Irisin as a mediator of the positive relationship between exercise and the brain in health and Alzheimer's disease

by

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B.S., Kansas State University, 2021
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AN ABSTRACT OF A DISSERTATION

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease impacting over 6 million Americans with cases projected to increase to over 14 million by 2060. The AD process creates difficulty completing everyday tasks or conversations, and ultimately, progresses to disrupt the most basic bodily functions and require full-time caretaking. While disease modifying therapy remains elusive, reducing the incidence of AD is crucial in order to mitigate the projected increase in cases. Exercise has emerged as an effective strategy to promote brain health in late adulthood and to protect against the onset of AD. Exercise opposes several disease processes including cognitive dysfunction, amyloid beta aggregation, tau phosphorylation, and deficits in hippocampal volume, mitochondrial function, cerebral blood flow, and neurogenesis through various pathways including the systemic release of exerkines. The exerkine irisin is an important mediator of the beneficial relationship between exercise and the brain. Previous work administering irisin therapeutically to healthy and preclinical AD mice has demonstrated irisin to replicate multiple exercise-induced effects in the brain and protect against AD-induced deficits. Although irisin is suggested as a promising strategy to promote brain health in late adulthood, our understanding of irisin signaling and irisin-induced protection against AD remains incomplete. Through behavioral testing and protein analyses in healthy and transgenic preclinical AD rats, this dissertation assessed the physiological role of irisin and the translatability of irisin-induced protection against AD. In our first investigation (chapter 2), we found that a single administration of irisin was sufficient for activation of irisin-hippocampus signaling. In our second investigation (chapter 3), we found that the cofactor extracellular heat shock protein 90 alpha, which had been previously demonstrated to be required for irisin signaling in adipose tissue, is not necessary for irisin-receptor interaction in the brain. In our third investigation

(chapter 4), we aimed to investigate AD phenotype in transgenic rats following an adenovirus intervention previously demonstrated to stimulate irisin production in AD mice. We found no increase in irisin levels or irisin signaling in our transgenic AD rats following vector administration. Taken together, these studies suggest irisin as an important, physiologically relevant promoter of brain health, however, the translatability of irisin-mediated neuroprotection in aged transgenic AD rats remains untested.

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Chapter 1 - Introduction and Literature Review

Alzheimer's Disease: Past, Present, and Future

One out of every nine people in the U.S. over the age of 65 is living with Alzheimer's disease (AD). By 2060, cases of AD in the U.S. are projected to increase by 127% to nearly 14 million, representing a rise in prevalence to approximately one out of every seven people (Rajan et al., 2021; U.S. Census Bureau, 2014). During this period, the annual cost of care for those with AD is expected to increase from $384 billion (2025) to $1 trillion (2050) in the absence of treatment breakthroughs. These figures represent a looming threat for caregivers, including both medical workers and family members, who currently report high levels of emotional and physical stress (Alzheimer's Association, 2025).

AD is a progressive neurodegenerative disease and the most common form of dementia. Those affected experience symptoms that disrupt daily life including memory loss, difficulties with problem solving and holding conversations, and changes in personality or behavior (Alzheimer's Association, 2025). AD exists on a continuum beginning in an asymptomatic preclinical stage, which may last for over 20 years prior to the onset of clinical symptoms (Gordon et al., 2018). During this period (clinical stages 1 and 2), changes in the brain begin and increase in severity over time. These changes include pathologic processing of amyloid beta (Aβ) and tau proteins, atrophy and neuronal loss, reductions in blood flow and mitochondrial function, and loss of ability to generate new neurons and neuronal connections (i.e., hippocampal neurogenesis) (Gordon et al., 2018; Iturria-Medina et al., 2016; Moreno-Jiménez et al., 2019). These deficits are accelerated in the hippocampus, a structure within the medial temporal lobe that is essential for memory function (Knierim, 2015). Consequently, the first sign of AD leading to a clinical diagnosis is often a decline in memory function. Once this occurs, patients are

considered to have mild cognitive impairment (clinical stage 3), a stage in which patients objectively display small declines in cognition and perform most activities of daily life independently (Alzheimer's Association, 2025). Subsequently, as disease severity worsens, patients are considered to have AD, and progress through mild, moderate, and severe AD (clinical stages 4-6). During this time, cognitive and functional impairments advance resulting in complete dependence on a caregiver for even the most basic tasks, and, ultimately, death (Alzheimer's Association, 2025).

Older age imposes the greatest risk of developing AD, and many of the brain changes that are present in AD also occur during physiologic aging, albeit at a later age and to a lesser extent. Prior to the transition from mild to moderate AD, brain changes that occur due to AD are only subtly different from those occurring during healthy aging (Alzheimer's Association, 2025). In addition to age, brain health in late adulthood is dictated by environment and genetics — this is summated neatly as modifiable and non-modifiable risk factors for AD and dementia.

The current report of the *Lancet* commission identifies 14 modifiable risk factors for dementia including education in early life; hearing loss, high LDL cholesterol, depression, traumatic brain injury, physical inactivity, smoking, diabetes, hypertension, obesity, and excessive alcohol consumption in midlife; social isolation, air pollution, and untreated vision loss in late life. Together, these risk factors are estimated to be associated with nearly half (45.3%) of worldwide cases of dementia (Livingston et al., 2024).

Non-modifiable risk factors are subdivided into deterministic and risk-associated genes. Deterministic genes for AD include amyloid precursor protein (APP), presenilin 1 (PSEN1), and PSEN2. Inheritance of a mutated version of any one of these genes virtually ensures AD will develop with symptoms typically developing before age 65; this represents approximately 1% of

AD cases (Bekris et al., 2010; Goldman et al., 2011). In addition to deterministic genes, there are 75 known genetic variants associated with the risk of developing AD (Bellenguez et al., 2022). Of these, the apolipoprotein E (APOE) gene, primarily involved in cholesterol transport in blood, holds the strongest association with either increased or decreased risk, depending on which alleles are present. Risk is increased by carrying one e4 allele and increased further when carrying two e4 alleles. Conversely, the e2/e3 genotype is protective against AD in comparison to the most common e3/e3 (Farrer et al., 1997; Loy et al., 2014; Qian et al., 2017). Our understanding of these genetic and environmental risk factors provides hope of preventing the onset of AD and creates opportunities for more accurate disease models.

As described above, AD is a progressive disease with a long dormant period and is closely tied to the aging process. As a result of these characteristics, investigators have faced challenges recruiting research participants prior to the onset of clinical symptoms to assess interventional candidates, provide a better understanding of early disease stages, or differentiate the timing and severity of brain changes due to aging and AD. One strategy to overcome these issues is through the use of animal models. AD research models are numerous and range in complexity from invertebrate animals, including C. elegans and Drosophila, to non-human primates, although mouse and rat rodent models are most commonly used (Drummond & Wisniewski, 2017). Importantly, in comparison to mice, rats are closer to humans in their behavior, development, genetics, and physiology (Do Carmo & Cuello, 2013). The TgF344-AD transgenic rat model of AD was generated using two known etiologic mutations in deterministic genes, specifically the “Swedish” mutant human APP and Δ exon 9 mutant human PSEN1 genes (Cohen et al., 2013). Similar to the human condition, TgF344-AD rats display progressive age-

dependent cognitive impairment, Aβ and tau pathology, neuronal loss, and deficits in cerebral blood flow and mitochondrial function (Cohen et al., 2013; Joo et al., 2017; Viel et al., 2021).

Exercise-Induced Neuroprotection in Aging and Alzheimer's Disease

Exercise promotes brain health in older adults and beneficial effects are present predominantly in the hippocampus. The following discussion will support this claim by providing evidence from older adults in health and disease and by discussing mechanisms through which this relationship is mediated. While both exercise and physical activity (PA) promote brain health, it is important to first distinguish between the two. Briefly, exercise is a subcategory of PA. Definitions of both include bodily movement via skeletal muscles resulting in energy expenditure, and both are positively correlated with physical fitness. The definition of exercise also extends to include being a planned bodily movement that is done to maintain or improve physical fitness (Caspersen et al., 1985). The current understanding of factors that influence brain health in late adulthood was formed by both retrospective studies, which often assess PA levels while statistically controlling for other variables, and prospective studies, which often assess an exercise intervention while methodologically controlling for other variables (i.e., age, sex, cognitive function, cardiovascular risk factors, etc.).

Exercise and PA are promising strategies to protect against and treat AD. This is supported by the fact that physical inactivity is a modifiable risk factor for AD (Livingston et al., 2024). Recent work analyzing longitudinal data collected in over 78,000 older adults found an optimal dose of ~10,000 steps per day to be associated with a 50% reduced risk (hazard ratio [HR] 0.49, 95% CI 0.39-0.62) of incident all-cause dementia (Del Pozo Cruz et al., 2022). Systematic review and meta-analysis of 58 investigations including cognitively healthy adult

cohorts provides stronger evidence that increased levels of PA reduces risk of incident all-cause dementia (relative risk [RR] 0.80, 95% CI 0.77-0.84, n=257983) and AD (RR 0.86, 95% CI 0.80-0.93, n=128261) (Iso-Markku et al., 2022). Similarly, meta-analysis of randomized control trials investigating the effect of exercise interventions on cognitive function in dementia patients found a positive effect in all-cause dementia (standardized mean difference [SMD] 0.42, 95% CI 0.23-0.62, n=691) and for interventions containing aerobic exercise in AD (SMD 0.59, 95% CI 0.32-0.86, n=691) (Groot et al., 2016). Observational studies in cognitively intact humans suggest beneficial associations of PA with Aβ and tau burdens (Liang et al., 2010; Rabin et al., 2019) in addition to protecting against other early AD-induced brain changes.

In healthy older adults, the beneficial relationship between PA and cognitive function was first demonstrated in the 1970s in a study demonstrating better reaction times in physically active older men in comparison to sedentary, age-matched controls (Spirduso & Clifford, 1978). Years later, these findings were expanded on in a landmark study from Erickson et al. (2011) who subjected 120 healthy older adults to either a year-long aerobic exercise program consisting of moderate-intensity walking (3 days per week, 40 minutes per day) or a stretching control treatment. Longitudinal MRI imaging demonstrated an average 2% increase in hippocampal volume in the exercise group. Opposingly, hippocampal volume decreased an average ~1.5% in the stretching control group. Further, changes in hippocampal volume positively correlated with changes in serum BDNF, cardiorespiratory fitness, and performance on a hippocampus-dependent task of spatial memory. These results are striking considering that non-demented older adults experience an annual 1-2% decrease in hippocampal volume (Raz et al., 2005). A second investigation subjecting healthy older adults to either aerobic exercise or stretching control groups found an increase in hippocampal blood flow and blood volume in the exercise group.

Similarly, changes in aerobic fitness, hippocampal blood flow, hippocampal volume, and a hippocampus-dependent task of recognition memory were positively correlated (Maass et al., 2015).

Neurogenesis occurs in the dentate gyrus subregion of the hippocampus and is an important process through which exercise promotes aging brain health. van Praag et al. demonstrated that both young and old mice with free running wheel access displayed increased neurogenesis in the dentate gyrus (van Praag, Christie, et al., 1999; van Praag et al., 2005; van Praag, Kempermann. Gerd, et al., 1999). Similarly, using dentate gyrus blood volume as a validated proxy for neurogenesis, Pereira et al. suggest that exercise promotes neurogenesis in humans and that increased neurogenesis correlates with aerobic fitness and cognitive function (Pereira et al., 2007). A crucial mediator of exercise-induced neurogenesis is brain-derived neurotrophic factor (BDNF). BDNF is upregulated by exercise (Coelho et al., 2013) and is a vital promoter of neuronal differentiation, synaptic plasticity, and overall neuronal health (Mattson et al., 2004).

In addition to volumetric, neurogenic, and blood flow changes, exercise maintains cerebral mitochondrial function in late adulthood. Analysis of post-mortem human hippocampal brain tissue demonstrates PA to oppose age-related gene transcription patterns. Genes conferring mitochondrial function, especially in the electron transport chain, were most prominently differentially expressed (Berchtold et al., 2019). Concordantly, animal studies demonstrate exercise to induce peroxisome proliferator-activated receptor-gamma coactivator (PGC1-α)-dependent hippocampal mitochondrial biogenesis as well as increases in respiratory capacity and formation of new synapses (Dietrich et al., 2008; Steiner et al., 2011). Of note, these respiratory and synaptic benefits are suggested to be dependent on uncoupling protein 2 (UCP; UCP2)-

regulated mitochondrial adaptation, as these outcomes are absent following exercise in UCP2 knock-out mice (Dietrich et al., 2008). Together, these results suggest that there is a significant link between structure and function in the aging brain, which manifests preferentially in the hippocampus and is positively impacted by aerobic exercise.

Exerkines, or exercise-induced factors that mediate systemic effects of exercise, have grown interest in recent years as important molecular mediators of these processes. Exerkines that help to mediate the beneficial relationship between exercise and the brain include lactate, cathepsin B, β-hydroxybutyrate, and, importantly, irisin. Irisin has gained special attention due to having a close relationship with BDNF and demonstrated potential to protect against AD.

Irisin Signaling Mediates the Effects of Exercise

Named after the Greek messenger goddess Iris, irisin is a 112-amino acid signaling peptide which systemically mediates influences of exercise (Boström et al., 2012). Following the discovery of irisin in 2012, research describing irisin physiology in health and disease, including effects of irisin in different tissue types, irisin-receptor interactions, relevant intracellular signaling pathways, and therapeutic potentials of irisin has flourished.

Irisin was first described by Boström et al. (2012) as a promoter of adipose browning and thermogenesis. It had been well characterized that, within muscle tissue, aerobic exercise promotes PGC1-α expression, which results in increased mitochondrial biogenesis and oxidative function (Choi et al., 2008; Puigserver et al., 1998; Wu et al., 1999). After finding increased adipose browning, a cellular process linked to thermogenesis involving mitochondrial uncoupling proteins (UCPs) and changes in oxidative metabolism, in mice with muscle-specific PGC1-α overexpression, Boström et al. reasoned that there must be exercise-linked, PGC1-α-

dependent factors secreted from muscle that can influence other tissues. To evaluate this, the authors first determined a list of candidate secreted proteins by probing culture media conditioned by PGC1-α expressing myocytes. They then applied commercially available candidates to white adipocytes and evaluated transcriptional changes. The candidate protein FNDC5 emerged as a robust promoter of thermogenic gene programing in white adipose tissue. Further analysis of the conditioned culture media determined that FNDC5, a type 1 transmembrane protein, had undergone proteolytic cleavage resulting in a portion of the extracellular domain being secreted, and it was this polypeptide that was responsible for inducing the browning of white adipocytes. The secreted polypeptide was named irisin (Boström et al., 2012).

Shortly thereafter, a role for irisin within the beneficial relationship between exercise and the brain gained recognition (Wrann et al., 2013). In both CNS neurons (including hippocampal neurons) and myocytes, PGC1-α (Lin et al., 2004) and FNDC5 (Dun et al., 2013) are expressed. Further, PGC1-α regulates FNDC5 expression and cleavage (Boström et al., 2012; Dun et al., 2013; Wrann et al., 2013) and aerobic exercise upregulates PCG1-α (Steiner et al., 2011) and FNDC5 expression (Wrann et al., 2013). Beneficial effects of exercise in the brain are mediated by irisin originating from either tissue type in two separate mechanisms. FNDC5/irisin transcribed in the hippocampus directly promotes BDNF transcription. Elevated BDNF expression in turn reduces FNDC5 expression creating a negative feedback loop (Wrann et al., 2013). Peripherally derived irisin crosses the blood brain barrier (Islam et al., 2021) and induces an intracellular signaling cascade resulting in transcription factor cyclic AMP-response element binding protein (CREB) phosphorylation at Ser133 and, subsequently, BDNF promotion (H. Guo et al., 2017; Lourenco et al., 2019). In addition, irisin has been reported to promote extracellular

signal-related kinase 1/2 (ERK1/2) phosphorylation in a pathway possibly downstream of CREB (Li et al., 2017; Lourenco et al., 2022).

Notably, irisin has been proposed as a therapeutic for AD. Previous attempts to administer irisin therapeutically in preclinical AD mouse models have demonstrated positive results on AD pathology. Irisin was shown to replicate portions of the benefits of exercise on the aging brain such as reduced glial cell activation, reduced tau pathology, and improved synaptic plasticity; ultimately, irisin therapy rescued AD-induced cognitive phenotypes (Bretland et al., 2021; Islam et al., 2021; Lourenco et al., 2019). While these results are promising, the aging- and AD-brain experience a plethora of deficits that may be reversed by exercise including declines in volume (Erickson et al., 2011), mitochondrial function (Berchtold et al., 2019; Steiner et al., 2011), blood flow (Maass et al., 2015; Palmer et al., 2023), and neurogenesis (Swain et al., 2003; Van Praag et al., 2005). Further work is needed to understand the possible influence of irisin on these mechanisms in AD.

In healthy adults, plasma irisin concentration increases as a result of exercise (Jedrychowski et al., 2015) and a positive association exists between plasma irisin concentration and cognitive performance (K. Y. Kim et al., 2022). In AD patients, however, while plasma irisin is unaltered (K. Y. Kim et al., 2022; Lourenco et al., 2019), cerebrospinal fluid (CSF) irisin is significantly reduced and increased plasma irisin is not associated with improved cognitive performance (K. Y. Kim et al., 2022). Indeed, CSF FNDC5/irisin and age are positively associated in cognitively normal adults and negatively associated in AD patients (Lourenco et al., 2019). In addition, FNDC5/irisin is reduced in the hippocampi of late-stage AD patients compared to both early-stage patients and healthy controls (Lourenco et al., 2020). Together, this suggests that AD disturbs irisin physiology by either disrupting irisin uptake at the blood-brain

barrier or hippocampal FNDC5/irisin expression. This relationship is also seen in mouse models of AD with reductions in hippocampal FNDC5/irisin and *Fndc5* mRNA compared to healthy controls (Islam et al., 2021; Lourenco et al., 2019). Importantly, both exercise and peripherally delivered therapeutic irisin restore hippocampal FNDC5/irisin levels and cognitive function in mouse models (Lourenco et al., 2019). Currently, the FNDC5/irisin dysregulation in the TgF344-AD rat remains unknown and the translatability of therapeutic irisin remains solely informed by pre-clinical mice.

Irisin was originally characterized to upregulate UCP1 in white adipose tissue, thereby contributing to adipocyte browning and thermogenesis (Boström et al., 2012). Subsequently, an acute dose of irisin given to healthy rats regulated transcription of UCP genes in the hippocampus and cortex and increased systemic metabolic rate (Erden et al., 2016). Investigations in pre-clinical models of depression (Wang & Pan, 2016), traumatic brain injury (P. Guo et al., 2021), and stroke (Tu et al., 2021) demonstrate the ability of irisin therapy to drive mitochondrial health and subsequently rescue cognitive function. Upregulated UCP2, PGC-1α, and adenosine monophosphate kinase (AMPK) phosphorylation as a result of irisin therapy improved mitochondrial biogenesis and dynamics, oxidative phosphorylation, ATP levels, brain glucose uptake, reactive oxygen species formation and antioxidant defense, and mitochondrial-mediated apoptosis. Further, AMPK (Wang & Pan, 2016) or UCP2 (P. Guo et al., 2021; Tu et al., 2021) inhibition abrogated irisin-induced mitochondrial and cognitive benefits. The congruence herein with exercise-induced benefits and AD-induced deficits to cerebral mitochondria suggest that the importance of mitochondria within irisin-mediated neuroprotection is currently underrepresented.

Effects of irisin in target tissues are elicited via signaling to αV/β5 integrin receptors (E. Kim et al., 2023; H. Kim et al., 2018). Previous work describes extracellular heat shock protein 90 alpha (HSP90α) as a cofactor that mediates irisin-receptor interaction. HSP90α binds and activates the αV/β5 receptor inducing the receptor to change conformational state and shift affinity for irisin from low to high. Separate in vivo experimentation validated the physiologic role of HSP90α in irisin signaling through biochemical analysis of mouse adipose tissue following injections of irisin and an inhibitory anti-HSP90 antibody (A et al., 2023).

Summary

Alzheimer's disease is the most common form of dementia and develops after years of multifaceted change in brain morphology and biochemistry. Aging brain health is dictated by many factors, and exercise has emerged as an effective strategy to promote brain health in late adulthood and to protect against the onset of AD. The exerkine irisin is an important mediator of the beneficial relationship between exercise and the brain. Previous work administering irisin therapeutically to healthy and preclinical AD mice has demonstrated irisin to protect against AD-induced deficits. This dissertation assesses the physiological role of irisin and the translatability of irisin-induced protection against AD using interventions to increase circulating irisin, behavioral tests to measure cognitive function, and biochemical analyses to assess protein expression in wild type and TgF344-AD rats.

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Chapter 2 - In vivo hippocampal CREB pathway activation via acute peripheral irisin injection

Abstract

Introduction. Skeletal muscle-brain crosstalk is an important contributor to the beneficial relationship between exercise and the brain. Irisin is a key crosstalk myokine due to the ability of irisin to cross the blood–brain barrier and mediate effects of exercise in the brain. Importantly, the extent of exercise-induced benefits in the brain promoted by a single irisin administration remain unclear. **Methods**. Twenty-four 2–3-month-old male and female F344 rats received an i.p. injection of irisin (100 μg/kg) or vehicle. Forty-eight hours following dosing, animals underwent cognitive testing, and brains were dissected and weighed. Hippocampal protein markers of irisin signaling, mitochondrial biogenesis, angiogenesis, and neurogenesis were quantified with Jess Simple Western automated western blot analysis. Values are mean ± standard deviation. **Results**. Greater hippocampal mass (59.95 ± 4.34 vs 53.84 ± 4.22 mg, $p = 0.0023$) and a 24.6% increase in p/t CREB (0.584 ± 0.131 vs 0.469 ± 0.088 a.u., $p = 0.0346$) presented in irisin animals. Significant differences in mitochondrial, angiogenic, and neurogenic markers were not found when analyzed with sexes combined. Sex-specific analysis revealed male-specific increases in hippocampal mass ($p = 0.0372$) and p/t CREB ($p = 0.0159$) and a female-specific increase in neurogenesis marker NeuroD ($p = 0.0183$) in comparison to within-sex controls. **Conclusions**. The present study demonstrates that a single injection of irisin is sufficient to increase hippocampal mass and CREB phosphorylation. This work supports irisin as an important, physiologically relevant mediator of the beneficial relationship between exercise, skeletal muscle, and the brain.

Introduction

Physical exercise is an effective strategy to promote brain health in advanced age and protect from the onset of dementia (Del Pozo Cruz et al., 2022). The aging brain is characterized by reduced hippocampal volume (Erickson et al., 2011), neurogenesis (Kuhn et al., 1996), blood flow (Heo et al., 2010), mitochondrial function (Boveris & Navarro, 2008), and cognitive performance. However, exercise opposes age-related cognitive dysfunction (Erickson et al., 2011; Maass et al., 2015) and deficits in hippocampal volume (Erickson et al., 2011), mitochondrial function (Berchtold et al., 2019; Steiner et al., 2011), blood flow (Maass et al., 2015; Palmer et al., 2023), and neurogenesis (Swain et al., 2003; Van Praag et al., 2005). This beneficial relationship between exercise and the brain is due, in part, to an endocrine role of skeletal muscle.

Irisin is a peroxisome proliferator-activated receptor-gamma coactivator (PCG1-α) dependent myokine cleaved from the extracellular domain of the fibronectin type III domain-containing 5 (FNDC5) transmembrane protein present on skeletal muscle in response to exercise (Boström et al., 2012). The concentration of irisin in plasma increases with exercise (Boström et al., 2012; Jedrychowski et al., 2015) and irisin signaling helps to mediate the beneficial relationship between exercise and the brain (Islam et al., 2021; Lourenco et al., 2019; Wrann et al., 2013). Previous mechanistic investigations using in vitro approaches demonstrate that irisin promotes brain derived neurotrophic factor (BDNF) (Wrann et al., 2013) and activates cAMP/PKA/CREB signaling (Lourenco et al., 2019), pathways which beget neurogenesis and memory formation.

Notably, previous attempts to administer irisin therapeutically in preclinical Alzheimer's disease (AD) mouse models have demonstrated positive results on AD pathology. Irisin was

shown to replicate portions of the benefits of exercise on the aging brain such as reduced glial cell activation, reduced tau pathology, and improved synaptic plasticity; ultimately, irisin therapy rescued AD-induced cognitive phenotypes (Bretland et al., 2021; Islam et al., 2021; Lourenco et al., 2019). While these results are promising, the aging- and AD-brain experience a plethora of deficits which may be reversed by exercise including declines in hippocampal volume (Erickson et al., 2011), mitochondrial function (Berchtold et al., 2019; Steiner et al., 2011), blood flow (Maass et al., 2015; Palmer et al., 2023), and neurogenesis (Swain et al., 2003; Van Praag et al., 2005).

Previous studies commonly use prolonged dosing regimens to investigate the influence of irisin on the brain (Bretland et al., 2021; Dehghan et al., 2021; Islam et al., 2021; Kam et al., 2022; Lourenco et al., 2019; Momenzadeh et al., 2021; Wrann et al., 2013). Taken together, these data suggest that there is a critical need to understand the potential in vivo influence of irisin on the brain, specifically the hippocampus. The current study aims to measure the influence of a single administration of irisin on hippocampal function and biochemistry in young, healthy male and female rats using behavioral tests and biochemical analysis.

Methods

Ethical approval

All protocols were approved by the Kansas State University Institutional Animal Care and Use Committee (No. 4466). Kansas State University is accredited by the AAALAC (000667), is assured by OLAW (A3609-01), and is registered with the USDA (48-R-0001). All protocols were in compliance with laboratory animal care and use policies and guidelines set forth by the US National Research Council and the US Public Health Service.

Experimental design

Fisher rats (Envigo) were housed three per cage in the Kansas State University Comparative Medicine Group facility on a 12-h light/dark cycle with food and water ad libitum. Animals were housed for two months prior to intervention. Three-month-old animals (n=24) were divided into two groups of 12 (50% male/female) which either received an irisin or vehicle injection. All animals underwent a four-day novel object recognition (NOR) test. Injections were administered directly following day 2 NOR trials, 48 hours prior to final NOR trials and sacrifice.

Interventions

Animals received one 250 μl intraperitoneal injection containing either 100 μg/kg irisin (Phoenix Pharmaceuticals 067-17) dissolved in 1% DMSO-saline or an equivalent amount of DMSO-saline. This dose was based on previous investigations in the brain following intraperitoneal irisin injection (Bretland et al., 2021; Dehghan et al., 2021; Momenzadeh et al., 2021).

Novel object recognition

NOR is a hippocampus-dependent measure of recognition memory (Squire et al., 2007) appropriate for rats older than 18 days (Contreras et al., 2019; Moreton et al., 2019). Animals were allowed 60 minutes in the testing room prior to initiating trials, and trials were conducted at the same time during the animals' dark cycle each day. The arena (78 x 52 x 36 cm) and objects were thoroughly cleaned (VersaClean, FisherBrand) and dried prior to each trial to remove odor.

Protocol is previously described (Antunes & Biala, 2012; Ennaceur & Delacour, 1988). Briefly, the four-day protocol included habituation, training, and testing phases. During the habituation phase (days 1 and 2), the animal was allowed to explore and become familiar with the empty testing arena for 5 (day 1) or 10 minutes (day 2). During the training phase (day 3), two identical objects were placed in the arena and the animal was allowed 10 minutes to explore. During the testing phase (day 4), one object is replaced with a new, novel object and the animal was allowed 5 minutes to explore. Novel object and novel object location were randomized to remove object and place preference effects. Trials were recorded and monitored in a room adjacent to the test room prior to quantifying exploration times (EthoVision, Noldus). All protocols were conducted by a blinded investigator. Exploration time was defined as directing the nose within 2 cm of the object. Animals with < 20 seconds of cumulative object exploration time during training (n=4) were excluded from analysis (Antunes & Biala, 2012).

Tissue harvest

Following completion of NOR testing trials, animals were euthanized via decapitation under 4% isoflurane gas. The brain was excised and weighed, and hippocampi were promptly isolated on an ice-cold metal dissection plate by a blind investigator. The right hippocampi, once isolated, were lightly blotted and weighed. Soleus skeletal muscles were then excised. Tissues were promptly flash frozen in liquid nitrogen before being transferred to -80°C freezer.

Tissue preparation

Hippocampus and soleus samples were lysed in ice-cold radio-immune precipitation assay (RIPA) buffer (Sigma-Aldrich) with protease inhibitor (Pierce Protease Inhibitor Mini

Tablet, ThermoFisher Scientific) in a glass Dounce homogenizer. Lysate protein concentrations were determined using the Qubit 4 Fluorometer with Protein Broad Range Assay kit (ThermoFisher Scientific).

Biochemical analysis

Western blot

Protein quantification in hippocampal lysates was completed with Jess Simple Western (ProteinSimple) automated western blot analysis with the following primary antibodies: cAMP-response element binding protein (CREB; NBP1-90364, Novus Biologicals, Centennial, CO, RRID:AB_11038978); Ser133 phosphorylated CREB (pCREB; NB300-273, Novus Biologicals, RRID:AB_10002306); nestin (MAB2736, R&D Systems, Minneapolis, MN, RRID:AB_2282664); neurogenic differentiation factor 1 (NeuroD; PA5-11889, ThermoFisher Scientific, Waltham, MA, RRID:AB_2149214); PCG1-α (PA5-72948, ThermoFisher Scientific, RRID:AB_2718802); vascular endothelial growth factor A (VEGF; NB100-664, Novus Biologicals, RRID:AB_10001947). Lysate protein concentrations were equalized prior to protein quantification and target proteins were assessed in the same experiment. Protein expression was quantified with Compass for Simple Western software.

ELISA

Levels of irisin (EK-067-20, Phoenix Pharmaceuticals, Burlingame, CA) and BDNF (DBNT00, R&D Systems) were quantified via commercial ELISA kits in hippocampal lysates. Assays were optimized to determine appropriate sample dilution factors and completed according to manufacturer's instructions.

Citrate synthase activity

Citrate synthase (CS) activity was quantified via CS Activity Assay Kit (NBP3-24481, Novus Biologicals) in hippocampal and soleus lysates. Assay was optimized to determine appropriate sample dilution factors and completed according to manufacturer's instructions. Units (U) of CS activity were defined as the amount of CS in one gram (g) of cell protein per one minute that produced 1 μmol CoA at 37°C (U g-1 protein).

Statistical analysis

Datasets were tested for normality prior to analysis. Between group comparisons were made via unpaired Student's or Welch's t-test. Recognition memory in the NOR task was tested against 50% (chance level) via one-sample t-test. Sex differences were identified via one-way ANOVA with Šídák's multiple comparisons test. All significance tests were two-sided using $p < 0.05$ as the level of significance. Data is presented as means and standard deviations. All analyses were performed using GraphPad Prism version 10.2.3 for macOS (GraphPad Software).

Results

Irisin injection resulted in increased hippocampus mass

In the present investigation, no influence of irisin on body mass (Figure 2.1A) or whole brain mass (Figure 2.1B) was detected. However, hippocampus mass of irisin animals (59.95 ± 4.34 mg) was significantly greater ($p = 0.002$) than vehicle (53.84 ± 4.22 mg). This finding, that hippocampal wet mass was increased only 48 hours following a single i.p. injection of irisin, is

interesting considering that changes in hippocampal volume are a strong metric of exercise- or age-induced brain health changes in late adulthood humans (Erickson et al., 2011).

Irisin injection did not influence recognition memory

NOR is a hippocampus-dependent task of recognition memory (Squire et al., 2007). Animals without sufficient exploration time (< 20 sec) during the familiarization phase were excluded from analysis (vehicle, n = 3; irisin, n = 1). Total object exploration times during training and testing phases were not influenced by irisin (Figure 2.2A). Novel object exploration as a percentage of total exploration time was not different than chance (50%) in irisin (57.7 ± 34.6%, p = 0.479) or vehicle (40.7 ± 24.4%, p = 0.285) animals (Figure 2.2B).

Acute irisin injection activated the hippocampal irisin signaling pathway

Peripheral irisin was previously demonstrated to activate the cAMP/PKA/CREB signaling pathway (Lourenco et al., 2019) and promote BDNF expression (Wrann et al., 2013) in the hippocampus. In the present investigation, an increase in hippocampal irisin was not present 48 hours following injection (Figure 2.3A). However, a significant 1.25-fold upregulation of hippocampal p/t CREB (0.584 ± 0.131 vs 0.469 ± 0.088 a.u., p = 0.035) presented in irisin animals compared to vehicle (Figure 2.3B), indicating activation of the irisin signaling pathway.

Activation of irisin signaling pathway did not influence hippocampal mitochondrial biogenesis, neurogenesis, or angiogenesis

We next quantified mitochondrial, neurogenic, and angiogenic markers as possible irisin signaling effectors. PCG1-α is considered the master regulator of mitochondrial biogenesis

(Puigserver et al., 1998). In the hippocampus, PCG1-α transcription is upregulated by exercise (Steiner et al., 2011) and regulates hippocampal FNDC5 transcription (Wrann et al., 2013). CS activity assays are commonly used as a proxy for mitochondrial mass (Marco et al., 1974). Although hippocampal PCG1-α expression was 21% higher in irisin animals compared to vehicle, hippocampal PCG1-α (1.86 ± 0.24 vs 1.54 ± 0.56 a.u., p = 0.1053) and CS activity (3.30 ± 0.50 vs 3.40 ± 0.40 U g-1 protein, p = 0.673) were not significantly different between groups (Figure 2.4). As expected, soleus skeletal muscle CS activity was not different between groups (Figure 2.4B).

Exercise promotes hippocampal neurogenesis (Swain et al., 2003; Van Praag et al., 1999), a process known to correlate with exercise-induced increases in cerebral blood volume and cognitive function (Pereira et al., 2007). In the present investigation, no differences in markers of neurogenesis (Figure 2.5A-B) or angiogenesis (Figure 2.5C) were detected between irisin and vehicle injection groups.

Males-specific induction of CREB signaling and female-specific neurogenic upregulation

We next separated our data into sex-specific datasets and analyzed via one-way ANOVA with Šídák's multiple comparisons test to investigate sex differences. One-way ANOVA revealed that there was a statistically significant difference in hippocampal mass ($F(3, 20) = 4.586$, $p = 0.013$), p/t CREB ($F(3, 17) = 5.725$, $p = 0.007$), and NeuroD expression ($F(3, 17) = 7.785$, $p = 0.002$) between at least two groups. Further, multiple comparisons tests revealed between group differences in each of these measures. Hippocampus mass of male irisin animals was greater than vehicle males ($p = 0.0372$), while no difference presented between female groups ($p = 0.216$, Figure 2.6A). Hippocampal p/t CREB was greater in male irisin animals compared to male

vehicle ($p = 0.016$) and female irisin ($p = 0.024$) groups (Figure 2.6B). Conversely, NeuroD protein expression was increased in female irisin animals in comparison to female vehicle ($p = 0.018$) and male irisin ($p = 0.002$) groups (Figure 2.6C).

Discussion

The present investigation assessed the effects of a peripheral administration of irisin on hippocampal structure, function, and biochemistry in young, male and female F344 rats. Our results demonstrate increased hippocampal mass and activation of hippocampal cAMP/PKA/CREB signaling 48 hours following a single dose of irisin. Further, irisin-induced increases in hippocampal mass and CREB phosphorylation were specific to males, while increases in neurogenesis marker NeuroD presented only in females.

Herein, we found a single administration of irisin resulted in increased hippocampal mass and p/t CREB 48 hours after injection. As previously mentioned, peripheral irisin signaling to the hippocampus activates the cAMP/PKA/CREB signaling pathway (Lourenco et al., 2019). Following phosphorylation and activation of the transcription factor CREB, there are multiple pathways which may be promoted including mitochondrial biogenesis (Herzig et al., 2001; St-Pierre et al., 2006), angiogenesis (Lee et al., 2010), and neurogenesis (H. Wang et al., 2018). Exercise has previously demonstrated to induce hippocampal mitochondrial biogenesis (Steiner et al., 2011), angiogenesis (Swain et al., 2003; Van Praag et al., 2005), and neurogenesis (Swain et al., 2003; Van Praag et al., 1999) in young adult rodents like those presented here. We sought to understand the mechanism of irisin-induced increased hippocampal mass by quantifying protein markers of these three processes.

PCG1-α and VEGF are crucial regulators of mitochondrial biogenesis (Puigserver et al., 1998) and angiogenesis (Ferrara & Henzel, 1989), respectively. Results reported here did not indicate promotion of mitochondrial biogenesis or angiogenesis 48 hours following a single injection of irisin in healthy animals (Figures 4A & 5C). However, influences of exogenous irisin on cerebral mitochondrial function and vascular control have been previously demonstrated. In preclinical models of stroke (Tu et al., 2021), traumatic brain injury (Guo et al., 2021), and depression (S. Wang & Pan, 2016), exogenous irisin administration successfully reversed disease phenotypes. In these investigations, therapeutic success in was dependent on irisin-induced attenuation of cerebral mitochondrial dysfunction via either AMPK phosphorylation (S. Wang & Pan, 2016) or upregulation of uncoupling protein (UCP) 2 (Guo et al., 2021; Tu et al., 2021). Further evidence of irisin-mediated regulation of hippocampal UCPs was given by Erden et al., who found changes in UCP 2–5 transcription 16, 24, and 48 hours following an intracerebroventricular irisin injection (Erden et al., 2016). Similarly, exogenous irisin given to spontaneous hypertensive rats rescued blood pressure phenotype (Fu et al., 2016; Huo et al., 2020; Zhang et al., 2015) by either acting directly on vascular endothelial cells to increase nitric oxide bioavailability (Fu et al., 2016) or by acting in the hypothalamic paraventricular nucleus to reduce sympathetic outflow (Huo et al., 2020). The above-described investigations support a mediating role for irisin in cerebral mitochondrial and cardiovascular exercise adaptations, it is important to note that these influences were demonstrated on preclinical models with inherent functional deficits. Although, as mentioned previously, exercise can promote hippocampal mitochondrial biogenesis (Steiner et al., 2011) and angiogenesis (Swain et al., 2003; Van Praag et al., 2005) in healthy rodents, we found no evidence of upregulation 48-hours following a single irisin administration. Additional work is needed to understand if a prolonged dosing

regimen may mimic the effects of exercise on these processes in the hippocampi of healthy rodents (Dehghan et al., 2021).

The dentate gyrus (DG) of the hippocampus is one specific area of the brain in which new neurons can be produced (Altman & Das, 1965; Kuhn et al., 1996). During adult neurogenesis in the DG, progenitor cells become functional neurons in a multi-stage process which includes proliferation, differentiation, migration, targeting, and synaptic integration (Von Bohlen Und Halbach, 2011). Alternatively, a differentiating progenitor cell may take a glial-specific lineage (Von Bohlen Und Halbach, 2011). Importantly, protein markers can be used to differentiate between neurogenesis stages and between cells in neuronal- or glial-specific lineages, including nestin and NeuroD. Nestin is expressed by progenitors in the proliferation phase; these cells may take on either neuronal- or glial-specific lineages (Fukuda et al., 2003; Lendahl et al., 1990). NeuroD is neuron-specific and expressed early in the neuronal lineage (Seki, 2002; Von Bohlen Und Halbach, 2011). In the present investigation, given the short 48 hour span from intervention to protein quantification, we did not seek to identify markers for migration, targeting, or synaptic integration stages. With combined sexes, we found no influence of irisin on hippocampal nestin or NeuroD expression, suggesting no increase in proliferation or differentiation stages of adult neurogenesis. Sex-specific irisin-induced increases in NeuroD expression was found in females and is discussed below. Utilizing an adeno-associated virus to force prolonged elevation of peripheral irisin levels, Islam et al. (2021) found no evidence of neurogenesis in the hippocampi of APP/PS1 mice two months after viral injections. Separate experimentation from these authors investigated adult born neurons in the DG of mice bred to have irisin depleted by genetic knockout (KO). Compared to wild-type litter mates, KO mice displayed increased number and dendritic complexity of adult born neurons in the DG. However,

running exercise promoted dendritic complexity and length of adult born neurons in WT mice while this response was absent in KO littermates (Islam et al., 2021). Together, these results do not suggest peripheral irisin signaling to promote adult hippocampal neurogenesis, but pose a role for either irisin or FNDC5 to regulate the dendritic morphology of adult born neurons.

In the present investigation, significant preference for the novel object, a hippocampus-dependent measure of recognition memory (Squire et al., 2007), was not displayed in vehicle or irisin groups. Previous studies of irisin physiology have found contrasting results following NOR testing. In preclinical mouse models of Alzheimer's disease, Lourenco et al. (2019) demonstrated that: (1) inhibition of peripheral irisin signaling ameliorated protective effects of exercise on NOR memory, and (2) upregulation of peripheral irisin signaling rescued AD-induced NOR memory deficits. In separate experiments using healthy mice, these authors found that inhibition of either cerebral irisin expression or peripheral irisin signaling impaired NOR memory after inhibition was present for four weeks (cerebral expression) or one week (peripheral signaling) (Lourenco et al., 2019). Regarding the results found in healthy mice, this suggests physiologic FNDC5/irisin is necessary to maintain cognitive function prior to age- or disease-mediated deficits. Islam et al. (2021) tested NOR memory in KO mice bred to not express irisin and WT littermates at young (8–10 weeks) and aged (21–24 months) time points. While genotype did not influence cognitive function at the young time point, deficits in NOR memory presented in aged KO mice (Islam et al., 2021). These results suggest that cognitive ability does not depend on the presence of physiologic FNDC5/irisin in adolescence; alternatively, because the KO was present at birth, the deficit seen in older animals may have resulted from increased treatment exposure time. The present investigation supports results from Islam et al. (2021) as there was no influence of irisin on NOR memory in our young adult cohort. Of note, in our investigation and

the previous work discussed herein, novel object preference was assessed by comparing novel object exploration time in each experimental group vs. chance (50% exploration) via one-sample t-test; these findings do not necessarily denote between-group differences. Accordingly, this statistical approach increases the viability of the inter-investigation comparisons detailed above. Together, these results suggest that irisin protects against age- or disease-induced cognitive deficits. Prior to the onset of these deficits (i.e. in a young, healthy animal), however, the influence of irisin on cognitive function remains inconclusive.

Sex differences in irisin physiology have been previously reported. In comparisons between young, healthy adult humans, females have been reported to have higher circulating levels of irisin (Koltun et al., 2024). Further, following an acute bout of anaerobic exercise, female plasma irisin levels increased while males did not differ from baseline (Wiecek et al., 2018). While these studies suggest young adult females to have higher levels of circulating irisin, concentrations of both plasma and cerebrospinal fluid (CSF) irisin were higher in males in a healthy cohort of late adulthood participants (Ruan et al., 2019). This post-menopausal disparity is significant, particularly in AD, considering that females have an increased risk of developing AD (Alzheimer's Association, 2024) and that CSF irisin is decreased in AD patients (Lourenco et al., 2019). Regarding AD, Bretland et al. (2021) investigated tau pathology amelioration in male and female preclinical AD mice in response irisin therapy. Irisin administration lowered phosphorylated tau in the hippocampi of female AD mice while no change presented in males (Bretland et al., 2021). In the present investigation, hippocampi of young adult rats given a single irisin injection, we found a female-specific increase in neurogenesis marker NeuroD and a male-specific increase in p/t CREB. Together, these results suggest sex differences in irisin homeostasis and hippocampal signaling that may be modulated by age, exercise, and

neurodegenerative disease. Further work is needed to replicate these findings to understand the influence of sex on irisin physiology.

Conclusions

We report increases in hippocampal mass and CREB phosphorylation 48 hours following a single administration of peripheral irisin. In addition, the present investigation adds to the growing body of literature aimed to understand sex differences in irisin physiology. Although this work supports irisin as a mediator of the positive influence of exercise on the brain, further investigation will be instructive in understanding the extent of irisin-mediated benefits of exercise in the brain.

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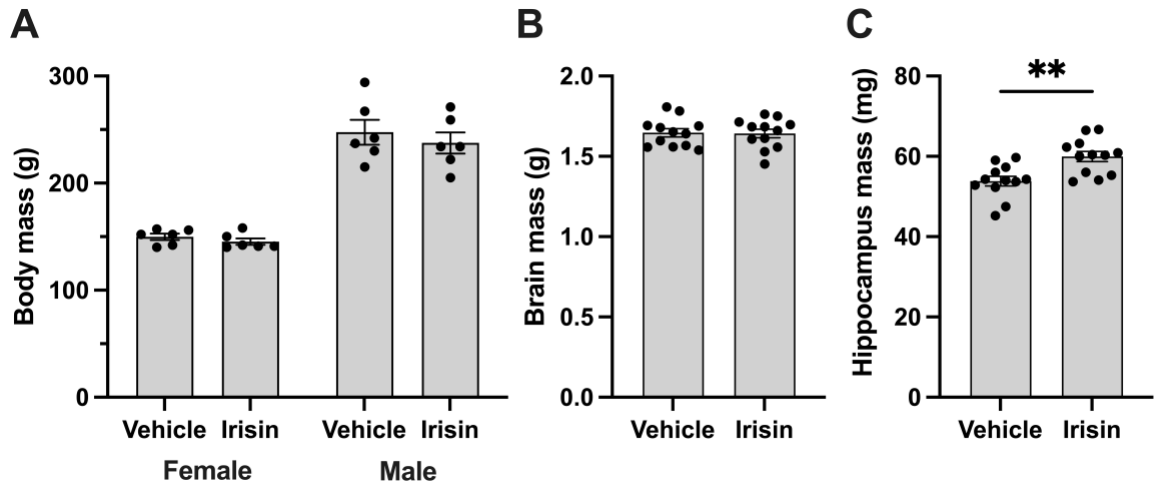
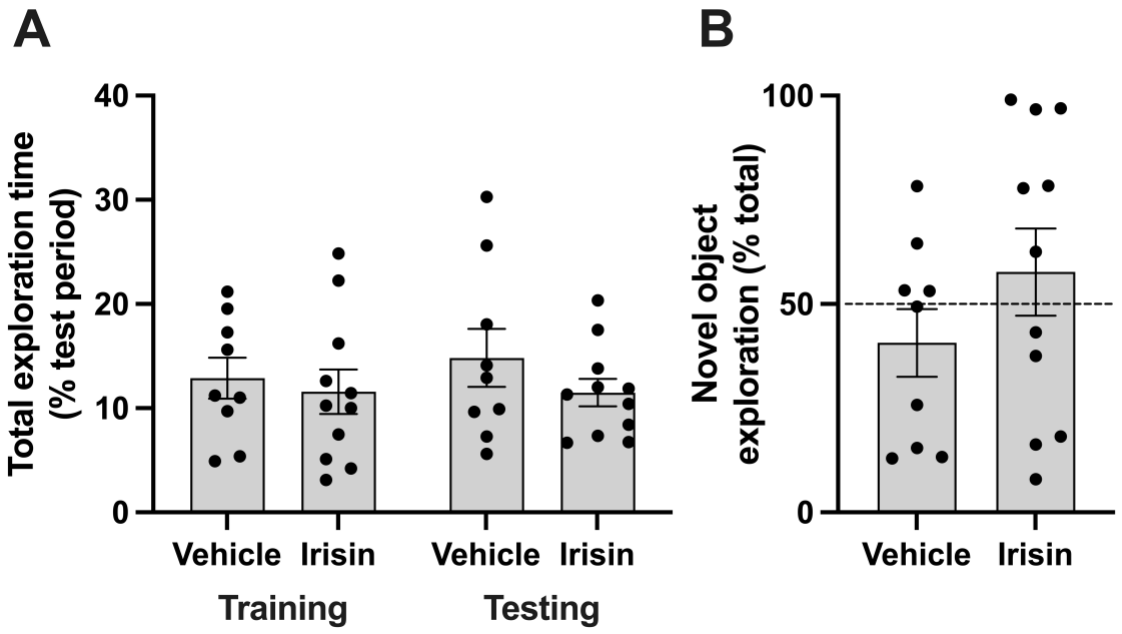
Figure 2.1. Body and brain mass.



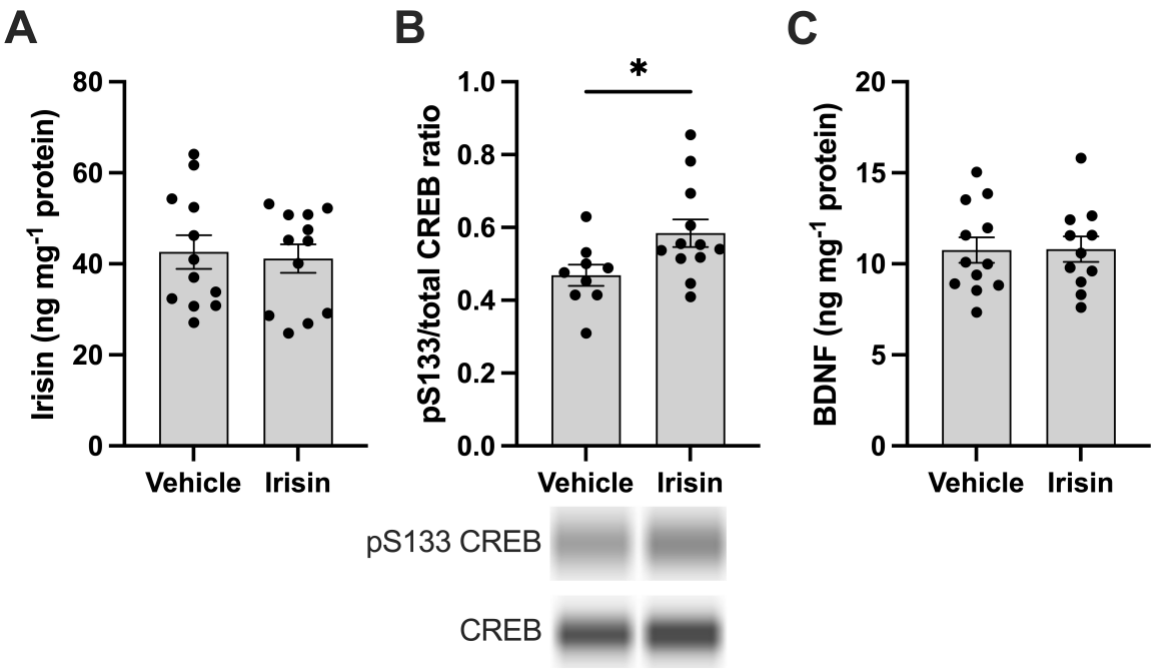
Comparisons of body mass (A), brain mass (B), and hippocampus mass (C) 48 hours following irisin or vehicle injections. Body mass was assessed independently in females and males due to the large disparity between sexes. Average hippocampus mass was greater in animals injected with irisin compared to vehicle. Two-sided unpaired Student's t-test (A, $n = 6$ animals per group; B–C, $n = 12$ animals per group). **$p < 0.01$. Bars are mean ± standard error of the mean.

Figure 2.2. Evaluation of hippocampus-dependent cognitive function.



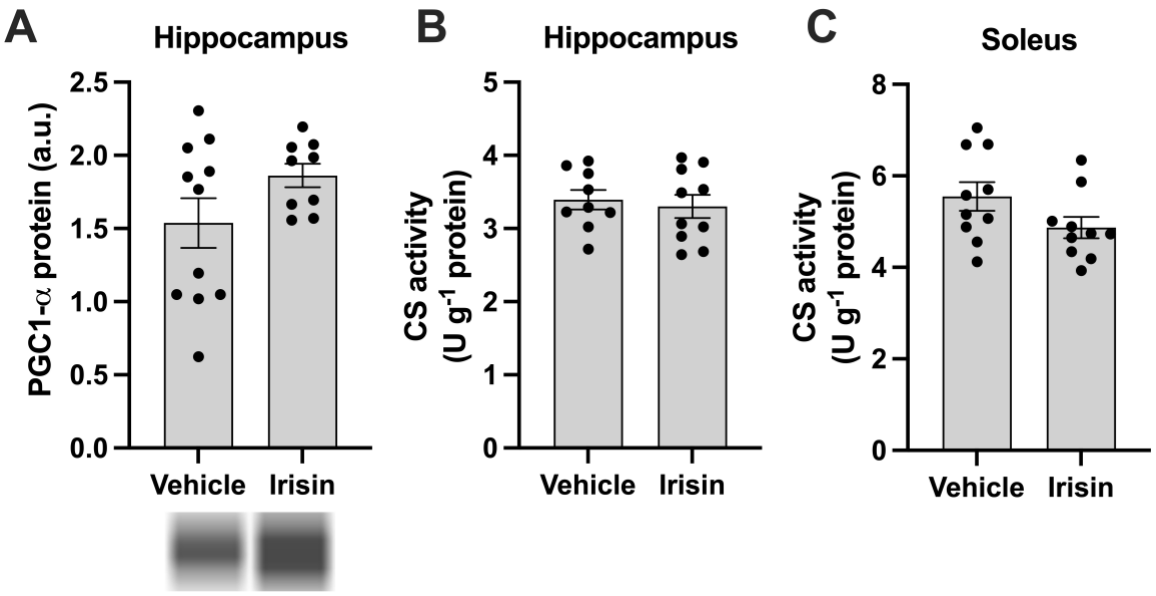
Between group comparisons of total object exploration time during training and testing phases of NOR testing revealed no influence of irisin on exploratory behavior (A). Novel object exploration was not statistically different than 50% (chance level) in either group (B). Two-sided unpaired (A) and one-sample (B) Student's t-tests (n = 9–11 animals per group). Bars are mean ± standard error of the mean.

Figure 2.3. Hippocampal irisin signaling pathway.



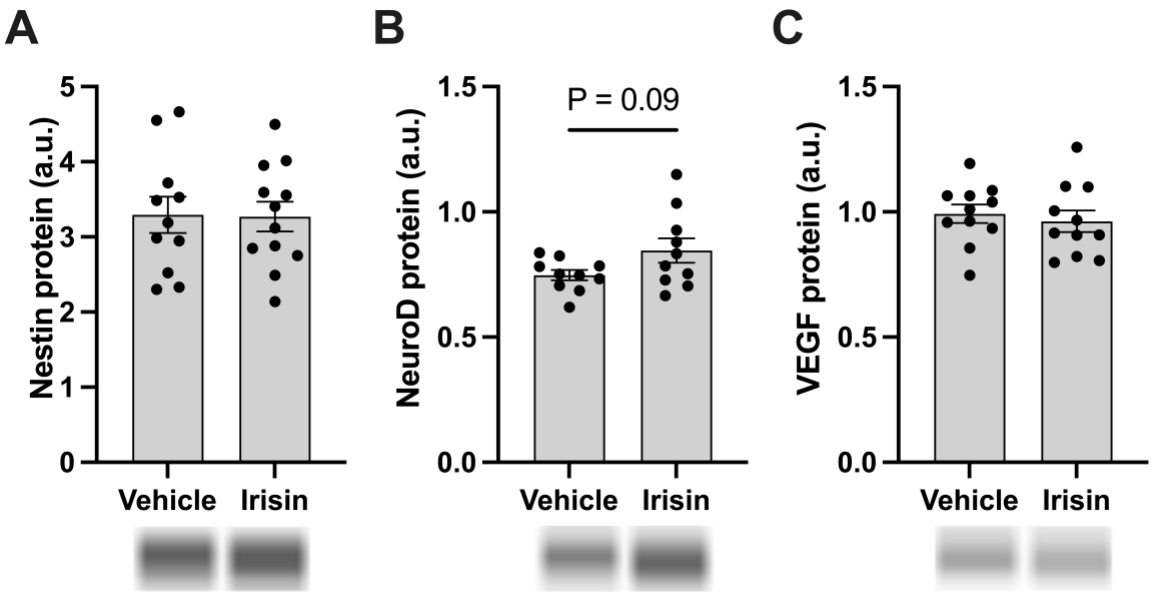
Quantification of hippocampal irisin (A), Ser133 phosphorylated to total CREB ratios (B), and BDNF (C) 48 hours following irisin or vehicle injection. Irisin injection increased p/t CREB in the hippocampus. Quantification completed by ELISA (A, C) or Jess automated western blot (B). *$p < 0.05$. Two-sided unpaired Student's t-test (n = 9–12 animals per group). Bars are mean ± standard error of the mean.

Figure 2.4. Markers of mitochondrial biogenesis.



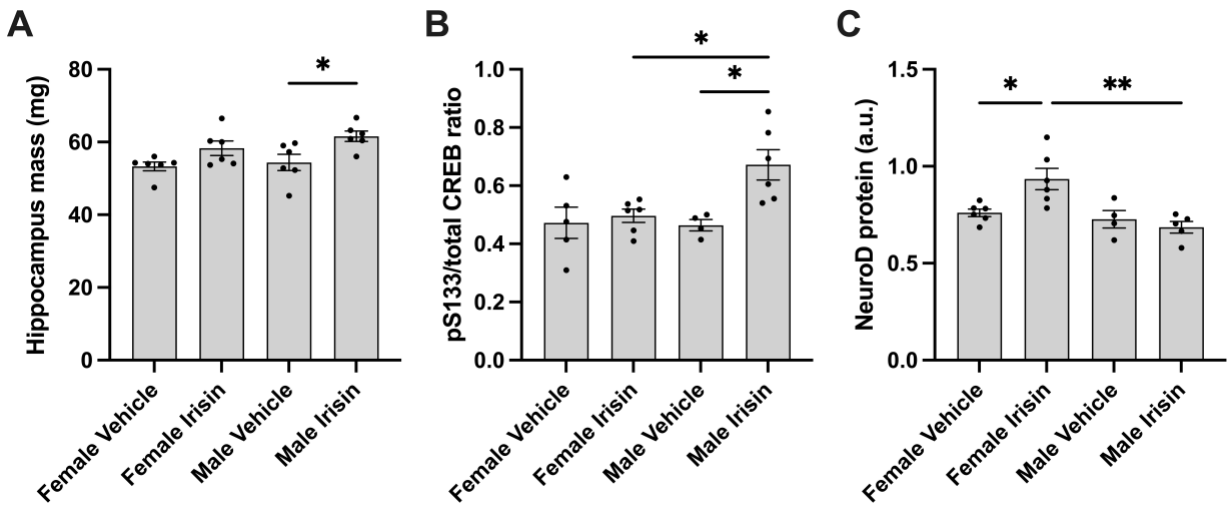
Quantification of hippocampal PCG1-α (A) and CS activity in hippocampi (B) and soleus skeletal muscles (C) 48 hours following irisin or vehicle injection. Quantification completed by Jess automated western blot (A) or ELISA (B, C). Two-sided unpaired Welch's (A) or Student's (B, C) t-test (n = 9–11 animals per group). Bars are mean ± standard error of the mean. CS, citrate synthase; a.u., arbitrary units.

Figure 2.5. Markers of hippocampal neurogenesis and angiogenesis.



Quantification of hippocampal nestin (A), NeuroD (B), and VEGF (C) protein expression 48 hours following irisin or vehicle injection. Quantification completed by Jess automated western blot. Two-sided unpaired Student's (A, C) or Welch's (B) t-test (n = 10–12 animals per group). Bars are mean ± standard error of the mean. a.u., arbitrary units.

Figure 2.6. Sex differences.



Comparisons of hippocampal mass (A), Ser133 phosphorylated to total CREB (B), and NeuroD protein expression (C) 48 hours following irisin or vehicle injection in male and female animals. Protein quantification completed via Jess automated western blot. *$p < 0.05$, **$p < 0.01$. One-way ANOVA with Šídák's multiple comparisons test (n = 4–6 animals per group). Bars are mean ± standard error of the mean. a.u., arbitrary units.

Chapter 3 - Influence of in vivo HSP90α inhibition on irisin signaling to the brain

Abstract

Introduction. Irisin is an exercise-induced myokine that elicits beneficial effects of exercise in fat, bone, and the brain. Although extracellular heat shock protein 90α (HSP90α) is suggested to mediate irisin-receptor interaction, the role of HSP90α in the irisin/brain interaction remains untested in vivo. **Methods**. Female F344 rats (3–4 months, n=32) were randomized into four treatment groups: irisin + HSP90α inhibition, irisin + control, control + HSP90α inhibition, and control + control. Forty-eight hours following interventions, animals underwent cognitive testing and tissue samples were collected. Protein markers of HSP90α inhibition, irisin signaling, and neurogenesis were quantified with Jess Simple Western automated western blot analysis and ELISA. Two-way ANOVA with Šídák's multiple comparisons test was used to identify treatment effects and group differences. **Results**. HSP90α inhibition was confirmed by a pAkt reduction in the livers of inhibited animals compared to vehicle ($p=0.0003$). In the novel object recognition task, only the irisin + control group displayed significant recognition memory (68.77±17.93% of exploration time with novel object; $p=0.009$, one-sample t-test vs 50%). Hippocampal levels of phosphorylated CREB ($p=0.0273$) and neurogenesis marker NeuroD ($p=0.0062$) were upregulated by irisin independent of HSP90α inhibition. **Conclusions**. This investigation suggests the beneficial relationship between irisin and the brain is independent of HSP90α activity.

Introduction

Physical exercise promotes systemic health and is an effective strategy to protect against brain aging and neurodegenerative disease (Del Pozo Cruz et al., 2022; Erickson et al., 2011). Several age-related processes, including increased neuroinflammation (Gomes Da Silva et al., 2013; Jensen et al., 2019) and reductions in neurogenesis (Swain et al., 2003; Van Praag et al., 2005), cerebral blood flow (Burdette et al., 2010; Maass et al., 2015), and cerebral mitochondrial function (Berchtold et al., 2019; Steiner et al., 2011) are attenuated by exercise. Exercise-induced promotions in brain health are mediated both indirectly and directly. Indirectly, brain health is benefited by ameliorating systemic conditions that disturb cognitive function and brain metabolism, including hypertension, obesity, diabetes, and depression (Nianogo et al., 2022). Direct promotion of brain health is due, in part, to exerkines, endocrine factors released into circulation in response to exercise.

A prominent exerkine that promotes brain health is irisin. Irisin is a peroxisome proliferator-activated receptor-gamma coactivator (PCG1-α) dependent exerkine cleaved from the extracellular domain of the fibronectin type III domain-containing 5 (FNDC5) transmembrane protein present on skeletal muscle (Boström et al., 2012). In addition to the brain, irisin mediates exercise effects in adipose and bone (Boström et al., 2012; Colaianni et al., 2015; Wrann et al., 2013). Irisin was first characterized to promote adipose browning by upregulating uncoupling protein 1 in adipocytes (Boström et al., 2012). In mice fed a high-fat diet and given an irisin treatment, the initiation of adipose browning was seen concomitantly with increased metabolic rate, reduced obesity, and improved insulin resistance (Boström et al., 2012). In bone, irisin acts to increase osteocytic survival and bone mass via sclerostin upregulation (Colaianni et al., 2015; H. Kim et al., 2018). In the brain, specifically the hippocampus, irisin crosses the

blood-brain barrier (Islam et al., 2021) and promotes long-term potentiation and memory formation through a mechanism involving the phosphorylation of cyclic AMP response element binding protein (CREB) (Lourenco et al., 2019; Morè et al., 2022; Schaefer et al., 2017).

Effects of irisin in target tissues are elicited via signaling to αV/β5 integrin receptors (E. Kim et al., 2023; H. Kim et al., 2018). Previous work describes heat shock protein 90 alpha (HSP90α), a protein that is upregulated with exercise, as a mediator of irisin-receptor interaction (A et al., 2023). Integrin receptors have distinct conformational states with differing ligand affinities, including a closed state with low affinity and an extended open state with high affinity (Springer & Dustin, 2012). HSP90α binding to an αV/β5 integrin receptor increases open state-likelihood, resulting in increased irisin binding affinity and irisin-receptor interaction at physiologically relevant irisin concentrations (A et al., 2023).

Although irisin works through αV/β5 integrin receptors in adipose, bone, and the brain, the requirement of HSP90α to mediate irisin-receptor interaction has only been shown in adipose tissue in vivo (H. Kim et al., 2018). Whether HSP90α is necessary for the physiological effects of irisin in the brain remains unknown. To address this gap, the present investigation assessed hippocampal function and irisin signaling following peripheral irisin administration with or without HSP90α inhibition.

Methods

Ethical approval

All protocols were approved by the Kansas State University Institutional Animal Care and Use Committee (No. 4466). Kansas State University is accredited by the AAALAC (000667), is assured by OLAW (A3609-01), and is registered with the USDA (48-R-0001). All protocols

were in compliance with laboratory animal care and use policies and guidelines set forth by the US National Research Council and the US Public Health Service.

Experimental design

Female Fischer-344 rats were purchased (Envigo) and housed three per cage in the Kansas State University Comparative Medicine Group facility on a 12-h light/dark cycle with food and water ad libitum. Only female rats were utilized based on previous work from our lab aimed at identifying sex differences which identified a more significant effect of irisin in females compared to males. Animals were housed for two months prior to intervention and entered the study at 3–4 months of age. Animals (n = 32) were randomly divided into four groups: irisin + HSP90α inhibition (n = 10); irisin + control (n = 10); control + HSP90α inhibition (n = 6); control + control (n = 6). Animals underwent a 4-day novel object recognition (NOR) protocol. Drug administrations were given directly following NOR day 2. Day 4 NOR testing and tissue collection occurred 48 hours following drug administrations.

Interventions

Irisin administration animals received one 250 µl intraperitoneal injection containing 100 µg/kg of irisin (067-17, Phoenix Pharmaceuticals, Burlingame, CA) dissolved in 1% DMSO-saline; control animals received equivalent-volume vehicle control (1%DMSO-saline). This dose was based on previous investigations in the brain following intraperitoneal irisin injection (Bretland et al., 2021; Dehghan et al., 2021; Momenzadeh et al., 2021). HSP90α inhibition animals received one 200 µl oral gavage containing 10 mg/kg of selective HSP90α inhibitor SNX-5422 (S2656, Selleck Chemicals, Houston, TX) dissolved in 0.25% Tween80 and 0.5%

carboxymethylcellulose (CMC)-saline; control animals received equivalent-volume vehicle control (0.25% Tween80 and 0.5% CMC-saline). SNX-5422 is the glycine ester prodrug of SNX-2112, a 2-aminobenzamide inhibitor that competitively binds to the N-terminal ATP-binding site of HSP90 (Huang et al., 2009). Previous in vivo and in vitro work demonstrated 10 mg/kg of SNX-5422 as the lowest tested dose expected to be detectable after 48 hours (Chandarlapaty et al., 2008; Huang et al., 2009).

Novel object recognition

Novel object recognition (NOR) is a hippocampus-dependent measure recognition memory (Squire et al., 2007) which can assess rats older than 18 days (Contreras et al., 2019; Moreton et al., 2019). Animals were allowed 60 minutes in the testing room prior to initiating trials, and trials were conducted at the same time during the animals' dark cycle each day. The arena (78 x 52 x 36 cm) and objects were thoroughly cleaned (VersaClean, FisherBrand) and dried prior to each trial to remove odor. Protocol is previously described (Antunes & Biala, 2012; Ennaceur & Delacour, 1988). Briefly, the four-day protocol included habituation, training, and testing phases. During the habituation phase (days 1 and 2), the animal was allowed to explore and become familiar with the empty testing arena for 5 (day 1) or 10 minutes (day 2). During the training phase (day 3), two identical objects were placed in the arena and the animal was allowed 10 minutes to explore. During the testing phase (day 4), one object was replaced with a new, novel object and the animal was allowed 5 minutes to explore. Novel object and novel object location were randomized to remove object and place preference effects. Trials were recorded and monitored in a room adjacent to the test room prior to quantifying exploration times (EthoVision, Noldus). All protocols were conducted by a blinded investigator. Exploration

time was defined as directing the nose within 2 cm of the object. Animals with < 20 seconds of cumulative object exploration time during training (n=4) were excluded from analysis (Antunes & Biala, 2012).

Tissue harvest

Animals were anesthetized (isoflurane, 4%) and blood was collected via left ventricular puncture then immediately transferred to silicone coated collection tube (367820, BD Vacutainer) for later centrifugation (1500 rpm, 20 min, 4°C) and serum extraction. Following blood collection, animals were promptly euthanized via decapitation. Brains were immediately excised on ice and weighed prior to the isolation of the frontal cortices and hippocampi. Brain dissections were done on a freezer-cold stainless steel block in ice. Samples were collected from the left lobe of the liver, rinsed with ice-cold PBS, and blotted dry. Tissues were snap frozen in liquid nitrogen then transferred to -80°C freezer to be later processed.

Tissue preparation

Whole-hippocampi and liver samples were lysed in ice-cold radio-immune precipitation assay (RIPA) buffer (Sigma-Aldrich) with protease inhibitor (Pierce Protease Inhibitor Mini Tablet, ThermoFisher Scientific) in a glass Dounce homogenizer. Lysate protein concentrations were determined using the Qubit 4 Fluorometer with Protein Broad Range Assay kit (ThermoFisher Scientific).

Biochemical analysis

Western blot

Protein quantification in hippocampal and liver lysates was completed with Jess Simple Western (ProteinSimple) automated western blot analysis with the following primary antibodies: cAMP-response element binding protein 1 (CREB; 1:10, NBP1-90364, Novus Biologicals, Centennial, CO, RRID:AB_11038978); Ser133 phosphorylated CREB (pCREB; 1:10, NB300-273, Novus Biologicals, RRID:AB_10002306); heat shock protein 90 alpha (HSP90α; 1:100, PA3-013, ThermoFisher Scientific, Waltham, MA, RRID:AB_2120934); nestin (1:100, MAB2736, R&D Systems, Minneapolis, MN, RRID:AB_2282664); neurogenic differentiation factor 1 (1:10, NeuroD; PA5-11889, ThermoFisher Scientific, RRID:AB_2149214); Ser473 phosphorylated Akt (pAkt; 1:100, AF887, R&D Systems, RRID:AB_355685). Lysate protein concentrations were equalized before protein quantification (1 or 2 μg/μl protein per sample). Protein expression was quantified with Compass for Simple Western software.

ELISA

Commercial ELISA kits were used to quantify irisin (EK-067-20, Phoenix Pharmaceuticals, Burlingame, CA) in hippocampal lysates and brain-derived neurotrophic factor (BDNF) (DBNT00, R&D Systems) in serum.

Statistical analysis

Datasets were tested for normality prior to analysis. Recognition memory in the NOR task was tested against 50% (chance level) via one-sample t-test. Two-way ANOVA with Šídák's multiple comparisons test was used to identify treatment effects and group differences. All significance tests were two-sided using $p < 0.05$ as the level of significance. Data is presented as

means and standard deviations. All analyses were performed using GraphPad Prism version 10.2.3 for macOS (GraphPad Software).

Results

Confirmation of HSP90α inhibition

We first confirmed the actions of HSP90α inhibitor SNX-5422 by quantifying phosphorylated Akt (pAkt) in liver samples collected 48 hours following drug administrations. HSP90 is important for the proper folding and stability of a group of cellular proteins, including the protein kinase Akt (Basso et al., 2002). Previous pharmacodynamic analysis of a single dose of SNX-2112 found relative levels of total Akt and pAkt to be approximately 67% and 55%, respectively, of baseline after 48-hours (Chandarlapaty et al., 2008). In the present study, mean levels of pAkt in animals receiving SNX-5422 were approximately 61% of those in non-inhibition animals, resulting in a main effect of HSP90α inhibition ($F(1, 13) = 24.49$, $p = 0.0003$, $\eta^2 = 0.631$; Figure 3.1). Further, significant reductions in pAkt were present in irisin + HSP90α inhibition (irisin + HSP90α inhibition vs irisin + control: [mean ± SD] 0.63 ± 0.13 vs 1.03 ± 0.15 a.u., $p = 0.017$) and control + HSP90α inhibition (control + HSP90α inhibition vs control + control: 0.62 ± 0.17 vs 1.00 ± 0.16 a.u., $p = 0.014$) groups compared to respective non-inhibition groups. These results confirm the inhibition of HSP90α by inhibitor SNX-5422.

HSP90α inhibition abrogated positive influence of irisin on recognition memory

NOR is a hippocampus-dependent task of recognition memory (Squire et al., 2007). Animals without sufficient object exploration time (<20 sec) during the familiarization phase were excluded from analysis (irisin + control, n = 1; control + control, n = 1; irisin + HSP90α

inhibition, n = 2) (Antunes & Biala, 2012). Novel object exploration time is commonly calculated as a percentage of total exploration time and tested against chance (50%) for each group. Animals receiving only irisin displayed novel object preference, indicative of high recognition memory (irisin + control: 68.7 ± 19.0%, p=0.009 vs 50%) while exploration in control + control (58.0 ± 38.1%, p = 0.662 vs 50%), control + HSP90α inhibition (62.6 ± 30.0%, p = 0.352 vs 50%), and irisin + HSP90α inhibition (67.7 ± 24.9%, p = 0.083 vs 50%) groups were not different than chance (Figure 3.2). These results suggest irisin to benefit recognition memory in a mechanism dependent on HSP90α activity.

Irisin upregulated total and phosphorylated hippocampal CREB in parallel

Peripheral irisin activates cAMP/PKA/CREB signaling in the hippocampus (Lourenco et al., 2019). In the present investigation, two-way ANOVA revealed main effects of irisin increasing both total CREB ($F(1, 26) = 4.47$, $p = 0.044$, $\eta^2 = 0.122$) and pCREB ($F(1, 25) = 5.50$, $p = 0.027$, $\eta^2 = 0.174$; Figure 3.3A-B). Further, our analysis of total CREB presented an interaction effect ($F(1, 26) = 6.40$, $p = 0.018$, $\eta^2 = 0.175$), suggesting irisin to influence CREB expression differently across HSP90α inhibition conditions. This is clarified by multiple comparisons testing revealing total CREB to be significantly increased in irisin + control compared to control + control (1.21 ± 0.15 vs 1.00 ± 0.16 a.u., p = 0.014) animals while irisin + HSP90α and control + HSP90α inhibition (1.09 ± 0.10 vs 1.11 ± 0.09 a.u, p = 0.997) animals were not different. Together, these results suggest irisin to upregulate pCREB independent of HSP90α inhibition and to upregulate total CREB predominantly in the absence of HSP90α inhibition. Due to similar magnitude increases in total and phosphorylated CREB, there was no change in the ratio of p:t CREB (Figure 3.3C).

Irisin increased hippocampal HSP90α discordantly across HSP90α inhibition conditions

Forty-eight hours following injections, levels of peripheral irisin were not influenced (Figure 3.4A). Despite this, analysis of hippocampal HSP90α (Figure 3.4B) presented a main effect of irisin with increasing levels of HSP90α ($F(1, 25) = 6.22$, $p = 0.020$, $\eta^2 = 0.169$) and an interaction effect ($F(1, 25) = 4.87$, $p = 0.037$, $\eta^2 = 0.132$), indicating that HSP90α inhibition did not have the same effect across irisin treatment conditions. This is clarified by multiple comparisons tests revealing significantly higher HSP90α protein levels in irisin + HSP90α inhibition compared to control + HSP90α inhibition animals (1.08 ± 0.16 vs 0.77 ± 0.15 a.u., $p = 0.012$) while irisin + control animals were similar to both control + control (1.02 ± 0.24 vs 1.00 ± 0.10 a.u., $p = 0.999$) and irisin + HSP90α inhibition groups (1.02 ± 0.24 vs 1.08 ± 0.77 a.u., $p = 0.931$). Although the disparity between control + control and control + HSP90α inhibition ($p = 0.117$) groups was statistically insignificant, it is likely that: 1) the interaction and irisin main effects were driven by an initial perturbation of HSP90α levels when the HSP90α inhibitor was given alone which was abrogated when the HSP90α inhibitor was given in combination with irisin and; 2) the irisin main effect was driven by average HSP90α levels being lower in non-irisin control animals rather than being higher in irisin animals. BDNF is a marker of neuronal plasticity and plays a significant role in memory. Irisin has been found to stimulate brain production of BDNF (Wrann et al., 2013). In the present investigation, irisin peripheral administration did not increase hippocampal BDNF levels (Figure 3.4C). Analysis of hippocampal BDNF revealed only an interaction effect ($F(1, 27) = 6.70$, $p = 0.015$, $\eta^2 = 0.195$), suggesting that HSP90α inhibition had different effects on hippocampal BDNF expression

across irisin and non-irisin conditions. Without significant between-group differences, this interaction effect remains difficult to interpret.

Irisin upregulated neurogenesis marker NeuroD

We next assessed if the ability of irisin to mediate the neurogenic effects of exercise in the brain depends on HSP90α. Nestin and NeuroD are developmental protein markers expressed by progenitor cells in the dentate gyrus of the hippocampus. Cells committed to either neuron- or glia-specific lineages may express Nestin; NeuroD is expressed by cells committed to a neuron-specific lineage (Von Bohlen Und Halbach, 2011). In the present study, NeuroD was upregulated by irisin administration regardless of HSP90α inhibition, as evidenced by a main effect of irisin ($F(1, 27) = 8.8$, $p = 0.0062$, $\eta^2 = 0.241$); NeuroD expression was increased in irisin + HSP90α inhibition compared to control + HSP90α inhibition animals (1.14 ± 0.12 vs 0.95 ± 0.16 a.u., $p = 0.039$; Figure 3.5A). We found no influence of either irisin or HSP90α inhibition on nestin expression (Figure 3.5B).

Discussion

The present investigation assessed the influence of peripheral irisin on hippocampal function and biochemistry with and without inhibition of HSP90α in female F344 rats. Our results demonstrate significant hippocampus-dependent recognition memory displayed by animals given irisin without HSP90α inhibition, confirming irisin as a promotor of hippocampal function. In addition, irisin induced increases in hippocampal CREB and NeuroD; however, these effects were largely independent of HSP90α activity. To our knowledge, this is the first investigation of the role of HSP90α on irisin signaling to the brain in vivo.

Tests of hippocampal function 48 hours following interventions found that animals given irisin without HSP90α inhibition were the sole group to display recognition memory. These results support the hypothesis that HSP90α is required for the functional effects of irisin in the brain. NOR is a common assessment of recognition memory, and irisin-induced improvement in hippocampal function via recognition memory has been previously reported (Islam et al., 2021; Lourenco et al., 2019). Of note, recognition memory was analyzed by the common method of comparing novel object exploration time to chance (50%) within each group. Thus, identifying between-group differences was not the objective of the analysis. Regarding the results reported herein, average exploration time spent with the novel object was approximately 1% lower in irisin + HSP90α inhibition compared to control + HSP90α inhibition animals. This small difference in group averages (in conjunction with each group's novel object exploration time variance) led to statistical significance for the irisin + control group and insignificance for the irisin + HSP90α inhibition group ($p=0.08$). Although irisin + control animals were the sole group to display significant recognition memory, this small differentiation of group means leads us to interpret the necessity of HSP90α for functional effects of irisin in the hippocampus as inconclusive.

Irisin-induced increases in hippocampal CREB, pCREB, and NeuroD expression were independent of HSP90α inhibition. Irisin signaling to the hippocampus activates the cAMP/PKA/CREB signaling pathway (Lourenco et al., 2019). We found irisin injection to have parallel increases in CREB and pCREB without an increase in the ratio of p:t CREB 48 hours after interventions. Although activation of the hippocampal irisin signaling pathway cannot be concluded without p:t CREB increases, the possibility that irisin injection animals had an increased ratio at an earlier time point during the experiment remains a strong possibility. CREB,

in addition to promoting gene transcription which underlies long-term potentiation and memory formation (Morè et al., 2022; Schaefer et al., 2017), helps to regulate adult hippocampal neurogenesis (Wang et al., 2018). Previous work suggests a role for irisin as a regulator of newborn hippocampal neuron number and morphology (Islam et al., 2021; E. Kim et al., 2023). Our results demonstrate upregulation of the neurogenesis marker NeuroD in animals who received irisin independent of HSP90α activity. Whether the upregulation of NeuroD expression in the present investigation was promoted by CREB is inconclusive. Nevertheless, these results do not support HSP90α as a crucial mediator of irisin signaling to the brain in vivo.

Inhibition of HSP90α by SNX-2112 was validated experimentally by quantifying levels of liver pAkt in the present investigation. After delivery via prodrug SNX-5422, SNX-2112 selectively binds the ATP pocket of HSP90α and HSP90α; this compound showed no significant activity against a panel of 75 off-target enzymes (Huang et al., 2009). An investigation of SNX-2112 in tumor-bearing mice found primary and secondary trafficking of the compound to the tumor and liver, respectively, for up to 48 hours after a bolus 50 mg/kg dose. During this time, tumor levels of pAkt fell below the detectable range in the first 10 hours after dosing then increased to ~55% of baseline by hour 48 (Chandarlapaty et al., 2008). In the present investigation, liver levels of pAkt in animals receiving SNX-2112 were 65% of control 48 hours after a 10 mg/kg bolus dose. These results confirm the inhibition of HSP90α during the intervention period of the present investigation.

Irisin has been proposed as a therapeutic for neurodegenerative conditions, including Alzheimer's disease (Bretland et al., 2021; Islam et al., 2021; Lourenco et al., 2019) and Parkinson's disease (Kam et al., 2022). Importantly, this may allow the beneficial effects of exercise on the brain to be extended to patient populations that are unable to regularly engage in

physical exercise or possibly to augment the impact of an exercise program. Regardless of prospective physical abilities, it is important for the field to understand the physiology required to promote irisin efficacy. Further work is needed in both sedentary and active subjects to understand the relationships between irisin therapy, HSP90α levels and activity, and brain health.

Conclusions

The present investigation supports irisin to promote brain health and provides conflicting evidence regarding the mediating role of HSP90α within the brain in vivo. As the number of individuals who are living into late adulthood continues to rise, a thorough understanding of the beneficial relationship between exercise and the brain is vital to combat the growing prevalence of neurodegenerative disease. These findings give important insight into the efficacy of irisin signaling to the brain under different physiological conditions.

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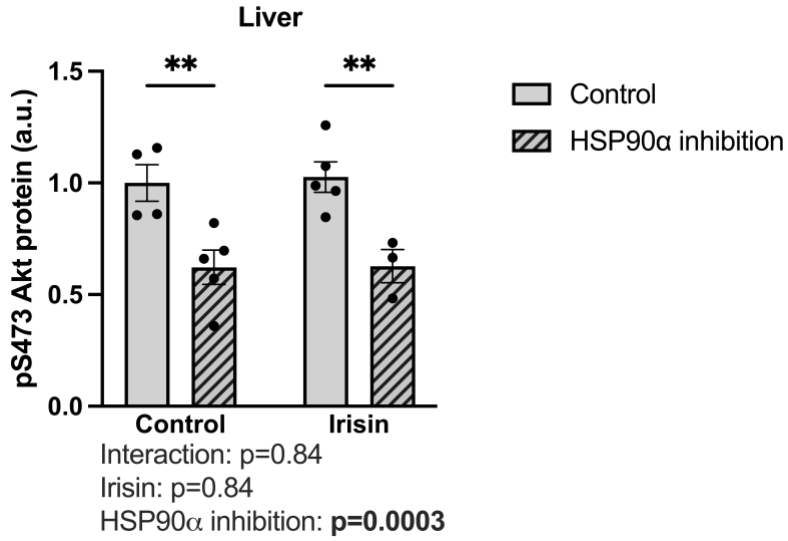
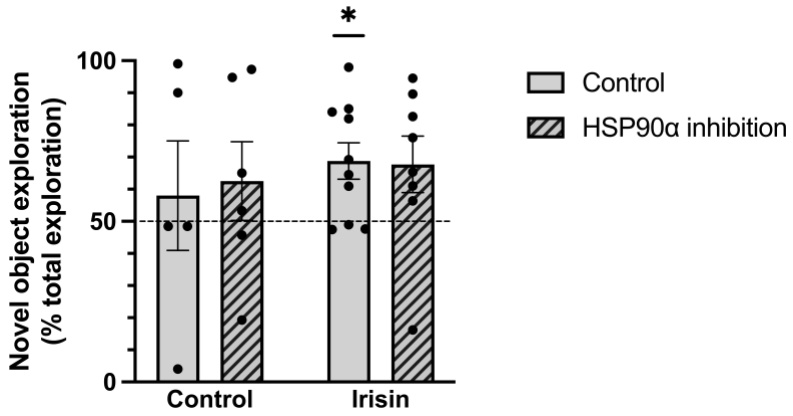
Figure 3.1. Confirmation of HSP90α inhibition.



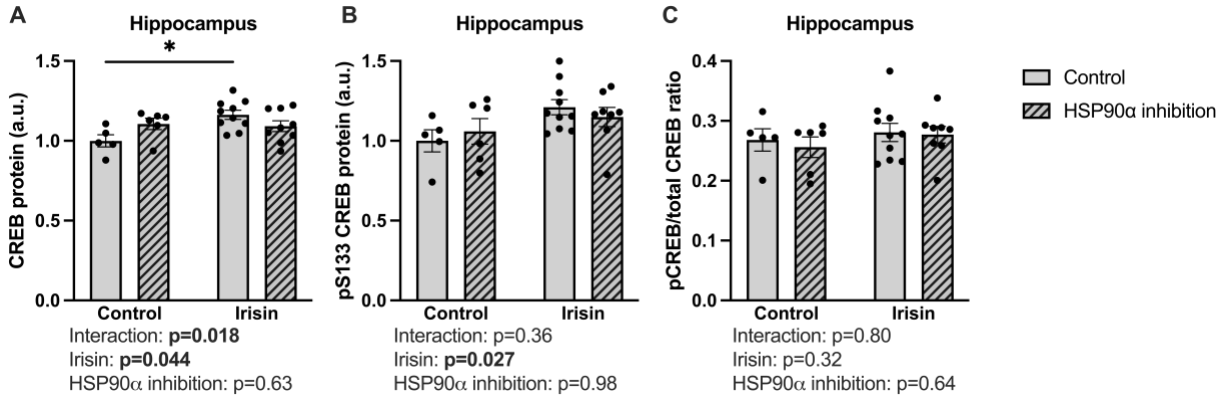
Levels of Ser473 pAkt were quantified in liver samples 48 hours following drug administrations to confirm the actions of HSP90α inhibitor SNX-5422. HSP90α inhibition resulted in decreased pAkt. **p < 0.01, two-way ANOVA with Šídák's multiple comparisons test (*n* = 3–5 animals per group). Bars are mean ± standard error of the mean. a.u., arbitrary units.

Figure 3.2. Evaluation of hippocampus-dependent cognitive function.



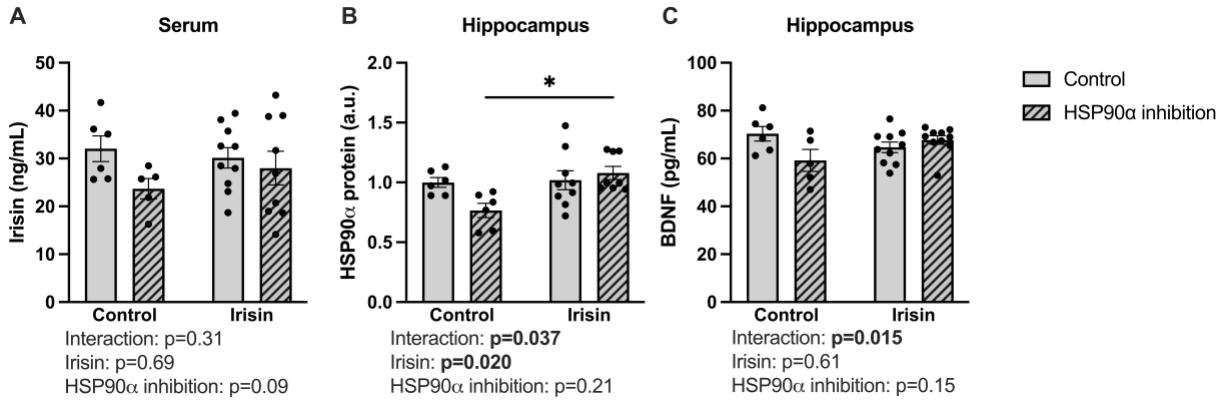
Novel object preference was displayed by animals receiving only irisin. Exploration was not statistically different than 50% (chance level) for animals not receiving irisin and animals receiving both irisin and HSP90α inhibition. *$p < 0.05$, one-sample Student's t-tests (n = 5–10 animals per group). Bars are mean ± standard error of the mean.

Figure 3.3. Hippocampal irisin signaling pathway.



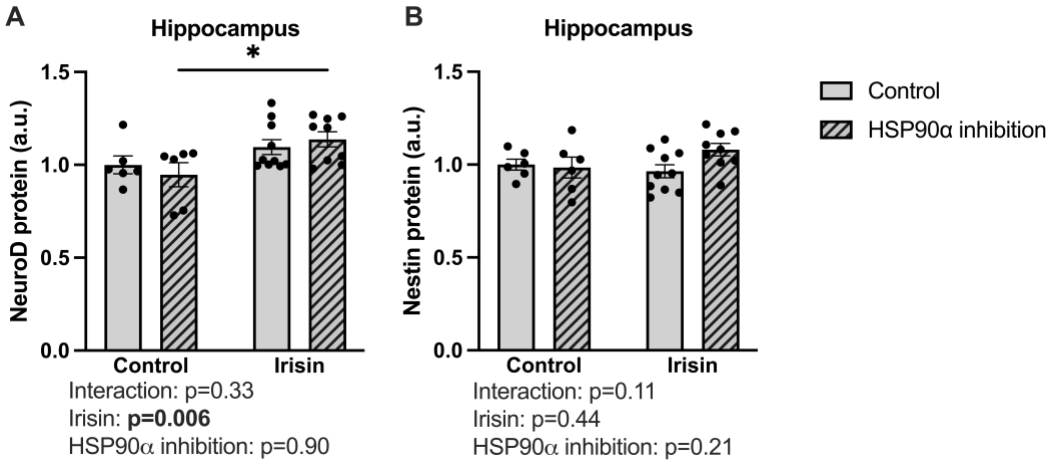
Levels of total CREB and pCREB-Ser133 were quantified in hippocampus samples 48 hours following drug administrations. Irisin administration Upregulation total CREB ($p = 0.044$) following irisin administration was driven by non-HSP90α inhibition animals (irisin + control vs control + control: 1.21 ± 0.15 vs 1.00 ± 0.16 a.u., $p = 0.014$). pCREB was increased by irisin ($p = 0.027$) independent of HSP90α inhibition. *$p < 0.05$, two-way ANOVA with Šídák's multiple comparisons test ($n = 5–10$ animals per group). Bars are mean ± standard error of the mean. a.u., arbitrary units.

Figure 3.4. Influence on hippocampal HSP90α.



Levels of serum irisin (A) and hippocampal HSP90α (B) and BDNF (C) were quantified 48 hours following drug administrations. Hippocampal HSP90α protein levels were increased following irisin administration ($p = 0.020$), predominantly between HSP90α inhibition animals (irisin + HSP90α inhibition vs control + HSP90α inhibition: 1.08 ± 0.16 vs 0.77 ± 0.15 a.u., $p = 0.012$). *$p < 0.05$, two-way ANOVA with Šídák's multiple comparisons test ($n = 5–10$ animals per group). Bars are mean ± standard error of the mean. a.u., arbitrary units.

Figure 3.5. Markers of hippocampal neurogenesis.



Levels of neurogenesis marker NeuroD (A) and nestin (B) were quantified in hippocampus samples 48 hours following drug administrations. Irisin administration upregulated NeuroD independent of HSP90α inhibition ($p = 0.006$), predominantly between HSP90α inhibition animals (irisin + HSP90α inhibition vs control + HSP90α inhibition: 1.14 ± 0.12 vs 0.95 ± 0.16 a.u., $p = 0.039$). *$p < 0.05$, two-way ANOVA with Šídák's multiple comparisons test ($n = 6–10$ animals per group). Bars are mean ± standard error of the mean. a.u., arbitrary units.

Chapter 4 - Evaluation of irisin-induced protection in a transgenic rat model of Alzheimer's disease

Abstract

Introduction. The exerkine irisin is a cleaved and secreted portion of the FNDC5 protein in skeletal muscle. Irisin mediates the beneficial relationship between exercise and the brain and is suggested as a therapeutic strategy against AD. Currently, the mechanisms of irisin-mediated protection against AD and therapeutic efficacy in rats remain unclear. The purpose of this study was to determine the therapeutic influence of irisin on behavioral outcomes and hippocampal mitochondria in TgF344-AD rats. **Methods.** Wild type and transgenic TgF344-AD rats received adenoviral vectors to express FNDC5 (adFNDC5), and subsequently increase systemic irisin, or GFP (adGFP) as control. Serial blood samples were taken, animals underwent behavioral and cognitive testing, and tissues were collected. Protein markers of hippocampal irisin signaling and mitochondrial function were quantified with Jess Simple Western automated western blot analysis and ELISA. **Results.** Serum levels of irisin were not different between adFNDC5 and adGFP animals at any time point following adenoviral intervention. Consequently, irisin-induced changes in behavior, cognitive function, and hippocampal protein expression were not demonstrated. **Conclusions.** The present investigation found no increase in systemic irisin using a strategy previously demonstrated in preclinical mice. The influence of irisin on cerebral mitochondria in AD and the efficacy of irisin-mediated protection against AD in rats remain inconclusive.

Introduction

Exercise is an effective strategy to maintain brain health in late adulthood and reduce the risk of Alzheimer's disease (AD) (Iso-Markku et al., 2022). This beneficial relationship between exercise and the brain in late-life is due, in part, to an endocrine role of skeletal muscle. Irisin is a peroxisome proliferator-activated receptor-gamma coactivator (PGC1-α) dependent myokine cleaved from the extracellular domain of the fibronectin type III domain-containing 5 (FNDC5) transmembrane protein present on skeletal muscle in response to exercise (Boström et al., 2012). The concentration of irisin in plasma increases with exercise and irisin signaling helps to mediate the beneficial relationship between exercise and the brain (Jedrychowski et al., 2015; Wrann et al., 2013).

Irisin is a proposed therapeutic strategy in AD. Administration of irisin in AD mouse models mimics many of the benefits of exercise on the aging brain and, ultimately, rescues AD phenotype (Bretland et al., 2021; Islam et al., 2021; Lourenco et al., 2019). Similarly, therapeutic irisin efficacy has also been demonstrated in preclinical models of stroke, traumatic brain injury, and depression, wherein neuroprotective effects depended upon attenuation of cerebral mitochondrial dysfunction (Guo et al., 2021; Tu et al., 2021; Wang & Pan, 2016). Mitochondrial dysfunction in the brain worsens with age, is a proposed AD etiology, and is opposed by exercise (Berchtold et al., 2019; Dietrich et al., 2008; Steiner et al., 2011; Swerdlow, 2023).

Currently, the therapeutic influence of irisin on mitochondrial health in AD remains unknown. Further, our understanding of irisin-mediated AD protection is informed solely by AD mice. Previous investigations into irisin physiology and irisin-mediated protection against AD have used an FNDC5 adenoviral virus (adFNDC5) to induce a prolonged upregulation of systemic irisin. This strategy results in an upregulation of liver and cerebral FNDC5 transcription

and increased levels of irisin in circulation and the brain (Boström et al., 2012; Lourenco et al., 2019; Wrann et al., 2013). Utilizing this strategy, the present investigation assessed cognitive function and hippocampal biochemistry in TgF344-AD rats.

Methods

Ethical approval

All protocols were approved by the Kansas State University Institutional Animal Care and Use Committee (No. 4466). Kansas State University is accredited by the AAALAC (000667), is assured by OLAW (A3609-01), and is registered with the USDA (48-R-0001). All protocols were in compliance with laboratory animal care and use policies and guidelines set forth by the US National Research Council and the US Public Health Service.

Experimental design

A breeding pair of TgF344-AD rats were obtained from Terrance Town at the University of Southern California. Animals were bred and later housed two-four per cage in the Kansas State University Comparative Medicine Group facility on a 12:12 light:dark cycle with food and water *ad libitum*. Animals were genotyped to determine carriers of the "Swedish" mutant human amyloid precursor protein (APP) and Δ exon 9 mutant human presenilin-1 (PSEN1) genes at three weeks of age. Nine-month-old gene-carrying (AD) and wild type (WT) littermates were selected at random to be injected with either a green fluorescent protein (GFP) control adenoviral vector (AdGFP) or with an adenovirus designed to force overexpression of FNDC5 (AdFNDC5) on experimental day 0. This design created four experimental groups (n=10 per group): WT adGFP, WT adFNDC5, AD adGFP, and AD adFNDC5. Following injections, animals underwent

a battery of behavioral tests. A separate group of WT animals (n=5) were injected with adGFP or adFNDC5 then underwent blood collections.

Interventions

FNDC5 and GFP adenovirus particles were purchased and amplified (ViraQuest Inc., Davis, CA). AdFNDC5 or AdGFP (400 μl; 4.4 x 10^{11} particles) were administered via tail vein injection. Briefly, animals were acclimated to plastic restraint cones restraints for 4 days prior to injections. Tails were applied with topical anesthetic (lidocaine and prilocaine cream, USP 2.5%/2.5%; NDC 62332-582-31; Alembic Pharmaceuticals Inc., Bedminster, NJ) for at least 30 minutes prior to injection. Tails were vasodilated in a warm water bath immediately prior to each injection and a heating pad was used to maintain body temperatures during the procedure. Injections were administered with a 24G IV catheter (No. CI+2420; Nipro Medical Corporation, Miami, FL). Animals were promptly returned to their cages and monitored for signs of distress.

Behavioral tests

Animals underwent a battery of behavioral testing including an open field test (OFT; experimental day 11), novel object recognition (NOR; experimental days 11–13), and Morris water maze (MWM; experimental days 15-18). Animals were allowed 60 minutes in the testing room prior to initiating trials. Trials were conducted under red light (OFT, NOR) or dimmed light (MWM) at the same time of the animals' dark cycle each day. Trials were recorded and monitored in a room adjacent to the test room prior to quantifying movements (EthoVision, Noldus). All protocols were conducted by a blinded investigator.

Open field test

OFT is a test of anxiety and locomotion. Protocol is previously described (Kraeuter et al., 2018; Voikar & Stanford, 2022). Briefly, animals were placed in the center of the arena (100 x 100 x 35 cm) and allowed 5 minutes to explore. The arena was thoroughly cleaned (95% ethanol) and dried prior to each trial to remove odor. The test area was digitally divided into center (75 x 75 cm) and border for quantification.

Novel object recognition

NOR is a hippocampus-dependent test of recognition memory (Squire et al., 2007). The arena (100 x 100 x 35 cm) and objects were thoroughly cleaned (95% ethanol) and dried prior to each trial to remove odor. Protocol is previously described (Antunes & Biala, 2012; Ennaceur & Delacour, 1988). Briefly, the three-day protocol included 5-minute habituation, training, and testing phases. During the habituation phase (day 1), the animal was allowed to explore and become familiar with the empty testing arena. During the training phase (day 2), two identical objects were placed in opposite corners of the arena and the animal was allowed to explore. During the testing phase (day 3), one object was replaced with a new, novel object and the animal was allowed to explore. Novel object and novel object location were randomized to remove object and place preference effects. Exploration time was defined as directing the nose within 2 cm of the object.

Morris water maze

MWM is a hippocampus-dependent test of spatial memory (Riedel et al., 1999). Protocol is previously described (Vorhees & Williams, 2006). Briefly, the four-day protocol included an

habituation phase (day 1), acquisition phase (days 2–3), and probe trial (day 4). The tank was filled with water to a level that is 1 inch below platform (habituation) or 1 inch above the platform with nonfat dry milk will be added to make the water opaque (acquisition, probe). All trials were completed under dim light and 26° C water. During habituation and acquisition phases, animals were placed in each pool quadrant and allowed 120 seconds to find the platform. The animal was guided to the platform if not located during the trial. Animals were allowed 15 seconds on the platform between trials. In the probe trial, the platform was removed, and animals were placed in the pool quadrant opposite of where the platform was learned to be. The first 30 seconds of the probe trial were used for analysis.

Tissue harvest

Animals were anesthetized (isoflurane, 4%) and euthanized via decapitation on experimental day 19. Brains were immediately excised on ice and weighed prior to the isolation of the frontal cortices and hippocampi. Brain dissections were done on a freezer-cold stainless steel block in ice. Tissues were snap frozen in liquid nitrogen then transferred to -80°C freezer to be later processed. In a subgroup of animals, blood samples were taken from the tail vein (experimental days -1, 7, 10, 14, and 17) or via left ventricular puncture at euthanasia (experimental day 20). Samples were collected into a microcentrifuge tube and allowed to coagulate for 30 minutes then centrifuged (1500 x g, 10 minutes, 4°C). Serum was separated then stored at -80°C.

Tissue Preparation

Whole-hippocampi were lysed in ice-cold radio-immune precipitation assay (RIPA) buffer (Sigma-Aldrich) with protease inhibitor (Pierce Protease Inhibitor Mini Tablet, ThermoFisher

Scientific) prepared according to manufacturer's guidelines. Lysate protein concentrations were determined using the Qubit 4 Fluorometer with Protein Broad Range Assay kit (ThermoFisher Scientific).

Biochemical analysis

Western blot

Protein quantification in hippocampal and liver lysates was completed with Jess Simple Western (ProteinSimple) automated western blot analysis with the following primary antibodies: AMP-activated protein kinase alpha (AMPK; 1:250, 10929-2-AP, Proteintech, Rosemont, IL, RRID:AB_2169568); Thr172 phosphorylated AMPK alpha (pAMPK; 1:250, 2535T, Cell Signaling Technology, Danvers, MA, RRID: AB_331250); cAMP-response element binding protein 1 (CREB; 1:10, NBP1-90364, Novus Biologicals, Centennial, CO, RRID:AB_11038978); Ser133 phosphorylated CREB (pCREB; 1:10, NB300-273, Novus Biologicals, RRID:AB_10002306); citrate synthase (1:50, NBP2-13878, Novus Biologicals, RRID:AB_3261453); cytochrome c (1:250, 126F3, Cell Signaling Technology, RRID:AB_10695410); extracellular signal-regulated kinase 1/2 (ERK1/2; 1:250, sc-514302, Santa Cruz Biotechnology, Dallas, TX, RRID:AB_2571739); Thr202/Tyr205 dually phosphorylated ERK1/2 (pERK1/2; 1:250, sc-136521, Santa Cruz Biotechnology, RRID:AB_10856869); PGC1-α (1/100, PA5-72948, ThermoFisher Scientific, RRID:AB_2718802). Lysate protein concentrations were equalized before protein quantification (1 or 2 μg/μl protein per sample). Protein expression was quantified with Compass for Simple Western software.

ELISA

Commercial ELISA kits were used to quantify irisin in hippocampal lysates and serum (EK-067-20, Phoenix Pharmaceuticals, Burlingame, CA). Assays were optimized to determine appropriate sample dilution factors and completed according to manufacturer's instructions.

Statistical Analysis

Datasets were first probed for outliers using the ROUT method (Q = 1%). Group differences were identified via one-way ANOVA with Šídák's multiple comparisons test (single pooled variance). Residuals were tested for normality (D'Agostino-Pearson omnibus K2 test) and homoscedasticity (Bartlett's test) to verify assumptions of ANOVA were met. If assumption of normality was not met, Kruskal-Wallis test with Dunn's multiple comparison's test was used. If the assumption of equal variance was not met, Welch's ANOVA with Dunnett's T3 multiple comparisons test (individual variances computed for each comparison) was used. All significance tests were two-sided using $p < 0.05$ as the level of significance. Values are presented as means and standard deviations. All analyses were performed using GraphPad Prism version 10.5.0 for macOS (GraphPad Software).

Results

Adenoviral upregulation of irisin

To quantify the expected upregulation of irisin, a subgroup of WT animals were injected with adFNDC5 or adGFP on experimental day 0 and blood samples were collected on experimental days -1, 7, 10, 14, 17, and 20. Serum irisin levels were not different between groups at any time point (Figure 4.1A). We additionally quantified irisin in the hippocampi of our primary cohort of

animals. Hippocampal irisin was decreased in AD adFNDC5 (16.95 ± 3.34 ng/mg) animals in comparison to WT adFNDC5 (23.65 ± 3.91 ng/mg, p = 0.003) and AD adGFP (23.28 ± 4.71 ng/mg, p = 0.006) groups (Figure 4.1B).

Behavioral and cognitive outcomes

Figure 4.2A is an experimental timeline of behavioral tests. The OFT is an assessment of spontaneous mobility and behavior. Less anxiety-like behavior is commonly represented as increased time spent exploring the center of the arena and higher locomotion. (Voikar & Stanford, 2022). Mood and behavioral changes, including heightened anxiety, are often seen with AD. Irisin is previously demonstrated to provide benefit in a rodent (Wang & Pan, 2016) model of depression, and we have previously shown that exercise ameliorates anxious-like behavior in the TgF344-AD rat (Lopez et al., 2024). In the present investigation, no group differences in anxiety-like behavior were present following adenovirus injections (Figure 4.2B-C).

The NOR test and MWM are hippocampus-dependent measures of spatial (Squire et al., 2007) and recognition (Riedel et al., 1999) memory, respectively. Previous work demonstrates therapeutic irisin to ameliorate AD-induced deficits in hippocampal function in mouse models (Islam et al., 2021; Lourenco et al., 2019). Due to low exploration times in the present investigation, NOR data could not be analyzed. Figure 4.2D-E shows time spent in each pool quadrant and distance traveled during the MWM probe trial. Quadrant times within each group were analyzed by one-way ANOVA with multiple comparisons against the target quadrant (SE). Time spent in the target quadrant was not significantly higher than all other quadrants in any

experimental group, suggesting that both AD-induced deficits and irisin-induced benefits in hippocampal function were either not present or not captured in the current investigation.

Irisin signaling and mitochondrial markers

Irisin signaling to the hippocampus activates the cyclic AMP/PKA/CREB signaling pathway, resulting in an increased ratio of phosphorylated to total (p/t) CREB and, subsequently, p/t ERK1/2 (Lourenco et al., 2019, 2022). In the present investigation, CREB and ERK1/2 phosphorylation was unaltered following adenoviral administration (Figure 4.3).

Previous work demonstrates therapeutic irisin to ameliorate stroke, depression, and traumatic brain injury phenotypes in mechanisms dependent on AMPK activation or UCP2 upregulation and improvement of cerebral mitochondrial health (Guo et al., 2021; Tu et al., 2021; Wang & Pan, 2016). We quantified these markers, as well as, PGC1-α, a key regulator of mitochondrial biogenesis and FNDC5 expression, and mitochondrial mass markers cytochrome C and citrate synthase to assess the effect of irisin on cerebral mitochondrial health in AD (Figure 4.4). ANOVA suggested significant differences between at least two groups in p/t AMPK ($F(3, 33) = 3.379$, $p = 0.030$), UCP2 ($F(3, 34) = 3.955$, $p = 0.016$), cytochrome C ($F(3, 22.6) = 3.127$, $p = 0.046$), and citrate synthase ($F(3, 36) = 3.034$, $p = 0.042$) expression. However, multiple comparisons tests did not reveal mean differences between groups.

Discussion

The present investigation primarily aimed to assess the influence of irisin on AD phenotype and cerebral mitochondria in TgF344-AD rats using a previously demonstrated

strategy to induce a prolonged irisin upregulation, behavioral tests, and protein analyses. Primarily, our results show no change in systemic irisin levels following adenoviral intervention. Consequently, influences of irisin on behavioral and biochemical outcomes in the present study remain inconclusive.

Intravenous administration of adFNDC5 in mice is previously demonstrated to induce a 15-fold increase in liver *Fndc5* mRNA resulting in a 3–4-fold increase in circulating irisin 10 days after injection (Boström et al., 2012). After 7 days, work from others report a similar increase in circulating irisin and hippocampal irisin without an upregulation of hippocampal or forebrain *Fndc5* gene expression (Lourenco et al., 2019; Wrann et al., 2013). Using this technique, roles of peripheral irisin as a promoter of white adipose browning and hippocampal *Bndf* expression were elucidated (Boström et al., 2012; Wrann et al., 2013). Further, elevated peripheral irisin due to adFNDC5 was used to reverse AD-induced deficits in recognition memory (Lourenco et al., 2022). In the present investigation, we found no increase in circulating irisin at 7 or 10 days in rats following intravenous adFNDC5 administration in rats.

In the present investigation, behavioral and cognitive tests did not indicate AD-induced deficits in 9-month-old TgF344-AD rats. Female-specific anxiety-like behavior and sex-combined deficits in spatial memory function in TgF344-AD rats as early as 6 months of age are previously demonstrated by our group (Lopez et al., 2024). Saré et al. (2020) showed anxious behavior in the OFT displayed by female transgenic TgF344-AD rats compared to WT at 6 and 12 months. Similarly, OFT tests in another study reported increased anxiety-like behavior in female transgenic animals at 9 months (Srivastava et al., 2023). Similar to the results reported herein, Srivastava et al. (2023) also found no spatial memory deficits during the MWM probe trial in female AD animals at 9 months. However, others have shown deficits in MWM

performance displayed by female transgenic rats as early as 6 months (Bernaud et al., 2022). Importantly, protection against behavioral and cognitive phenotypes from exercise is demonstrated in TgF344-AD rats (Lopez et al., 2024; Yang et al., 2022) and from irisin in preclinical mouse models (Islam et al., 2021; Lourenco et al., 2019; Wang & Pan, 2016). Without an upregulation of irisin in the present study, irisin-mediated protection against behavioral and cognitive deficits in the TgF344-AD model remains untested.

Mitochondrial dysfunction in the brain develops early in AD and is previously described in TgF344-AD rats. The AD mitochondrial cascade hypothesis holds that mitochondrial dysfunction is present early in the AD continuum. This deficit drives and is driven by changes in histology (e.g., amyloid beta plaques, tau tangles) and brain systems (e.g., CBF alterations, neuroinflammation). In concert, these neurodegenerative mechanisms lead to AD dementia (Swerdlow, 2023). Mitochondrial alterations in AD include reduced TCA cycle (Gibson et al., 1998) and electron transport chain complex enzyme activities, altered mitochondrial morphology (Hirai et al., 2001), increased mitochondrial DNA oxidation (Mecocci et al., 1994), and reduced hippocampal protein and mRNA levels of PGC1-α (Sheng et al., 2012). The TgF344-AD model displays deficits in complex I activity and oxidative phosphorylation as early as 6-7 months (Viel et al., 2021), impairments in whole-body and hippocampal and glucose uptake at 9 months (Joo et al., 2022; Srivastava et al., 2023), and reduced hippocampal PGC1-α (Yang et al., 2022). Investigations in pre-clinical models of depression, traumatic brain injury, and stroke demonstrate irisin therapy to drive mitochondrial health and subsequently rescue cognitive function. Upregulated UCP2, PGC1-α, and AMPK phosphorylation as a result of irisin therapy improved mitochondrial biogenesis and dynamics, oxidative phosphorylation, ATP levels, brain glucose uptake, reactive oxygen species formation and antioxidant defense, and mitochondrial-

mediated apoptosis (Guo et al., 2021; Tu et al., 2021; Wang & Pan, 2016). In the present investigation, irisin-induced changes in hippocampal mitochondrial protein markers were not present. Further work is needed to investigate the influence of irisin therapy on cerebral mitochondria in AD.

Conclusions

Interventions to upregulate systemic irisin mimic a portion of the beneficial effects of exercise on the brain and are proposed as a therapeutic strategy against AD. The present study found no increase systemic irisin using a strategy previously demonstrated in preclinical mice. Further work is needed to understand the applicability of adFNDC5 in rats and to assess overlapping mechanisms in irisin- and exercise-mediated protection against AD.

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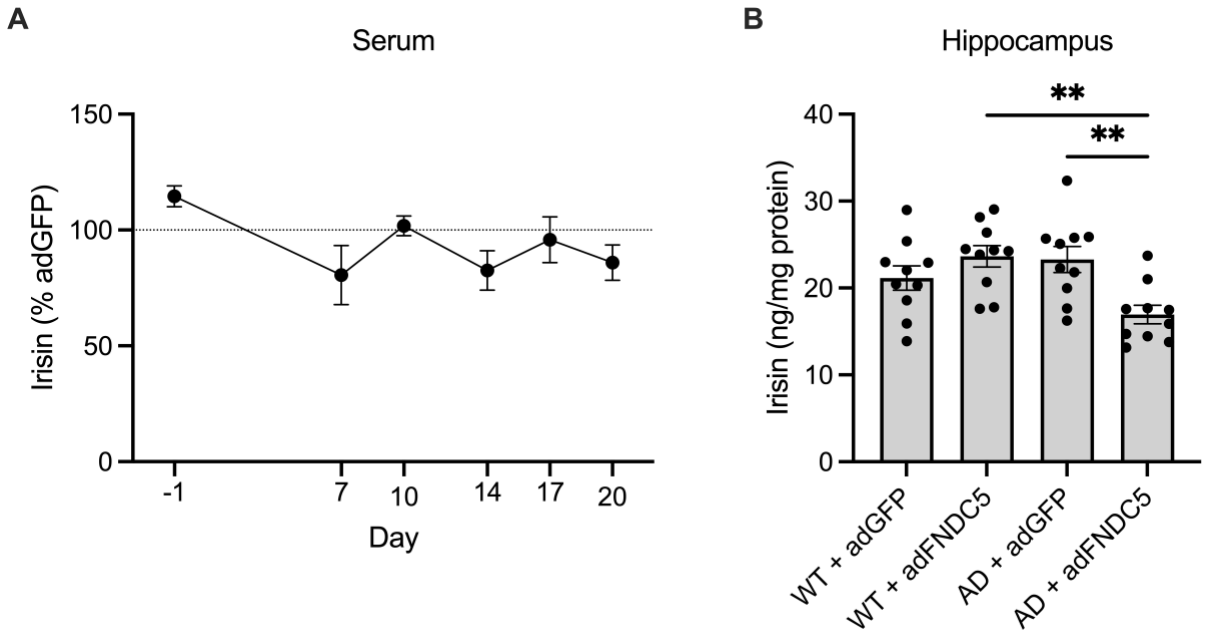
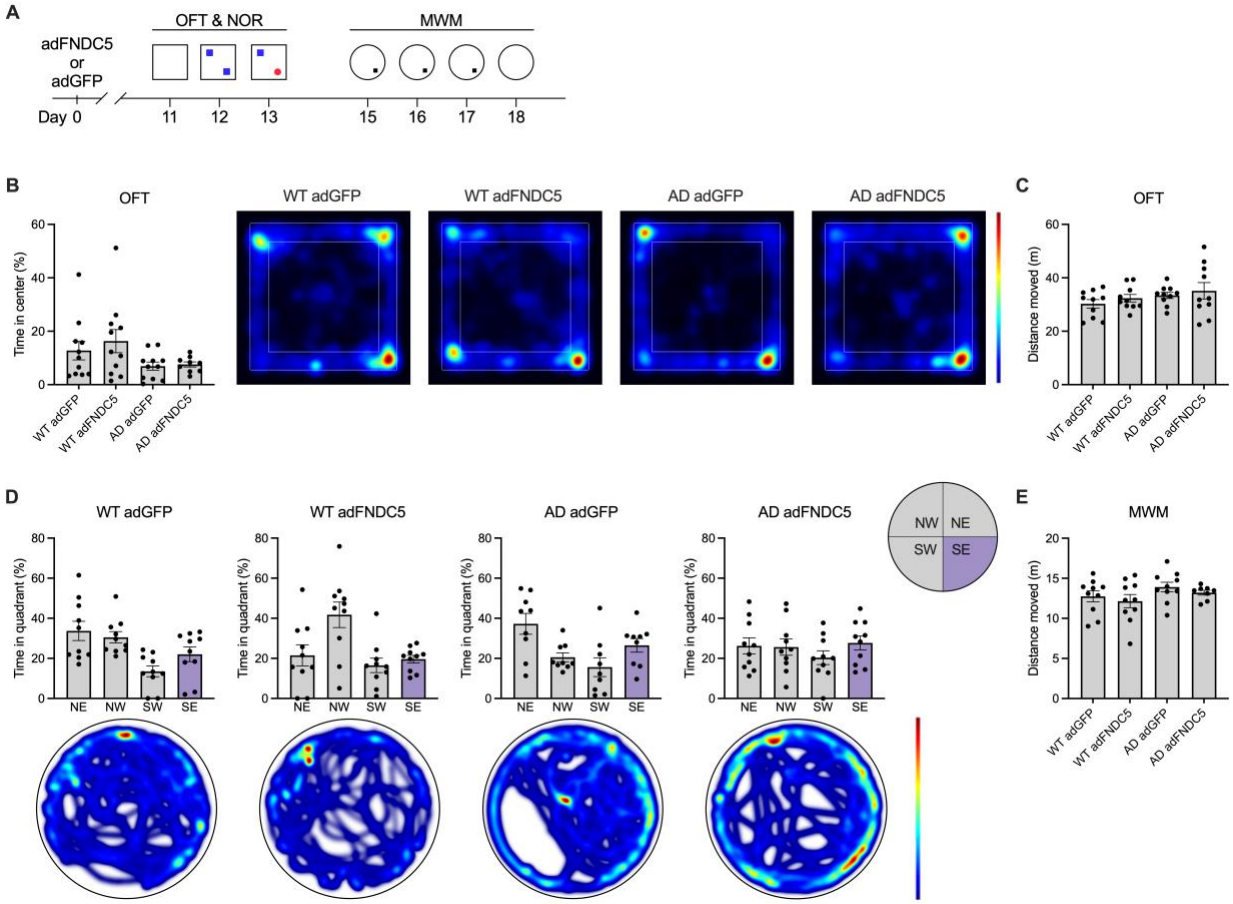
Figure 4.1. Serum and hippocampal irisin following adenovirus administration.



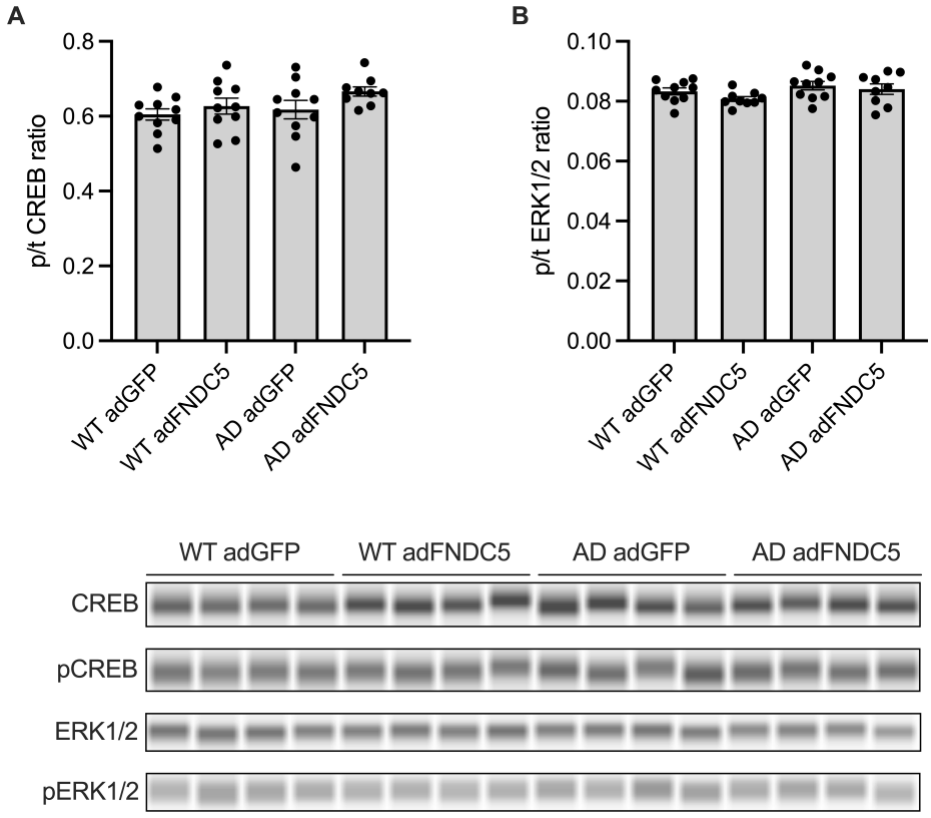
Comparisons of serum and hippocampal irisin after adenovirus administration. A, Serum irisin was not different between groups at baseline or in the days following adenovirus injection (two-sided unpaired Student's t-tests, *n* = 2-3 animals per group). B, Hippocampal irisin on experimental day 19 was lower in AD adFNDC5 in comparison to WT and AD counterparts. Two-sided unpaired Student's t-tests, *n* = 2-3 animals per group (A) or one-way ANOVA with Šídák's comparisons test, *n* = 10 animals per group (B). **p < 0.01. Values are mean ± standard error of the mean.

Figure 4.2. Behavioral and cognitive outcomes.



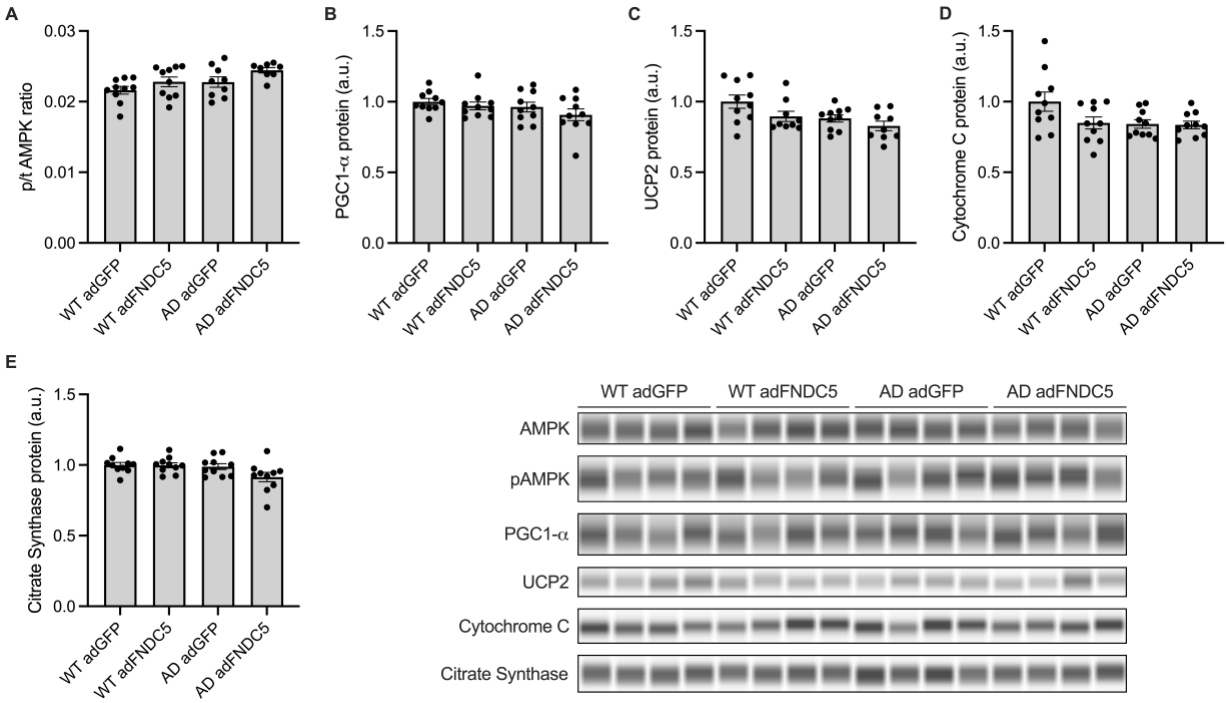
Behavioral outcomes following adenoviral administration. A, Schematic depicting the experimental timeline of behavioral tests. B-C, Time spent in the center of the arena (B) and distance traveled (C) during OFT. Heatmaps are group exploration averages with white lines denoting the outer edge and center area of the test arena. D-E, Time spent in each pool quadrant with graphical legend (D) and swimming distance (E) during the MWM probe trial. Heatmaps are group exploration averages with black circle denoting the outer edge of the test arena. One-way ANOVA with Šídák's comparisons test (B-C, E) or one-way ANOVA with Šídák's comparisons test vs SE quadrant (D), n = 8-10 per group. Bars are mean ± standard error of the mean. OFT, open field test; NOR, novel object recognition; MWM, Morris water maze.

Figure 4.3. Hippocampal mediators of irisin signaling.



Protein quantification was completed in hippocampi collected on experimental day 19 to assess the hippocampal irisin signaling pathway. Ratios of total and S133 phosphorylated CREB (A) and total and Thr202/Ty2205 dually phosphorylated ERK1/2 (B). Group differences CREB and ERK phosphorylation were not present. One-way ANOVA with Šídák's comparisons test, n = 9-10 per group. Bars are mean ± standard error of the mean.

Figure 4.4. Mitochondrial markers.



Protein quantification was completed in hippocampi collected on experimental day 19 to assess the influence of adenoviral intervention on markers of mitochondrial biogenesis and function. Quantities of total and Thr172 phosphorylated AMPK ratio (A), PGC1-α (B), UCP2 (C), cytochrome C (D), and citrate synthase (E). One-way ANOVA with Šídák's comparisons test (A-C, E) or Brown-Forsythe ANOVA with Dunnett's T3 comparisons test (D), n = 8-10 per group. Bars are mean ± standard error of the mean. a.u., arbitrary units.

Chapter 5 - Conclusions

This dissertation aimed to assess the physiological role of irisin and mechanisms of irisin-induced protection against AD. In chapter 2, we found that a single administration of irisin was sufficient for activation of irisin-hippocampus signaling. In chapter 3, we found animals receiving irisin displayed significant hippocampus-dependent cognitive function and that the cofactor extracellular heat shock protein 90 alpha was not necessary for irisin-receptor interaction in the brain. In chapter 4, we aimed to investigate the influence of irisin on AD phenotype and cerebral mitochondria in transgenic rats following an adenovirus intervention previously demonstrated to upregulate systemic irisin. We found no increase in irisin levels or irisin signaling in our transgenic AD rats following vector administration.

Taken together, these studies suggest irisin as an important, physiologically relevant mediator of the beneficial relationship between exercise and the brain. As the number of individuals who are living into late adulthood continues to rise, a thorough understanding of the beneficial relationship between exercise and the brain is vital to combat the growing prevalence of neurodegenerative disease. These findings give important insight into the efficacy of irisin signaling to the brain under different physiologic conditions. However, further work is needed to understand the translatability of irisin-mediated neuroprotection and mechanisms through which irisin may confer protection against AD.