Early Metabolic Differences in Alzheimer's Disease Mouse Model and Exercise Intervention Treatment

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Alzheimer's disease (AD) is a neurodegenerative disorder that affects the central nervous system (CNS). In this study, we characterized and examined the early metabolic changes in the triple transgenic Alzheimer's disease (3xtg-AD) mouse model. We examined its relationship with the hypothalamus, a key region of metabolism in the CNS. We observed that the 3xtg-AD model exhibited significantly increased oxygen consumption as well as food intake before previously reported amyloid plaque formation, indicating that metabolic abnormalities occurred at early on-set in the 3xtg-AD model compared to their control. Analysis of gene expression in the hypothalamus showed increased mRNA expressions of inflammation and apoptosis related genes and decreased gene expression of orexigenic neuropeptide Y (NPY) at 12 weeks. Immunofluorescence analysis revealed that anorexigenic pro-opiomelanocortin (POMC) neurons were reduced at 24 weeks in 3xtg-AD model. Exercise has been known to stimulate positive effects throughout the CNS and has been studied as a possible treatment for many different neurological disease. Our study wanted to understand the effect of exercise on the 3xtg-AD model. Four-weeks of voluntary exercise treatment was sufficient to reduce several inflammation and apoptosis related gene expression in the hypothalamus. Eight-weeks of voluntary exercise in the 3xtg-AD mice increased POMC and NPY neuron populations compared to sedentary

conditions. Our results indicated that early on-set of metabolic abnormalities may have contributed to the pathology of AD. These metabolic abnormalities appeared during the same interval with increased inflammation and decreased neuronal populations and key neuropeptides in the hypothalamus.

Early Metabolic Differences in Alzheimer's Disease Mouse Model and Exercise Intervention Treatment

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Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

General Information

Alzheimer's disease (AD) is a debilitative neurological disease that becomes more prevalent with aging. It is characterized by neurodegeneration associated with amyloid beta plaque formation and neurofibrillary tangles (NFTs) derived from tau phosphorylation in the CNS. It is currently unknown whether or not the buildup of proteins like amyloid beta and tau phosphorylation are the main cause of AD or symptomatic. Plaque formation in the CNS leads to reduced synaptic signaling and deterioration of neurons. The pathology usually occurs earliest in the cortex and hippocampus, areas responsible for spatial coordination and memory, and slowly spreads throughout the central nervous system (CNS). The average age of diagnosis in AD is 65 years with a small subset, around 5% of the population, obtaining symptoms earlier which is referred to as early onset [1].

Metabolism and the Hypothalamus – Roles of NPY/POMC

The hypothalamus is a region of the forebrain responsible for many different functions including coordination of the autonomics nervous system, pituitary activity, body temperature regulation, homeostatic regulation, and metabolism. Metabolic function in the CNS is mainly attributed to signaling in the hypothalamus. Some neurons in the hypothalamus have highly specific functions that express and release neuropeptides to control feeding behavior and energy expenditure. Two of the important populations of neurons that release appetite controlling neuropeptides are POMC and AgRP/NPY neurons [32]. POMC neurons release several different neuropeptides including α-MSH which is responsible for decreased appetite [33]. AgRP/NPY neurons release neuropeptide Y and Agouti-related peptides to increase appetite [34]. AgRP and NPY neuropeptides are expressed within the same cells and are often synonymously referred to

as AgRP/NPY neurons. These populations are located in the arcuate nucleus along the third ventricle. The third ventricle is located between the two hemispheres of the brain and is the interspace between the hypothalamus and the thalamus, allowing for the flow of cerebral spinal fluid in the interim space.

Exercise in Relation to AD

Metabolic etiologies have always been a focus in the study of AD. Current research has sought to combine the beneficial aspects of exercise to counteract the negative effects of AD. There have been many different studies that have sought to utilize exercise therapy to slow down or reverse AD with varying degrees of success [19, 20, and 21]. Exercise has also been shown to improve cognitive decline associated in the mouse models along with improving synaptic function and decreasing amyloid-beta aggregation in the brain [22]. On the other end of the metabolic spectrum, it has been reported that increased weight and obesity is correlated with an increase risk in developing AD [23, 24].

3xtg-AD Mouse Model

Studying the early metabolic interactions of AD can be difficult. The largest risk factor for AD is age and many of the early symptoms such as memory deficits and decreased coordination are similar symptoms to aging. These symptoms make it difficult for early diagnosis without invasive procedures. There is a body of knowledge in this area of AD research trying to identify a preclinical stage of AD, the stage before mild cognitive impairment. Behavioral tests are difficult due to their subjective nature and the lack of noticeable cognitive decline in the preclinical stage. Most methods of identification involve analyzing cerebral spinal fluid (CSF) for biomarkers in conjunction with PET scans or MRI [37, 38]. There are still a lot of

uncertainty in the current methods for early detection such as biomarker threshold, whether or not these biomarkers will lead to AD, length of time until development, and more [36].

My research utilizes a mouse model to study the effects of early metabolic irregularities in AD. The 3xtg-AD strain of mice provides a model that exhibits both amyloid beta plaque pathology and NFTs [18]. The model proliferates key factors relating to AD. It expresses three human genes that work to proliferate amyloid beta and tau phosphorylation. The model has been reported to display early signs of AD and is well defined through behavioral studies as an adequate model for AD studies [35]. In previous studies it has been reported that the model displays cognitive impairments in 4-6 months of age due to amyloid plaque pathology [28]. As a result, this is the normal time frame many studies utilize as the start of their experiment and research.

My study is more interested in the preclinical stages of AD, before cognitive impairments are reported. There has been far less studies relating to preclinical symptoms of AD. The model has well defined periods of AD impairments making it easy to isolate and observe the preclinical time points in the model. Utilizing the model it may be possible to observe metabolic changes in preclinical stages of AD and whether or not different treatments such as an exercise intervention can alter the preclinical stage.

Hypothesis

It is well regarded that metabolic dysfunction plays a role in AD. It has been shown in different mice models that metabolic deficiencies precedes traditional AD pathology (39). Utilizing the 3xtg-AD model we pinpointed a time period where significant metabolic discrepancies occurred such as increased oxygen consumption, food intake, and changes in body composition, between 10-12 weeks. We hypothesize that these effects are related to the

hypothalamic neuronal populations involved in metabolic regulation, POMC and NPY/AgRP. We also want to investigate if exercise has any effect on early metabolic changes.

We hypothesize that early metabolic differences is partly a result of changing POMC and NPY/AgRP neuron populations. We implemented a voluntary exercise regimen, between the age of 6-14 weeks, as a potential treatment to alter metabolic dysfunction and observe CNS differences in the hypothalamus as exercise has been shown to have many positive benefits relating to dementia and metabolic function.

Chapter 2: Literature Review

History of Alzheimer's Disease

The etymology of dementia comes from the Latin roots "de" for to depart and "mens" for the mind. Throughout history, dementia has always been a synonymous marker with aging but the causes were unknown. In 1901, Alois Alzheimer identified the first case of what would be aptly named AD. He followed a fifty year old woman's case until her death in 1906 and wrote the first reported case on dementia. At this time, advancements in microscopy were being made and new histochemical techniques were being developed that helped broaden the understanding of nervous system disorders [9]. In Alois Alzheimer's report he noted, "Remarkable changes in neurofibrils appeared. In the interior of a cell that otherwise appeared normal, one or several fibrils stood out due to their extraordinary thickness and impregnability." He describes the neurofibril as abnormal from other patients with dementia that he had observed in which he did not note such structures to exist [40]. The structure he described is known today as phosphorylated tau protein which makes up the cytoskeleton of neurons in the form of microtubules, but in the AD process becomes phosphorylated losing their rigid structure, collapsing the microtubules, and subsequently forming neurofibrillary tangles (NFTs). We also know the reason he did not see these structures in some of his other patients that he documented because many of his previous dementia cases were from patients who suffered from neurosyphilis and vascular disease such as brain ischemia instead of AD [9]. In his most famous report he further commented on the mechanistic nature of the fibrils saying, "At a late stage, many fibrils appeared, situated side by side and altered in the same way. Then they merged into dense bundles and gradually reached the surface of the cell. Finally, the nucleus and the cell disintegrated, and only a dense bundle of fibrils indicated the site where a ganglion cell had

been." This perfectly fits into our modern understanding of tau and the dysfunction that appears later in AD in which the tau proteins phosphorylate and destabilize microtubules collapsing neurons [40].

The official endorsement of the disease came from Emil Kraepelin, considered today as one of the founders of modern psychiatry, who included the term "Alzheimer's Disease" in the eighth edition of his *Textbook of Psychiatry* published in 1910. The identification of AD would become a major turning point in scientific literature on the understanding of senile dementia [9]. For the remainder of the century, AD would be used to label individuals with dementia within a narrow age range, 45-65, who displayed dementia. It would not be until 1977 at the conference on AD that many researchers and clinicians agreed that pre-senile and senile dementia were almost identical leading them to determine that AD was independent of age [42]. Soon after, AD, would be formally adopted in medical terminology to describe individuals with common characteristics, disease course, and neuropathology regardless of age.

Basic Pathology

AD is characterized by several distinct pathological elements that eventually lead to cognitive decline, neuronal degradation, and atrophy. The gradual buildup of amyloid beta oligomers is one of the hallmark symptoms, often preceding cognitive decline by as long as a decade. Amyloid beta overexpresses creating oligomers that disrupts neuronal transmission and triggers immune response [7]. The main contributor of amyloid plaque buildup is a specific isoform of the amyloid protein called amyloid beta 42, which has the unique property of being water insoluble. The water insolubility leads to higher amounts of plaque buildups in areas between synapses inhibiting neuronal transmission.

Another pathological element of AD and a major contributor of neuronal degradation is the phosphorylation of tau proteins. Tau proteins are integral to the cytoskeleton since they form microtubules, a key component of the cytoskeleton. When phosphorylated, tau proteins disassociate from microtubules forming into NFTs. The process of phosphorylation destroys the structural integrity of microtubules as it destabilizes them through the loss of tau protein. Eventually the cell collapses without microtubule support leading to apoptosis. Amyloid beta aggregation and tau phosphorylation are classic symptoms of AD pathology. Amyloid pathology can occur decades before diagnosis while tau phosphorylation usually occurs later with age [36]. Plaque buildup effects early in areas such as the cortex and hippocampus affecting spatial awareness and memory leading to early symptoms of cognitive decline and later on tau phosphorylation causes neuronal degeneration.

There are many symptoms of AD that have not fully been explored. Many of these symptoms do not directly relate to the buildup of detrimental protein. It has been observed that many biological functions change during the onset of AD such as alteration of circadian rhythm and mitochondrial function [2]. There is an undeniable genetic component to AD. In 2006, one of the largest Swedish twin studies for AD utilizing approximately twelve thousand twin pairs reported that the heritability of AD was between 58-79% [3]. Many families of genes seem to be involved in the onset and aggressiveness of AD and new genes are constantly being discovered through widespread studies and meta-analysis. In 2013, a meta-analysis of approximately seventy five thousand individuals identified eleven new loci that are affected or susceptible to AD [4]. While there is undeniably a genetic component to AD, there are many hypotheses relating to the cause of AD.

Amyloid Hypothesis

The most widely accepted hypothesis on the pathology of AD is the amyloid hypothesis. It postulates that the aggregation of extracellular amyloid beta is the cause of AD [5, 25, and 26]. Amyloid beta is a protein produced in the brain derived from the enzymatic cutting of amyloid precursor protein (APP). Amyloid beta has many isoforms but the two most studied for AD research is amyloid beta 40 and 42. These two are the most common forms of amyloid beta in AD patients with 42 being the cause of a lot of neurological damage since it is water insoluble. The origin of this hypothesis came from an observational correlation between those with Down syndrome and AD. It was found that the gene for amyloid precursor protein (APP) is located on chromosome 21. It was also observed that individuals with Down syndrome, a genetic disease in which they have an extra chromosome 21, exhibited higher rates of AD and at earlier ages, usually developing the disease by age 40, being within the 5% that exhibit symptoms before age 65. These two observations lead to the first conclusion that AD is related to an overexpression of amyloid beta [6].

Metabolic Irregularities

There are many symptoms relating to AD that have not fully been explored that do not directly relate to the buildup of detrimental protein. It has been observed that many biological functions change during the onset of AD such as mitochondrial function and immune function [2, 7]. There is an undeniable genetic component to AD [3]. Many families of genes are associated and suspected to be involved in the onset of AD through unknown mechanisms [4]. While there is undeniably a genetic component to Alzheimer, there are many hypothesis relating to the cause of Alzheimer although the scientific consensus currently focuses on an overexpression of amyloid beta and tau phosphorylation [11, 13].

To date there has been few studies on the metabolic effects of AD in the early stages even though there are studies suggesting that early damage is done with regards to the mitochondria that precedes the normal pathology of previous AD models [2]. However, it has been shown that late stages of AD exhibit reduced glucose transport through impairments in the cerebrovascular system [14, 15], this phenomenon has been so prevalent that AD has been theatrically referred to as Type III Diabetes Mellitus. Mitochondrial data suggest that there are many differing irregularities in mitochondrial function in AD early on that can be attributed to these changes. It has been observed that a sudden infusion of amyloid beta is directly related to a decrease in mitochondrial membrane viscosity and associated ATP decrease and inhibition of the respiratory chain [8]. These studies suggest an early inhibition in mitochondrial function relating to an increase in amyloid beta. Mitochondrial function is critical to the metabolic functionality of the body as the primary producer of ATP but even more so in the brain which uses an estimated 20% of the total energy output of the body. Any disruption or inhibition in the process could be enough to exacerbate the symptoms of AD.

Metabolic Neurons and Whole Body Interactions

The activation and inhibition of NPY and POMC neurons are derived from several different hormones. The two largest contributors are the hormones leptin and ghrelin. Leptin is secreted from adipose tissue and activates POMC neurons while simultaneously inhibiting AgRP/NPY neurons [43]. When POMC is activated it releases α-MSH to GABAergic neurons signaling the body to decrease food intake and increase energy expenditure. Ghrelin is another hormone that plays a large role in food intake. Ghrelin is secreted from the stomach to induce hunger and when it circulates to the hypothalamus it activates AgRP/NPY neurons while

simultaneously inhibiting POMC neurons [44]. The activation of AgRP/NPY neurons releases AgRP/NPY neuropeptides which signals the body to increase food intake.

AgRP, NPY and α-MSH are neuropeptides which are highly linked to food intake responses. A release of α-MSH peptides from POMC neurons leads to a decrease in food intake through the promotion of a "satiety" response in the paraventricular nucleus (PVN) through melanocortin 4 receptors (MC4R) on GABAergic neurons. NPY activity in the hypothalamus, on the other hand, is responsible for the "hunger" response. NPY also has a major role as a metabolic regulator apart from producing a hunger response. It has been observed in mice and humans that repeated stress will cause NPY activation to occur. NPY activation causes reduced energy expenditure and stimulation of fat growth, especially abdominal fat [27]. The response explains a part of why some people binge-eat/ gain weight during periods of high stress.

Alzheimer's Disease and Early Screening Methods and Medications

Currently there is no medical treatment that will delay or halt the symptoms of AD. A majority of the current medications seek to treat the symptoms of AD and focus on personal care for the patients. There are five primary medications on the market that deal with the cognitive symptoms of AD. Four of these are cholinesterase inhibitors and one is a NMDA receptor antagonist. The medications work by inhibiting the enzyme that breaks down acetylcholine or activates receptors leading to an increase in signaling. The benefits derived from these medications are minimal, which speaks to the severity of the disease and division of knowledge within the field on how to treat it [16, 17]. The primary focus on AD has, historically, been towards care giving for patients. A lot of scientific effort has been put towards treatment and detection of AD as early as possible [16].

Metabolism and Alzheimer Interconnections

As of date there has been relatively little study into the metabolic effects of AD in the early stages even though there are studies suggesting that early mitochondrial dysfunction precedes the normal pathology of previous AD models [2]. Previous mitochondrial studies suggest that there are many differing irregularities in mitochondrial function in AD early on that can be attributed to AD pathology. It has been observed that a sudden infusion of amyloid beta is directly related to a decrease in mitochondrial membrane viscosity and associated ATP decrease and inhibition of the respiratory chain in neuronal cell cultures [8]. Studies suggest an early inhibition in mitochondrial function could be related to an increase in amyloid beta. Mitochondrial function is critical to the metabolic functionality of the body as the primary producer of ATP but even more so in the brain which contributes an estimated 20% of the total energy output.

AD shares many similar characteristics to metabolic diseases such as Type 2 Diabetes Mellitus (T2DM). Similar symptoms include inflammation, oxidative stress, impaired insulin signaling, and cognitive deficits [31]. In T2DM, obesity and high fat diets lead to peripheral inflammation which adds to cellular stress leading to insulin resistance. In AD, amyloid beta leads to microglia and astrocyte activation which promotes inflammation in the CNS [7]. Inflammation in the CNS could lead to insulin resistance in a similar fashion to the peripheral system, ultimately this contributes to synapse deterioration and cognitive decline which are also seen in T2DM.

Exercise as Viable Treatment for AD

Exercise interventions in the 3xtg-AD model have traditionally been administered at older ages and for longer than the proposed 2 months since cognitive dysfunction aren't reported

until at least 4 months of age which is the starting point of many studies regarding the model [28]. A majority of studies also have a heightened focus on cortex and hippocampus because amyloid beta plaque, in late stages, build up in the folds of the cortex and induce cognitive impairments associated with the hippocampus. Additionally it is well established that exercise decreases amyloid beta load in the frontal cortex and hippocampus [29]. The effects of exercise in the hypothalamus has not been explored nearly as well. There are some studies that indicate that exercise training increases excitability in the hypothalamus [30] contributing to increase peptide signaling but there is no literature about the effects of exercise on the hypothalamus at the early stages, pre-cognitive decline. The novelty of the proposed study is that we have identified an early time point in which metabolic differences have been observed and will try to alter it with an exercise regimen to see if there is a change or metabolic response in the hypothalamus.

Chapter 3: Results

3xtg-AD Mice Exhibit Amyloid Pathology

Amyloid pathology is one of the earliest indicators of AD. Amyloid beta, through several mechanisms, is overexpressed in the form of oligomers. These oligomers left to their own devices go on to interfere with synaptic transmission and form amyloid plaques. In the 3xtg-AD model we observe amyloid beta pathology in the cortex as early as 12 weeks into the model but no amyloid plaques (Fig 1). This suggest that oligomers are present and could have an adverse effect upon neurons before symptoms of cognitive decline.

Changes in Energy Balance in the 3xtg-AD Model

Oxygen consumption measurements provided insight into metabolic changes in the 3xtg-AD model at 12 weeks of age compared to controls, differences persisted at 24 weeks of age (Fig 2B). Increased oxygen consumption indicated that the 3xtg-AD mice were expending more energy. Locomotion data showed a decrease at 12 weeks for the 3xtg-AD group but not the control group (Fig 2C). Locomotion is predicted to be approximately 10-15% of energy expenditure. The lack of drastic increase from the 3xtg-AD over the control indicates that the increased energy expenditure is not a result of increased locomotion and could be related to metabolic functions. At every time point, the 3xtg-AD model consumed significantly more food than their counterparts (Fig 2A). Taken together this demonstrates that the 3xtg-AD consume and expend more energy than their control counterpart. The lack of increased locomotion indicates that there could be a metabolic driver for the energy abnormality since they are not expending it through increased locomotion.

Body Composition Changes

When body composition was taken into account there was no difference in total weight and fat mass between both groups (Fig 3A and 3C). There was a decrease in lean mass in the 3xtg-AD mice that was significant at 12 weeks and 24 weeks of age (Fig 3B).

Hypothalamus Related mRNA Differences at 12 Weeks

At 12 weeks there was a difference in AgRP mRNA expression between groups (Fig 4A). The 3xtg-AD model displayed decrease AgRP and MC4R mRNA expression (Fig 4A and C). This decrease was not significant in the mRNA expression of POMC (Fig 4B). AgRP mRNA expression could indicate a decrease in the synthesis of AgRP and MC4R neurons in the hypothalamus. AgRP neurons are responsible for food seeking behaviors and ablating AgRP neurons in mice have been effective in reducing weight significantly [13]. In young mice there are compensatory mechanisms that can stop the detrimental weight loss attributed to reduced NPY/AgRP neurons [12]. MC4R are receptors on GABAergic neurons in the PVN, an area that POMC and NPY/AgRP neurons project to. A 4 week exercise treatment was seen to be sufficient in increasing mRNA of AgRP and MC4R expression but not POMC expression.

Hypothalamus Related Degenerative Markers

At 12 weeks there was a noticeable difference in the two biomarkers utilized to examine apoptosis and inflammation. Tumor Necrosis Factor Alpha (TNF-α) mRNA expression was approximately four times the level of the control (Fig 5A). Similarly Interleukin-6 (IL 6), a pro-inflammatory cytokine linked with AD, showed an increase approximately 2.5x higher than the control (Fig 5B) [10]. IKKB, an inflammatory and apoptosis marker was decreased in the 3xtg-AD (Fig 5C). IKKB inhibits inflammation and apoptosis. The decrease in IKKB was concurrent with the increase in inflammation and apoptosis biomarkers. The upregulation in mRNA

expression of these biomarkers suggest apoptosis and inflammatory responses are occurring in the hypothalamus as early as 12 weeks when the metabolic differences are occurring. A 4 week voluntary exercise treatment was shown to have a positive effect in reducing TNF-α and IL-6 to similar levels as control.

After 4 weeks of exercise intervention there was no gene expression difference in mitochondrial biogenesis markers PGC1A, TFAM, and NRF1 between the 3xtg-AD and control (Fig 5D, 5E, and 5F). Although 3xtg-AD group had a decreasing trend it was non-statistically significant. The exercise intervention did not alter mRNA expression of mitochondrial biogenesis markers.

POMC Neuron Differences through Immunofluorescence

At 12 weeks of age there was no difference in POMC expressing neuron populations (Fig 6A and 6B). At 24 weeks of age there was a significant decreased in the POMC expressing neuronal populations in the 3xtg-AD compared to controls (Fig 6A and 6C), this decrease could be partly responsible for increased food intake since POMC neurons are anorexigenic.

Eight Weeks of a Voluntary Exercise Intervention and Effects on POMC

Mice with eight weeks of exercise significantly increased the populations of POMC expressing neurons. (Fig 7B). This indicates that exercise could have a beneficial protective effect on the POMC neurons in the hypothalamus.

Eight Weeks of a Voluntary Exercise Intervention and Effects on NPY

Eight weeks of voluntary exercise in the 3xtg-AD group showed significantly increased amounts of NPY expressing neuronal populations than 3xtg-AD group without an exercise treatment (Fig 8B). NPY expressing neuronal populations in the 3xtg-AD group with exercise indicating that exercise could have a neuroprotective effect on NPY neurons in the early stages

of AD. It was seen that AgRP mRNA expression decreased in the 3xtg-AD but normalized to control after a 4 week exercise intervention (4A). It seems that 8 weeks of voluntary exercise may have a beneficial effect on NPY neuronal populations in AD.

Chapter 4: Discussion

AD is traditionally thought of as a degenerative disease that affects memory and coordination. This is due to its most prominent effects in the cortex and hippocampus. Based on the present study, we have found that AD is not just a neurodegenerative disease, metabolically significant changes occur before the neurodegeneration, which suggests that it may also be a metabolic disorder. The 3xtg-AD mouse model showed an increase in energy expenditure through heightened oxygen consumption and an increase in energy intake through food consumption. What the 3xtg-AD model did not show was a locomotion increase to utilize the excess energy nor a significant difference in weight. The energy intake was not converted to adipose tissue nor expended through locomotion. This indicates that there are metabolic mechanisms that have not been identified. A metabolic change in this model at early stages have not yet been reported and demands further study.

At the same time as observed metabolic changes in the 3xtg-AD mouse model there were indications of neurodegeneration as early as 12 weeks. This is before reports of amyloid plaque formation but amyloid oligomers cannot be discounted as playing a role [28]. Amyloid beta oligomers have been shown to exacerbate inflammation and neurodegeneration through synaptic impairment [7, 45]. Amyloid beta has been observed in the cortex of mice at 12 weeks of age although no plaque formation was found. Gene expression of inflammation and apoptosis markers were shown to increase in the hypothalamus of 3xtg-AD mice in the current study, possibly leading to early degeneration. These two pieces of information seem to suggest that there is neurological damage linked to amyloid beta oligomers occurring as early as 12 weeks of age.

During the same time hypothalamic neuronal populations were being affected, decreased mRNA expression of AgRP and MC4R were also observed. The decrease in mRNA of these neuropeptides and their mediators at 12 weeks suggest that they may be more susceptible to neuronal damage. The decrease in AgRP mRNA expression suggests that AgRP neuropeptides were decreased. A decrease in AgRP neuropeptides, which are orexigenic, should indicate that the mice would have a lower food intake which was not the case at 12 weeks. At 12 weeks, the 3xtg-AD model displayed higher food intake than the control. An underlying mechanism for this could have been that there is a compensatory effect acting on the AgRP neurons. It has been shown that if NPY/AgRP populations in adult mice are ablated that they rapidly lose weight but if they're ablated when young the mice weight develops normally [13]. It has also been shown that there are a select population of neurons in the hypothalamus that can alter AgrP/NPY and POMC activation. These neurons are tyrosine hydroxylase (TH) expressing neurons and release dopamine which can bind to receptors on the NPY/AgRP and POMC neurons producing an orexigenic effect.

The effect is thought to be the origin of pleasure derived from eating, a hedonic effect [12]. It could be that at 12 weeks of age when mRNA expression for AgRP neuropeptides were decreased the compensatory mechanism helped maintain their hunger drive. At 24 weeks, POMC neurons decreased. These neurons are responsible for an anorexic effect and increased energy expenditure and their decrease would lead to increased food intake and decreased energy expenditure. At 12 weeks, there was an increase in food intake and energy expenditure through increased oxygen consumption. An increase in food intake suggest that NPY activation was occurring while an increase in energy expenditure suggests POMC activation. A possible avenue

for exploration is whether or not the increased food intake and energy expenditure is a result of shifting populations of TH, NPY, and POMC neurons.

An exercise intervention has been shown to have protective benefits in several models, including metabolic and cognitive models [22, 29, and 46]. In our model we saw a protective effect on the POMC neurons from an eight week-long voluntary exercise treatment. There was also a significant increase in AgRP neurons with eight weeks of voluntary exercise. This suggests that eight weeks of voluntary exercise could have significant benefits to early AD. Even four weeks of exercise was sufficient to show changes in hypothalamic mRNA expression. Four weeks of exercise was sufficient to show an increase in mRNA expression of MC4R and AgRP (4A and 4C). In addition there was a decrease in biomarkers for apoptosis and inflammation (5A and 5B). There was no change in mRNA expression of mitochondrial biogenesis biomarkers indicating that a four week exercise program may not play a significant role in mitochondrial biogenesis in this particular model. These changes in mRNA expression suggest that exercise was sufficient to change outcomes related to hypothalamic function in the 3xtg-AD model at a critical point before development of reported cognitive decline.

Conclusion and Further Study

It is clear that metabolic abnormalities are occurring prior to the reported pre-cognitive decline in the 3xtg-AD model that expresses three human transgenes for proliferation of amyloid beta and tau phosphorylation. An increased energy expenditure through oxygen consumption along with an increased energy intake through increased food consumption without an outlet for energy expenditure, such as locomotion or increased fat mass, implies other internal mechanisms are involved. We saw that there were differences in POMC neurons at 24 weeks in the 3xtg-AD model (Fig 6C), a population of neurons that drives metabolism. This indicates that at 24 weeks

of age there were already significant changes in the model, which is near the beginning of amyloid plaque pathology. There is enough evidence to further investigate metabolic changes pre-AD and is a research area that should be further explored.

The next step in studying AD through a metabolic lens would be to see if exercise in the model affects cognitive function. It would be worth investigating through different behavioral tests after exercise treatments to see if cognitive function is rescued by a voluntary exercise treatment. In addition it would be worth investigating if hypothalamic changes in neuronal populations related to metabolism such as POMC or AgRP/NPY populations could be normalized with a longer exercise treatment and if this has a dose-dependent effect.

Chapter 5: Methods

Transgenic animal model and Voluntary Exercise Treatment

The triple transgenic AD model mouse displays three mutations associated with AD (APP Swedish, MAPT P301L, and PSEN1 M146V) was gift from The Harriet and John Wooten Laboratory for Alzheimer's and Neurodegenerative Diseases Research at East Carolina University. All animals were housed under controlled temperature and lighting conditions of 20-22 degrees and 12-h light-darkness cycle. For the study of voluntary wheel running, age-matched male mice in the 3xtg-AD + EX group were placed in cages equipped with running wheels for mice (TSE PHENOMASTER, Bad Homburg, Germany), whereas mice in the control group and AD group were housed in cages without running wheels for 4, and 8 weeks respectively. Each cage accommodated one mouse. All aspects of animal care and experimentation were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of East Carolina University.

Metabolic Measurements

Food intake was measured over a 14 day period. Mice were given ~10-12g of food every day and cages were changed every time when the food weight was measured, any residual bits of food in the bedding were not included in measurements. The data was combined, averaged, and analyzed, cumulative food intake data was obtained by adding all intake measurements during the study. Fat and lean body mass were assessed using Echo MRI (Echo Medical Systems Houston, USA). Energy expenditure was measured by assessing oxygen consumption and carbon dioxide production with indirect calorimetry by using metabolic chambers (TSE

PHENOMASTER, Germany). Mice were acclimated in the metabolic chambers for 72 hours before data collection, and had free access to food and water for the duration of the studies.

Tissue Collection

Mice were euthanized by isoflurane and perfused intracardially with phosphate buffered saline PBS followed by 10% formalin, then the brains were collected and fixed in 10% formalin overnight, then transferred in 30% sucrose solution. For the gene expression analysis, all samples were isolated and removed, and submerged in liquid nitrogen to preserve for qPCR at a later date.

Immunofluorescence staining (IF)

Coronal tissue sliced with Leica VT1000 vibrotome at 30um thickness and preserved in an anti-freeze solution at -20ºC until needed.

Tissue were washed in PBS, then blocked with 3 percent normal donkey serum solution. Tissue were then transferred to primary antibody solutions (POMC – Phoenix Pharmaceutical Catalog# H-029-30, amyloid-beta 4g8 - Signet Catalog# 9220-02, NPY – Santa Cruz Catalog # sc-133080, and NeuN – Cell Signaling Catalog # 12943) at recommended concentrations overnight. Then they were washed in PBS next day and transferred to secondary (1:500) for ~2 hours and washed again. Tissue samples were then plated and stained with DAPI solution (Vector H-1200) and imaged using an optical microscope (Leica DM6000). NeuN, POMC, NPY-positive neurons throughout the mediobasal hypothalamus were counted using ImageJ software. At least three serial sections were analyzed in each mouse.

Quantitative PCR

Isolation of mRNA was done utilizing a standard Trizol protocol. The expression of specific mRNAs was assayed using fluorescence-based quantitative real-time PCR (RT-qPCR)

(POWER-SYBR GREEN PCR Master Mix; Applied Biosystems). Quantification reactions were performed in duplicate for each sample using the 'delta-delta Ct' method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as a reference (housekeeping) gene along with appropriate primers for genes of interest (Table 1). At the end of the assay, a melting curve was constructed to verify the specificity of the reaction. To determine the expression levels of relevant RNA expression, cDNA was pre-amplified for 20 PCR cycles, according to the manufacturer's protocol (PrimeScript RT Master Mix Kit; TaKaRa, RR036A), thereby increasing the sensitivity of the subsequent real-time PCR reaction.

Statistical Analysis

Data represent means ± SEM, significance determined through non-paired t-test, after one-way ANOVA when applicable. Significance was set at $P \leq .05$.

Tables VI

Table 1 - qPCR Primer List

POMC	GCG AGA GGT CGA GTT TGC ACC TCA CCA CGG AGA GCA AC
AgRP	GCG GAG GTG CTA GAT CCA CA AGG ACT CGT GCA GCC TTA CAC
MC4R	GCG TTT CGA ATG GGT CGG AAA CCA CCG CAA TGG AAA GCA GGC TGC AA
TNF-α	CAG GCG GTG CCT ATG TCT C CGA TCA CCC CGA AGT TCA GTA G
IL-6	AGT GGT ATA GAC AGG TCT GTT GG CTG CAA GAG ACT TCC ATC CAG
IKKβ	CGG CCC TTC CTC CCT AAC GGT GCC ACA TAA GCA TCA GC
GAPDH	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA

Figures VII

Figure 1 – Amyloid Beta Images

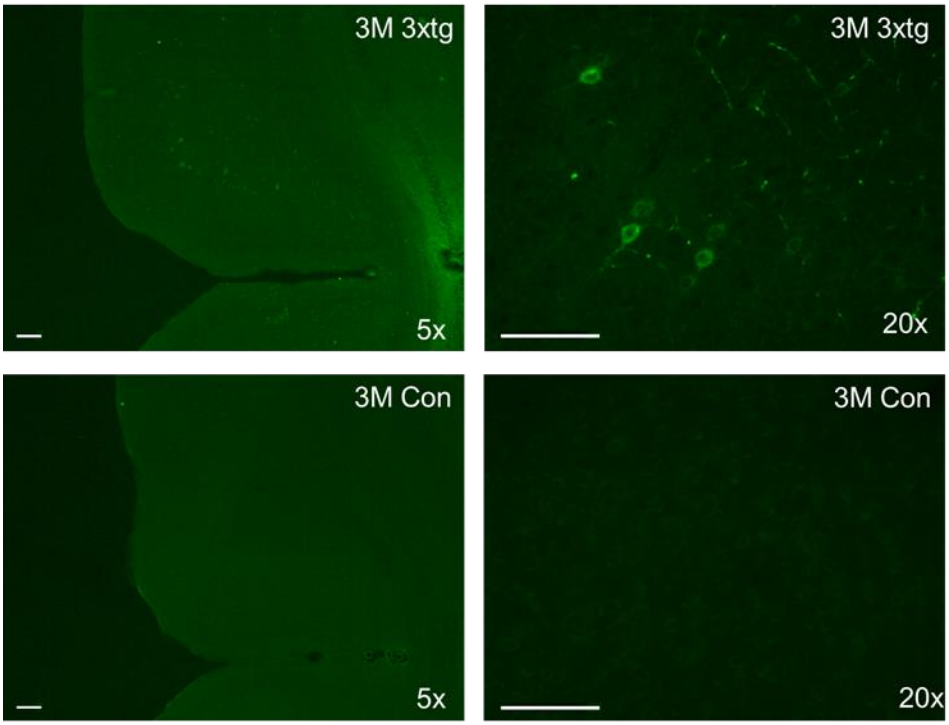

Fig 1. A comparison of amyloid beta load between the triple transgenic (3xtg-AD) and control. All scale bars set at 200 microns.

Mice with the 3xtg-AD genes (top) shows amyloid beta under fluorescent microscopy compared to control (bottom). Representative image of cortex (left) shows amyloid beta at 5x objective. 20x objective (right) shows a close up of amyloid beta congregation around neurons in the 3xtg-AD and none in the control.

Figure 2 – Metabolic Markers

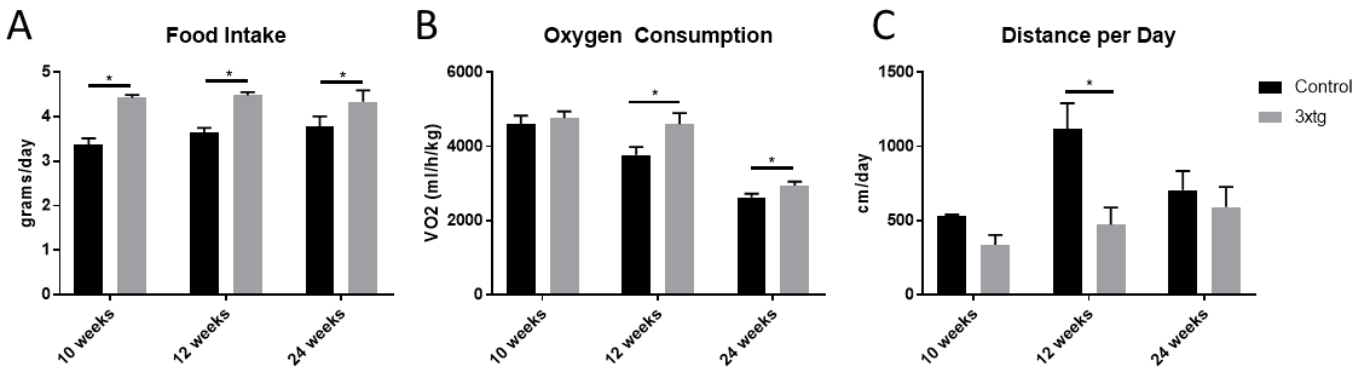

Fig 2. Metabolic Markers at different age points (10, 12, and 24 weeks).

2A. Food intake at 10, 12, and 24 weeks between 3xtg-AD and control group.

2B. Resting oxygen consumption at 10, 12, and 24 weeks between groups.

2C. Locomotion between groups at 10, 12, and 24 weeks between groups.

N = 4-6 per group, *p< 0.05 vs Control. Data represent mean ± SEM.

Figure 3 – Body Composition

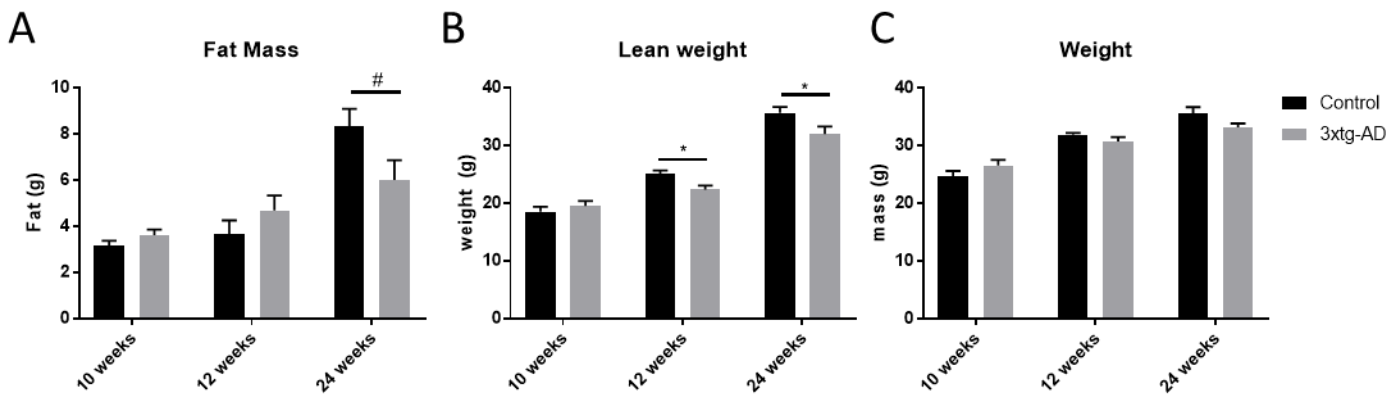

Fig 3. Body composition at different age points (10, 12, and 24 weeks).

3A. Fat mass at 10, 12, and 24 weeks between groups. 3B. Lean weight at 10, 12, and 24 weeks between groups. 3C. Total weight at 10, 12, and 24 weeks between groups. N = 4-6 per group. *p< 0.05 vs Control. Data represent mean ± SEM.

Figure 4 - mRNA expression of Hypothalamic Neuropeptides

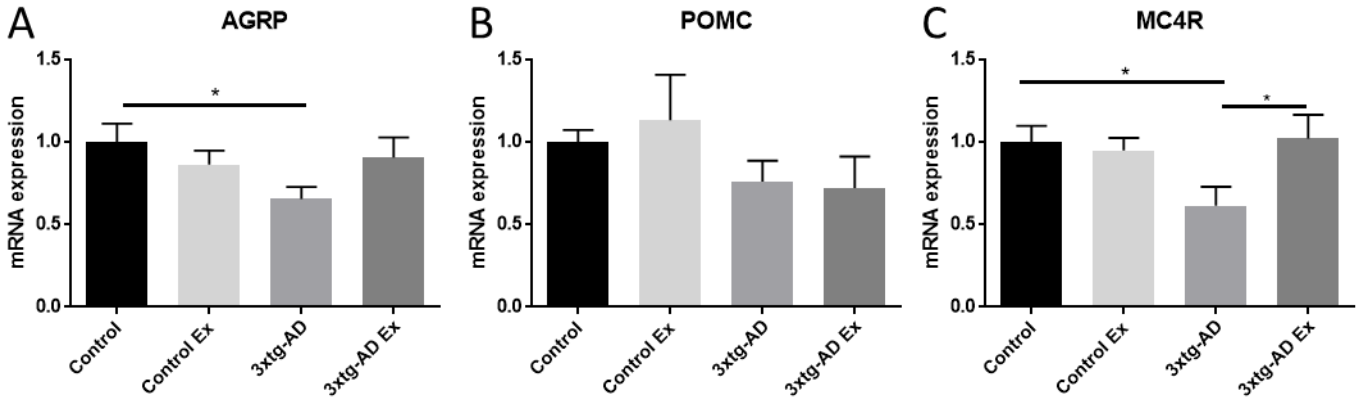

Fig 4. mRNA expression of key neuron groups in the hypothalamus at 12 weeks after a 4-week voluntary exercise treatment.

4A. mRNA expression of AgRP at 12 weeks between control, 3xtg-AD, and a 3xtg-AD group given a voluntary exercise treatment. 4B. mRNA expression of POMC at 12 weeks between control, 3xtg-AD, and a 3xtg-AD group given a voluntary exercise treatment. 4C. mRNA expression of MC4R at 12 weeks between control, 3xtg-AD, and a 3xtg-AD group given a voluntary exercise treatment. N = 3-4 per group, *$p < 0.05$ vs Control. Data represent mean ± SEM.

Figure 5 - mRNA Expression of Apoptotic/Inflammation Markers and Biogenesis in the Hypothalamus

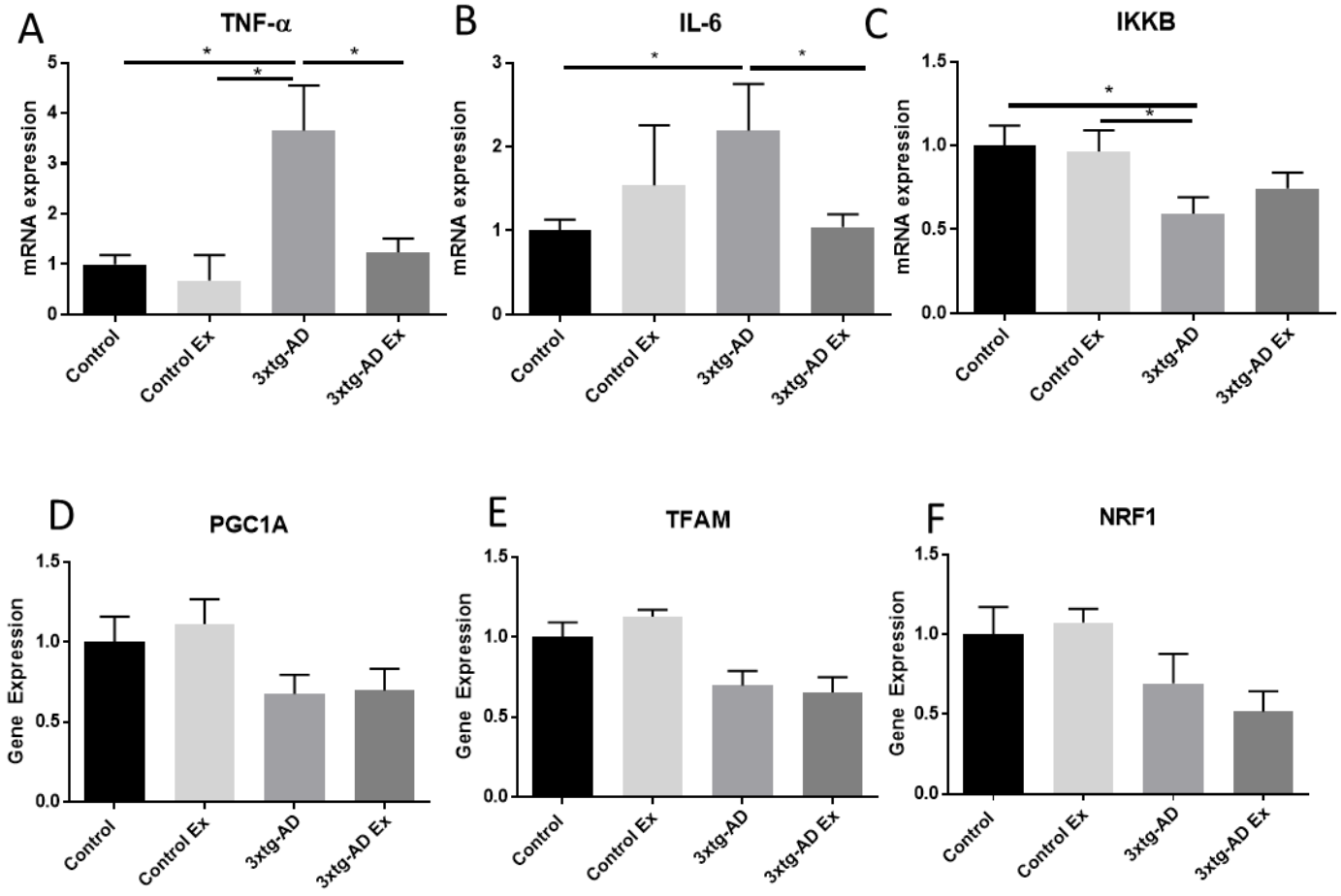

Fig 5. mRNA expression of apoptotic/ inflammation and mitochondrial biogenesis biomarkers in the hypothalamus at 12 weeks after a 4-week voluntary exercise treatment.

5A. mRNA expression of apoptotic biomarker, TNF-α, at 12 weeks after a voluntary exercise treatment. 5B. mRNA expression of inflammation biomarker, IL-6, at 12 weeks after a voluntary exercise treatment. 5C. mRNA expression of inflammation and apoptosis biomarker, IKKB, at 12 weeks after a voluntary exercise treatment. 5D. There was no difference between groups with respect to PGC1A. 5E. There was no difference between groups observed in TFAM. 5F. There was no difference between groups observed in NRF1. N = 3-4 per group,

Figure 6 - POMC Neuron Populations at Different Time Points

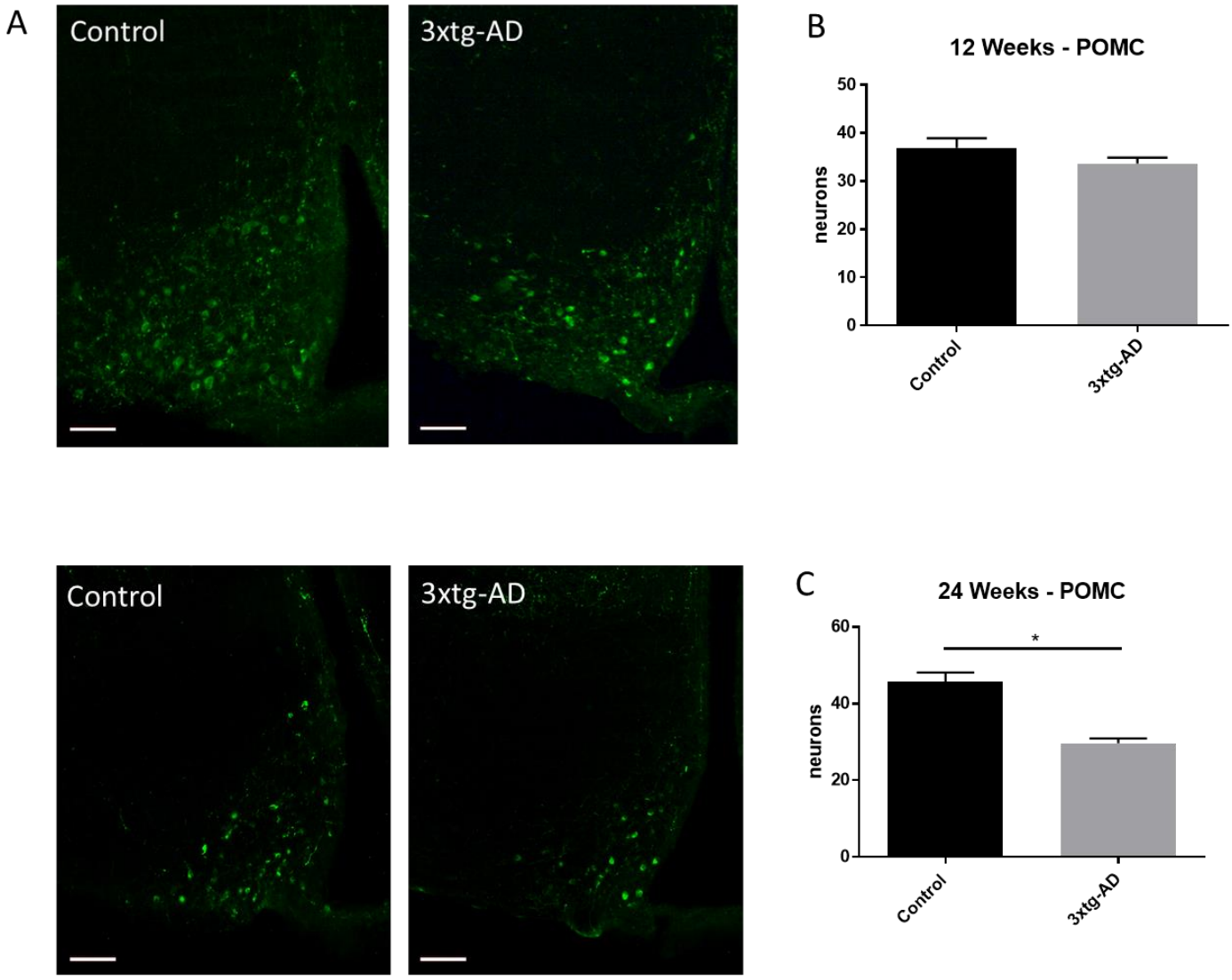

Fig 6. POMC neuron populations at time points 12 and 24 weeks between 3xtg-AD and control. All scale bars set at 200 microns. Images taken at 10x objective.

6A. Representative image of POMC neuron populations at 12 (top) and 24 (bottom) weeks between control and 3xtg-AD group. 6B. Quantification of POMC neuron population at 12 weeks between control and 3xtg-AD group. 6C. Quantification of POMC neuron population at

24 weeks between control and 3xtg-AD group. N = 4-6 per group, *p< 0.05 vs Control. Data represent mean ± SEM.

Figure 7 - POMC Neuron Populations after Exercise Treatment

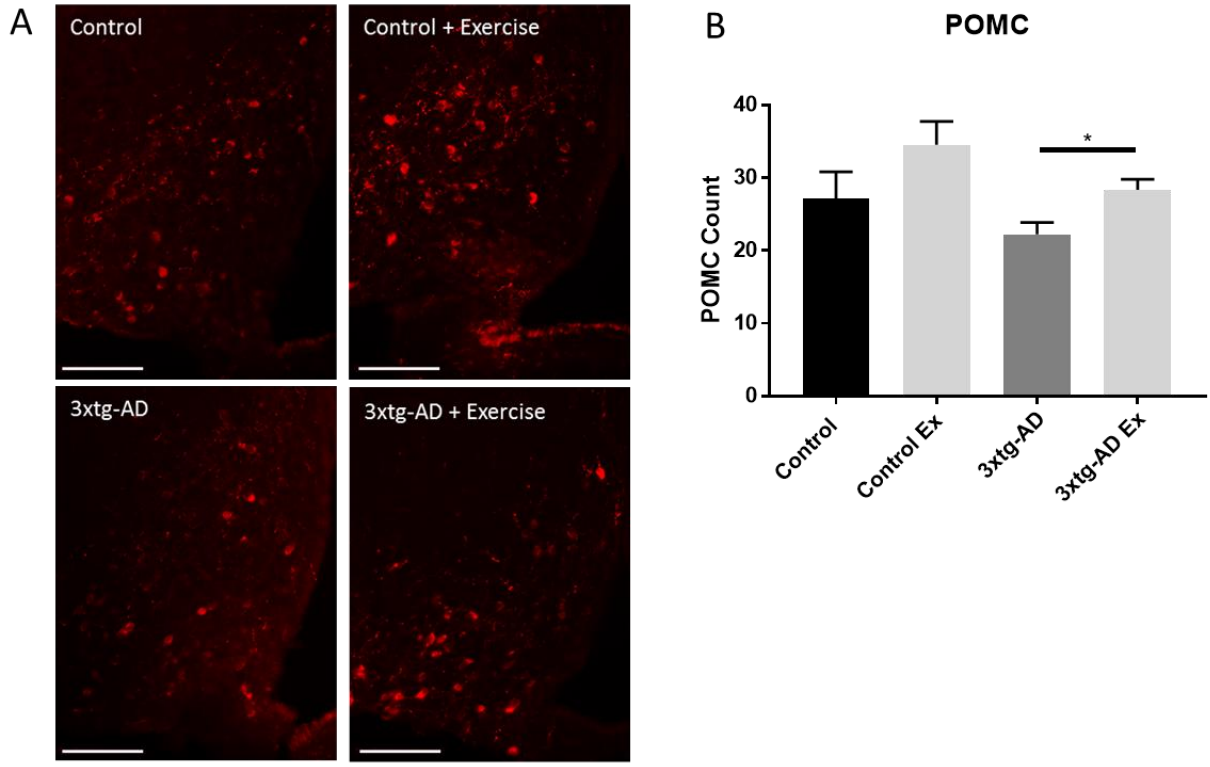

Fig 7. POMC neuron populations after an 8-week voluntary exercise treatment between control and 3xtg-AD. All scale bars set at 200 microns. Images taken at 20x objective.

7A. Representation of POMC neuron population after 8 weeks of voluntary exercise between control, 3xtg-AD, and 3xt-AD group with exercise. 7B. Quantification of POMC neuron populations after 8 weeks of voluntary exercise treatment. N = 3-5 per group, *p< 0.05 vs Control. Data represent mean ± SEM.

Figure 8 - NPY Neuron Populations after Exercise Treatment

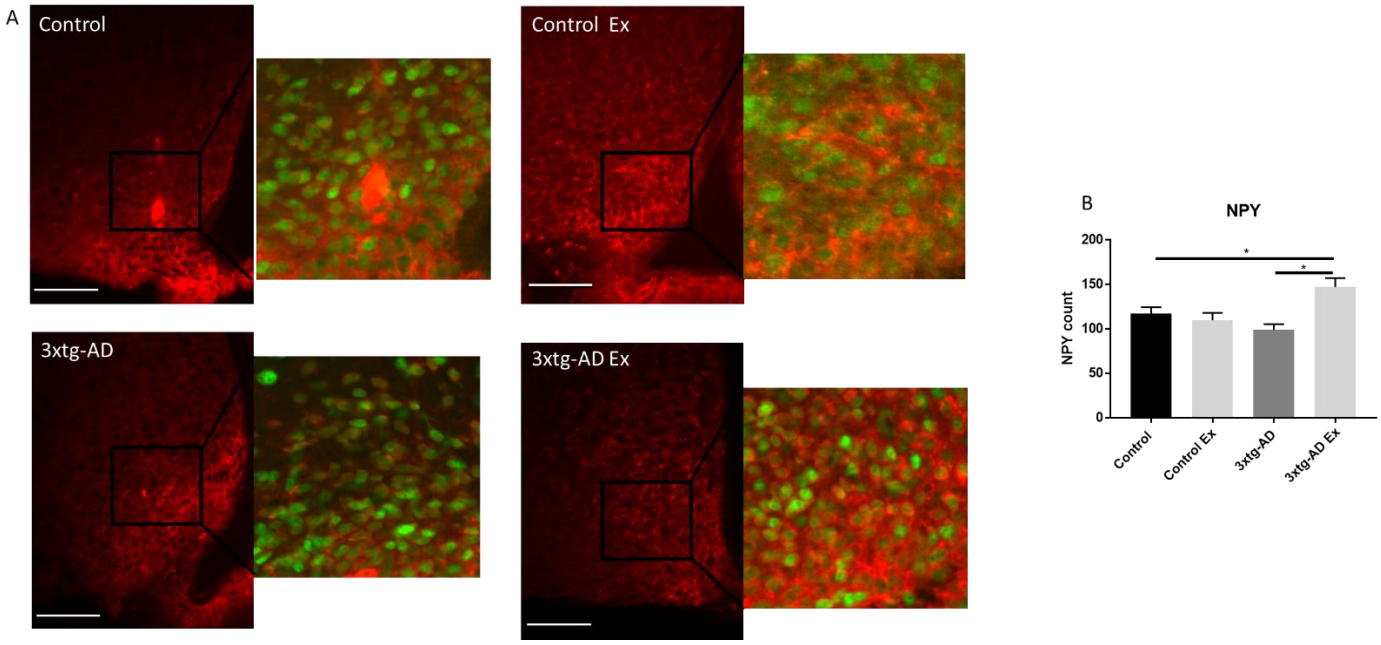

Fig 8. NPY neuron population after 8 weeks of voluntary exercise between control and 3xtg-AD group. All scale bars set at 200 microns. Images taken at 20x objective.

8A. Representation of NPY neuron population after 8 weeks of voluntary exercise between control, 3xtg-AD group, and 3xt-AD group with exercise. Quantification of NPY neurons after 8 weeks of voluntary exercise treatment. 8B. Representative image of NPY neuron co-localization.

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Appendix A

East Carolina University

Animal Care and
Use Commitee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

October 18, 2016

Hu Huang, Ph.D.
Department of Kinesiology
ECDOI
ECU Brody School of Medicine

Dear Dr. Huang:

Your Animal Use Protocol entitled, "Central Nervous System Control of Metabolism Responses to Exercise and Diet – Experiments" (AUP #P085a) was reviewed by this institution's Animal Care and Use Committee on October 18, 2016. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834

252-744-2436 office
252-744-2355 fax

MEMORANDUM

TO: Khoa Do
Department of Kinesiology

FROM: Dorcas O'Rourke, D.V.M.
University Veterinarian

SUBJECT: Certificate of Training - Experimental Surgery

DATE : March 27, 2015

This letter is provided to certify that you have participated in Species Specific Skills Training: Aseptic Surgical Techniques in Stereotaxic Procedures on 3/25/15.

Training was provided in accordance with the provisions of regulations of the U.S. Department of Agriculture (9 CFR 2.32) and the Policy and Guidelines of the National Institutes of Health. The training was provided by the East Carolina University Department of Comparative Medicine.

We suggest that you retain this letter in your training file for future reference.