UNIVERSITY OF CALIFORNIA,
IRVINE

Regulation of Repressive Histone Methylation by BDNF and Oxidative Stress

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Neurobiology and Behavior

by

Andra Ionescu Tucker

Dissertation Committee:
Professor Carl Cotman, Chair
Professor Marcelo Wood
Professor Andrea Tenner

2021

DEDICATION

To my sister Laura

Thank you for being my best friend, my angel of light. Follow your own dreams and no one else's. You alone know what is best for you. I will always be there for you.

And

To my husband Thomas

You've made me the person I am today, a much calmer, happier and more balanced person than I was 7 years ago. You are my life's greatest blessing. Thank you for supporting me unconditionally. I promise to continue doing the same for you.

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It has been my greatest privilege to work with undergraduate students and be amazed by their growth. Thank you Maria, Brandon, James and Katherine for learning with me and being great friends. I can't wait to see what you will accomplish.

Everything I have accomplished is because of my family. To my parents, who suffered greatly to build a life for us in a new country, thank you. To my sister Laura, who is a pure ray of sunshine in my life, thank you. To my husband Thomas, who always forces me to take care of myself, thank you. And to my in laws and extended family in Romania, thank you for continuing to believe in me.

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Curriculum Vitae

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Education

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Publications

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Ionescu-Tucker A, Tong L, Berchtold N, Cotman CW (2021). *In Review*. BDNF Inhibition Upregulates Hippocampal H3K9me3 via an Oxidative Stress Dependent Mechanism.

Ionescu-Tucker A, Cotman CW (2021). Emerging Roles of Oxidative Stress in Aging and Alzheimer's Disease. Neurobiology of Aging. ISSN 0197-4580, https://doi.org/10.1016/j.neurobiolaging.2021.07.014.

Research Experience

Dissertation, University of California, Irvine 2016-2021
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Investigated changes in H3K9me3 (a repressive epigenetic mark) in *in vitro* models of aging and oxidative stress to determine the relation between gene repression and neurodegeneration

Volunteer Research Assistant, University of California, San Diego 2013-2015
Advisor: Dr. Darwin Berg

Investigated the effects of cholinergic nicotinic signaling on astrocytic endosome uptake utilizing *in vitro* astrocyte cultures, immunostaining and confocal microscopy

Regent's Scholar Research Assistant, University of California, San Diego 2012-2013
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Analyzed MRIs from 200 patients for Alzheimer's clinical morphometry project using Excel

Oral Presentations

Ionescu Tucker, A (2021, May). How exercise changes gene expression. CNLM Awards Ceremony Elevator Pitch, University of California, Irvine.

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Ionescu, A (2018, May). Regulation of Repressive Histone Methylation: A Novel Approach to Alzheimer's Disease Research. Neuroblitz seminar, University of California, Irvine.

Ionescu, A (2018, April). Reversing the Genetic Off Switch Behind Alzheimer's Disease. Presentation at the Associated Graduate Student Symposium, University of California, Irvine.

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Ionescu A, Tong L, Tseung K, Berchtold B, Cotman CW (2019, February). Oxidative stress regulates repressive histone methylation involved in age-related cognitive decline. Poster presented at ReMIND Emerging Scientists Symposium, University of California, Irvine.

Tseung K CE, Tong L, **Ionescu Tucker A**, Wiley EA., Cotman CW (2018, December). Epigenetic Effects of BDNF on H3K9me3 in Hippocampal Neurons. Poster presented at Keck Senior Thesis Symposium, Claremont.

Ionescu A, Tong L, Tseung K, Berchtold N, Cotman CW (2018, November). The age-associated stressor oxidative damage induces repressive histone methylation in cultured hippocampal neurons. Poster presented at the Society for Neuroscience Meeting, San Diego.

Tong L, **Ionescu A**, Butler C, Snigdha S, Tseung K, Overman L, Cotman CW (2018, November). A SUV39H1 selective inhibitor enhances synaptic plasticity by prolonging ERK activation. Poster presented at the Society for Neuroscience Meeting, San Diego.

Abidi SA, Chakoumakos M, Chhor B, **Ionescu A**, Kwong K, Winslow T, et al. (2013, June). Lytic Genes in MycoBacteriophage QBert. Poster presented at UC San Diego Undergraduate Research Showcase, University of California, San Diego.

Teaching Experience

University of California, Irvine January 2020-December 2020
Pedagogical Fellow, Division of Teaching Excellence and Innovation

- Trained 25 graduate students across 3 biology departments in pedagogical techniques
- Led virtual workshops in diversity and inclusion, online teaching, active learning, backwards lesson design, office hours, grading and conflict management.
- Developed and led an accelerated virtual training program for summer session teaching assistants in two days

University of California, Irvine January 2018-September 2020
Teaching Assistant, School of Biological Sciences

- Molecular Biology (Bio99, 200 students, virtual): facilitated online Zoom classes, held virtual office hours, created project rubrics, and graded exams and final projects
- Brain Dysfunction (Bio37, 400 students): designed and graded exams, proctored exams, input iClicker scores into Canvas and led review sessions
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University of California, San Diego January 2015-June 2016
Teaching Assistant, Division of Biological Sciences

- Organismic and Evolutionary Biology (BILD 3, 200 students): created and administered quizzes, led discussion sections and review sessions, hosted office hours, graded exams
- The Beginning of Life (BILD 7, 200 students): reviewed homework assignments during weekly discussion sections, led review sessions, held office hours, graded exams
- Neurobiology and Behavior (BILD 12, 100 students): created and proctored exams, led weekly discussion section, held office hours, graded exams

University Service

PROMISE Taskforce (Promoting Inclusion in the Student Experience) 2021
Worked to make undergraduate education in the School of Biological Sciences more equitable and inclusive while maintaining high standards of academic rigor

Pedagogical Hiring Committee 2020
Virtually interviewed and selected the 2021 cohort of pedagogical fellows

Teaching Consultant 2019
Observed and reviewed the teaching of fellow graduate teaching assistants

Pedagogical Liaison 2019
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Honors and Awards

Dean's Recent Alumni Fellowship	2021
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Certificate in Course Design	Spring 2019
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Certificate in Mentoring Excellence	Spring 2017
Teaching Assistant Professional Development Program	Fall 2017

ABSTRACT OF THE DISSERTATION

Regulation of Repressive Histone Methylation by BDNF and Oxidative Stress

By
Andra Ionescu Tucker

Doctor of Philosophy in Neurobiology and Behavior
University of California, Irvine, 2021
Professor Carl W. Cotman, Chair

Emerging evidence suggests that histone modifications contribute to age-related cognitive decline. Our lab previously demonstrated that elevated H3K9me3 in aged mice leads to synaptic loss, cognitive impairment and a reduction in brain derived neurotrophic factor (BDNF). Treatment with ETP69, a selective inhibitor of H3K9me3's catalyzing enzyme (SUV39H1), was shown to restore synapses, BDNF and cognitive performance. However, the mechanism underlying H3K9me3 regulation is poorly understood. In this study, we investigated the role of age-associated stressors such as oxidative stress in H3K9me3 elevation. The oxidative stressor hydrogen peroxide elevated the SUV39H1 regulator SIRT1 but did not increase H3K9me3. The aged brain is also marked by reduced BDNF, and we found that inhibiting BDNF signaling by blocking the BDNF receptor TrkB elevates H3K9me3 in an age-dependent fashion. Antioxidant treatment prevented the H3K9me3 elevation by TrkB-Fc, suggesting that inhibiting BDNF signaling regulates H3K9me3 via an oxidative stress-based mechanism. We further investigated if exercise, which stimulates BDNF production, regulates repressive H3K9me3 at the promoters of neuronal plasticity genes. Exercise decreased H3K9me3 at BDNF promoter VI in aged mice and stimulated BDNF production. Similarly, SUV39H1 inhibition decreased H3K9me3 at BDNF promoter VI in aged mice and showed a corresponding increase in BDNF VI expression. Exercise and SUV39H1 inhibition differentially affected BDNF and GABRA2 expression in young and old mice. H3K9me3 promoter binding at all neuronal plasticity genes except for

GABBR1 decreased as mice aged. Overall, our data suggests that H3K9me3 and BDNF are engaged in a negative feedback mechanism that is affected by physical activity, oxidative stress and age.

Introduction

Cognitive functions in aging are constrained by histone methylation, and investigating these blockades may clarify the mechanism of age-related cognitive decline (Lardenoije et al., 2015). A highly regulated site is histone 3, lysine 9 (H3K9) which is associated with gene silencing when di or tri-methylated (Barter and Foster, 2018; Peters et al., 2003; Stewart et al., 2005). Previous work by our lab has shown that H3K9me3 increases in the mouse hippocampus with age and Alzheimer's disease pathology. To investigate if H3K9me3 was associated with age-related cognitive decline, we reduced H3K9me3 in aged mice using a selective SUV39H1 inhibitor (ETP69), which improved performance in object location memory (OLM) and fear conditioning tasks. ETP69 further increased the expression of brain derived neurotrophic factor (BDNF) while decreasing H3K9me3 at BDNF promoter 1(Snigdha et al., 2016). This suggests that H3K9me3 regulates the expression of a neurotrophic factor that is critical for memory, neuron survival and brain plasticity (Berchtold et al., 2002; Cotman and Berchtold, 2002). Our work indicates an important role for H3K9me3 in the regulation of brain health and demonstrates a clear association between hippocampal H3K9me3 and age. However, the mechanism underlying H3K9me3 elevation in the aging hippocampus has yet to be explored.

Recent studies have begun to examine how exercise affects the regulation of neuronal plasticity genes. Of particular interest are BDNF, which enhances learning and memory, and GABA receptors 1 and 2, which maintain the balance between excitatory and inhibitory neurotransmitter signaling (Cotman and Berchtold, 2002). Exercise also correlates with the expression of 8 GABA genes in the aged hippocampus, including the receptors GABBR1 and GABRA2 (Berchtold et al., 2019). H3K9me3 may play a role in the repression of GABA receptors as well as BDNF; a study of postmortem cortical samples showed an increase in

H3K9me3 at the promoters of BDNF, GABBR1 and GABRA2 with Alzheimer's disease (Lee et al., 2020). Current research has just started to explore how exercise regulates the epigenetics of the aging brain.

My dissertation investigates how H3K9me3 is regulated by oxidative stressors and inhibition of BDNF signaling. I further examined how exercise and ETP69 affects H3K9me3 regulation of neuronal plasticity genes. My results suggest that H3K9me3 is regulated by oxidative stress, BDNF and neuronal age and that it is a critical repressor of BDNF expression. Additionally, age differentially affects neuronal gene expression in response to exercise and H3K9me3 inhibition.

Oxidative Stress in the Aged Brain

The healthy brain is resilient enough to retain most cognitive abilities as it ages, but it remains marked by slight declines in gene expression, neuroplasticity and cognitive abilities. Synaptic genes are downregulated with age beginning at age 40, with the greatest decline occurring after age 70, corresponding to a decline in synaptic density (Lu et al., 2004; Benavides-Piccione et al., 2013). Neurogenesis is also reduced throughout the aged brain. In the hippocampus, there is a decline in progenitor cells along the dorsal-ventral axis, which compromises spatial learning (Bannerman et al., 2004). Due to these changes, there is a decline in short-term recall, episodic memory and processing speed with age (Nyberg et al., 2012; Fjell et al., 2014). Many cognitive functions are preserved, including long term memory, implicit memory, attention span and vocabulary, distinguishing brain aging from more severe age-related dementias (Yankner et al., 2008). The causes of brain aging have been continually debated, yet one prevailing theory is that oxidative stress drives age-related cognitive decline.

In 1954, Denham Harman proposed that free radical reactions "may be involved in production of the aging changes associated with the environment, disease and an intrinsic aging process" (Harman, 1992). Time has confirmed that free radicals are heavily involved in the aging process, as well as diseases associated with advanced age such as Alzheimer's disease (AD) (Butterfield and Halliwell, 2019; Grimm and Eckert, 2017; Guillaumet-Adkins et al., 2017). As the population continues to age, cases of this tragic disease will increase, making it critical to find effective therapies for age-associated oxidative stress.

Harman's free radical theory of aging is most relevant to the central nervous system, which consumes 20% of the body's oxygen and is highly vulnerable to oxidative stress (Bonda et al., 2014). Neurons are especially sensitive since they are non-dividing, post mitotic cells and cannot be replaced in the event of damage, leading to mitochondrial dysfunction late in their lifespan (Grimm and Eckert, 2017; Wang and Michaelis, 2010). Additionally, mitochondria in presynaptic terminals are exposed to high levels of calcium insult from voltage gated calcium channels, accelerating synaptic oxidative damage (Grimm and Eckert, 2017). The brain's susceptibility to oxidative stress leads to an increase in oxidative biomarkers with age, including toxic levels of metals, DNA damage and deficits in protein metabolism (Grimm and Eckert, 2017; Mecocci et al., 2018; Thanan et al., 2014;).

Oxidative stress is a byproduct of mitochondrial respiration

Mitochondria are the main hub of neuronal oxidative stress, as most free radicals are generated as byproducts of the mitochondrial electron transport chain. Superoxide is a byproduct of mitochondrial respiration which is produced from oxygen when there is an excess of ATP and electron transport is diminished (Sinha et al., 2013). Superoxide dismutase can then convert

superoxide to hydrogen peroxide, which is further broken down into hydroxyl radicals and anions by the Fenton reaction (Singh et al., 2019). Mitochondrial ROS can lead to redox imbalance, neurotoxicity, genomic instability, pro-inflammatory gene transcription and cytokine release, such as IL-1, IL-6 and TNF alpha (Islam, 2017). In a positive feedback cycle, ROS species can damage and inactivate parts of the electron transport chain, leading to increased electron reduction of oxygen to superoxide (Islam, 2017; Sies et al., 2017; Sinha et al., 2013). Mitochondrial ROS are particularly damaging to mitochondrial DNA (mtDNA), which are not protected by histones and mutate at a higher rate. The frequent damage to mtDNA severely impairs the function of postmitotic neurons, making removal of damaged mitochondria via mitophagy critical (Islam, 2017: Lee et al., 2012). Mitophagy is the degradation of mitochondria due to signals such as starvation or oxidative stress. Oxidative stress can induce mitophagy by decreasing the mitochondrial membrane potential but can also impair mitophagy by interacting with its regulators. Parkin, a key mitophagy regulator involved in Parkinson's disease, can be S nitrosylated by ROS, inhibiting it and preventing the removal of damaged mitochondria (Lee et al., 2012). The central role of mitochondria in ROS production has led to a working theory of aging that combines ROS, DNA damage and mitochondrial theories (Maynard et al., 2015). The theory posits that DNA damage activates kinases and PARP, which deplete NAD+ levels, a cofactor for many metabolic pathways and a donor in the production of ATP. A reduction in NAD+ increases the need for oxygen consumption and ATP production, causing mitochondria to couple to meet high energy demands. Mitochondrial coupling increases membrane potential, decreases mitophagy, and elevates free radicals, the last damage (Fig. 0.1; Guillaumet-Adkins et al., 2017; Maynard et al., 2015). The close relationship

between harmful ROS and mitochondria lends weight to the hypothesis that mitochondria play a critical role in aging and associated neurodegenerative diseases.

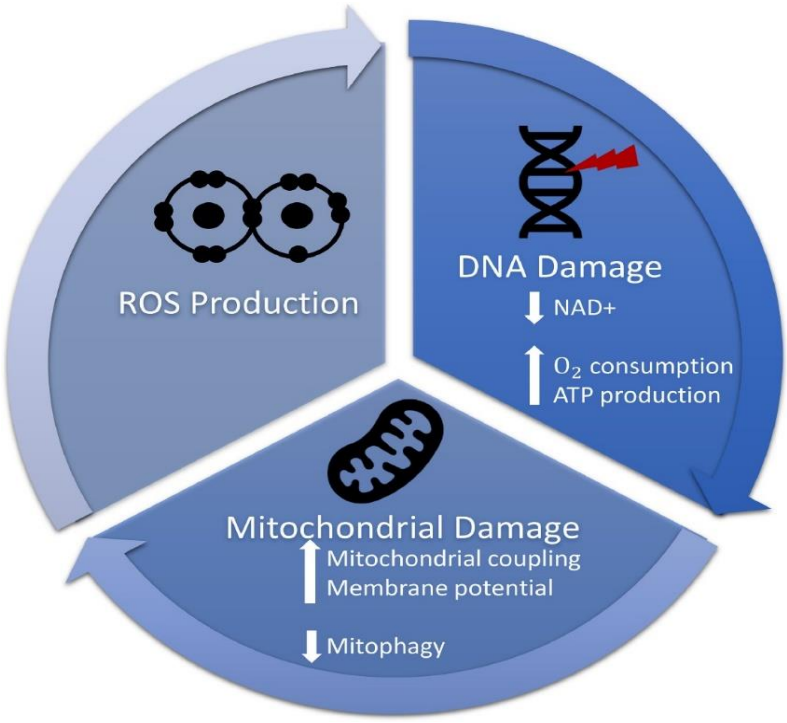

Figure 0.1. Combined Theory of Aging. This theory combines the DNA damage, mitochondrial and free radical theories of aging. DNA damage activates kinases and PARP, which consume NAD+. The decline in NAD+ increases energy production needs, elevating oxygen consumption and ATP production. Compensatory mitochondrial coupling leads to increased membrane potential, a decline in mitophagy and higher levels of free radicals, which further elevate DNA damage. Taken from Ionescu-Tucker and Cotman, 2021.

Mitochondrial oxidative stress and dysfunction increase in the aged brain

Mitochondrial dysfunction is prevalent in aging and improving mitochondrial function can have far reaching effects on systemic age-associated oxidative stress. In healthy aging, there are marked deficits in mitochondrial metabolism, specifically a reduction in the α subunit of the mitochondrial F1 ATP synthase, which couples oxidative phosphorylation to ATP synthesis. This leads to decreased ATP production, increased ROS production and increased

DNA, protein and lipid oxidation (Grimm and Eckert, 2017; Lu et al., 2004; Mecocci et al., 2018). Mitochondrial impairment leads not only to mtDNA damage but nuclear DNA damage as well, including in the promoter region of age-downregulated genes involved with vesicular function, synaptic plasticity and mitochondrial function (Lu et al., 2004).

Antioxidant overexpression has been tested as a potential means of mitigating the deleterious effects of age but has proven ineffective in extending the lifespan except in superoxide dismutase (SOD) overexpressing flies (Pérez et al., 2009; Tower, 2000). However, overexpression of mitochondrial catalase (mCAT) significantly extends the mouse lifespan. Mitochondrial catalase overexpression provides additional benefits including an overall reduction in oxidation, preserved insulin signaling and delayed cardiac and cataract pathology (Dai et al., 2009; Paglialunga et al., 2015; Schriner et al., 2005). DNA damage was similarly reduced in mCAT mice, both in aged skeletal muscle and mtDNA from asbestos exposed lungs (Campisi et al., 2019; Kim et al., 2016). These findings suggest that mCAT mice might prevent DNA damage in other tissue types, and in nuclear as well as mitochondrial DNA. A focused increase in mitochondrial catalase effectively mitigates oxidative stress, indicating the critical role of mitochondria in regulating ROS with age.

Catalase

Catalase reduces oxidative stress by converting hydrogen peroxide to water and oxygen (Ahn et al., 2016; Lockrow et al., 2012). It is critical for preventing ROS toxicity, as most individuals born with near-total catalase deficiency (acatalesmia) develop age and oxidative stress related diseases. A larger than normal proportion of these patients (18.5%) develop diabetes due to increased oxidative damage to sensitive pancreatic beta cells (Góth and Nagy, 2013). Catalase expression patterns in humans and animals directly support the free radical

theory of aging, showing that higher levels of ROS breakdown correlate with increased longevity and lower risk of age-related pathologies.

Catalase is restricted to peroxisomes in most cells, unlike other antioxidant enzymes such as superoxide dismutase and glutathione peroxidase which are prevalent in the cytoplasm and mitochondria (Mavelli et al., 1982). These peroxisomes are found next to mitochondria, indicating catalase's role in reducing metabolic stress. Catalase active peroxisomes are not restricted to one cell type in the brain, but are found in catecholamine cell bodies, oligodendrocytes and astrocytic processes (McKenna et al., 1976; Geremia et al., 1990). In the brain, the hypothalamus and substantia nigra are richest in catalase, but the hippocampus contains a moderate level of catalase equal to 65% of hypothalamic levels (Brannan et al., 1981). On a systemic scale, catalase levels peak in early adulthood (2 to 6 months in rats) and decline again in later life to very low levels (21 to 24 months in rats) (Mavelli et al., 1982; Ahn et al., 2016; Tsay et al., 2000). Catalase levels are lower in the hippocampus than other parts of the body, such as the liver, yet they remain high enough to form a significant defense system against cerebral oxidation.

The catalase inhibitor 3 amino-1,2,4-triazole (3AT) prevents the breakdown of endogenous hydrogen peroxide, increasing intracellular levels of reactive oxygen species (ROS). Catalase inhibition by 3 amino-1,2,4-triazole makes neurons more vulnerable to oxidative stressors such as amyloid beta oligomers (Milton, 2001). In the presence of hydrogen peroxide, the inactivation of catalase is irreversible, although in its absence, as in purified catalase experimThe effect of 3AT is thus only detected in tissues where hydrogen peroxide is present.

Current literature featuring 3AT gives a false impression of delayed kinetics, as most studies treat cultures for 12 to 24 hours (Ruiz-Ojeda et al., 2016; Santos et al., 2005). However, a

classic study showed that treatment of rat liver suspensions with 3AT led to a 95% reduction in catalase activity in only 2 hours. Similarly, treatment of blood lysates led to an 80% reduction in catalase activity in 2 hours and a complete loss of activity in 4 hours (Margoliash et al., 1958). The kinetics of catalase inactivation by 3AT are thus rapid as well as irreversible. In our studies, we examined how catalase inhibition by 3AT affected cell survival, synaptic density and epigenetics in aged neuron cultures.

Types of DNA Damage

Oxidative stress causes various types of DNA damage, including apurinic and apyrimidinic DNA sites, oxidized purines and pyrimidines, and DNA breaks. These DNA breaks are either single strand breaks (SSBs) or double strand breaks (DSBs), with DSBs being the more toxic of the two. SSBs are caused by the breakdown of the sugar phosphate backbone of DNA following ROS oxidation, while DSBs are longer lasting and can lead to changes in the transcription of gene promoters near break sites (Shanbhag et al., 2019; Thanan et al., 2014). DNA repair capacity is determined by levels of NAD+; while higher levels of NAD+ correlate with increased DNA repair, lower levels are indicative of ROS accumulation. NAD+ activates SIRT1, which decreases oxidative stress and resultant DNA damage by upregulating autophagy, mitophagy and protective histone methylation (Bosch-Presegué et al., 2011; Fang et al., 2017). NAD+ intermediates such as nicotinamide mononucleotide (NMN) were also found to increase SIRT1 activity, increasing NAD+ and MnSOD levels in turn (de Picciotto et al., 2016). While neuronal DNA is generally more vulnerable to damage than

DNA in other cell types, mitochondrial DNA is particularly susceptible due to its proximity to the electron transport chain and its lack of protective histones (Mecocci et al., 2018).

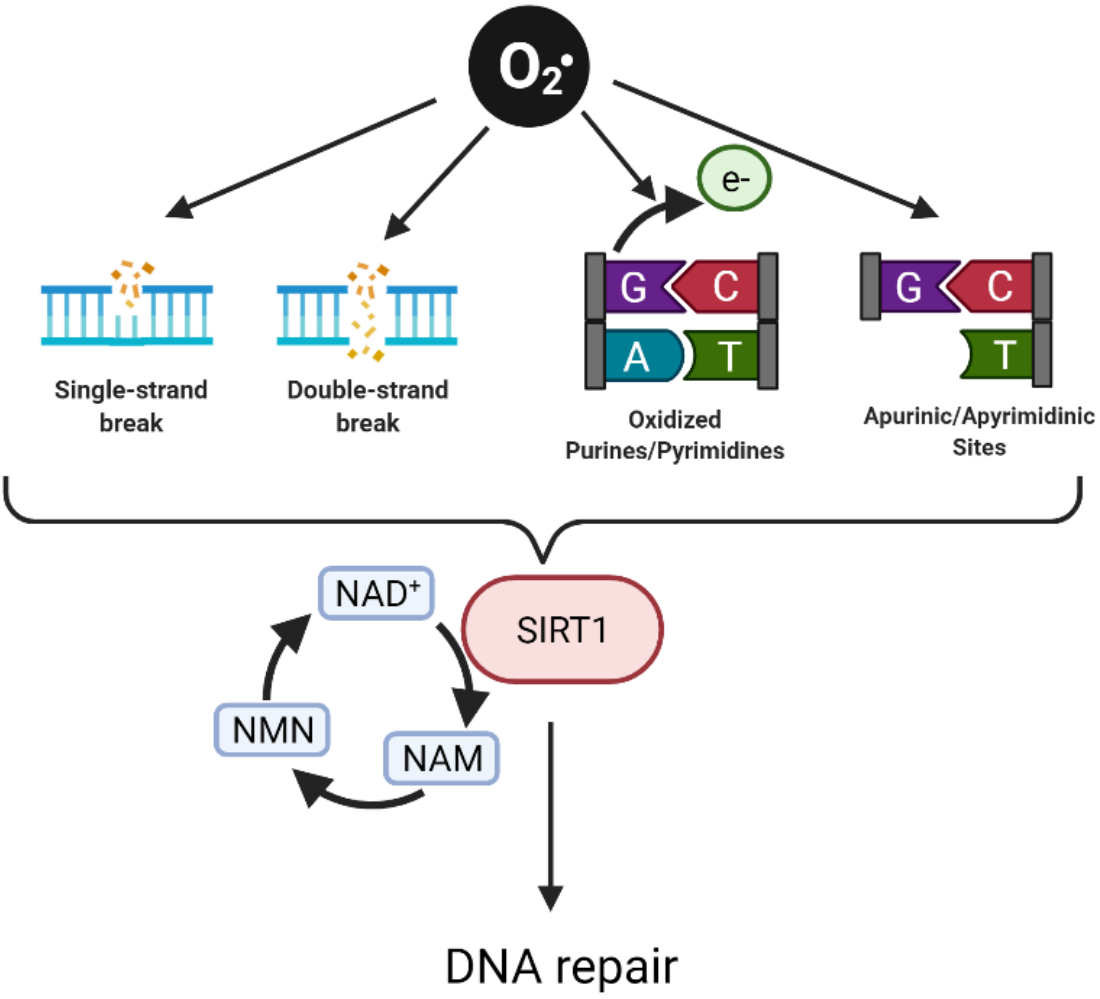

Figure 0.2. Oxidative Stress Causes DNA Damage. Oxidative stress causes single and double strand breaks, oxidized purines and pyrimidines, apurinic sites, and apyrimidinic sites. NAD+ (nicotinamide adenine dinucleotide) activates SIRT1, which is involved in multiple DNA repair pathways. SIRT1's consumption of NAD+ generates NAM (nicotinamide). Nicotinamide mononucleotide (NMN) is generated from NAM, and NMN is recycled back into NAD+. Created with Biorender.com.

DNA damage in the aged brain impairs cognition

DNA damage is elevated in both healthy aged and Alzheimer's disease brains, and some theorize that unrepaired DNA damage is a root cause of the aging process (Maynard et al., 2015; Thanan et al., 2014). A foundational paper found that the promoters of many cortical genes

are downregulated with age, beginning after age 40 and reaching peak levels after age 70 (Lu et al., 2004). These downregulated genes are mainly involved in synaptic plasticity, vesicular transport and mitochondrial function, indicating that DNA damage directly impairs neuronal and synaptic health. Brain aging also leads to increased expression of genes that can compensate for age-associated deficits, including genes involved in protein folding (heat shock protein 70 and alpha crystallin), antioxidant defense (nonselenium glutathione peroxidase, paraoxonase and selenoprotein P) and metal ion homeostasis. Increased expression of the base-excision repair enzymes 8-oxoguanine DNA glycosylase and uracil DNA glycosylase are indicative of both neuroprotective changes in gene expression as well as increased single strand DNA damage. Lu further examined if promoters of downregulated genes have impaired DNA repair. Promoter reporter plasmids were damaged in vitro with H2O2 and transfected into SH-SY5Y cells. After damage with H2O2, reporters derived from promoters of age-downregulated genes had much lower activation rates than reporters from promoters of age-stable genes. This suggests that age-downregulated genes have reduced base excision repair, indicating an increase in single strand breaks. (Lu et al., 2004). Increased single strand breaks have also been observed in aged hippocampal pyramidal and granule cells as well as cerebellar granule cells, but not in cerebellar Purkinje cells (Rutten et al., 2007). While DNA damage is elevated with age in nuclear DNA, it is 10-fold higher in mitochondrial DNA from subjects 42 years and older, and 15-fold higher in subjects over 70 (Mecocci et al., 2018). An elevation in age-associated single strand breaks impairs the expression of genes involved in synaptic plasticity, learning and memory, but preferentially affects mitochondrial DNA involved in metabolism, energetics and neuron survival.

Oxidative stress generates epigenetic modifications

Recent studies have shown that oxidative stress directly affects epigenetic chromatin modifications that control how genes are expressed. These changes may drive physiological responses to oxidative stress and facilitate the progression of diseases, including neurodegeneration (Kreuz, 2016). Oxidative stress can directly reduce methylation of DNA by oxidizing DNA, increasing TET-mediated hydroxymethylation, and interfering with binding of DNA methyltransferases that produce the methyl donor S-adenosylmethionine (Chia et al., 2011; Thanan et al., 2014). ROS can also form oxidized DNA lesions by hydroxylating pyrimidines and 5-methylcytosine (5mC), which can interfere with 5-hydroxymethylcytosine (5hmC) epigenetic signals (Guillaumet-Adkins et al., 2017; Lewandowska and Bartoszek, 2011). Oxidative stress alters post translational histone modifications, which can change chromatin structure, gene expression, gene stability and replication. These effects are often indirect, as ROS impair metabolic efficiency, reducing levels of metabolites such as acetyl-CoA, Fe, NAD+ and ketoglutarate that are essential for histone-modifying enzymes (Guillaumet-Adkins et al., 2017). H2O2, the most common means of ROS induction *in vitro*, has been shown to increase H3K9me3, H3K4me3 and H3K27me3 while decreasing H3K9ac and H4K8ac in bronchial epithelial cells. Preincubation with ascorbate prevented this elevation, indicating that antioxidants can prevent ROS-induced epigenetic changes. The methylation effects of oxidation were transient and did not persist after H2O2 washout (Niu et al., 2015). In neuronal SH-SY5Y cells, H2O2 increased histone acetyltransferases and downregulated histone deacetylases, as well as hypomethylating APP and BACE1. H2O2 thus caused the upregulation of the APP and BACE1 gene promoters, leading to amyloid beta peptide overproduction (Gu et al., 2013). These findings suggest that H2O2 generates transient epigenetic modifications which, among its wide-reaching effects, can accelerate amyloid beta pathology.

Our group has recently begun studying the epigenetics of aging, in which oxidative stress plays a principal role. We have shown that at least 1 specific repressive epigenetic mark, H3K9me3, increases with age in the hippocampus. We also reduced H3K9me3 activity by inhibiting the enzyme that produces it, SUV39H1, with a specific inhibitor (ETP69) and found that this improved object location memory, increased synaptic density and elevated BDNF levels in aged mice (Snigdha et al., 2016). Work by the Vaquero group suggests that oxidative stress may be one cause of H3K9me3 elevation. They demonstrate that H2O2 upregulates SIRT1, which protects against oxidative stress by increasing mitophagy and autophagy (Fang et al., 2017). SIRT1 stabilizes the histone methyltransferase SUV39H1, and SIRT1's upregulation thus leads to upregulation of SUV39H1 and its product H3K9me3 (Bosch-Presegué et al., 2011; Vaquero et al., 2007). This finding suggests that the increase in H3K9me3 we observed in our research may in fact be due to a protective SIRT1 upregulation caused by oxidative stress. A recent paper found that inhibition of the histone methyltransferase G9a is similarly protective in AD mice. The G9a inhibitor UNC0642 improved memory of objects and locations, reduced amyloid beta plaques, reduced 5-methyl cytosine (5-mc) levels, and increased levels of antioxidant heme oxygenase 1 in 5XFAD mice (Griñán-Ferré et al., 2019). Unlike SUV39H1, G9a does not specifically catalyze one methylated form of H3K9 but can produce mono- di- or tri-methylated H3K9. Other studies using the UNC0642 inhibitor have led to a decline in both di- and tri-methylated H3K9 (Kim et al., 2017). Overall, there is emerging evidence that the methylation of H3K9 may be upregulated by oxidative stress, and that inhibiting this methylation may prevent cognitive aging and reduce Alzheimer's pathology (Fig. 0.3). However, many

questions remain about the different roles of methylated histones, and how oxidative stress regulates their expression.

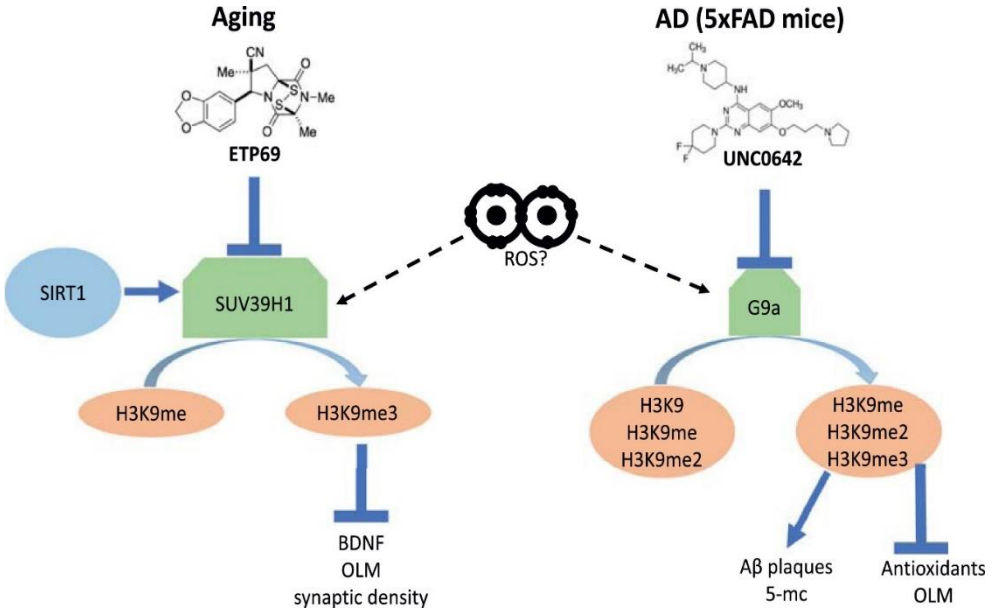

Figure 0.3. Histone TriMethylation Leads to Cognitive Decline in Aging and AD. SIRT1 stabilizes SUV39H1, the histone methyltransferase that catalyzes H3K9me3 formation. The SUV39H1 inhibitor ETP69 reduces levels of H3K9me3, while increasing BDNF, synaptic density and the performance of aged mice in an object location memory (OLM) task. The G9a inhibitor UNC0642 has a similar effect in 5xFAD mice, reducing mono-, di- and trimethylated H3K9 along with amyloid beta plaques and 5-methylcytosine (5mc). Conversely, UNC0642 increases levels of antioxidants and improves OLM task performance. H3K9 methylation may underlie cognitive decline in both aging and AD, and the upregulation of SUV39H1 and G9a may be a compensatory response to oxidative stress. Taken from Ionescu-Tucker and Cotman, 2021.

Antioxidant Therapeutics

Perhaps the most convincing evidence that oxidative stress is key to brain aging and AD is the plethora of therapeutics that target neuronal oxidative stress. The most common means of reducing oxidative stress is antioxidant treatment, which in some cases has been effective in reducing age-associated neuronal impairments and markers of neurodegeneration. The combination of Vitamins C and E (ascorbic acid and α-tocopherol) has been shown to decrease lipid peroxidation in neuronal cells (Li et al., 2003). When 24 month old Wistar

rats were fed N acetyl-l-cysteine (NAC), which increases glutathione levels in the brain, there was a coincident reduction in ROS and inflammation markers such as TNF alpha, IL-1β and IL-6, as well as a protective upregulation in SIRT1 (Garg et al., 2018). 1 month of daily NAC and alpha-lipoic acid injections also improved the performance of SAMP8 (senescence accelerated prone 8) mice in learning and memory tasks (Farr et al., 2003). Our lab has similarly demonstrated that antioxidants are effective at improving performance in cognitive tasks. We found that supplementation with antioxidants and mitochondrial cofactors (Vitamins E and C, L-carnitine, alpha lipoic acid and 1% fruit and vegetable granules) improved the performance of aged beagles in a size and black and white discrimination task, but that this improvement was greater when coupled with behavioral enrichment (Milgram et al., 2005). In humans, Vitamin E consumption from food and supplements was associated with a lower rate of cognitive decline in a large-scale longitudinal study, although Vitamin C and carnitine consumption was not (Morris et al., 2002). Although the efficacy of different antioxidants varies across models, they remain a powerful tool for reducing oxidative stress and improving cognition in the aged brain. In our lab, we use antioxidant deprivation in neuron cultures to simulate oxidative stress and increase neuronal susceptibility to exogenous stressors.

Physical Activity as an Oxidative Stress Therapeutic

Physical activity is a proven means of improving learning and memory by increasing levels of BDNF (Berchtold et al., 2002; Cotman and Berchtold, 2002; Neeper et al., 1995; Neeper et al., 1996;). Exercise promotes hormetic conditions, subtoxic levels of oxidative stressors that protect against more severe stressors, by initially increasing free radicals to a low, physiologically necessary level (Birringer, 2011; Coskun and Busciglio, 2012). Exercise also

induces Nrf2, as it protects against oxidative damage through a BDNF mediated pathway (Pall and Levine, 2015) . Exercise induced BDNF activates CREB, which upregulates APE1, involved in the base excision repair pathway. Through increased BDNF, physical activity protects neurons from oxidative DNA damage by enhancing DNA repair (Yang et al., 2014). At the same time, contracting muscles produce free radicals to promote the exercise training response. Exercise also activates the transcriptional coactivator PGC1 alpha, involved in exercise induced BDNF elevation. Endurance exercise furthermore increases the amount of antioxidant enzymes in cardiac and skeletal muscles (Powers et al., 2016). Physical activity thus signals the brain to compensate for exercise induced ROS by producing additional neuroprotective factors.

The length of an exercise program is the determining factor in its efficacy as an oxidative stress therapeutic. While short term exercise tends to increase ROS, long term exercise increases antioxidant enzymes, reducing overall oxidative stress (Belviranli and Gökbel, 2006). When 12-month-old female Wistar rats were exercised for 15 weeks on a treadmill at moderate intensity, hippocampal levels of ROS decreased while levels of PGC1 alpha, phospho-adenosine monophosphate protein kinase (p-AMPK), SOD1, SOD2 and glutathione peroxidase (GPX) increased (Marosi et al., 2012). In a clinical study, long term exercise decreased advanced oxidation protein products (AOPP) in the blood of older adults who engaged in aerobic exercise 3 times a week for 6 months (Rytz et al., 2020). Exercise has even been shown to accelerate DNA repair. A recent study of sedentary and active male volunteers found that while all participants had radiation induced DNA damage, trained individuals had more rapid repair of radiation-induced DNA strand breaks after exhaustive exercise. In addition, exhaustive exercise only produced DNA strand breaks in the lymphocytes of sedentary individuals (Moreno-Villanueva et al., 2019).

Long term exercise was also shown to reduce oxidative stress in models of AD. When 12-month-old 3xTgAD female mice had access to a running wheel for 3 months, levels of lipoperoxide, glutathione disulfide (GSSG), GPX and glutathione reductase (GR) were reduced to control levels while the antioxidant CuZn-SOD was increased. A network analysis showed that this reduction in oxidative stress was central to other behavioral and pathological changes, such as improved spatial memory, reduced amyloid beta and p-tau, and reduced anxiety (García-Mesa et al., 2016). Similar findings were reported in a streptozotocin (STZ) induced rat model of AD that were exercised on a treadmill for 4 weeks. The rats displayed a reduction in oxidative stress, mitochondrial dysfunction, amyloid beta and p-tau (Lu et al., 2017). These studies both demonstrate that the reduction in neurodegeneration observed with exercise is at least partially due to a reduction in oxidative stress. A recent paper suggests that long term exercise does not intrinsically reduce oxidative stress, but rather makes the brain more resilient to other stressors. 8 to 9-month-old Lewis rats were treated with rotenone, a pesticide that inhibits complex 1 of the mitochondrial respiratory chain to mimic sporadic neurodegeneration. By itself, 6 weeks of exercise increased hydrogen peroxide in the motor cortex of these rats. However, in the presence of rotenone, exercise increased the activity of the antioxidant GPX and reduced hydrogen peroxide. Additionally, prior exercise training reduced hydrogen peroxide levels in the spinal cords of both DMSO and rotenone exposed rats (Melo et al., 2019). In the presence of age, toxins, or neurodegeneration, long term exercise reduces oxidative stress and helps restore homeostatic conditions in the damaged brain.

BDNF Regulation

Introduction to BDNF Signaling

BDNF is a critical neurotrophin that promotes the survival, maintenance and growth of neurons (Berchtold et al., 2002). It is the most abundant, widely distributed and widely studied neurotrophin in the central nervous system. The BDNF gene consists of 9 5' non-coding exons and one 3' coding exon (exon IX), all of which are differentially expressed in the hippocampus presumably under the control of specific promoters. Transcription of the gene results in BDNF transcripts containing one of the eight 5' exons spliced to the protein coding exon. While BDNF is present in all neuronal and some non-neuronal tissues, it is most prevalent in the hippocampus, designating its important role in memory formation (Aid et al., 2007).

BDNF enhances synaptic plasticity and transmission through excitatory signaling pathways (Cotman and Berchtold, 2002). During neuronal activity, both glutamate and BDNF are secreted from synapses. BDNF is secreted more slowly than glutamate, and it tends to bind to its receptor TrkB after glutamate has activated it's postsynaptic signaling pathway. TrkB activation by BDNF binding can excite neurons by releasing calcium from internal stores, which activates the synaptic regulator CAMKII. BDNF also affects signaling cascades downstream of neuronal activation, leading to long term potentiation and other changes in synaptic plasticity. When BDNF and glutamate are released within a similar time window in the CA1, they produce repeated action potentials which can initiate timing dependent long term potentiation. BDNF may regulate LTP via different mechanisms depending on the hippocampal area; for instance, in mossy fiber neurons LTP is presynaptic, suggesting that BDNF and TrkB signaling enhance neurotransmitter release. BDNF has multiple pathways by which it regulates synaptic plasticity, and the precise mechanisms of these pathways continue to be explored. (Fig. 0.4; Sasi et al., 2017).

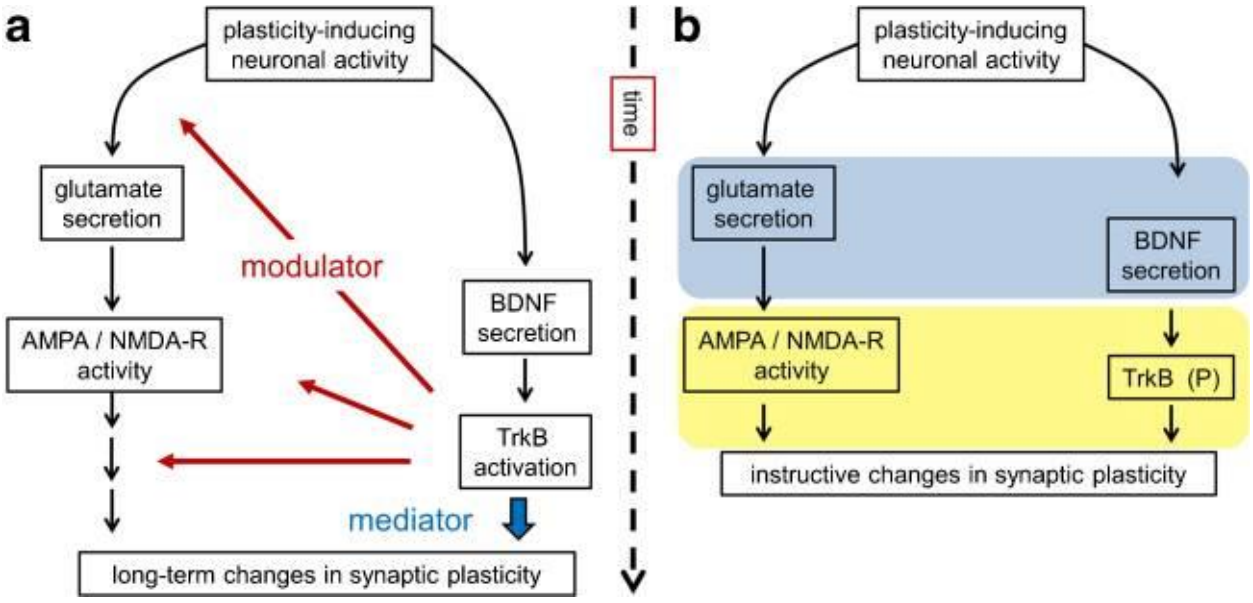

Figure 0.4. Overview of BDNF signaling. A major source of BDNF in the brain is the excitatory glutamatergic synapse, a principle synapse of synaptic plasticity, learning, and memory. During plasticity-inducing neuronal activity, BDNF and glutamate are released at synapses. **a** BDNF secretion occurs at a slower timescale than glutamate release. BDNF binds to its receptor TrkB to activate modulatory signaling cascades. BDNF and TrkB modulate signaling cascades downstream of neuronal excitation. BDNF is a mediator that can directly influence late effects in synaptic plasticity, for instance, local protein synthesis, spine remodeling, or gene transcription. **b** BDNF as an instructor of synaptic plasticity. Glutamate and BDNF are released within a critical time window (*bright blue*) and TrkB activation by BDNF serves as an instructive signal for associative postsynaptic long-term potentiation (*bright yellow window*). Modified from Sasi et al., 2017.

BDNF Epigenetic Regulation

BDNF expression is modulated by epigenetic changes at exon transcripts, such as CpG island methylation and histone modifications (acetylation, phosphorylation and methylation) (Intlekofer et al., 2013; Lubin et al., 2008; Martinowich et al., 2003). Various environmental stimuli can change BDNF expression levels by modifying epigenetic marks at its promoter regions, including neuronal activity, fear conditioning and environmental enrichment. Neuronal stimulation triggered by NMDA application (which causes long term depression) was recently shown to increase H3K27ac at BDNF II and VI promoters and increase transcription of these exons, signifying their involvement in long term depression (Palomer et al., 2016). In contrast, a

fear conditioning study highlighted how BDNF exons were differentially regulated by environmental stimuli. Contextual fear conditioning increased H3 and H4 acetylation at BDNF promoter IV and increased BDNF IV expression, but context exposure alone was sufficient to upregulate BDNF I and VI (Lubin et al., 2008). These findings suggest that BDNF IV is more involved in fear and emotion-based learning than BDNF I and VI, and that different exons may be regulated by different mechanisms. Two separate studies found that environmental enrichment combined with behavioral training improved BDNF exon regulation in aged rats, demonstrating that environmental enrichment can restore BDNF transcription even in old age. A combination of environmental enrichment and novel object training increased H3K4me3 levels at the BDNF exon IV promoter in the aged rat hippocampus and rescued memory deficits. Unlike H3K9me3, H3K4me3 upregulates gene expression (Morse et al., 2015). Similarly, environmental enrichment and Morris water maze testing in old rats increased H3ac at BDNF promoter 1 and increased BDNF I expression (Neidl et al., 2016). These studies establish that environmental stimuli can regulate BDNF expression through epigenetic modifications (Fernandes et al., 2018).

Epigenetic Regulation of BDNF by Exercise

Recent studies on the cognitive benefits of exercise have increasingly focused on its ability to affect the epigenetics of BDNF. Exercise was found to stimulate DNA demethylation at BDNF promoter IV in the rat hippocampus and elevate levels of the activated methyl CpG-binding protein 2. Exercise further increased acetylated H3 at BDNF promoter IV and elevated pCREB and CREB binding protein, both of which are key players in BDNF transcription (Gomez-Pinilla et al., 2010; Vecsey et al., 2007). This is supported by a study in mouse hippocampus showing that exercise increases H3 acetylation at exons I, II, III, IV, VI and VII and increases the expression of these

transcripts. Exercise prevented the effects of acute restraint stress, which reduced the expression of BDNF transcripts but had no effect on H3 modifications at their promoters (Ieraci et al., 2015). Sleiman et al. (2016) have also identified histone deacetylase (HDAC)2 and 3 as key BDNF modulators. They observed that 30 days of voluntary exercise reduced total HDAC2 levels in the mouse hippocampus and specifically reduced binding of HDAC2 and 3 to BDNF promoter 1, increasing total BDNF expression. They further found that the metabolite beta-hydroxybutyrate, produced after prolonged exercise, induces the activity of BDNF promoter I by reducing HDAC2 and HDAC3 binding. Our own lab has also established that 3 weeks of exercise or sodium butyrate (an HDAC inhibitor) increase H4K8ac at BDNF promoters I and IV while increasing transcript expression. Exercise and sodium butyrate further enhanced discrimination in an object location memory task after a subthreshold training paradigm (3 minutes training instead of 10) (Stefanko et al., 2009; Intlekofer et al., 2013; Fernandes et al., 2018). Taken together, these studies demonstrate that exercise induces a permissive chromatin state at BDNF promoter regions via a range of epigenetic modifications. Of note is that compounds that mimic the effects of exercise, such as sodium butyrate and beta-hydroxybutyrate, have similar effects on epigenetic modifications and BDNF transcription. Exercise and its mimetics are thus an effective means of modulating BDNF by epigenetic activation of its promoter regions.

Many studies have proven that exercise is a potent tool for increasing BDNF transcription (Neeper et al., 1995; Adlard et al., 2004). However, the majority of these studies focus on the effects of exercise in young mice, as BDNF expression declines with age. Although there are some exceptions in highly active individuals, advanced age is correlated with decreased serum BDNF, hippocampal volume and cardiorespiratory fitness (Erickson et al., 2010). In mice, more than one week of exercise does not significantly increase BDNF in mature (15 month old) and aged (24

month old) mice, although degree of exercise is proportional to BDNF expression in young mice (Adlard et al., 2004). Specific BDNF exons are also only increased in young mice (exons IIb, IIc and VI) (Adlard et al., 2005). The existing evidence suggests that exercise-induced regulation of BDNF is impaired with age. However, studies examining the epigenetic effects of exercise in aged animals are limited. In my research, I will examine if BDNF's epigenetic modulation by exercise is impaired in aged mice.

GABA Receptor Regulation

Introduction to GABA Signaling

GABA is the primary inhibitory neurotransmitter in the mammalian brain, and moderates neuronal excitability throughout the central nervous system. GABA receptors also regulate BDNF secretion. The two main classes of GABA receptor are GABA A and GABA B. GABA A receptors are ionotropic and facilitate rapid chloride ion influx. They mediate the majority of rapid inhibitory transmission in the vertebrate brain and have been studied to a much greater extent than their counterpart. GABA B receptors are coupled to G proteins and mediate slow hyperpolarization through potassium channels (Olsen and DeLorey, 1999). Although their composition varies, GABA A receptors are generally made up of two α and β subunits and one γ subunit (Li and De Blas, 1997). Presently, at least twenty genes encoding distinct receptor subunits have been identified. These subunits are grouped according to their degree of sequence identity and include the α1–6, β1–4, γ1–3, ρ1–3, δ, ε, π, and θ subunits (Rissman et al., 2007; Olsen and Tobin, 1990). GABA B receptors are generally composed of an R1 and an R2 subunit. The R2 subunit mediates interactions with the G protein and facilitates receptor complex expression at the plasma membrane (Robbins et al., 2001; Margeta-Mitrovic et al., 2000). The R1 subunit is expressed as one of two

isoforms (A and B). While isoform A targets the receptor complex to axons and presynaptic terminals, isoform B targets dendrites and postsynaptic terminals. Together, the isoforms of the R1 subunit ensure an even distribution of pre and postsynaptic inhibitory signaling (McQuail et al., 2011; Biermann et al., 2010; Vigot et al., 2006)

Age Related Declines in Hippocampal GABA Signaling

Studies show that GABA signaling is reduced in the hippocampus with age, contributing to overexcitation and memory impairment (McQuail et al., 2015; Stanley et al., 2004). A study in rats demonstrated that GABAergic interneuron numbers declined with age in both the dentate gyrus and CA3 region. However, only neuron loss in the hilus of the dentate gyrus was correlated with impaired performance in a water maze task. Treatment with the antiepileptic drug leviteracetam, which was previously shown to improve learning and memory, restored hilar gabaergic neuron expression (Spiegel et al., 2013). Whole cell patch clamp recording similarly showed a reduction in spontaneous IPSPs in granule cells of the aged dentate gyrus (Patrylo et al., 2007). Overall, these studies illustrate that reduced GABA signaling in the dentate gyrus is involved in age-related cognitive decline.

In contrast to GABAergic signaling, GABA receptor expression is largely preserved within the aged hippocampus. A study of 21-month-old mice found that hippocampal GABA A receptor levels were preserved with age. However, there was a localized decrease in the alpha 2 subunit (GABRA2) around neuronal cell bodies and proximal dendrites in the CA1 and CA3 (Palpagama et al., 2019). These findings indicate that receptor composition may be locally altered to compensate for an age-related decline in GABAergic signaling. GABA B receptor expression was similarly preserved in the hippocampi of aged mice, but reduced GABA B receptor 1 (GABBR1)

expression was observed in aged rats with impaired learning of a Morris water maze (McQuail et al., 2011). This suggests that GABBR1 expression is directly linked to hippocampal-dependent spatial learning. Although GABA receptors may not be globally reduced with age, their hippocampal expression patterns are altered by the synaptic and cognitive decline observed in aged animals.

Exercise Regulates GABA Receptor Expression

Our lab recently found that exercise elevates the expression of synaptic genes that oppose expression changes in aging and Alzheimer's disease. Among these are 8 GABA genes including GABBR1 and GABRA2, which we identified as targets for future epigenetic studies (Berchtold et al., 2019). Exercise may increase GABA receptor expression to compensate for an age-associated decline in inhibitory signaling (McQuail et al., 2015; Spiegel et al., 2013). In contrast, exercise may have an opposing effect on GABA receptor expression in young mice where inhibitory signaling is fully functional. This hypothesis is supported by the finding that 4 weeks of wheel running decreased GABRA2 in the forebrain of young rats (Hill et al., 2010). While studies on a broader range of receptor subtypes are needed, the existing literature suggests that exercise differentially moderates GABA receptor expression in an age-dependent manner.

Studies on the epigenetic regulation of GABA receptor are limited, but emerging evidence suggests that reduced GABA signaling is associated with a repressive chromatin state. Bicuculline treatment, a GABA A receptor inhibitor, increases HDAC activity in the hippocampi of both sedentary and exercised mice, reducing chromatin accessibility (Maejima et al., 2018). This is supported by a recent study showing that GABBR1 and GABRA2 have increased H3K9me3 at their promoters in Alzheimer's diseased brains, corresponding to reduced gene transcription (Lee et al., 2020). Reduced GABA signaling is thus strongly correlated with increased gene repression.

In our studies, we will examine if exercise and a drug that reduces H3K9me3 (ETP69) can reduce repressive H3K9me3 at neuronal plasticity genes such as GABBR1 and GABRA2 and increase gene expression.

Chapter 1: Regulation of Hippocampal H3K9me3 by Catalase Inhibition and Hydrogen Peroxide

Rationale

Histone modifications are emerging as a key contributor to the cognitive decline that occurs in aging and neurodegeneration (Lardenoije et al., 2015). A highly regulated site is histone 3, lysine 9 (H3K9) which is associated with gene silencing when di or tri-methylated (Barter and Foster, 2018; Peters et al., 2003; Stewart et al., 2005). H3K9 trimethylation (me3) has been shown to have a critical role in aging, including vascular inflammation and diabetes (Sedivy et al., 2008; Villeneuve et al., 2010). Previous work by our lab has shown that H3K9me3 increases in the mouse hippocampus with age, and that inhibiting SUV39H1, the enzyme that produces H3K9me3, reduces this repressive mark and improves performance in object location memory and fear conditioning tasks (Snigdha et al., 2016). Although H3K9me3 plays a key role in regulating cognitive function, the mechanism underlying H3K9me3 elevation in the aging hippocampus has yet to be explored.

In this study, we investigated H3K9me3 regulation in the aged hippocampus by determining which age-associated stressors lead to its elevation. Work by the Vaquero group suggests that oxidative stress, which is elevated with age, may be one cause of H3K9me3 elevation (Harman, 1992; Butterfield and Halliwell, 2019). Oxidative stress in the form of hydrogen peroxide increased H3K9me3 in human embryonic kidney cells by upregulating SIRT1, a sirtuin that promotes protective responses to oxidative stress. SIRT1 stabilizes the histone methyltransferase SUV39H1, and SIRT1's upregulation activates SUV39H1 and increases H3K9me3 levels (Vaquero et al., 2007). Similar studies found that H2O2 elevates SUV39H1 and H3K9me3 in fibroblasts and ventricular monocytes (Bosch-Presegué et al., 2011; Yang et al., 2017). We thus examined if the oxidative stressors 3 amino-1,2,3-triazole (3AT) or

hydrogen peroxide (H_2O_2) led to an upregulation in H3K9me3. Overall, our data suggests that 3AT and H_2O_2 are insufficient to change H3K9me3 levels in the hippocampus.

Materials and Methods

Hippocampal Neuron Cultures

Primary cultures of dissociated hippocampal neurons were prepared from E17-19 Sprague-Dawley rats by dissection in calcium- and magnesium- free buffer, then digested in 0.125% trypsin at 37 °C for 7 minutes with inversions every minute. The neurons were gently pelleted and re-suspended in 1mL of growth media, containing neurobasal media with penicillin-streptomycin, B27 (Fisher 17504044), and glutamax. Cells were triturated using three fire-polished pipettes and strained through a 40uM cell strainer. Cells were plated at 6×10^5 cells/9.5cm^2 on plastic plates coated with 0.125mg/ml poly-D-lysine and maintained at 37°C in a 5% CO_2 atmosphere in growth media. Cells were fed twice weekly by 50% media exchange. Prior to treatment, 50% of the culture media was replaced twice with antioxidant deprived treatment media consisting of neurobasal media with B27-AO (without antioxidants, Fisher 10889038), penicillin-streptomycin and glutamax.

Organotypic Cultures

Rat pups (7–10 d old) were decapitated, and transverse hippocampal slices (350 μm) were obtained using a tissue chopper. Slices were separated and transferred to 30 mm Millicell-CM 0.4-μm-thick sterile tissue culture plate inserts (Millipore). The inserts were placed on six-well tissue culture plates, each containing 1 ml of culture medium. The slices were maintained for 7 days in horse serum-containing medium (50% Neurobasal medium, 25% HBSS, 0.5% GlutaMax, pH 7.2, and 25% horse serum), then for 2 days in Neurobasal B27-containing

medium plus 10% horse serum, 2 days in Neurobasal B27-containing medium plus 3% horse serum, and finally in neurobasal medium containing B27, glutamax and penicillin-streptomycin. Slices were cultured for 12 days at 37°C in a 5% CO2 atmosphere and fed twice weekly by 50% medium exchange. Prior to treatment, 50% of the culture medium was replaced twice with antioxidant deprived media consisting of neurobasal media with B27-AO (without antioxidants), penicillin-streptomycin and glutamax.

Western Blot

Cells were plated at 2.5 x 10^5 cells/ml in 6 well plates coated with 0.125 mg/ml poly-l-lysine and washed. Media was removed from all wells and ice-cold PBS was used to wash the cells. Cells were lysed in RIPA buffer containing protease inhibitor cocktails from Pierce. Cell homogenates were harvested, sonicated for 6 seconds and spun at 12,000xg for 12 minutes. The supernatant was removed for microBCA assay (Fisher 23235) and western blot analysis. Equal amounts of protein were loaded into 10 or 15% Bio-Rad Tris-HCl gels and transferred onto PVDF membranes using a Bio-Rad Turbo Transfer system. Membranes were washed in tris-buffered saline with 0.1% tween 20 (TBS-T), blocked in 5% bovine serum albumin (BSA) in TBS-T, incubated in primary antibodies overnight in 2.5% BSA in TBS-T at 4°C (anti-H3K9me3, ab8898; anti-beta actin, Cell Signaling Technologies #4967; anti-PSD-95, ab18258) washed, and incubated in horseradish peroxidase secondary antibodies in TBS-T at room temperature. Membranes were developed using Pierce ECL detection kit and immunoreactivity was quantified using Image J.

MTT

Cells were plated at 2.5 X 10^5 cells/ml in 24 well plates coated with 0.125mg/ml poly-L-lysine and washed. Cells were maintained and treated according to the dissociated culture protocol. After treatment, 25uL of thiazolyl blue (MTT) stock solution was added to each well and incubated at 37°C for 1 to 4 hours. The media was removed and 100uL of 0.04M hydrogen chloride in isopropanol was added to each well. After 5 minutes, plates were read on a microplate reader with absorbance at 595 nm.

LDH

Cells were plated at 2.5 X 10^5 cells/ml in 24 well plates coated with 0.125mg/ml poly-L-lysine and washed. LDH release was analyzed using a CyQyant LDH assay according to the manufacturer's instructions (C20301). In brief, 50uL 10x lysis buffer was added to maximum LDH control wells and 50uL ddH20 was added to spontaneous LDH control wells 45 minutes before the assay. 50uL of sample medium was transferred from each culture well to a 96 well plate, and samples were measured in duplicate. Media was incubated with 50uL reaction mixture for 30 minutes before the addition of 50uL stop solution. Absorbance was measured at 490 and 680 nm. To determine LDH activity, the 680 nm value was subtracted from the 490 nm absorbance before calculation of percent cytotoxicity [(compound treated LDH activity - spontaneous LDH activity)/(maximum LDH activity-spontaneous LDH activity) *100] and normalization to control conditions (100% survival) to determine relative survival.

Propidium Iodide

Organotypic cultures were plated, maintained and treated according to the organotypic culture protocol. After treatment, 5ug/mL propidium iodide was added to culture media for 5 minutes at

room temperature. Cultures were washed with antioxidant deprived media and imaged 1 hour after treatment.

Glutathione Assay

Reduced glutathione (GSH) was quantified using a detection assay kit (ab138881) according to the manufacturer's instructions. In brief, cells or tissue were washed in PBS and homogenized in 0.5% NP-40 in PBS. Samples were centrifuged for 15 minutes at 14,000 x g at 4°C and the supernatant was collected. Protein was removed from samples by treatment with trichloroacetic acid (TCA), centrifuged at 12,000 x g for 5 minutes at 4°C and neutralized with $NaHCO_3$ to a pH of 4-6. Samples were centrifuged at 13,000 x g for 15 minutes at 4°C and the supernatant was collected. 50uL of sample was placed in the wells of a black 96 well plate and incubated with 50uL of GSH assay mixture (1x thiol green solution) for 1 hour. Fluorescence was measured using a microplate reader at an excitation of 490 and an emission of 520 nm.

Dichlorofluorescein Assay

Dissociated neurons were plated at 6 x 10^5 cells/9.5cm^2 in 96 well plates coated with 0.125mg/ml poly-L-lysine. At 12 DIV, culture media was replaced twice with antioxidant deprived media and reduced to 100uL per well. Wells were treated with 2mM 3AT for the appropriate lengths of time. 45 minutes before the end of treatment, 100uL of 0.02mg/mL dichlorofluoroscein (DCF) in antioxidant deprived media was added to each well. After treatment, fluorescence was read on a microplate reader with an excitation of 485 nm and an emission of 535 nm.

Statistical Analysis

The results of ANOVA analysis are as reported. In all cases, $p \leq 0.05$ was considered significant. Image J was used for image analysis, and Prism was used for data analysis. All graphs represent means $\pm$ SEM.

Results

We began by examining if the catalase inhibitor 3 amino-1,2,4-triazole (3AT) increased H3K9me3 levels in hippocampal neurons. We first optimized a concentration of 3AT that did not induce significant cell death. We tested 2 and 10mM 3AT as these concentrations are commonly

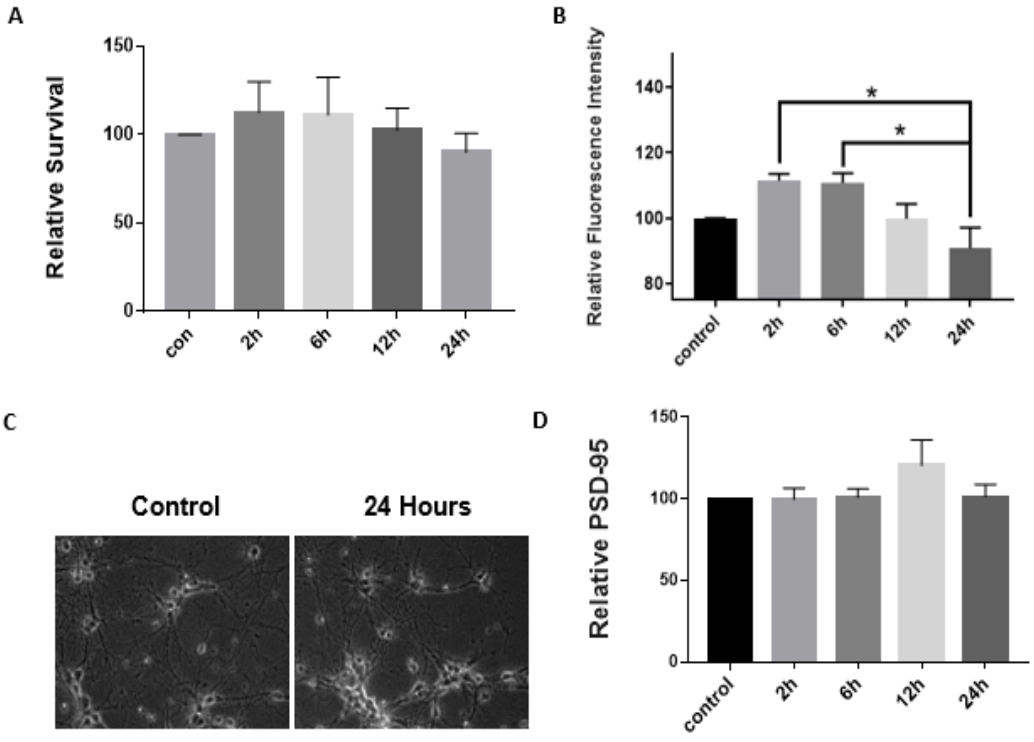

Figure 1.1 The age-associated stressor 3AT does not change survival, oxidative stress or synaptic density. A, Time dependent MTT survival assay of 12-13 DIV hippocampal neuron cultures treated with 2mM 3AT. N=3 for 6h and 24h, n=4 for control, 2h and 12h, $p>0.05$. B, Time dependent dichlorofluorescein assay of 12 DIV rat hippocampal neuron cultures treated with 2mM 3AT. One-way ANOVA *$p=0.016$, n=3; Tukey's multiple comparisons test, * $p<0.05$ relative to 24h. C, Representative images of negative control cultures and cultures treated with 2mM 3AT for 24 hours. D, Time dependent PSD-95 western blot analysis of 12 DIV rat hippocampal neuron cultures treated with 2mM 3AT. N=4, $p>0.05$. N represents number of independent experiments.

used for inducing oxidative stress in vitro (Ruiz Ojeda et al., 2016; Ueda et al., 2003; Bayliak et al., 2008). In preliminary studies 10mM 3AT reduced neuron survival and led to degeneration (data not shown). In contrast, 2mM 3AT did not reduce neuron survival or change neuron morphology following treatment of mature neurons for up to 24 hours (Fig. 1.1A,C). However, this concentration did not produce significant oxidative stress relative to control conditions as measured by a dichlorofluorescein assay, although 2 and 6 hours of treatment produced significantly more oxidative stress than 24 hours (One way ANOVA, *p=0.016; Tukey's post hoc test, *p<0.05 for 2h and 6h vs. 24h; Fig. 1.1B). This suggests that long term treatment with 3AT reduces oxidative stress as neurons compensate for the exogenous stressor. There was also no significant reduction in synaptic density as measured by PSD-95 (Fig. 1.1D). We concluded that 3AT was not an effective means of inducing oxidative stress in our dissociated hippocampal neurons.

In parallel, we assessed if 3AT could affect H3K9me3 in organotypic hippocampal cultures without reducing cell survival. Organotypic cultures are more resilient than dissociated neurons due to their more complex structure and the presence of glial cells. Thus, we tested a range of doses from 2-20mM to see which concentration could change H3K9me3 levels without inducing cell death. While none of the concentrations induced significant cell death (Fig. 1.2A, B,C), none of the concentrations significantly changed H3K9me3 levels either (Fig. 1.2D). We concluded that 3AT did not generate a significant change in H3K9me3 at the concentration used.

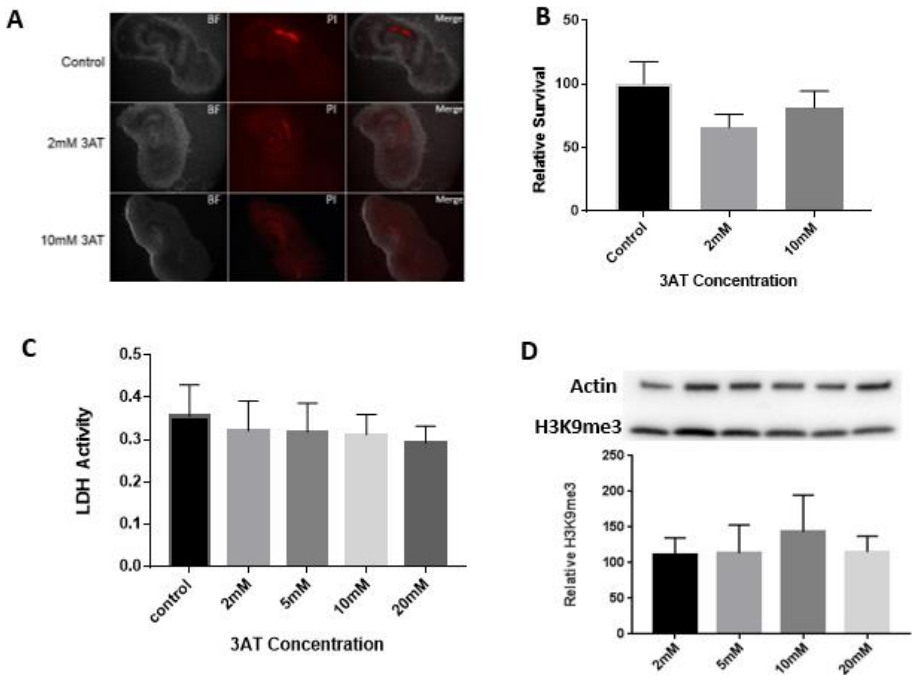

Figure 1.2. Catalase inhibition does not affect H3K9me3 in organotypic hippocampal cultures. A, Representative images of propidium iodide treated 12 DIV organotypic cultures after 24 hours of treatment with varying doses of 3AT. BF= bright field, PI=propidium iodide, 2.5x magnification. B, Treatment with 2 or 10mM of 3AT does not reduce neuron survival. Graph shows relative toxicity compared to the negative control. N=10 for the negative control, n=9 for 2mM 3AT, n=13 for 10mM 3AT, $p>0.05$, where n represents number of organotypic slices. C, Treatment with 3AT does not affect neuron survival. LDH activity of 12DIV organotypic cultures treated with varying concentrations of 3AT for 24 hours. Kruskal-Wallis test, n=4, $p>0.05$, where n is the number of independent experiments. D, Representative western blot and western blot analysis of 12 DIV organotypic cultures treated with 5-20mM 3AT for 24 hours. N=6, $p>0.05$, where n is the number of independent experiments.

We turned to an oxidative stressor that is more commonly used in the literature, H_2O_2. Exogenous application of H_2O_2 directly induces oxidative stress, and has been shown to activate SUV39H1 and increase H3K9me3 in a variety of non-neuronal cell types (Vaquero et al., 2007; Bosque-Presegué et al., 2011; Yang et al., 2017). We first conducted a dose-response study to determine a concentration of hydrogen peroxide that significantly elevated oxidative stress. We used 12 DIV hippocampal neuron cultures and treatment with varying concentrations of hydrogen peroxide previously shown to induce oxidative stress by our lab (Whittemore et al., 1995). We treated cultures with H_2O_2 for 5 minutes, replaced the media and harvested cultures 8 hours later, a treatment paradigm that induces epigenetic changes in mouse hippocampal neuron

cultures (Gräff et al., 2012). LDH assay quantification indicated a significant decrease in survival with 200uM H2O2 (One way ANOVA, *p=0.0025; Tukey's post hoc test, ***p=0.0010 relative to control) which corresponds to approximately a 7% decrease in neuron survival (Fig. 1.3A). 100 and 200uM H2O2 led to a significant decrease in the antioxidant reduced glutathione (GSH) (One way ANOVA, *p=0.041; Dunnett's post hoc test, *p=0.02 100uM relative to control, **p=0.012 200uM relative to control), indicating that these concentrations of H2O2 significantly increase oxidative stress (Fig. 1.3B). However, H2O2 did not significantly change H3K9me3 levels (One way ANOVA, p=0.68) (Fig. 1.3C). While we optimized two concentrations of hydrogen peroxide that induced oxidative stress (100 and 200uM), neither of these concentrations were sufficient to elicit a change in hippocampal H3K9me3. We concluded that hydrogen peroxide was insufficient to elicit a change in H3K9me3 in mature neuron cultures.

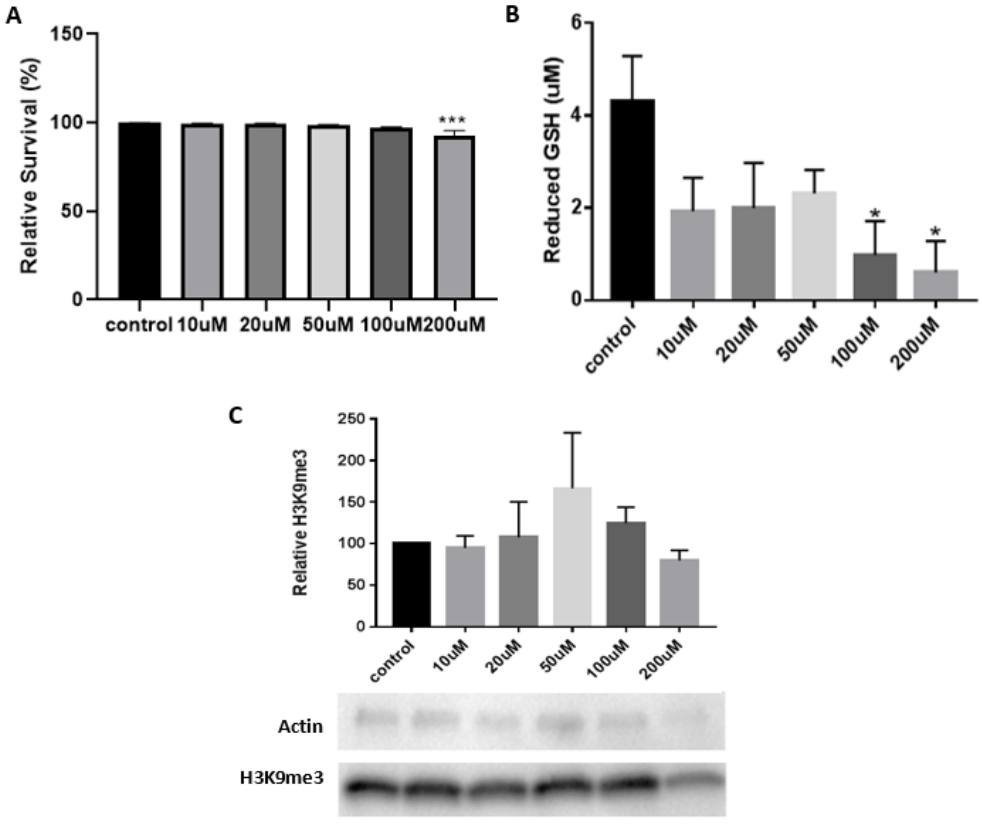

Figure 1.3. H2O2 induces oxidative stress without increasing repressive histone methylation in mature (12 DIV) hippocampal neurons. A, LDH assay quantification of toxicity in 12DIV hippocampal neuron cultures treated with 10 to 200uM H2O2. One-way ANOVA, ***p=0.0025, n=4. Tukey's multiple comparisons test, ***p=0.0010 control vs 200uM. B, Glutathione assay for 12DIV hippocampal neuron cultures treated with 10 to 200uM H2O2. One way ANOVA, *p=0.041, n=4. Dunnett's multiple comparisons test,*p=0.02 100uM vs. control, *p=0.012 200uM vs control. C, Quantification of H3K9me3 in 12 DIV hippocampal neuron cultures. One way ANOVA, n=4, p=0.68. All cultures were treated with H2O2 for 5 minutes and harvested 8 hours later.

Discussion

We found that neither 3AT nor H2O2 significantly changed H3K9me3 in neuronal cultures. While previous studies in human embryonic kidney cells, mouse embryonic fibroblasts and rat neonatal ventricular monocytes demonstrated that H2O2 affected H3K9me3 levels, this is not the case in neurons (Vaquero et al., 2007; Bosque-Presegué et al., 2011; Yang et al., 2017). Epigenetic regulation may differ in neurons since they have greater levels of endogenous oxidative stress than other cell types. Neurons are post-mitotic cells that cannot be replaced and accumulate oxidative and mitochondrial damage as they age (Grimm and Eckert, 2017). Out of all brain regions, oxidative stress is highest in the hippocampus, partly due to high levels of reactive oxygen species (ROS) in the CA1 (Wang and Michaelis, 2010). Neurons are protected from excess oxidation by neurotrophic factors such as BDNF, which increases levels of superoxide dismutase (SOD) and glutathione reductase (GRX) in cultured rat hippocampal neurons (Mattson et al.,1995; Chen et al., 2017). To elevate oxidative stress sufficiently, it is possible that the activity of neuroprotective factors such as BDNF should be suppressed. In the following chapter, we investigate if inhibiting BDNF signaling can change H3K9 trimethylation levels.

Chapter 2: Inhibiting BDNF Signaling Upregulates Hippocampal H3K9me3 via an Age and Oxidative Stress Dependent Mechanism

Rationale

In this study we investigated if the age-associated stressor inhibiting BDNF signaling increases H3K9 trimethylation. BDNF is reduced in the aged brain and promotes antioxidant production in hippocampal neurons, suggesting that its inhibition might increase oxidative stress to the levels necessary for epigenetic changes (Chen et al, 2017; Erickson et al, 2012; Mattson et al.,1995). A study in cortical neurons found that BDNF signaling leads to SUV39H1 degradation, a pathway which may also be active in hippocampal neurons (Sen and Snyder, 2011). We treated cultures with the BDNF inhibitor TrkB-Fc, H_2O_2 or a combination of the two stressors and found that TrkB-Fc alone or with H_2O_2 elevated H3K9me3 in mature hippocampal neurons.

We next examined if H3K9me3's susceptibility to stressors was age-dependent by examining H3K9me3 levels in young (6 DIV) and aged (21 DIV) hippocampal neurons in addition to mature (12 DIV) cultures. We hypothesized that hippocampal neurons would become more susceptible to modifications with age due to the accumulation of oxidative damage (Wang and Michaelis, 2010). Surprisingly, H3K9me3 elevation was greater in 12 DIV neurons than 21 DIV neurons, suggesting that H3K9 regulation may be impaired with increased age.

Lastly, we determined the role of oxidative stress in H3K9 trimethylation induced by inhibiting BDNF signaling. We tested if antioxidants could prevent H3K9me3 elevation in cultures treated with both H_2O_2 and TrkB-Fc. We further examined if SIRT1 levels correlated with H3K9me3 elevation, as SIRT1 mediates oxidative stress-induced epigenetic changes in other cell types (Vaquero et al., 2007; Bosque-Presegué et al., 2011; Yang et al., 2017).

Antioxidant treatment prevented H3K9me3 elevation in 12 DIV neurons treated with TrkB-Fc and H_2O_2, demonstrating that inhibiting BDNF signaling elevates H3K9me3 via an oxidation-based mechanism. However, SIRT1 was only elevated in young, H_2O_2 treated neurons and did not correlate with H3K9me3, suggesting that SIRT1 is not the primary driver of H3K9 trimethylation in hippocampal neurons. Overall, this study establishes that inhibiting BDNF signaling promotes hippocampal H3K9 trimethylation in an oxidative stress and age dependent manner.

Methods

Hippocampal Neuron Cultures

Primary cultures of dissociated hippocampal neurons were prepared from E17-19 Sprague-Dawley rats by dissection in calcium- and magnesium- free buffer, then digested in 0.125% trypsin at 37 °C for 7 minutes with inversions every minute. The neurons were gently pelleted and re-suspended in 1mL of growth media, containing neurobasal media with penicillin-streptomycin, B27 (Fisher 17504044), and glutamax. Cells were triturated using three fire-polished pipettes and strained through a 40uM cell strainer. Cells were plated at 6×10^5 cells/9.5cm^2 on plastic plates coated with 0.125mg/ml poly-D-lysine and maintained at 37°C in a 5% CO_2 atmosphere in growth media. Cells were fed twice weekly by 50% media exchange. Prior to treatment, 50% of the culture media was replaced twice with antioxidant deprived treatment media consisting of neurobasal media with B27-AO (without antioxidants, Fisher 10889038), penicillin-streptomycin and glutamax.

Culture Treatments

Hippocampal neuron cultures were treated with varying concentrations (10uM to 200uM) of H2O2 diluted in treatment media for 5 minutes. 50% of the culture media was replaced twice with treatment media and cultures were harvested for western blot or LDH analysis 8 hours later. Alternatively, cultures were treated with 200uM H2O2 for 4 hours. Lyophilized TrkB-Fc (R&D Systems, 688-TK-100) was diluted to a stock solution of 100ug/ml in sterile PBS. Cultures were treated with 1ug/ml TrkB-Fc for 24 hours. For antioxidant treatment, 50% of culture media was replaced twice with growth media containing B27 at the time of treatment with exogenous stressors.

Western Blot

Cells were plated at 2.5 x 10^5 cells/ml in 6 well plates coated with 0.125 mg/ml poly-l-lysine and washed. Media was removed from all wells and ice-cold PBS was used to wash the cells. Cells were lysed in RIPA buffer containing protease inhibitor cocktails from Pierce. Cell homogenates were harvested, sonicated for 6 seconds and spun at 12,000xg for 12 minutes. The supernatant was removed for microBCA assay (Fisher 23235) and western blot analysis. Equal amounts of protein were loaded into 10 or 15% Bio-Rad Tris-HCl gels and transferred onto PVDF membranes using a Bio-Rad Turbo Transfer system. Membranes were washed in tris-buffered saline with 0.1% tween 20 (TBS-T), blocked in 5% bovine serum albumin (BSA) in TBS-T, incubated in primary antibodies overnight in 2.5% BSA in TBS-T at 4°C (anti-H3K9me3, ab8898; anti-beta actin, Cell Signaling Technologies #4967; anti-SIRT1, ab110304; anti-H3, ab1791) washed, and incubated in horseradish secondary antibodies in TBS-T at room temperature. H3K9me3 was normalized to actin and SIRT1 was normalized to H3. Membranes were developed using Pierce ECL detection kit and immunoreactivity was quantified using Image J.

LDH

Cells were plated at 2.5 X 10^5 cells/ml in 24 well plates coated with 0.125mg/ml poly-L-lysine and washed. LDH release was analyzed using a CyQyant LDH assay according to the manufacturer's instructions (C20301). In brief, 50uL 10x lysis buffer was added to maximum LDH control wells and 50uL ddH20 was added to spontaneous LDH control wells 45 minutes before the assay. 50uL of sample medium was transferred from each culture well to a 96 well plate, and samples were measured in duplicate. Media was incubated with 50uL reaction mixture for 30 minutes before the addition of 50uL stop solution. Absorbance was measured at 490 and 680 nm. To determine LDH activity, the 680 nm value was subtracted from the 490 nm absorbance before calculation of percent cytotoxicity [(compound treated LDH activity - spontaneous LDH activity)/(maximum LDH activity-spontaneous LDH activity) *100] and normalization to control conditions (100% survival) to determine relative survival.

Statistical Analysis

The results of ANOVA analysis are as reported. In all cases, $p \leq 0.05$ was considered significant. Image J was used for image analysis, and Prism was used for data analysis. All graphs represent means $\pm$ SEM. N represents number of independent experiments.

Results

We first investigated if BDNF signaling with or without additional oxidative stress could change H3K9me3 levels in 12 DIV hippocampal neurons. We used a concentration of a BDNF receptor inhibitor (TrkB-Fc) that had previously been optimized in our lab for cell culture (24 hours, 1ug/mL; data not shown). We further tested if a longer H2O2 treatment time (4 hours) could change H3K9me3 levels as a brief treatment period had no effect. Our findings show that

both TrkB-Fc alone and in combination with 4h H2O2 treatment induced a significant increase in H3K9me3 (Welch's ANOVA, p=0.033, n=9; Dunnett's post hoc test,*p=0.033 control vs TrkB-Fc + 4h H2O2, *p=0.037 control vs TrkB-Fc) (Fig. 2.1A). Our findings show that inhibiting BDNF signaling significantly elevates H3K9me3 in mature (12 DIV) hippocampal neurons.

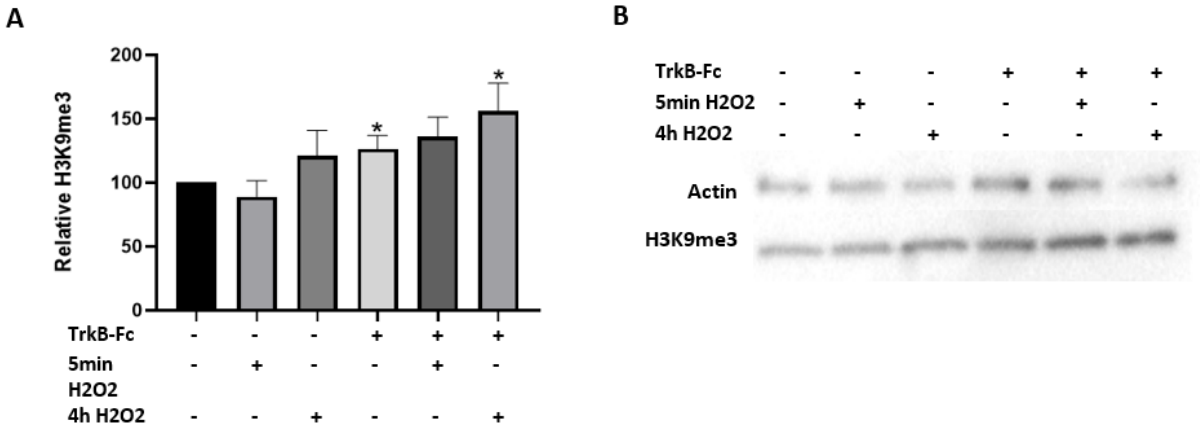

Figure 2.1. TrkB-Fc and 4h H2O2 Significantly Elevate H3K9me3 in Mature (12 DIV) Neuron Cultures. A, H3K9me3 western blot analysis of 12 DIV hippocampal neuron cultures threated with 200uM H2O2 for 5 minutes 8h before harvest or 4h, 1ug/mL TrkB-Fc for 24h. Welch's ANOVA, p=0.033, n=9 for control, TrkB-Fc, 4h H2O2 and TrkB-Fc+4h H2O2; n=4 for 5min H2O2 and TrkB-Fc + 5min H2O2 . Dunnett's T3 multiple comparisons test,*p=0.033 control vs TrkB-Fc + 4h H2O2, *p=0.037 control vs TrkB-Fc. B, Representative western blot images for H3K9me3 and actin (loading control).

To determine if stress induced changes in H3K9me3 were age dependent, we examined the effects of inhibiting BDNF signaling and oxidative stress across three culture ages; 6 DIV (young), 12 DIV (mature) and 21 DIV (aged). As 5 minutes of H2O2 treatment did not change H3K9me3 in 12 DIV neurons (Fig. 2.1A), we did not use this treatment condition in later experiments. We further examined if antioxidant supplementation could prevent an elevation in H3K9me3 when inhibition of BDNF signaling was combined with oxidative stress (H2O2). Antioxidant treated cultures were fed with B27 growth media as opposed to standard treatment media (B27 without antioxidants) 24 hours before harvesting. Neuron survival was not affected by TrkB-Fc, H2O2 or antioxidants at any culture age (Fig. 2.2)

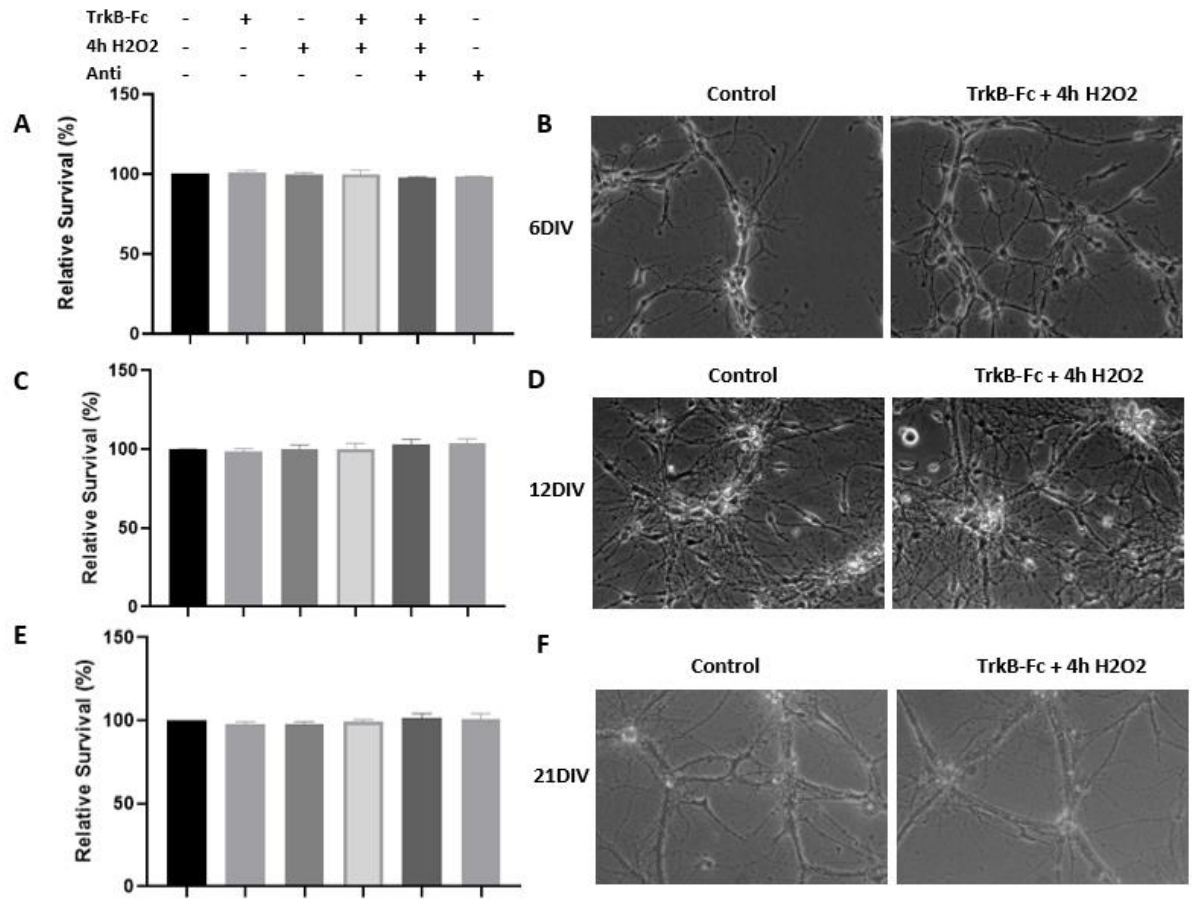

Figure 2.2. TrkB-Fc and H2O2 Do Not Affect Survival in Hippocampal Neuron Cultures. A, LDH survival assay of 6 DIV hippocampal neuron cultures shows no significant change in survival with TrkB-Fc, H2O2 and antioxidants. One way ANOVA, p=0.36, n=4. B, Representative images of 6 DIV negative control cultures and cultures treated with TrkB-Fc + 4h H2O2. C, LDH survival assay of 12 DIV hippocampal neuron cultures shows no significant change in survival with TrkB-Fc, H2O2, or antioxidants. One way ANOVA, *p=0.039, n=4. D, Representative images of 12 DIV negative control cultures and cultures treated with TrkB-Fc and 4h H2O2. E, LDH survival assay of 21 DIV hippocampal neurons treated with TrkB-Fc, H2O2 and antioxidants. One way ANOVA, p=0.714, n=4. F, Representative images of 21 DIV negative control cultures and cultures treated with TrkB-Fc and 4h H2O2.

TrkB-Fc treatment significantly increased H3K9me3 in 21 DIV neuron cultures and 12 DIV neuron cultures compared to 6 DIV cultures (Fig. 2.3A, Welch's ANOVA, *p=0.018 6 DIV vs 21 DIV, *p=0.048 6 DIV vs 12 DIV). TrkB-Fc in combination with 4h H2O2 increased H3K9me3 in 12 DIV cultures relative to 6 DIV cultures (Fig. 2.3C, Welch's ANOVA, *p=0.032 6 DIV vs 12 DIV), while H3K9me3 levels in 21 DIV cultures failed to reach statistical

significance (p=0.095). Consistent with our finding that H2O2 does not affect H3K9me3 in 12 DIV neurons (Fig. 2.1A), 4h of H2O2 treatment did not affect H3K9me3 levels at any culture age (Fig. 2.3B, Welch's ANOVA, *p=0.12). These findings suggest that while inhibiting BDNF signaling can induce H3K9me3 in aged hippocampal neurons (Fig. 2.3A), additional oxidative stress may prevent regulation of H3K9me3 by BDNF (Fig. 2.3C). The mechanism of H3K9me3 regulation is thus highly age dependent, and 12 DIV neurons display the greatest elevation in H3K9me3 when treated with exogenous stressors.

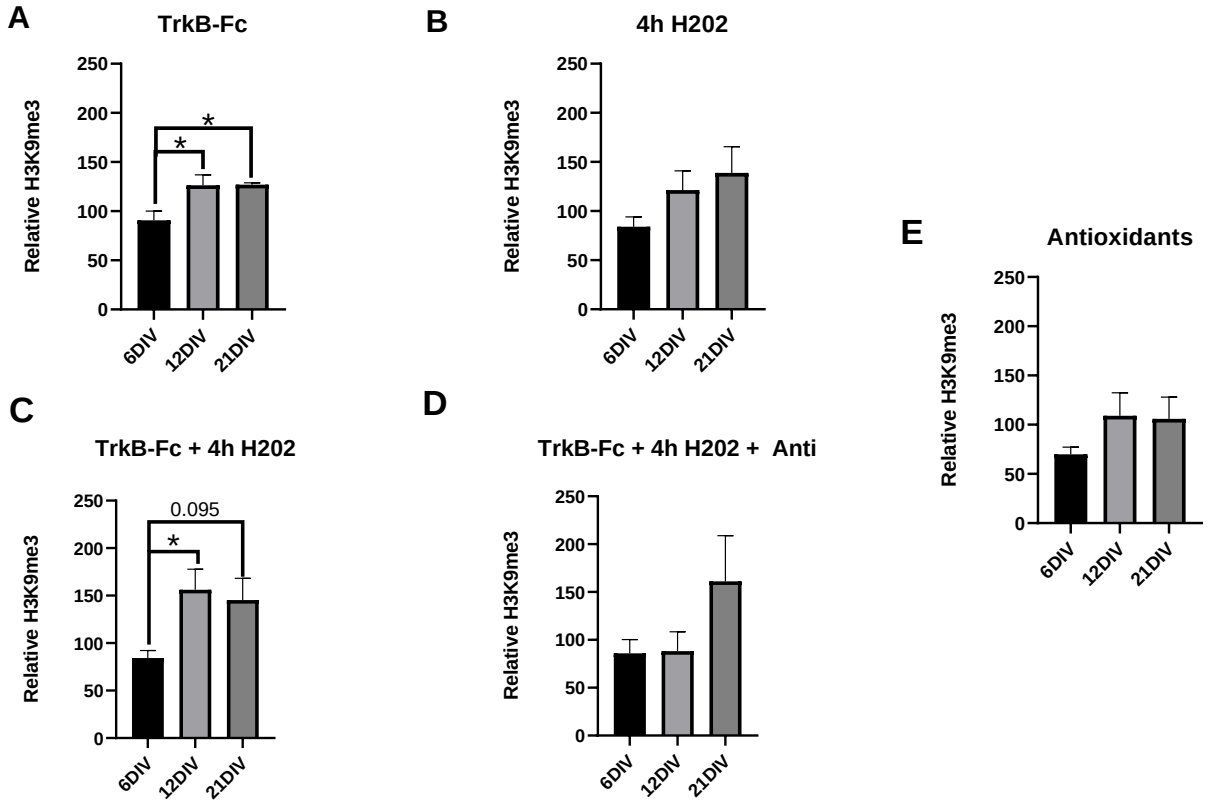

Figure 2.3. TrkB-Fc Significantly Elevates H3K9me3 in Mature and Aged Hippocampal Neuron Cultures. A, H3K9me3 western blot analysis of hippocampal neuron cultures treated with 1ug/mL TrkB=Fc for 24h. Welch's ANOVA, *p=0.015; n=7 for 6 DIV, n=9 for 12 DIV, n=5 for 21 DIV. Dunnet's T3 multiple comparisons test, *p=0.018 6 DIV vs. 21 DIV, *p=0.048 6 DIV vs. 12 DIV. B, H3K9me3 western blot analysis of hippocampal neuron cultures treated with 200uM H2O2 for 4h. Welch's ANOVA, p=0.12; n=7 for 6 DIV, n=9 for 12 DIV, n=5 for 21 DIV. C, H3K9me3 western blot analysis of hippocampal neuron cultures treated with 1ug/ml TrkB-Fc for 24h and 200uM H2O2 for 4h. Welch's ANOVA, p=*0.018, n=7 for 6 DIV, n=9 for 12 DIV, n=5 for 21 DIV. Dunnett's T3 multiple comparisons test, *p=0.022 6 DIV vs. 12 DIV, p=0.095 6DIV vs. 21 DIV. D, H3K9me3 western blot analysis of hippocampal neurons treated with 1ug/mL TrkB-Fc for 24h, 200uM H2O2 for 4h and antioxidants for 24h. Welch's ANOVA, p=0.44; n=7 for 6 div, n=4 for 12 DIV and n=3 for 21 DIV. E, H3K9me3 western blot analysis of hippocampal neurons treated with antioxidants for 24h. Welch's ANOVA, p=0.27; n=7 for 6 DIV, n=4 for 12 DIV and n=3 for 21 DIV.

We examined if antioxidant treatment could change H3K9me3 levels in hippocampal neurons treated with TrkB-Fc and H2O2. We hypothesized that antioxidant treatment would ameliorate the oxidative stress produced by H2O2, elevating H3K9me3 in aged neurons in a manner similar to TrkB-Fc alone (Fig. 2.3A). Antioxidant treatment did not significantly change H3K9me3 levels in 21 DIV neurons, but it did prevent the elevation in H3K9me3 observed in 12 DIV neurons with TrkB-Fc and H2O2 (Fig. 2.3C, D). This suggests that antioxidants reduced oxidative stress produced by TrkB-Fc as well as H2O2, and that this oxidative stress is involved in the regulation of H3K9me3. While antioxidant treatment had no effect on H3K9me3 in unstressed hippocampal neurons (Fig. 2.3E), it effectively decreased the H3K9me3 produced by TrkB-Fc and H2O2 (Fig. 2.3D).

Studies in non-neuronal tissues indicate that SIRT1 mediates oxidative stress-induced epigenetic changes (Vaquero et al., 2007; Bosque-Presegué et al., 2011; Yang et al., 2017). We measured SIRT1 levels in our stressed cultures to determine if SIRT1 correlated with H3K9me3 elevation. We found no correlation between SIRT1 and H3K9me3 elevation, although SIRT1

was significantly increased in 6 DIV neurons compared to 12 DIV neurons treated with H2O2 (Fig. 2.4B, Welch's ANOVA, *p=0.037). Similarly, there was no change in SIRT1 levels in antioxidant treated cultures (Fig. 2.5). While hydrogen peroxide does elevate SIRT1 in 6 DIV neurons, SIRT1 upregulation does not lead to H3K9 trimethylation in neurotrophically deprived cultures.

Representative western blot images of H3K9me3 (normalized to actin) and SIRT1 (normalized to H3) are displayed in Figure 2.6.

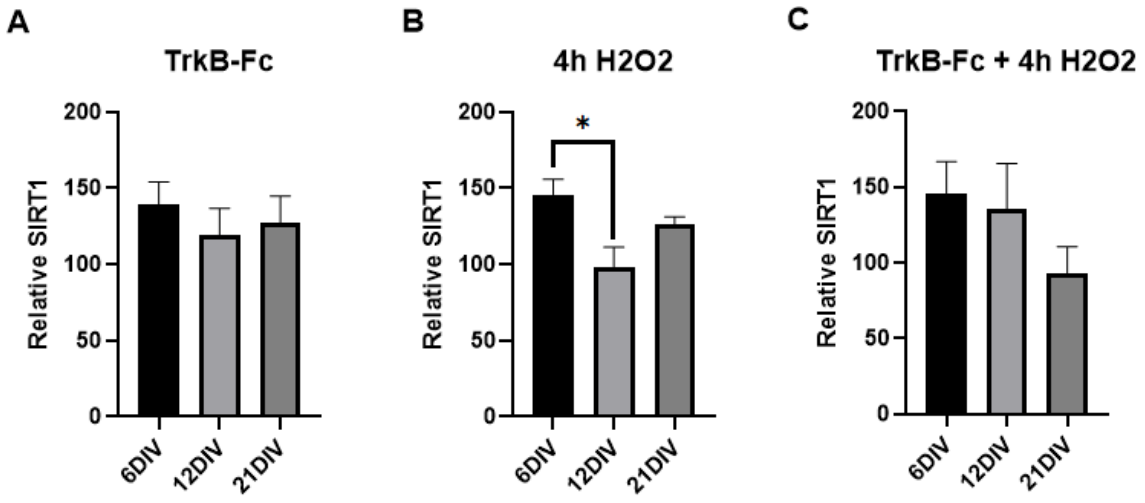

Figure 2.4. SIRT1 is Elevated in Young Neurons Treated with Hydrogen Peroxide. A, SIRT1 western blot analysis of hippocampal neurons treated with 1ug/ml TrkB-Fc for 24 h. Welch's ANOVA, p=0.72. B, SIRT1 western blot analysis of hippocampal neurons treated with 200uM H2O2 for 4h. Welch's ANOVA *p=0.011; Dunnett's T3 multiple comparisons test, *p=0.037 6DIV vs. 12 DIV. C, SIRT1 western blot analysis of hippocampal neurons treated with 1ug/ml TrkB-Fc for 24h and 200uM H2O2 for 4h. Welch's ANOVA, p=0.21. N=5 for 6 DIV, n=9 for 12 DIV, n=4 for 21 DIV.

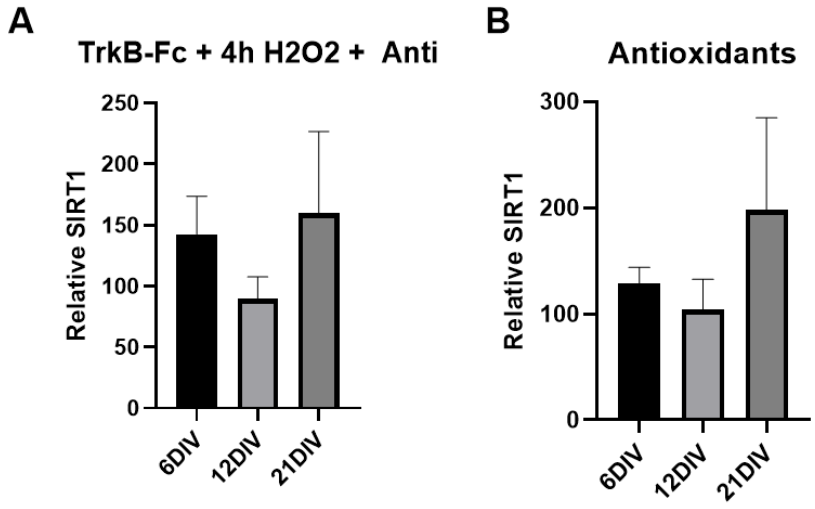

Figure 2.5. Antioxidant Treatment Does Not Change SIRT1 Levels. A, SIRT1 western blot analysis of hippocampal neurons treated with 1ug/ml TrkB-Fc for 24h, 200uM H2O2 for 4h and antioxidants for 24h. Welch's ANOVA, p=0.38. B, SIRT1 western blot analysis of hippocampal neurons treated with antioxidants for 24h. Welch's ANOVA, p=0.55. N=7 for 6 div, n=4 for 12 DIV and n=3 for 21 DIV

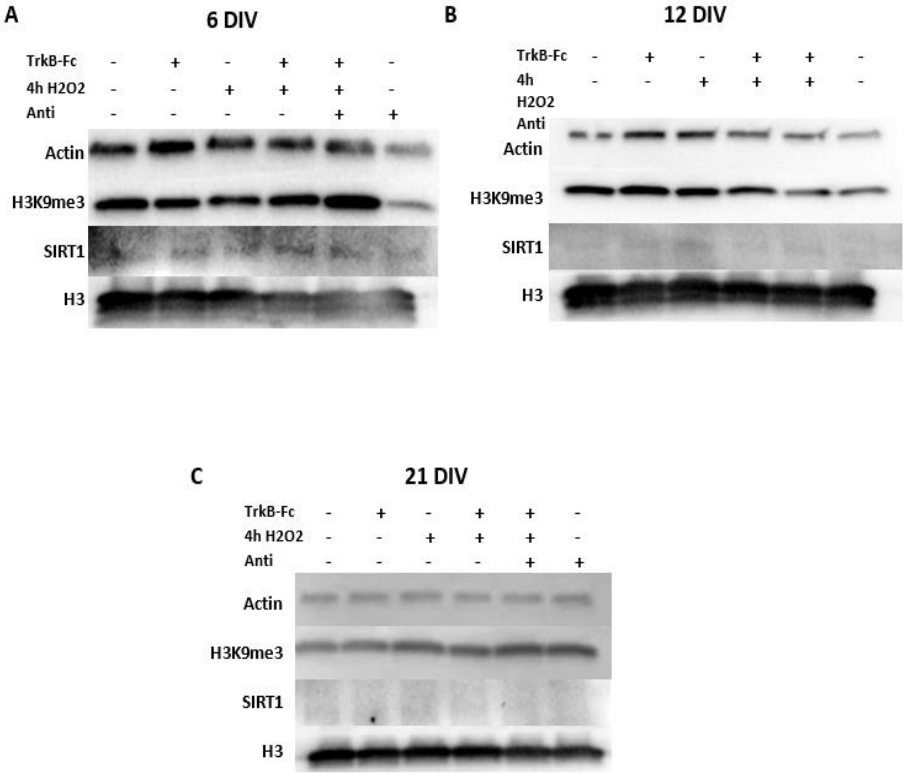

Figure 2.6. Representative Western Blot Images for 6, 12 and 21 DIV Cultures. Representative western blot images for 6 DIV(A), 12 DIV (B) and 21 DIV neurons (C) stained for SIRT1, actin, and H3.

Discussion

In this study we found that inhibiting BDNF signaling elevates H3K9me3 in mature and aged, but not young, hippocampal neurons. While H2O2 alone did not affect H3K9me3 levels at any culture age, antioxidant treatment prevented H3K9me3 elevation in mature neurons treated with TrkB-Fc and H2O2. Surprisingly, SIRT1 is not correlated with H3K9 trimethylation caused by inhibiting BDNF signaling. Our findings suggest that neurotrophic deprivation elevates H3K9me3 in an age and oxidative stress dependent manner.

Inhibition of BDNF signaling (TrkB-Fc) elevated H3K9me3 in mature neurons by itself and in combination with H2O2 (Fig. 2A). We hypothesized that hippocampal neurons would become more susceptible to stress induced epigenetic changes with age due to the accumulation of oxidative damage (Wang and Michaelis, 2010). We thus expected that H3K9me3 would not be elevated in young neurons by TrkB-Fc or H2O2, while aged neurons would show a similar or greater increase in H3K9me3 with TrkB-Fc and H2O2. As predicted, stressors did not increase H3K9me3 in young neurons, and TrkB-Fc increased H3K9me3 in aged neurons (Fig. 6A). However, the combination of TrkB-Fc and H2O2 did not significantly increase H3K9me3 in aged neurons, although there was an increasing trend (Fig. 6C). This suggests that high levels of oxidative stress in the absence of BDNF may activate a different signaling pathway in aged neurons. Regulation of H3K9me3 may be impaired in aged neurons by high levels of oxidative stress, such that excess stress does not stimulate H3K9 trimethylation. A recent study found that although hippocampal H3K9me3 was elevated in both adult and aged mice, the greatest elevation was in adult mice (Kushwaha and Thakur, 2020). This is consistent with our previous research since we did not examine the adult mouse population and found that H3K9me3 was

elevated in aged mice in comparison to young mice (Snigdha et al., 2016). While neurons are more susceptible to epigenetic changes as they age, the regulation of H3K9me3 may be impaired in aged neurons due to high levels of oxidative stress. Further research is needed to determine how the mechanism of H3K9me3 regulation changes with neuronal age.

Although antioxidants had no effect on H3K9me3 in unstressed cultures, antioxidant treatment prevented H3K9me3 elevation in mature neurons treated with TrkB-Fc and H2O2 (Fig. 3D). This shows that antioxidants by themselves are not sufficient to reduce H3K9me3. Indeed, H3K9me3 appears to be relatively stable due to its critical role in the maintenance of heterochromatin stability and double strand break repair (Ayrapetov et al., 2014). While antioxidants cannot reduce H3K9me3 from baseline levels, they can prevent an elevation in H3K9me3 in stressed cultures where BDNF is inhibited. Impairment of BDNF signaling reduces antioxidants and antioxidant supplementation prevents H3K9me3 from increasing, demonstrating that the oxidative stress caused by inhibiting BDNF signaling elevates H3K9me3 (Mattson et al., 1995; Chen et al., 2017). Oxidative stress is responsible for H3K9me3 elevation in neurons as in other tissue types, but a critical source of protective antioxidants (BDNF) must be inhibited to reach a ROS threshold that can increase H3K9me3.

Our findings suggest that inhibiting BDNF signaling upregulates H3K9me3 via an oxidative stress dependent mechanism. Various studies have shown that oxidative stress (specifically H2O2) elevates SIRT1 and in turn H3K9me3 in non-neuronal cells (Vaquero et al., 2007; Bosque-Presegué et al., 2011; Yang et al., 2017). However, we found no positive correlation between SIRT1 and H3K9me3 levels in hippocampal neuron cultures (Fig. 4). While SUV39H1 and H3K9me3 can repress SIRT1 expression, there was also no negative correlation indicative of a negative feedback mechanism (Yang et al., 2017). SIRT1 was only significantly

elevated in young neurons treated with H_2O_2, suggesting that SIRT1 transcription may be impaired in older neurons. Previous studies examining SIRT1 have all used H_2O_2 as an oxidative stressor, and have not explored the effects of alternate stressors such as BDNF signaling inhibition. Although SIRT1 may still play a role in SUV39H1 activation, it is not the primary driver of H3K9me3 upregulation in neurotrophically deprived neurons.

In summary, we found that inhibiting BDNF signaling via TrkB-Fc upregulates H3K9me3 in an age dependent manner, with the greatest elevation occurring in mature hippocampal neurons. This elevation is at least partially mediated by oxidative stress, as antioxidant treatment prevented H3K9me3 from increasing in TrkB-Fc and H_2O_2 treated cultures. Although SIRT1 is elevated by H_2O_2, its expression does not correlate with H3K9me3 upregulation, suggesting that a SIRT1 independent pathway is the primary regulator of hippocampal H3K9me3. Our findings give insight into the epigenetic effects of BDNF, and suggest that interventions to increase BDNF, such as exercise, may effectively reduce hippocampal H3K9me3.

Chapter 3: Exercise Reduces H3K9me3 and Regulates BDNF and GABRA2 Expression in an Age Dependent Manner

Rationale

Exercise has been shown to improve cognition in the aged brain across a range of clinical and animal studies (Snigdha et al., 2014; Kirk-Sanchez et al., 2014; van Praag et al., 2005). Underlying these improvements are changes to the expression of genes related to neuronal plasticity. Our lab was the first to show a dose-dependent effect of exercise on hippocampal brain derived neurotrophic factor (BDNF), a neurotrophic factor that is critical for memory, neuron survival and brain plasticity (Berchtold et al., 2002; Cotman and Berchtold, 2002; Neeper et al., 1995). Recent studies suggest that exercise changes gene expression via epigenetic mechanisms, which are also critical contributors to age-related cognitive decline (Fernandes et al., 2017; Lardenoije et al., 2015). A dynamic repressive mark is trimethylated histone 3, lysine 9 (H3K9me3), which is associated with age-related gene repression and regulates genes critical for cognitive function (Sedivy et al, 2008; Villeneuve et al, 2010; Snigdha et al., 2016).

Our lab has focused on elucidating the role of H3K9me3 in cognitive function. We first determined that H3K9me3 is elevated in the hippocampi of aged mice. To investigate if H3K9me3 is involved in cognitive decline, we inhibited the histone methyltransferase (SUV39H1) that catalyzes H3K9 trimethylation. Treatment with a selective SUV39H1 inhibitor (ETP69) improved the performance of aged mice in object location memory (OLM) and fear conditioning tasks. ETP69 also increased hippocampal spine density as measured by Golgi staining and flow cytometry. Importantly, ETP69 treatment before or after acquisition of an OLM task increased total BDNF levels and decreased H3K9me3 at the BDNF 1 promoter

(Snigdha et al., 2016). These findings demonstrate that H3K9me3 leads to memory decline, reduces synaptic plasticity, and represses BDNF expression in aged mice. While this study examined the relationship between H3K9me3 and memory, the effects of exercise on H3K9me3 gene repression have not been investigated. Further research is needed to determine if exercise-induced increases in neuronal gene expression correlate with a reduction in H3K9me3 at promoter regions.

Recent studies have begun to examine how exercise affects the regulation of neuronal plasticity genes such as BDNF, which enhances learning and memory (Cotman and Berchtold, 2002). The BDNF gene consists of 9 5' non-coding exons and one 3' coding exon (exon IX), all of which are differentially expressed in the hippocampus (Aid et al., 2007). We have shown that the degree of exercise is proportional to hippocampal BDNF expression in young mice and rats (Neeper et al., 1995; Adlard et al., 2004). However, more than 1 week of exercise does not significantly increase BDNF in mature (15 month old) and aged (24 month old) mice, suggesting that BDNF expression is impaired in older mice (Adlard et al., 2005). Reduced GABA signaling may play a role in the age-associated decline of BDNF. GABA B receptors phosphorylate α-CAMKII, involved in BDNF release, and BDNF secretion triggered by GABA B receptors increases levels of GABA A receptors (Kolarow et al., 2007; Fiorentino et al., 2009; Porcher et al., 2018). GABA A receptor activation also increases BDNF secretion via an ERK dependent pathway (Brady et al., 2018). GABA receptors are thus involved in the regulation of BDNF signaling, and GABA signaling is also reduced in the hippocampus with age (McQuail et al., 2015; Stanley et al., 2004). We recently found that exercise correlates with the expression of 8 GABA genes in the aged hippocampus, including GABA B receptor 1 (GABBR1) and the alpha

2 subunit of the GABA A receptor (GABRA2) (Berchtold et al., 2019). H3K9me3 may play a role in the repression of GABA receptors as well as BDNF. A study of postmortem cortical samples showed an increase in H3K9me3 at the promoters of BDNF, GABBR1 and GABRA2 in Alzheimer's diseased brains (Lee et al., 2020). The parallels between GABA receptor and BDNF expression with age and exercise suggests that GABA signaling may be involved in exercise-induced BDNF regulation.

In this study, we examined how exercise, SUV39H1 inhibition and age affect H3K9me3 promoter repression and expression of BDNF and GABA receptors. We first separated aged and young mice into sedentary, exercised and ETP69 treatment groups. We then measured levels of H3K9me3 at BDNF exon I, IV and VI promoters, and at the promoters of GABBR1 and GABRA2. Lastly, we quantified transcript mRNA to determine the correlation between promoter H3K9me3 levels and total gene expression. Exercise and ETP69 decreased H3K9me3 at BDNF promoter VI in aged mice, corresponding with an increase in BDNF VI expression with ETP69. In young mice, exercise increased BDNF I expression, suggesting an age-dependent change in BDNF regulation. Exercise increased GABRA2 expression in aged mice while both exercise and ETP69 reduced GABRA2 expression in young mice. Overall, H3K9me3 repression at BDNF and GABA receptor promoters decreased with age. Our findings suggest that exercise and SUV39H1 inhibition differentially modulate BDNF and GABRA2 expression in an age dependent manner.

Methods

Animals

All experiments were conducted in accordance with the National Institutes of Health guidelines for animal care and use, and were approved by the Institutional Animal Care and Use Committee of the University of California at Irvine. Male 18 month old and 3 month old C57Bl/6J mice (n=21 for each age; Jackson Laboratory) were individually housed with food and water ad libitum, and allowed 1 wk acclimation to the vivarium prior to experiments. Lights were maintained on a 12 h light–dark cycle. Mice were sacrificed by carbon dioxide gas and cervical dislocation, and brains were rapidly frozen on isopentane cooled in liquid nitrogen. 1mm punches of the dorsal hippocampus were collected from 500µm slices.

Exercise and ETP69 Treatment

All mice were single housed in either standard cages (sedentary and ETP69 injected mice) or cages with a running wheel (exercised mice). Exercise cages consisted of 24 × 35 × 20 cm clear plastic, containing a running wheel 40 cm in circumference, 12.7 cm diameter (Lafayette). Aged exercised mice had access to a running wheel for 6 weeks and young exercised mice had access to a running wheel for 4 weeks, as 3 weeks was previously shown to improve learning and memory in young mice (Intlekofer et al., 2013). Mice were i.p. injected with 10mg/kg ETP69 dissolved in 50% weight/volume β cyclodextrin 24 hours prior to sacrifice.

Chromatin Immunoprecipitation

ChIP was performed as described previously based on the protocol from the Millipore Magna ChIP kit (Malvaez et al., 2011; Rogge et al., 2013). Tissue was cross-linked with 1% formaldehyde (Sigma), lysed and sonicated, and chromatin was immunoprecipitated overnight with 2uL of H3K9me3 (ab8898) or 5 µL of anti-mouse IgG (negative control, Millipore). The

immunoprecipitate was collected using magnetic protein A beads (Millipore). After washing, chromatin was eluted from the beads and reverse cross-linked in the presence of proteinase K before column purification of DNA. BDNF, GABBR1 and GABRA2 promoter enrichment in ChIP samples was measured by quantitative real-time PCR using the Roche 480 LightCycler and SYBR green. Primer sequences for BDNF promoters were taken from Intlekofer et al. (2013) and GABA receptor primers were designed by the PrimerBLAST program (Supplementary Table 1). Five μL of input, anti-H3K9me3, or anti-mouse IgG immunoprecipitate from mice in each condition were examined in duplicate. To normalize ChIP-qPCR data, we used the percent input method. The input sample was adjusted to 100% and both the IP and IgG samples were calculated as a percent of this input using the formula: 100*AE^(adjusted input –Ct (IP)). An in-plate standard curve determined amplification efficiency (AE). Primers are listed in Table 1.

Quantitative qRT-PCR

qRT-PCR was performed as previously described (Rogge et al., 2013). RNA was isolated from punches using an RNA Easy Mini Kit (Qiagen) and cDNA was created using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher). Primer and probes sequences were designed by Integrated DNA Technologies and are listed in Supplementary Table 2. All probes were conjugated to the dye FAM except for GAPDH, which was conjugated to the dye HEX. Samples were measured in duplicate using the Roche 480 LightCycler and Gene Expression Master Mix (Integrated DNA Technologies). All values were normalized to GAPDH expression levels. Analysis and statistics were performed based on the Pfaffl method (Pfaffl, 2001). Primers and probes are listed in Table 2.

Table 1. qPCR Primer Sequences for ChIP

Promoter and Refseq Accession Number	Primer Sequence 5'-3'	Corresponding to Position	Amplicon (bp)
BDNF 1 NT_039207	TGATCATCACTCACGACCACG	50555972-50555992	134
	CAGCCTCTCTGAGCCAGTTAC	50556105-50556085	
BDNF IV NT_039207	TGCGCGGAATTCTGATTCTGG	50573782-50573802	107
	GTCCACGAGAGGGCTCCACG	50573888-50573871	
BDNF VI NT_039207	ACTCACACTCGCTTCCTCCT	50574691-50574710	171
	GCACTGGCTTCTCTCCATTT	50574861-50574842	
GABBR1 NC_000083.7	CAGGAGAGCGAAAGGGGAAG	37383295-37383314	299
	AACAGCGCCAAGAGAATGGA	37383593-37383574	
GABRA2 NC_000071.7	TTCTGGGGAGGGACATTGGA	71252739-71252758	141
	TGCTCATTCCCCTCTGCTTC	71252879-71252860	

Table 2. qPCR Primer and Probe Sequences for mRNA

Gene Name and Refseq Accession Number	Primer and Probe Sequence 5'-3'	Corresponding to Position	Amplicon (bp)
BDNF I NM_007540.4	GACACATTACCTTCCTGCATCT	564-586	108
	GGATGGTCATCACTCTTCTCAC	650-629	
	Probe: ACAGCAAAGCCACAATGTTCCACC	624-648	
BDNF IV NM_001048141.1	TCCCCTTCTCTTCAGTTAAAAGG	177-197	73
	TTGCTGCAGAACAGGACTACA	249-227	
	Probe: TATCGGCCACCAAAGACTCGCC	198-219	
BDNF VI NM_001048142.1	CTGAGCGTGTGTGACAGTATTA	786-808	112
	CTTTGGATACCGGGACTTTCTC	876-898	
	Probe:TTTATCTGCCGCTGTGACCCACTC	810-834	
GABBR1 NM_019439.3	GCTCTTGGGCTTAGGCTTTA	2325-2345	109
	GGTCTTCCTCCATTCCTTCTTC	2412-2434	
	Probe:TTGTGAAGACTGTGTGGACCCACC	2378-2402	
GABRA2 NM_008066.4	AGAGAGAGCTCAGAGGATAACA	4107-4129	115
	CAGAGCTCCTACAGCACATATC	4200-4222	
	Probe:AGAGGGCGTGATTTCAAGTTCCCA	4146-4170	
GAPDH NM_008084.3	AATGGTGAAGGTCGGTGTG	240-258	150
	GTGGAGTCATACTGGAACATGTAG	389-366	
	Probe:TGCAAATGGCAGCCCTGGTG	307-288	

Statistical Analysis

Results were analyzed using one way ANOVA, Welch's ANOVA, or two way ANOVA, followed by post hoc analysis using Prism software. In all cases, $p \leq 0.05$ was considered significant. Excel was used for percent input analysis of ChIP data and Pfaffl analysis of qRT-qPCR data.

Results

ETP69 and Exercise Reduce H3K9me3 at BDNF Promoter VI in Aged Mice

We first investigated if ETP69 and exercise affected H3K9 trimethylation levels at BDNF promoter regions. We examined BDNF I, IV and VI as these regulatory exons are highly expressed in the hippocampus (Aid et al., 2007). Our lab previously found that ETP69 treatment before or after the acquisition phase of an OLM task improved performance in aged mice and reduced H3K9me3 at the BDNF I promoter (Snigdha et al., 2016). Building on this study, we investigated if ETP69's effect on BDNF repression was dependent on neuronal stimulation or a behavioral task. Treating sedentary, non-behaving aged mice with ETP69 reduced H3K9me3 at BDNF promoter VI instead of promoter I (Fig. 3.1C, One way ANOVA **p=0.0036; Dunnett's post hoc test, *p=0.011 ETP69 vs. sedentary). This suggests that the neuronal activity caused by an OLM task changes the regulation of BDNF promoter regions.

We also explored the effect of physical activity on H3K9 trimethylation at BDNF promoter regions, as BDNF production is increased in young but not aged mice after long term exercise (Neeper et al., 1995; Adlard et al., 2004; Adlard et al., 2005). Our lab previously found that exercise increases H4K8ac at BDNF I and IV promoters in young mice, suggesting that exercise may also influence H3K9me3 levels at these promoters (Intlekofer et al., 2013). Exercise reduced H3K9me3 levels at the BDNF VI transcript in aged mice (Fig. 3.1C;

**p=0.0021 exercise vs. sedentary), displaying a specific and parallel effect of exercise and ETP69 on BDNF promoters. Surprisingly, neither ETP69 nor exercise changed H3K9me3 at BDNF promoter transcripts in young mice (Fig. 3.2), suggesting that the stability of promoter repression decreases with age.

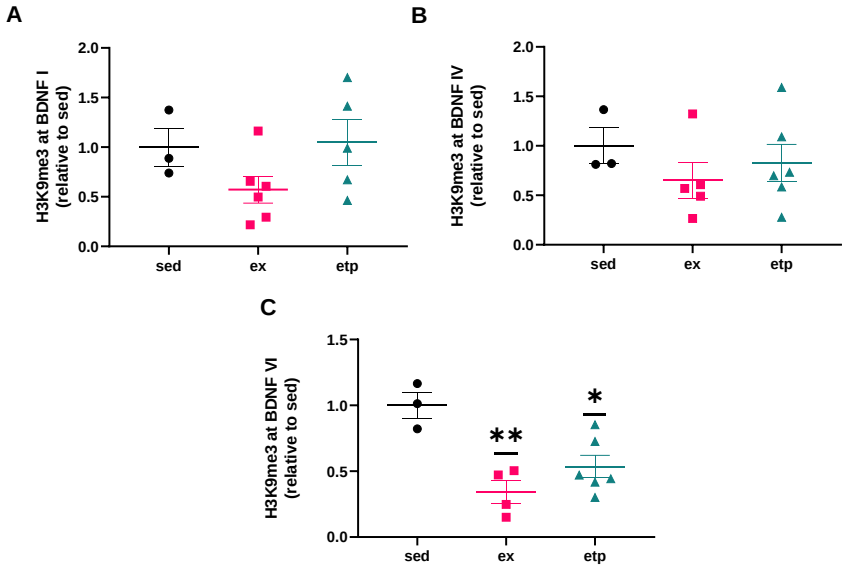

Figure 3.1. Exercise and ETP69 Reduce H3K9me3 at BDNF Promoter VI in Aged Mice. Exercise and ETP69 treatment significantly reduce H3K9me3 expression at BDNF promoter VI (C), but not at promoter 1(A, One way ANOVA, p=0.16) or IV(B, One way ANOVA, p=0.17). C. One way ANOVA **p=0.0036; Dunnett's post hoc test (**p=0.0021 exercise vs. sed. *p=0.011 ETP69 vs. sedentary).

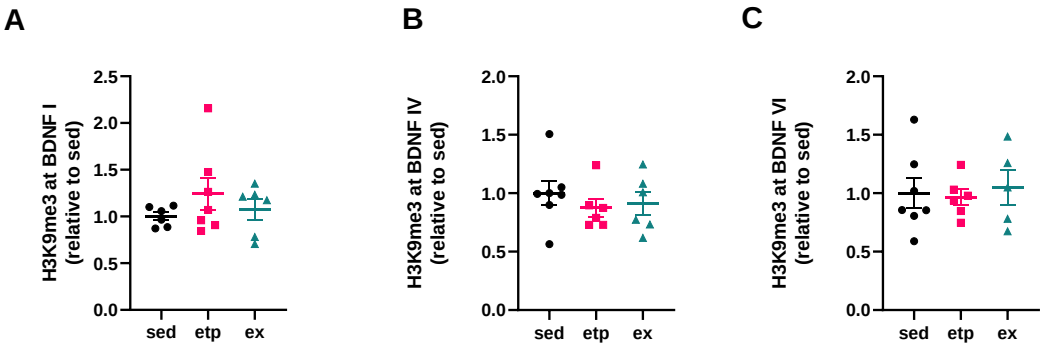

Figure 3.2. Exercise and ETP69 Do Not Affect H3K9me3 at BDNF Promoters in Young Mice. A, H3K9me3 at BDNF I promoter, one way ANOVA, p=0.41. B, H3K9me3 at BDNF IV promoter, one way ANOVA, p=0.6. C, H3K9me3 at BDNF VI promoter, one way ANOVA, p=0.89.

Exercise and ETP69 Differentially Regulate BDNF Exon Expression in Young and Aged Mice

To determine if a reduction in H3K9me3 is associated with increased BDNF expression, we measured BDNF exon mRNA levels in aged and young mice. Reduced H3K9me3 at BDNF promoter VI correlated with an increase in BDNF VI expression in aged mice treated with ETP69, but not aged exercised mice (Fig. 3.3C; Welch's ANOVA **p=0.007; Dunnett's post hoc test, etp vs. sed *p=0.012). This finding is supported by previous work from our lab showing that exercise did not increase expression of BDNF exons I, IV or VI in the aged mouse hippocampus (Adlard et al., 2005). These results suggest that BDNF VI levels are regulated by a direct reduction in H3K9me3 repression in aged mice. In contrast, exercise activates multiple signaling pathways that modulate BDNF expression, and its reduction of H3K9me3 does not drive an increase in exon VI expression.

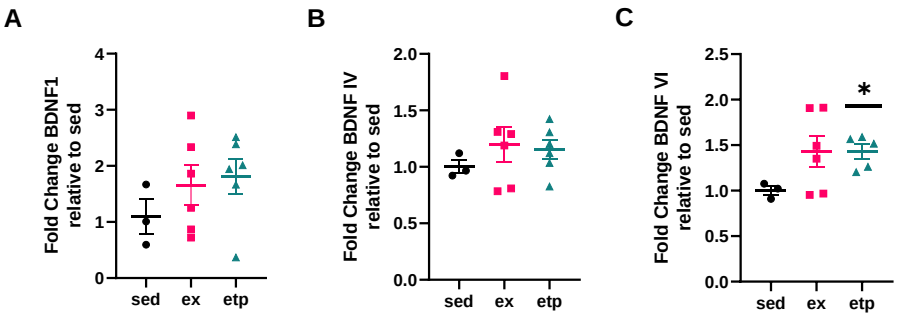

Figure 3.3. ETP69 Increases BDNF VI Expression in Aged Mice. C, Welch's ANOVA, **p=0.007; Dunnett's post hoc test, etp vs. sed *p=0.012. Exercise and ETP69 did not affect levels of BDNF I (A) or BDNF IV (B) in aged mice.

Neither exercise nor ETP69 affected H3K9me3 binding to BDNF promoters in young mice (Fig. 3.2), yet exercise significantly increased BDNF I expression (Fig. 3.4A; One way ANOVA, *p=0.012; Tukey's post hoc test, *p=0.018 sed vs. ex, *p=0.03 ex vs. etp). Exercise significantly elevated BDNF IV levels in comparison to ETP69 treatment, but failed to reach significance relative to sedentary controls (Fig. 3.4B; Welch's ANOVA, *p=0.049; Dunnett's

post hoc test, *p=0.056 ex vs. etp). This is in accordance with previous findings that exercise induces BDNF I and IV expression but not BDNF VI expression in young mice after 3 weeks of exercise (Intlekofer et al., 2013). Exercise has a greater effect on BDNF exon expression in young mice than in aged mice, suggesting that BDNF induction by exercise is less prevalent with age.

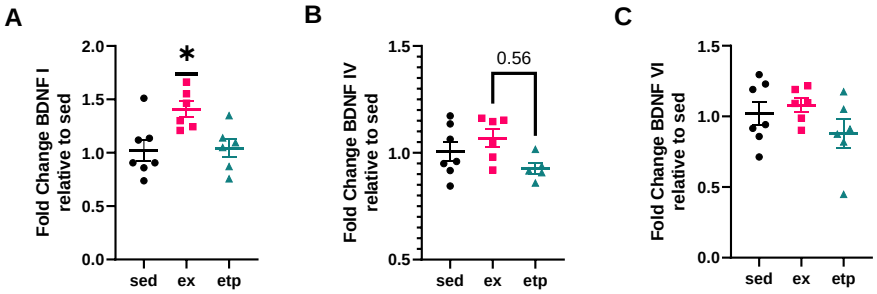

Figure 3.4. Exercise Increases BDNF I Expression in Young Mice. A, One way ANOVA, *p=0.012; Tukey's post hoc test, *p=0.018 sed vs. ex, *p=0.03 ex vs. etp. B, Welch's ANOVA, *p=0.049; Dunnett's post hoc test, *p=0.056 ex vs. etp. C, One way ANOVA, p=0.24.

Exercise and ETP69 Modulate GABRA2 Expression in an Age Dependent Manner

We next investigated how the repression and expression of GABA receptors correlate with BDNF, as GABA receptor activation increases BDNF signaling (Kolarow et al., 2007; Fiorentino et al., 2009; Brady et al., 2018; Porcher et al., 2018). We previously found that both BDNF and GABA receptor expression are regulated by exercise, suggesting that GABA receptors may be involved in regulating exercise-induced BDNF expression (Adlard et al., 20014; Berchtold et al., 2019). We first examined if H3K9me3 levels at the GABBR1 and GABRA2 promoters were changed by exercise and ETP69. Promoter repression was not significantly different from sedentary mice in either young or aged animals (Fig. 3.5, 3.6). These results suggest that GABA receptor promoters are resilient to epigenetic changes by exercise and global reductions in H3K9me3.

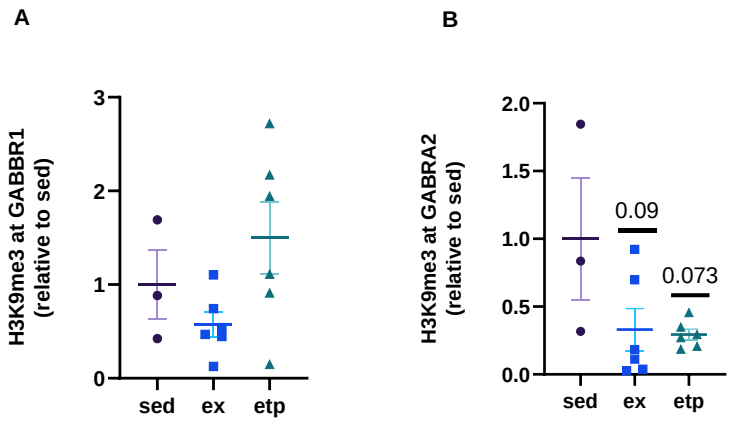

Figure 3.5. Exercise and ETP69 Do Not Change H3K9me3 at GABA Receptor Promoters in Aged Mice. A, H3K9me3 at GABBR1 promoter, One way ANOVA, p=0.11. B, H3K9me3 at GABRA2 promoter, One way ANOVA, p=0.066; Dunnett's post hoc test, p=0.09 sed vs. ex, p=0.073 sed vs. etp.

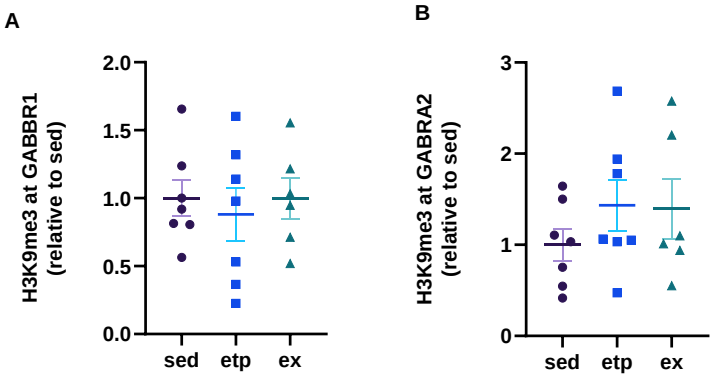

Figure 3.6. Exercise and ETP69 Do not Change H3K9me3 at GABA Receptor Promoters in Young Mice. A, H3K9me3 at GABBR1 promoter, one way ANOVA, p=0.89. B, H3K9me3 at GABRA2 promoter, one way ANOVA, p=0.44.

We next asked if exercise and ETP69 changed GABA receptor expression levels and found that the direction of change was highly dependent on mouse age. Exercise significantly increased GABRA2 expression in aged mice (Fig. 3.7B; One way ANOVA, *p=0.024; Tukey's post hoc test, ex vs. sed *p=0.052, ex vs. etp *p=0.044). In contrast, exercise and ETP69 reduced GABRA2 expression in young mice (Fig. 3.8B; One way ANOVA, **p=0.0012; Tukey's post hoc test, **p=0.001 sed vs. ex, *p=0.28 sed vs. etp). ETP69's effect on GABRA2 is surprising considering it does not reduce H3K9me3 at its promoter, suggesting that a global reduction in H3K9me3 activates a pathway that decreases inhibitory signaling. The exercise-induced increase in GABRA2 in aged mice is supported by our research in aged postmortem brains, suggesting that the need for inhibitory signaling increases in the aged brain (Berchtold et al., 2019). There was no treatment effect on GABBR1 expression, although there was a non-significant decrease

with ETP69 in young mice (Fig. 3.8A; One way ANOVA, p=0.13; Tukey's post hoc test, p=0.12 sed vs. etp). Therefore, exercise and ETP69 regulate GABRA2 expression independently of promoter H3K9me3 in an age-dependent manner.

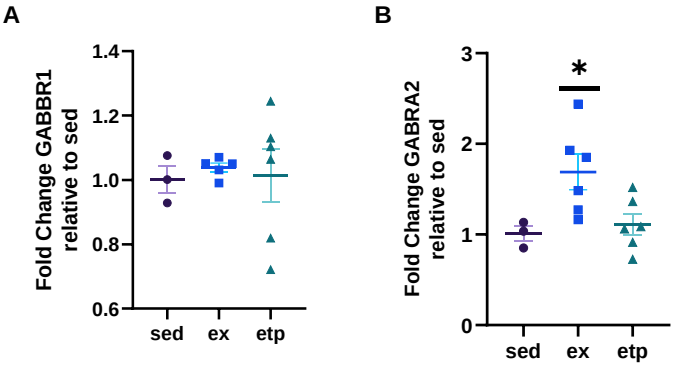

Figure 3.7. Exercise Increases GABRA2 Expression in Aged Mice. B, One way ANOVA, *p=0.024; Tukey's post hoc test, ex vs. sed *p=0.052, ex vs. etp *p=0.044. Exercise and ETP69 did not affect levels of GABBR1 (A) in aged mice (One way ANOVA, p=0.93).

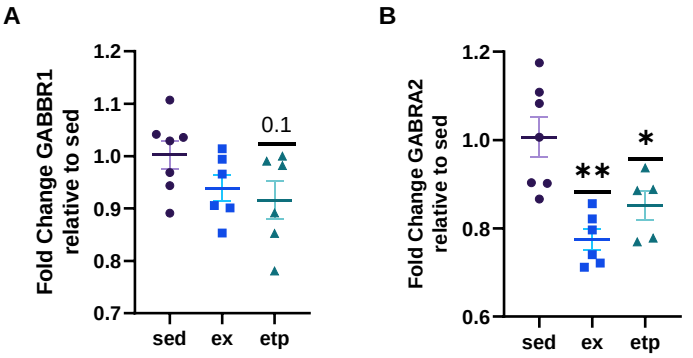

Figure 3.8. Exercise and ETP69 Decrease GABRA2 Expression in Young Mice. A, One way ANOVA, p=0.13; Tukey's post hoc test, p=0.12 sed vs. etp. B, One way ANOVA, **p=0.0012; Tukey's post hoc test, **p=0.001 sed vs. ex, *p=0.28 sed vs. etp.

H3K9me3 Repression of BDNF and GABA Receptors Decreases with Age

Our lab previously found that H3K9me3 is increased with age in the hippocampus of aged mice, leading us to ask if promoter bound H3K9me3 was also elevated with age in our study (Snigdha et al., 2016). We compared percent input levels from ChIP experiments measuring H3K9me3 binding in young and aged mice. Surprisingly, we found that H3K9me3 promoter binding was greater in young mice than in aged mice at BDNF promoters I, IV and VI, as well as at the GABRA2 promoter (Fig. 3.9A, B, C, E). There was no effect of age on H3K9me3 binding at the GABBR1 promoter (Fig. 3.9D). This finding suggests that H3K9me3

promoter repression is impaired with increasing age, which is associated with reduced chromatin stability and compromised transcriptional regulation.

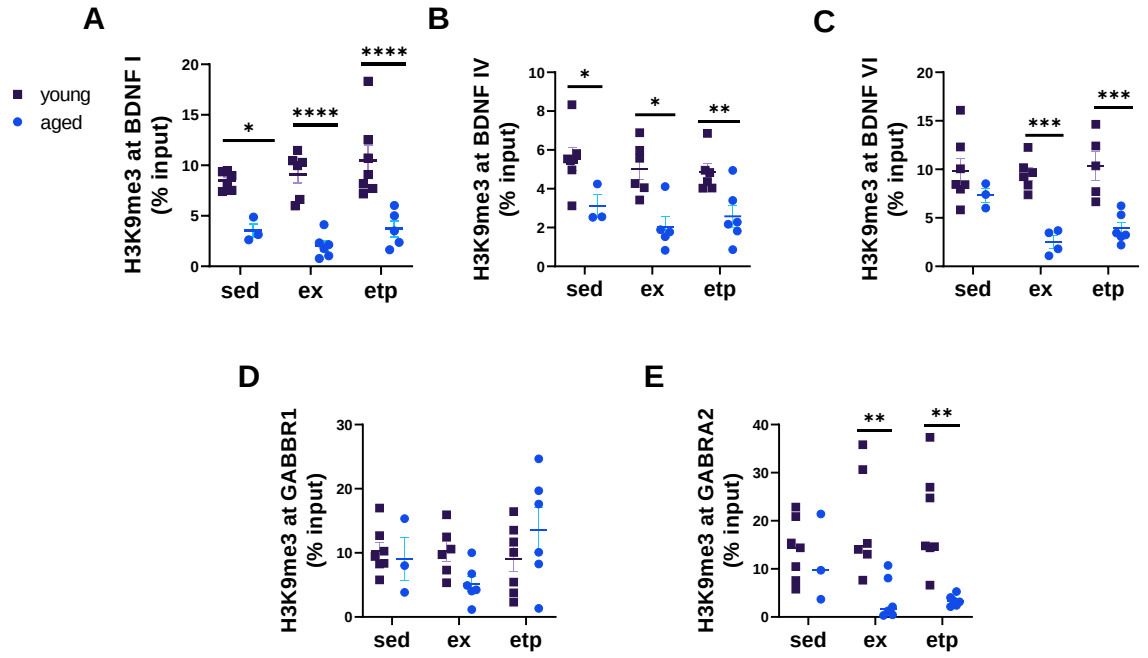

Figure 3.9. H3K9me3 Repression of BDNF and GABA Receptors Decreases with Age. Comparison of ChIP % input data from young and aged mice. A, Two way ANOVA main effect of age F(1,27)=56.73, ****p<0.0001. No significant effect of treatment [F(2,27)=1.369, p=0.27] or interaction [F(2,27), p=0.58]. Sidak's multiple comparisons test sed young vs. aged t(7)=3.03, *p=0.016; ex young vs. aged t(10)=5.33, ****p<0.0001; etp young vs. aged t(10)=5.041, ****p<0.0001. B, Two way ANOVA main effect of age F(1,27)=29.23, ****p<0.0001. No significant effect of treatment [F(2,27)=0.41, p=0.41] or interaction [F(2,27)=0.24, p=0.79]. Sidak's multiple comparisons test sed young vs. aged t(8)=2.67, *p=0.037; ex young vs. aged t(9)=3.78, **p=.0024; etp young vs. aged t(10)=2.99, *p=0.017. C, Two way ANOVA main effect of age F(1,25)=34.9, ****p<0.0001. No significant effect of treatment [F(2,25)=2.23, p=0.13] or interaction [F(2,25)=2.552, p=0.098]. Sidak's multiple comparisons test sed young vs. aged t(8)=1.54, p=0.36; ex young vs. aged t(8)=4.49, ***p=0.0004; etp young vs. aged t(9)=4.41, ***p=0.0005. D, Two way ANOVA no significant efect of age [F(1,29)=0.09, p=0.77], treatment [F(2,29)=1.48, p=0.24] or interaction [F(2,29)=2.65, p=0.088]. E, Two way ANOVA main effect of age F(1,29)=17.28, ***p=0.0003. No significant effect of treatment [F(2,29)=0.06, p=0.94] or interaction [F(2,29)=2.442, p=0.10]. Sidak's multiple comparisons test sed young vs. aged t(8)=0.43, p=0.96; ex young vs. aged t(10)=3.45, **p=0.0052; etp young vs. aged t(11)=3.79, **p=0.0021.

Discussion

Our findings demonstrate that exercise and SUV39H1 inhibition affect the expression of BDNF exons and GABRA2 in an age dependent manner. Exercise and ETP69 significantly decreased H3K9me3 at BDNF promoter VI in aged mice, corresponding to an increase in BDNF VI expression with ETP69 treatment. Apart from this important exception, exercise and ETP69 did not significantly alter H3K9me3 levels at the promoters of neuronal plasticity genes, although they did regulate mRNA levels. These findings suggest that H3K9me3 repression is not a primary driver of neuronal plasticity gene expression. Age had a strong effect on H3K9me3 promoter binding, as H3K9me3 repression of BDNF and GABA receptors decreased significantly in old mice. Exercise and ETP69 altered neuronal gene expression in an age-dependent manner and reduced H3K9me3 recruitment at the BDNF VI promoter region.

Of the promoters we examined, exercise and ETP69 only reduced H3K9me3 at the BDNF VI promoter in aged mice, suggesting that H3K9me3 is a largely stable epigenetic modification. This finding differs from previous research showing that ETP69 reduces H3K9me3 at BDNF I following an OLM task (Snigdha et al., 2016). OLM increases the expression of the immediate early gene Nr4a2, which activates BDNF promoters and modulates BDNF expression (Kwapis et al., 2019; Volpicelli et al., 2007). Inhibiting SUV39H1 thus had a disparate effect on BDNF regulation in home cage mice as opposed to cognitively engaged mice. ETP69 increased BDNF VI expression in aged mice, potentially as a means of improving plasticity in a system where synaptic genes are downregulated (Lu et al., 2004). BDNF VI is localized to distal dendrites while BDNF IV is restricted to the soma, and BDNF I is present in both soma and dendrites (Boulle et al., 2012; Chiaruttini et al., 2009; Pattabiraman et al., 2005). BDNF VI is also involved in governing the complexity of dendritic spines (Maynard et al., 2017). This

regulatory exon may be selectively upregulated by SUV39H1 inhibition to improve plasticity in the aged brain.

In addition to increasing BDNF VI levels in aged mice, exercise also elevated BDNF I expression in young mice. This finding is supported by previous studies in our lab showing that three weeks of wheel running increase BDNF I levels in the hippocampi of young rats and mice (Intlekofer et al., 2013; Tong et al., 2001). There was no change in BDNF IV in young or aged mice, a finding which is supported by an exercise study in young mice (Intlekofer et al., 2013). As previously mentioned, BDNF IV is localized at the soma as opposed to dendrites like BDNF I and VI and does not play a significant role in synaptic plasticity (Boulle et al., 2012; Chiaruttini et al., 2009; Pattabiraman et al., 2005). BDNF IV may also be more involved in fear and emotion based learning than BDNF I and VI. A fear conditioning study showed that consolidation of fear learning upregulated BDNF IV expression, while context exposure alone was enough to upregulate BDNF I and VI (Boulle et al., 2012; Lubin et al., 2008). Based on these studies, it is possible that BDNF IV expression is not regulated by the same mechanism as BDNF I and VI.

Intriguingly, exercise and SUV39H1 inhibition significantly affected GABRA2 expression, yet the direction of change was dependent on mouse age. While exercise increased GABRA2 expression in aged mice, ETP69 and exercise both reduced GABRA2 levels in young mice. Studies show that GABA signaling is reduced in the hippocampus with age, contributing to overexcitation and memory impairment (Mcquail et al., 2015; Spiegel et al, 2013; Stanley et al., 2004). A study of 21 month old mice found a specific decrease in GABRA2 around neuronal cell bodies in the CA1 and CA3 (Palpagama et al., 2019). GABA A receptor activity is also correlated with cognitive performance in aged mice (Koh et al., 2013, 2020). Exercise may induce an increase in GABRA2 in the aged hippocampus to compensate for an age-associated

decline in inhibitory signaling, improving cognition in the process. In contrast, exercise may reduce GABA receptors in young mice to increase neuronal excitation. This is supported by a study showing that 4 weeks of wheel running decreased GABRA2 in the forebrain of young rats (Hill et al., 2010). The ability of ETP69 to reduce GABRA2 indicates that it must decrease repressive H3K9me3 at a transcriptional suppressor. SUV39H1 inhibition can dynamically impact neuronal plasticity, but the pathway by which it regulates neuronal gene expression remains to be established.

Exercise induced changes in BDNF and GABRA2 expression in our models, yet GABRA2's role in BDNF regulation remains unclear. In aged and exercised mice, the increase in GABRA2 correlated with an increase in BDNF VI expression, suggesting that GABRA2 may upregulate BDNF in aged models of exercise. In contrast, exercise induced a significant decrease in GABRA2 and an increase in BDNF I expression in young mice. GABRA2 may only be involved in BDNF upregulation in systems where BDNF and GABA signaling is impaired, such as models of aging. Future studies should examine the signaling pathway between GABRA2 and BDNF in models of aging and exercise to determine if GABRA2 differentially upregulates BDNF expression.

Although promoter H3K9me3 was largely unaffected by our manipulations, its stability and binding affinity appear to decline with age. With the exception of GABBR1, H3K9me3 binding was greater in young mice than aged mice at the promoters of neuronal plasticity genes. Our findings show that H3K9me3 at promoter regions decreases with age even as overall H3K9me3 increases, which might indicate a change in H3K9me3's function (Snigdha et al., 2016). Increasing age is associated with a global reduction in heterochromatin and impaired genome stability (Kane et al., 2019). Apart from repressing genes involved in cognition,

H3K9me3 is also involved in double strand break repair and maintains heterochromatin integrity (Ayrapetov et al., 2014). Future studies should explore if H3K9me3 is increasingly recruited for these alternate functions with age.

This study opens up avenues for future investigation into the regulation of neuronal plasticity genes. The role of GABRA2 in BDNF regulation has yet to be explored, and studies involving GABRA2 inhibition or overexpression could elucidate its effect on BDNF expression. Studies on H3K9me3 localization throughout the lifespan will further suggest how its function changes with age. Our SUV39H1 inhibitor, ETP69, globally reduces H3K9me3 but cannot modify this marker at specific genes. ChIP-sequencing could be used to identify how H3K9me3 binding changes with age and exercise, and RNA-sequencing can determine if these changes are correlated with gene expression. A comprehensive examination of H3K9me3 will clarify its effect on neuronal gene expression and specific sites of pharmacological manipulation.

In conclusion, we have demonstrated that H3K9me3 regulates the expression of BDNF VI at its promoter in aged mice. Reducing H3K9me3 with ETP69 increases BDNF VI expression, thus increasing synaptic plasticity at distal dendrites. Increased age reduces H3K9me3 promoter binding and changes the regulation of BDNF and GABRA2 by exercise and ETP69. Overall, exercise and SUV39H1 inhibition can effectively modify neuronal gene expression and are dynamic tools for improving cognition across the lifespan.

Chapter 4: Conclusions

By the year 2050, the proportion of the world's population over 60 will nearly double from 12% to 22% (World Health Organization). It is estimated that 40% of these individuals will be affected by age related cognitive decline, or ARCD (Juan and Adlard, 2019; Vanguilder and Freeman, 2011). Although most of their cognitive functions are preserved, those with ARCD suffer from a decline in recall and processing speed that correlates with a downregulation of synaptic genes (Yankner, 2008; Lu, 2004). ARCD significantly decreases quality of life and increases the burden on caregivers, making it imperative to research its mechanism and preventative measures.

Emerging evidence suggests that brain aging is affected by epigenetic mechanisms, particularly histone modifications (Lardenoije et al., 2015). One highly regulated site is histone 3, lysine 9 (H3K9), which is associated with gene repression when di or trimethylated (Barter and Foster, 2018; Peters et al., 2003; Stewart et al., 2005). H3K9 is trimethylated by the histone methyltransferase SUV39H1, and our lab has previously shown that H3K9me3 is correlated with cognitive aging and synaptic loss (Snigdha et al., 2016). In our studies, we sought to elucidate the mechanism of H3K9 trimethylation and determine which age-associated stressors lead to its elevation.

In **Chapter 1**, we determined that H3K9me3 in hippocampal neurons is not regulated by a direct reduction in oxidative stress as it is in other cell types. This led to our discovery in **Chapter 2** that inhibiting BDNF signaling elevates H3K9me3, and that this elevation is dependent on both age and oxidative stress. In **Chapter 3** we explored how an intervention that elevates BDNF, physical activity, affects H3K9me3 repression of genes critical for neuronal plasticity. We found that exercise and SUV39H1 inhibition reduce H3K9me3 repression of

BDNF promoter VI, but only ETP69 increases BDNF VI expression. However, both interventions differentially affect BDNF and GABA receptor 2 expression depending on mouse age. Overall, we have identified a negative feedback mechanism between hippocampal H3K9me3 and BDNF in which H3K9me3 represses BDNF expression and is in turn suppressed by BDNF signaling.

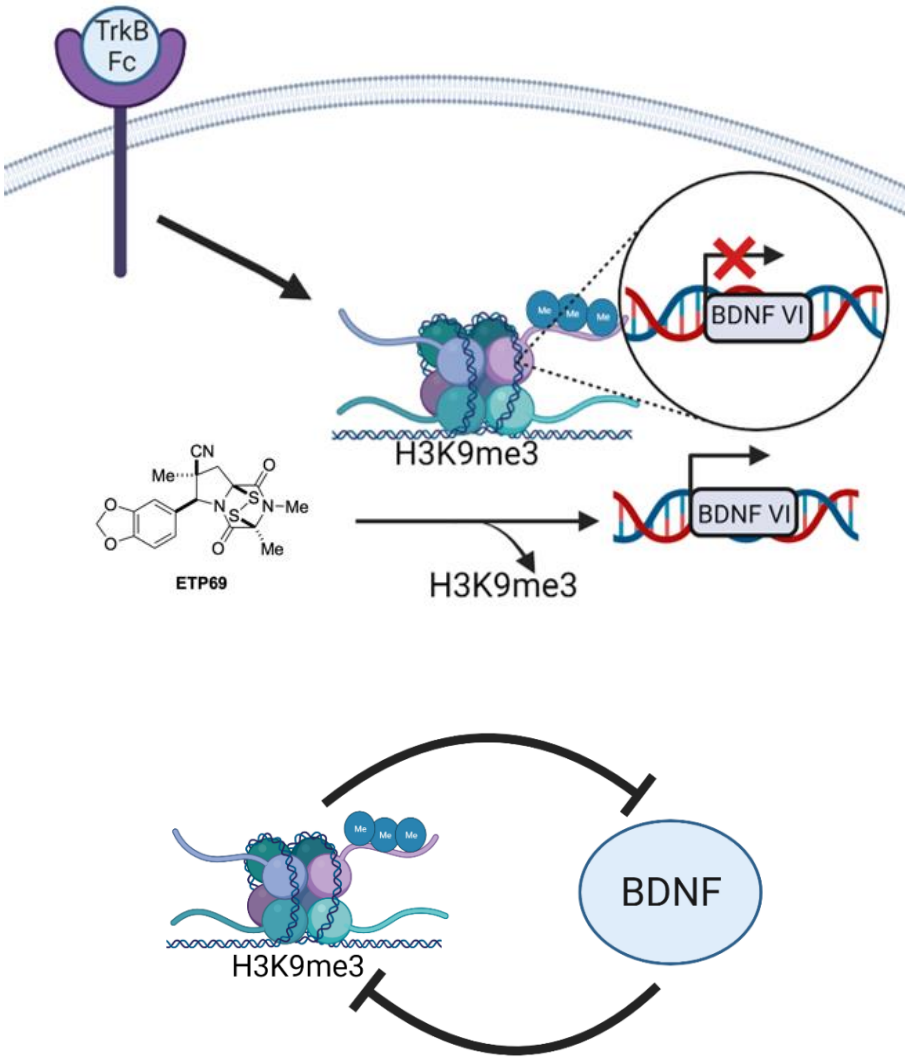

Figure 4.1 Negative Feedback Loop Between H3K9me3 and BDNF. Inhibiting BDNF signaling increases H3K9me3 in an age and oxidative stress dependent manner. H3K9me3 represses BDNF exon VI at its promoter, and ETP69 can reduce H3K9me3 at the BDNF VI promoter and increase BDNF VI expression. There is a negative feedback mechanism between hippocampal H3K9me3 and BDNF in which H3K9me3 represses BDNF expression and is in turn inhibited by BDNF signaling. Created with Biorender.com.

Our research has shed light on a novel mechanism of H3K9me3 regulation. However, we have not confirmed the regulatory factors that mediate the interaction between BDNF and H3K9me3. The Snyder lab found that BDNF degrades SUV39H1 and H3K9me3 via a nitric oxide dependent pathway in cortical neurons (Sen and Snyder, 2011). Future investigations should determine if this mechanism is also at play in the rodent hippocampus.

The studies in this dissertation are grounded in the discovery that H3K9me3 inhibition improves cognitive performance in aged mice (Snigdha et al., 2016). H3K9me3 thus emerged as a critical epigenetic mark that could have lasting implications for the field of brain aging. However, H3K9me3 also plays beneficial roles by maintaining heterochromatin integrity, regulating gene expression, and repairing double strand breaks (Hongjie et al., 2019; Ayrapetov et al., 2014). Thus our current global SUV39H1 inhibitor, ETP69, has utility as a mechanistic tool but not as a therapeutic. Future studies should examine the localization and function of hippocampal H3K9me3 across the lifespan in clinical as well as rodent models. Only then can we identify sites of H3K9me3 overexpression that can be targeted without impacting its homeostatic functions.

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