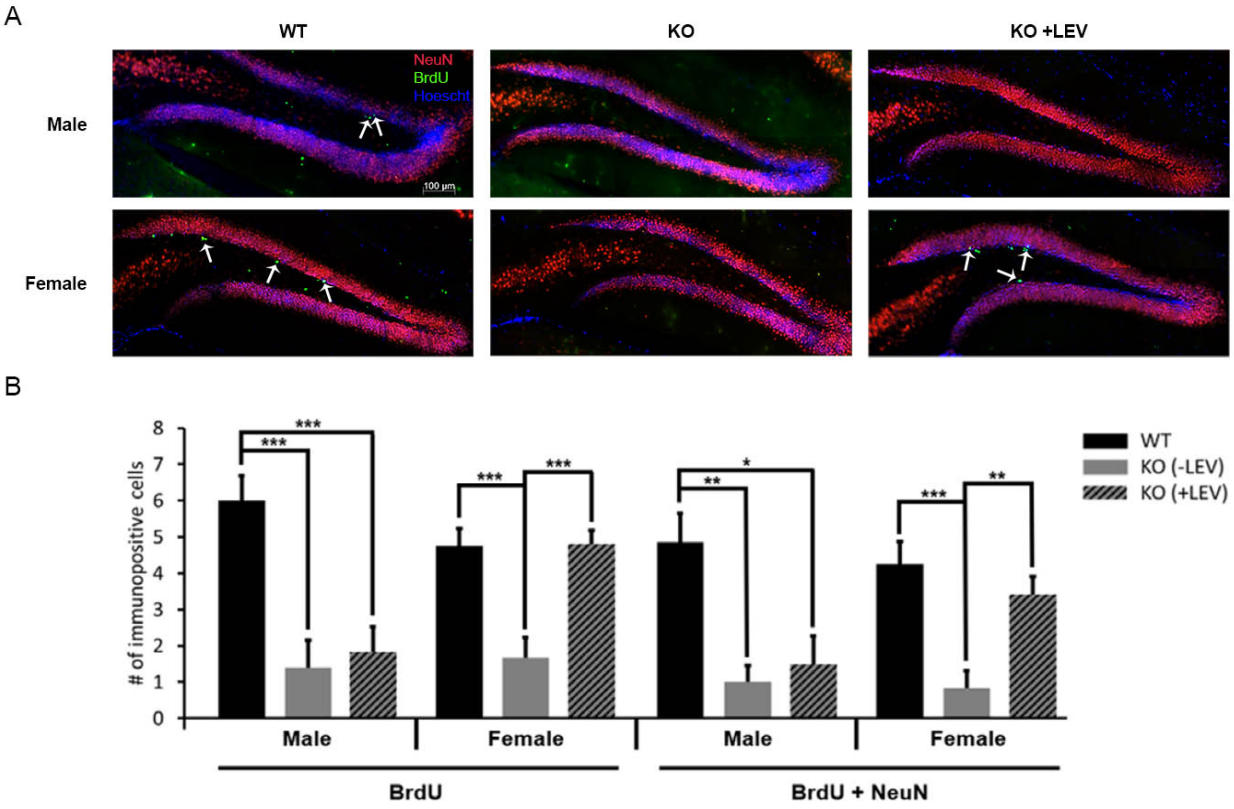

Figure 3-10. Chronic LEV restores neurogenesis only in female *ZnT3*$^{-/-}$ mice. Six-month old WT and *ZnT3*$^{-/-}$ mice were given oral BrdU for seven days. Afterwards, brains were extracted and coronally sectioned, followed by immunohistochemistry. **(A)** Immunolabeling of the hippocampus showing BrdU (green), NeuN (red), and Hoescht (blue). Arrows indicate examples of positive immunolabeling. Quantification of image analysis indicates **(B)** chronic LEV treatment completely restores neurogenesis in female *ZnT3*$^{-/-}$ mice, but does not have an effect in male *ZnT3*$^{-/-}$ mice. One-way ANOVA with Tukey's *post hoc* test, *p<0.05, **p<0.01, ***p<0.001, n=5-6/group.

Table 3-10. Summary of BrdU and BrdU+NeuN immunopositive cell counts±SEM for sex-matched WT mice, *ZnT3*$^{-/-}$ mice, and *ZnT3*$^{-/-}$ mice treated with LEV.

	BrdU	BrdU + NeuN
3mo WT	8.60±0.74	6.30±0.80
3mo KO	6.75±0.46	4.25±0.37
6mo Male WT	6.00±0.68	4.83±0.79
6mo Male KO	1.40±0.75	1.00±0.45
6mo Male KO +LEV	1.83±0.70	1.50±0.76
6mo Female WT	4.75±0.48	4.25±0.63
6mo Female KO	1.66±0.56	0.83±0.48

Hyperexcitability is an upstream mechanism leading to neurodegeneration, and a high acute dose of LEV (200mg/kg) reduced spiking in the hAPP mouse model (Sanchez et al., 2012). These researchers also showed that a low chronic dose of LEV (75mg/kg/day for 4 weeks) improves performance on the elevated plus maze, the Morris Water Maze, and the novel object recognition memory tasks, with behavioral abnormalities returning within 35 days after the end of treatment. Because of the uncertainty of the involvement of the hippocampus in the novel object recognition memory task (Broadbent et al., 2010, Barker and Warburton, 2011, Cohen and Stackman, 2015), we decided to use the object location memory task, which requires the hippocampus for encoding, consolidation, and retrieval (reviewed in Vogel-Ciernia and Wood, 2014). Our data demonstrate that six-month-old *ZnT3*$^{-/-}$ mice have impaired hippocampal cognition and that a low chronic dose, but not an acute dose, of LEV prevents this cognitive decline. The observation that only the chronic dose prevents impairment suggests that the mechanism of action of LEV in cognition may not be related to its antiepileptic properties. Our electroencephalogram analysis indicates that a low chronic dose of LEV does not significantly change spiking activity in sex-matched *ZnT3*$^{-/-}$ mice; however, there is a more

profound effect in female *ZnT3*$^{-/-}$ mice, reducing spiking when compared to treated male *ZnT3*$^{-/-}$ mice. However, chronic LEV did not have an effect on the overall power characteristics in *ZnT3*$^{-/-}$ mice, suggesting that the dose of LEV given was not sufficient enough to invoke its antiepileptic properties in the behavioral rescue. Because of this, we sought to characterize gene expression changes resulting from chronic LEV treatment to understand how this dose of LEV prevents cognitive impairment.

mRNA analysis using NanoString and a custom 150 gene CodeSet indicated that not only are there significant differences between sex-matched *ZnT3*$^{-/-}$ mice and WT mice, but that LEV has significant effects in both *ZnT3*$^{-/-}$ and WT mice in a sex-specific manner. Chronic LEV treatment modified the gene targets for female *ZnT3*$^{-/-}$ mice more than male *ZnT3*$^{-/-}$ mice. Among the significant gene changes were genes involved in excitability, cell signaling, neurogenesis, cognition, and the epigenetic regulation of gene expression. The upregulation of the gene *Hdac3* is particularly intriguing, as Hdac3 deacetylates chromatin and represses gene expression, and inhibition of *Hdac3* is typically associated with improved memory (Stefanko et al., 2009, McQuown and Wood, 2011). Thus, the upregulation of *Hdac3* along with upregulation of *Crest* and the histone acetyltransferase *Crebbp*, known to interact and positively regulate gene expression (Aizawa et al., 2004, Mellstrom et al., 2008, Qiu and Ghosh, 2008), in six-month-old female *ZnT3*$^{-/-}$ mice offers the intriguing possibility that the chromatin landscape may be more tightly regulated to ensure proper expression of genes involved in learning and memory (Wood et al., 2006, Caccamo et al., 2010, Barrett et al., 2011, Haettig et al., 2011). Future studies should explore these possible epigenetic mechanisms for chronic LEV-mediated prevention of cognitive decline.

One of the genes significantly increased following chronic LEV in female *ZnT3*$^{-/-}$ mice was *Dcx*, a gene known to be involved in adult neurogenesis and expressed in areas of continuous neurogenesis, such as the hippocampus (des Portes et al., 1998, Nacher et al., 2001, Brown et al., 2003, Couillard-Despres et al., 2005). Interestingly, basal *Dcx* expression was higher in male *ZnT3*$^{-/-}$ mice compared with female *ZnT3*$^{-/-}$ mice, yet chronic LEV only changed *Dcx* in female *ZnT3*$^{-/-}$ mice. Our neurogenesis data corroborates this finding, as only in female *ZnT3*$^{-/-}$ mice was the number of BrdU- and BrdU+NeuN-immunopositive cells restored to WT levels following chronic LEV treatment. The total number of cells changed by chronic LEV suggests that instead of LEV acting primarily through increasing the total number of new neurons in the hippocampus, LEV may contribute to the correct integration of newly formed neurons into the existing hippocampal network important for hippocampus-dependent tasks (van Praag et al., 2002, Ramirez-Amaya et al., 2006, Zhao et al., 2006, Toni et al., 2007, Song et al., 2012).

In summary, in this chapter, I presented experiments that demonstrate that *ZnT3*$^{-/-}$ mice exhibit age-related impairment in hippocampal-dependent memory tasks which can be prevented by chronic, but not acute, treatment with the antiepileptic drug LEV, suggesting that a chronic low dose of LEV does not act through its antiepileptic properties to prevent cognitive decline. Chronic LEV modified gene expression in a sex-specific manner, and specifically rescued neurogenesis in aged female *ZnT3*$^{-/-}$ mice.

Chapter 4

Summary and Future Directions

There are two pressing issues related to Alzheimer's Disease (AD) research and treatment: 1) the absence of effective ways to establish when the disease starts as well as methods to follow up the effectiveness of potential treatments, and 2) the absence of successful therapies for AD. This second issue was the focus of this project. In recent years it has become clear that one of the abnormal changes occurring in the brains of AD patients is a progressive increase of abnormal neuronal activity, or neuronal "hyperactivity". This hyperactivity is a serious complication that is expressed as a malfunction of not only individual neurons, but also of groups of hundreds or thousands of neurons forming neuronal circuits in the brain. One of the most serious consequences of excessive neuronal hyperactivity is the development of seizures. In fact, the occurrence of seizures is significantly increased in AD. When hyperactivity and seizures occur, the function and the survival capacity of affected neurons is compromised, interfering with learning and memory processes and resulting in cognitive decline. This dissertation was aimed at preventing neuronal hyperactivity in the *ZnT3*$^{-/-}$ mouse model of AD by using Levetiracetam (LEV), a compound currently marketed as an antiepileptic drug.

Although there are plenty of studies examining the efficacy of LEV on attenuating cognitive deficits (reviewed in Xiao, 2016), the mechanisms by which LEV acts to prevent cognitive impairment remain unknown. Previous research indicates that acute LEV does not suppress seizure activity through mediating GABAergic and calcium signaling, unlike classical antiepileptic drugs (Margineanu and Klitgaard, 2003). In fact, in the clinic, the chronic effects of LEV are not primarily on suppressing seizure activity (Piazzini et al.,

2006); rather, it suppresses hippocampal hyperactivity in the dentate gyrus and CA3 regions associated with cortical thinning (Putcha et al., 2011; Bakker et al., 2012). In the hAPP mouse model, chronic LEV improves cognition by suppressing subclinical epileptiform activity as opposed to clinical seizures (Sanchez et al., 2012). These studies suggest that chronic LEV may have additional effects to enhance and stabilize neuroplasticiity leading to prevention of cognitive decline, which was the focus of this study.

To begin understanding how a low chronic dose of LEV prevents cognitive decline, we first performed behavioral analysis on six-month old *ZnT3*$^{-/-}$ mice treated with either acute or chronic LEV. Using the hippocampus-dependent object location memory task, we found that a low chronic dose, but not a high acute dose typically used for antiepileptic studies, of LEV prevented age-related cognitive decline. These results suggest that chronic LEV treatment can modify the abnormal behavioral patterns seen in *ZnT3*$^{-/-}$ mice. To assess whether the effects of LEV might be through reduction of spiking activity in *ZnT3*$^{-/-}$ mice, we developed a novel electroencephalogram (EEG) electrode implantation method which has the advantages over other methods of being low cost, not relying on screws for anchorage which may cause electrical interference, and can be left in place for weeks without signal degradation (Vogler et al., 2017). We saw increased spiking in male *ZnT3*$^{-/-}$ mice compared with WT, and male *ZnT3*$^{-/-}$ mice and WT mice consistently had more spiking than their female counterparts. In addition, chronic LEV treatment reduced spiking in female *ZnT3*$^{-/-}$ mice when compared to male *ZnT3*$^{-/-}$ mice. However, chronic LEV did not change the power characteristics in the different treatment groups,

suggesting again that the effects of chronic LEV on prevention of cognitive impairment may be through mechanisms not necessarily associated with its antiepileptic properties.

mRNA analysis indicated sex-specific effects of LEV which include changes in expression of genes involved in cognition, neurogenesis, and epigenetics, among others. Specifically, the results of the NanoString data suggest that chronic LEV may lead to a tighter regulation of the expression of genes involved in learning and memory and synaptic plasticity by increasing chromatin compaction, while upregulating expression of factors that promote gene expression. This combination implies that during aging, the chromatin landscape becomes dysregulated, and promotes the expression of age-related deleterious genes. LEV corrects this by increasing chromatin compaction, which may allow for less abnormal gene transcription by inhibiting the ability of transcription factors to bind and enhance expression of deleterious genes. This epigenetic effect of LEV is intriguing and merits further study. Specifically, the seemingly paradoxical result of behavioral amelioration and increased expression of *Hdac3*, typically associated with increased chromatin compaction and decreased gene expression, following chronic LEV, as well as the reduction in *MeCP2*, typically associated with decreased chromatin compaction and increased gene expression, further suggest a complex interplay of epigenetic factors leading to improved behavioral performance after chronic LEV treatment. Examining *MeCP2* occupation at different promoter regions of genes involved in epigenetics and learning and memory might shed further light on potential mechanisms of LEV-mediated prevention of cognitive decline.

Another result coming from the NanoString analysis is the upregulation of *Dcx*, a gene involved in neurogenesis, only in aged female *ZnT3*$^{-/-}$ mice. Adult hippocampal

neurogenesis is required for formation of new memories (reviewed in Deng et al., 2010). Because chronic LEV prevented cognitive impairment in aged *ZnT3*$^{-/-}$ mice, we next explored its effects on neurogenesis and found that it increased the amount of new neurons being created, but only in aged female *ZnT3*$^{-/-}$ mice, further suggesting sex-specific actions of chronic LEV. The amount of new neurons following chronic LEV suggests that it is the integration of a few neurons into the existing hippocampal network that is important for supporting memory. How these neurons are integrated, and how they might differ from basal neurogenic cells, could be an avenue of future research.

In conclusion, these experiments indicate that a chronic low dose (50mg/kg/day versus 200mg/kg used to reduce spiking) of LEV prevents age-related cognitive impairment in the *ZnT3*$^{-/-}$ mouse model in a sex-specific manner (Figure 4-1). Although LEV is currently marketed as an antiepileptic, its ability to enhance cognition through changes in gene expression and increased neurogenesis when applied chronically suggests that it can act as an epigenetic and neurogenic regulator as well. Further examination of these mechanisms may give insight into the debilitating disorder that is AD and perhaps allow for the repurposing of LEV for prevention of cognitive impairment.

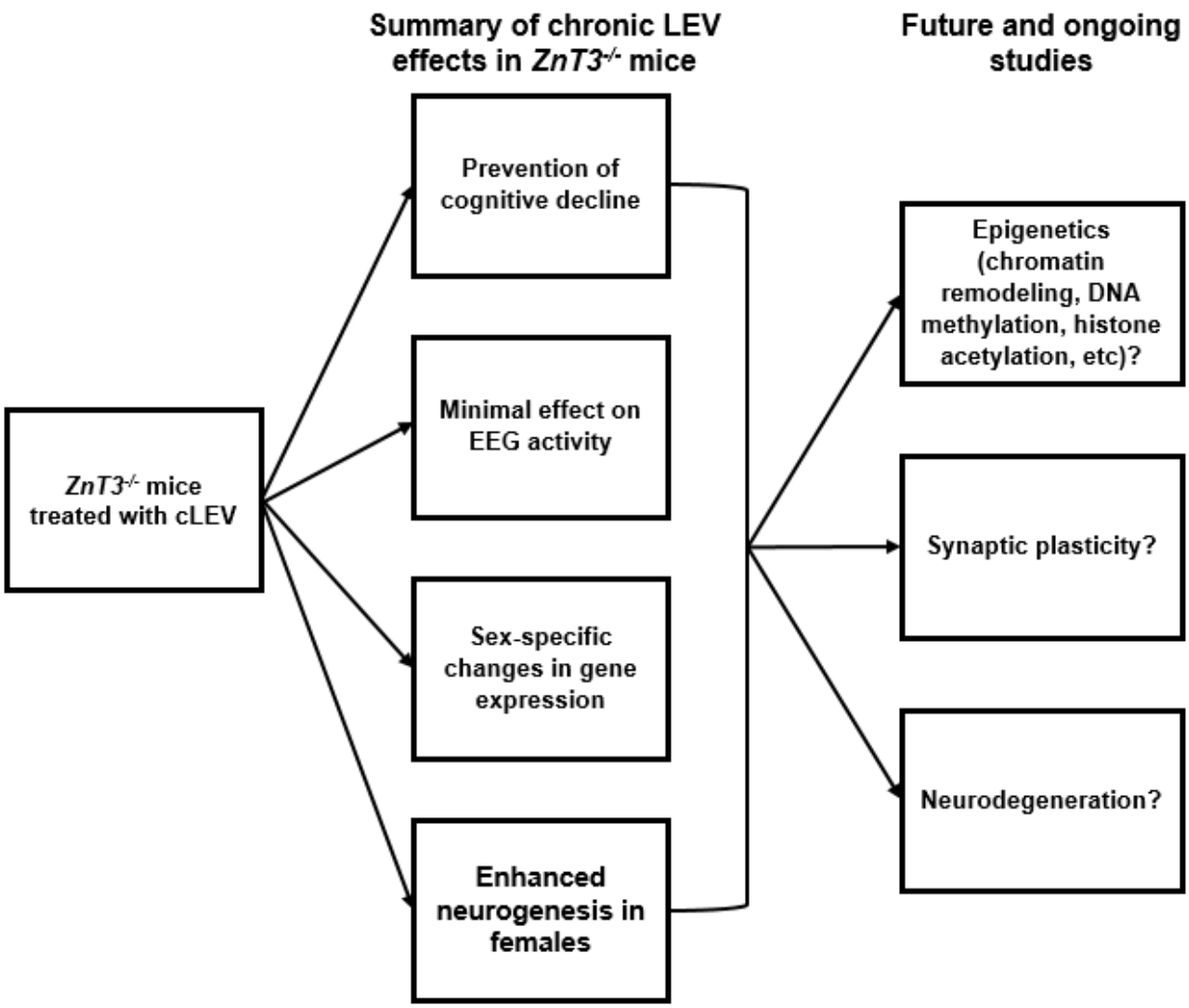

Figure 4-1. Summary of chronic LEV effects on *ZnT3*$^{-/-}$ mice. This dissertation investigated the effects of a chronic low dose of Levetiracetam (cLEV, 50mg/kg/day) in preventing cognitive decline in *ZnT3*$^{-/-}$ mice. The effects on electroencephalogram (EEG) activity were minimal, suggesting that this dose does not work through its antiepileptic mechanisms to reduce neuronal excitability. Instead, it sex-specifically enhances hippocampal neurogenesis. NanoString analysis indicates that it also sex-specifically alters mRNA levels, including genes involved in the epigenetic regulation of gene expression, synaptic plasticity, and neurodegeneration. Some of the genes altered (ex. *MeCP2, Dnmt1, Hdac3, Crest, Crebbp*) suggest that the low chronic dose of LEV may promote chromatin compaction though affecting remodeling complexes and decreasing histone acetylation. In addition DNA methylation may be decreased allowing for the promotion of genes involved in synaptic plasticity and cognition, which may ultimately lead to enhanced plasticity and reduced neurodegeneration.

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Appendix

Gene data used in the construction of the 150 gene custom CodeSet

#	Gene	Accession	Position	Target Sequence
1	Abca1	NM_013454.3	6866-6965	CTCCTTGTCATCTCTAGCCAGGATATTCAG CATCCTCTCCCAGAGCAAAAAGCGACTCCA CATAGAAGACTACTCTGTCTCTCAGACAAC ACTTGACCAA
2	Ache	NM_009599.3	1074-1173	GCCTCAAGAAAGTATCTTCCGATTTTCCTT CGTGCCTGTGGTAGACGGGGACTTCCTCA GTGACACACCGGAGGCTCTCATCAATACTG GAGATTTTCAA
3	Adam10	NM_007399.3	2391-2490	CATTCAGCAGCCCCCGCGTCAGAGGCCCC GAGAGAGTTATCAAATGGGACACATGCGA CGCTAATGCAGCTTTTGCCTTGGTTCTTCC TAGTGCCTACAG
4	ADARB2	NM_052977.4	967-1066	TTCCCAGACACGTTGTTCAAGGAGTTTGAA CCATCCTCAAAAAACGAAGACTTCCCAGGC TGTCATCCCGTTGATACTGAGTTTCTGTCC TCTGCCTATC
5	apbb1	NM_001253885.1	2007-2106	TATTACCTGGGAAATGTGCCAGTTGCTAAA CCTGTTGGGGTAGACGTGATTAATGGGGC CCTGGAGTCAGTCCTGTCTTCCAGTAGCC GTGAGCAGTGGA
6	aplp1	NM_007467.3	2301-2400	CTGGGTCCCAGGTATGTATGTCACTCCCTG GAATTCACCATCCCACGTTTCTTCACTAAC ATCCCAATAAAGTCCTCTTTCCCACCCGGC CAAAAAAAAA
7	apoa1	NM_009692.3	226-325	GTGAAGGATTTCGCTAATGTGTATGTGGAT GCGGTCAAAGACAGCGGCAGAGACTATGT GTCCCAGTTTGAATCCTCCTCCTTGGGCCA ACAGCTGAACC
8	Apoe	NM_001305844.1	904-1003	CCTGCAGGCGGAGATCTTCCAGGCCCGCC TCAAGGGCTGGTTCGAGCCAATAGTGGAA GACATGCATCGCCAGTGGGCAAACCTGAT GGAGAAGATACAG
9	App	NM_007471.2	512-611	GTGAGCGACGCCCTTCTCGTGCCCGACAA GTGCAAGTTCCTACACCAGGAGCGGATGG ATGTTTGTGAGACCCATCTTCACTGGCACA CCGTCGCCAAAG
10	Arc	NM_018790.2	2716-2815	TGCAGTCTGAGTAGGCCTTGTTGCGGTTCC TCCATCTGCCTGGTCTATTGGTGTTCTGAG ACCAATTCCACTGATGTTCTGACAGATCCT CCACCCTGTG
11	Atg12	NM_026217.1	1801-1900	CATGGCAGCACTCCTAGCATTGAGGAAGT GGAAACAAGCAGACTGAAAGTTTAAGACTG TGTGGGCTACAGAGTGAGATTGTCTCACCT CTGGGGGAAAA

12	Bace1	NM_011792.4	3108-3207	CTTTATGGAATACTGAGTGGGTTCATTCCTCTCTTGCCCTCTCCAATGGCCCCTCTATTTATCTGGCTAAGGAAACACCACGCATTGGCTAGTATTAAAC
13	BCL2	NM_009741.3	1845-1944	GGCCTTCTTTGAGTTCGGTGGGGTCATGTGTGTGGAGAGCGTCAACAGGGAGATGTCACCCCTGGTGGACAACATCGCCCTGTGGATGACTGAGTACCTG
14	Bdnf	NM_007540.4	3261-3360	AGTCCCGTCTGTACTTTACCCTTTGGGGTTAGAAGTCAAGTTGGAAGCCTGAATGAATGGACCCAATGAGAACTAGTGTTAAGCCCATTTCCCTAGTCAG
15	Becn1	NM_019584.3	1146-1245	TCTGGAGTCTCTGACAGACAAATCTAAGGAGTTGCCGTTATACTGTTCTGGGGGTTTGCGGTTTTTCTGGGACAACAAGTTTGACCATGCAATGGTAGCT
16	Bin1	NM_001083334.1	1401-1500	GATTCATGTTCAAGGTTCAAGCCCAGCATGATTACACGGCCACTGACACTGATGAGCTGCAACTCAAAGCTGGCGATGTGGTGTTGGTGATTCCTTTCCA
17	Cacna2d2	NM_020263.3	1403-1502	GGGGTGAGGATCGCGTGCAGGATGTCTTCGAAAAGTACAATTGGCCCAATCGGACGGTACGTGTGTTCACGTTCTCCGTAGGACAGCATAACTATGATGT
18	Cacng2	NM_007583.2	645-744	GACGCGGACTACGAAGCTGACACCGCAGAGTATTTCCTCCGGGCCGTGAGGGCCTCGAGTATCTTCCCGATCCTGAGTGTGATCCTGCTTTTCATGGGTG
19	Calb1	NM_009788.4	344-443	ATGGAAAAATAGGAATTGTAGAGTTGGCTCACGTCTTACCCACAGAAGAGAATTTCTTGCTGCTCTTTCGATGCCAGCAACTGAAGTCCTGCGAGGAATT
20	Camk2a	NM_177407.4	3291-3390	TCAAGGTAATAGTCTCAGTGTCCAACTTGGACTTACGTTGCTGCCTCTCCGTGCTTTTGGTCTCTCTGTGGCTATGTTTTGCCAGCATGAGACCCTGTTC
21	CamkIV	NM_009793.3	3281-3380	GTTTCTCCAGGCTGTTAGAGTGTCAGCACTTCAGCAGGTCAAGCACAAACCTCTGACAGCTTTTGGGGAAGGAGACAGTGTGGTGCTTTGAGCATTTTGT
22	Casp3	NM_009810.2	631-730	GAGGCTGACTTCCTGTATGCTTACTCTACAGCACCTGGTTACTATTCCTGGAGAAATTCAAAGGACGGGTCGTGGTTCATCCAGTCCCTTTGCAGCATGC
23	Ccl2	NM_011333.3	416-515	TCTTCAGCACCTTTGAATGTGAAGTTGACCCGTAAATCTGAAGCTAATGCATCCACTACCTTTTCCACAACCACCTCAAGCACTTCTGTAGGAGTGACCA

24	Cdk5	NM_007668.3	78-177	GGAAGGCACCTATGGAACTGTGTTCAAGG CTAAAAACCGGGAAACTCATGAGATTGTGG CTCTGAAGCGTGTCAGGCTGGATGATGAC GATGAGGGTGTG
25	CDK5R1	NM_009871.2	3281-3380	TTTTGAGAAGGTGCATATTTCAGAAGTGGT CGGCAACTGAGGAAAGAGCTTCCTTGGTT CCATTGTGAAGCCCGTGTCCTCTCTGCTTG CGCTGGCTCTC
26	CHAT	NM_009891.2	585-684	TTGGGTCTCTGAATACTGGCTGAATGACAT GTATCTAAACAACCGCCTGGCCCTGCCAGT CAACTCTAGCCCTGCTGTGATCTTTGCTCG GCAGCACTTC
27	Clcn2	NM_009900.2	1579-1678	TCTTCAAAACTCGGTTCCGACTCGACTTCC CATTTGACCTGCAAGAGCTGCCAGCCTTTG CTGTCATTGGCATTGCTAGTGGCTTCGGG GGAGCCCTCTT
28	Clu	NM_013492.2	355-454	TTAATAAGGAGATTCAGAACGCCGTCCAGG GAGTGAAGCACATAAAAACTCTCATAGAAA AAACCAACGCAGAGCGCAAGTCCTTGCTC AACAGTTTAGA
29	Creb1	NM_001037726.1	2735-2834	TTCTGTCTGGACAGTTCACCAGATTCTCCA GAAGGCTTTCAAACGGCTAAAGTTTGATCT TTGTCCTGCTGAGCTTGCTGGGAAGGAGA TAGCATAAAAG
30	Crebbp	NM_001025432.1	3771-3870	TTGGATATTGCTGTGGACGAAAGTATGAGT TCTCCCCACAGACTTTGTGCTGTTACGGAA AGCAGCTGTGTACAATTCCTCGTGATGCAG CCTACTACAG
31	ctcf	NM_181322.3	1606-1705	AATGTGGCCAAATTTCATTGTCCCCATTGT GACACTGTCATAGCCCGAAAAAGTGATTTG GGTGTCCACTTGCGAAAGCAGCATTCCTAT ATTGAACAGG
32	Ctsb	NM_007798.2	2016-2115	TGTCACCCTCAGAAATGGTTCTCAAAACCG TACCTGTAACTGCTCACACCTCGGAGCACT TCCTCCTGCTTAGAGATAAGGGAGCTACAC TTGTTTAATT
33	Dcx	NM_010025.2	8576-8675	GAGTGATACAAAGGCAGTGTTTCAACAAGA CACTCTCTTCTTTGGTTTGCTGTTCTCTCTT GGACTGGAATGTTTGGCAAGGCCCATGTA AACTGGTTAG
34	DLG4	NM_001109752.1	1867-1966	CTGAGCTATGAGACGGTGACGCAGATGGA AGTGCACTACGCTCGCCCCATCATCATCCT TGGGCCTACCAAAGACCGTGCCAACGATG ATCTTCTCTCCG
35	Dnmt1	NM_010066.3	2381-2480	CTCGGTCATTCCAGATGATTCCTCCAAACC ACTCTATCTAGCCAGGGTCACAGCTCTGTG GGAAGACAAAAATGGTCAGATGATGTTCCA TGCGCACTGG

36	Dnmt3a	NM_007872.4	7161-7260	TCCAGAGAGTAGAAGAAAATTCCTGGGCCTGTCTGGTTGGTGCAGAGCTTTTATGGCACTACAAGGTCTTCAGGAACTGTCACTGTAAGAGCCCTGGCTG
37	drd2	NM_010077.2	631-730	GAATGAGTGTATCATTGCCAACCCTGCCTTCGTGGTCTACTCCTCCATCGTCTCGTTCTACGTGCCCTTCATCGTCACCCTGCTGGTCTATATCAAAATC
38	DYRK1A	NM_001113389.1	3235-3334	GAGGTCTGAGTGGTGGTCCTTGGGATAACCTTTGGCCTTACGGATTTGGACTCGGAGTTAGAAGAGCCCACCATTTCAGATGCAATCACTTGTGGACATG
39	Egr1	NM_007913.5	516-615	CGGCAGCAGCGCCTTCAATCCTCAAGGGGAGCCGAGCGAACAACCCTATGAGCACCTGACCACAGAGTCCTTTTCTGACATCGCTCTGAATAATGAGAAG
40	Ehmt1	NM_001109686.2	2387-2486	CTCCACATGTTTGCATTTGGCTGCCAAGAAAGGCCACTATGATGTGGTTCAGTATCTGCTTTCAAATGGACAGATGGATGTCAACTGCCAGGATGACGGT
41	EIF4B	NM_145625.3	633-732	GTGCTCTGAGTCTCAATGAAGAGTCTCTAGGTAACAGGAGAATTCGTGTGGATGTTGCTGATCAAGCACAGGATAAAGACAGGGATGACCGTTCTTTTGG
42	ELK1	NM_007922.4	3071-3170	AGGGTCTCATCTTTGCCTAAACTTACCAGTTGGGATACACTTGCTGGCCATCAAGTCTCAAGGATCTACCTGGTTCTACCTTGGGATTACAAGCATGTAC
43	ep300	NM_177821.6	4306-4405	TGGGACCTTTCTGGAGAATCGAGTGAATGACTTTCTGAGGCGACAAAATCACCCTGAATCAGGAGAGGTCACTGTTCGGGTTGTTCATGCTTCTGACAAA
44	ERBB4	NM_010154.1	2291-2390	TCCTGGTGATCATGGCTTTGACATTTGCTGTCTATGTCAGAAGAAAGAGCATCAAAAAGAAACGTGCTTTGAGGAGATTCCTGGAGACAGAGCTGGTAGA
45	fas	NM_007987.2	96-195	GGCTCACAGTTAAGAGTTCATACTCAAGGTACTAATAGCATCTCCGAGAGTTTAAAGCTGAGGAGGCGGGTTCGTGAAACTGATAAAAACTGCTCAGAAG
46	Fgf2	NM_008006.2	288-387	CTCTACTGCAAGAACGGCGGCTTCTTCCTGCGCATCCATCCCGACGGCCGCGTGGATGGCGTCCGCGAGAAGAGCGACCCACACGTCAAACTACAACTCC
47	FMR1	NM_008031.2	766-865	GGTAATTTTGTCCATCAATGAAGTCACCTCAAAGCGAGCCCACATGTTGATTGACATGCACTTTCGAAGTCTGCGCACCAAGTTGTCTCTTATACTGAGA

48	fto	NM_011936.2	635-734	CCTACAACGTGACTTTGCTAAACTTCATGG ATCCTCAGAAGATGCCCTACTTGAAAGAGG AGCCCTATTTCGGCATGGGGAAGATGGCG GTGAGCTGGCA
49	FYN	NM_008054.2	1031-1130	TTCTTATCCGCGAGAGCGAAACCACCAAAG GTGCCTACTCACTTTCCATCCGTGATTGGG ATGATATGAAAGGGGACCACGTCAAACATT ATAAAATCCG
50	Gabbr1	NM_019439.3	1367-1466	TGCCTGTTAAAAACCTGAAGCGTCAAGATG CTCGAATCATCGTGGGACTTTTCTATGAGA CCGAAGCCCGGAAAGTTTTTTGTGAGGTCT ATAAGGAACG
51	Gabrg2	NM_177408.5	1614-1713	TTAGAACAAGAGCTGTTACACTGAGCAAGA TACCTTTGAGCAACAGCAATGAAAACAGTG GAAGCTGGGAGGGTTTAAAGTGGCATTATC AGTCTTTGAC
52	gal	NM_010253.3	527-626	TGTCCACTGTGCACGTGTGTCCTGTGCTGT AATTTAAAGTCATTCTAGGCTAAGAAGAATC TTCTGCCAACTCCTCAAGCCAATCGTCTGT TCTCTGCTT
53	gap43	NM_008083.2	143-242	TACCACCATGCTGTGCTGTATGAGAAGAAC CAAACAGGTTGAAAAGAATGATGAGGACCA AAAGATTGAACAAGATGGTGTCAAGCCGGA AGATAAGGCT
54	gapdh	NM_001001303.1	891-990	AGGTTGTCTCCTGCGACTTCAACAGCAACT CCCACTCTTCCACCTTCGATGCCGGGGCT GGCATTGCTCTCAATGACAACTTTGTCAAG CTCATTTCCTG
55	gck	NM_010292.4	863-962	TGTACGAAAAGATCATTGGCGGAAAGTACA TGGGCGAGCTGGTACGACTTGTGCTGCTC AAGCTGGTAGAGGAGAATCTTCTGTTCCAC GGAGAGGCCTC
56	GFAP	NM_001131020.1	611-710	CACCCTGGCTCGTGTGGATTTGGAGAGAA AGGTTGAATCGCTGGAGGAGGAGATCCAG TTCTTAAGGAAGATCTATGAGGAGGAAGTT CGAGAACTCCGG
57	gephyrin	NM_145965.2	1213-1312	AAAGCCTTCATTACAGTCCTGGAGATGACT CCGGTGCTTGGTACAGAAATCATCAATTAC CGAGATGGAATGGGGCGAGTCCTTGCTCA AGATGTATATG
58	GRIA2	NM_001039195.1	301-400	GACCTCAAAATGCAGAGGATCTAATTTGCT GAGGAAAACGGTCAAAGAAGGAAAAGGAG GAAAGGGAAACGAGGGGATATTTTGTGGA TGCTCTACTTTT
59	Grin1	NM_008169.2	493-592	ACAGATGGCCCTGTCAGTGTGTGAGGACC TCATCTCTAGCCAGGTCTACGCTATCCTAG TTAGTCACCCGCCTACTCCCAACGACCACT TCACTCCCACC

60	GRIN2A	NM_008170.2	1789-1888	CCTTCTTTTACCATTGGAAAAGCTATATGGCTCCTCTGGGGCCTGGTCTTCAACAATTCTGTGCCCGTCCAGAATCCTAAAGGCACAACCAGCAAGATAA
61	Grin2b	NM_008171.3	6341-6440	GGGAAAGCTCTTCGTATAAGGCTTTGTGAAAGAGCCATTACAGTAGGGTGAGAGAGGGGGATGTTTTTAGTCATTAACGGTAGGGTTAGTGAGAAAGGGG
62	gsk3a	NM_001031667.1	2151-2250	CCCATCCTGGAAGGAGGGGAGTAGAGAGAGTCCCTGGTGTCTTAGTTTCCACAGTAAGGTTTGCCTGTGTACAGACCTCTGTTCAATAAATTATTGGCAT
63	Gsk3b	NM_019827.3	2216-2315	CCACTGATTACACGTCCAGTATAGATGTATGGTCTGCAGGCTGTGTGTTGGCTGAATTGTTGCTAGGACAACCAATATTTCCTGGGGACAGTGGTGTGGA
64	Hdac2	NM_008229.2	1011-1110	CTGGGGACAGGCTTGGTTGTTTCAATCTAACTGTCAAAGGTCATGCTAAATGTGTAGAAGTAGTGAAAACTTTTAACTTGCCATTGCTGATGCTCGGTGG
65	HDAC3	NM_010411.2	1026-1125	GGCCATTAGTGAGGAACTTCCCTATAGTGAATACTTCGAGTACTTTGCCCCAGATTTCACACTCCATCCAGATGTCAGCACCCGCATCGAGAATCAGAAC
66	hprt	NM_013556.2	31-130	TGCTGAGGCGGCGAGGGAGAGCGTTGGGCTTACCTCACTGCTTTCCGGAGCGGTAGCACCTCCTCCGCCGGCTTCCTCCTCAGACCGCTTTTTGCCGCGA
67	hsd17b10	NM_016763.2	226-325	CAAGCAGCTTTGACTCTAGCAAAAGAAAAGTTTGGCCGTATAGATGTGGCTGTCAACTGTGCAGGTATTGCAGTGGCCATTAAGACATACCACCAAAAGA
68	Hsf1	NM_008296.2	868-967	GGCCCATACTCAGCTCCATCTCCAGCCTACAGCAGCTCTAGCCTTTACTCCTCTGATGCTGTCACCAGCTCTGGACCCATAATCTCCGATATCACTGAGC
69	htr1a	NM_008308.4	3171-3270	CACACAAATTTCGCAGATCTCTGGGCTCTCATTGTGAAGGGTATCAACACCTCACCATTACCTACCTAGGATCTTGATCTCAGAGACCTAAAAGAGAGAG
70	Ide	NM_031156.2	501-600	TTTCCCATGAACACTTGGAAGGAGCCCTGGACAGGTTTGCGCAGTTTTTCCTGTGCCCCTTGTTTGATGCAAGTTGTAAAGACAGAGAGGTGAACGCTGT
71	ikbke	NM_019777.3	619-718	TGGGAGCCTGCTGAGCGTGCTGGAAGACCCTGAGAACACGTTCGGGCTTTCTGAAGAGGAGTTCCTAGTGGTGCTGCGCTGTGTGGTGGCTGGCATGAAC

72	Il10	NM_010548.1	986-1085	GGGCCCTTTGCTATGGTGTCCTTTCAATTG CTCTCATCCCTGAGTTCAGAGCTCCTAAGA GAGTTGTGAAGAAACTCATGGGTCTTGGGA AGAGAAACCA
73	Il1b	NM_008361.3	1121-1220	GTTGATTCAAGGGGACATTAGGCAGCACTC TCTAGAACAGAACCTAGCTGTCAACGTGTG GGGGATGAATTGGTCATAGCCCGCACTGA GGTCTTTCATT
74	Il6	NM_031168.1	41-140	CTCTCTGCAAGAGACTTCCATCCAGTTGCC TTCTTGGGACTGATGCTGGTGACAACCACG GCCTTCCCTACTTCACAAGTCCGGAGAGG AGACTTCACAG
75	ins	NM_008387.3	267-366	AACTGGAGCTGGGTGGAGGCCCGGGAGC AGGTGACCTTCAGACCTTGGCACTGGAGG TGGCCCAGCAGAAGCGTGGCATTGTAGAT CAGTGCTGCACCAG
76	insr	NM_010568.2	8315-8414	CAGCTGGGTGTGGTGCCACCAACACAAGT AAAAACTATCCTTACTATCTTATCAGGACAG ACGTTCTACCATGCACAGTGATTATTGGGT TAAGGGCTGT
77	JAK2	NM_008413.2	1050-1149	GATCCAAGACTATCACATTTTAACCCGGAA GCGAATCAGGTACAGATTTCGCAGATTCAT TCAGCAATTCAGTCAATGTAAAGCCACTGC CAGGAACCTA
78	Jun	NM_010591.2	2213-2312	CGCGACCAGAACGATGGACTTTTCGTTAAC ATTGACCAAGAACTGCATGGACCTAACATT CGATCTCATTCAGTATTAAAGGGGGGTGG GAGGGGTTACA
79	pcaf	NM_001190846.1	623-722	TACCGCAGTTCTGTGACAGCTTACCTCGGT ACGAAACCACAAAGGTGTTTGGGAGAACAT TGCTTCGCTCGGTCTTCACCATCATGAGAC GACAGCTCTT
80	Kcnq2	NM_001003824.1	661-760	GATTGCCTCCATTGCTGTGCTGGCTGCTG GTTCCCAGGGCAATGTCTTTGCCACATCTG CGCTTCGGAGCTTGCGGTTCTTGCAAATCT TGCGGATGATC
81	lsd1	NM_133872.1	1264-1363	AACAAGAATTTAACCGGTTGCTAGAAGCCA CTTCTTACCTTAGTCACCAGTTAGACTTCAA CGTCCTCAATAATAAACCTGTATCCCTTGG CCAGGCATT
82	Kif3a	NM_008443.3	2475-2574	TGTCTGTCACACTGCTCTGCAGGAAGTAAG GGTTCCGTTGTTGTACGTGAATGTGCGTTT CTTAGCTATGTATTCAACTACCGTGGCTCT AGACAAGCAG
83	Klf14	NM_001135093.1	2306-2405	GTACATTGTTGTTTCTGTTACAGGTGAATTG GTGGGTGAGGGGGAAAAGTGAGAGATTGG TTAATTTTAGAGAGCTGCTGTCTTGGATTTG GGGTGAGAG

84	Mll	NM_001081049.1	2081-2180	TTCTGAGAGCTCCCAGATTTACTCCAAGTGAGGCACACTCTAGAATATTTGAGTCTGTGACCTTGCCTAGTAATCGAACTTCTTCTGGAGCGTCCTCTTC
85	Lgi1	NM_020278.2	2911-3010	CTCCTTCTCTTATAATAAACTCCACGGGTCCCTCTGACCTATCGAGTACTTACTGGCTACTTGTTCAGAATATGGTGTGGTTGTGGGTAACTAATACAGG
86	Lrp1	NM_008512.2	1311-1410	GAGCAGATGGCAATCGACTGGCTGACGGGAAACTTCTACTTTGTCGACGACATTGACGACAGGATCTTTGTCTGTAACCGAAACGGGGACACCTGTGTCA
87	lrp8	NM_001080926.1	5291-5390	GACCGACCTCCTTCTATCCTTGGAAACTTTTTGAGCCTCATTCTGAGGTGTAATTCAGACTTCTGTTGCCTTTCGCCTCTGCCTATGTATCCACAGCCAA
88	MAP2K1	NM_008927.3	1696-1795	CTTTGTGCTTGGGGCTATTTGTCTGTTCATCAAACACATGCCAGGCTGAACTACAGTGAAACCCTAGTGACCTGGGTGGTCGTTCTTACTGATGTTTGCA
89	MAPK14	NM_011951.2	1421-1520	GAAGACCTTCTCATGGGAACTCTCCAAATACCATTCAAGTGCCTCTTGTTGAAAGATTCCTTCATGGTGGAAGGGGGTGCATGTATGTGTTAGTGTTTGT
90	Mapt	NM_001038609.2	1203-1302	CAGTCGAAGATTGGCTCCTTGGATAATATCACCCACGTCCCTGGAGGAGGGAATAAGAAGATTGAAACCCACAAGCTGACCTTCAGGGAGAATGCCAAAG
91	mecp2	NM_010788.2	756-855	AAAGGGAGCGGCACTGGGAGACCAAAGGCAGCAGCATCAGAAGGTGTTCAGGTGAAAAGGGTCCTGGAGAAGAGCCCTGGGAAACTTGTTGTCAAGATGC
92	ki67	XM_006507412.2	3829-3928	CCCGGCAAGCACAAAGAGACTCTCCAAGACAGATCTCAGTAAGGTGGATGTGAGAGAAGACCCTTCAATACTTGGGAAAAAAACAAAGTCACCAGGCAGA
93	Mmp2	NM_008610.2	2377-2476	AGTTAACCAGCCTTCTCCTTCACCTGGTGACTTCAGATTTAAGAGGGTGGCTTCTTTTTGTGCCCAAAGAAAGGTGCTGACTGTACCCTCCCGGGTGCTG
94	Mmp9	NM_013599.2	1571-1670	CCTCTACAGAGTCTTTGAGTCCGGCAGACAATCCTTGCAATGTGGATGTTTTTGATGCTATTGCTGAGATCCAGGGCGCTCTGCATTTCTTCAAGGACGG
95	mpo	XM_006532405.1	1375-1474	CAGTACCGATCTTACAACGACTCAGTAGACCCTCGAATCGCCAATGTCTTCACCAACGCTTTCCGTTATGGCCACACCCTCATCCAACCCTTCATGTTCC

96	Mtor	NM_020009.2	2433-2532	CATGGAGCCTATCCTGAAGGCTTTAATTTTGAAACTGAAAGATCCAGACCCTGACCCAAACCCGGGCGTGATCAATAACGTGTTGGCCACTATAGGAGAA
97	ncor1	NM_011308.2	212-311	GTTGGACTTGATCTCACAGCCCAGTGAGGACGTCTTTACTGATAATGTCAAGTTCAGGTTACCCTCCCAACCAAGGAGCTTTCAGCACAGAGCAAAGTCG
98	Nfkb1	NM_008689.2	2126-2225	GTCTTACACTTAGCCATCATCCACCTCCACGCTCAGCTTGTGAGGGATCTGCTGGAAGTCACATCTGGTTTGATCTCTGATGACATCATCAACATGAGAA
99	Ngf	NM_001112698.1	631-730	CAAGGAGGTGACAGTGCTGGCCGAGGTGAACATTAACAACAGTGTATTCAGACAGTACTTTTTTGAGACCAAGTGCCGAGCCTCCAATCCTGTTGAGAGT
100	NPAS4	NM_153553.4	581-680	TGCTGATCGCCTTTTCCGTTGTCGATTCAACACCTCCAAGTCCCTCCGGCGCCAGAGTTCAGGAAACAAACTGGTGCTTATTCGAGGTCGATTCCATGCT
101	Ntrk1	NM_001033124.1	1482-1581	ACTCCAGGGCCACATCATGGAGAACCCACAGTACTTCAGTGATACCTGTGTCCATCACATCAAGCGCCAGGACATCATTCTCAAGTGGGAGCTAGGGGAG
102	polr1b	XM_006499025.1	1957-2056	CCCTTCTCGGATCACAACCAGAGTCCTCGGAACATGTACCAGTGCCAGATGGGTAAGCAGACCATGGGCTTTCCGCTGCTCACTTACCAAAACCGATCAG
103	polr2a	NM_009089.2	2221-2320	GTGAATCGTATTCTCAATGATGCTCGAGACAAAACTGGCTCCTCTGCACAGAAATCCCTCTCTGAATATAACAACTTCAAGTCTATGGTGGTGTCTGGAG
104	Pparg	NM_011146.1	1061-1160	ACCAAGTGACTCTGCTCAAGTATGGTGTCCATGAGATCATCTACACGATGCTGGCCTCCCTGATGAATAAAGATGGAGTCCTCATCTCAGAGGGCCAAGG
105	PPP3CA	NM_008913.4	1676-1775	AGGGCATGATGGGATTGCTGCATCTCAGCAGTTGGATGTTCTTGCCTCTGAAGGTAGCTTGTTTGCTCTGGGGGCCAGGAATTGGATTCAGTTTACACTA
106	prc1	NM_145150.3	989-1088	ATGCAAAACATAAAGCAAGTGATTGAGAAAATCCGAGTGGAGCTGGCTCAATTCTGGGACCAGTGTTTTTATAGCCAGGAACAGAGGCAGGCTTTTGCCC
107	prkcg	NM_011102.3	1581-1680	TTTAATGTACCACATCCAGCAACTGGGCAAGTTTAAGGAGCCTCATGCAGCATTCTACGCTGCGGAAATCGCCATAGGCCTCTTCTTCCTTCACAACCAG

108	prkcz	NM_008860.2	3871-3970	GGGGGCTTATTTGGATACTGCCCTATTGCCAAATGACTTGCTGGTTACTGGTTCGGCTGCTCATGTACAAATGTGTGAATAATATTGTGCCCACCAGAGG
109	Prox1	NM_008937.2	2113-2212	AGCACCGCAGAAGGACTCTCTTTGTCACTCATAAAGTCTGAGTGTGGAGATCTTCAAGATATGTCCGACATCTCACCTTATTCAGGAAGCGCAATGCAGG
110	PTEN	NM_008960.2	5161-5260	CAGCCTTACCCCGATTCAGCCTCTTCAGATACTCTTGTGCTGTGCAGCAGTGGCTCTGTGTGTAAATGCTATGCACTGAGGATACACAAATATGACGTGT
111	Ptk2	NM_007982.2	1061-1160	TGTACAGATTTGACAAAGAGTGCTTCAAGTGTGCCCTTGGGTCAAGTTGGATCATTTCTGTGGAATTGGCAATCGGCCCAGAAGAAGGGATCAGTTACCT
112	Pvalb	NM_013645.3	61-160	GAAGGCGATAGGAGCCTTTGCTGCTGCAGACTCCTTCGACCACAAAAAGTTCTTCCAGATGGTGGGCCTGAAGAAAAAGAACCCGGATGAGGTGAAGAAG
113	Pycard	NM_023258.4	1655-1754	TGAAGATCGTATTGTGTGAATATAAACACGGGTGCATGTCTGCACAGAAGCTTGAAGACAAACTCCAGAGTTCTGTTTCTTACCTTGAGGTAGGGAGGAG
114	RCAN1	NM_001081549.1	1061-1160	ACACAAGGACACTGGGGACATCCTGAGAAAACTGATAGTTCTTGTAATTGCTCATTTCTAGGTTCTGTTTTTGGCAAGGACAGGTTGACTGGTGGCCCAG
115	rcor1	NM_198023.2	1231-1330	AGATACGCATCTGCCTCATGAAAACTGTTGGTAGCTTTGAACACTTGGTATGGACGACACGTTATCCAGGTATCAGGTATTACAAGACATCACCTAGCCA
116	Rela	NM_009045.4	646-745	GAGGCTGACCTCTGCCCAGACCGCAGTATCCATAGCTTCCAGAACCTGGGGATCCAGTGTGTGAAGAAGCGAGACCTGGAGCAAGCCATTAGCCAGCGAA
117	Reln	NM_011261.2	2546-2645	TCAACAAAGATGGGAGGCGGCAGCTAATCACGTCCTTTCTGGACAGCTCGCAGTCCAGGTTTCTTCAGTTTACACTGAGGCTGGGGAGCAAGTCTGTGCT
118	Rest	NM_011263.1	886-985	GAACGCCCGTATAAATGTGAACTTTGTCCTTACTCAAGCTCTCAGAAGACTCATCTAACGCGACACATGCGGACTCATTCAGGTGAGAAGCCATTTAAAT
119	rpl19	NM_009078.1	21-120	GAAGAGGCTTGCCTCTAGTGTCCTCCGCTGCGGGAAAAAGAAGGTCTGGTTGGATCCCAATGAGACCAATGAAATCGCCAATGCCAACTCCCGTCAGCAG

120	RPS6	NM_009096.3	1171-1270	ATTCTTGGTGAATGGTAGTGGAAGGGATTAAACCTAGGCCTCAGTCATGCTTCCCAGTCACTGGTACTGATTTGTATGCACCCGCTTAGGTGTGAAGGTA
121	rxra	NM_011305.3	3741-3840	CCTGATTGGATTGTTGCCACCAATGGTCATGTGACTTCCACATTGCAGCTTCTGGGCCATATTCCTCATGTGTGTCACCACCCTGCTTGGAGATTCATCT
122	S100b	NM_009115.3	1091-1190	ACCGAGAATCAAAATTCTGCTCGGCAGACTTCTCCTTTCAGGATGATCGCTTTGTTCCTGGAGGACAGAGGAGGGGAATGGCCAGAGTCTTTTTCTAGTT
123	Scn1a	NM_018733.2	1031-1130	CCCACCAACGCTTCCCTTGAGGAACATAGCATAGAGAAGAATATAACTATGGATTACAATGGCACACTTGTAAATGAAACCGTGTTCGAGTTTGACTGGA
124	SHANK	NM_001081370.2	4931-5030	TGTGGACAGGATCGTGCCCTGCTTGCTTAACTTGCTGTCTTTATTTCTGCAAACAGATTAGCATAATTCCAGGGTGGCCAAAATGAAGTCACACGGAGTT
125	Sirt1	NM_019812.2	844-943	GGATTCCTGACTTCAGATCAAGAGACGGTATCTATGCTCGCCTTGCGGTGGACTTCCCAGACCTCCCAGACCCTCAAGCCATGTTTGATATTGAGTATTT
126	Sirt6	XM_006513863.2	136-235	TTCGAGAATGCTCGGCCCTCGAAGACCCACATGGCCCTGGTTCAGCTAGAACGCATGGGCTTCCTCAGCTTCCTGGTCAGCCAGAACGTAGACGGGCTGC
127	kcc2	NM_020333.2	5619-5718	TAGCTAGATGTTCCATGTCCATCCAGGTGACTTCTACTCTGAGTGCAATATTTCAGTAGCCTGGTAGTGAGAAGAGTGTCGCTTCTGTTTCAGCAGACCT
128	znt3	NM_011773.3	1111-1210	GAGTCCGAGCAACCCATGATCTCCATCTGTGGGCCCTGACGCTTACTTACCACGTTGCCTCTGCACACCTGGCTATTGACTCCACGGCTGACCCTGAAGC
129	Slc30a8	NM_172816.3	861-960	CATGATCACTGTTTCAGGCTGTGCAGTGGCAGCCAACATTGTACTAACTATGATTTTGCACCAACGGAACTTTGGCTACAACCACAAGGATGTACAAGCT
130	smad3	NM_016769.3	1846-1945	GTGTATCGCCACCTGACTCCTTGTTTAATGACAGAGGTCTGGGATGTCACAGTCCAAAAGGAAAGTGCCTTTCTCCATGGCTGGAGTATGGAGTTTACCT
131	SNCA	NM_009221.2	286-385	TGTGGCAGAGGCAGCTGGAAAGACAAAAGAGGGAGTCCTCTATGTAGGTTCCAAAACTAAGGAAGGAGTGGTTCATGGAGTGACAACAGTGGCTGAGAAG

132	sncb	NM_033610.2	677-776	CCCAAACACGAGATCTTCCTTCGGCTCTGAGGCACTTCCTCAGGGCCTGTGTTAGTGTCTGCCCTGCCCTTCCGTCTGTCCTACTTGCCCCGCGTTCATCC
133	Sod1	NM_011434.1	557-656	TGGAGTGATTGGGATTGCGCAGTAAACATTCCCTGTGTGGTCTGAGTCTCAGACTCATCTGCTACCCTCAAACCATTAAACTGTAATCTGAAAAAAAAAA
134	Sorl1	NM_011436.3	2721-2820	GTGGATGCCGGCTTTAAAAAGATCGAGGTAGCTAATCCAGATGGTGACTTCCGACTGACAATCGTCAATTCCTCTGTGCTTGATCGACCCAGGGCTTTAG
135	Sphk1	NM_011451.3	563-662	TGCACACTGCTTCTGGGCTGCGGCTCTATTCTGTGCTCAGTCTGTCCTGGGGCTTTGTTGCTGACGTGGACCTCGAGAGTGAGAAGTACAGGCGCTTGGG
136	crest	NM_178750.5	1533-1632	AGCATTGTCTTTGGACCCTTCATAGTAGTATGTTCTGGACAAGCCGGTGGCAGTTCTGATGAGTAGCGACATGTTGGTCACCCTCTCTGCCCAGTGCCGT
137	Stat1	NM_009283.3	1591-1690	ACGCTGGGAACAGAACTAATGAGGGGCCTCTCATTGTCACCGAAGAACTTCACTCTCTTAGCTTTGAAACCCAGTTGTGCCAGCCAGGCTTGGTGATTGA
138	Sv2a	NM_022030.3	2036-2135	GATGTTACATCCAGCAACACATTCTTCCGCAACTGCACGTTCATCAACACTGTGTTCTATAACACTGACCTATTTGAGTACAAGTTCGTGAACAGCCGGC
139	Synpo	NM_177340.2	1099-1198	CGCCCACTAAGGTGTATAGTGAAGTACATCTCACACTAGCCAAGCCTGCATCCGTGGTCAACAGGACCGCCAGGCCTTTTGGGATCCAGTCGCCAGGGAC
140	tbp	NM_013684.3	71-170	GTGGCGGGTATCTGCTGGCGGTTTGGCTAGGTTTCTGCGGTCGCGTCATTTTCTCCGCAGTGCCCAGCATCACTATTTCATGGTGTGTGAAGATAACCCA
141	Tbxa2r	NM_009325.3	447-546	TCGCTGGGGGCCTGCTTTCGCCCGGTGAACATCACGCTGCAGGAGCGACGTGCCATCGCGTCGCCATGGTTCGCTGCGTCCTTTTGCGCGCTGGGCCTGG
142	Tcf7l2	NM_001142924.1	664-763	CCCATCACACTCTGCACACGACCGGCATCCCTCACCCGGCCATCGTCACACCGACAGTCAAGCAGGAATCCTCCCAGAGTGACGTCGGCTCACTCCACAG
143	tet3	NM_183138.2	6546-6645	GAAACACGCCCATCCTAAAATCCTGGTGCATTCATTCAGCTCTTTGAAAATGAGAACGTTGGTGCTTTAATTTCTGTGACATTGAGAGGTTGGGCCTGAC

144	Tgfb1	NM_011577.1	1471-1570	GGAGTTGTACGGCAGTGGCTGAACCAAGGAGACGGAATACAGGGCTTTCGATTCAGCGCTCACTGCTCTTGTGACAGCAAAGATAACAAACTCCACGTGG
145	Tlr2	NM_011905.2	256-355	GCAGGCGGTCACTGGCAGGAGATGTGTCCGCAATCATAGTTTCTGATGGTGAAGGTTGGACGGCAGTCTCTGCGACCTAGAAGTGGAAAAGATGTCGTTC
146	Tlr3	NM_126166.2	1166-1265	TTTCTCTGGGCTGAAGTGGACAAATCTCACCCAGCTCGATCTTTCCTACAACAACCTCCATGATGTCGGCAACGGTTCCTTCTCCTATCTCCCAAGCCTG
147	Trem2	NM_031254.2	8-107	GGGCGCCTACCCTAGTCCTGACTGTTGCTCAATCCAGGAGCACAGTTCCTGTGGGCTGAGCCTGACTGGCTTGGTCATCTCTTTTCTGCACTTCAAGGGA
148	Vcam-1	NM_011693.2	1441-1540	GGTTTTGAGGATGAACACTCTTACCTGTGCGCTGTGACCTGTCTGCAAAGGACACTGGAAAAGAGAACCCAGGTGGAGGTCTACTCATTCCCTGAAGATC
149	Vegfa	NM_001025250.3	3016-3115	TCTCTCTCTCCCAGATCGGTGACAGTCACTAGCTTGTCCTGAGAAGATATTTAATTTTGCTAACACTCAGCTCTGCCCTCCCTTGTCCCCACCACACATT
150	yy1	NM_009537.3	761-860	CGGTCACCATGTGGTCCTCGGATGAAAAAAAAGATATTGACCATGAAACAGTGGTTGAAGAGCAGATCATTGGAGAGAACTCACCTCCTGATTATTCTGA