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2011

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Effect of AMPK Activation on Alzheimer's-like Symptoms in APP Mice

A dissertation submitted in partial satisfaction of the requirements for
the degree Doctor of Philosophy

in

Biology

by

Kacee DiTacchio

Committee in charge:

Professor Stephen Heinemann, Chair

Professor Amy Kiger

Professor Edward Koo

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2011

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University of California, San Diego

2011

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Acknowledgements

I would like to thank Stephen Heinemann for the opportunity to conduct my dissertation research in his lab. He first accepted me into his lab as a research assistant in 2003. During that period, the experience in his lab taught me a lot about how research is conducted and inspired me to continue my education. After joining the lab as a graduate student, Steve allowed me to pursue my own ideas while providing whatever support I required. His approach to science is an honest and unbiased, yet critical and skeptical, search for truth. I feel very fortunate to have had the opportunity to study under his guidance.

I would also like to thank Gustavo Dziewczapolski for training on behavioral studies with the mice, advice about my project, consideration of data, reviewing my written works and general support in my many times of need. He always has a frank perspective and a kind word, which have been invaluable to me throughout the course of my study.

I would like to acknowledge Russell Nofsinger for training on the insulin tolerance testing that was used in the metformin-treated animals. I would like to acknowledge Ann Atkins for measurement of plasma levels of metformin. Also, although not included in this work, Eliezer Masliah provided me with training and support in histology, but also with opinions and suggestions about the project.

Finally, I would like to thank my family. My children, Kayla and Sydney, have given me purpose, fulfillment, happiness and the desire to be better. In

particular, Kayla has grown into an intelligent and beautiful young lady through these years and has been my joy and my helper and made me very proud. My husband, Luciano DiTacchio, has been there for me through these years that have not always been easy, has listened to my ideas and doubts, and has been a friend when I needed and a colleague and critic at times as well. He has demonstrated personal and professional qualities that I admire and hope to emulate.

Chapter 2, in part is currently being prepared for submission for publication of the material. DiTacchio, Kacee; Atkins, Ann; Heinemann, Stephen. The dissertation author was the primary investigator and author of this material.

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ABSTRACT OF THE DISSERTATION

The Effect of AMPK Activation on Alzheimer's-like Symptoms in APP Mice

by

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Doctor of Philosophy in Biology

University of California, San Diego 2011

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Research toward understanding Alzheimer's disease has revealed that it is strongly linked with metabolic dysfunction, such as that found in Type II Diabetes. For instance, diabetic patients have a doubled risk of developing AD, while Alzheimer's patients have disrupted insulin signaling in the brain. Moreover, some lifestyle modifications beneficial to diabetic patients - specifically, increased physical activity and caloric restriction - have shown promise as tools to manage AD. Both of these interventions create an energy deficit whereby the cellular energy sensor AMPK becomes activated. In turn, AMPK activation promotes energy availability, insulin sensitivity and cellular survival during times of stress. We activated AMPK both pharmacologically and genetically in a transgenic mouse model of AD and measured the effect on learning and memory function by the Morris water maze. Surprisingly, our results revealed a gender-specific response in Alzheimer's phenotypes. Pharmacological activation of AMPK by the anti-diabetic drug metformin in

male APP mice worsened learning and memory function. This finding was reproduced with a genetic model of AMPK activation, requiring only liver-specific activity to occur. Remarkably, activation of AMPK in this murine model, either by genetic means or metformin treatment, had beneficial effects for female animals.

The results herein demonstrate the effect of AMPK activation in the APP mouse model of AD, the gender-specific differences in cognitive function in response to treatment, the importance of liver function in mental health and the need to assess these factors in human patients as there are currently millions of patients already taking metformin that may be altering their risk of Alzheimer's disease.

CHAPTER 1:

Introduction and Hypothesis

Alzheimer's disease (AD) is a neurodegenerative illness characterized by loss of mental abilities affecting memory, language and cognition, eventually progressing to dementia and ultimately to death. This ailment has significant social and economic ramifications, as the memory loss of those affected eventually becomes so extensive that they can no longer recognize family members or care for themselves. There are currently over 5 million Alzheimer's disease sufferers in the U.S. alone, but this figure is likely to increase significantly as our population ages along with an increasing life expectancy. Some estimates predict that by the year 2025, we will have a 50% increase in the number of cases in the US [1]. Currently there is neither a cure nor even any treatment shown to slow the disease progression significantly.

The AD brain is characterized by the presence of amyloid plaques, neurofibrillary tangles, synaptic degeneration and neuronal death. A cleavage product of the amyloid precursor protein (APP), the beta amyloid peptide ($A\beta$), is found in the amyloid plaques and is thought to play a causative role in the development of the disease. This has been supported by the generation of mouse models of AD overexpressing human variants of APP: those that carry mutations favoring the generation and aggregation of $A\beta$ have successfully recreated many of the phenotypes observed in human patients [2] both in terms of pathology and cognitive decline.

APP processing and generation of the $A\beta$ peptide

APP is a ubiquitously-expressed, single-transmembrane protein. It undergoes proteolytic cleavage events that release fragments into both the

extracellular and intracellular space. The first cleavage is mediated by either α - or β -secretase at one of two mutually exclusive sites, which leads to the extracellular release of the N-terminal domain. The alpha cleavage generates the longer sAPP α , which is thought to be involved in numerous beneficial functions including synaptic plasticity and protection from excitotoxic and oxidative insults (reviewed in [3]). Moreover, alpha cleavage precludes the production of the toxic A β fragment upon later cleavage within the membrane by γ -secretase. On the other hand, the β -site cleavage releases a shorter fragment extracellularly without known benefits and furthermore enables the production of A β . This cleavage pathway is considered amyloidogenic and is initiated by the β -site APP cleavage enzyme 1, BACE1 (reviewed in [4]).

Following β -site proteolysis, a second cleavage event within the membrane by γ -secretase can occur at one of multiple sites. This event results in the generation of A β peptides of which the predominant variants are A β_{40} and A β_{42} . These peptides are found in cerebrospinal fluid in healthy individuals throughout life without apparent detrimental effect. Evidence suggests that they become toxic upon accumulation and oligomerization [5]. The A β_{42} species is more aggregation-prone. Increased production of A β , especially A β_{42} , or reduced clearance, may promote its accumulation and eventual aggregation. Larger aggregates form insoluble amyloid plaques that are a characteristic feature of the AD brain.

Neuropathological features of AD

Many adverse changes have been noted in the AD brain, which are thought to arise as a downstream consequence of A β oligomerization. For instance, the activation of reactive astrocytes and microglia has been reported [6], which leads to the production of proinflammatory cytokines and reactive oxygen species. Such activation is generally a protective mechanism by which the brain removes foreign bodies, but in the case of AD it appears that this process exacerbates the damage [7]. In healthy brain, glial cells remain quiescent until an event such as neuronal injury leads to glial cell activation and inflammatory response. In the AD-afflicted brain, additional stimuli are present for induction of glial cell activation, including A β , but in this case, the resultant inflammatory response becomes chronic [8, 9]. The activated cells, together with the cytokines and reactive oxygen species they produce, efficiently destroy and clear invading bodies and debris, but when persistently active may be cytotoxic to neighboring neurons [6, 10].

Other changes found in the AD brain include mitochondrial dysfunction, energy depletion and oxidative stress, which are thought to play important roles in the progression of AD as well as other neurodegenerative diseases [11]. The need for energy is high in neuronal cells, as the maintenance of ion concentration gradients and synaptic vesicle cycling requires ATP and is essential to neuronal function. Aerobic respiration is utilized to meet the usual energy needs, but the brains of AD patients show decreased cytochrome c oxidase activity and reduced mitochondrial oxygen

consumption (reviewed in [12]). Exposure to A β is thought to be the cause of this dysfunction and has also been demonstrated to induce oxidative damage. It is unclear if the A β -induced oxidative damage causes the mitochondrial dysfunction or if A β interferes directly with mitochondrial function which then leads to oxidative stress. Paradoxically, oxidative damage has been reported to promote the production of additional A β [13], thereby creating a vicious positive feedback cycle with further damage.

Finally, an interesting difference between the brains of Alzheimer's patients and those of healthy individuals is in the decreased CSF-to-plasma insulin ratios and decreased insulin signaling that have been observed in the former [14, 15]. One potential cause of this alteration is peripheral hyperinsulinemia, which is thought to interfere with the transport of insulin across the blood brain barrier if the associated receptor-dependent transporters are desensitized (reviewed in [16]). Reduced insulin levels in the brain may be problematic for at least two reasons. First, it has lately been reported that insulin signaling is important for the clearance of A β , possibly by stimulating the secretion of the insulin degrading enzyme, which degrades both insulin and A β , or by enhancing internalization of A β , which in combination with insulin signaling causes no impairment to neuronal function or plasticity [17]. Additionally, insulin has been demonstrated to enhance synaptic plasticity and, when given in conjunction with glucose, enhances cognitive function in humans and animal models [18] – reducing this signaling pathway may, therefore, have the opposite effects. To counteract the insulin

signaling deficiency in AD, recent studies have administered intranasal insulin and reported some acute improvements in memory and attention in early AD patients without affecting plasma insulin or glucose levels [19].

Metabolic dysfunction and AD

Epidemiological data have correlated obesity and diabetes with a two- to three-fold increased risk of developing AD [20, 21]. Although epidemiological data have demonstrated the correlation between these factors, an additional causal relationship has been suggested by studies in animal models. For instance, mouse models of AD fed a high-fat diet or high-fat/high cholesterol diet exhibit accelerated AD-like phenotypes [22, 23]. In particular, when the fat content of food offered to wild type mice is increased from 10% to 60%, they become obese and develop insulin resistance. When this diet is given to APP mice, they generate more A β and demonstrate greater cognitive impairment on the Morris water maze behavioral test of learning and memory. Such evidence of a strong relationship between obesity and insulin resistance with Alzheimer's has prompted exploration into the treatment of AD with anti-diabetic medication [23].

Therapeutic strategies – drug treatment and clinical trials

Currently, there are five drugs falling into two classes that have been approved by the FDA for treatment of AD. Four of the drugs are cholinesterase inhibitors. Early Alzheimer's research found a loss of cholinergic neurons that provide input to the regions most affected in the AD brain [24]. Inhibiting cholinesterase action allows acetylcholine to remain present for a

longer time at the synapse, thus enhancing the effects of what remains. Patients taking these drugs experience modest improvements in memory, with no alteration to the long-term progression of the disease.

The last of the five FDA-approved drugs is the NMDA receptor antagonist memantine. The rationale behind the use of this drug is to protect neurons from glutamate-induced excitotoxicity. Similar to cholinesterase inhibitors, this drug has shown benefits against the symptoms of the disease, but does not alter the progression. In fact, either treatment only benefits roughly half of patients for an average of six months to one year.

Recently, strategies to reduce A β levels have been attempted by various mechanisms. Inhibition of β - or γ - secretases would prevent the production of A β , but it has proven difficult to design drugs small enough to cross the blood brain barrier but sufficiently large to bind the catalytic sites specifically and without significant side effects or safety concerns [25]. More than one γ -secretase inhibitor has made it to phase III clinical trials only to fail for lack of efficacy [26, 27]. However, if specificity and safety can be designed, β - or γ -secretase inhibition remain promising treatments. Indeed, both are still being pursued at various levels of clinical testing.

In addition to preventing the production of A β , another approach has been to use antibodies to aid in its removal. Early studies with active immunization in mice were very encouraging [28], but when attempted in clinical trials, a small percentage of participants developed auto-immune meningoencephalitis [29, 30]. Later investigation suggested that inflammation

was centered around antibodies binding to A β amyloid angiopathy [31]. Passive immunotherapy is currently being pursued with caution in three current phase III clinical trials.

Given the connection between diabetes and Alzheimer's disease, the anti-diabetic drug rosiglitazone was proposed for use and demonstrated positive outcomes in murine models [32]. The drug is a peroxisome proliferator-activated receptor (PPAR) γ agonist and functions throughout the body to improve insulin sensitivity. A subset of human patients – those without ApoE4 – demonstrated improvement in cognitive function in early clinical trials [33, 34]; however, larger, phase III clinical trials demonstrated no benefit and further testing has been discontinued [35].

One other potential treatment involves intranasal insulin. This mode of administration avoids potential hypoglycemia or insulin resistance that could occur if it were given systemically. It is thought that diabetes may exacerbate AD by disrupting insulin signaling within the brain – this could occur if neuronal insulin receptors become insulin resistant along with peripheral receptors; alternatively, it has been suggested that transport of insulin across the blood brain barrier is a receptor-dependent mechanism which can itself become insulin resistant, leading to hypoinsulinemia within the brain [16]. Indeed, AD patients have reduced insulin signaling [36], and also lower insulin levels within the brain [15]. Intranasal insulin has previously been shown to confer acute benefits to memory [37] and a recent study showed similar benefits over the

course of a three-week period providing another avenue of hope for AD patients and paving the way for more extensive clinical trials [38].

Therapeutic strategies – lifestyle changes

Although no pharmaceutical treatment has yet been shown to slow the progression of AD significantly, some lifestyle modifications have been suggested to be successful in this regard or to reduce the risk of developing the disease. One such modification is caloric restriction (CR). Reducing caloric intake to 60-70% of normal food levels while meeting nutritional needs has been demonstrated in a wide variety of animals to promote health and longevity, including an increase in both mean and maximum lifespan (reviewed in [39]). This highly conserved survival strategy protects organisms against many age-related diseases and neurodegenerative disorders such as Alzheimer's. Studies specifically considering CR in mouse models of AD in APP mice have shown that this regimen leads to decreased production and deposition of A β [40, 41] and improved performance in behavioral tasks measuring learning and memory [42].

Besides CR, regular physical activity is another environmental variable that may protect against AD. Physical activity is known to increase an organism's "health-span" by delaying the onset of many age-related disorders [39]. Some particular benefits of regular exercise include: improved cardiovascular health, reduced obesity, improved anxiety and depression, and many studies now suggest advantages for cognitive function throughout life as well [43]. Several studies in human patients with AD have shown a

decrease in the loss of cognitive abilities and activities of daily living (ADL) with regular exercise [44-47]. These studies have typically been carried out with patients in nursing homes and included 30-60 min of exercise 2-3 times per week. Additional studies have been conducted in mouse models of AD, in which exercise has been shown to improve disease phenotypes. In such studies, exercise has been shown to significantly decrease the decline in cognitive function typically found in these mice. Pathologically, this is accompanied by a reduction in A β load and alterations to neuroinflammatory profiles [48-50]. The mechanism by which exercise exerts these effects remains unknown.

AMPK: the cellular energy sensor

In addition to promoting general health and neuroprotection in AD, CR and exercise share a molecular effect: both are conditions in which AMP-dependent protein kinase (AMPK) becomes active. AMPK is a heterotrimeric protein consisting of α , β , and γ subunits that serves as a cellular energy sensor. When ATP levels decrease and AMP levels rise, as occurs during exercise or in low nutrient conditions, AMP binds to the γ subunit of AMPK causing a conformational change that allows for the activating phosphorylation of the catalytic α subunit by an upstream kinase [51]. Active AMPK induces a reduction in energy-consuming and energy-storage pathways along with an increase in energy-producing pathways and generally favors cell survival in times of stress. Consistently, during exercise or while experiencing caloric

restriction, AMPK becomes activated in the periphery, particularly in liver, skeletal muscle and adipocytes.

In the liver, the effects of AMPK activation are to decrease gluconeogenesis and improve glucose tolerance [52, 53]. A study in transgenic mice reported that constitutive AMPK activation in the liver decreased weight gain with age, decreased fat stores, reduced plasma insulin levels and provided resistance to obesity when the mice were given a high fat diet [53]. This report suggests that AMPK activation in the liver leads to beneficial metabolic effects throughout the body, which serve to protect against obesity and diabetes, and may thereby reduce two widespread risk factors for AD.

In adipocytes, AMPK activation increases beta-oxidation of fatty acids and inhibits lipogenesis, thus mobilizing energy stores and reducing additional triglyceride synthesis [54]. Utilization of fat stores counteracts obesity, which alone may serve to mitigate AD risk. Moreover, obesity alters expression of at least two adipocyte-derived hormones: adiponectin and leptin. Adiponectin, which is itself an AMPK activator and is considered an anti-diabetic hormone, is severely reduced in obese individuals [54-56]. Reduction of fat stores through frequent exercise or CR, therefore, could restore this signaling pathway, which in turn affects various target cells throughout the periphery as well as within the brain. Furthermore, adiponectin has been demonstrated to cross the blood brain barrier where it acts to increase energy expenditure and decrease body weight [57].

Unlike adiponectin, obesity increases expression of leptin [58]. Also an AMPK activator, this hormone has diverse roles within the body including stimulation of energy utilization and induction of inflammatory processes [59, 60]. Within the brain, leptin is considered an anorexigenic hormone for its ability to reduce hunger [61]. This action should counteract obesity, yet leptin receptors become resistant and do not respond to the usual levels of the hormone. Thus, reducing fat stores in adipocytes may be beneficial by restoring leptin sensitivity. Interestingly, leptin has recently been implicated in AD. Its receptors are expressed in the hippocampus [62] and AD patients have serum levels inversely correlated with the disease [63]. Evidence from a mouse model and neuronal culture suggest that restoring leptin signaling may reduce A β levels and tau phosphorylation [64-66].

AMPK activation in the brain

AMPK activation has also been observed in various regions of the brain following CR and exercise. In the hypothalamus AMPK stimulates hunger, which prompts organisms to replenish nutrients [67]. Interestingly, AMPK activation has also been observed in the hippocampus – a key brain region in memory formation and also a key AD-damaged structure – following physical exertion or under food restriction [68, 69]. However, it is unclear what the effects of AMPK activity may be within this region, since what has been observed appears to be dependent on the level of activation achieved or the conditions under which it becomes activated. For example, low levels of AMPK activation following moderate CR correlate with enhanced

neurogenesis and improved learning and memory, but, in contrast, higher levels of activated AMPK after severe caloric restriction correlate with impaired cognitive performance and induction of neuronal apoptosis [69]. Activation following exercise also occurs in this region of the brain and correlates with improved cognitive function and enhanced neurogenesis [70, 71], but it is unclear how the level of activation after exercise compares to that seen in CR. Furthermore, after hypoxic conditions, such as those encountered during a stroke, AMPK signaling appears to be a detrimental factor [72]. Taken together it appears that AMPK activation serves to boost energy production and promote survival during times of mild to moderate stress, while in conditions in which the nutrient deprivation or other neuronal stressor cannot be overcome, its activation becomes detrimental.

In the context of Alzheimer's disease, conflicting reports have been published regarding the effect of AMPK activation within neurons in cell culture. In one such study, AMPK activation protects a neuronal cell line from A β -induced oxidative stress and reduces the activity of BACE1, one of the enzymes that cleaves APP to generate the A β fragment [64]. However, another report suggests that AMPK activation enhances the production of A β through the increased expression of BACE1 [73]. Given such diametrically opposed conclusions, further studies are required to better understand the consequences of AMPK activation, and the extent to which it occurs, in the AD brain *in vivo*.

Finally, activation of AMPK in astrocytes and microglia may also alter the progression of AD. It is unclear whether or not these cell types are affected following CR or exercise. However, if AMPK activation in the brain is achieved via pharmacological agents (e.g., metformin or resveratrol), these cell types may be affected as well. AMPK activation in these cells prevents their induction of an “activated” state wherein they produce proinflammatory cytokines, including $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 [74, 75]. Such an activated state may occur in response to the accumulation of amyloid plaques, but chronic activation may cause toxicity to neighboring neurons. Counteracting the effects of proinflammatory molecules using NSAIDs or other anti-inflammatory drugs has been hypothesized to be protective, but has been met with limited success in the treatment of AD [76, 77]. Preventing or reducing glial cell activation, however, may decrease the production of these molecules and circumvent the need to treat their negative effects. On the other hand, it also remains possible that glial cell activation is, albeit imperfect, beneficial in the diseased, Alzheimer’s brain. In this case, reducing such activation may remove one source of protection and exacerbate the progression of degeneration in AD.

Purpose of the Thesis Research

As our population ages and life expectancy increases, the number of Alzheimer’s cases will undoubtedly rise. Although our knowledge of the disease has advanced, many questions remain unresolved and treatment options are limited. The aim of the work outlined in this dissertation is to further

our understanding of the molecular mechanisms that either protect against the development of disease symptoms or accelerate the symptoms of AD with the goal of contributing to better future treatments for this devastating illness.

Study Design and Hypothesis

Upon noting that caloric restriction and exercise are two of the most promising interventions that may protect against AD, we sought to activate AMPK to determine if this change may be the relevant molecular effect that occurs during these conditions and therefore may protect against cognitive decline in the disease. We chose to activate AMPK both pharmacologically and genetically in a mouse model of AD and subsequently measure learning and memory function. We hypothesized that AMPK activation would mimic a healthy metabolic state and protect AD mice against the development of AD-like phenotypes.

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CHAPTER 2:

The Effect of Metformin Treatment on APP Mice

Abstract

Widespread reports of the anti-aging benefits of caloric restriction have led to the search for a caloric-restriction mimetic that may be of use in humans for the treatment of aging-related diseases such as Alzheimer's disease. The well-tolerated and widely-prescribed anti-diabetic drug metformin has been hypothesized to be such a drug based on its effect of activating the cellular energy sensor, AMPK. Studies in cell culture have yielded mixed results and an *in vivo* functional effect of this drug relating to the disease has yet to be reported. We therefore tested whether metformin treatment of a mouse model of Alzheimer's disease led to effects on behavioral phenotypes. Surprisingly, our findings indicate that, in male mice, metformin treatment exacerbates the learning and memory deficiencies characteristic of APP-transgenic mice. Just as remarkable, we also found that this effect is sex-specific, as female mice displayed an improved cognitive phenotype. These findings suggest that, if metformin has a similar effect in humans, its potentially negative impact should be taken into account for a large portion of patients receiving this drug.

Introduction

The risk and progression of Alzheimer's disease is influenced by several genetic factors. Among these, those that increase either the production or accumulation of the A β fragment of the amyloid precursor protein (APP) have demonstrated the importance of this peptide in the disease (reviewed in [1]). However, their mere presence is insufficient to explain the etiology of the disease, given that these fragments are found in cerebrospinal fluid of healthy individuals throughout life [2-4]. Thus, additional factors have been proposed to exist in order to account for the progression to pathogenicity. Indeed, mounting evidence indicates that lifestyle factors may also influence the course of the disease. For instance, diabetic patients have a two- to three-fold higher risk of developing AD [5, 6]. Consistent with this connection, murine Alzheimer's models subjected to metabolic stress induced by a high-fat or high-sugar diet exhibit an accelerated disease phenotype [7-9]. In contrast, factors that counteract obesity and diabetes have shown promise in combatting AD. For example, nursing home patients under an exercise regimen exhibit a slower rate in the decline of their cognitive function and consequently are capable of self-care for a longer period of time than patients that lead a sedentary lifestyle [10-13]. Similarly, in mouse models, exercise reduces cognitive impairments characteristic of the disease [14, 15]. Additionally, as Alzheimer's disease is considered to be an aging-related illness, caloric restriction – which seems to be able to slow the aging process in every organism so far tested [16] – reduces A β production and decreases

plaque load [17-19]. Yet, given the inherent difficulty in maintaining a calorically restricted dietary regimen, whether such an approach has any benefits in human patients remains unclear.

At a physiological level, a feature common to both exercise and caloric restriction is a lowered energetic status. At the cellular level, this energy reduction is apparent as a decrease in levels of ATP and a concomitant increase in levels of AMP. This shift is detected by the AMP-activated protein kinase (AMPK); a central sensor of cellular energetic status and regulator of metabolic processes [20]. Under conditions of limited nutrient availability, such as found under decreased caloric intake or during periods of increased physical activity, AMPK acts to promote energy availability and suppress its storage and nonessential consumption. Pharmacological activation of AMPK is known to have some metabolically favorable properties and may serve as an exercise- and/or caloric restriction- mimetic [21, 22].

The anti-diabetic drug metformin derives part, if not all, of its therapeutic benefits from its ability to activate AMPK [23]. In the liver, AMPK activation suppresses gluconeogenesis [24, 25], which, in turn, serves to reduce fasting blood glucose levels. Metformin also increases insulin sensitivity and, although poorly understood, is thought to do so through AMPK-mediated mechanisms such as glucose transporter translocation to the membrane [26, 27].

We hypothesized that metformin treatment may serve as an exercise- or caloric restriction- mimetic and thereby provide protection against the

development of Alzheimer's disease. To address this possibility, we treated APP mice with metformin for up to one year and tested their cognitive function by Morris water maze.

Results

Metformin Treatment and Insulin Sensitivity

Long-term treatment of mice with metformin in drinking water at 2mg/mL has previously been reported (for example [28, 29]). To examine the effects of metformin treatment on AD-like cognitive decline, we treated APP mice with metformin in their drinking water at this dose over a 6 to 12 month treatment period (Figure 2.1). We confirmed that metformin in the drinking water did not alter water consumption or body weight (Figure 2.2, Figure 2.3). Mice carrying the APP transgene were measured smaller than wild type animals, as reported previously [30] in another AD model, but no differences were found between treatment groups. Average mass spectrometry measurements of metformin in the blood plasma were found to be 3.4mg/L. For comparison, human patients have plasma levels between 1-4mg/L [31].

Since metformin is an insulin-sensitizing drug, we measured insulin tolerance in male mice. Female mice were not tested since hormonal fluctuations alter blood glucose levels leading to difficulties determining the source of variations. Male mice were fasted for six hours prior to testing and an initial baseline fasting blood glucose measurement was taken. Each mouse was then weighed and given an intraperitoneal injection of 1U/kg insulin. Subsequent blood glucose measurements were recorded over the following two hours.

Although another mouse model of AD has been reported to develop insulin resistance [30, 32], we were unable to detect a difference by genotype

in baseline blood glucose levels or in insulin responsiveness (Figure 2.4). Similarly, within each genotype group specifically, no differences in insulin sensitivity were detected by treatment. However, a two-factor, repeated-measures ANOVA did uncover a statistically significant main effect of treatment whereby metformin-treated animals were more responsive to insulin than control-treated animals regardless of genotype. No differences were observed in fasting baseline blood glucose levels between treatment groups.

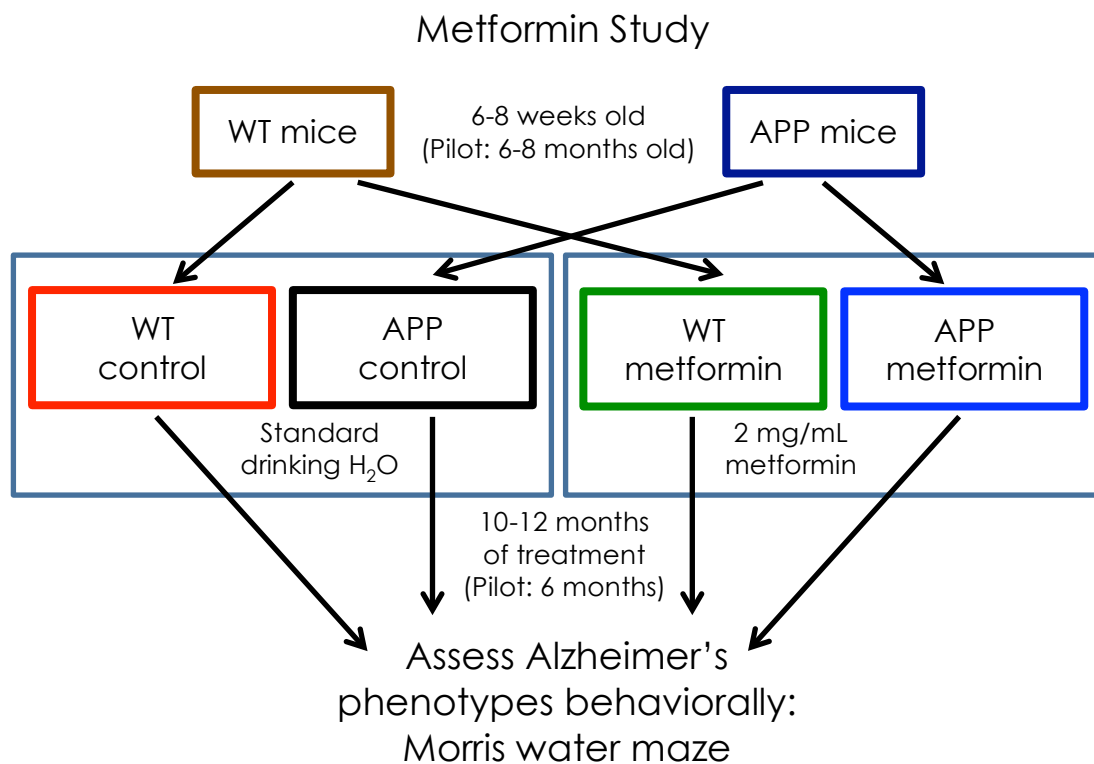


Figure 2.1: Diagram of Metformin Study. Mice of both WT and APP genotypes were divided into groups receiving 2mg/mL metformin in their drinking water or standard drinking water. Treatment continued for the duration of the life of the mice, including behavioral testing at 12-14 months of age. A total of 4 cohorts of mice were included with 155 mice. The first cohort of 24 mice in our Pilot study began treatment at 6-8 months of age; all others began between 6-8 weeks of age.

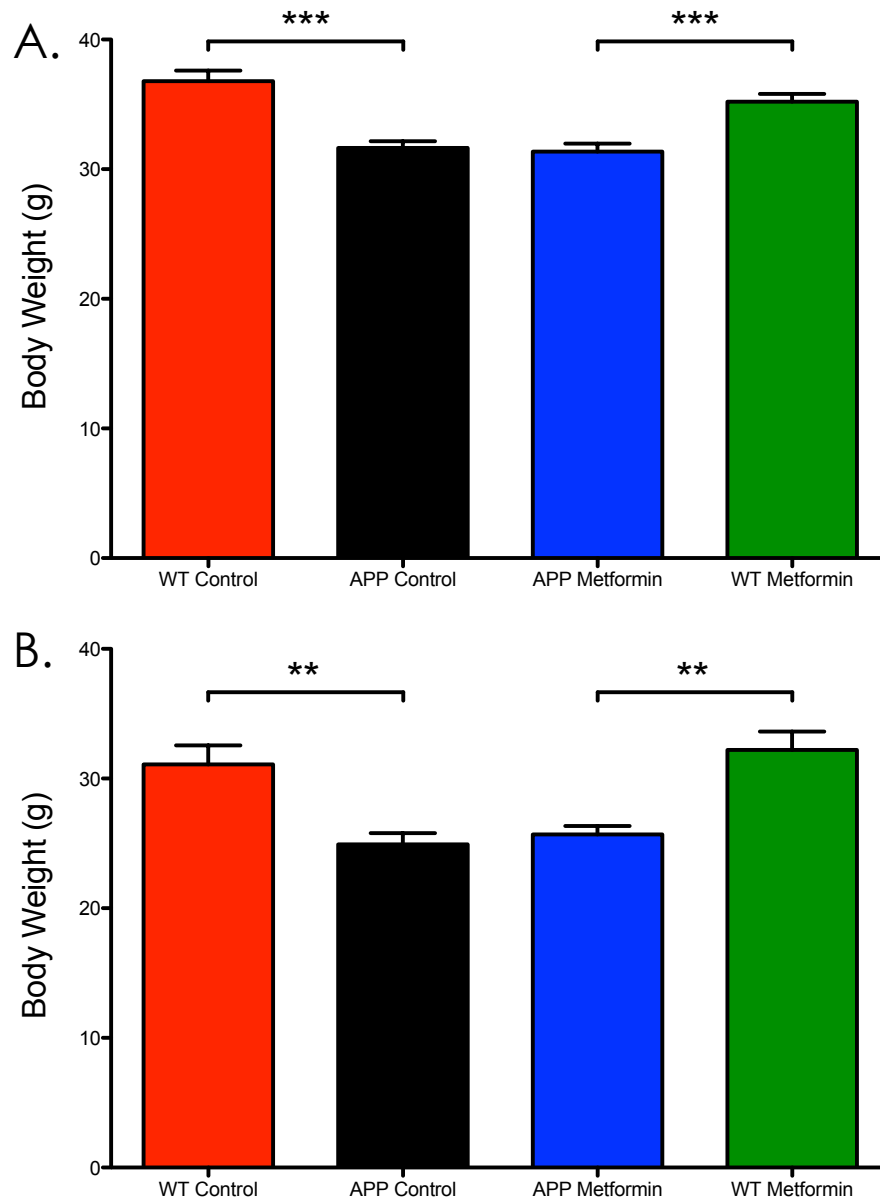


Figure 2.2: Body weight is unchanged by metformin treatment. Mice were 14-16 months old at the time of measurement. Data are depicted as mean; error bars represent SEM. Three-factor ANOVA indicated a main effect of gender and genotype on body weight, with no effect of treatment. Male mice (A) are larger than female mice (B) and within each group WT mice are larger than APP mice. $n = 20 - 38$ mice per group for males; $n = 6 - 11$ mice per group for females. (**) indicates $p < 0.01$; (***) indicates $p < 0.001$.

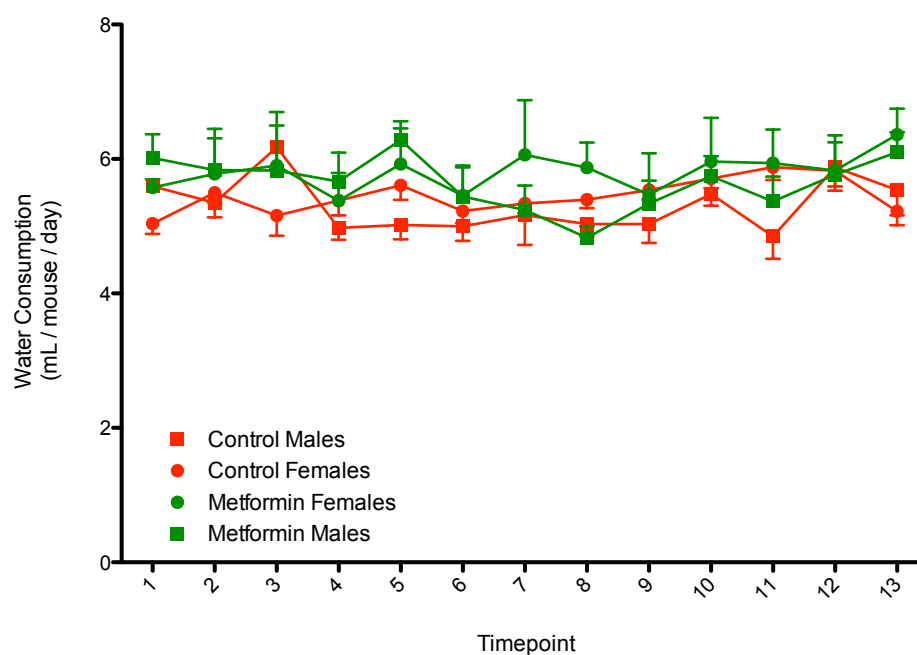


Figure 2.3: Metformin in the drinking water does not change water consumption. Water consumption was periodically assessed throughout the treatment period by measuring the amount of water remaining in the water bottle offered to the cage and subtracting from the starting volume – no distinction between genotypes could be made as multiple mice were housed together in each cage. No difference was detected between gender or treatment groups.

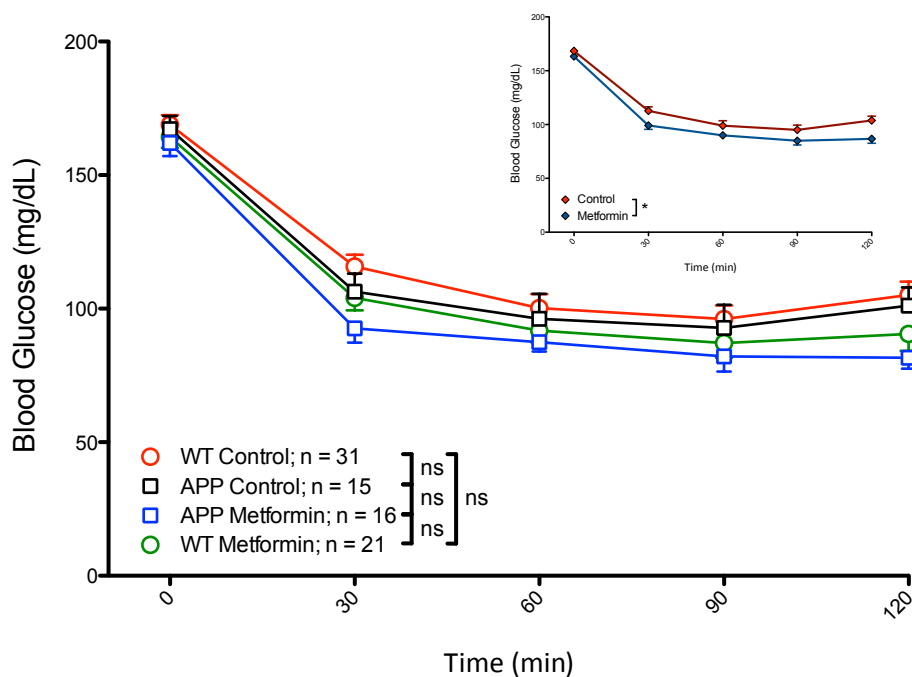


Figure 2.4: Metformin treatment enhances insulin sensitivity. Male mice were tested for insulin tolerance following a six-hour fast by measuring blood glucose levels just before and at 30-minute intervals following intraperitoneal injection of 1U/kg insulin. Within each genotype group, a consistent trend was observed in which metformin-treated mice had a greater drop in blood glucose levels following insulin administration, however, no statistical distinction could be made between individual groups. Using a two-factor, repeated measures ANOVA (graphically represented in the inset) a main effect of treatment was discovered ($p = 0.023$), while genotype had no effect. No differences were found between baseline fasting blood glucose levels.

Learning and Memory

To determine if metformin treatment resulted in a functional consequence, we tested the mice on the Morris water maze. We used a three-stage protocol including (1) a three-day visible platform training phase in which the mice were acclimated to the test and which also served as a control to ensure that mice were able to both see and swim; (2) a hidden platform training phase in which mice learned to find a hidden platform to measure spatial memory acquisition; and (3) a probe test in which the platform was removed to measure memory retention.

During the three-day visible platform phase of testing, mice were acclimated to the maze and tested for ability to participate. Each day, they participated in four trials and swam to find a submerged platform whose location was marked by a visible flag. Criteria for inclusion in further testing included successful location and mounting of the escape platform within 60 seconds in more than 50% of trials on the final day of testing. No differences were found between groups in their ability to meet these criteria. Three mice were excluded from testing for refusal to swim and one mouse was excluded with a missing eye.

The hidden platform phase of training required the mice to learn to find the platform from different starting positions using visual cues within the room since its location was no longer marked with a flag – performance during this phase served as a measure of memory acquisition. At the conclusion of training, all mice were tested for memory retention during a probe trial in

which the platform was removed and the mice were observed for the number of times they crossed the area where the platform had previously been located. A difference in latency to locate the escape platform was detected between male and female APP mice (Fig 1.5) with a statistically significant interaction of genotype, treatment and gender ($p = 0.009$). Although all mice remain part of the statistical model (three factor, repeated measures ANOVA), males and females will be discussed and illustrated separately for simplicity.

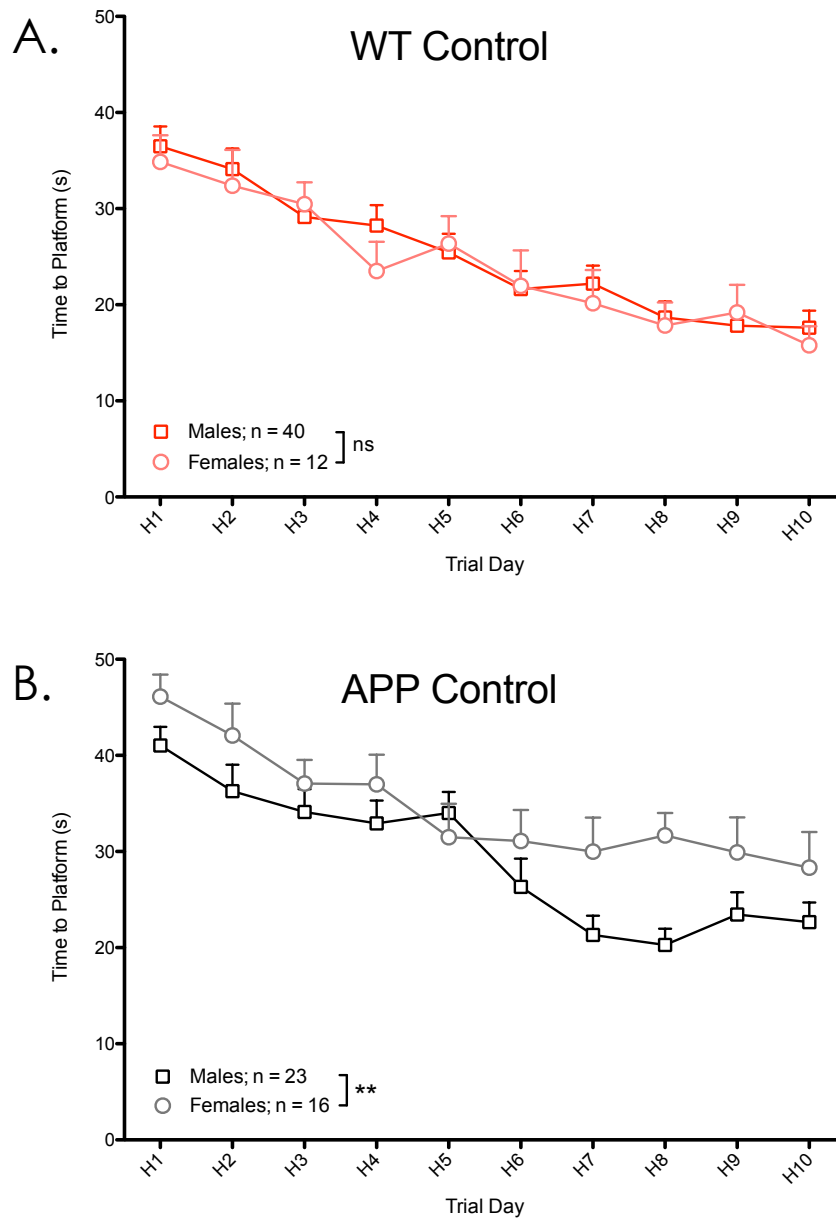
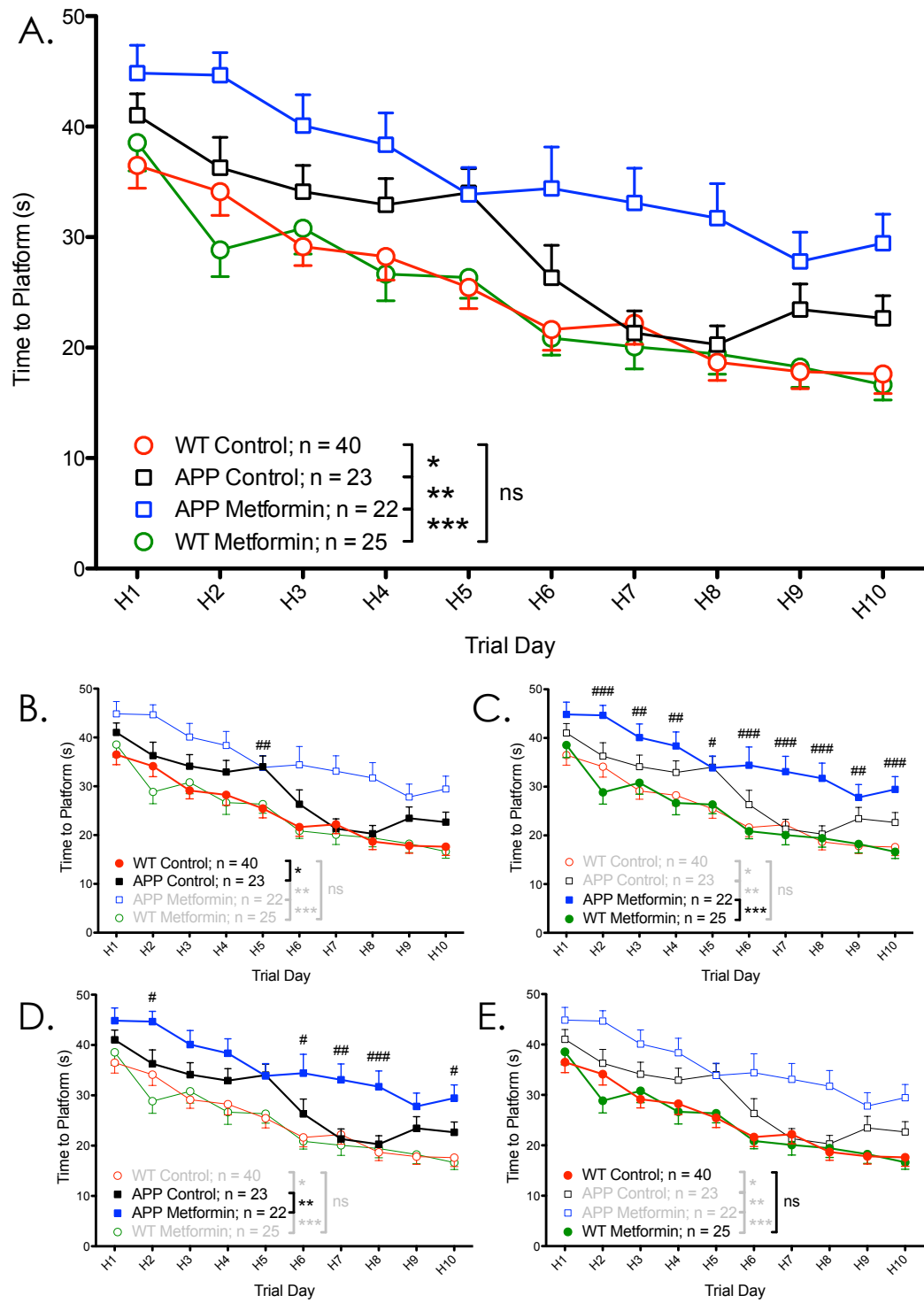


Figure 2.5: Female APP mice perform worse than male APP mice in spatial learning. Mice were trained to find a hidden platform for 10 days in the Morris water maze. A) No difference was observed between male and female WT mice. B) Female APP mice demonstrated longer latencies to find the escape platform than male APP mice. Data points represent average latencies of 4 trials per day for each group; error bars represent SEM; ns = not significant; (**) represents $p < 0.01$.

Male mice expressing the APP transgene in the control treatment group, demonstrated learning difficulties compared to their WT counterparts as indicated by longer time-to-platform measurements during the hidden platform phase (Figure 2.6 A, B) ($p = 0.009$). After 10 days of learning, the APP mice in the control group had reached a level of learning such that they were no longer statistically distinguishable from wild types on the probe trial (Figure 2.7) ($p = 0.0696$). To our surprise, male APP mice receiving metformin treatment demonstrated further impairments over the control-treated APP mice during the training phase, consistently requiring longer amounts of time to locate the platform (Figure 2.6 A, C, D) ($p = 0.0003$). Additionally, their memory retention as measured by the probe trial was decreased compared to similarly-treated wild type mice (Figure 2.6) ($p = 0.0051$). Wild type mice were unchanged by metformin treatment (Figure 2.6 A, E; Figure 2.7). Since the time required to locate the platform depends in part on swim velocity, average swim velocity was measured and APP mice were found to exhibit slower swim speeds than WT mice with no difference found between treatment groups (Figure 2.8). To ensure that the observed latency differences resulted from deficits in memory acquisition rather than slower swim speeds, measurements of the path length traveled to find the platform were also obtained and similar results were found (Figure 2.9).

Figure 2.6: Metformin treatment impairs spatial learning in male APP mice. Mice were trained for 10 days to find a hidden platform in the Morris water maze. A) All groups of male mice are depicted. B) Within the control treated-groups, APP mice perform worse than WT mice. C) Within the metformin-treated groups, APP mice perform worse than WT mice. D) Metformin-treated APP mice perform significantly worse than control-treated APP mice. E) WT mice are unimpaired by metformin treatment. Asterisks represent significance of the entire curve by repeated measures ANOVA; (*) represents $p < 0.05$, (**) represents $p < 0.01$, (***) represents $p < 0.001$. Pound signs represent significance of individual points on the curve; (#) represents $p < 0.05$, (##) represents $p < 0.01$, (###) represents $p < 0.001$.



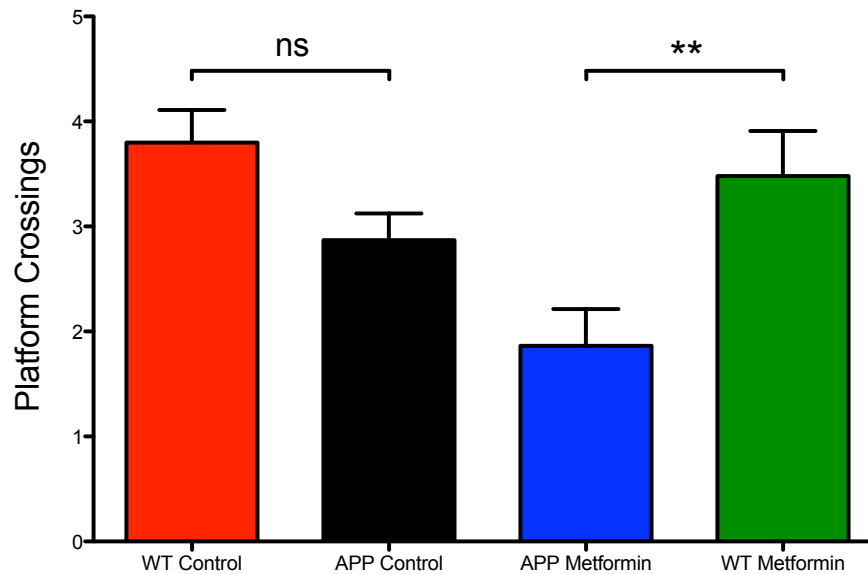


Figure 2.7: Metformin treatment impairs memory retention in male APP mice. Following hidden platform training, mice were tested for memory retention in a probe trial in which the platform was removed and the mice swam for one minute. Within the control group, APP mice crossed the platform location fewer times than WT mice on average, but the difference did not quite meet the criteria for statistical significance ($p = 0.0696$). Within the metformin-treated group, APP mice demonstrated significantly impaired spatial memory retention. Data represent the average number of times the mice in each group crossed the area where the platform had previously been located; error bars represent SEM; ns = not significant; (**) represents $p < 0.01$. WT control, $n = 40$; APP control, $n = 23$; APP metformin, $n = 22$; WT metformin, $n = 25$.

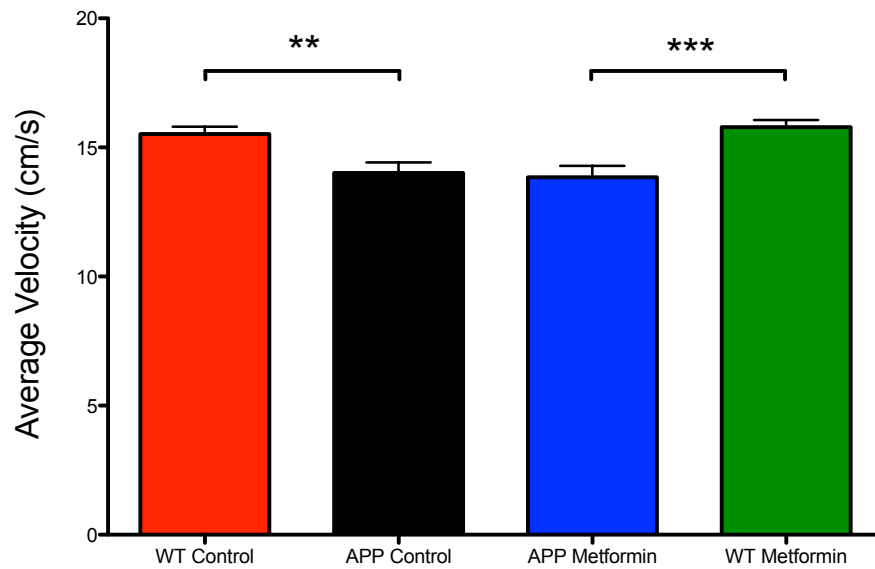
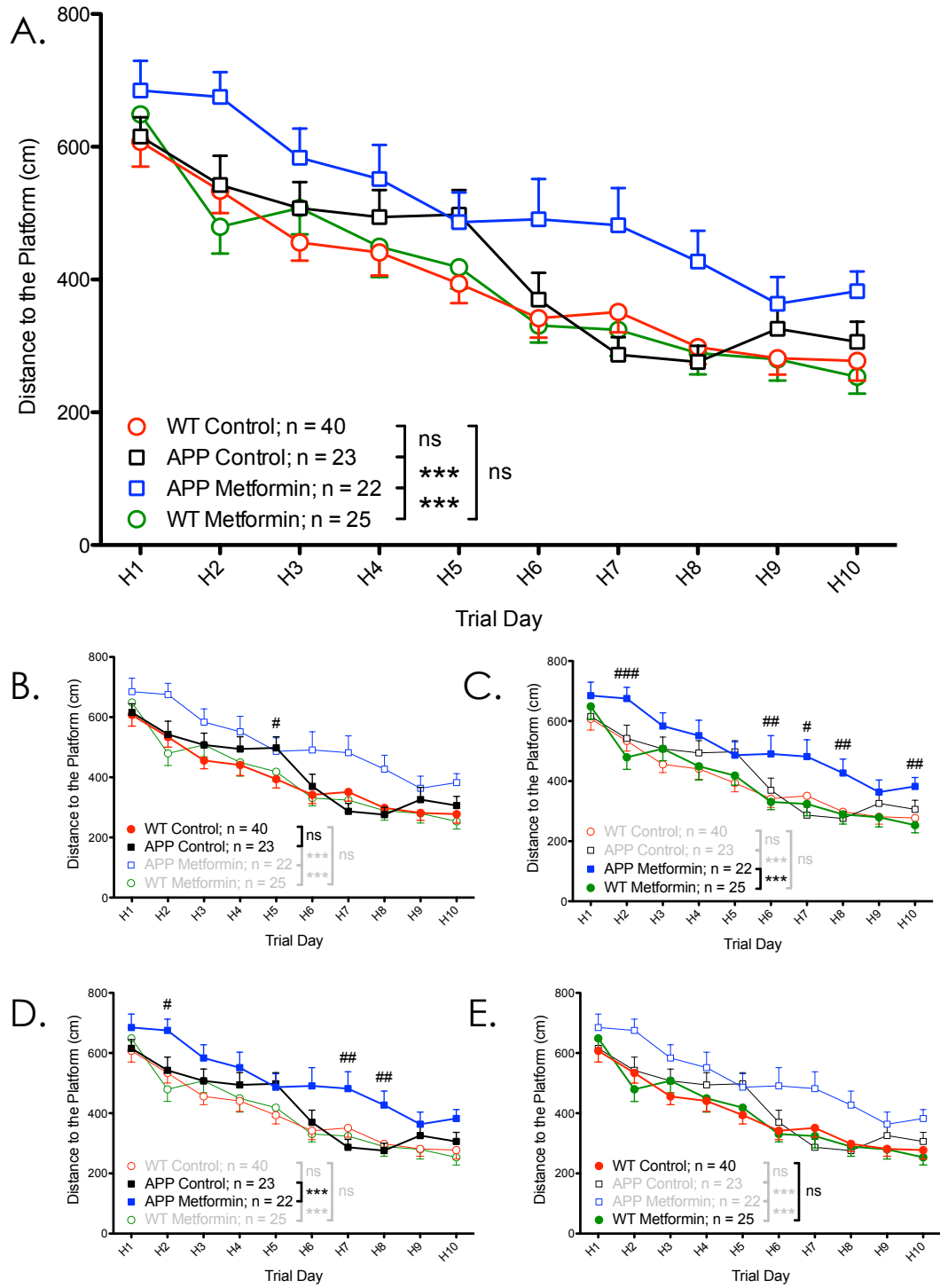


Figure 2.8: Swim velocity is decreased in male APP mice. Within each treatment group, APP mice swim slower than WT mice. The average swim velocity for each mouse was determined using data from all swim trials over the course of the 14-day testing period. Data graphed represent the average swim velocity for each group; error bars represent SEM; (*) represents $p < 0.01$; (**) represents $p < 0.001$.

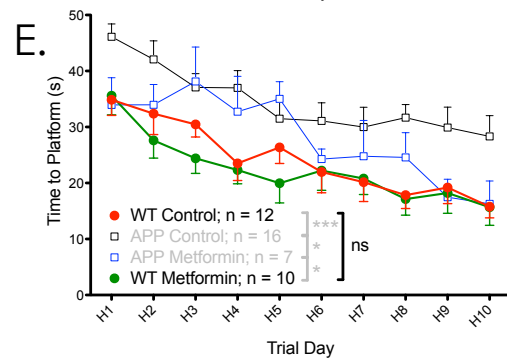
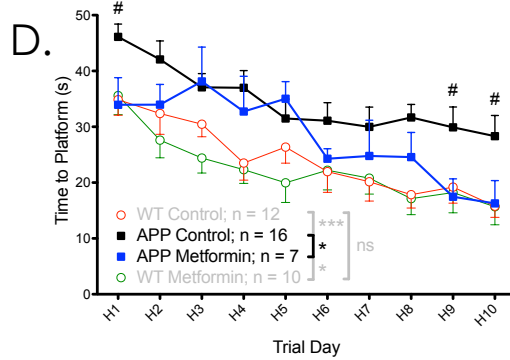
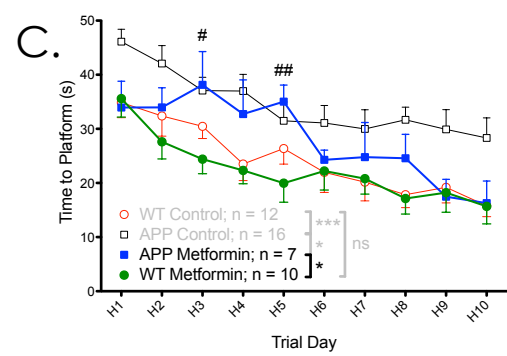
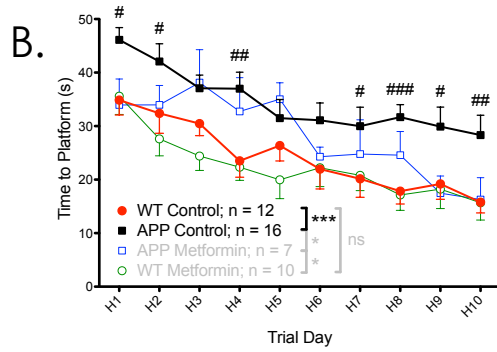
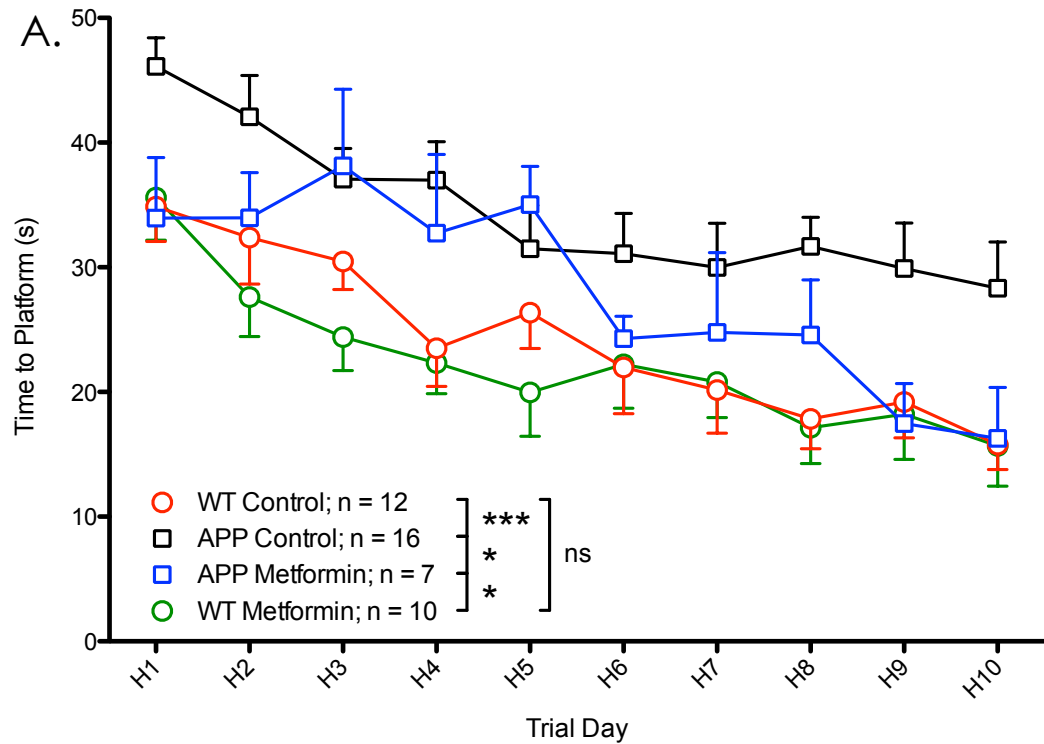
Figure 2.9: Metformin-treated male APP mice have longer path lengths to the platform. A) All male mice. B) By measure of path length, APP mice in the control group are not statistically distinguishable from their WT counterparts by repeated measures ANOVA. When looking at individual days, a difference could be detected between these groups on day 5. C) Within the metformin-treated group, APP mice swam longer distances before finding the escape platform. D) Male APP mice receiving metformin treatment had longer path lengths than control-treated APP mice. E) No difference was detected between WT mice receiving standard drinking water and metformin drinking water. Asterisks represent significance of the entire curve by repeated measures ANOVA; (***) represents $p < 0.001$. Pound signs represent significance of individual points on the curve; (#) represents $p < 0.05$, (##) represents $p < 0.01$, (###) represents $p < 0.001$.



To a greater extent than the males, female APP mice in the control group also exhibited difficulties learning to find the platform as compared to wild type mice (Figure 2.10 A, B) ($F_{(1,147)} = 20.54$; $p < 0.0001$). Consistent with this larger deficit, female APP mice were unable to remember the platform location as well as wild types when tested on the probe test (Figure 2.11) ($p = 0.0073$). Contrary to the findings in the male mice treated with metformin, female APP mice receiving metformin treatment out-performed the control-treated APP mice ($p = 0.019$), although they still appeared worse than wild types in this measure of memory acquisition (Figure 2.10 A, C, D) ($p = 0.051$). During the probe trial, female APP mice that had received metformin treatment were rescued of the deficit observed in the female APP mice in the control group (Figure 2.11).

Across all mice, there was a main effect of genotype on swim velocity, with APP mice swimming slower than WT mice. Similar to the males, this effect was observed between genotype groups within the female population; however, it was only statistically significant in the metformin treatment group (Figure 2.12). Again, there was no distinction between treatment groups and the difference was not large enough to account for the increased latencies to find the platform since the APP control females demonstrated longer path lengths to find the platform and the APP metformin females were improved in function by this measure as well (Figure 2.13).

Figure 2.10: Metformin treatment improves spatial memory acquisition in female APP mice. Mice were trained for 10 days to find a hidden platform in the Morris water maze. A) All groups of female mice are depicted. B) Female APP mice had longer latencies to find the escape platform than WT mice in the control group. C) Within the metformin-treated groups, APP mice took longer to find the platform in the early days of testing. D) Metformin-treated APP mice were significantly improved over control-treated APP mice. E) No difference was detected between WT mice in the control and metformin treatment groups. Asterisks represent significance of the entire curve by repeated measures ANOVA; (*) represents $p < 0.05$, (***) represents $p < 0.001$. Pound signs represent significance of individual points on the curve; (#) represents $p < 0.05$, (##) represents $p < 0.01$, (###) represents $p < 0.001$.



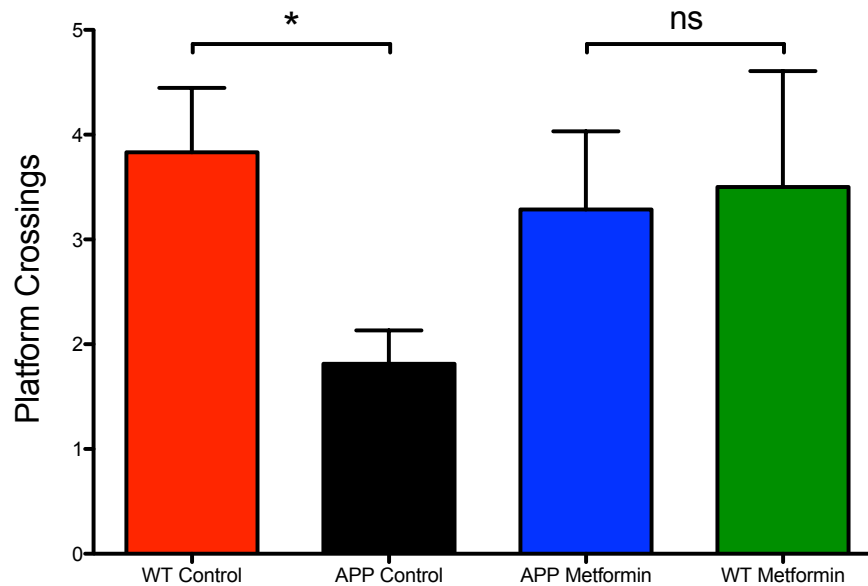


Figure 2.11: Metformin treatment improves memory retention in female APP mice. Following hidden platform training, mice were tested for memory retention in a probe trial in which the platform was removed and the mice swam for one minute. Within the control treatment group, APP mice crossed the platform location fewer times than WT mice. APP mice in the metformin treatment group, however, were indistinguishable from WT mice. Data represent the average number of times the mice in each group crossed the area where the platform had previously been located; error bars represent SEM; ns = not significant; (*) represents $p < 0.05$. WT control, $n = 12$; APP control, $n = 16$; APP metformin, $n = 7$; WT metformin, $n = 10$.

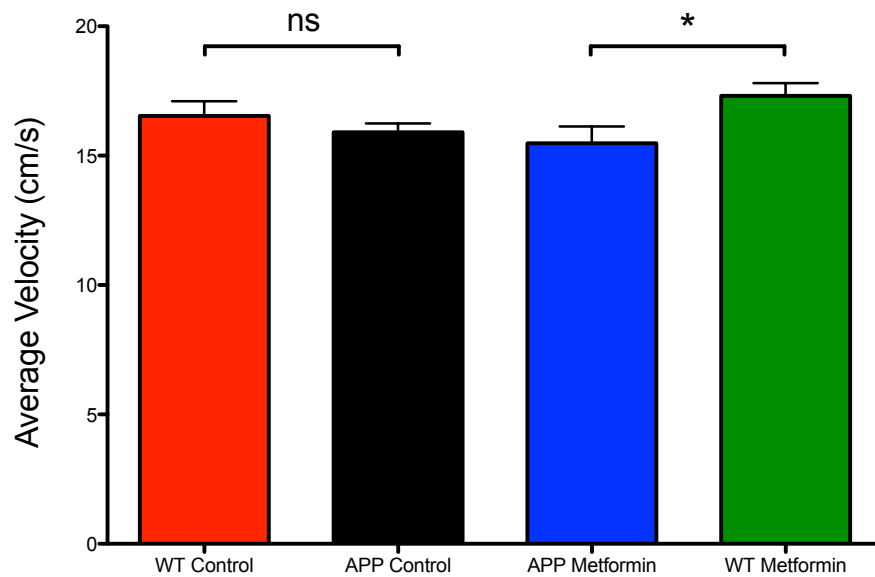
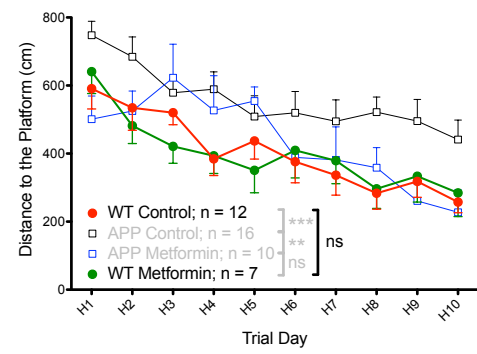
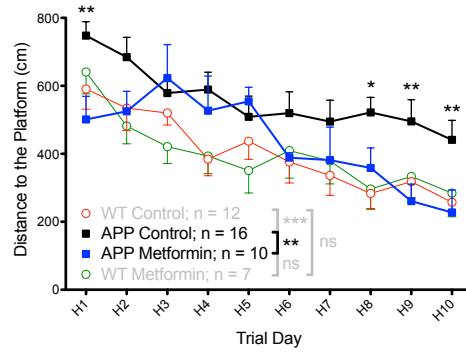
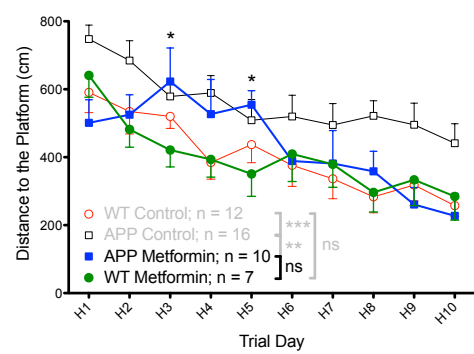
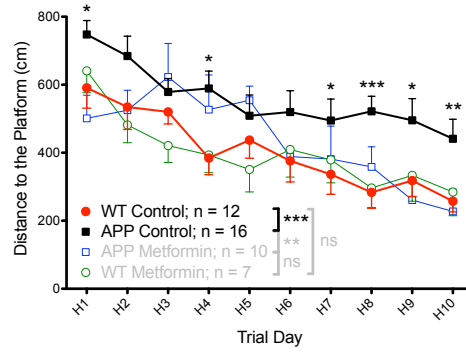
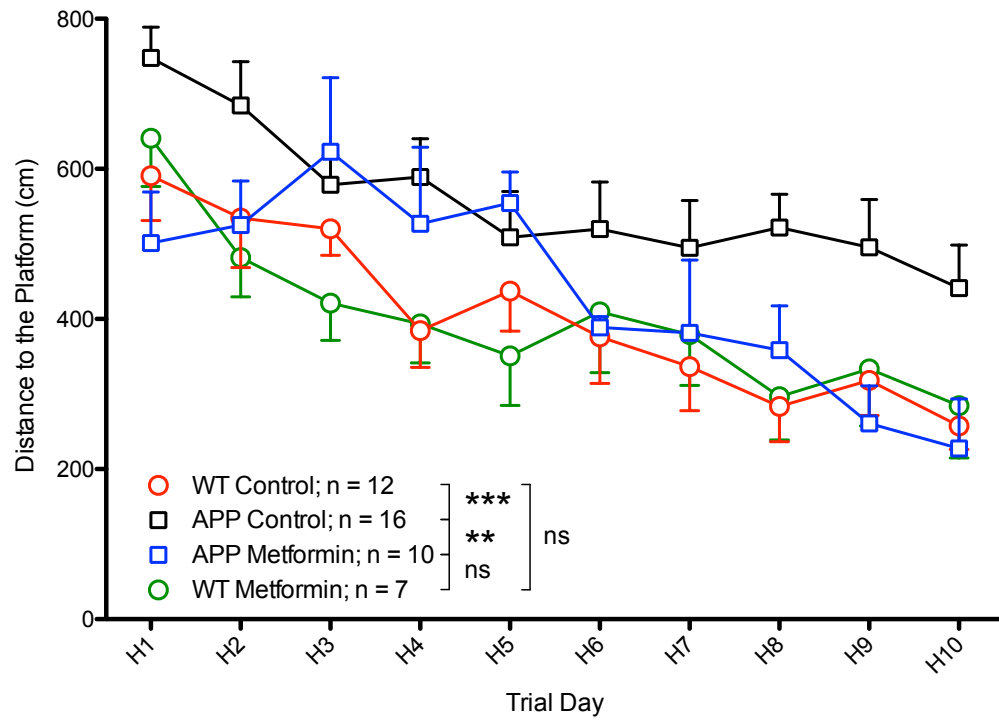


Figure 2.12: Swim velocity is decreased in female APP mice. An overall main effect of genotype was found whereby APP mice swam slower than WT mice. Within individual treatment groups, this effect was only apparent in metformin-treated animals. The average swim velocity for each mouse was determined using data from all swim trials over the course of the 14-day testing period. Data graphed represent the average swim velocity for each group; error bars represent SEM; (*) represents $p < 0.05$; ns = not significant.

Figure 2.13: Metformin-treated female APP mice have shorter path lengths to the platform. A) All male mice. B) By measure of path length, APP mice in the control group have longer path lengths to find the platform. C) Within the metformin-treated group, APP mice were indistinguishable from WT mice when considering overall performance by repeated measures ANOVA, however, when considering individual days, APP mice swam further before finding the escape on days 3 and 5. D) Female APP mice receiving metformin treatment were significantly improved in path length over control-treated APP mice. E) No difference was detected between WT mice receiving standard drinking water and metformin drinking water. Asterisks represent significance of the entire curve by repeated measures ANOVA; (**) represents $p < 0.01$; (***) represents $p < 0.001$. Pound signs represent significance of individual points on the curve; (#) represents $p < 0.05$, (##) represents $p < 0.01$, (###) represents $p < 0.001$.



Discussion

Metformin derives most, if not all, of its anti-diabetic benefits from its activation of AMPK, including enhanced insulin sensitivity and reduced hepatic gluconeogenesis [23]. This drug is generally considered safe with very few and minor side effects except in rare cases such as in patients with reduced kidney function [33]. Metformin is the first-resort prescription drug given to type II diabetics when diet and exercise alone fail to get their blood sugar under control. If this treatment is additionally found to be insufficient, other medications are frequently prescribed *in addition to* metformin, but the metformin treatment continues. Currently, over 48 millions prescriptions are filled for metformin each year in the U.S. alone [34].

Although a causal relationship has not been established between diabetes and AD, epidemiological studies have indicated that diabetics have a two- to three- fold higher incidence of the disease [5, 6]. It is also known that Alzheimer's patients have disrupted insulin signaling and some reports have even begun to refer to AD as "type III diabetes" [35]. On the surface, it seems that an insulin-sensitizing, anti-diabetic drug would be an excellent candidate to treat AD, or, at the very least, to treat diabetes thereby reducing or eliminating an important risk factor.

Several studies of metformin treatment in relevant models have been recently reported with mixed results. For instance, during the course of the present study, another report found that metformin treatment in a triple transgenic mouse model of AD led to an increase in the expression of *bace1*,

one of the two enzymes that cleave APP to generate A β , which was accompanied by an increase in A β production and small plaque formation [36]. Another report looking at the effects of AMPK activation in neuronal function [37] found that metformin application reduced late long-term potentiation in hippocampal slices, which is an electrophysiological correlate of memory. Despite the information leading us to initially hypothesize that metformin treatment would be beneficial, these studies would predict that metformin treatment would be harmful to Alzheimer's patients and worsen their memory function.

On the other hand, both of these reports found that their results depended on metformin's effect of AMPK activation; this same activation has been shown to have beneficial results in other studies. For instance, AMPK activation has been shown to protect against the production of A β [38] and to promote survival in the face of A β -induced stress [39]. Moreover, a recent study has generated a neuronal model of insulin resistance with characteristics of AD, which were reversed with metformin treatment [40]. These differential effects may be due to different doses and levels of AMPK activation or because different models were tested; prior to the present study, none have addressed the functional, *in vivo* effect of metformin treatment in any model of AD.

We chose to explore the effects metformin in AD for two principle reasons: first, because metformin is an AMPK activator and we hypothesized that AMPK activation was a key mechanism by which exercise and caloric

restriction protected against AD; and second, because it is already approved for use in humans and believed to be relatively safe. We first explored the likelihood of our hypothesis in a pilot study comprised of just 24 male mice that were already 6-8 months old at the start of treatment. We restricted our study to males at this point in order to keep the sample as homogeneous as possible and thereby reduce variation in the small sample size we had available. Furthermore, males do not have hormonal fluctuations to the same extent as females, which would facilitate assessment of their insulin response. This small group of animals exhibited a striking result that was the opposite of our hypothesis, with metformin worsening learning and memory function rather than bringing about improvement. We realized that this finding had potentially alarming implications if it held true in human patients as well, and decided to increase our sample size to ensure we were not observing an effect that was due to just a few mice that happened to be outliers.

We treated and tested another three cohorts of mice, this time including females, and found that the result from our pilot study held true, albeit only in the male mice. Herein we have reported that male mice receiving metformin in their drinking water reach steady-state plasma levels of metformin in a range comparable to that of human patients taking the drug for treatment of diabetes, that they exhibit improved insulin sensitivity as would be expected from this anti-diabetic drug and, yet, their Alzheimer's-related behavioral phenotypes are worsened. In contrast, we found that female APP

mice receiving the same treatment are improved in learning and memory function, consistent with our initial hypothesis.

Gender-specific effects of metformin treatment are not entirely unprecedented. Anisimov and colleagues have recently reported that metformin treatment in 129/Sv mice extended lifespan in females while shortening it in males [Anisimov 2010]. Additionally, they found a benefit to females with metformin in that they developed fewer malignant tumors, while there was no change in tumorigenesis in males. On a related note, metformin treatment can modulate hormonal systems in certain situations in human patients, however, it is not clear if this would be true if the systems were not disrupted to begin with. For instance, in females with polycystic ovary syndrome, metformin treatment improves insulin sensitivity and, likely as a consequence, reduces hyperandrogenism, regulates menstrual cycles and improves fertility [41, 42]. Moreover, insulin resistant males experience reduced testosterone levels that can be normalized by improving insulin sensitivity with metformin treatment [43].

There is some data to support the possibility that hormonal disturbances contribute to Alzheimer's disease, at least in females. As it turns out, more women are affected with AD than men and the discrepancy goes beyond what can be explained by the longer life expectancy of females [44-46]. Similarly, in several murine models, females have greater Alzheimer's phenotypes than males [47-50] and, indeed, herein we have reported this to be true in our APP mice as well. Estrogen has several protective effects within

the brain and post-menopausal drops in estrogen may contribute to the higher rate of disease in women [51, 52]. There have been reports that indicate that estrogen signaling can activate AMPK [53, 54]; therefore, metformin treatment may replace a protective signaling pathway lost in older females. Alternatively, in mice, metformin treatment has been shown to prolong regular estrous cycles [55, 56], suggesting that the full gamut of estrogen-related benefits to the brain may be functioning longer, which would certainly include AMPK-independent effects as well.

Unlike females, males do not experience a precipitous drop in estrogen. While this may help explain the baseline gender differences in Alzheimer's disease, it does not predict a difference in response to metformin treatment. Recently, it has been reported that testosterone inhibits AMPK activity in adipocytes [53, 54]. This raises the possibility that while metformin may be enhancing or replacing part of a hormonal signaling pathway in females, it may be counteracting one in males.

In summary, our results indicate that metformin treatment may have therapeutic potential against Alzheimer's disease in women. However, men may have a worsened outlook with the same treatment. These findings suggest that the effects of metformin treatment on cognitive function and Alzheimer's disease in human patients urgently needs to be explored with special attention given to gender differences since millions of patients within a population already at risk for AD already take this drug.

Acknowledgements

I would like to acknowledge Ann Atkins for measurement of plasma levels of metformin. Chapter 2, in part is currently being prepared for submission for publication of the material. DiTacchio, Kacee; Atkins, Ann; Heinemann, Stephen. The dissertation author was the primary investigator and author of this material.

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CHAPTER 3:

The Effect of Peripheral Genetic Activation of AMPK on APP Mice

Introduction

The AMP-activated protein kinase (AMPK) is a cellular energy sensor whose activity throughout the body favors insulin sensitivity and inhibits fat storage. Recently, the US has been experiencing an obesity epidemic, with an associated increase of type 2 diabetes [1]. Since these health problems, in turn, increase the risk and complications of other ailments – ranging from cardiovascular disease [2] to Alzheimer's disease (AD) [3, 4] to cancer [4] – finding ways to counteract them is of paramount importance. AMPK is already a target of anti-diabetic drugs and it has been suggested for use against metabolic syndrome [5], AD [6], cancer [7, 8] and even aging [9, 10].

Although the effects of AMPK activation are generally favorable, its role in different cell types varies. For instance, in the liver, AMPK activation serves to inhibit gluconeogenesis [11, 12]; in other tissues functions overlap, but importantly, in adipose tissue, it activates fatty acid oxidation and inhibits fat storage [13]; in muscle, it stimulates translocation of glucose transporters to the cell membrane [14]. The role of AMPK within the brain may be even more diverse and, in certain conditions of stress, may even be detrimental. For example, AMPK becomes activated in conditions of low nutrient or oxygen supply, two conditions that are present during a stroke. In such a case AMPK inhibition prevents the neuronal tissue damage that would otherwise occur [15]. Additionally, under harsh caloric restriction conditions, AMPK becomes strongly activated in the brain and memory function is impaired [16].

The ubiquity of this protein and the variety of possible effects and outcomes of its activation make AMPK a somewhat risky target of pharmaceutical therapies. For instance, the AMPK-activating, anti-diabetic drug metformin has clear metabolic benefits, yet has no tissue specificity and the relative contribution of its many targets remains unclear. We recently treated our mouse model of AD with metformin and found a surprising impairment of learning and memory function in male mice. It is unclear if this result was due to AMPK activation in the brain, the periphery, or – another potential problem with drug treatment – if it involved AMPK-activation at all.

Since many of the metabolic advantages of metformin depend upon AMPK activation in the periphery, we wondered if restricting activation from the brain might allow males to benefit metabolically without any harmful effects to learning and memory function. Furthermore, it is feasible that a state of improved metabolic function in the body, such as that found in caloric restriction and following regular exercise, has indirect benefits in the brain independent of AMPK activation. If so, developing drugs that target AMPK without crossing the blood brain barrier may pharmacologically reproduce a healthy metabolic state that would be beneficial in diabetes without posing a danger to AD or that may even be valuable in the treatment of both diseases.

A transgenic mouse model has been developed that expresses a constitutively active version of AMPK in the liver. Mice harboring such a mutation are lean and have a lower fasting blood glucose levels and improved insulin sensitivity [12]. By breeding these mice with the APP mice, we

reasoned that we could test the specific effects of AMPK activation in the periphery without affecting the brain. Moreover, using a genetic model would allow us to examine the specific effects of AMPK activation without any off-target effects that might be found with drug treatment. Herein, we report the effect of liver-specific constitutively active- (CA-) AMPK expression on learning and memory function in APP mice.

Results

To determine if CA-AMPK expression in the liver altered cognitive function in APP mice, we first bred PDGF β -APP, line J9 mice with liver-specific CA-AMPK mice. Four genotypes were generated: nontransgenic “wild type” mice (WT / WT), single transgenic APP mice (APP / WT), single transgenic CA-AMPK mice (WT / AMPK), and double transgenic APP / AMPK mice. Once the mice reached the age at which we expected behavioral deficits to be apparent in APP mice based on previous findings (12-14 months), we tested them on the Morris water maze.

For comparison to our previous metformin study, we used the same three-stage protocol including (1) a three-day visible platform training phase in which the mice were acclimated to the test and which also served as a control to ensure that mice were able to both see and swim; (2) a 10-day hidden platform training phase in which mice learned to find a hidden platform to measure spatial memory acquisition; and (3) a probe trial on the final day of testing in which the platform was removed to measure memory retention.

No differences were found between groups in the visible platform-training phase; however, several mice failed to pass this control following a disturbance in the room prior to the final day of visible platform training in the first cohort tested. A total of seven mice were excluded from testing including mice from both genders and with and without both APP and AMPK expression; no bias to any group was observed.

During the hidden platform training, the difference between WT / WT and APP / WT female mice could not be distinguished by repeated measures ANOVA when considering the entire curve (Figure 3.1 A, B)($p = 0.068$). However, since these were our control groups where we expected to see a difference based on observations from previous experiments and since we noticed that the learning curves diverged for the latter part of testing, we repeated the statistical analysis focusing on the last half of the data. Through this analysis, the predicted difference between WT / WT and APP / WT mice could be detected ($p = 0.007$). Similar to our findings with metformin treatment, AMPK expression in APP female mice improved spatial learning compared to APP / WT mice (Figure 3.1 A, D) whether considering the last half of the testing period ($p = 0.008$) or the entire curve ($p = 0.038$). In fact, APP / AMPK female mice were indistinguishable from WT / AMPK mice (Figure 3.1 A, C) and WT mice were unchanged with AMPK expression (WT / WT versus WT / AMPK mice; Figure 3.1 A, E).

At the end of the training period, mice were tested for memory retention. Similar to the training phase, we had difficulty distinguishing between WT / WT and APP / WT mice by our usual measure of platform crossings (Figure 3.2 A). We looked instead at the time spent in each quadrant and discovered that WT / WT mice had learned the general area where the platform should have been found as indicated by their performance being better than chance (Figure 3.2 B). APP / WT mice, however, were not statistically better than chance by this measure, indicating impaired spatial

memory retention. Within the AMPK groups, both WT / AMPK and APP / AMPK mice performed equally well by platform crossings and both groups learned the target quadrant better than chance (Figure 3.2 A, B). No differences were found between any groups in swim velocity (Figure 3.3).

Figure 3.1: AMPK expression improves learning in female APP mice. Mice were trained for 10 days to find a hidden platform in the Morris water maze. A) All groups of female mice are depicted. B) Female APP / WT mice had longer latencies to find the escape platform than WT / WT mice. C) Within the AMPK groups, APP / AMPK mice were unchanged from WT / AMPK mice. D) APP / AMPK mice were significantly improved over APP / WT mice. E) No difference was detected between WT mice and WT mice with AMPK expression. Asterisks represent significance by repeated measures ANOVA; the first symbol represents comparison of the entire curve, the second symbol represents the last half; (ns = not significant, * $p < 0.05$, ** $p < 0.01$). Pound signs represent significance of comparisons of individual points on the curve; (# $p < 0.05$, ## $p < 0.01$).

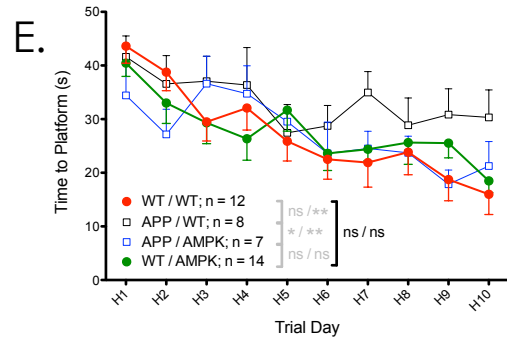
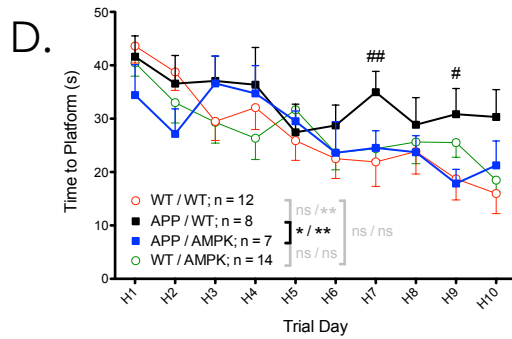
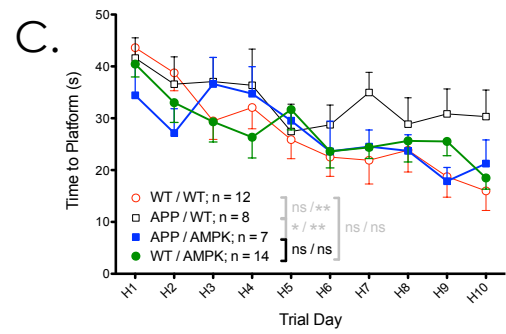
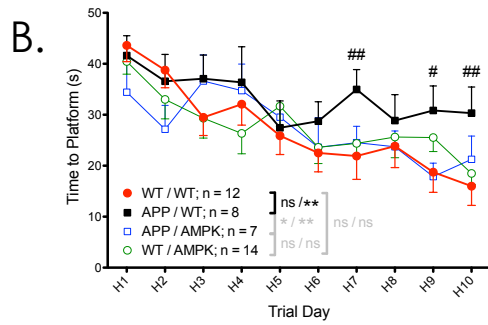
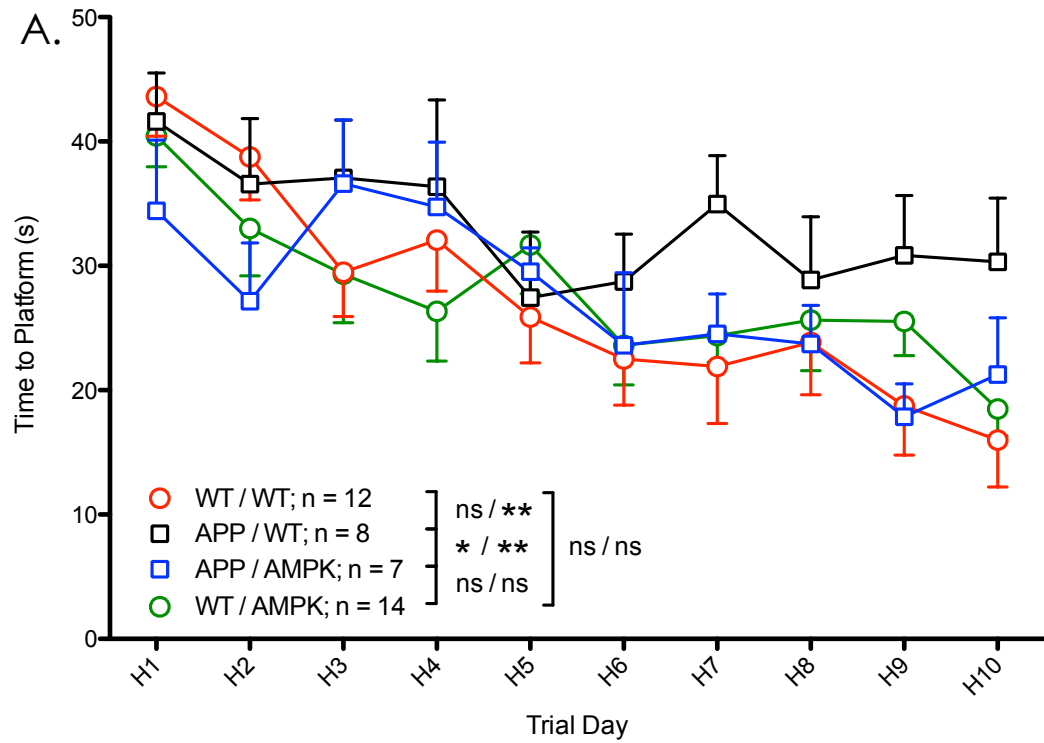
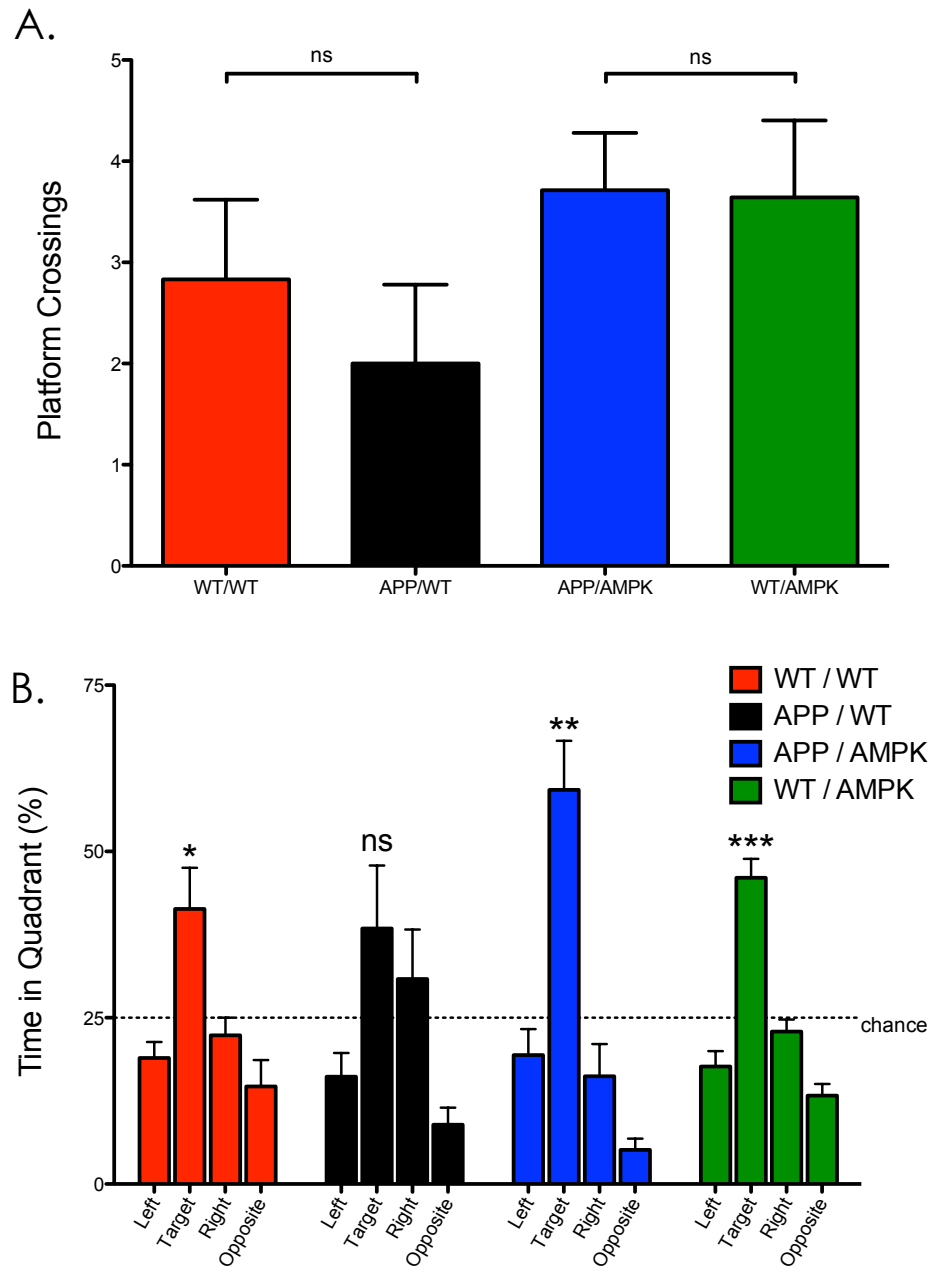


Figure 3.2: AMPK expression rescues spatial memory retention in female APP mice. Following hidden platform training mice were tested for memory retention in a probe trial in which the platform was removed and the mice swam for one minute. A) By the measure of platform crossings, APP / WT mice performed poorly, but were not statistically different from WT / WT mice. APP / AMPK mice demonstrated no memory impairment. B) By measuring the percentage of time spent in each quadrant of the water maze, WT / WT mice remembered the location of the platform and spent more time swimming in the target quadrant than would be expected by chance. However, APP / WT mice did not perform better than chance by this measure. With AMPK expression, both WT and APP mice preferred the target quadrant. Data represent the average values for each group; error bars represent SEM; (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$); WT / WT, $n = 12$; APP / WT, $n = 8$; APP / AMPK, $n = 7$; WT / AMPK, $n = 14$.



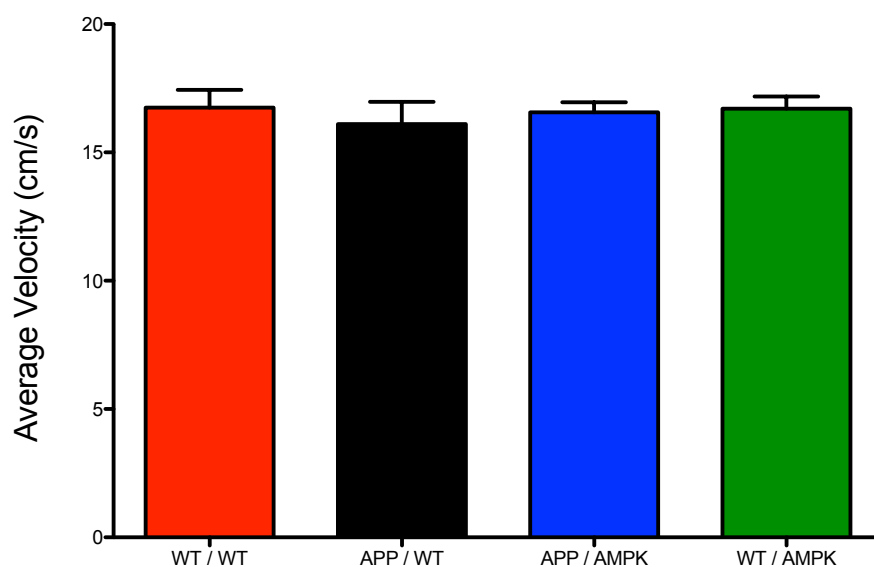


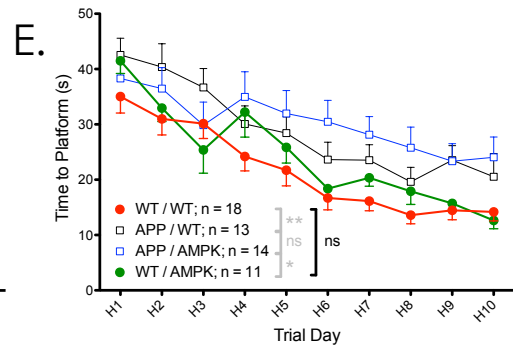
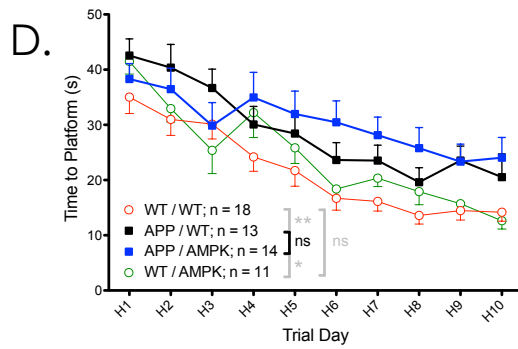
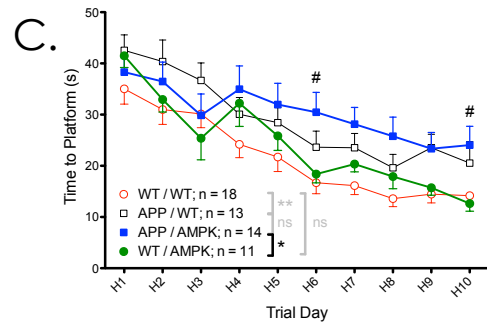
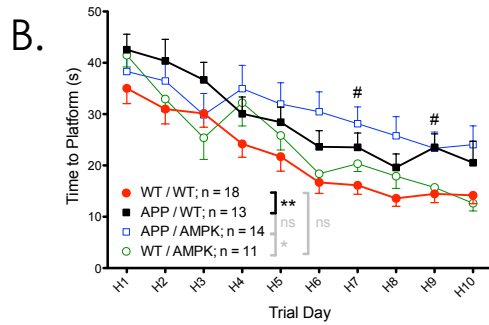
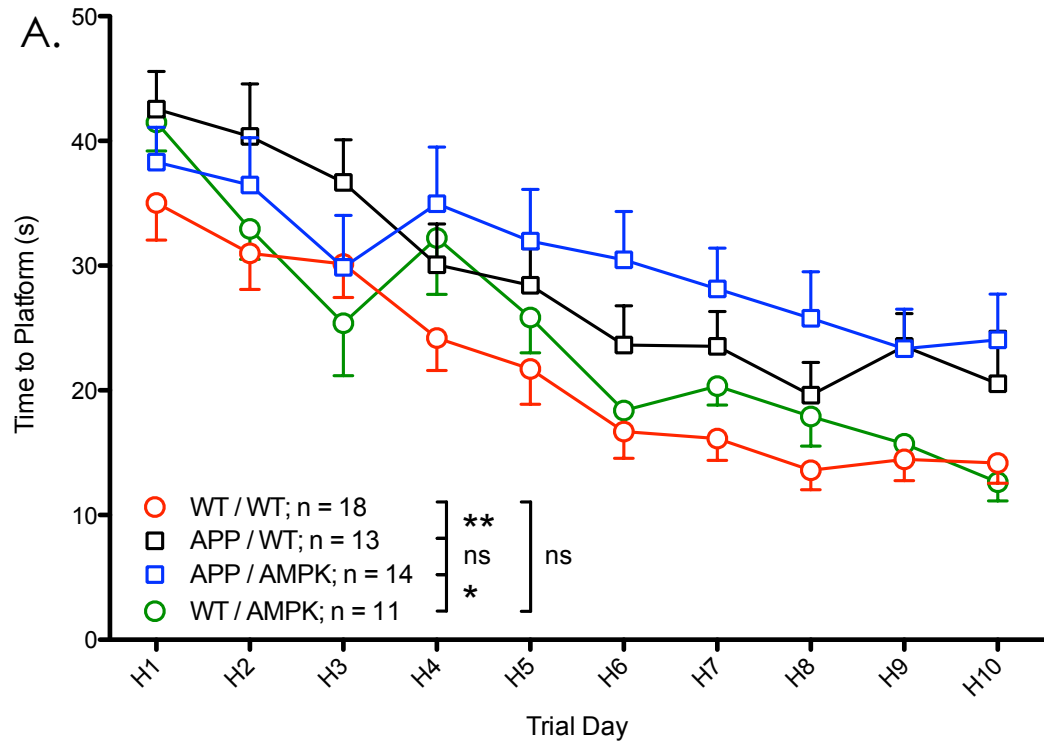
Figure 3.3: Swim velocity is unchanged by genotype in female mice. No differences in average swim velocity were detected between APP or AMPK groups. Data for each individual mouse was determined by averaging velocities from all swim trials for that mouse over the course of the 14-day testing period. Data graphed represent the average swim velocity for each group; error bars represent SEM.

Within the groups of male mice tested, APP / WT mice displayed difficulty learning the location of the escape platform compared to WT / WT mice (Figure 3.4 A, B)($p = 0.0068$). Similarly, within the AMPK groups, APP mice also demonstrated impaired performance (Figure 3.4 A, C)($p = 0.0379$). Interestingly, starting at day four, APP / AMPK mice consistently had a longer average latency to find the escape platform compared to APP / WT mice, however, the difference was not statistically significant (Figure 3.4 A, D). WT mice were unchanged in spatial learning performance by AMPK expression (Figure 3.4 A, E).

Following conclusion of the training phase, we tested the mice for memory retention on the probe trial. APP / WT mice were able to learn the location of the platform to a level such that they were not statistically distinguishable from WT / WT mice (Figure 3.5)($p = 0.333$). APP / AMPK mice, however, displayed worsened memory retention compared to their WT counterparts (Figure 3.5)($p = 0.003$). Swim velocity in male mice was unchanged by APP or AMPK genotype. These results indicate that memory function is impaired in male APP mice with peripheral expression of CA-AMPK.

All mice were weighed to determine their body weights following behavioral testing at an age of between 16-18 months. Both gender ($p < 0.001$) and APP genotype ($p < 0.001$) significantly reduced body weight, while no effect was found by AMPK genotype. Between individual groups, the difference by APP genotype was only observed within the AMPK mice (Figure 3.7 A, B).

Figure 3.4: Peripheral AMPK expression slightly alters spatial memory acquisition in male mice. Mice were trained for 10 days to find a hidden platform in the Morris water maze. A) All groups of male mice are depicted. B) APP / WT mice perform worse than WT / WT mice. C) APP / AMPK mice perform worse than WT / AMPK mice. D) APP / AMPK mice were not statistically distinguishable from APP / WT mice although a slight but consistent trend toward impaired performance with AMPK expression was apparent in later part of training. E) WT mice are unimpaired by AMPK expression. Asterisks in the reference legend of each graph represent significance of the entire curve by repeated measures ANOVA; (ns = not significant, * $p < 0.05$, ** $p < 0.01$). Pound signs represent significance of comparisons of individual points on the curve (# $p < 0.05$).



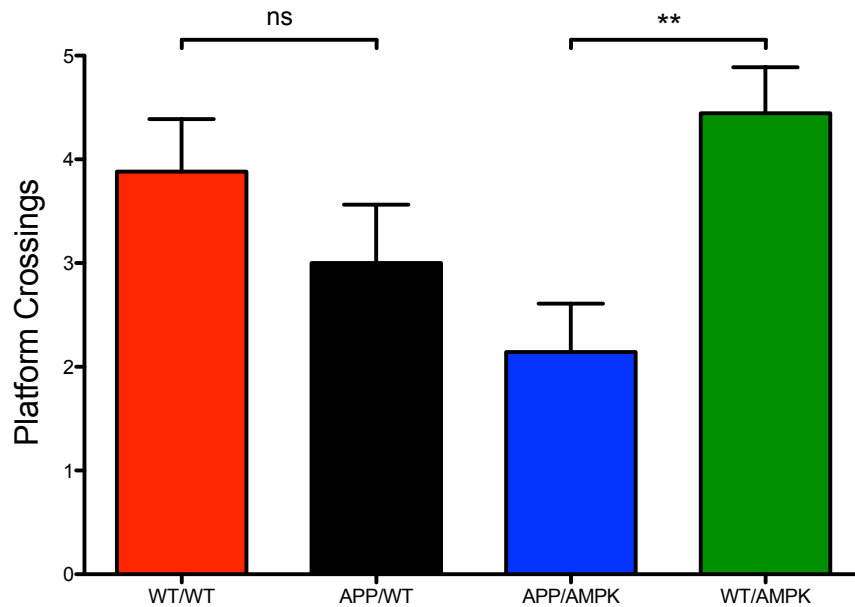


Figure 3.5: AMPK expression impairs spatial memory retention in male APP mice. Following hidden platform training, mice were tested for memory retention in a probe trial in which the platform was removed and the mice swam for one minute. Although on average APP / WT mice crossed the platform location fewer times than WT / WT mice, the difference was not statistically significant. With AMPK expression, APP mice demonstrated significantly impaired spatial memory retention. Data represent the average number of times the mice in each group crossed the area where the platform had previously been located; error bars represent SEM (ns = not significant, ** $p < 0.01$); WT / WT, $n = 18$; APP / WT, $n = 13$; APP / AMPK, $n = 14$; WT / AMPK, $n = 11$.

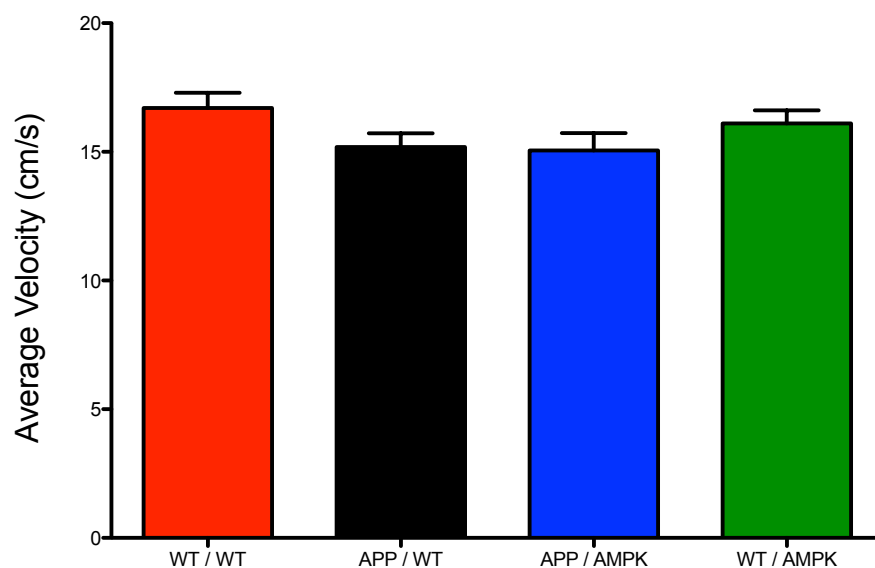


Figure 3.6: Swim velocity is unchanged by genotype in male mice. No differences in average swim velocity were detected between APP or AMPK groups. Data for each individual mouse was determined by averaging velocities from all swim trials for that mouse over the course of the 14-day testing period. Data graphed represent the average swim velocity for each group; error bars represent SEM.

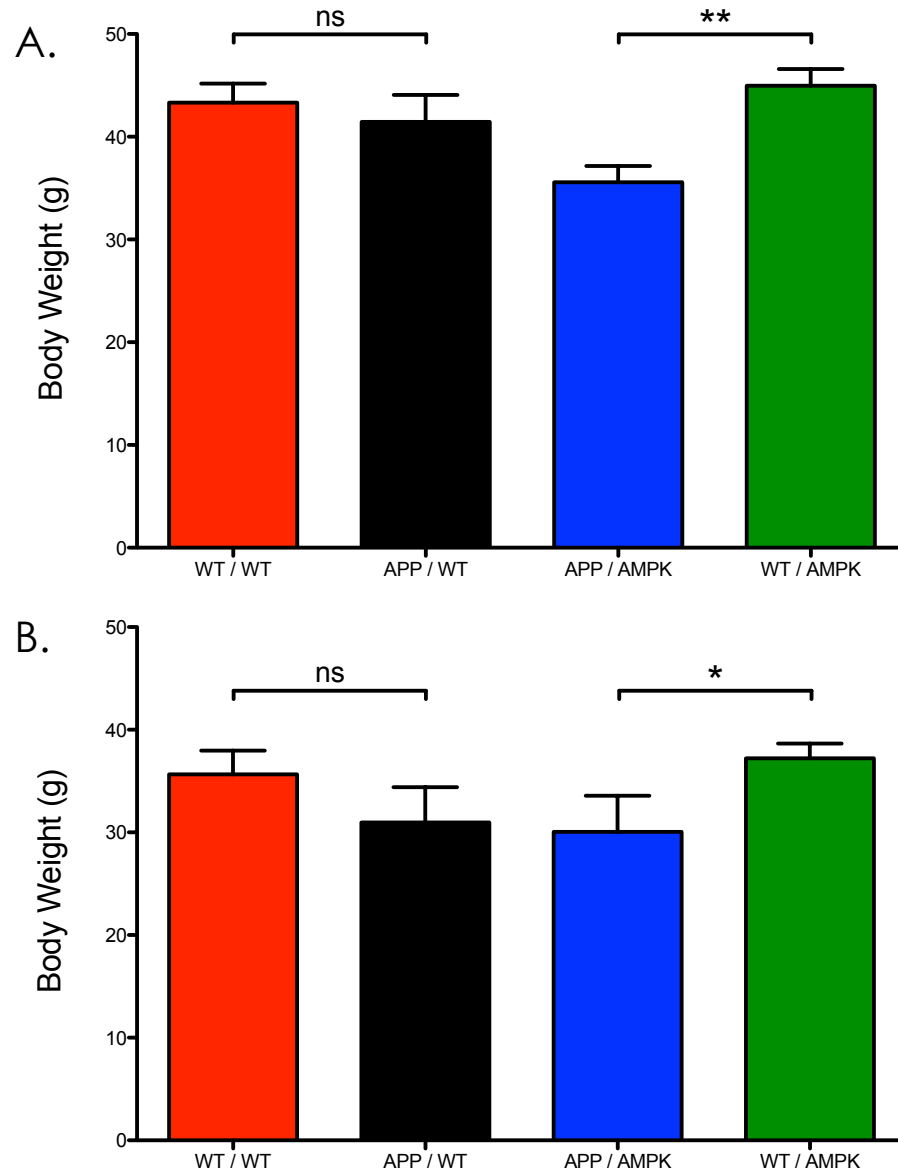


Figure 3.7: Body weight is unchanged by AMPK expression. Data are depicted as mean; error bars represent SEM. Three-factor ANOVA indicated a main effect of gender and APP genotype on body weight, with no effect of AMPK expression. Male mice (A) are larger than female mice (B) and within the AMPK groups, WT mice are larger than APP mice. $n = 11-18$ mice per group for males; $n = 7-14$ mice per group for females (* $p < 0.05$, ** $p < 0.01$).

Discussion

The liver is the main energetic homeostatic organ of the body. It plays a role in digestion, detoxification, cholesterol synthesis, distribution of fatty acids, regulation of blood sugar and storage of readily available energy and other nutrients. Each and every one of these effects is intimately connected to all other systems of the body, yet originates in processes at the molecular level within individual hepatocytes.

One key molecular regulator within the liver is the AMP-activated protein kinase. AMPK is a heterotrimeric protein capable of sensing changes in the ratio of AMP/ATP. Under conditions of energetic scarcity, ATP levels drop with a concomitant rise in levels of AMP. As such, this ratio is a main marker of cellular energetic status. AMPK senses this shift in ratio by binding AMP through its Bateman domains, upon which it undergoes a conformational change allowing activation by an upstream kinase [17]. Upon activation AMPK acts to increase energy availability while preventing its unnecessary use. For example, it then serves to stimulate fatty acid oxidation, glucose transport and glycolysis; processes that will function to increase energy availability. Simultaneously, it inhibits gluconeogenesis, protein and fatty acid synthesis; activities which are all costly in terms of ATP.

Hepatic AMPK also responds to signals indicating the metabolic status of the entire body, not just of that within the cell, and its effects have consequences for the entire body as well. For instance, the fat-derived, anti-obesity hormones, adiponectin and leptin, both reduce blood sugar levels

and gluconeogenesis through activation of AMPK in the liver [18]. In addition, AMPK activation is also regulates processes such as lipid metabolism and insulin sensitivity [17], having effects throughout the body.

Given the importance of the liver in maintaining energy homeostasis, disruption of its ability to maintain balance is likely to have impacts on the whole body, including the brain. A growing amount of data is supporting a connection between the metabolic status of the body and cognition. For instance, diet-induced obesity has been reported to decrease memory function and diabetes is a risk factor for AD [19, 20]. However, since many changes occur within an obese or diabetic individual – disrupted insulin signaling, hyperglycemia, oxidative stress, inflammation, damaged blood vessels, altered circulating hormone levels – it is unclear which factors contribute to cognitive alterations or what molecular mechanisms underlie those factors. In the present study, we have demonstrated that constitutive AMPK activation in the liver is sufficient to alter learning and memory function in APP mice.

We previously reported cognitive consequences of metformin treatment in APP mice and now we report that liver-specific activation of AMPK is sufficient to bring about these results. Additionally, our results demonstrate a gender-bias favoring female mice that we also observed with metformin treatment. Genetic activation of AMPK in the liver of female APP mice resulted in improved learning and memory function. Male APP mice, however, had worsened memory retention by this same genetic modification.

We initially considered the possibility that the results from our metformin study depended upon AMPK activation within the brain or that off-target, non-AMPK-related activity was to blame for the negative consequences in male APP mice; however, this new data suggests that metformin's effects on cognition involved the same molecular target that is beneficial in diabetes. Moreover, both studies involved mice with improved insulin sensitivity, yet the males exhibited worsened cognitive capacity. Other studies using genetic- or dietary-induced models of insulin resistance have also shown detriment or no benefit to AD [21-23], however controversy remains as the opposite result has also been reported [24, 25]. Although disrupted insulin signaling may be part of AD, the results of the current study indicate this correlation alone may be too simplistic and that synergism with other signaling pathways or hormonal systems may be important factors for which a better understanding is needed.

Acknowledgements

Chapter 3, in part is currently being prepared for submission for publication of the material. DiTacchio, Kacey; Heinemann, Stephen. The dissertation author was the primary investigator and author of this material.

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CHAPTER 4:

Discussion and Summary

Alzheimer's disease (AD) is a neurodegenerative disorder that affects cognition and although our understanding of Alzheimer's disease has advanced significantly, many questions remain unresolved and treatment options are limited. The aim of the work outlined in this dissertation was to further our understanding of the molecular mechanisms that either protect against the development of disease symptoms or accelerate the symptoms of AD, with the goal of contributing to better future treatments for this devastating illness.

The etiology and risk of Alzheimer's disease is multivariate, with genetic, age and environmental components. Importantly, one of the factors that seem to increase the risk for this debilitating disease is metabolic dysfunction. Obese and diabetic individuals have a two- to three-fold enhanced rate of AD [1, 2]. This has tremendous implications for the incidence of Alzheimer's disease; our population is currently aging in an era when not only life expectancy is increasing, but when we are facing an obesity and excess body weight epidemic. Two non-pharmacological interventions believed to diminish the incidence and slow down the progression of AD are caloric restriction and exercise, both of which ameliorate diabetes and metabolic dysfunction and, additionally, may slow down the aging process.

Based on these observations, we decided to explore the possibility that a pharmacological agent known to activate the master energy homeostatic regulator, AMPK, which is of critical importance in mediating the effects of caloric restriction and exercise (reviewed in [3]), would have an impact on the

occurrence and progression of Alzheimer's disease. To this end we used the anti-diabetic and AMPK-activator metformin, which is currently taken by millions of patients, and monitored its effect on a murine model of Alzheimer's disease.

We initially hypothesized that metformin treatment would act as a caloric restriction and exercise mimetic and, as such, would have a beneficial effect on the cognitive function of AD mice. To our surprise, in a pilot study performed with a small number of male mice we found metformin to have the opposite effect of what we had expected; metformin-treated, APP-positive mice presented a more pronounced cognitive decline (as assessed by their spatial memory) than the controls counterparts. Repetition of the study using a larger number of mice and also including animals of both genders gave us a better view of the effects of metformin. Male mice, as we had seen in our initial study, were negatively affected by the drug. Remarkably, female mice significantly benefited from metformin treatment.

A secondary aim of this work was to define whether AMPK activation in the liver – a major site of action in the treatment of diabetes – would account for the observed effects of metformin. To answer this question, we made use of a murine model harboring a constitutively active AMPK transgene. Much like the pharmacological portion of this work, genetic activation of this key energetic regulator led to a greater decline of cognition in male mice while protecting females.

Although our results have important implications for both genders, it remains unclear if the effects we observed would hold true in human patients as well. First, our mouse model is a good and convenient model in that it replicates many of the characteristics of the disease and shares most basic physiological processes with humans, yet it is not perfect. Mice do not develop Alzheimer's phenotypes or pathologies naturally. By overexpressing mutant, human APP, the model generates A β and many amyloid-related impairments; however, tau pathologies are missing, we do not observe significant neuronal loss, nor did we observe diabetic-like symptoms in the current model. Since we were interested in exploring the connection between Alzheimer's and diabetes and general metabolic health, the lack of insulin resistance is one relevant way in which our model may not have perfectly replicated the human disease. Additionally, carrying the APP transgene with familial mutations makes the APP mouse a better model for familial AD, whereas the majority of Alzheimer's cases are sporadic, likely involve different mechanisms and may be amenable to different interventions.

Another anti-diabetic drug, rosiglitazone, has shown many benefits in mouse models, including reduction of A β levels, improved memory function and reversal of insulin resistance [4]; however, when administered to human patients in clinical trials, the drug failed for lack of efficacy [5]. Thus, although the mouse is a good model from which we can learn many things, additional testing in human patients is required before conclusions can be made.

One factor that was not addressed in the current study was the level of AMPK activation. The general function of AMPK activation provides protection to cells in situations of stress (reviewed in [6]). Nonetheless, there is evidence that AMPK activation is detrimental in times of extreme stress [7, 8]. The current study did not explore different doses of metformin or varying levels of AMPK activation. AMPK activation has been shown to provide protection against A β -induced stress [9], but it is not clear how the level of stress in the cell culture model in which that was shown compares to what the mouse is experiencing. Moreover, the level of AMPK activation may be different and any number of other factors carefully controlled in cell culture may be affecting the outcome in vivo. It is possible that males and females have differing baseline stress levels, different stress responses to A β or different interactions of AMPK with the stress system contributing to the gender differences observed in our model. This may explain the fact that WT mice, without A β -stress, are unchanged by AMPK activation. In one possible scenario, AMPK activation would be suboptimal in female APP mice or the level of stress experienced would not be beyond what activation could assist; therefore, activating AMPK would be beneficial. On the other hand, in male APP mice, activation of AMPK may overshoot the ideal level or the mice may already be experiencing stress to the extent that additional AMPK activation would be detrimental.

One final consideration following the results of the present study is the interrelatedness of the brain and the body. In understanding physiological processes and disease, tissues and organs are frequently considered in

isolation. As demonstrated herein, peripheral manipulations can affect cognitive processes. Despite the growing connection between diabetes and AD, there are no examples currently in the literature reporting cognitive testing following treatment with metformin – the most commonly prescribed anti-diabetic medication. Perhaps tests of mental function should become part of routine physicals and clinical trials.

In conclusion, we have shown that metformin treatment is beneficial to female APP mice in Alzheimer's-related behavioral phenotypes. Given the wide array of benefits reported following metformin treatment by various measures in different models – improved insulin sensitivity, reduced cancer, anti-aging – this drug should be considered for treatment or potentially prevention of AD in females. On the contrary, metformin treatment was detrimental to male APP mice in learning and memory function. The fact that AMPK activation in the liver was sufficient to bring about this effect suggests that a different approach to diabetic treatment may be needed if human patients are similarly affected. Nevertheless, male patients taking metformin should be observed for changes in mental function and future research should explore the basis of this gender difference so that the negative effects in memory function can hopefully be overcome.

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CHAPTER 5:

Methods

Mice

We used APP mice on a C57Bl/6 background for our Alzheimer's model. Briefly, the mice used in the present study are line J9 from the transgenic mouse expressing a 695-amino acid version of human APP containing mutations found in familial AD (Swedish (K670N, M671L), and Indiana (V717F)) and driven by the platelet derived growth factor (PDGF)- β promoter. This line has previously been shown to express A β and develop plaques [1], to have deficits in input/output curves and long-term potentiation in electrophysiology measures [2] and to develop deficits in learning and memory as measured in the Morris water maze [3-5].

For our genetic model of peripheral AMPK activation, we used a transgenic model expressing constitutively active (CA)-AMPK- α 1 in the liver under the human Apo E promoter [6]. The transgene is a truncated version of the AMPK- α 1 gene (residues 1-312) lacking regulatory domains and containing an activating mutation (T172D). These mice were reported to have resistance against obesity, have lower fasting blood glucose levels and improved insulin sensitivity.

Metformin Treatment

Metformin was obtained from various sources: Sigma, cat# D150959; Spectrum Chemical, cat# M1566; a local pharmacy, metformin hydrochloride, 1000mg. Mice in the treatment group were given drinking water with 2 mg/mL metformin, changed every 4-5 days on average. Treatment began between 6-8 weeks of age, except for a small group of

animals in our pilot study that began treatment at 6-8 months of age. Control animals received standard drinking water changed on the same schedule as the treatment group.

Insulin Tolerance Test

Insulin was obtained from Eli Lilly (Humulin R, Hi-210) and diluted to 10 U/mL in sterile phosphate-buffered saline. For one to two weeks prior to testing, mice were conditioned to handling by scruffing to pick them up and then mimicking the procedure by poking the abdomen and scratching the tail to reduce the stress response on the day of testing. On the day of testing, mice were transferred to clean cages with Alpha-dri bedding and a water bottle, but with no access to food six hours before the test began. Prior to insulin administration, mice were weighed to determine their fasted body weight and a baseline blood glucose measurement was made by collecting a small drop of blood from a small nick towards the end of the tail. Blood glucose levels were measured using a NovaMax glucose meter and test strips. Each mouse was given insulin at the dose of one U/kg fasted body weight. Blood glucose responses to the insulin challenge were measured at 15 – 30 minute intervals over the subsequent two hours. Mice were then returned to a standard cage with access to food again.

Morris Water Maze

Learning and memory were assessed using the Morris water maze with a three-phase protocol: (1) Visible Platform training, (2) Hidden Platform training and (3) Probe Test (Figure 5.1). The tank that was used had a diameter

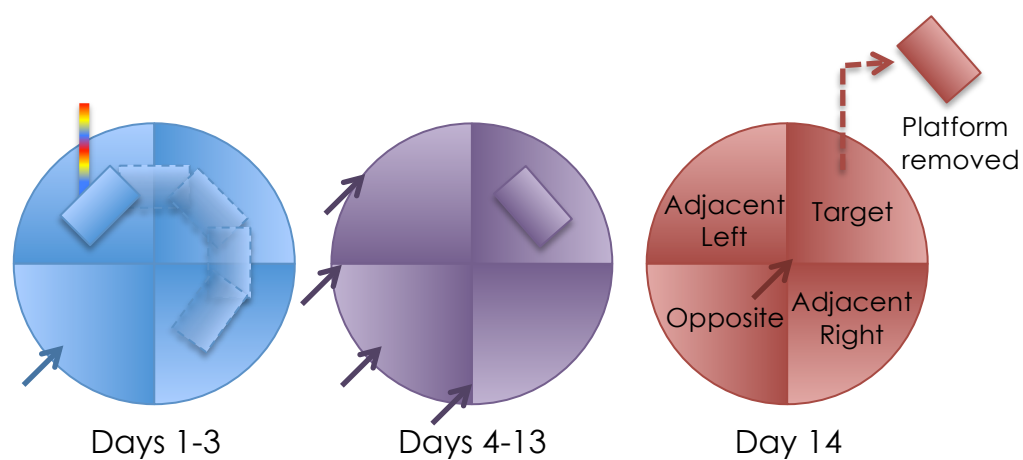
of 1.16m and was filled with water at $24 \pm 1.5^{\circ}\text{C}$ made opaque by the addition of non-toxic paint. Mouse movements were tracked and parameters calculated by Ethovision software (Noldus). For the training phases, the platform was submerged approximately one cm below the surface of the water. Mice were lowered gently into the tank facing the wall and were given up to one minute of swim time to locate the platform. When the platform was found in less than one minute, the trial was ended and the mouse was given 15 seconds to stand on the platform before being removed. Mice that did not find the platform within one minute were placed on the platform by the investigator and also given 15 seconds to stand and look around to ensure that all mice had the same learning opportunity in each trial regardless of whether they successfully found the platform on their own. At the conclusion of each trial, mice were gently towel-dried and returned to the home cage. The mice were given 4 trials for training each day.

The Visible Platform phase of training lasted for 3 consecutive days, during which the platform was marked in location by the presence of a flag. The location of the platform varied between trials with no two successive trials having the platform in the same position, however, the location of entry to the watermaze by the mice remained constant. This phase of testing allowed the animals to acclimate to the test and ensured that they could both swim and see: impairment in either would make it impossible to determine if any underperformance was due to learning or memory deficits or rather motor or

visual deficits. Animals that failed to find the platform >50% of trials on the final day of this phase were excluded from further testing.

The Hidden Platform phase of training began on the day following the conclusion of the Visible Platform and lasted for 10 subsequent days. The location of the platform was no longer marked, however, its position remained constant for the remainder of testing, as did visual cues within the room allowing the mice to orient themselves in space. The entry point for each subsequent trial varied thus requiring mice to use spatial memory to learn to find the platform. A few mice passed the Visible Platform test and qualified at that time for inclusion, but in later trials would not swim and spent the duration of the test floating. It was decided to exclude these mice since there was no way to determine if and to what extent any learning or memory deficit contributed to the refusal to participate. This phase of training served as a measure of memory acquisition.

The day following the conclusion of the Hidden platform training, mice were tested on the Probe Test. In this test, the escape platform was removed and mice were given one minute to swim in the water maze. Each mouse was gently lowered into the center of the tank so to avoid bias to any particular quadrant.



	Trials per day	Number of days	Flag	Platform location	Entry location	Purpose
Visible	4	3	Yes	Varied	Fixed	Acclimation/control
Hidden	4	10	No	Fixed	Varied	Memory acquisition
Probe	1	1	No	Absent	Center	Memory retention

Figure 5.1: Description of the Morris water maze protocol.

Statistical Analysis

GraphPad Prism was used for graphing and SPSS was used for statistical analysis. For analysis of variance with three factors, the GLM command with full factorial analysis was used to assess the main effects of each factor and the significance of any interaction; the MANOVA command was subsequently used to identify differences between levels of factors. For the Morris water maze hidden platform phase of training, individual trial data were averaged for each mouse to yield the average time to platform or distance to platform for the day. Day was then used as the Within Subjects factor; gender, genotype and treatment were the Between Subjects factors in the case of the metformin study. For the CA-AMPK study, the treatment group was replaced with a second genotype factor for the presence or absence of the AMPK transgene. For analysis of the insulin tolerance test, a similar model was used with the only exception being the lack of the gender factor since only males were tested. For analysis of the platform crossings on the probe trial, average swim velocity and body weight, the same design was used, except using univariate analysis without a repeated measures design.

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