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Transfusion of Plasma from Young Exercise mice ameliorates Aging-associated Cognitive Impairments on Learning and Memory through activation of Autophagy.

By

Reine Khoury

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THESIS APPROVAL FORM

Student Name: Reine Khoury I.D. #: 201704733

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The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment of the requirements for the degree of:

Master of Science in the major of Biological Sciences

Thesis Advisor's Name: Dr. Sama Sleiman // Co-advisor's Name: Dr. Joseph Stephan

Signature: [redacted] Date: 18 / 7 / 2022 (Day / Month / Year)

Committee Member's Name: Dr. Sandra Rizk

Signature: [redacted] Date: 18 / 7 / 2022 (Day / Month / Year)

Committee Member's Name: Dr. Costantine Daher [redacted]

Signature: Date: 18 / 7 / 2022 (Day / Month / Year)

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Transfusion of Plasma from Young Exercise mice ameliorates Aging-associated Cognitive Impairments on Learning and Memory through activation of Autophagy.

Reine Khoury

Abstract

The brain's cognitive skills gradually decline with aging. In old animals, damaged proteins accumulate in neurons since autophagy, a catabolic process responsible for organelle and protein degradation, decreases. Physical exercise is a known lifestyle factor that promotes learning and memory formation in the hippocampus. The beneficial effects of exercise are mediated through the induction of the brain derived neurotrophic factor (BDNF). Previous work identified that exercise promotes cognition by inducing autophagy. In this study, we report that voluntary exercise increases autophagic activity in the hippocampus of adult C57BL/6 mice. This increase in autophagy is correlated with enhanced spatial learning and memory formation in the Morris water maze. Inhibition of autophagy in adult exercise mice with chloroquine phosphate (CQ) during the behavioral test showed impaired learning and memory formation, as well as decreased BDNF levels in the hippocampus as compared to the control exercise group. Activation of BDNF signaling in mice treated with CQ did not rescue learning and memory deficits. Hence, our results suggest that BDNF signaling is upstream of autophagy in the hippocampus. The same exercise paradigm did not promote learning and memory formation in middle-aged and old male mice. Interestingly, we show that systemic administration of adult exercise plasma into middle-aged mice rejuvenates learning and memory in an autophagy-dependent manner. Our results are consistent with autophagy playing central roles in promoting exercise-induced effects on cognition. Among the plasma factors, we identified

β-hydroxybutyrate, a liver-derived molecule, as an exercise-induced factor that promotes learning and memory in an autophagy-dependent manner. The results reveal the potential therapeutic benefits of plasma factors released in response to exercise in an autophagy-dependent manner.

Keywords: Aging, Autophagy, BDNF, Brain, Learning, LC3B, Memory, Plasma factors, Voluntary exercise.

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List of Abbreviations

MWM: Morris Water Maze

DBHB: D-β-Hydroxybutyrate

BDNF: Brain derived neurotrophic factor

VEGF: Vascular endothelial growth factor

IL-6: Interleukin 6

IL-10: Interleukin 10

IGF-1: Insulin-like growth factor

PI3K: Phosphatidylinositol 3-kinase

MAPK: Mitogen-activated protein kinase

PLC-γ: Phospholipase C-γ

ERK: Extracellular signal-regulated kinase

IRS1/2: Insulin receptor substrates ½

CRE: cAMP-calcium response element

CREB: cAMP-calcium response element binding protein

Ras: GTP binding protein

Raf: Ras associated factor

MEK: MAP/Erk kinase

mTOR: Mammalian target of rapamycin

Gpld1: Glycosylphosphatidylinositol (GPI)-specific phospholipase D1

FNDC5: Fibronectin type III domain-containing protein 5

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

CMA: Chaperone-mediated autophagy

HSC70: Heat-shock protein 70

LC3B: 11 microtubule-associated protein light chain 3

HDAC: Histone Deacetylase

SQSTM1: Autophagy receptor sequestosome 1

CQ: Chloroquine diphosphate

7,8-DHF: 7,8 Dihydroxyflavone

Chapter One

Literature Review

1.1 Exercise

1.1.1 The benefits of physical exercise on the brain

Physical exercise is one of the most effective means to sustain a healthy mind and body. Accumulating research on human and animal models supports the evidence that physical activity promotes hippocampal plasticity, improves cognitive function (Cooper et al., 2018), increases learning and memory formation (Hötting and Röder, 2013), delays the onset of neurodegenerative symptoms (Valenzuela et al., 2020), and reduces depression and anxiety (Gujral et al., 2017). MRI scans of mice subjected to voluntary wheel running show increased hippocampal volume and connectivity compared to sedentary mice (Islam et al., 2020). Furthermore, it was demonstrated that one month of voluntary exercise causes positive changes in new neuron physiology and circuitry which leads to better memory performance in young adult mice (Vivar et al., 2016, Nauer et al., 2020). Research also shows that chronic treadmill exercise can stimulate the mTOR pathway (Chen et al., 2019), hence increasing the motor skill learning in exercise mice (Bergeron et al., 2016). Investigations on the effects of voluntary exercise in a transgenic mice model of Alzheimer's disease show increased cognitive functioning (Berardi et al., 2007), reduced tau depositions (Adlard et al., 2005), ameliorated amyloid-β levels, and delayed onset of neurodegenerative disease progression (Brini et al., 2018). Moreover, exercise induces expression of the brain-derived neurotrophic factor (BDNF), which was shown to help treat major depressive disorder (Russo-Neustadt et al., 2000) and anxiety disorder among many other psychiatric disorders (Carek et al., 2011). Indeed, physical exercise is a strong lifestyle factor that is highly correlated with maintaining healthy functions of the cognitive brain, and preventing a wide range of mental disorders, neurodegenerative diseases, as well as acquired brain injuries such as depression, Alzheimer's disease, and stroke (Gubert and Hannan, 2021; Liu et al. 2019; Bliss et al., 2021).

1.1.2 The mechanisms of action of physical exercise

Current studies on the different mechanisms underlying the beneficial outcomes of exercise on the brain's health suggest that the mechanism of action is related to crosstalk between the brain and the periphery (Gonçalves and De Felice, 2021). Both central and peripheral factors enhance neural plasticity and improve learning and memory performance (Cooper et al., 2018). Growth factors such as BDNF and the vascular endothelial growth factor (VEGF) are associated with exercise-induced neurogenesis, vascular plasticity, and improved learning and memory (Cotman et al., 2002; Udo et al., 2008). Interestingly, an increase in the levels of such neurotrophins in the brain could be due to an exercise-induced increase in peripheral factors such as D-β-hydroxybutyrate (DBHB) secreted by the liver (Sleiman et al., 2016; Wang et al., 2020), osteocalcin secreted by the bones (Obri et al., 2018; Nicolini et al., 2020), lactate, cathepsin B, IL-6, and irisin secreted by the muscles (Cooper et al., 2018; Descalzi et al., 2019), adiponectin and IL-10 secreted by adipose tissues (Figure 1.1) (Cooper et al., 2018).

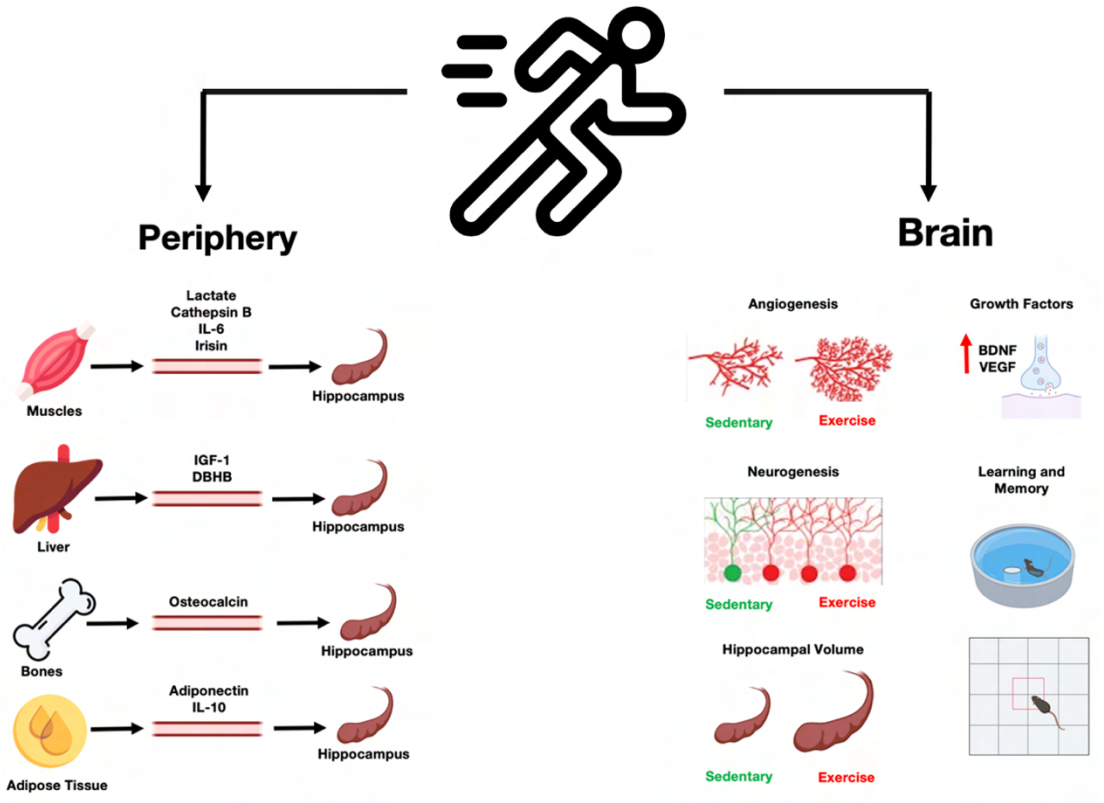

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Figure 1.1: Exercise-induced release of central and peripheral factors and their effects on brain plasticity, learning and memory.

IL-6, interleukin 6; DBHB, D-β-hydroxybutyrate; IGF-1, insulin-like growth factor 1; IL-10, interleukin 10; BDNF, brain derived neurotrophic factor; VEGF, vascular endothelial growth factor.

1.2 Neurotrophins

1.2.1 Overview on Neurotrophins

Signals from the environment cause constant structural remodeling of the brain. At the molecular level, these changes are mediated by protein growth factors in the nervous system which are called neurotrophins (Dechant and Neumann, 2002). Neurotrophins constitute a highly conserved family of different growth factors and are prime mediators of neuronal plasticity. The neurotrophin family includes the nerve growth factor (NGF), the brain derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 (Bibel and Barde, 2000). Neurotrophins are associated with neuronal proliferation, differentiation, synaptic transmission, and plasticity. In addition, they are important for facilitating higher-order activities such as learning and memory (Chao, 2003). The four mammalian neurotrophic factors exert their positive effects on the brain through activating two classes of receptors: the tropomyosin receptor kinase family of receptor tyrosine kinases (TrkA, TrkB, and TrkC), and the p75 neurotrophin receptor (Skaper, 2018). Interestingly, decreased expression of neurotrophins is observed in neurodegenerative diseases and psychiatric disorders, and ectopic expression of these factors is a viable approach to treating patients with such diseases (Mitre et al., 2017). Of the four neurotrophins mentioned above, BDNF is the most widely researched factor since it has a wide range of functions in health and disease states of the brain. BDNF has several important functions in the adult brain such as stimulation of neurotransmitter release, regulation of synapse structure and connections, as well as plasticity (Song et al., 2017; Huang and Reichardt, 2001). Moreover, decreased levels of BDNF in the central nervous system are associated with gene expression changes observed in Alzheimer's disease and Huntington's disease pathogenesis (Berchtold et al., 2013). Transcription of the BDNF gene leads to the formation of a precursor molecule known as proBDNF, which is then converted into a mature form known as mBDNF. Both forms bind to distinct receptors and can stimulate separate signaling pathways. mBDNF binds to TrkB, to promote neurogenesis, and cell survival. ProBDNF binds to p75, to stimulate long-term depression and apoptosis (Erickson et al., 2012).

1.2.2 The brain derived neurotrophic factor structure and signaling

BDNF Structure

The BDNF rodent gene comprises nine exons. All nine exons are alternatively spliced to exon IX, which is the common coding exon. This leads to alternatively spliced transcripts which all translate eventually into one identical BDNF protein (Aid et al., 2007; Liu et al., 2006). The regulation of the BDNF gene is as complex as its structure. Several promoters and different transcription factors govern the regulation of the many BDNF transcripts' expression. This leads to specific expression trends in different tissues and in response to different stimuli. For example physical activity and electrical stimulation specifically increase the expression of BDNF and NGF in the hippocampus (Sleiman et al., 2016, Neeper et al., 1995; Patterson et al., 1992). Expression of the BDNF gene was also shown to be decreased in several mood disorders such as major depressive disorder (Caviedes et al., 2017), and schizophrenia (Huo et al., 2021).

BDNF Signaling

mBDNF binds with high affinity to the Trk-B receptor located in the membrane of intracellular vesicles. BDNF binding to Trk-B stimulates its dimerization and autophosphorylation at its tyrosine residues. The phosphorylated tyrosine residues recruit a series of signaling molecules to the docking sites of the receptor bound to BDNF (Kaplan and Miller, 2000). As a result, three major signaling pathways are activated: phosphatidylinositol 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and phospholipase C-γ (PLC-γ) pathways (Huang and Reichardt, 2003). First, the PI3K pathway activates AKT, which then activates the transcription factor cAMP response element binding protein (CREB) to mediate anti-apoptotic effects (Baydyuk and Xu, 2014). Second, the MAPK signaling pathway activates the extracellular-signal-regulated kinase 1/2 (ERK 1/2) which then phosphorylates CREB to regulate protein synthesis during neuronal growth (Reichardt, 2006). Both AKT and ERK stimulate the activity of mTOR which regulates protein translation (Takei and Nawa, 2013). Third, The PLC γ pathway causes an increase in the levels of cytoplasmic calcium which induces the

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), to eventually activate CREB, and enhance synaptic plasticity (Gonzalez et al., 2016). Hence, all three pathways activate CREB which increases transcription of genes associated with learning and memory formation, and synaptic plasticity such as BDNF forming a transcriptional positive feedback loop, and c-fos (Gallo et al., 2018). (Figure 1.2) (Lamprecht, 1999; Cunha et al., 2010).

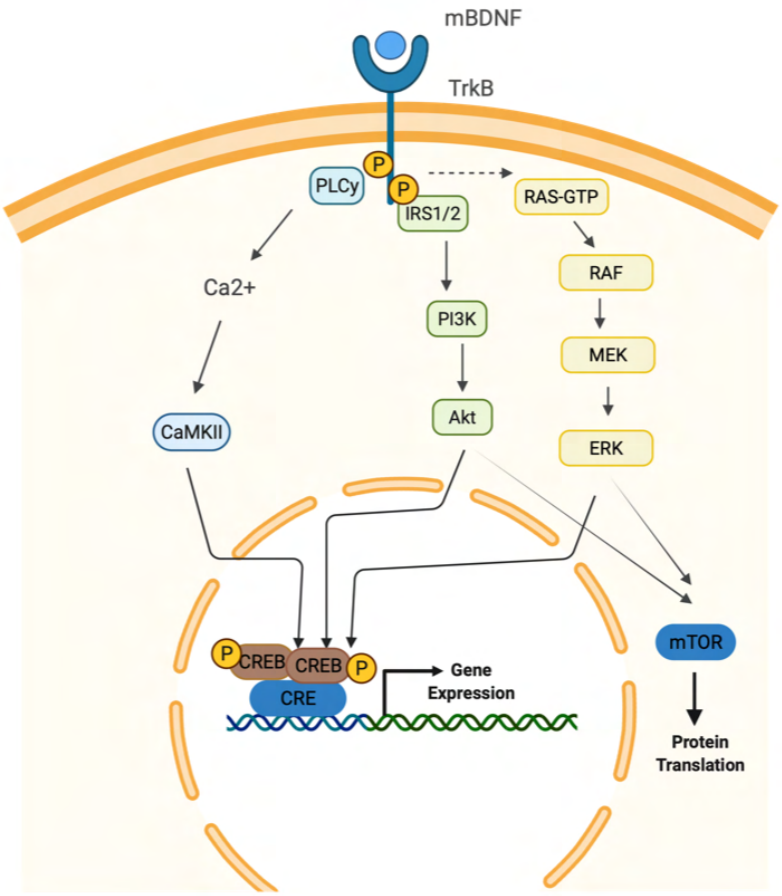

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Figure 1.2: The different signaling pathways activated by BDNF-TrkB interaction

PLCγ, phospholipase Cγ; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; IRS1/2, insulin receptor substrates 1/2; CRE, cAMP-calcium response element; CREB, cAMP-calcium response element binding protein; Ras, GTP binding protein; Raf, Ras associated factor; MEK, MAP/Erk kinase; mTOR, mammalian target of rapamycin.

1.2.3 The role of BDNF in learning and memory performance

The nervous system depends on neurotrophins for survival, growth, maturation, and maintenance. BDNF is highly expressed in the developing brain, and plays a significant role in promoting synaptic plasticity, learning and memory, and neurogenesis (Miranda et al., 2019). Various studies show the vital role of BDNF in learning. Mice subjected to different learning-associated training models exhibit increased BDNF mRNA levels in the hippocampus (Kesslak et al., 1998; Mizuno et al., 2000). Administration of BDNF into the hippocampus of rats led to enhanced performance in a spatial memory training test (Cirulli et al., 2004); on the other hand, a hippocampus-specific knockout of BDNF or infusion of antisense BDNF causes a significant decline in learning, memory, and novel object recognition, as well as decreased extinction of conditioned fear (Heldt et al., 2007; Linnarsson et al., 1997; Mizuno et al., 2000). Interestingly, major depressive disorder patients who suffer from reduced verbal memory performance show low levels of plasma BDNF (Grassi-Oliveira et al., 2008; Erickson et al., 2012). Increases in BDNF levels are associated with stimulation of the Trk-B signaling pathways. For example, aged rats subjected to a water maze learning paradigm show increased levels of the Trk-B receptor expression (Silhol et al., 2007). Similarly, spatial learning causes Trk-B phosphorylation to increase in the hippocampus of rats (Mizuno et al., 2003). On the other hand, blocking Trk-B receptor in the hippocampus causes significant loss of memory formation (Blank et al., 2016; Minichiello et al., 1999). Furthermore, strong evidence suggests that physical exercise mediates its positive effects on synaptic plasticity, learning and memory formation via BDNF induction in multiple brain regions (Oliff et al., 1998; Cotman and Berchtold et al., 2002; Neeper et al., 1996). Blocking the BDNF/Trk-B signaling pathway in animal models diminishes all the exercise-induced effects on learning and memory (Vaynman and Gomez-Pinilla, 2004).

Taken together, BDNF plays a critical role in hippocampus-specific neuronal plasticity, as well as learning and memory formation.

1.3 Peripheral Factors

1.3.1 The beneficial effects of plasma factors on the aged brain

The benefits of exercise on the aging brain are associated with increased neurogenesis, and plasticity, as well as improved learning and memory, but little information is known about the released factors that promote these effects. Corroborative evidence on plasma transfusions, parabiosis, and blood transfer studies show that young blood could possibly reduce age-related loss of excitability and plasticity, and can cause brain rejuvenation in aged animals, such as improving learning and memory performance in the hippocampus (Bouchard and Villeda, 2015; Kang et al., 2020). Pairing one young and one aged mouse in parabiosis led to several transcriptional changes linked to synaptic plasticity in the hippocampus of aged mice. Exposure to young blood caused improved spatial learning and memory, and contextual fear conditioning in aged mice. The effects seen in the aged mice were mediated by the activation of the cyclic AMP response element binding protein (CREB) in the hippocampus (Villeda et al., 2014; Castellano et al., 2017). These findings were replicated in triple-transgenic AD mice treated with young plasma. These animals had decreased tau and amyloid-β pathologies, as well as improved cognitive function (Zhao et al., 2020). Similarly, a study by Gan and Südhof (2020) showed that certain factors present in young mouse serum can stimulate neural plasticity, increase the release of neurotransmitters, and augment N- methyl-D-aspartate (NMDA) receptors in neuronal cells (Gan & Südhof, 2020). Interestingly, another recent study shows that the infusion of plasma from exercise mice, ameliorates the neurological and cognitive decline seen in an Alzheimer's disease mouse model (Kim et al., 2020). Taken together, these investigations indicate that young blood contains youthful factors, that can rejuvenate several organs in the body both in healthy and diseased states (Conboy & Rando, 2012). Manipulation of such factors can lead to promising therapeutic advances for treating aging-associated diseases. The challenge remains in the discovery of the different mechanisms and the circulating factors involved peripherally and in the brain.

1.3.2 The beneficial effects of exercise-induced metabolites

Elderly patients with nervous system disorders might not be able to exercise due to their physical condition, hence it is of utmost importance that we discover the different therapeutic approaches that mimic the beneficial effects of physical exercise. Consequently, a large body of the research is focused on understanding the underlying mechanisms, and the key molecules that mediate the induction of BDNF expression through exercise. Various exercise-induced factors of proteins and metabolites released by the muscles, liver, and bones into the blood, have been shown to cross the blood-brain barrier (BBB), enhance BDNF signaling, and promote learning and memory formation in the brain.

Exercise factors released by the liver

Physical exercise induces the liver to release several metabolites and proteins into the blood, such as the ketone body DBHB (Sleiman et al., 2016), and glycosylphosphatidylinositol (GPI)-specific phospholipase D1 (Gpld1) (Horowitz et al., 2020). D-β-hydroxybutyrate is released into the bloodstream, it crosses the blood-brain barrier, blocks the recruitment of histone deacetylase 2 and 3 (HDAC) to the promoter of BDNF, hence activates the transcription of BDNF and its expression (Sleiman et al., 2016). Interestingly, ketogenic diets that increase DBHB levels improve neurogenesis and memory performance in aging mice (Newman et al., 2017; Roberts et al., 2018) and in a Kabuki syndrome mouse model through HDAC inhibition (Benjamin et al., 2017). Additionally, DBHB slows the progression of Parkinson's disease (Norwitz et al., 2019), and improves cognition in an Alzheimer's mouse model (Yin et al., 2016; Broom et al., 2019). The levels of the liver derived Gpld1 also increase after exercise in the plasma only. Gpld1 induces BDNF expression in the hippocampus, and rescues weakened neurogenesis and cognition in aged mice (Horowitz et al., 2020).

Exercise factors released by the muscle

Exercise also induces the muscles to release factors that can regulate BDNF expression such as Cathepsin B, lactate and FNDC5/irisin. Proteomic analyses showed that exercise increases the levels of Cathepsin B (CTSB) in mouse muscle and plasma, however exercise in CTSB knockout mice did not enhance learning and memory or neurogenesis (Moon et al., 2016). Treatment of hippocampal progenitor cells with CTSB caused enhanced expression of BDNF in these cells. (Moon et al., 2016). Previously, our lab demonstrated that exercise increases the expression levels of the muscle derived lactate molecule through a PGC1a/FNDC5/BDNF pathway (El Hayek et al., 2019). Lactate crosses the blood-brain barrier, increases expression of the lysine deacetylase Sirtuin 1 (SIRT1), which activates PGC1alpha/ERRa complex, leading to an increase in the levels of the myokine Fibronectin type III domain-containing protein 5 (FNDC5) in the hippocampus (Wrann et al., 2013). Through intermediate pathways, FNDC5 and its cleavage product irisin induce activation of BDNF transcription, hence upregulating learning, and memory formation (El Hayek et al., 2019). Indeed, peripheral delivery of FNDC5 to the liver increases irisin levels in the blood, and induces BDNF expression in the hippocampus (Wrann et al., 2013). Additionally, FNDC5 and irisin delivery to the brain, increases BDNF levels and rescues cognitive impairments in AD mouse models (Lourenco et al., 2019).

Exercise factors released by the bones

In response to endurance exercise, osteoblasts release a protein called osteocalcin into the bloodstream (Mera et al., 2016). Remarkably, it was shown that when osteocalcin crosses the BBB, it inhibits anxiety and depressive-like behaviors in mice, as well as promote learning and memory formation (Oury et al., 2013; Khrimian et al., 2017). One session of high-intensity interval exercise in healthy individuals caused a surge in corticospinal excitability, BDNF levels and uncarboxylated osteocalcin levels (Nicolini et al., 2020). These beneficial effects are mediated by activating Gpr158, an orphan G protein-coupled receptor, which regulates osteocalcin to eventually lead to an increase in BDNF levels (Khrimian et al., 2017).

1.4 Autophagy

Aging is associated with the accumulation of dysfunctional and damaged proteins. Failure of the regulatory mechanisms to maintain and repair the damaged structures leads to the impairment of cellular proteosasis and increases the probability of cell senescence and death (Madeo et al., 2015; Escobar et al., 2018). Autophagy is a highly conserved cellular housekeeping phenomenon involved in the degradation of misfolded proteins, and aggregates, as well as malfunctioning organelles. It also plays a critical role in the maintenance of protein balance and homeostasis (Escobar et al., 2019). With age, autophagic activity decreases (Kaushik and Cuervo a, 2015), making it harder to achieve cellular proteostasis, especially with the increase in aggregated protein structures (Madeo et al., 2015). There exist three different primary pathways which are (1) macroautophagy, (2) microautophagy, and (3) chaperone-mediated autophagy (CMA) (Figure 1.4). In macroautophagy, the phagophore takes up part of the cytoplasm which includes proteins and organelles, forming the autophagosome. The autophagosome fuses with a lysosome and degrades all the substances present inside the autolysosome. In microautophagy, the cytosolic particles are taken up through invagination by the lysosome. In chaperone-mediated autophagy, proteins containing the KFERQ-like sequence are recognized by chaperones, then are translocated into the lysosome lumen via binding to the Lamp-2a receptor, leading to the protein's unfolding or degradation (Mizushima and Komatsu, 2011; Escobar et al., 2019). Because of the surge in interest in macroautophagy and its role in neurodegenerative disease, we decided to focus on macroautophagy since there is limited information on how environmental factors such as caloric restriction and physical exercise affect macroautophagy in the brain.

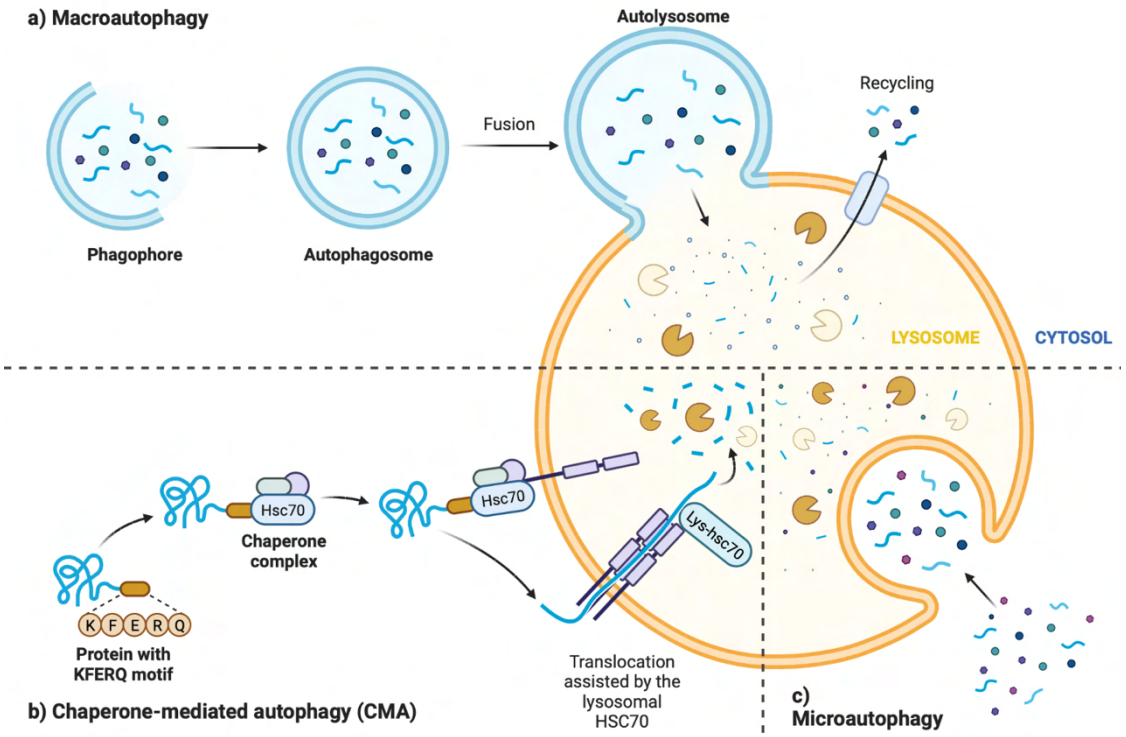

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Figure 1.4: The three main pathways of autophagy

CMA, Chaperone-mediated autophagy; HSC70, Heat-shock protein 70.

1.4.1 The different autophagy markers

Macroutophagy necessitates the creation of a double-membrane vesicle, called the autophagosome, which carries the aggregated, and damaged material to the lysosome. Their fusion results in the formation of an autophagolysosome, within which the internalized material is destroyed by hydrolases. This process is divided into five stages: (1) initiation, (2) elongation, (3) maturation, (4) fusion, and (5) degradation (Sun et al., 2013). Proteins involved in the first phase, whose end product is the autophagosome, are utilized as biomarkers in the study of autophagic activity. In the first stage, a complex involving Vps34, Beclin-1, ambra1 and other proteins is generated for initiation, while elongation takes place in two pathways analogous to ubiquitin conjugation (Mizushima, 2007). The "11 microtubule-associated protein light chain 3" (LC3B) is one protein whose

levels are utilized as an autophagy marker. It is found in the cytoplasm of most cells, when autophagy is induced, it is cleaved into LC3B-I, which is subsequently transformed from the activated LC3B-I form to LC3B-II, which is integrated into the autophagosome membrane by autophagy proteins (ATGs). LC3B-II is a protein that aids in membrane fusion and the selects molecules to be destroyed; its levels rise during autophagy (Glick et al., 2010). Another protein that is employed as an autophagy marker is the autophagy receptor sequestosome 1 (SQSTM1, p62). It attaches to ubiquitinated proteins that are misfolded and discards them to reduce toxicity or degrades them (Zatloukal et al., 2002; Moscat and Diaz-Meco, 2009). Additionally, the cargo is physically linked to the membrane of the autophagosome via p62, which has a binding site to LC3B-II which is is incorporated into the autophagosome membrane (Glick et al., 2010). Both proteins' expression levels have been investigated extensively as molecular indicators of autophagy.

1.4.2 The role of autophagy in the brain

Autophagy is essential for neuronal health. Macroautophagy is a highly conserved crucial pathway to dispose of misfolded, dysfunctional proteins and organelles in the brain (Bourdenx and Dehay, 2017). It is critical for autophagy to function properly in the brain, since it plays major roles in neurodevelopment, neuronal activity, plasticity, memory, and aging. In addition, autophagy can influence neuronal growth, differentiation, and synaptic formation (Stavoe and Holzbaur, 2019). Recently, it was demonstrated that the autophagic activity in the hippocampus declines with age and that increasing it is enough to treat age-related memory loss. Injecting youthful plasma into elderly mice rejuvenates memory in an autophagy-dependent manner, implying that autophagy plays an important role in promoting communication between systemic factors and neurons in the development of cognition (Glatigny et al., 2019). Furthermore, osteocalcin was identified as an important factor that mediates the effects of the plasma from young mice (Glatigny et al., 2019). Additionally, compelling evidence show the role of autophagy in regulating synaptic plasticity (Vijayan and Verstreken, 2017), and memory formation (Shehata and Inokuchi, 2014). Inducing autophagy in the hippocampus leads to novel memory formation

(Glatigny et al., 2019) and loss of autophagy related genes in mice leads to synaptic pruning (Tang et al., 2014). Moreover, autophagic dysfunction is a common component of the pathogenic process in several disorders, according to findings from both experimental models and post-mortem study of human tissues (Boland et al., 2018). Indeed, loss of critical autophagic proteins, such as the autophagy-related gene 7 leads to the accumulation of damaged proteins and resulted in a neurodegenerative phenotype and neural synapse defects in mice (Komatsu et al., 2006; Liang, 2019). Autophagy has a critical role in degrading proteins prone to aggregation that are linked to neurodegenerative disorders, such as polyglutamine expansions (Ravikumar et al., 2002), mutant alpha-synuclein seen in Parkinson's disease (Webb et al., 2003), and the different forms of the tau aggregates seen in dementia (Berger et al., 2006). Indeed, induction of autophagy in neuronal cells alleviates symptoms of neurodegeneration (Barmada et al., 2014).

1.4.3 The role of autophagy in the mechanisms of physical exercise

The link between autophagy and exercise is being studied to a great extent. Physical activity has a variety of health advantages, and many of these benefits are similar to known protective effects of macroautophagy. As a result, scientists speculated that activation of autophagy may be responsible for some of the health advantages of physical exercise (He et al., 2012). It was demonstrated that endurance exercise upregulates the expression levels of several autophagy-related genes and increases autophagic activity in skeletal muscle cells (Jamart et al., 2012). Additionally, exercise augments autophagic activity in the brain by increasing levels of the autophagy marker LC3B and decreased levels of SQSTM/p62 in the cortex of mouse models (He et al., 2012). Activation of the different autophagic routes is highly dependent on the intensity and the duration of the physical activity (Schwalm et al., 2015). These routes entail an increase in autophagic flux or upregulation of thirteen key autophagy genes in response to exercise (Halling and Pilegaard, 2017). Short-term aerobic exercise using running wheels for two weeks led to an increase in the LC3B marker in the cerebral cortex of mice (Rocchi and He, 2017). Interestingly, physical activity could enhance cognition, and prevent early onset of neurodegenerative

illnesses by promoting autophagy in the brain (Xing et al., 2019). Indeed, it was demonstrated that exercise improves autophagy-lysosomal efficiency and decreases amyloid beta plaques in transgenic AD mice (Zhao et al., 2018)

1.5 Aim of the study

In this study, we hypothesized that voluntary physical exercise promotes spatial learning and memory formation in an autophagy-dependent manner, through activation of hippocampal BDNF expression in adult animals. We also aimed to examine whether autophagy regulates the release of exercise factors that promote spatial learning and memory. To assess this, we conducted plasma transfusions experiments from exercise young adult mice into sedentary middle-aged mice and observed the effects of exercise-induced plasma factors on learning and memory formation in aged mice. In addition, we intended to identify the exercise-induced molecule responsible for the effects on the brain observed in plasma transfusions.

Chapter Two

Materials and Methods

2.1 Animal housing

Young (10-week-old), middle-aged (32-week-old) and old (1-year-old) male C57BL/6 mice were individually housed in cages with food and water *ad libitum* and maintained on a 12-hour light-dark cycle. The mice were divided according to the different experimental groups. Animal use and care is in accordance with the guidelines and as approved by the Lebanese American University Animal Care and Use Committee (ACUC).

2.2 Exercise paradigm

Young (10-week-old), middle-aged (32-week-old), and old (1 year old) male C57BL/6 mice were individually housed with food and water ad libitum in cages and divided into two groups based on the treatments they were receiving: sedentary animals or exercising animals. Exercising mice were provided with free access to running wheels for the duration of 14 days. Before day zero of the Morris Water Maze, the running wheels were removed from their cages. Sedentary mice were housed in the same conditions but without running wheels.

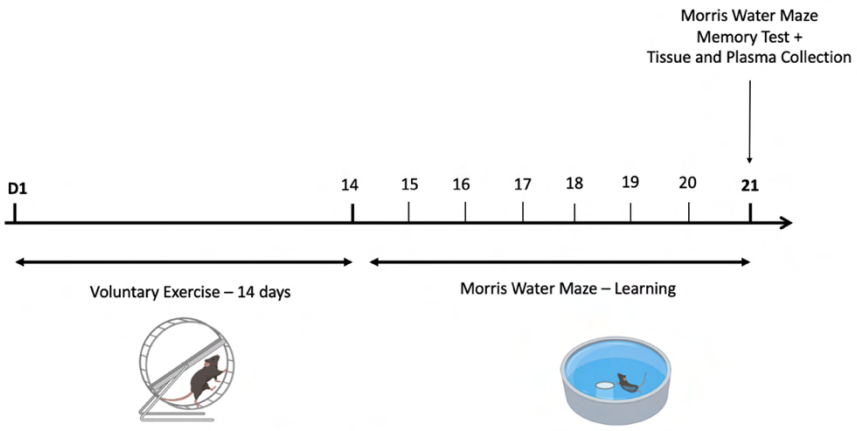

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Figure 2.1: Exercise Paradigm for 10-week-old, 32-week-old, and 1-year-old C57BL/6 male mice followed by the Morris Water Maze Test.

2.3 Treatments

2.3.1 Chloroquine and 7,8 Dihydroxyflavone Injections:

Young C57BL/6 (10-week-old) male mice received daily intraperitoneal injections of Chloroquine diphosphate (50mg/kg), or 7,8 Dihydroxyflavone (5mg/kg) alone, or 7,8 Dihydroxyflavone in combination with Chloroquine diphosphate. Control groups received saline injections. All intraperitoneal injections were performed fifteen minutes prior to their behavioral training during the 7 days of Morris Water Maze. On the memory test day of the Morris Water Maze, the mice were sacrificed, the brains were dissected on dry ice, and their nucleus accumbens, hippocampi, and cortices were harvested and stored at -80°C for further molecular analysis.

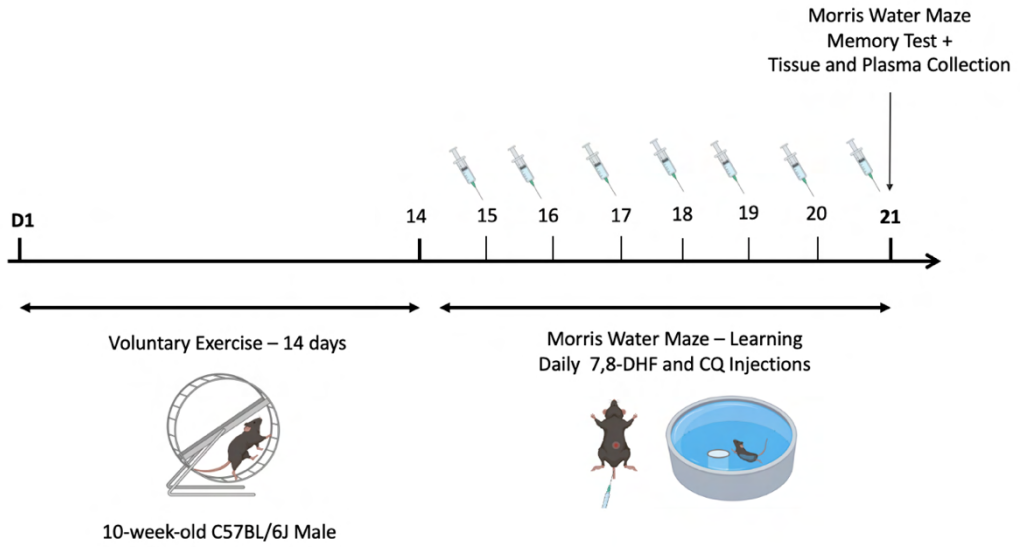

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Figure 2.2: Exercise paradigm, followed by chloroquine 50mg/kg injections and the Morris Water Maze test.

2.3.2 Lactate Injections:

Young male C57BL/6 male mice received daily intraperitoneal injections of lactate (180mg/kg) or saline four days prior to behavioral training and during the 7 days of the Morris Water Maze test. On the memory test day of the Morris Water Maze, the mice were sacrificed and their nucleus accumbens, hippocampi, and cortices were harvested and stored at -80°C for further molecular analysis.

2.3.3 D-β-Hydroxybutyrate Injections:

Young male C57BL/6 male mice received daily intraperitoneal injections of D-β-Hydroxybutyrate (DBHB - 100mg/kg) or saline, 4 days prior to behavioral training and during the 7 days of the Morris Water Maze test. On the memory test day of the Morris Water Maze, the mice were sacrificed and their nucleus accumbens, hippocampi, and cortices were harvested and stored at -80°C for further molecular analysis.

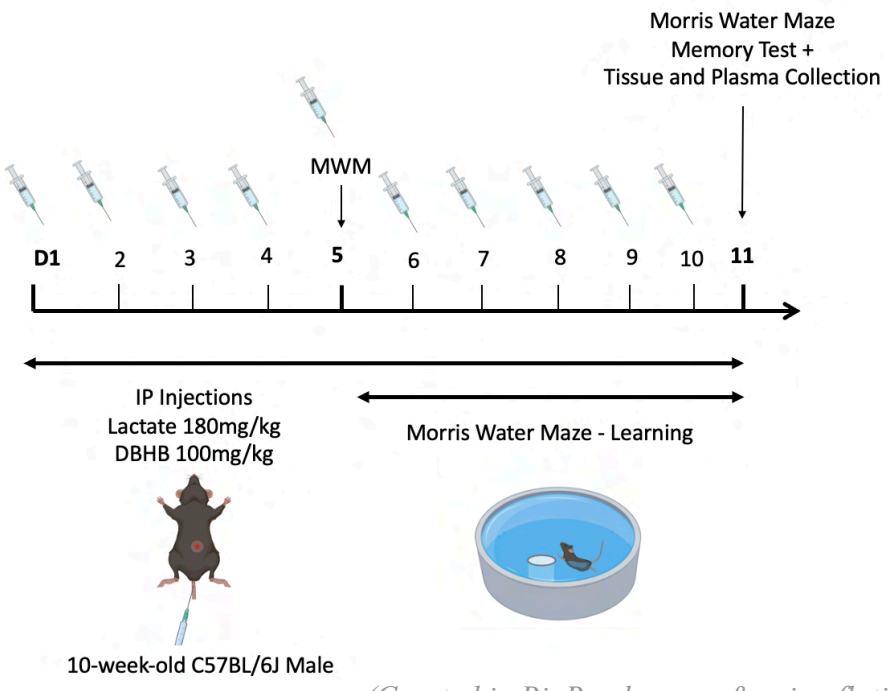

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Figure 2.3: Lactate 180 mg/kg and DBHB 100mg/kg injections followed by the Morris Water Maze Test.

2.4 Plasma donation from young and middle-aged mice

Young (10-week-old) and middle-aged (32-week-old) C57BL/6 male mice that were eventually used for the plasma donation exercised for 14 days and underwent the Morris Water Maze for 7 days. These animals either received saline or Chloroquine diphosphate. Sedentary mice were housed in the same housing conditions, but without running wheels in their cages. On the last day of the Morris Water Maze, the mice were sacrificed, and trunk blood was collected in EDTA (0.5 M) coated tubes, and centrifuged at 3000 rpm, 4°C, for 15 minutes. Pooled plasma was separated from the blood and stored at -80°C for transfusions into 32-week-old (C57BL/6J) male mice.

2.5 Plasma transfusions to 32-week-old mice

Middle-aged (32-week-old) C57BL/6J male mice received intraperitoneal injections of plasma collected from young (10-week-old) or middle-aged (32-week-old) exercise male mice, or sedentary male mice treated with saline or the autophagy inhibitor Chloroquine diphosphate. The plasma was injected in 100 µL doses every three days, for a total of eight doses over the period of 22 days. On the 23rd day, the mice underwent the Morris Water Maze, on the memory test day the mice were sacrificed and their nucleus accumbens, hippocampi, and cortices were harvested and stored at -80°C for further molecular analysis (Protocol adapted from Horowitz et al., 2020).

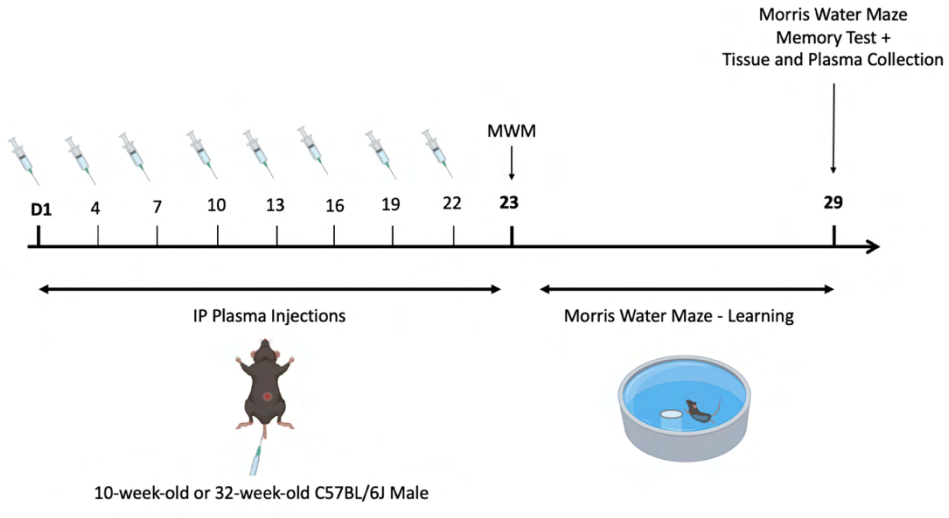

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Figure 2.4: Plasma Injections into 10-week-old and 32-week-old male mice followed by the Morris Water Maze Test.

2.6 Morris Water Maze test

Fourteen days after voluntary exercise, the mice underwent the behavioral test "Morris Water Maze" for 7 consecutive days. This test is performed to assess the mice's spatial learning and memory. Mice use different cues present on the maze at its borders, to escape the water, and reach a hidden platform (Morris, 1984). The Morris water maze consists of a small pool divided into 4 quadrants of the same size named Q1, Q2, Q3, and Q4. A hidden platform is placed in Q1. The perimeter of the pool is lined with five different spatial cues representing distinct shapes and colors. At day 0, the mice were allowed three trials (60 seconds) to reach the platform in a clear water. The purpose of this trial is to familiarize the mice with the setup. From day 1 till day 5, the mice's latency to reach the platform was recorded via the ANY-maze video tracking system. From day 2 till day 5, white paint was gradually added to hide the platform. On day 6, (memory test) the platform was removed from the pool, and the tracking system recorded the time spent by the mouse in Q1 searching for the missing platform.

2.7 Preparation of Tissue and Protein Extraction

After the Morris Water Maze test, the animals were sacrificed, and the hippocampus, prefrontal cortex, and nucleus accumbens was harvested into separate microcentrifuge tubes. Cellular proteins were isolated from brain tissues by homogenizing and lysing the tissues on ice with lysis buffer *RIPA-B* (1% Triton X-100, 1% SDS, 50mm Tris-Cl, pH 7.4, 500mm NaCl, and 1mm EDTA), in the presence of protease inhibitors (Sigma, Bio-world), phosphatase inhibitors (Sigma, Bio-world), and proteasome inhibitors Mg-132 (Sigma). The samples were centrifuged for 15 minutes, at 15000 rpm and 4°C and the supernatant was collected.

2.8 Western Blot

Samples were prepared in Laemmli buffer and heat-shocked at 95°C for five minutes. Next, the samples were loaded into wells for electrophoresis on acrylamide gels (Bio-Rad). Using the Trans-Blot SD Semi-Dry transfer cell (Bio-Rad), the proteins were transferred to a PVDF or nitrocellulose membrane. Next, the membranes were incubated overnight at 4°C or for 2 hours at room temperature with non-fat milk diluted in TBS-Tween to prevent non-specific binding. The membranes were washed with TBS-T (TBS-Tween20) three times for 5 minutes after blocking, then incubated overnight at 4°C or for 2 hours at room temperature with primary antibodies against LC3B (1:1000; Abcam), BDNF (1:500; N-20, Santa Cruz Biotechnology), PGC1a (1:100; Abcam), GAPDH (1:1000; Abcam) and Actin (1:1000; Santa Cruz Biotechnology). Membranes were washed with TBS-T three times for 5 minutes, and then incubated with secondary antibodies (1:5000; Bio-Rad) for 1 hour at room temperature. Signals from the secondary antibody were visualized using Clarity Western ECL Substrate (Bio-Rad) or Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific) by chemiluminescence using the ChemiDoc (Bio-Rad). ImageJ was used for band quantification.

2.9 D-β-Hydroxybutyrate Assay

Plasma levels of D-β-hydroxybutyrate were measured using the D-β-hydroxybutyrate assay kit (Sigma) according to the manufacturer's protocol.

2.10 Statistical Analysis

Data was analyzed using Prism GraphPad with unpaired T-test, one-way ANOVA, and two-way ANOVA followed by Tukey post-hoc tests. p-values <0.05 was considered statistically significant. *: $p<0.05$, **: $p<0.01$, ***: $p<0.005$

Chapter Three

Results

3.1 Voluntary exercise induces autophagy in the hippocampus of adult mice

To assess how voluntary exercise affects autophagy in the hippocampus of mice, we subjected adult 10-week-old male mice to a voluntary exercise paradigm of 14 days (Diederich et al., 2017). Consequently, we divided 10-week-old male mice into 2 groups: one that received access to a running wheel for 14 days, and one that did not have access to a running wheel, serving as a sedentary control. Fourteen days after voluntary exercise, mice were subjected to a behavioral test called the Morris water maze (MWM) that assesses hippocampal-dependent spatial learning and memory. At the end of the MWM, the mice were sacrificed, and their hippocampi were harvested. To examine how exercise affects autophagy in the brain of 10-week-old male mice, we compared the hippocampal proteins levels of the autophagy marker LC3B in sedentary versus exercise animals. Exercising mice in this paradigm have significantly higher LC3B protein levels compared to the sedentary mice as seen in the western blot figure and quantification (n=4 sedentary, n=4 exercise) (Figures 3.1A and 3.1B). This data is consistent with voluntary exercise promoting the induction of autophagy in the hippocampus. These results led us to test whether the same voluntary exercise paradigm can promote spatial learning and memory formation in the brain of adult 10-week-old mice in an autophagy dependent manner.

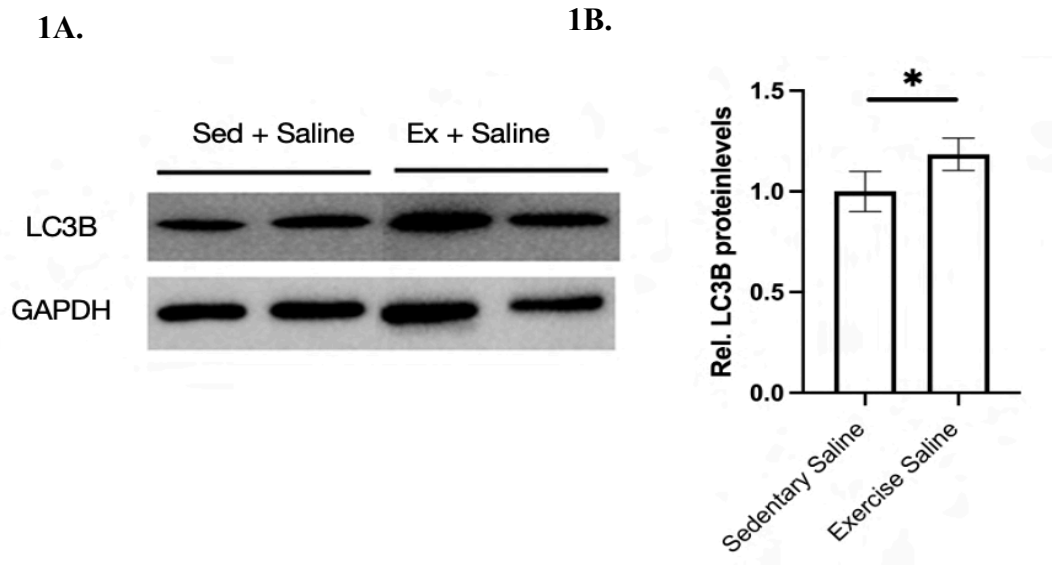


Figure 3.1: Voluntary exercise induces increase in the autophagy marker LC3B in the hippocampus of adult 10-week-old mice.

(1A) Representative western blot image depicting the increase in the autophagy marker LC3B protein levels in the hippocampus of adult 10-week-old exercise animals as compared to sedentary mice. (1B) Quantification of the LC3B western blot. Statistical significance was measured by unpaired t-test * $p<0.05$. The n number for the sedentary group receiving saline is 4, and for the exercise group receiving saline is 5.

3.2 Voluntary exercise promotes learning and memory formation in the brain of adult mice in an autophagy dependent manner

Previous work has shown that exercise promotes learning and memory formation (Cassilhas et al., 2016). Having demonstrated that short-term voluntary exercise induces autophagy in the hippocampus of young male mice, we next tested whether voluntary exercise promotes learning and memory formation in the brain of adult 10-week-old mice in an autophagy dependent manner. Thus, we used a brain-permeable autophagy inhibitor, known as chloroquine phosphate, to inhibit exercise-induced autophagy. 10-week-old mice were subjected to the exercise paradigm for 14 days. These mice were divided into groups that either received saline or chloroquine phosphate (CQ; 50mg/kg). The intraperitoneal injections were performed every day during behavioral testing. In addition

to these two groups, control groups consisted of 10-week-old sedentary mice that were also either treated with saline or with CQ. This allowed us to compare the effect of chloroquine on learning and memory in the sedentary and exercising states. We observed that exercise improved spatial learning in adult male mice by significantly reducing the escape latency period ((n= 55 sedentary saline, n=42 sedentary CQ, n =76 exercise saline, n =51 exercise CQ). Moreover, inhibition of autophagy by chloroquine phosphate abolishes the exercise-induced spatial learning in exercising mice (Figure 3.2A). Furthermore, exercise animals receiving saline exhibited enhanced memory formation compared to sedentary saline mice, and treatment with the autophagy inhibitor chloroquine phosphate abolished this effect (n= 55 sedentary saline, n=42 sedentary CQ, n =74 exercise saline, n =50 exercise CQ) (Figure 3.2B). Our results were consistent with voluntary exercise inducing learning and memory formation in an autophagy dependent manner.

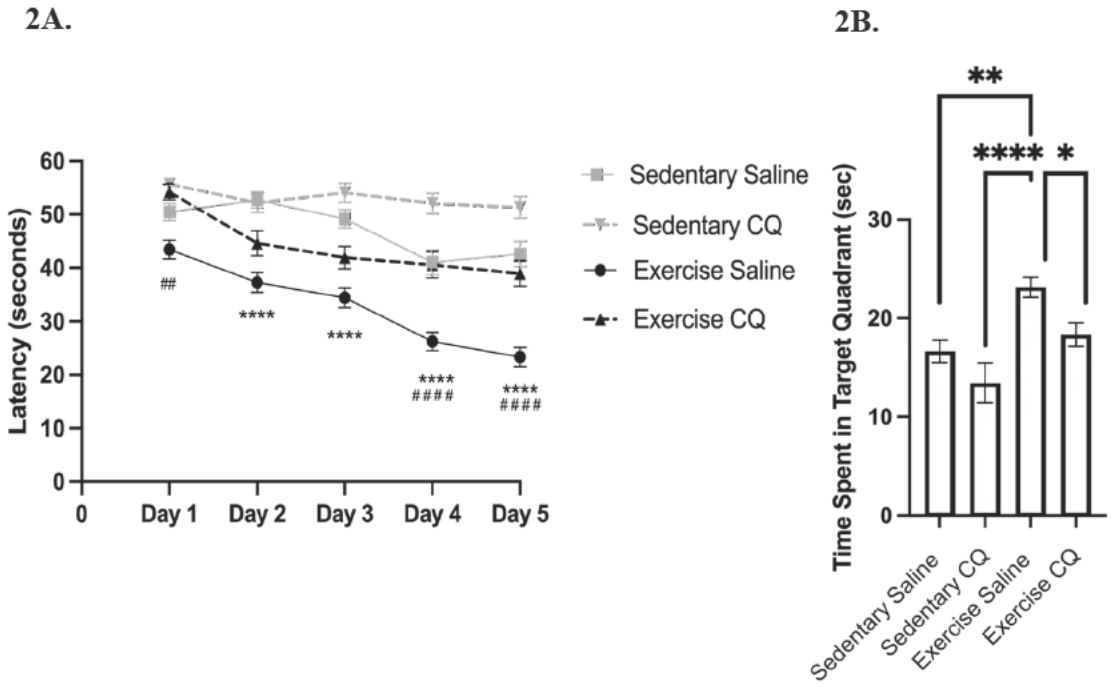


Figure 3.2: Voluntary exercise promotes learning and memory in an autophagy dependent manner.

(2A) Adult 10-week-old male mice were trained for five days for spatial learning in the Morris water maze test. Exercise animals receiving intraperitoneal injections of saline showed significantly reduced escape latency compared to sedentary saline animals, and to animals receiving intraperitoneal injections of chloroquine phosphate (50mg/kg). Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. The asterisk sign (*) shows significance between exercise saline mice versus sedentary saline mice. The octothorpe sign (#) shows significance between exercise saline animals versus exercise chloroquine phosphate animals. (n= 55 sedentary saline, n=42 sedentary CQ, n =76 exercise saline, n =51 exercise CQ) (2B) Adult 10-week-old exercise mice spent more time in the target quadrant during the memory test day of the Morris water maze as compared to sedentary saline mice. Results are expressed as Mean +/- SEM. (n= 55 sedentary saline, n=42 sedentary CQ, n =74 exercise saline, n =50 exercise CQ) Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ ****$p<0.00001$

3.3 Voluntary exercise increases BDNF levels in an autophagy dependent manner

Several studies show that physical exercise increases BDNF levels in the hippocampus of mice (Cotman and Berchtold et al., 2002; Sleiman et al, 2016; El Hayek et al., 2019). Since we showed that inhibition of autophagy using CQ causes a decrease in spatial learning and memory performance in adult mice (Figures 3.2A and 3.2B), we next tested whether autophagy regulates hippocampal BDNF levels in exercising mice to improve learning and memory performance. Consequently, we compared hippocampal BDNF protein levels in mice that received either intraperitoneal injections of saline or CQ. Our results demonstrate that hippocampal BDNF protein levels significantly decrease in exercise animals that received CQ as compared to exercise animals that received saline (n= 3 exercise saline, n=4 exercise CQ). These results suggested that exercise mediated BDNF induction is autophagy dependent (Figures 3.3A and 3.3B).

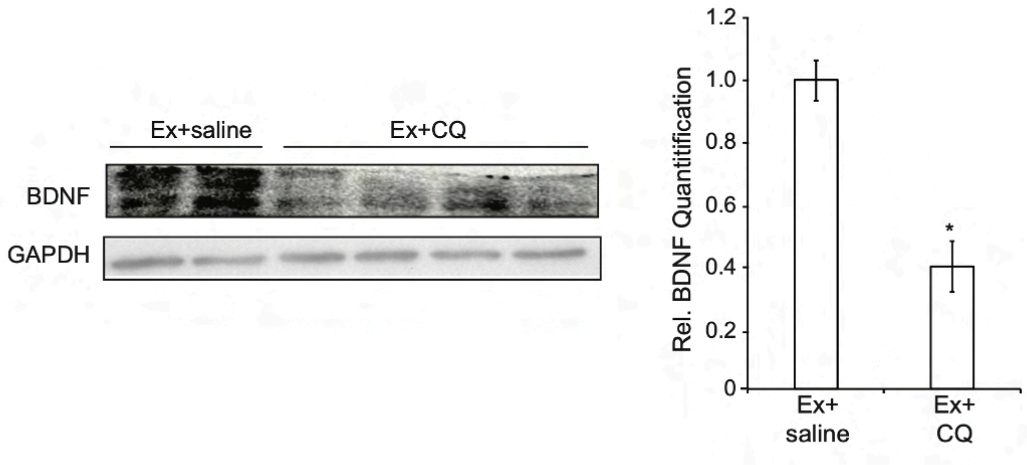


Figure 3.3: Exercise mice receiving Chloroquine phosphate exhibit a decrease in the brain-derived neurotrophic factor (BDNF) protein levels.

(3A) Representative western blot image depicting the decrease in BDNF protein levels in the hippocampus of adult 10-week-old exercise animals receiving intraperitoneal injections of Chloroquine phosphate (50mg/kg) compared to exercise saline animals. (3B) Quantification of the BDNF western blot. Statistical significance was measured by unpaired t-test * $p<0.05$ (n= 3 exercise saline, n=4 exercise CQ).

3.4 Activation of Trk-B with 7,8-dihydroxyflavone does not bypass inhibition of autophagy.

Since our results suggest that short-term voluntary exercise increases hippocampal BDNF protein levels and induces spatial learning and memory formation in an autophagy dependent manner, we suspected that activating the BDNF receptor Trk-B can bypass the inhibition of autophagy and induce spatial learning and memory formation in animals receiving CQ. Thus, we decided to use a brain-permeable Trk-B receptor agonist, 7,8-dihydroxyflavone (7,8-DHF) which can activate the BDNF signaling pathway to determine if activation of BDNF signaling can bypass the inhibition of autophagy in exercising animals and can induce spatial learning and memory formation. For that reason, exercise mice were divided into two groups: one that received 7,8-DHF alone, and another

group that received 7,8-DHF and CQ together. We also included exercise mice that received saline or chloroquine phosphate alone as controls. The intraperitoneal injections were performed every day during behavioral testing. We observed that exercising mice that received 7,8-DHF and CQ combined showed a significant increase in the latency to reach the platform. Hence, these mice showed a worsened spatial learning performance compared to exercising mice that received saline injections during their behavioral training (n= 76 exercise saline , n=51 exercise CQ, n =15 exercise 7,8-DHF, n =16 exercise CQ + 7,8-DHF) (Figure 3.4A). Furthermore, exercise animals that received saline alone spend a significantly longer time in the target quadrant Q1 as compared to exercise mice that received the combinatorial treatment of 7,8-DHF and CQ (n= 74 exercise saline, n=50 exercise CQ, n = exercise 7,8-DHF, n =16 exercise CQ + 7,8-DHF) (Figure 3.4B). These results show that activation of BDNF signaling can't bypass the inhibition of autophagy to mediate exercise-induced spatial learning and memory formation. From here, our results are consistent with a model by which exercise induces BDNF signaling in the hippocampus of 10-week-old mice. Activation of BDNF signaling in turn activates autophagy. Finally, activation of autophagy leads to a positive feedback mechanism that increases BDNF expression and signaling and in turn learning and memory formation.

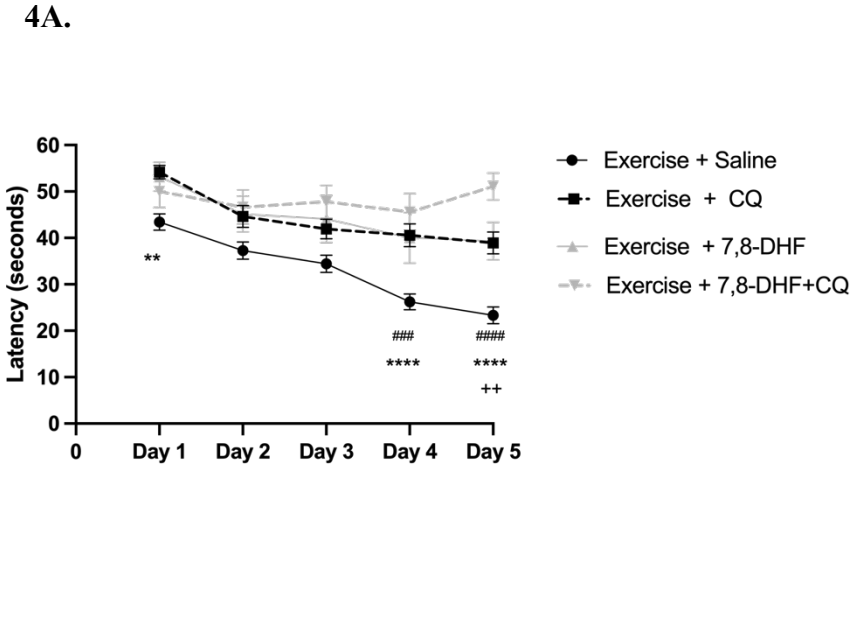


Figure 3.4: Activation of the BDNF receptor Trk-B with 7,8- dihydroxyflavone (7,8-DHF) could not bypass the inhibition of autophagy trough treatment with Chloroquine phosphate.

(4A) Adult 10-week-old exercising mice were trained for five days for spatial learning in the Morris water maze test. Exercise animals receiving intraperitoneal injections of 7,8-dihydroxyflavone (7,8-DHF - 5 mg/kg) and Chloroquine phosphate (CQ - 50mg/kg) showed significantly increased escape latency compared to exercise saline animals. Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. The asterisk sign (*) shows significance between exercise saline mice versus exercise 7,8-DHF mice. The octothorpe sign (#) shows significance between exercise saline animals versus exercise 7,8-DHF and CQ combined animals, the plus (+) sign shows significance between exercise saline animals versus exercise 7,8-DHF (n= 76 exercise saline , n=51 exercise CQ, n =15 exercise 7,8-DHF, n =16 exercise CQ + 7,8-DHF). (4B) Adult 10-week-old exercise mice spent more time in the target quadrant during the memory test day of the Morris water maze as compared to exercise animals receiving CQ alone or in combination with 7,8-DHF (n= 74 exercise saline, n=50 exercise CQ, n = 15 exercise 7,8-DHF , n =16 exercise CQ + 7,8-DHF). Results are expressed as Mean +/- SEM. Statistical significance was measured by Ordinary One-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, and ***p<0.001 ****p<0.00001

3.5 Voluntary exercise does not induce learning and memory formation in middle-aged and old mice

Next, we tested whether the same paradigm of voluntary exercise enhances spatial learning and memory formation in middle-aged (32-week-old) and old (1-year-old) mice. Hence, we subjected 32-week-old and 1-year-old mice to fourteen days of voluntary wheel exercise. Sedentary 32-week-old and 1-year-old mice served as controls. Fourteen days after voluntary exercise, mice underwent the Morris water maze test to assess hippocampal-dependent spatial learning and memory. The results show no difference in spatial learning and memory formation in 32-week-old exercise mice versus sedentary

mice (Figures 5A and 5B). Indeed, middle-aged (32-week-old) and old (1-year-old) mice that exercised did not have a decrease in escape latency across the five days of training (Figures 3.5A), nor did they show any significant increase in the time spent in Q1 searching for the platform (n=36 sedentary, n=66 exercise saline). (Figures 3.5B). We saw similar results in 1-year-old mice in which exercise mice did not show any enhanced spatial learning and memory formation compared to 1-year-old sedentary mice (n=6 sedentary, n=7 exercise) (Figures 3.5C and 3.5D).

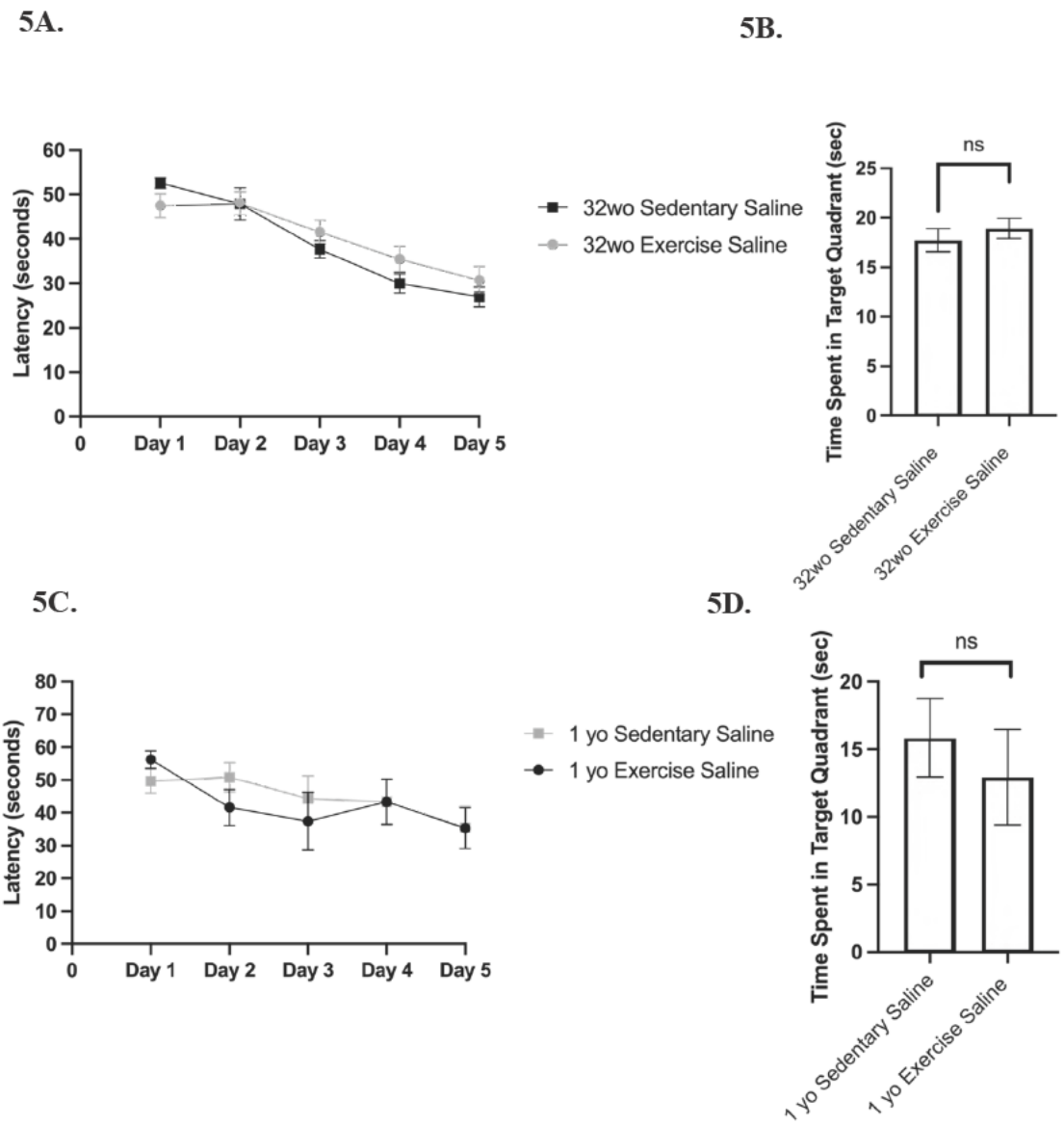


Figure 3.5: Voluntary exercise does not induce spatial learning and memory formation in 32-week-old middle-aged 1 year-old male mice

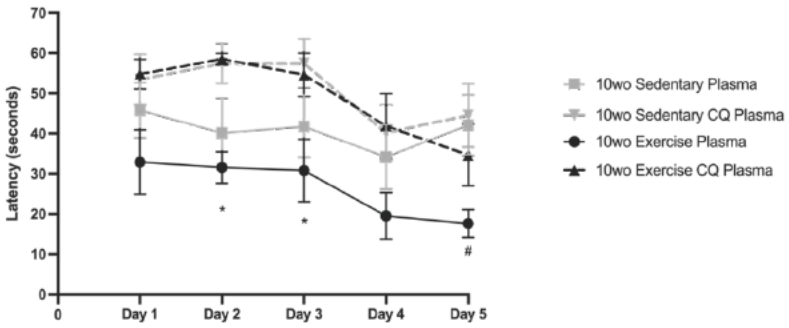
(5A and 5C) Middle-aged 32-week-old and 1 year-old exercising mice were trained for five days for spatial learning in the Morris water maze test. Middle-aged and old exercising animals did not show any enhanced escape latency compared to sedentary animals. Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test). (5B and 5D) Middle-aged and old exercising mice did not spend more time in the target quadrant during the memory test day of the Morris water maze as compared to sedentary animals. Results are expressed as Mean +/- SEM. Statistical significance was measured by unpaired t-test (1 year old: n=6 sedentary, n=7 exercise // 32-week-old: n=36 sedentary, n=66 exercise saline).

3.6 Plasma transfusions from young exercise mice ameliorate spatial learning and memory in middle-aged sedentary mice in an autophagy dependent manner

Similar to our results in 1-year-old, exercise did not enhance spatial learning and memory formation in 32-week-old mice. For that reason, we decided to focus on the 32-week-old mice to understand whether plasma transfusions from young exercise (10-week-old) mice induce spatial learning and memory formation in middle-aged (32-week-old) sedentary mice. To perform the plasma transfusions, we first collected plasma from 10-week-old exercise or sedentary mice that were treated with saline or CQ during their behavioral training. Next, middle-aged sedentary mice received injections with plasma collected from the young mice. The middle-aged sedentary mice received 8 plasma injections over the period of 22 days. On the 23rd day, the middle-aged mice treated with plasma from the young mice underwent behavioral testing. Interestingly, the results show that middle-aged sedentary mice receiving intraperitoneal injections of plasma from young exercise mice showed significantly reduced escape latency as compared to sedentary middle-aged mice receiving plasma from sedentary young mice. When the sedentary middle-aged mice received plasma of exercise mice treated with the autophagy

inhibitor CQ, we observed a significant increase in the escape latency period on day 2 and 3 compared to sedentary mice receiving exercise plasma (n= 7 10wo exercise plasma, n=6 10wo sedentary plasma, n =7 10wo exercise CQ plasma, n =6 10wo sedentary CQ plasma). (Figure 3.6A). Additionally, we observed similar effects in the memory performance test, whereby sedentary middle-aged mice that received exercise plasma from young exercise mice spent significantly more time in the target quadrant as compared to the animals that received sedentary plasma from young mice. Whereas sedentary middle-aged animals that received exercise CQ plasma spent significantly less time in the target quadrant as compared to the sedentary middle-aged mice that received exercise saline plasma from adult mice (n= 6 10wo exercise plasma, n=7 10wo sedentary plasma, n =7 10wo exercise CQ plasma, n =6 10wo sedentary CQ plasma) (Figure 3.6B). This suggests that plasma from young exercise mice enhances spatial learning and memory in middle-aged sedentary mice in an autophagy dependent manner. Similarly, as a control group to the previous experiment, we collected plasma from 32-week-old sedentary and exercise mice and injected them into 32-week-old sedentary mice. Animals receiving intraperitoneal injections of plasma from middle-aged exercise mice did not show any enhanced spatial learning or memory formation compared to animals receiving intraperitoneal injections of plasma from middle-aged sedentary mice (n=7 32wo exercise plasma, n=4 32wo sedentary plasma). (Figures 3.6B and 3.6C). Consequently, our results suggest that exercise induces peripheral tissues to release factors into the circulation in an autophagy dependent manner in young mice. These factors are sufficient to promote learning and memory formation in middle-aged mice that normally fail to release such factors in response to exercise. To further confirm our results, we compared the hippocampal protein levels of the autophagy marker LC3B in sedentary middle-aged mice that received exercise plasma, exercise CQ plasma, sedentary plasma, and sedentary CQ plasma from adult mice. Middle-aged mice that received exercise plasma have significantly higher LC3B protein levels compared to mice that received exercise CQ plasma as seen in the western blot figure and quantification (n=5 exercise plasma, n=5 exercise CQ plasma) (Figures 3.6E and 3.6F). The data indicates the presence of exercise-induced factors found in the plasma, which can induce an increase in spatial learning and memory formation in middle-aged sedentary mice.

6A.



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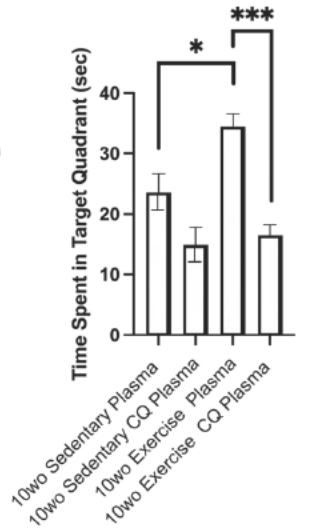

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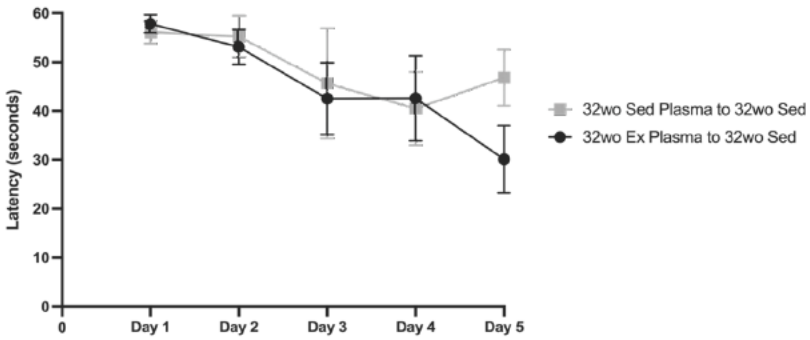

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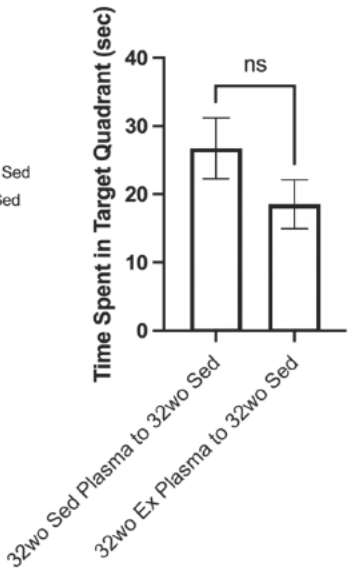

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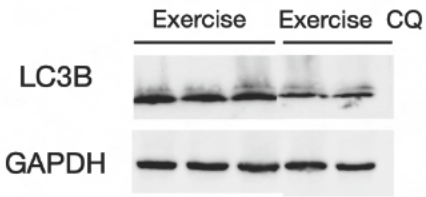

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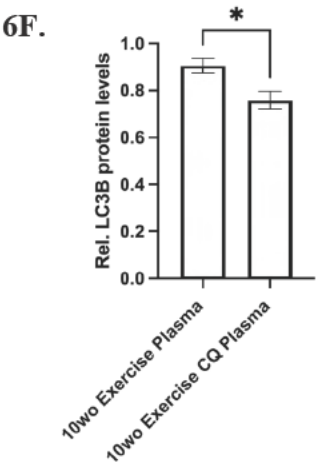

Figure 3.6: Plasma Transfusions from adult exercise mice ameliorates spatial learning and memory in middle-aged sedentary mice in an autophagy dependent manner.

(6A) Middle-aged 32-week-old sedentary mice received 8 plasma injections over the period of 22 days from 10-week-old mice and then the middle-aged mice were trained for five days for spatial learning in the Morris water maze test. Middle-aged sedentary animals receiving intraperitoneal injections of plasma from the adult exercising animals significantly reduced escape latency compared to the middle-aged sedentary animals which received plasma from adult sedentary mice. Middle aged sedentary animals receiving plasma from adult exercise chloroquine animals showed significantly increased escape latency compared to sedentary animals receiving plasma from adult exercise saline animals. Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. The asterisk sign (*) shows significance between animals receiving plasma from adult exercise saline mice versus plasma from adult exercise CQ mice. The octothorpe sign (#) shows significance between animals receiving plasma from adult exercise animals versus plasma from adult sedentary animals (n= 7 10wo exercise plasma , n=6 10wo sedentary plasma, n =7 10wo exercise CQ plasma, n =6 10wo sedentary CQ plasma). (6B) Middle-aged 32-week-old sedentary that received plasma from adult exercise mice spent more time in the target quadrant during the memory test day of the Morris water maze as compared to sedentary animals receiving plasma from adult exercise CQ mice, and from adult sedentary saline mice. Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, and ***p<0.001 ****p<0.00001 (n= 6 10wo exercise plasma , n=7 10wo sedentary plasma, n =7 10wo exercise CQ plasma, n =6 10wo sedentary CQ plasma). (6C) Middle aged sedentary animals receiving plasma from middle-aged exercise animals do not show any difference in escape latency compared to sedentary animals receiving plasma from middle-aged sedentary saline animals. Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test (n=7 32wo exercise plasma, n=4 32wo sedentary plasma). (6D) Middle-aged 32-week-old sedentary that received plasma from middle-aged exercise mice did not

show any difference in the time spent in the target quadrant during the memory test day of the Morris water maze as compared to sedentary animals receiving plasma from middle-aged sedentary animals. Results are expressed as Mean +/- SEM. Statistical significance was measured by unpaired t-test (n=7 32wo exercise plasma, n=4 32wo sedentary plasma). (6E) Representative western blot image depicting the increase in the autophagy marker LC3B protein levels in the hippocampus of middle-aged sedentary animals that received plasma from adult exercise animals as compared to the middle-aged mice that received plasma from adult exercise CQ mice. (6F) Quantification of the LC3B western blot. Statistical significance was measured by unpaired t-test * $p<0.05$ (n=5 exercise plasma, n=5 exercise CQ plasma).

3.7 Lactate does not mediate its positive effects on spatial memory in an autophagy dependent manner

The beneficial effects of exercise on the brain's cognitive functions are mediated by multiple factors secreted by the bones, the muscles, and the liver (Stephan & Sleiman, 2021). The muscles secrete lactate which significantly increases BDNF protein levels and enhances spatial learning and memory formation in mice (El Hayek et al., 2019). Subsequently, we investigated whether lactate was the factor that promotes spatial learning and memory formation in an autophagy dependent manner. We injected 10-week-old mice with either lactate, lactate CQ, saline or saline CQ four days prior and during their behavioral testing. The concentration of lactate used mimics the levels of lactate in the plasma after exercise (El Hayek et al., 2019). Lactate didn't induce LC3B levels in the hippocampus (n=10 saline, n = 5 lactate, n = 5 lactate CQ, n=6 saline CQ) (Figures 3.7A and 3.7B). Consistent with previous observations (El Hayek et al. 2019), lactate promoted learning and memory formation. Indeed, mice receiving lactate had a significantly reduced escape latency as compared mice receiving saline on day five. In addition, these mice spent significantly more time in the target quadrant. While inhibition of autophagy by CQ abolished lactate's ability to promote learning (n= 54 saline, n=41 saline CQ, n =43 lactate, n =38 lactate CQ). (Figure 3.7C), it did not have any effects on memory formation (n= 52 saline, n=41 saline CQ, n =42 lactate, n =38 lactate CQ). (Figure

3.7D). Thus, our results suggest that lactate does not induce autophagy in the hippocampus, nor does it promote spatial memory formation in an autophagy dependent manner.

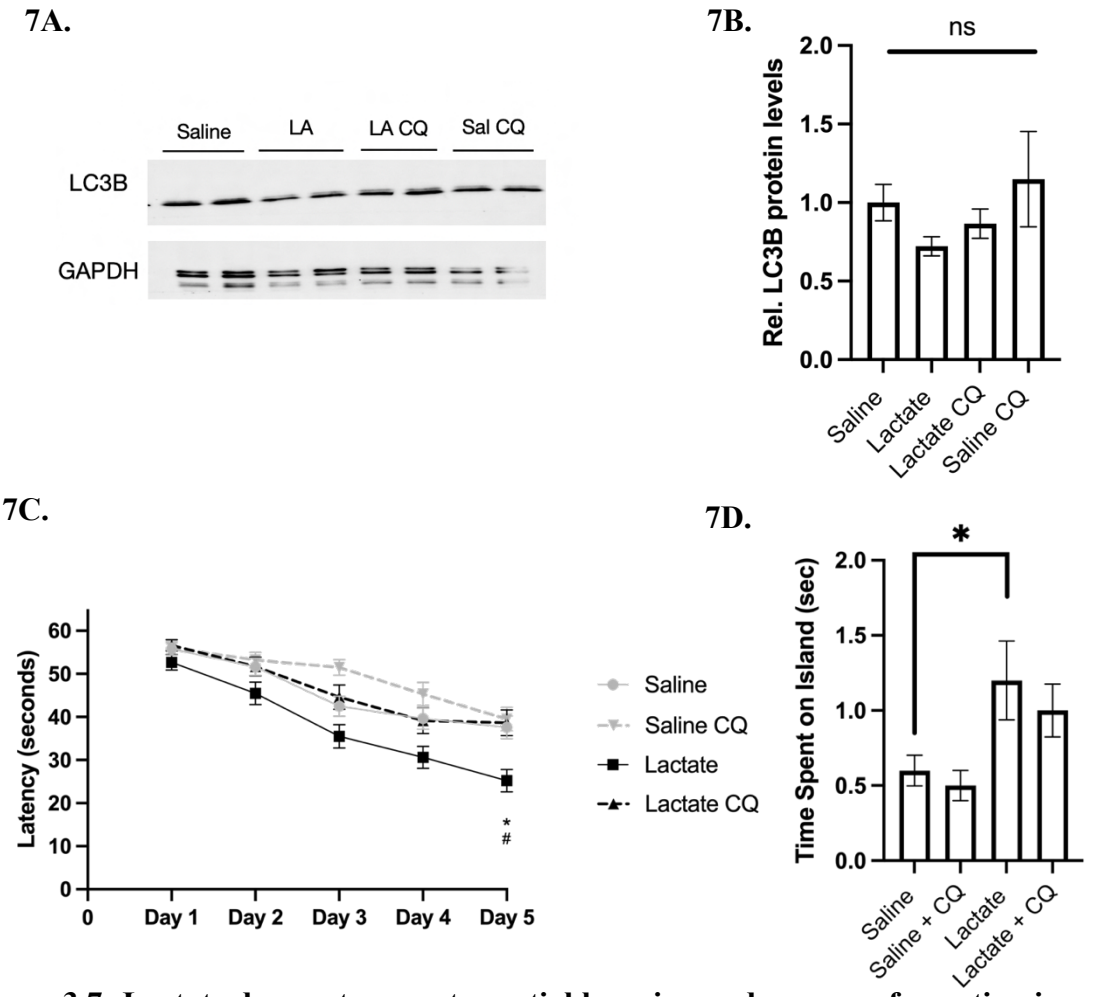

Figure 3.7: Lactate does not promote spatial learning and memory formation in an autophagy dependent manner.

(7A) Representative western blot image of the autophagy marker LC3B protein levels in the hippocampus of adult 10-week-old lactate, lactate CQ, saline, and saline CQ animals. (7B) Quantification of the LC3B western blot. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test (n=10 saline, n = 5 lactate, n = 5 lactate CQ, n=6 saline CQ). (7C) Adult 10-week-old mice received intraperitoneal injections of lactate (180 mg/kg), or lactate combined with Chloroquine phosphate, or saline and saline CQ, four days prior and during their behavioral training for the Morris water maze. Animals receiving intraperitoneal injections of lactate

(180mg/kg) showed significantly reduced escape latency on day 5 compared to animals receiving saline, and lactate CQ injections. Results are expressed as Mean +/- SEM. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. The asterisk sign (*) shows significance between animals receiving lactate versus animals receiving saline. The octothorpe sign (#) shows significance between animals receiving lactate versus lactate CQ animals (n= 54 saline , n=41 saline CQ, n =43 lactate, n =38 lactate CQ). (7D) Adult 10-week-old mice receiving lactate injections spent more time in the target quadrant during the memory test day of the Morris water maze as compared to animals receiving saline injections only. Results are expressed as Mean +/- SEM (n= 52 saline , n=41 saline CQ, n =42 lactate, n =38 lactate CQ). Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, and ***p<0.001 ****p<0.00001.

3.8 D-β-hydroxybutyrate (DBHB) mediates its positive effects on learning and memory in the brain in an autophagy dependent manner

Another exercise-induced factor, the ketone body DBHB is secreted by the liver, inhibits histone deacetylases such as HDAC2 and HDAC3, and increases the levels of BDNF *in vitro* and *in vivo* in the hippocampus of mice. (Sleiman et al. 2016). Therefore, we investigated whether DBHB was the factor that promotes spatial learning and memory formation in an autophagy dependent manner. Interestingly, young mice that underwent voluntary exercise showed significantly higher levels of DBHB in their plasma as compared to exercise mice treated with the autophagy inhibitor CQ (n=4 xercise, n=4 exercise CQ). (Figure 3.8A). Indeed, DBHB also induced LC3B levels in the hippocampus (Figures 3.8B and 3.8C), and this increase was abolished by CQ treatment (n= 10 saline, n=10 DBHB, n= 6 DBHB CQ, n=6 saline CQ). Consistent with these results, DBHB promoted learning and memory formation. Indeed, mice receiving DBHB had a significantly reduced escape latency on day 2 and this effect was abolished by CQ treatment (n= 52 saline , n=41 saline CQ, n =12 DBHB, n =7 DBHB CQ) (Figure 3.8D). Moreover, mice receiving DBHB spent significantly more time in the target quadrant as compared to control mice. CQ abolished DBHB's effect on memory formation (n= 52 saline, n=41 saline CQ, n =12 DBHB, n =6 DBHB CQ) (Figure 3.8E). Thus, our results

suggest that DBHB is an exercise factor that is released into the circulation in an autophagy dependent manner. DBHB also induces autophagy in the hippocampus to promote learning and memory formation.

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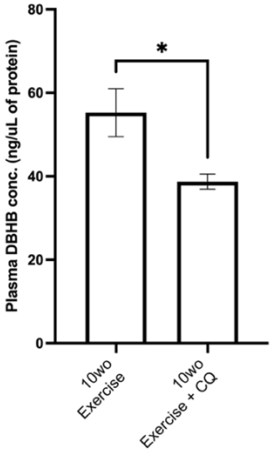

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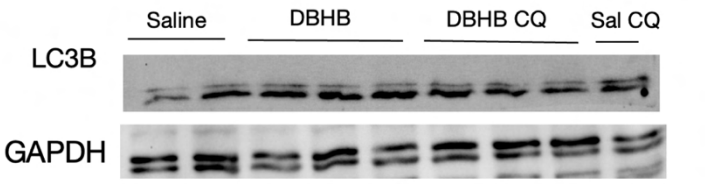

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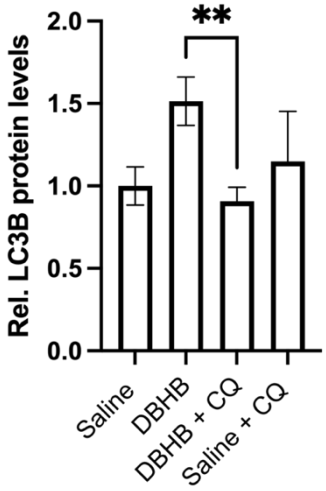

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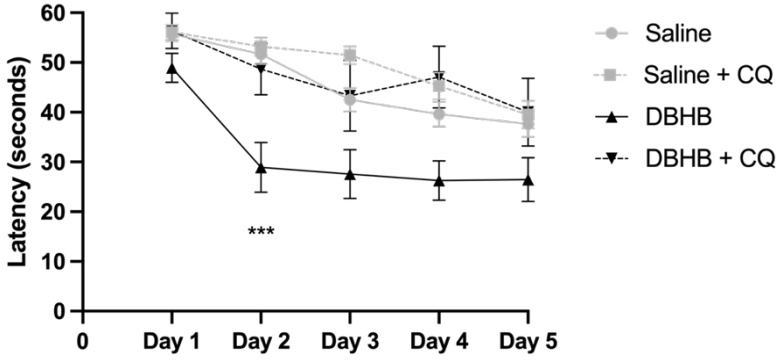

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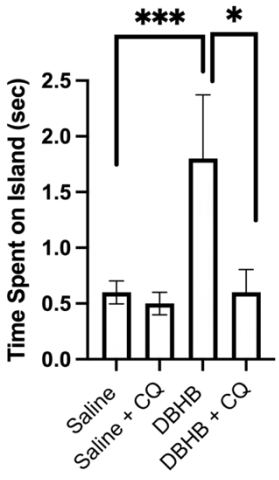

Figure 3.8: DBHB does promote spatial learning and memory formation in an autophagy dependent manner.

(8A) Adult 10-week-old exercise mice that received intraperitoneal injections of Chloroquine phosphate (50mg/kg) during their behavioral training show a significant decrease in the plasma DBHB levels. The number of plasma samples used is 4 for each group (exercise versus exercise CQ). Statistical significance was measured by unpaired t-test. *p<0.05. (8B) Representative western blot image depicting the increase in the autophagy marker LC3B protein levels in the hippocampus of adult 10-week-old DBHB animals as compared to DBHB CQ animals. (8C) Quantification of the LC3B western blot. Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test (n= 10 saline, n=10 DBHB, n= 6 DBHB CQ, n=6 saline CQ). (8D) Adult 10-week-old mice received intraperitoneal injections of DBHB (100 mg/kg), or DBHB combined with Chloroquine phosphate, or saline and saline CQ, four days prior and during their behavioral training for the Morris water maze. Animals receiving intraperitoneal injections of DBHB (100mg/kg) showed significantly reduced escape latency on day 2 compared to animals receiving saline, and DBHB CQ injections. Results are expressed as Mean +/- SEM (n= 52 saline , n=41 saline CQ, n =12 DBHB, n =7 DBHB CQ). Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. (8E) Adult 10-week-old mice receiving DBHB injections spent more time in the target quadrant during the memory test day of the Morris water maze as compared to animals receiving saline or DBHB CQ injections. Results are expressed as Mean +/- SEM (n= 52 saline , n=41 saline CQ, n =12 DBHB, n =6 DBHB CQ). Statistical significance was measured by Two-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, and ***p<0.001 ****p<0.00001.

Chapter Four

Discussion

Our results provide a link between voluntary exercise, autophagy, and exercise-induced plasma factors that can enhance spatial learning and memory formation in aging mice. Physical exercise has shown promise as an effective treatment for a wide range of neurodegenerative diseases (Bliss et al., 2021). Previous work on physical exercise shows that exercise intervention promotes neurogenesis via increasing exercise-induced factors such as lactate, D-β-Hydroxybutyrate, irisin, and cathepsin-B. These factors converge on inducing the expression of BDNF (Valenzuela et al., 2020; El Hayek et al., 2019; Sleiman et al., 2016), hence promoting learning and memory formation, as well as resilience to depression and anxiety (van Praag, 2005; Gujral et al., 2017).

Consequently, physical exercise could potentially promote healthy aging through the activation of several pathways that are yet to be fully discovered. The risk of developing neurodegenerative diseases such as Alzheimer's or Parkinson's disease and cognitive impairments increase with aging (Hou et al., 2019), hence it is central we uncover the underlying molecular pathways of exercise and its benefits on the aging population. One of the molecular hallmarks of aging includes buildup of dysfunctional and damaged intracellular proteins in the brain (Kaushik & Cuervo, 2015).

On the other hand, caloric restriction has been shown to stimulate BDNF signaling in the hippocampus and prevent the transcription of autophagy genes, leading to an improved memory performance (Nikoletopoulou et al., 2017). Thus, because autophagy plays an important role in protein homeostasis and since caloric restriction and physical exercise share similar molecular signaling pathways (Kaushik et al., 2021), we decided to test whether autophagy plays a role in exercise-induced spatial learning and memory formation, through regulating the release of different exercise-induced plasma factors. Interestingly, studies on juvenile plasma show that the effects of aging could be potentially reversed or prevented especially in the brain by regulating numerous biological pathways and changing the track of age-associated diseases (Kheifets & Braithwaite, 2019).

First, we tested whether voluntary exercise induces autophagy in the hippocampus of young 10-week-old male mice. Previous studies demonstrated exercise as an inducer of autophagy in peripheral tissues and in the brain (He et al., 2012; Brandt et al., 2018). Indeed, we discovered that exercise induces autophagic activity in the hippocampus of young mice (Figures 3.1A and 3.1B). These observations suggest a possible regulation role for autophagy in response to exercise.

Next, we wanted to examine whether voluntary exercise can promote learning and memory formation in the brain of young mice in an autophagy dependent manner. After fourteen days of voluntary exercise, we inhibited autophagy daily for eight consecutive days during the animals' behavioral training, using a brain permeable molecule chloroquine phosphate (50mg/kg). Our results demonstrated that inhibition of autophagy decreases spatial learning and memory performance in young mice (Figures 3.2A and 3.2B). These findings suggest that autophagy could potentially regulate learning and memory formation in the hippocampus.

Previous work shows that exercise increases BDNF levels and that BDNF is necessary for exercise's positive effects on spatial learning and memory formation (Kesslak et al., 1998; Mizuno et al., 2000). For that reason, we examined the relationship between exercise-induced autophagy and BDNF protein levels. We found that inhibition of autophagy with CQ drastically decreased BDNF protein levels in the hippocampus of adult exercise mice (Figure 3.3A and 3.3B). A simple conclusion from these results is that exercise induces BDNF in an autophagy-dependent manner. As a result, one could predict that if BDNF signaling is downstream of autophagy, activating the signaling pathway would bypass the inhibition of autophagy by CQ and could promote learning and memory formation in the presence of the autophagy inhibitor. We, therefore assessed whether BDNF signaling is upstream or downstream of autophagy. We activated BDNF signaling with 7,8-DHF, a Trk-B receptor agonist, to look at whether activation of BDNF can bypass the inhibition of autophagy with CQ. Our results show that exercise mice perform better than exercise mice treated with 7,8-DHF and CQ in the learning and memory test. (Figures 3.4A and 3.4B). There was no difference in performance between mice treated with 7,8-DHF combined with CQ during the probe memory test. These findings suggest that

activation of BDNF signaling using 7,8-DHF could not bypass inhibition of autophagy and did not rescue the attenuation of learning and memory formation seen with inhibition of autophagy. Our results support the hypothesis that BDNF signaling is upstream of autophagy and that autophagy is exerting a positive feedback loop on BDNF signaling to promote learning and memory formation.

We were next interested in understanding whether the same exercise paradigm could promote learning and memory formation in old (1-year-old) and middle aged (32-week-old) mice. Unlike young mice, voluntary exercise did not increase spatial learning and memory formation in old and middle-aged mice (Figures 3.5A-3.5D). Previous studies that showed beneficial effects of exercise on learning in aged mice allowed mice to exercise for a longer time (van Praag et al., 2005; Brett et al., 2020).

Recent studies show that delivering blood factors in plasma from exercised aged mice could transfer the benefits of physical exercise to inactive old mice. Scientists identified a liver to brain connection by which plasma factors can enhance the effects of exercise on neurogenesis (Horowitz et al., 2020). Thus, we decided to focus on middle-aged (32-week-old) mice to test whether plasma transfusions from young exercising mice induce learning and memory formation in sedentary middle-aged mice. Interestingly, sedentary middle-aged mice receiving plasma from young exercise mice showed improved spatial learning and memory performance compared to mice receiving young sedentary plasma. However, when sedentary middle-aged mice received plasma of young exercise mice treated with CQ, we could clearly observe attenuation of learning and memory (Figures 3.6A and 3.6B). Similarly, we observed an induction of autophagic activity in the hippocampus of sedentary aged mice that received plasma from young exercise mice compared to sedentary aged mice that received plasma from young exercise mice treated with CQ (Figures 3.6E and 3.6F). These results suggest that plasma from young exercise mice ameliorates spatial learning and memory in middle-aged mice in an autophagy dependent manner.

To demonstrate that exercise and young plasma are both factors in mediating the positive effects on cognition in middle-aged mice, we administered middle-aged sedentary and exercise plasma into middle-aged sedentary mice. Results show no difference in

learning and memory performance in animals receiving sedentary or exercise plasma from middle-aged mice (Figures 3.6C and 3.6D). The data supports our hypothesis that in young animals, exercise induces peripheral tissues to release factors into the circulation in an autophagy dependent manner and that these plasma factors induce spatial learning and memory formation in middle-aged mice.

In the brain, lactate is required for long-term memory formation (Suzuki et al., 2011). It is an exercise-induced metabolite that promotes learning and memory formation in a BDNF-dependent manner (El Hayek et al., 2019). Another exercise-induced metabolite is the ketone body DBHB, which was shown to inhibit histone deacetylases, and increase BDNF levels (Sleiman et al., 2016). For this reason, we tested whether lactate and DBHB mediate the positive effects of exercise in an autophagy-dependent manner. Our results show that there is no difference in the levels of the autophagy marker LC3B in lactate treated mice versus saline treated mice (Figures 3.7A and 3.7B). While lactate induces learning and memory formation in the brain, it does not do so in an autophagy dependent manner (Figures 3.7C and 3.7D). In contrast, our results are consistent with DBHB promoting learning and memory formation in an autophagy-dependent manner. Exercise mice show increased plasma DBHB levels compared to exercise mice that received CQ. This suggest that the release of DBHB into the plasma is autophagy dependent. Moreover, DBHB increased the levels of LC3B in the hippocampus, as well as enhanced learning and memory performance compared. Inhibition of autophagy attenuated the positive effects of DBHB (Figures 3.8A – 3.8E). Thus, these findings are consistent with DBHB mediating its positive effects on learning and memory in an autophagy-dependent manner. These results also suggest DBHB could be one of the factors responsible for the positive effects on learning and memory observed in plasma transfusions. One could argue if all these different molecules and metabolites (Figure 1.1) have a synergistic effect on exercise-induced learning and memory performance. Moon et al. (2016), demonstrate that a knockout of cathepsin B in exercise mice models impairs learning and memory formation (Moon et al., 2016). Hence, it will be of interest to look at whether DBHB knockout in young exercise mice could also impair

learning and memory or is it a synergistic effect of several different molecules that enhance learning and memory performance.

In conclusion, this study supports the evidence that there is a separation from what's happening inside the brain versus what's happening in the peripheral tissues regarding exercise and autophagy. The data suggests that exercise induces the release of different factors such as DBHB from different organs such as the liver. This release is dependent on the activation of autophagy. These factors in turn induce BDNF expression in the hippocampus. BDNF signaling itself activates autophagy in the hippocampus. This activation creates a positive feedback loop between BDNF and autophagy in the hippocampus leading to enhanced spatial learning and memory formation (Figure 4.1).

For future studies, we still need to further identify the different exercise factors present in the plasma that are autophagy and BDNF dependent, and that can promote spatial learning and memory formation.

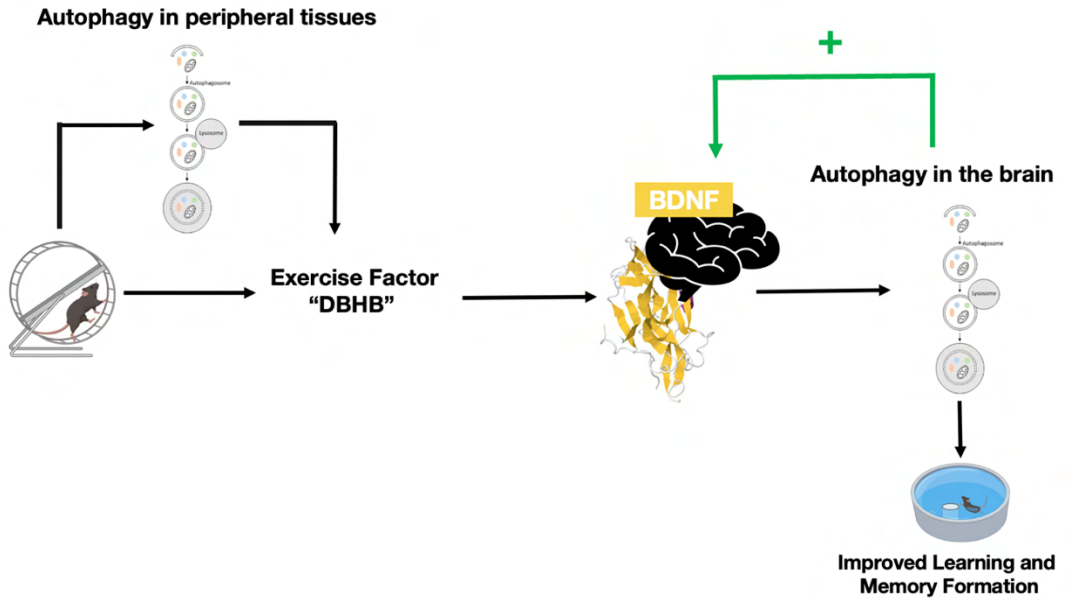

Figure 4.1: Proposed mechanism of exercise induced factors and autophagy on the brain's cognitive abilities

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