Kiran Chaudhari, Antioxidants, Exercise, *APOE* Genotype and Brain Function. Doctor of Philosophy (Biomedical Sciences), November, 2014, 183 pages, 30 illustrations, 71 titles.

Apolipoprotein E4 (*APOE*4) is a well-established and extensively prevalent genetic risk factor for the development of Alzheimer's disease (AD). The presence of *APOE*4 allele accelerates the pathophysiology and symptomology of AD. A large set (36%) of the population suffering from AD expresses *APOE*4.

Being a chronic progressive disease with very few pharmaco-therapeutic agents approved by FDA, non-drug lifestyle modifications have been an important part of management of AD. People often eat healthy diet rich in antioxidants and focus on healthy living habits such as exercise. Health care providers frequently suggest combining antioxidants with physical activity for higher benefits. Antioxidants have been beneficial in counteracting oxidative stress and improving learning and memory. Similarly, different regimens of exercise also improved cognition and delayed development of AD.

However, the nature of the interaction between antioxidants and exercise remain elusive and complicated. While some studies reported additive effects, others have also shown a concerning antagonistic action of the antioxidants on the beneficial effects of exercise. In the context of *APOE* genotype, we set our study to determine the nature of such interaction between antioxidants and exercise. Using vitamins C and E and a treadmill-based forced exercise in a genetically modified mouse model expressing human *APOE*3 and *APOE*4 (GFAP-*APOE*3, GFAP-*APOE*4), we explored the nature of that interaction on functional and biochemical outcomes. We examined the mice for spatial learning and

memory, working memory and executive function, coordinated running performance, muscular reflexes, spontaneous locomotor activity, anxiety and muscle strength. Interestingly, we observed that the young adult mice expressing *E*4 allele performed better on higher brain functions including spatial learning and memory and short term memory in contrast to middle age mice, which developed a cognitive deficit as expected. Motor functions, reflexes and coordination were poor among all the mice carrying *E*4 allele irrespective of age. Antioxidants and exercise interventions led to outcomes that were dependent on genotype, age and the brain function under consideration. There was additive beneficial effect of combination of antioxidants and exercise on cognitive outcomes but not on motor outcomes in middle age groups. However, in young adults, an antagonistic interaction was observed on motor outcomes but no such interaction was observed on cognitive outcomes.

Hence we can conclude that, combination of antioxidants and exercise is not a “fit for all” approach and needs to be tailored base on individual’s age and genotype.

ANTIOXIDANTS, EXERCISE, *APOE* GENOTYPE AND BRAIN FUNCTION

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ANTIOXIDANTS, EXERCISE, *APOE* GENOTYPE AND BRAIN FUNCTION

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

For the Degree of

DOCTOR OF PHILOSOPHY

By

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Fort Worth, Texas

November, 2014

ACKNOWLEDGMENTS

I would like to express my utmost gratitude and respect to my outstanding mentor Dr. Nathalie Sumien. I thank her for her guidance, patience, generosity, encouragement and support I received from her during my time as her student. She will always be one of my role models with her noticeable traits like honesty, sincerity, hard work, approachability and the most, reliability. I would like to extend my gratitude to my committee members Dr. Michael Forster, Dr. Eric Gonzales, Dr. Robert Luedtke and Dr. Jerry Simecka for their kind support, valuable time and the knowledge they shared with me.

I thank my colleagues from Dr. Sumien' s lab, Jessica Wong, Philip Vann, Dr. Samuel Ikonne, Akram Sidhu, James Mock, Haydee Izurieta Munoz and Robbyn Kindle for their friendship and encouragement. I would also like to thank Dr. Ritu Shetty, Dr. Nopporn Thangthaeng and Dr. Margaret Rutledge for their wonderful suggestions and guidance. I thank Dr. Gourav Roy Choudhury, Ali Winters and Dr. Yang for their help and support.

I would like to thank my wonderful family, my wife, Sarika Chaudhari her patience, understanding and love for me and my daughter, Shourya Chaudhari for making every single day special for me. They were, are and always will be my biggest strength and weakness.

I thank everyone in the Department of Pharmacology & Neuroscience and University of North Texas Health Science Center for helping me achieve my goals and for making my experience as a student, a memorable one.

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INTRODUCTION

Alzheimer's disease

Alzheimer's disease (AD) is a major neurodegenerative disorder commonly starting after the age of 65 years. Aging is one of the highest risk factor for AD, which is supported by the fact that, after the age of 65 years, the incidence of AD doubles every 5 years (1). This irreversible and progressive brain disease initiates gradually with cognitive decline, loss of thinking and reasoning abilities eventually leading to an inability to perform simple daily activities. Currently, AD affects 5.2 million American people, including 200,000 individuals younger than 65 years of age suffering from early-onset Alzheimer's disease. In the near future, with the baby boomers reaching the age of 65 and beyond, the estimated rise in the number of people with AD may triple from 5.2 million to 16 million by 2050 (2).

The mortality due to AD is approximately 500,000 per year, making AD the 6^{th} leading cause of death among high income countries and 5^{th} leading cause of death in elderly >65 years of age in the United States. This occurred due to the medical advancement and improvement in the quality and span of life, that led to an observable reduced mortality from other major

diseases, and therefore, indirectly increasing the prevalence of AD and rise in concurrent death up to 68% between 2000 and 2010 (3).

Furthermore, the slow nature of disease progression requires long-term management of AD patients, giving rise to huge socioeconomic impacts. In 2013, 15.5 million caregivers expended 17.7 billion unpaid hours, which was approximately equal to $220.2 billion in care value. Among the leading diseases, AD is the most expensive medical condition in the United States with estimated cost of care around $214 million for the year 2014 and escalating towards 1.2 trillion dollars by 2050. In addition to this, a neglected socioeconomic impact involves the health of the caregivers themselves costing about $9.3 billion. This socioeconomic burden compels researchers to focus on discoveries of effective and economical management options for AD patients in coming years (4).

Interestingly, women are more commonly affected by the disease and comprise 66.66% (3.2 million) of the AD population compared to men (1.8million). This puts 1 in every 6 women compared to 1 in every 11 men at the lifetime risk of developing AD at age 65. Furthermore, women are the largest population involved in care giving too. Currently, 3 in 5 unpaid AD caregivers are women, including as many as 2.5 times than men in 24 hour services (3). Hence we need to focus on the risk factors that predispose women to AD.

The pathophysiology of AD involves risk factors like aging, specific genotypes, immune-inflammatory reactions and environmental factors including but not limited to the level of education, brain trauma and oxidative stress (5,6). Many hypotheses have arisen from the various pathophysiology associated with AD: (1) genotype influence: amyloid precursor protein (APP), presenilin 1, presenilin 2 and apolipoprotein E (*APOE*), (2) oxidative stress, (3) inflammation, (4) cell cycle disturbance, and (5) hyperphosphorylation of Tau proteins and amyloid-β (Aβ)

peptides metabolism. Extracellular deposition of Aβ forming senile plaques is considered the most common pathology in AD, also known as the amyloid cascade hypothesis (7,8). Our focus is on the largest subset of the AD population and the major genetic risk factor for sporadic AD: Apolipoprotein E.

Apolipoprotein E in AD

Apolipoprotein E (*APOE*) transports lipids through the lymphatic and into the blood for lipid homeostasis, repairing neurons, synaptic connections, and toxin removal in the brain. APOE is produced primarily by astrocytes with the function to transport cholesterol to the neurons, which have APOE receptors. In humans, a single nucleotide polymorphism (SNP) at either location 112 or 158 or both leads to 3 distinct alleles: ε2, ε3 and ε4. APOE3 has Cys-112 and Arg-158, APOE4 has Arg-112 and Arg-158 and APOE2 has Cys-112 and Cys-158. The alleles have exhibited different properties: *APOE*2 has been established as the neuroprotective allele whereas the presence of *APOE*4 is a risk factor for development of cognitive decline in late onset familial and sporadic Alzheimer's disease (9,10), early-onset AD as well as amyloid deposition, plaque formation in cognitively normal aging brain (11,12).

Among all genetic risk factors, *APOE*4 is over-represented and strongly related to early - onset AD among population of different racial origin (13). Furthermore, *APOE*4 has been associated with an acceleration of the pathophysiology and cognitive decline in an allele dose dependent manner with the lowest at 0 alleles of *APOE*4 and highest at 2 alleles of *APOE*4. Similarly, *APOE*4 contributes to the exacerbation of β-amyloid deposition in AD (14) as well as in healthy aging brain (15). This effect on AD pathophysiology in the presence of *APOE*4 could be due to increased oxidative stress (16).

When compared to *APOE*2 and *APOE*3, *APOE*4 is more susceptible to oxidative stress, forming neuron-specific toxic C-terminal fragment (17). Various Aβ forms bind to APOE and undergo structural changes leading to toxicity and deposition in an isoform-specific (APOE4 > APOE3 > APOE2) manner (18). Furthermore, the proteolytic degradation of Aβ via modulating Aβ degrading enzyme activity is also APOE isoform dependent (18).

Considering the prolonged course of disease progression and vast genetic variation among humans, various rodent models have been developed and extensively studied for understanding the mechanisms involved in pathogenesis of AD. Unlike humans, the animals used for experimental purposes do not have *APOE* 2, 3, 4 alleles, thereby humanized transgenic mouse model expressing human *APOE* alleles under either a mouse promoter (targeted replacement, TR-*APOE*) (19), or a human neuron specific enolase promoter (NSE-*APOE*) (20), or a human glial fibrillary acidic protein promoter (GFAP-*APOE*) (21) have been developed as unique tools to study the cellular source-dependent roles of human *APOE* isoforms in neurobiology and in the pathogenesis of AD (22). It is noteworthy that, in the brain, astrocytes are the major source of APOE (23), while neurons contribute only a minute amount of APOE (24).

One hypothesis that arose to explain why the presence of *APOE4* leads to increased risk of developing AD is an increase in oxidative stress. This is supported by the association of oxidative stress and the presence of *APOE*4 in healthy humans as well as AD patients. In AD patients' hippocampus, *APOE*4 was associated with an imbalance between oxidants and antioxidant defenses (25). In healthy subjects carrying the *APOE*4 allele, higher levels of isoprostanes in CSF were found compared to non-*APOE*4 carriers (26). Furthermore, the antioxidant activity of APOE protein is higher with APOE2 than APOE3 than APOE4 (27).

Oxidative stress, AD and cognitive dysfunction

Oxidative stress and AD

Over the last decade, accrual of oxidative stress has been projected as a pathogenic factor that appears earlier than the other pathogenic mechanisms in AD (28); which is supported by studies in transgenic rodent models exhibiting an increase in oxidative stress markers prior to Aβ accumulation (29). The central nervous system has a very high metabolic turnover with 20% of total body oxygen consumption and 25% of total body glucose consumption (30) making it more vulnerable to oxidative stress and damage. This is exacerbated by the presence of radical-susceptible polyunsaturated fatty acids (PUFA) in the brain (31). The inadequate antioxidant defense levels in the brain promote the sensitivity of neurons to reactive oxygen species (ROS) (32). This is further worsened by higher content of redox active/sensitive metals like iron, which can promote the formation of ROS (33)

Oxidative stress has been implicated as an important pathogenic factor in AD. During the early stages of AD, brain regions showed higher levels of lipid peroxidation markers like 4-hydroxyhexenal (34), F2-isoprostane and F4-neuroprostane (35) when compared to controls. Similarly, higher levels of 8-OHG (measure of RNA oxidation) were observed in the hippocampus and temporal lobe in the early stage of AD (36). Interestingly, mitochondrial DNA oxidation occurred at higher level than oxidation of nuclear DNA (37). Protein carbonyls and 3-nitrotyrosine (3-NT) were also higher in frontal cortex of individuals with mild cognitive impairments (38),(39).

Oxidative stress and cognition in AD

The next interesting question will be identifying the association of oxidative stress and functional decline in AD. Mitochondrial H_2O_2 levels were positively associated with cognitive

decline in AD transgenic mouse model; supported by the underlying mitogen-activated protein kinase pathway alteration (40). This was also observed in humans, as oxidative stress was found to mediate mild cognitive impairments (41). The oxidative stress hypothesis underlies studies on the utilization of antioxidants to slow the progression of AD and improve the functional outcome. Antioxidants, endogenous and exogenous, have been vastly used and studied for the cognitive benefits in AD. Vitamin E, vitamin C and β-carotene are exogenous antioxidants that act through chain breaking mechanism and decreasing free-radical-mediated damage and help to inhibit dementia pathogenesis in mammalian cells (42). Other antioxidants include metal chelators, glutathione peroxidases (GPx), and superoxide dismutase (SOD) enzymes, cytoplasmic Cu-,Zn-SOD (43) and a mitochondrial Mn-SOD, repair enzymes such as lipases, proteases, and DNA repair enzymes (44) providing neuronal protection against oxidative damages (45). The focus of our research was on vitamin C and vitamin E as they are two of the most commonly consumed antioxidants.

AD management

The progressive nature of AD and the lack of complete understanding of the pathophysiology, had led to the development of very few drugs to tackle this problem. None of these currently FDA-approved drugs can treat AD in its entirety, but they do slow down the progression of some of the signs and symptoms of AD. The most common part of any management protocol designed for AD patients include lifestyle modifications, which includes health eating habits with diets rich in antioxidants, vitamins, and micro-nutrients. In this study we focus on dietary supplements rich in antioxidant like vitamins C and vitamin E.

Vitamin C in AD

Ascorbic acid (AA; Vitamin C) is a strong water-soluble antioxidant. Vitamin C is a major player in various metabolic reactions and normal physiological functioning of brain among animals and humans. Unlike mice and some rodents, humans cannot synthesize vitamin C due to lack of enzyme gulonolactone oxidase (46) and are therefore dependent on dietary sources and "carrier" transport across the gut and the blood brain barrier to meet the demands of the brain. Regardless of this, the brain and the CSF have higher vitamin C concentration than other organs and plasma due to the Na-dependent saturable active transporter, sodium dependent vitamin C transporter 2 (SVCT2) present at the choroid plexus (47,48). This active transport builds a 4 fold vitamin C plasma gradient in rats (200-400 μmol/L in CSF, 60 μmol/L in plasma) and 3 fold in human (160 μmol/L in CSF, 40 to 60 μmol/L in plasma)(49). Once in the CSF, vitamin C diffuses into brain interstitium, and is then transported to neurons and glia by SVCT2 located on the membranes and via GLUT transporter when it has been oxidized into dehydroascorbate (DHA). This DHA is then reduced back to vitamin C within the cells. Interestingly, neurons use both SVCT2 and GLUT transporters, while astrocytes do not have SVCT2 and use solely the GLUT pathway (50,51). Furthermore, there is evidence that DHA also crosses the blood brain barrier using GLUT1 (52). All these transport capabilities support the usefulness of vitamin C-dietary model as an intervention capable of reaching the target organ: the brain.

Vitamin C is accumulated and retained among the cerebellum, hippocampus and cortex during acute, short-term inadequate synthesis or intake depletion at the cost of other organs using SVCT2 (53). However, during the process of long term/chronic deficiency the brain is unable to maintain the requisite levels, which could be an important factor in aging and neurodegenerative diseases.

In the brain, vitamin C is one of the first lines of antioxidant defense that detoxifies free radicals in extracellular fluid and cytosol (54). Vitamin C neutralizes a variety of ROS by donating one electron and being converted to semidehydroascorbate or ascorbyl radical; a relatively stable compound. DHA can be recycled back to its antioxidative form, ascorbic acid, by the cells.(55) Vitamin C also functions as an antioxidant indirectly by having a role in the recycling of oxidized vitamin E and glutathione (GSH) (56). Interestingly, GSH is also involved in reducing semidehydroascorbate to ascorbic acid if the latter is involved as the first response to oxidative stress (56). Furthermore, vitamin C also has the ability to inhibit LDL oxidation (57), recycle endothelial nitric oxide synthase (eNOS) cofactor, tetrahydrobiopterin, and therefore alter arterial elasticity and blood pressure (58). Because of these properties, vitamin C is a prime candidate for therapeutic effects by lowering oxidative stress, and thereby improving brain function.

In AD, vitamin C plasma levels were found to be low irrespective of dietary intake when compared to normal subjects. Interestingly, higher accumulation of vitamin C was observed in AD brain with the CSF to plasma ratio 5:1 when compared to 3:1 in the control after vitamin C supplementation (59). This can be explained as the AD brain has a higher need and higher consumption of vitamin C and will therefore drain the pool of vitamin C present in the plasma. There are mixed reports on therapeutic effect of vitamin C in AD. Vitamin C administration in amyloid precursor protein / presenilin protein 1 (APP/PSEN1) transgenic mice did not ameliorate AD-like neuropathy or reduce oxidative stress, but did benefit cognitive function (60) indicating its nootropic effect. However, other studies have indicated benefits of vitamin C supplementation on oxidative stress and proinflammatory cytokines in hippocampus (61). Further, vitamin C has been reported to prevent apoptosis and cell death due to β-amyloid (62). Despite mixed results,

most studies support vitamin C as a good candidate for therapeutic effects on cognitive function in AD.

Vitamin E in AD

Vitamin E is a powerful, lipid-soluble chain-breaking antioxidant. It resides in the lipid bilayer, circulating lipoproteins and low-density lipoprotein (LDL) particles (63). Among the various isoforms of vitamin E, α-tocopherol is preferentially absorbed and stored in humans (64). Vitamin E supplementation decreased amyloid toxicity along with improved cognition in rats as well as humans (65,66). Interestingly, vitamin E exerts a preventive role in terms of lowering lipid peroxidation and diminishing Aβ levels and senile plaque deposition when administered prior to the initiation of AD pathology; however, when supplemented after the formation of amyloid plaques, vitamin E reduced oxidative stress, but did not reduce the plaques (67). Alpha-tocopherol suppressed and/or delayed tau pathology, improved health and motor function and decreased carbonyls and 8-OHdG in transgenic mouse (68). In Drosophila, α-tocopherol suppressed tau-induced neurotoxicity (69). This is supported with increased longevity in human AD subjects on vitamin E supplementation compared to those taking no drug or a choline esterase inhibitor alone (70). Vitamin E in the form of α-tocopherol (2,000 IU a day) reduced neuronal damage and decelerated progression of moderate AD, predicting therapeutic benefit of α-tocopherol in slowing worsening in AD patients.

Combination of vitamin C and vitamin E

Vitamin C and vitamin E target aqueous and lipophilic compartments of the body respectively. Therefore, combining vitamin C and vitamin E could have a more pronounced antioxidant effect by covering both compartments. In addition, vitamin C has been shown to be involved in the regeneration of vitamin E from its oxidized form. Interestingly, vitamin C and

vitamin E interact synergistically against lipid peroxidation (71,72) in AD patients (73). In the context of *APOE4* allele, when combined with non-steroidal anti-inflammatory drugs, this combination of vitamin C and vitamin E was able to lower cognitive decline and lower risk for AD in elderly population (74). Hence the combination of vitamin C and vitamin E could be the best nutritional option for prevention and treatment of oxidative stress in AD. Furthermore, preliminary data from our laboratory have shown a potential synergistic antioxidant effect of vitamin C and vitamin E against the cognitive decline in aging C57BL/6 mice.

Exercise in AD

Lately, exercise has proven to have beneficial effects on brain function (75) supported by improved markers of cellular aging (76). Exercise is one of the major interventions that have been constantly proven to improve cognitive performance in adults and older individuals. Different forms of exercise (single episode, short term acute bouts, longer term from 3- to 6-month) interventions have been associated with improved simple reaction time and creative thinking in young adults (77), reallocation of attention and memory resources towards executive functioning (78), increased gray and white matter in brain regions (MRI scan in older adults 60-79 years) (79). In humans, increased brain volume in various regions of the brain has been used as an indicator relating to improved cognitive performance as an effect of exercise (80,81). Interestingly, these exercise associated cognitive benefits are more pronounced in presence of APOE4 allele (82,83).

The molecular mechanisms underlying the beneficial effects of exercise on cognition in AD and other conditions are ambiguous. One of the proposed mechanisms for such beneficial effect is by lowering oxidative stress. Exercise-induced cognitive improvement was associated with reduced oxidative stress due to reduced ROS production (84-86), decreased hydroxyl

radical generation (significant in plasma and trend in brain) (87), and increased levels/activity of antioxidant enzymes (86,88-90) in rats. Interestingly, exercise lowered lipid peroxidation and protein oxidation products, but did not affect DNA oxidation product, 8-OHdG and 8-oxoguanine-DNA glycosylase (OGG1) activity (84).

Another mechanism of exercise-induced beneficial effect of brain function could be via an anti-inflammatory effect of exercise. Inflammatory biomarkers are clinically associated with functional and pathological development of AD. Measured in either plasma, cerebrospinal fluid or directly in the brain regions, interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1β, tissue necrosis factor α (TNF-α) and keratinocyte-derived cytokine/ growth regulated oncogene (KC/GRO) have been related to AD (91). Exercise lowered the levels of interleukin-6 (IL-6) and tissue necrosis factor α (TNF-α), pro-inflammatory cytokine markers, in mild cognitive impairment (MCI) subjects (92) along with lowering of KC/GRO in runners (93) . Hence exercise might be helpful to lower the peripheral levels of IL-6 (from peripheral blood mononuclear cells) which has been higher in AD compared healthy adults (94).

Another aspect of exercise-induced functional improvements that has been well described in AD and dementia conditions is its effect on neurotrophic factors. Exercise induces a variety of neurotrophins including but not limited to brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell line-derived neurotrophic factor (GDNF). Exercise with moderate to high intensity up-regulates BDNF production (95,96). These exercise-initiated benefits on cognition and BDNF are more pronounced, especially in the presence of *APOE4* genotype (97). Similar to BDNF, exercise induced GDNF which has been shown to be involved in cell survival and neuronal plasticity, and neuroprotective effects (98). Treadmill exercise

training significantly attenuated the age-associated decrease in BDNF and NGF level modulating long term potentiation (LTP).

Combination of Vitamin E, C and / or Exercise

Very often, health conscious individuals focus on physical activity and eating a healthy diet rich in vitamin, mineral and other micro/macro nutrients (that includes antioxidant vitamins). Vitamin C and vitamin E are antioxidant vitamins that are part of such healthy diet accompanying exercise. Previously a number of studies were done to identify the interaction of antioxidants and exercise in various contexts. Interestingly the outcome was never repetitive, but context dependent. Nalbant *et.al*. (2009) observed that 6 months of aerobic exercise improved indices of physical performance and body composition in older adults (71.5+/-7.5 years) when compared to sedentary controls; while the addition of Vitamin E supplementation to this exercise regimen did not have any additive effect (100). When an elderly population (71+/-2 years) with mild hypertension underwent exercise in addition to being supplemented with a combination of antioxidants (vitamins C and E, and alpha-lipoic acid), the overall effect of this combination was antagonistic and deleterious for cardiovascular health nullifying the benefits from exercise alone (101). Similarly combination of antioxidants (vitamin C and vitamin E) blunted the exercise-induced beneficial effects on insulin sensitivity in young adults (25-35years) (102). Improved cognitive outcome in old adults (60-85 years) after exercise was not further enhanced when supplementation with vitamin E (900IU/day) was added (103). Interestingly, when studied in rodents, DHA diet enhanced exercise-associated improvement in cognition and BDNF-related synaptic plasticity (104).

Functional assessment of mice

While previous studies have tested similar mouse model using a few behavioral test, this study will be the first to provide a comprehensive behavioral phenotype of the GFAP-APOE mice. The battery will not only study different domains of cognitive function but also aspects of affective and motor function that could bias the outcomes on cognitive function. While previous studies have shown moderate effects of the APOE4 allele on aspects of spatial memory (105), the results may have been biased by running the visible platform test prior to the Morris water maze test, and more difficult aspect of learning were not studied. The current study will present a more representative picture of what the actual phenotype of these mice are, and a more specific picture as to which aspects of motor and cognitive functions are affected by the presence of APOE4. All our protocols have been enhanced to provide reliable and stable data under varying conditions such as diet and genotype. These tests have proven sensitive to interventions with antioxidant supplementation and exercise in a mouse model of aging.

The mice were tested for spontaneous activity, coordination, strength, balance, musculoskeletal reflexes, spatial learning and memory, discriminative learning and cognitive flexibility, and anxiety assessment. Unlike previous studies, cognitive tests will be focused on hippocampal function (Morris Water Maze: spatial discrimination) with visible platform being tested after to avoid confounding effect on the water maze, and cortical function (active avoidance: T-maze), allowing us to study two different domains. The complete battery of behavioral tests to be used in this project has been detailed in several publications from our group (106-110).

Goals of the current research

Antioxidant supplementation and exercise are two interventions that have shown promise individually in reducing cognitive and motor dysfunction associated with aging and

neurodegenerative diseases. Based on the premise that more is better, health conscious individuals as well as physicians have been combining these two interventions in the anticipation of additive beneficial effects. However, recent studies and preliminary work in our laboratory had shown that there is a potential for a negative interaction of antioxidants and exercise. In these studies, the beneficial effects of exercise on markers of diabetes (102) or oxidative stress (unpublished data) have been antagonized when antioxidants were added to the treatment. It is important to point out that these studies were completed in young subjects/animals, and that there might be an environmental factor that influences the outcome of the combination of the two interventions. In the presence of *APOE*4 genotype, with higher oxidative stress and higher cognitive impairment, the interaction of antioxidants and exercise has never been studied. The focus of our research was to identify the nature of such interaction in context of cognition, motor function, *APOE* genotype, age and sex of the mice. The working hypothesis of this major study is '***antioxidant and exercise will reduce APOE4-associated declines in cognitive function to a larger extent that each intervention alone***'.

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CHAPTER 2

EXERCISE TRAINING AND ANTIOXIDANT SUPPLEMENTATION INDEPENDENTLY IMPROVE COGNITIVE FUNCTION IN ADULT MALE AND FEMALE GFAP-*APOE* MICE

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Running title: Cognitive effects of exercise and antioxidant intake

Published in Journal of Sport and Health Science, Volume 3, Issue 3, September 2014, Pages 196-205

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ABSTRACT

The purpose of this study was to determine if antioxidant supplementation, moderate exercise, and the combination of both treatments could ameliorate cognitive performance in adult mice and whether the apolipoprotein E (*APOE*) genotype as well as sex could influence the functional outcomes of the treatments.

For a period of 16 weeks, separate groups of male and female mice expressing either the human *APOE*3 or *APOE*4 isoforms were fed either a control diet (NIH-31) or the control diet supplemented with vitamins E and C (1.12 IU/g diet α-tocopheryl acetate and 1.65 mg/g ascorbic acid). The mice were further separated into a sedentary group or a group that followed a daily exercise regimen. After 8 weeks on the treatments, the mice were administered a battery of functional tests including tests to measure cognitive and affective function.

There was no effect of genotype or treatment on the learning performance in the Morris water maze. In the discriminated avoidance task, *APOE*4 mice performed better in learning the discrimination component of the task. Overall, exercise improved performance of *APOE*4 and *APOE*3 mice on various aspects of the active avoidance task. Antioxidant supplementation improved performance only in the *APOE*4 mice. On the test for anxiety, *APOE*4 mice spent more time in the open arms and supplementation with antioxidant reversed that effect.

Exercise was the most effective treatment at improving cognitive function in both genotypes and sex, while antioxidants seemed to be effective only in the *APOE*4. In young adult mice only non-spatial learning and memory were improved. The combination of the two treatments did not yield further improvement in cognition, and there was no antagonistic action of the antioxidant supplementation on the beneficial effects of exercise.

INTRODUCTION

Apolipoprotein E (*APOE*) is a soluble protein and an integral part of the lipid transport and distribution system (1). In humans, there exist three alleles coding for the three major isoforms of *APOE*: E2, E3, and E4. In the central nervous system, *APOE* has an important role in neurogenesis and neuroprotection (2). The most commonly found isoform is the *APOE*3, present in 79% of the population, while the *APOE*2 and E4 are lower with 14% and 7% presence, respectively. Although not a determinant of the disease, the *APOE*4 presence has been established as a major genetic risk factor for development of late-onset sporadic Alzheimer's disease (AD) (3-5). *APOE*4 has also been associated with exacerbated cognitive declines during non-pathological non-AD dementia (6-8).

While there remains some controversy, it is believed that women expressing *APOE*4 have an increased risk of developing AD when compared to men (9, 10). Furthermore, *APOE*4 presence has been associated with cognitive declines during normal aging in women only (11). Many studies in rodents expressing human *APOE* genes have supported an interaction of sex and *APOE* genotype. Using different rodent models, females expressing human *APOE*4 had impaired spatial learning and memory, which seemed to be exacerbated when compared to males (2, 12).

In middle-aged individuals, *APOE*4 has been associated with cognitive deterioration and memory loss, (13, 14) however reports on differences within younger population are conflicted (15-18). While some studies have reported a lack of difference between *APOE*4 carriers and non-carriers, (17, 18) others have described a worsening associated with *APOE*4 (16). More interestingly others have related an improved cognitive performance associated with the presence of *APOE*4 (19-22). Studies in human *APOE*-expressing mouse models also remain inconclusive regarding the effect of *APOE*4 on cognitive function in young mice, however most studies hinted

at a better performance in young *APOE*4 mice compared to *APOE*3 (12, 23, 24). This type of antagonistic pleiotropy (age-dependent shift from beneficial to deleterious outcomes) associated with the *APOE* isoforms may also impact the outcome of interventions.

Human and animal studies have suggested that lifestyle factors may play an important role in preventing cognitive deterioration and dementia (25-34). *APOE*4 has been associated with an increase in oxidative stress levels, (35, 36) and oxidative stress has been associated with brain dysfunction (37). Therefore antioxidant intake should decrease *APOE*4-associated oxidative stress and improve cognitive function, an interaction that has been demonstrated in several studies (25, 38-40). As another factor, physical activity has been shown to reduce the risk of AD, (41-43) delay onset, (30) and improve AD symptoms in an activity intensity- (dose) and duration-dependent manner (44). Recent studies have established the existence of a potential interaction between *APOE* genotype and exercise on cognition. Most studies have reported that the beneficial effects of exercise are more pronounced in *APOE*4 carriers when compared to non-carriers, (45, 46) however one study reported the opposite (47). Exercise training has also been shown to lower oxidative stress while improving cognition (34, 48). Based on these studies and the potential existence of a common mechanism of action, it can be hypothesized that combining antioxidant with exercise training will lead to a synergistic or additive beneficial effect, (49-52) a therapeutic approach employed by many health conscious individuals and recommended by healthcare professionals. However, the occurrence of such outcome has not been fully established in relation to age, sex/gender, and genetic make-up. For example, a recent study demonstrated an antagonistic action of antioxidant supplementation on beneficial effects of exercise (53).

Even though, antioxidant intake and exercise training have been previously studied, there are no data available evaluating the effect of these factors in both sexes, in two genotypes in young adult mice within the same study. The goals of the current study were 1) to characterize the cognitive and anxiety phenotypes of the adult glial fibrillary acidic protein (GFAP)- *APOE*3 and *APOE*4 mice (human *APOE* expressed under a GFAP promoter); 2) to determine whether antioxidant intake and exercise training led to beneficial improvements in these young mice, same as previously reported in older ones; 3) to determine whether the combination of antioxidant and exercise yield a synergistic or additive beneficial effect; and lastly 4) to determine whether the beneficial outcomes are genotype-dependent.

MATERIAL AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center at Fort Worth. Separate groups of male and female GFAP-*APOE**3 (B6.Cg-Tg(GFAP-*APOE**3)37Hol *APOE*tm1Unc/J) and GFAP-*APOE**4 (B6.Cg-Tg(GFAP-*APOE**4)1Hol *APOE*tm1Unc/J) mice were obtained from Jackson Laboratories (catalog numbers 004633 and 004631; total n of 180) at the age of 2 months and subsequently maintained in the UNT Health Science Center vivarium. The mice were housed in groups of 3 or 4 in standard polycarbonate cages (28 × 17 × 12.5 cm) with corncob bedding and ad libitum access to food and water, and were maintained at ambient temperature (23 ± 1 °C), under a 12-h light/dark cycle starting at 06:00. The mice were weighed weekly, and survival was monitored throughout the study. A group of young (2 months, n = 12) male and female C57BL/6 mice (wild-type) was used as a control to compare the *APOE*3 and E4 controls to determine whether the behavioral differences between *APOE*3 and E4 were due to an altered phenotype of the transgenic mice.

Treatment

The mice were fed, ad libitum, either a control diet (LabDiet® R&M 5LG6 4F, cat #: 5S84) or the control diet supplemented with vitamins E and C (modified 5LG6 with1.65 mg/g diet of ascorbic acid and 1.12 IU/g diet of α-tocopheryl acetate, cat#: 5SH0). Furthermore, the mice were either sedentary or following a moderate exercise regimen. Based on this, the mice were randomly assigned to one of four experimental groups: (1) sedentary fed the control diet (SedCon), (2) sedentary fed the vitamins E and C supplemented diet (SedEC), (3) forced exercise fed the control diet (ExCon), (4) forced exercise fed the vitamins E and C supplemented diet

(ExEC). Each experimental group was balanced for sex of the mice. The moderate exercise regimen was introduced progressively using treadmills (AccuPacer Treadmill; Omnitech Electronics Inc., Columbus, OH, USA). Over a 12-day period, the training was gradually incremented in time and speed to reach a maximal exercise of 1 h (6, 8, 10, and 12 m/min for 5 min each, and then at 14 m/min for 40 min). The training protocol used was a modification of previously published exercise protocols (54). Forced exercise was implemented via transient 0.29 mA electric foot shock to the feet. Each exercise mouse was paired with a control which received the same number of shock for each training day.

The mice were on their respective treatments for 8 weeks prior to and throughout behavioral assessments for a total of 16 weeks. The mice received a series of behavioral tests and the results of the cognitive tests are presented in this manuscript. Morris water maze (MWM) and discriminated avoidance were used to measure different aspects of cognitive function. The mice were about 5–6 months old when tested for cognitive function.

MWM

Spatial learning and memory were measured using an MWM test slightly modified from described previously (55). On a given trial, the mouse was allowed to swim in a tank filled with opacified water and maintained at 24 ± 1 °C. The mice were able to escape the water by means of a hidden platform (1.5 cm below the surface of the water). A computerized tracking system recorded various measures such as path length and swimming speed (Any-maze; Stoelting Co., Wood Dale, IL, USA).

The test consisted of four phases: (1) pre-training phase: the tank was covered by a black curtain to hide surrounding visual cues. The mice learned the components of swimming and climbing onto a platform using a straight alley that had a platform at one end. The mice were

allowed to swim until they reached the platform or a maximum of 60 s had elapsed. The mice received two sessions consisting of five trials with an intertrial interval of 5 min; (2) acquisition phase: the black curtain was removed and the mice were tested for their ability to locate a hidden platform using spatial cues around the room. Each daily session consisted of five trials, at 2-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. The mice were allowed to swim until they reached the platform or a maximum of 90 s had elapsed. Testing was conducted over nine sessions (Tuesday-Friday and Monday–Friday). On sessions 2, 4, 5, 7, and 9, a probe trial was conducted as the fifth trial during which the platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it; (3) retention phase: one 60-s probe trial session was conducted 1 week after the ninth session of the previous phase; (4) visible platform phase: the mice were given a total of eight sessions (2/day separated by 2 h), each consisting of five trials with a 10-min inter-trial interval. The platform was identified by a triangular flag that was raised above the surface of the water. On each trial the mouse had to swim to the platform from a different starting point and the platform was moved to a different location before each trial. Thus, the mouse had to learn to associate the location of the flag with location of the platform.

Path length (distance taken to reach the platform) over sessions was used as the primary measure of performance. The path-independent swim speed was calculated by dividing distance by the latency to reach the platform. On probe trial, spatial bias for the platform location was evaluated in terms of the percentage of time spent within a 40-cm diameter annulus surrounding the platform location.

Discriminated avoidance

A T-maze constructed of acrylic (black for the sides and clear for the top) was utilized for the discriminated avoidance task. The maze was divided into three compartments: a start box (10 × 6.3 × 6 cm), a stem (17.5 × 6.3 × 6 cm), and two goal arms (14.5 × 6.3 × 6 cm), each separated by clear acrylic doors. The maze rested on a grid floor wired to deliver 0.69-mA scrambled shock to the feet.

The test consisted of three sessions separated by 1 h. On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning of the trial. On the first trial of the first session (information trial), the mouse received shock in the first arm entered (preference arm) and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 s after the opening of the start door if the mouse had not entered the correct goal arm or immediately upon entry into the incorrect arm. In either case, the shock continued until the correct goal arm was entered or a maximum of 60 s had elapsed. Upon the mouse's entry into the correct arm, the door was closed (to prevent departure), and, after 10 s, the mouse was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training in this fashion continued at 1-min intervals until the mouse had met the criterion of a correct avoidance (defined as running directly to the correct arm within 5 s) on four of the last five training trials of which the last two must be within 5 s. The second session of avoidance training was a reversal such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous session. Two measures were considered to show the ability of the mice to learn the discrimination and avoidance components of the task. Their ability to learn was considered inversely proportional to the number of trials required to reach the avoidance

criterion aforementioned and the number of trials required to reach the discrimination criterion (4 out 5 correct turns regardless of the time taken).

Elevated plus maze

To measure anxiety, an elevated plus maze test was conducted using a plus maze elevated three feet above the floor in a dimly lit test room (60 W) consisting of two arms opened to the room and two arms enclosed such that the floor is not visible. A computerized tracking system was used to monitor the position of the mice in the maze (Any-maze). The mice were positioned in the center of the plus facing an open arm and were given 5 min to explore the maze. The amount of time spent in the closed vs. open arms was recorded.

Statistical analysis

Functional performance of the mice on the behavioral tests was assessed using two-way analyses of variance (ANOVA) with Genotype and Treatment as between-group factors. Planned individual comparisons between different genotype groups (E3 vs. E4) and treatment groups (SedCon vs. SedEC vs. ExCon vs. ExEC) were performed using a single degree-of-freedom F tests involving the error term from the overall ANOVA. Performances were also considered in three-way with Session as the repeated measure. The effects of strain within the SedCon groups were analyzed using a one-way ANOVA with Strain (wild-type vs. E3 vs. E4) as a factor. Planned individual comparisons were performed using a single degree-of-freedom F tests involving the error term from the overall ANOVA. Pooling male and female data was not responsible for driving any of the main results. The α level was set at 0.05 for all analyses. The software used for the analyses was Systat 13 (Systat Software Inc., San Jose, CA, USA).

RESULTS

MWM

The performance of the mice as measured by path length and swimming speed is presented in Fig. 1. Path length of all wild-type, E3, and E4 mice decreased as a function of sessions (Fig. 1A). The effect of testing session on path length was confirmed by an analysis of variance with Session as repeated measure ($p < 0.05$). There was no effect of Strain or Treatment on the performance of the mice as supported by a lack of significant main effects or interaction of Strain and Treatment (all $p > 0.259$). The wild-type C57BL/6 SedCon group took shorter path length than the E3 or E4 mice, especially between sessions 3 and 7. This was supported by an ANOVA revealing a main effect of Strain ($p < 0.05$).

Overall, the E4 mice swam faster than the E3 ones, which was supported by a main effect of Strain ($p < 0.05$). The SedEC and ExEC mice seemed to swim slower than the controls throughout the sessions, however this was not supported by the analysis of the path-independent swimming speed yielding no main effect of Treatment ($p = 0.057$) or interaction of Strain and Treatment ($p = 0.359$). The wild-type and E4 mice swam faster than the E3 mice, which was supported by a main effect of Strain ($p < 0.01$) following a one-way ANOVA.

Accuracy for spatial memory was measured by conducting a probe trial as the last trial of sessions 2, 4, 5, 7, and 9 (Fig. 2). All the mice tested developed a strong bias for the platform location ($p < 0.05$), however there was no difference between the performance of the E3 and E4 strains ($p = 0.052$) and no effect of Treatment ($p = 0.067$). Within the SedCon groups, the wild-type mice seemed to develop a strong bias on session 5 which remained for the rest of the sessions; however the difference between E3 and E4 was only seen on session 5. This

observation was supported by a significant interaction between Session and Strain ($p < 0.01$), and no main effect of Strain was detected ($p = 0.346$).

Spatial retention was also tested 1 week after the last session in a single probe trial (session 10). All the mice retained the previously learned information well. In comparing genotypes within the SedCon groups, there was no effect of Strain ($p = 0.97$) on the performance of the mice. There was no difference between the performance of the E3 and E4 mice and no effect of Treatment (all ps > 0.221).

The mice were also tested on a visible platform test to determine whether their vision may have affected their performance in the MWM. A composite measure, learning index, was calculated by averaging the path length taken by the mice to the flagged platform during sessions 2, 3, and 4 (Fig. 3). There was no discernable effect of Strain or Treatment on the performance of the mice, which was supported by a lack of main effect or interaction between Strain and Treatment (all ps > 0.164). Swimming speed on sessions 2, 3, and 4 was also averaged and considered for analysis. The speed of the SedCon E4 mice was 25% faster than the SedCon E3 ones, and there was no effect of the Treatment on the speed of the E3 or E4 mice. These observations were supported by a significant main effect of Strain ($p < 0.05$) and a lack of main effect of Treatment or an interaction (all ps > 0.386). There were no differences in performance between the wild-type, E3, and E4 mice when analyzing the learning index ($p = 0.989$). The speed of the wild-type was comparable to the one of the E4 mice, which was significantly higher than the swimming speed of the E3 mice. This was supported by a significant effect of Strain ($p < 0.05$) following a one-way ANOVA.

Discriminated avoidance test

Components of the discriminated avoidance learning were considered for effects of Strain and Treatment during the acquisition and reversal sessions. Learning of the preemptive response is shown in Fig. 4, whereas the discriminative component is shown in Fig. 5. During acquisition, the SedCon E4 mice took 13% more trials than their E3 counterparts. The ExCon and ExEC E3 mice took 27% and 22.5% less trials to reach the avoidance criterion compared to the SedCon E3 mice. Number of trials taken to make a correct avoidance response was reduced by 18%–20% in the SedEC, ExCon, and ExEC E4 mice in comparison to their genotype-matched control (SedCon). Analysis of the trials to avoidance criterion for session 1 yielded a significant main effects of Strain and Treatment (all ps < 0.021) but no interaction of Strain and Treatment (p = 0.63). In the reversal session, there was no difference between the SedCon E3 and SedCon E4. Similar improved performances were observed with the mice on antioxidant supplementation and/or exercise regimen, however they were not as large in the E4 groups (11%–16%) while they remained in the same range for the E3 (26%). Analysis of the data from session 2 indicated only a significant main effect of Treatment (p = 0.002), and did not yield a significant Strain × Treatment interaction (p = 0.38). There were no significant differences between the SedCon groups from each genotype in acquisition (p = 0.390) and reversal (p = 0.371).

Perusal of the discriminative component (Fig. 5) during acquisition and reversal revealed a strain-related difference in performance. Interestingly, the SedCon E4 mice learned the discriminative component of the active avoidance task taking less trials that the SedCon E3 ones. Furthermore, significant effects of Treatment were only observed in the E3 mice. In the acquisition session, the ExCon and ExEC mice took 32% less trials to reach the criterion compared to the SedCon E3 mice while it was only about 15% less trials for the E4 mice.

Analysis of the trials to the discriminative component for session 1 yielded main effects of Strain and Treatment (all *p*s < 0.008) but did not reveal a significant interaction between Strain and Treatment ($p = 0.23$). In the reversal session, the all treated E3 mice took 25%–42% less trials than the SedCon mice while the E4 treated mice improved only by 8%–16%. An analysis of the data during session 2 indicated significant main effects of Strain and Treatment as well as an interaction between Strain and Treatment (all *p*s < 0.036). For the discriminative component of the active avoidance, a one-way ANOVA yielded only a main effect during the reversal phase ($p = 0.039$), however this main effect was solely driven by the significant difference between E3 and E4. There were no significant differences between the genotypes in both phases.

Elevated plus maze

The effect of Strain and Treatment were analyzed in terms of percent time spent in the closed arms and open arms of the plus maze (Fig. 6). In the E3 group, there was no effect of Treatment on either measure; however it seems that the supplementation with EC diet reduced the amount of time spent in the open arms by the E4 mice. Furthermore, overall the E4 mice spent more time in the open arms compared to the E3 ones. Analyses of the data revealed a significant main effect of Strain for percent time in open arms ($p < 0.05$), however no effect of Treatment or an interaction between Strain and Treatment were found (all *p*s > 0.109). When comparing the SedCon treatments groups across wild-type, E3, and E4 genotype there was no difference in their time spent in open arms ($p = 0.071$) and closed arms ($p = 0.052$).

DISCUSSION

The main findings of this study were (1) *APOE* genotypes showed differential behavioral phenotype on the discriminative avoidance task, however there was no interaction of sex; (2) *APOE*4 mice exhibited higher swimming speeds and lower anxiety levels; (3) *APOE*3 and *APOE*4 mice performance was different from wild-type controls in the MWM, but not on the non-spatial cognitive task; (4) supplementation with vitamins E and C decreased swimming speed and anxiety; (5) exercise training improved cognitive function in both genotypes, while antioxidant supplementation primarily improved function in the *APOE*4 mice; and (6) no synergism/additive or antagonism effect was detected between antioxidant and exercise treatments.

Our study aimed at cognitively phenotyping the GFAP- *APOE*3 and *APOE*4 mice using two different tests including spatial and non-spatial tasks. While *APOE*4 have been associated with accelerated cognitive declines (6,7,56) and neurodegenerative diseases, (24,57,58) reports regarding cognitive outcomes in young *APOE*4 population have remained inconclusive. In humans, *APOE*4 has been associated with better performance in young individuals which then shifts to a negative outcome in older individuals (20-22). This antagonistic pleiotropy has not been well studied and has remained elusive. Studies in animal models have led to conflicting results with some studies showing early signs of deleterious effects with *APOE*4, (16) and others showing improvements (12, 19, 23, 24). Some of the differences may be due to the mouse model chosen: targeted replacement model vs. hAPP-Yac/*APOE*-TR model, as well as the different behavioral tests conducted. In our study we opted to use the GFAP-*APOE* mice, in which the expression of the human *APOE* isoforms is under glial promoter control (56). Our findings suggested that *APOE*4 performed better on the discriminative component of the active avoidance

but not on the avoidance component, which is more difficult to learn and achieve. Furthermore, even though there was no main effect of Sex on any of the measures, it is noteworthy that on the MWM, female *APOE*4 in the SedCon group seemed to perform better than the *APOE*3 SedCon ones. Our data suggested that indeed *APOE*4 may confer some type of beneficial effect at a younger age. Our mice were about 5–6 months when tested for cognitive function, and it is possible that the *APOE* effect would have been larger if tested at a younger age.

Interestingly, in the current study, the *APOE*4 mice exhibited a behavioral profile that seemed to match the one of the wild-type mice on activity- and affective-related tasks. The speed measured in the water maze task and the anxiety levels of the *APOE*4 mice were similar to the wild-type ones, while the *APOE*3 mice were less active in the water and seemed more anxious. Studies of older mice showed that E3 and E4 mice were more anxious than the wild-type (56). Furthermore, while our study yielded a better performance on the MWM for the wild-type compared to *APOE*3 and E4 mice, other studies have indicated a lack of effect of genotype on this particular task (56). While the methodology was different, it is noteworthy that E3 and E4 mice did not differ in their performance in both studies. Interestingly, both studies showed differences in working memory with Hartman et al. (56) showing impairments associated with *APOE*4 while our study yielded a better performance associated with E4 when compared to E3. These differences may be due to the age of the mice used in the study (young adult in the present one vs. older mice in Hartman's study), which are in support of the antagonistic pleiotropy that has been associated with *APOE*4 expression.

While our analyses did not reveal any interactions of Sex and Treatment on any of the measures presented, we conducted full analyses including Sex as a factor. The resulting analyses did not show any interaction of Sex with Strain, supporting that the performance of each strain

on the behavioral tests was not influenced by the sex of the mice. This was in contradiction with previous reports that clearly indicated a further impairment in *APOE*4 females when compared to *APOE*4 males (12). It is noteworthy that these studies showing impairments were done in a different model expressing human *APOE* isoforms (12) or in mice that were relatively older (2). Furthermore, epidemiological studies looking at the association between *APOE*4 and AD risk or cognitive declines have been done in relatively old populations and have also demonstrated that age is an influencing factor. For example, a study by Qiu et al. (59) has identified a strong association between *APOE*4 and AD risk that was stronger in men than in women. Despite a lack of sex interaction in these young adult mice, it is noteworthy that the females and males responded to the same extent to the Treatment and that sex was not a driving factor of the observed treatment effects.

The *APOE*4 mice also exhibited another interesting and unexpected behavior in this study. Interestingly, the *APOE*4 mice had higher swimming speed in the hidden and visible platform tests. Even though this observation could be a sign of higher motivation, it did not translate to an improvement in spatial learning and memory. Furthermore, the lack of an effect of Strain or Treatment on the visible platform phase indicated that motivation was not a factor influencing the performance of the mice. While other studies in humans have reported hyperactivity being associated with the presence of *APOE*4, (60) the mice in our study did not exhibit increased locomotion or exploration during open field test or elevated plus maze (data not shown). Other studies have actually reported decreased locomotor activity in *APOE*4-TR mice (61) and even slower swimming speed in the MWM (62). The *APOE*4 swimming speed was also higher during the visible platform phase of the swim maze. Vitamin E is transported via the same transporter as *APOE* which is defective in *APOE*4 mice, therefore vitamin E levels should be

lower in *APOE*4 mice compared to *APOE*3 ones. Antioxidant intake has been associated in some instances with increased swimming speed and spontaneous activity, (63) and with hyperactivity (64). Though the mechanisms by which antioxidants may affect hyperactivity remain unknown, there seemed to be a definite influence.

Though this increase in activity was only observed on the swim maze task, a test for anxiety was performed to determine whether it could be linked to an increase in anxiety-related behavior as noted in previous studies (65, 66). Overall, the *APOE*4 mice spent more time in the open arms than their *APOE*3 counterpart, results that are in contradiction with previous studies (65). Furthermore, supplementation with vitamins influenced in a genotype-dependent manner the behavior of the mice on this task. In the *APOE*4 mice, the supplementation with antioxidants increased anxiety. This was in definite contrast with several studies relating that vitamin E depletion increases anxiety (67). Furthermore, other studies have demonstrated a decrease in anxiety in rats supplemented with vitamins E and C (68).

In our active avoidance paradigm, the results differed depending on whether we analyzed the discriminative component or the avoidance component of the task. In the discriminative component, which is the component of active avoidance that the mice learned first, there was a definite improvement in performance following exercise and antioxidant Treatment in the *APOE*3 mice. The lack of significant improvement in the *APOE*4 mice could be due to a maximum plateau of performance due to the set criterion. The criterion was set as the number of trials to reach four out of five correct turns, therefore four trials would be the minimum number of trials than a mouse could take. On average the SedCon *APOE*4 mice took between six and eight trials, thereby making it difficult to detect a significant effect of Treatment. The effects of

Treatment were mostly due to exercise Treatment as the performance of the *APOE*3 mice remained largely unaffected by supplementation with antioxidants.

In the avoidance component of the task, exercise training improved performance of the *APOE* mice, irrespective of genotype in the acquisition phase. Interestingly, supplementation with antioxidants was only effective in the *APOE*4 mice. This is most likely due to the transporter protein being dysfunctional (69) and *APOE*4 mice having lower vitamin E levels, (70) therefore more responsive to antioxidant supplementation. Physical activity has been shown to reduce AD risk, (41-43) to improve cognitive function and to have a positive impact on functional plasticity (44). Interestingly, *APOE*4 allele carriers with a sedentary life style have been shown to be more vulnerable to excessive amyloid deposition in brain (45,71). Physical activity levels have been strongly positively associated with cognitive function in individuals carrying *APOE*4 (72,73) supported by transgenic mouse model carrying human *APOE*4 (33). These studies focused on individuals or mice in which cognitive dysfunction was present, while our study demonstrated that improvement can also be attained without apparent cognitive dysfunction and did not seem to be dependent upon the *APOE* genotype.

Studies on combination of antioxidant and exercise have led to conflicting results. In aged rodents, studies have reported an additive effect combining vitamin E with swimming exercise on markers of oxidative stress in various brain regions (50,52). In young diabetic patients, antioxidant intake abolished the activation of molecular regulators of endogenous antioxidant enzymes by a moderate exercise regimen (53). *APOE*4 has been associated with lower antioxidant activity (74), decreased capacity to remove by-products of oxidative stress (75) and increased oxidative stress (76). Therefore, a combination of antioxidants to lower oxidative stress and exercise to boost antioxidant defenses should lead to a further improvement than each

intervention independently. Our study did not reveal such a beneficial additive interaction; in fact most effects observed with the combined Treatment mimicked the effects seen with exercise. The lack of an additive/synergistic effect on cognitive function may have been due to reaching a maximum ceiling of performance. While each intervention independently improved the performance of the mice, it may have improved to a maximal level of performance and further improvements by combining Treatments cannot be detected. Further studies will be needed to determine whether the combination had an additive/synergistic effect at the molecular level which did not translate to further improvements due to a ceiling effect being reached.

CONCLUSION

Even though the effects were minor and in select domains of cognition, our study supported previous reports of *APOE*4 mice performing better than *APOE*3 mice at a young age. While the beneficial effect of exercise training on learning and cognitive flexibility was found in both genotype and in both males and females, the beneficial effect of antioxidant supplementation seemed to be genotype dependent. Lastly, in young adult mice the combination of exercise and antioxidant did not lead to additive or antagonistic effects.

FIGURES AND LEGENDS

Figure 1. Effects of genotype, antioxidant intake, and moderated exercise on Morris water maze performance as measured by path length (A) and swimming speed (B) in adult GFAP-APOE3, GFAP-APOE4, and C57BL/6 (wild-type) mice.

Each value represents the mean ± SEM. For APOE3, sedentary with control diet (SedCon) n = 20, sedentary with diet rich in vitamins E and C (SedEC) n = 21, exercise with control diet (ExCon) n = 21, exercise with diet rich in vitamins E and C (ExEC) n = 21; for APOE4, SedCon n = 20, SedEC n = 21, ExCon n = 20, ExEC n = 19; for wild-type SedCon n = 12.

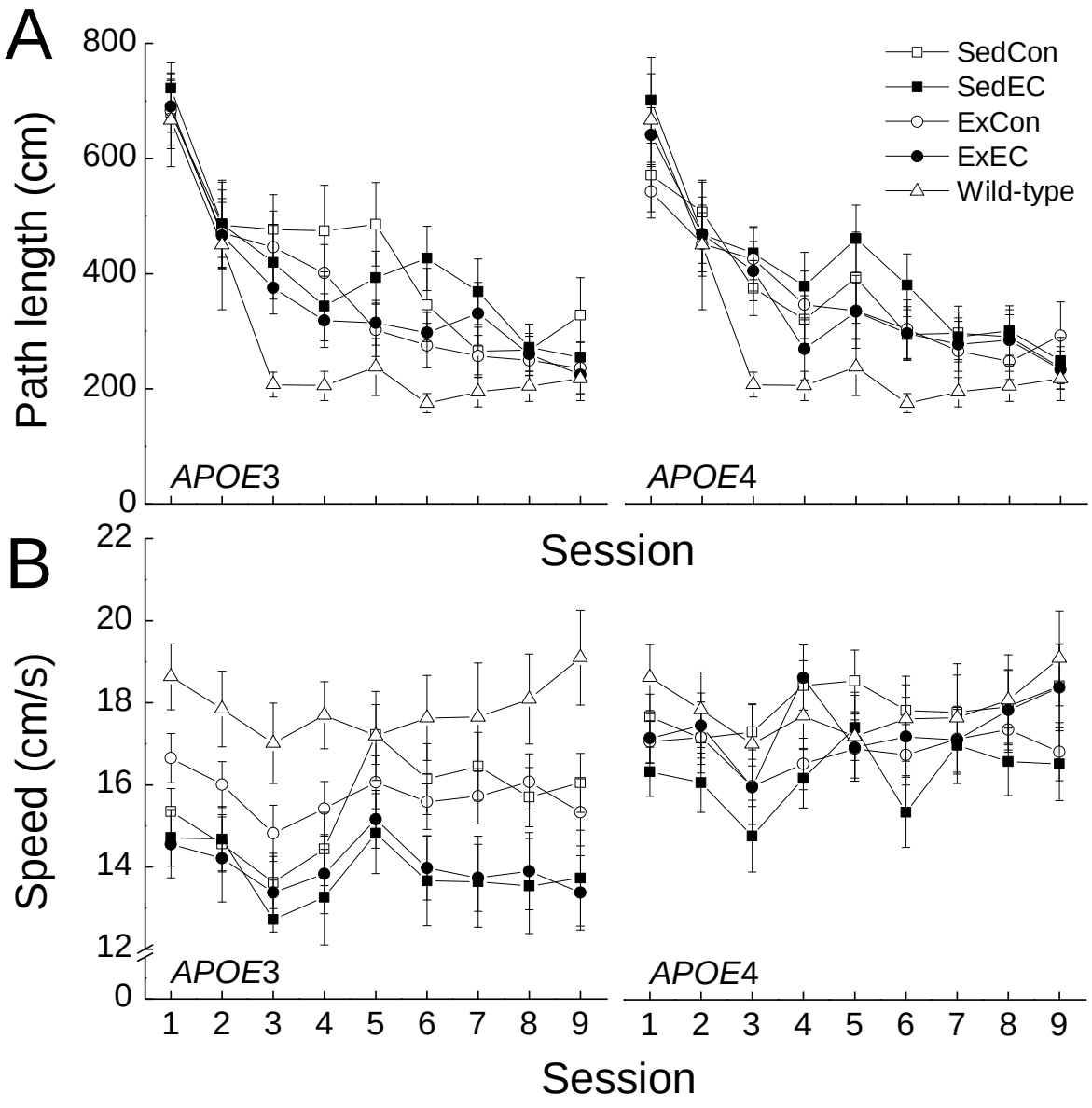

A
Path length (cm)
800
600
400
200
0
SedCon
SedEC
ExCon
ExEC
Wild-type
APOE3
APOE4
Session
B
Speed (cm/s)
22
20
18
16
14
12
0
APOE3
APOE4
1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
Session

Figure 2. Effects of genotype, antioxidant intake, and moderated exercise on Morris water maze performance as measured by percent time spent in a 40-cm area around the hidden platform location in adult GFAP-APOE3, GFAP-APOE4, and C57BL/6 (wild-type) mice. Each value represents the mean ± SEM. For APOE3, sedentary with control diet (SedCon) n = 20, sedentary with diet rich in vitamins E and C (SedEC) n = 21, exercise with control diet (ExCon) n = 21, exercise with diet rich in vitamins E and C (ExEC) n = 21; for APOE4, SedCon n = 20, SedEC n = 21, ExCon n = 20, ExEC n = 19; for wild-type SedCon n = 12.

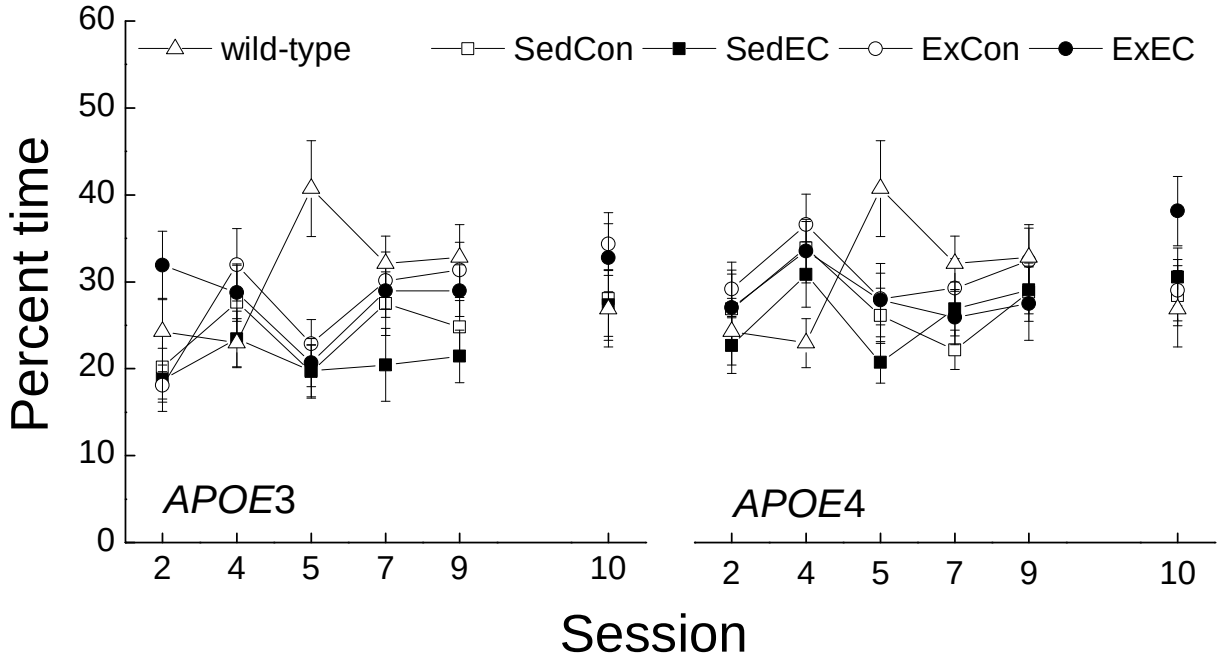

wild-type
SedCon
SedEC
ExCon
ExEC
Percent time
60
50
40
30
20
10
0
APOE3
APOE4
2
4
5
7
9
10
Session

Figure 3. Effects of genotype, antioxidant intake, and moderated exercise on visible platform performance as measured by learning index (A) and swimming speed (B) in adult GFAP-APOE3, GFAP-APOE4, and C57BL/6 (wild-type) mice.

The dotted line represents the performance of the SedCon wild-type mice. Each value represents the mean ± SEM. For APOE3, sedentary with control diet (SedCon) n = 20, sedentary with diet rich in vitamins E and C (SedEC) n = 19, exercise with control diet (ExCon) n = 21, exercise with diet rich in vitamins E and C (ExEC) n = 21; for APOE4, SedCon n = 20, SedEC n = 20, ExCon n = 19, ExEC n = 18; for wild-type SedCon n = 12. $*p < 0.05$, compared with wild-type; $\#p < 0.05$, compared with SedCon E4.

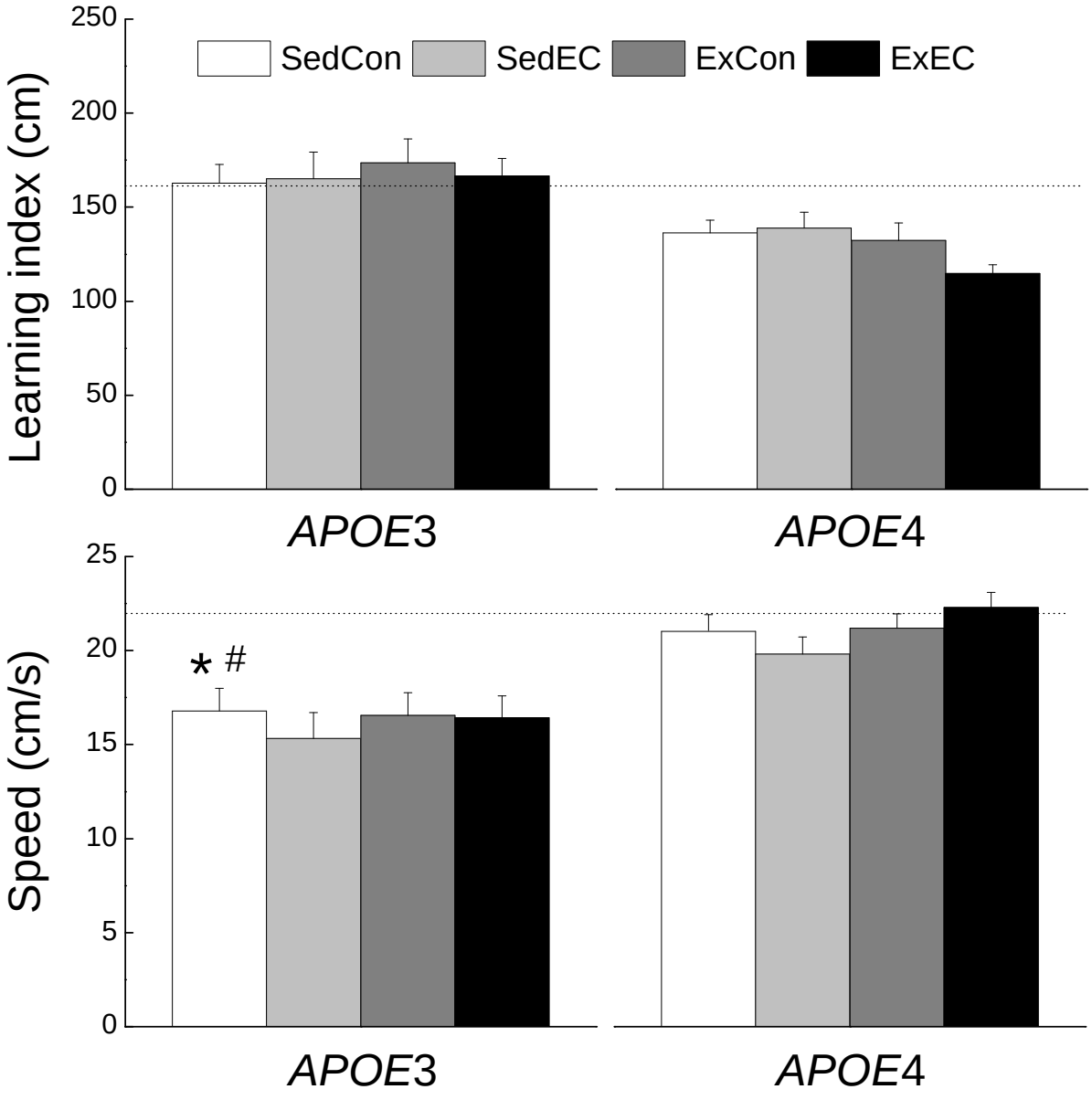

SedCon
SedEC
ExCon
ExEC
Learning index (cm)
250
200
150
100
50
0
APOE3
APOE4
Speed (cm/s)
25
20
15
10
5
0
* #
APOE3
APOE4

Figure 4. Effects of genotype, antioxidant intake, and moderated exercise on discriminated avoidance performance as measured by the number of total trial taken to reach discriminative criterion during acquisition (A) and reversal (B) sessions in adult GFAP-APOE3, GFAP-APOE4, and C57BL/6 (wild-type) mice.

The dotted line represents the performance of the SedCon wild-type mice. Each value represents the mean ± SEM. For APOE3, sedentary with control (SedCon) n = 20, sedentary with diet rich in vitamins E and C (SedEC) n = 20, exercise with control diet (ExCon) n = 20, exercise with diet rich in vitamins E and C (ExEC) n = 20; for APOE4, SedCon n = 20, SedEC n = 20, ExCon n = 15, ExEC n = 17; for wild-type SedCon n = 12. $*p < 0.05$, compared with genotype-matched SedCon.

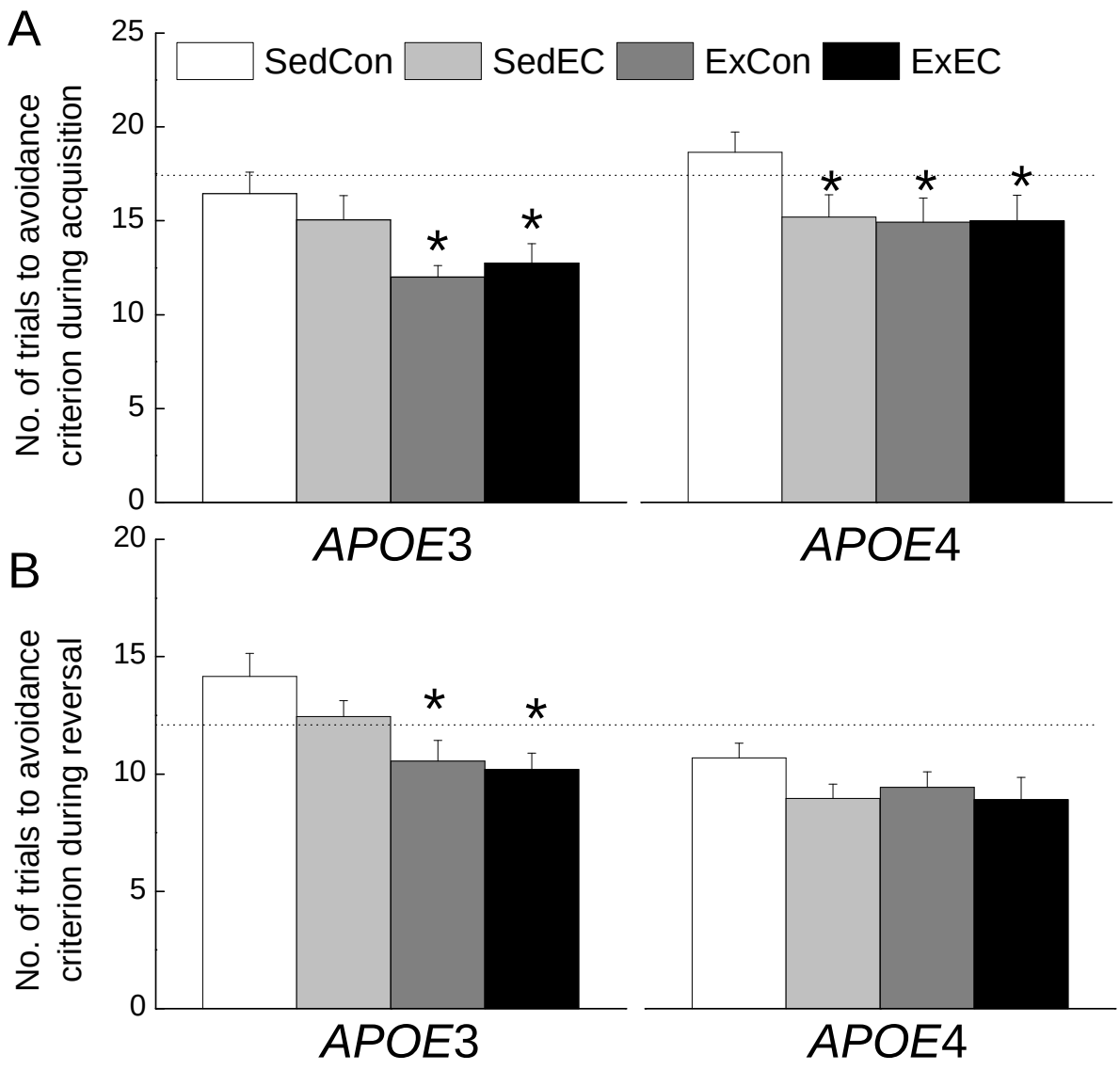

A
SedCon
SedEC
ExCon
ExEC
No. of trials to avoidance criterion during acquisition
25
20
15
10
5
0
*
*
*
*
APOE3
APOE4
B
No. of trials to avoidance criterion during reversal
20
15
10
5
0
*
*
APOE3
APOE4

Figure 5. Effects of genotype, antioxidant intake, and moderated exercise on discriminated avoidance performance as measured by the number of total trial taken to two consecutive correct turns during acquisition (A) and reversal (B) sessions in adult glial GFAP-APOE3, GFAP-APOE4, and C57BL/6 (wild-type) mice.

The dotted line represents the performance of the SedCon wild-type mice. Each value represents the mean ± SEM. For APOE3, sedentary with control diet (SedCon) n = 20, sedentary with diet rich in vitamins E and C (SedEC) n = 20, exercise with control diet (ExCon) n = 20, exercise with diet rich in vitamins E and C (ExEC) n = 20; for APOE4, SedCon n = 20, SedEC n = 20, ExCon n = 15, ExEC n = 17; for wild-type SedCon n = 12. $*p < 0.05$, compared with genotype-matched SedCon; $\#p < 0.05$, compared with SedCon E3; $\dagger p < 0.05$, compared with genotype-matched SedEC.

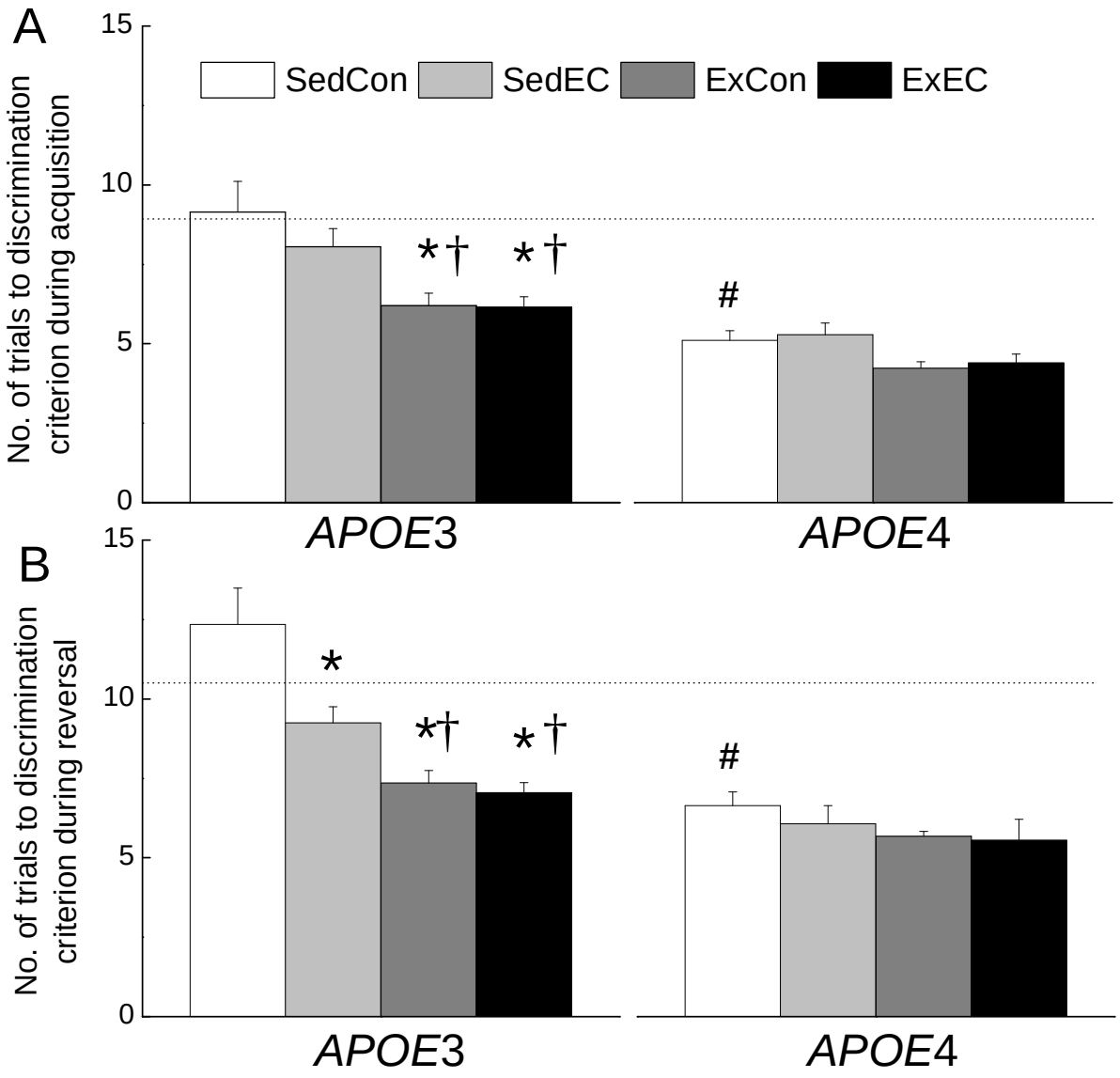
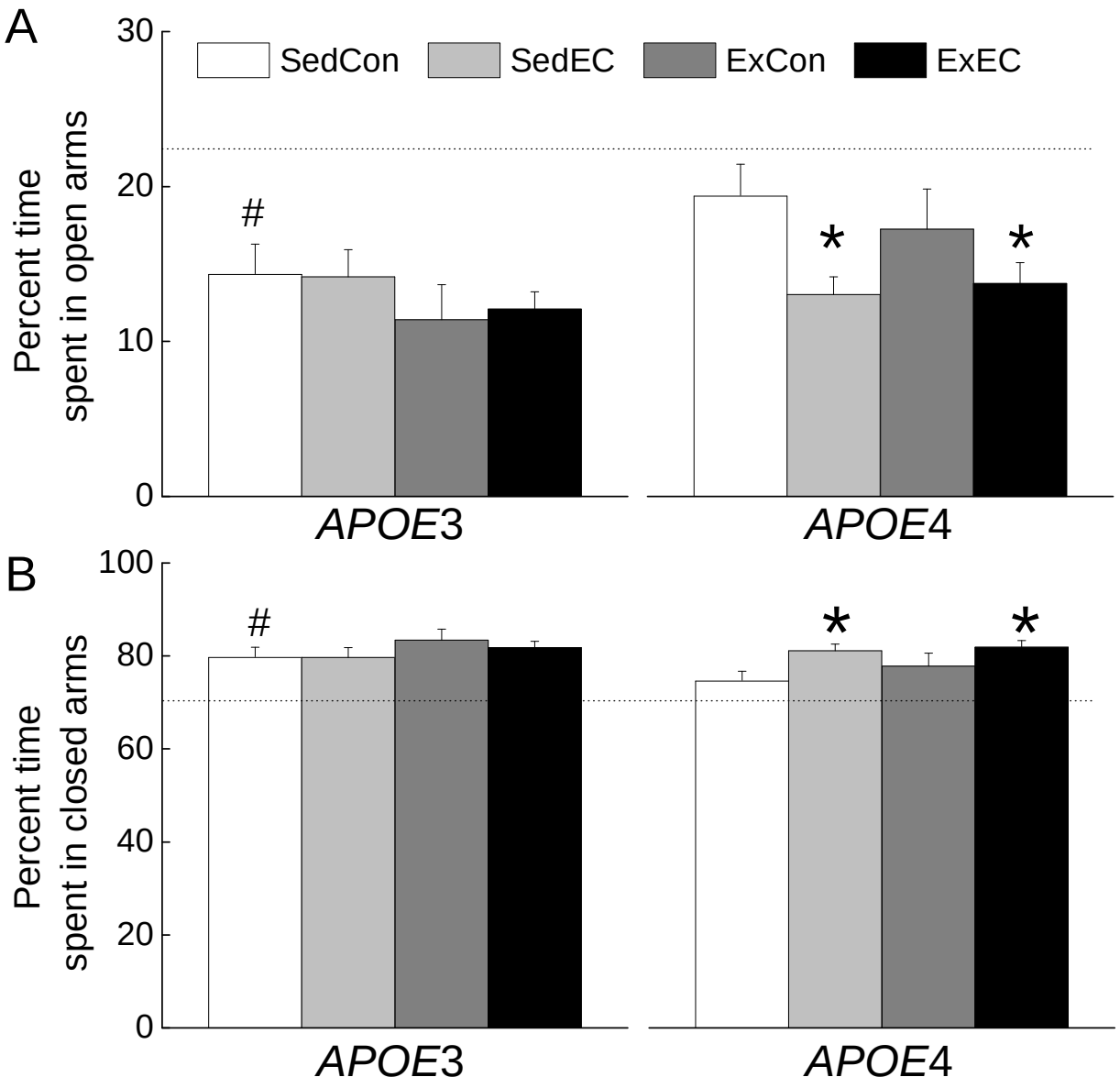

A
15
10
5
0
SedCon
SedEC
ExCon
ExEC
No. of trials to discrimination criterion during acquisition
*†
*†
#
APOE3
APOE4
B
15
10
5
0
No. of trials to discrimination criterion during reversal
*
*†
*†
#
APOE3
APOE4

Figure 6. Effects of genotype, antioxidant intake, and moderated exercise on elevated plus maze performance as measured by the percent time spent in the open arms (A) and closed arms (B)sessions in adult GFAP-APOE3, GFAP-APOE4, and C57BL/6 (wild-type) mice. The dotted line represents the performance of the SedCon wild-type mice. Each value represents the mean ± SEM. For APOE3, sedentary with control diet (SedCon) n = 20, sedentary with diet rich in vitamins E and C (SedEC) n = 20, exercise with control diet (ExCon) n = 21, exercise with diet rich in vitamins E and C (ExEC) n = 21; for APOE4, SedCon n = 20, SedEC n = 20, ExCon n = 17, ExEC n = 18; for wild-type SedCon n = 12. $*p < 0.05$, compared with genotype-matched SedCon; $\#p < 0.05$, compared with wild-type.


A
30
20
10
0
SedCon
SedEC
ExCon
ExEC
Percent time spent in open arms
#
*
*
APOE3
APOE4
B
100
80
60
40
20
0
Percent time spent in closed arms
#
*
*
APOE3
APOE4

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CHAPTER 3

EXERCISE REVERSED *APOE*4-ASSOCIATED MOTOR IMPAIRMENTS IN ADULT MICE

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Running title: Motor effects of exercise and antioxidant intake

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ABSTRACT

This study was designed to determine the beneficial impact of antioxidant supplementation, moderate exercise, and the combination of both treatments on various motor functions in adult mice and whether the apolipoprotein E (*APOE*) genotype could influence the functional outcomes of the treatments.

For a period of 9-10 weeks, separate groups of male and female mice expressing either the human *APOE*3 or *APOE*4 isoforms were fed either a control diet (NIH-31) or the control diet supplemented with vitamins E and C (1.12 IU/g diet α-tocopheryl acetate and 1.65 mg/g ascorbic acid). The mice were further separated into a sedentary group or a group that followed a daily exercise regimen. After 8 weeks on the treatments, the mice were administered a battery of functional tests including tests to measure reflexes, strength, coordination and balance.

Overall, *APOE*4 mice had poorer performance when compared to *APOE*3. *APOE*4 had impaired motor learning and diminished strength, while spontaneous activity and balance were not different between the two strains. Interestingly antioxidant supplementation improved motor learning and balance only in the *APOE*3 mice, and only marginal improvements in both strains on reflexes. Exercise improved motor learning in both strains and balance only in the *APOE*3 group. Contrarily to expected outcomes the combination of the two treatments did not yield further improvement in motor functions, and rather supported an antagonistic action of the antioxidant supplementation on the beneficial effects of exercise on strength and motor learning.

Keywords: Antioxidant, *APOE*, motor function, exercise, vitamin C, vitamin E.

INTRODUCTION

Alzheimer's disease (AD) is a major neurodegenerative disease. In AD, cognitive decline is often accompanied (and often overlooked in studies) by motor function disability and inability to learn new motor skills (1-4). Motor functions and skills are governed by distal effector muscle units and a central motor control system regulating the initiation, planning and execution of a movement. This motor control system is spread in multiple interconnected cortical and subcortical motor regions namely motor cortex, basal ganglia, and cerebellum (5-8). Any pathological changes occurring in these brain regions might be responsible for motor function decline in AD (9-11). Additionally, AD has associated with loss of distal dendritic segments and decreased total number of dendritic spines within the Purkinje cells in cerebellum (12,13). This could explain the coordination, balance dysfunction and fall tendency appearing with the initial stages of AD whether or not there is presence of cognitive deficit. Risk factors (such as apolipoprotein E4; *APOE4*) predisposing and aggravating AD might also exacerbate these motor symptoms along with accelerated cognitive pathology. The presence of *APOE*4 allele was associated with a two-fold increase in the rate of global motor function decline (14).

The progressive nature and non-availability of specific counter-measures to combat AD are conducive to the development of preventive approaches rather than therapeutics. Lifestyle modification such as physical activity and dietary alterations are undisputable part of AD management. Exercise /physical activity benefits AD by reducing the risk of AD (15-17), delaying onset (18), improving AD symptoms (19) in an activity intensity (dose) and duration dependent manner. Interestingly, these exercise benefits are more pronounced in *APOE*4 allele carriers (20,21). Further, long term exercise lowers oxidative stress and improves cognition (22,23) in accordance to the effect of antioxidant on oxidative stress and brain functions (24,25).

Interestingly, various exercise regimes improve motor function in cognitively impaired geriatric population (26-28). The rate of injurious fall has been dramatically reduced among such AD patients with motor training (29-31).

The brain of AD patient is more vulnerable to accumulation of oxidative stress as evidenced in animal models and humans (32,33). *APOE*4, a major genetic risk factor present in approximately 36% of AD cases, exaggerates AD pathophysiology possibly via exacerbated oxidative stress (34,35). Therefore, AD symptomology in presence of *APOE*4 allele should respond to antioxidant therapy such as vitamin E supplementation alone (36,37) or with combination of antioxidants (38,39).

Based on the oxidative stress lowering potential of low intensity aerobic exercise (40-43) and antioxidant supplementation, it can be assumed that when simultaneously implemented, such combination will have additive beneficial effects on AD pathology and outcome (44-47). Most AD studies have put cognition at the center of management strategies, and often motor components are sidelined and considered as part of normal aging rather than another symptom of AD. The combination of these two interventions have shown promising effects on cognitive function (48), however effect of such combination on the motor functions in *APOE3* and *APOE4* adults has never been evaluated. The goals of the current study were 1) to characterize the motor learning and performance, strength and reflexes phenotypes of the adult GFAP-*APOE*3 and *E4* mice (*APOE* expressed under a GFAP promoter); 2) to determine whether antioxidant intake and exercise training led to beneficial improvements in these young mice; 3) to determine whether the combination of antioxidant and exercise yields a synergistic or additive beneficial effect; and lastly 4) to determine whether the beneficial outcomes are genotype-dependent.

MATERIAL AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the UNT Health Science Center at Fort Worth. Separate groups of male and female GFAP-*APOE**3 (B6.Cg-Tg(GFAP-*APOE**3)37Hol *APOE*tm1Unc/J) and GFAP-*APOE**4 (B6.Cg-Tg(GFAP-*APOE**4)1Hol *APOE*tm1Unc/J) mice were obtained from Jackson Laboratories (catalog numbers 004633 and 004631; total n of 180) at the age of 2 months and subsequently maintained in the UNT Health Science Center vivarium. The mice were housed in groups of 3 or 4 in standard polycarbonate cages (28 x17 x 12.5 cm) with corncob bedding and *ad libitum* access to food and water, and were maintained at ambient temperature (23±1°), under a 12-h light/dark cycle starting at 0600. The mice were weighed weekly, and survival was monitored throughout the study. A group of male and female C57BL/6 mice (wild-type, n=12) was used as a control to compare the *APOE*3 and *E*4 controls to determine whether the behavioral differences between *APOE*3 and E4 were due to an altered phenotype of the transgenic mice.

Treatment

Upon arrival, the mice were randomly assigned to one of four experimental groups: (1) sedentary fed the control diet (SedCon), (2) sedentary fed the vitamins E and C supplemented diet (SedEC), (3) forced exercise fed the control diet (ExCon), (4) forced exercise fed the vitamins E and C supplemented diet (ExEC). Each experimental group was balanced for sex of the mice. The mice were fed, *ad libitum*, either a control diet (LabDiet® R&M 5LG6 4F, cat #: 5S84) or the control diet supplemented with vitamins E and C (modified 5LG6 with1.65mg/g diet of ascorbic acid and 1.12 IU/g diet of α-tocopheryl acetate, cat.#: 5SH0). Furthermore, the mice were either sedentary or following a moderate exercise regimen. The moderate exercise

regimen was introduced progressively using treadmills (AccuScan Instruments Inc, AccuPacer Treadmill Sr No: AN5817629). Over a 12-day period, the training was gradually incremented in time and speed to reach a maximal exercise of 1h (6, 8, 10, and 12 m/min for 5 min each, and then at 14m/min for 40 min). The training protocol used was a modification of previously published exercise protocols (49). Forced exercise was implemented via transient 0.29 mA electric foot shock to the feet. Each exercise mouse was paired with a control which received the same number of shock for each training day.

The mice were on their respective treatments for 8 weeks prior to and throughout behavioral assessments for a total of 16 weeks. The behavioral tests were conducted in the following order: locomotor activity (spontaneous activity), coordinated running (maximum motor performance and motor learning), motor function tests (spontaneous walk initiation, reflexive turn in blind alley, reflexive turn over negative geotaxis, muscle strength in wire suspension and balance in elevated bridge walk.

Food intake

Food intake was monitored three times during the course of the study: during the first week of treatment; a week prior to the start of the behavioral testing and after the completion of the behavioral testing. Each time point was an average of food consumption during five consecutive days measured at the same time of day to control for any diurnal variation. The data was presented as the averaged over the 3 time points.

Locomotor activity

Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously (50). Each mouse was placed in a clear acrylic test cage (40.5×40.5×30.5 cm) that was surrounded by a metal frame lined with

photocells. The test cage was enclosed in a dimly lit, sound-attenuating chamber equipped with a fan that provided background noise (80 dB). Each mouse was placed in a chamber for 4 consecutive sessions, each 4 minutes in duration for a total of 16 minutes. During a 16-min period, movements in the horizontal plane, as well as a vertical plane 7.6 cm above the floor, were detected by the photocells and processed by a software program to yield different variables describing distance, vertical, and spatial components of spontaneous activity in the apparatus.

Coordinated running

Motor learning and maximum running performance were measured using an accelerating rotorod test described previously (51). The apparatus was a motor-driven treadmill (Omnitech Electronics, Model # AIO411RRT525M) that consisted of a 3-cm diameter nylon cylinder mounted horizontally at a height of 35 cm above a padded surface. On a given trial, the mouse was placed on the cylinder, which then began rotating with increasing speed until the animal fell to a well-padded surface. Ability of the mice to improve running performance was assessed in a series of training sessions (two per day), each consisting of four trials at 10-min intervals. The training sessions continued until the running performance (the average latency to fall from the cylinder) failed to show improvement over three consecutive sessions. The treatment groups were compared for their average latency to fall on the first seven sessions and for the final session on which each mouse had reached its maximum stable level of performance.

Reflexive musculoskeletal responses

Over four consecutive daily sessions, the mice were administered three simple reflex tests. The first test consisted of placing the mouse on a flat smooth surface and recording the latency to move one body length (walk initiation). The second test measured the latency to reverse direction when the mouse was placed in a 3.5-cm wide, 14-cm long, dead-end alley

(alley turning). For the third test, the mouse was placed facing downward on a flat surface that was tilted 45°, and the latency to turn 180° in either direction was measured (negative geotaxis). The mouse was allowed to grip a horizontal wire with the front paws when suspended 27 cm above a padded surface (wire suspension). The latency to tread (reach the wire with their hind legs) and the latency to fall were recorded and averaged over four consecutive daily sessions (two trials/day).

Bridge Walking

Each mouse was tested for the latency to fall or reach a safe platform after being placed on one of four acrylic bridges, each mounted 50 cm above a padded surface. The bridges differed in diameter (small or large) and shape (round or square), providing four levels of difficulty. Each bridge was presented three times, and the measure of performance was the latency to fall, either examined as the average latency to fall (of three trials) for each bridge individually, or a single overall mean representing the average latency to fall from all four types of bridges.

Statistical analysis of data

Functional performance of the mice on the behavioral tests was assessed using two-way analyses of variance (ANOVA) with Genotype and Treatment as between-groups factors. Planned individual comparisons between different genotype groups (E3 vs. E4) and treatment groups (SedCon vs. SedEC vs. ExCon vs. ExEC) were performed using a single degree-of-freedom F tests involving the error term from the overall ANOVA. Performances were also considered in three-way with Sessions as the repeated measure. The alpha level was set at 0.05 for all analyses.

RESULTS

Body weight and food intake

Weekly body weights were taken throughout the study and presented in Figure 1A and food intake was measured at the end of the study and presented in Figure 1B. Overall, the *APOE*3 mice weighed more than the *APOE*4 mice, and none of the treatments affected the weights of the mice from either genotype. An ANOVA yielded a main effect of Strain ($p<0.05$) and no main effect of Treatment or interaction of Strain by Treatment (all ps>0.774). Food intake averaged across the 3 time points was lower in the ExCon mice than any other treatment group regardless of genotype, which was supported by a main effect of Treatment ($p=0.001$).

Locomotor activity

Measures for spontaneous locomotor activity were analyzed and presented in Figure 2. The *APOE*3 ExEC mice travelled 19% more distance than their SedCon, while in the *APOE*4 group the ExCon and ExEC travelled ~10 % less than their SedCon (Figure 2A). These observations were supported by a significant interaction between Treatment and Strain ($p=0.012$). Rearing counts were lower in the mice that received exercise training with or without antioxidants (Figure 2B). A two-way ANOVA did yield a significant effect of Treatment ($p=0.056$). Time spent in the center is an indirect measure of anxiety and was affected by exercise (Figure 2C). An ANOVA revealed a main effect of treatment ($p=0.021$), and almost a main effect of Strain ($p=0.056$). Overall, the *APOE*4 mice seemed to spend more time in the center than the *APOE*3 mice, and exercise with or without antioxidants increased that time though only significantly in the *APOE*3 group. Interestingly while the horizontal activity of *APOE*3 and E4 mice did not differ from that of the wild-type mice, the transgenic mice exhibited significantly less rearing counts than the wild-type mice ($p<0.05$).

Coordinated running

The performance of the mice as measured by latency to fall from an accelerated rotating rod as a function of coordinated motor learning and maximum running performance are shown in Figure 3. All groups improved performance over sessions supported by a significant effect of Session ($p< 0.01$). However, *APOE*4 mice had ~ 30% shorter latencies that the *APOE*3 mice (Figure 3A), which was supported by a significant Session and Strain interaction ($p< 0.001$) and an overall main effect ($p<0.01$). The mice receiving the treatment were taking longer latencies to fall from the rotating rod than the SedCon in both strains, though it seems that the differences were more pronounced during the learning phase (Sessions 1 through 3) than when a plateau was reached. An ANOVA done with all 7 sessions did not reveal any significant effect though the main effect of Treatment was close ($p=0.067$). The data were further scrutinized by analyzing the effects on the average performance during the learning phase (Figure 3B) and when the mice reached a plateau of performance (Figure 3C). In *APOE*3 mice, all treatments improved the performance of the mice while only exercise alone improved it in the *APOE*4 group. A two-way ANOVA yielded a significant effect of Treatment and Strain (all ps <0.05) but not interaction between the two factors ($p=0.485$). Upon reaching a plateau of performance, the *APOE*4 still had lower latencies than their *APOE*3 counterparts ($p<0.05$), however there was no significant effect of Treatments ($p=0.16$).

Reflexive musculoskeletal responses

Performance was measured daily and averaged over 4 sessions, and presented in Figures 4 and 5. The SedCon *APOE*4 mice took longer latencies to initiate walking when compared the SedCon *APOE*3, however there was no main effect of Strain ($p=0.126$). Only the SedEC mice had shorter latencies than the SedCon in the *APOE*4 group, while there were no observable

differences in the *APOE*3 group. A two-way ANOVA did not yield a main effect of Treatment or an interaction between Strain and Treatment (all *p*s>0.142). The latency to turn in a dead-end alley was affected differently by treatments depending on the strain, as supported by a significant interaction between Strain and Treatment (p=0.006). This interaction was mainly driven by the ExEC group in the *APOE*4 mice that exhibited higher latencies that the other groups. Overall, the *APOE*4 mice took longer latencies to turn when compared to the *APOE*3 mice, supported by a main effect of Strain (p<0.01). The initiation of negative geotaxis was affected by Strain, with the *APOE*4 mice taking 60% longer latencies that their *APOE*3 counterparts. The ExCon and ExEC group took shorter latencies to turn, though only the ExCon was significantly different from the SedCon in the *APOE*4 group. A two-way ANOVA revealed a main effect of Strain (p<0.01), and a close to significance effect of Treatment (p=0.055) but not interaction between the two factors (p=0.757).

When suspended from the wire, the latency to tread (reflex) and the latency to fall were measured and are presented in Figure 5. Latency to tread was longer in *APOE*4 than *APOE*3 mice, supported by a main effect of Strain (p<0.01). The SedEC *APOE*4 mice had latencies 45% shorter than the SedCon (p<0.05), but did not differ in the *APOE*3 group. While the ExCon group had shorter latencies in both *APOE*3 and *APOE*4, these differences did not reach significance. The ExEC groups did not differ from the SedCon in either strain. A two-way ANOVA did not yield a main effect of Treatment or an interaction between Treatment and Strain (all *p*s>0.156). Latency to fall from the wire seemed to be shorter in the *APOE*4 group than the *APOE*3, however a two-way ANOVA did not yield a significant effect of Strain (p=0.091). Only the ExCon group in the *APOE*4 mice had significantly longer latencies than the SedCon. A two-

way ANOVA did not yield a main effect of Treatment or a Strain by Treatment interaction (all *p*s> 0.074).

Bridge walking

The latency to fall from a high bridge were averaged over the 4 sessions and analyzed, but also analyzed across increasing level of difficulty are shown in Figure 6. Overall, the *APOE*4 mice fell faster from the bridge, but effects of treatments seem marginal (Figure 6A). A two-way ANOVA revealed a main effect of Strain (p=0.018), but no effect of Treatment or an interaction between Strain and Treatment (all *p*s>0.058). The data were further analyzed across sessions and presented in Figure 6B. As the level of difficulty increased, the latencies to fall from the bridge were shorter, as supported by a significant effect of Session (p<0.01). While there were little differences between any of the groups during sessions 1 and 2, effects of strain and treatment were observable on session 3 and 4 (p=0.032). Even though a main effect of treatment did not reach significance (p=0.076), individual comparisons supported that ExCon and ExEC took 7-9% longer latencies to fall from the bridge on session 3. On session 4, *APOE*3 mice performed better than *APOE*4, supported by a main effect of Strain (p=0.02). The ExCon and ExEC mice took 12-17% longer latencies than the SedCon in the *APOE*4 mice, however only the ExEC came close to significance (p=0.059).

DISCUSSION

The main findings of this study were , 1) *APOE*3 and *APOE*4 mice exhibited differential motor phenotypes; 2) *APOE*4 mice had impaired motor learning and musculoskeletal reflexes, and decreased strength; 3) Exercise only affected spontaneous activity, improved coordinated running in both strains; 4) Exercise improved reflexes and increased strength in *APOE*4 mice, while no such effect was observed in *APOE*3 mice; 5) Antagonistic effects of antioxidants on the beneficial effects of exercise were observed in tests for strength and coordination in *APOE*4 mice.

Though cognition is mainly the focus of research and management in AD, motor symptoms are equally necessary to study. Motor function disability and inability to learn new motor skills has been established in AD (1-4). As a major genetic risk factor for development of AD, *APOE*4 is associated with acceleration in the cognitive decline. Similarly, *APOE*4 has been correlated with global motor function decline (14). The brain regions involved in the motor control systems, namely motor cortex, basal ganglia, and cerebellum govern (5-8) initiation, planning and execution of motor function. Pathological changes occurring in these brain regions could be responsible for motor function decline seen in AD (9-11). Our study set out to determine whether *APOE*4 was indeed associated with motor declines and whether they could be reversed with the use of antioxidant or exercise regimen.

Horizontal and spatial components of spontaneous activity were unaffected by the presence of human *APOE* while rearing was significantly diminished. Similar reports have been made in other studies using NSE-*APOE* mouse model (52) and GFAP-*APOE* mouse model (53). Interestingly, exercise lowered horizontal and vertical activity in the *APOE*4 mice. Furthermore, the ExCon or ExEC mice seemed to spent more time in the center of the apparatus, reflecting a

lower degree of anxiety. All these observations associated with exercise could be related to lesser anxiety among these mice as reported previously (48).

The *APOE*4 mice had impaired motor learning and coordination, with lower initial performance, slower slope of learning and lower maximum performance being reached compared to the *APOE*3 mice. This is in accordance with previous study by Meer et.al.(54), that has also reported motor impairment associated with the presence of *APOE*4. The cerebellum is a brain region which is responsible for balance and coordination function. Loss of distal dendritic segments along with decreased total number of dendritic spines in the Purkinje cells in cerebellum was reported in AD (12,13). This could be the explanation for the deficit in coordination, and balance AD and might also be aggravated in presence of *APOE*4 allele.

While antioxidant treatment had a marginal effect on coordination in the *APOE*3 mice, the exercise treatment was more successful at improving coordination and balance in this study. These indicate that exercise may influence cerebellar components (55).

Muscular reflex, plantar responses and muscle tone deficiencies have been studied for many years as cognition independent neurologic symptoms in AD (56). We observed that *APOE*4 mice had poorer musculoskeletal reflexes compared to the *APOE*3 mice. Treatments had a differential influence based on genotype and the test in use. Antioxidants alone quicken the walk initiation and tread reflexes only in *APOE*4 mice. While, exercise alone increased muscle strength and make the negative geotaxis reflex faster in *APOE*4 mice. Interestingly, combination of antioxidants and exercise failed to improve any motor function in both genotypes.

After carefully considering the treatment effects, it was evident that the combination of exercise and antioxidants did not have any synergistic or additive effect; but rather an antagonistic effect across all of the motor function tests performed. In *APOE*3 mice, exercise

decreased rearing activity and improved latency to fall on high bridge walk test was nullified with (the addition antioxidants) ExEC treatments. Similarly, among *APOE*4 mice, exercise led lowered spontaneous locomotion, improved learning phase latency in coordinated running performance task, faster reflexes achieved in negative geotaxis test and lastly improved muscle strength in wire suspension test became not significant with ExEC treatment. Further, antioxidant treatment directed improved treading reflex and shorter spontaneous walk initiation were cancelled with combination of antioxidants and exercise.

Our study supported the previous reports of *APOE*4 mice exhibiting motor function deficits, which could be an early indicator of AD among people with *APOE*4 genotype. Overall, exercise was the only life style modification that could successfully ameliorate most of the components of motor decline associated with *APOE*4 at an early age. Most importantly, antioxidant supplementation antagonized the beneficial effects of exercise on motor function in APOE4 mice. Therefore, one should be careful when implementing lifestyle changes to fend off the effects of aging and risk for neurodegenerative diseases.

FIGURES AND LEGENDS

Figure 1: Effect of exercise and/or antioxidant regimen on body weight (A) and food intake (B) in adult GFAP-*APOE*3 and GFAP-*APOE*4 mice; *APOE*3 (left panel) and *APOE*4 (right panel).

Each value represents mean ± SEM, n=9-21 for body weights and n=8-12 for food intake. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C; *$p<0.05$ compared to SedCon.

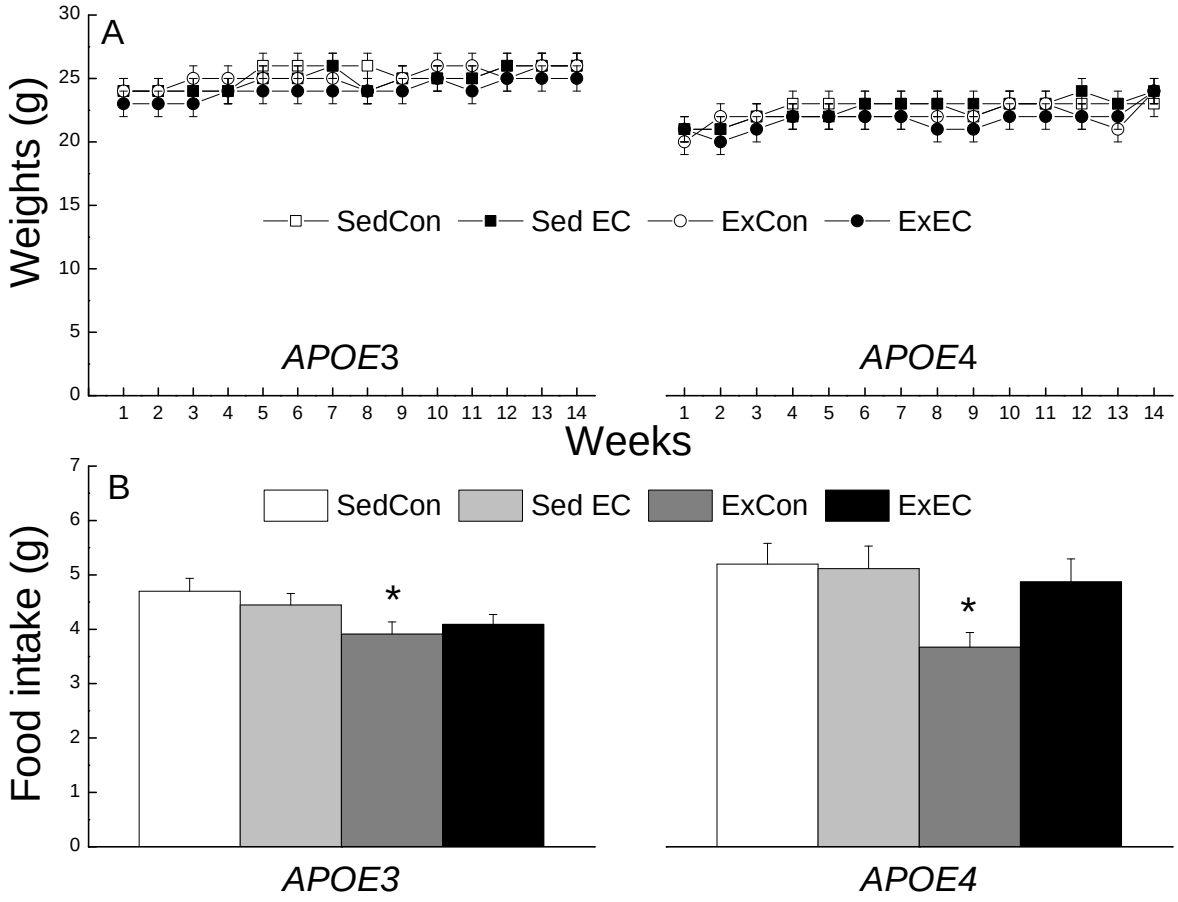

A
Weights (g)
SedCon
Sed EC
ExCon
ExEC
APOE3
APOE4
Weeks
B
Food intake (g)
SedCon
Sed EC
ExCon
ExEC
*
*
APOE3
APOE4

Figure 2: Effect of exercise and/or antioxidant regimen on locomotor activity as measured by (A) total distance travelled, (B) rearing activity, and (C) time spent in the center in adult GFAP-*APOE*3 (left panel), GFAP-*APOE*4 (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=19-21. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE3;* *$p<0.05$ compared to SedCon; †$p<0.05$ compared to genotype-matched SedEC; +$p<0.05$ compared to SedCon wild-type.

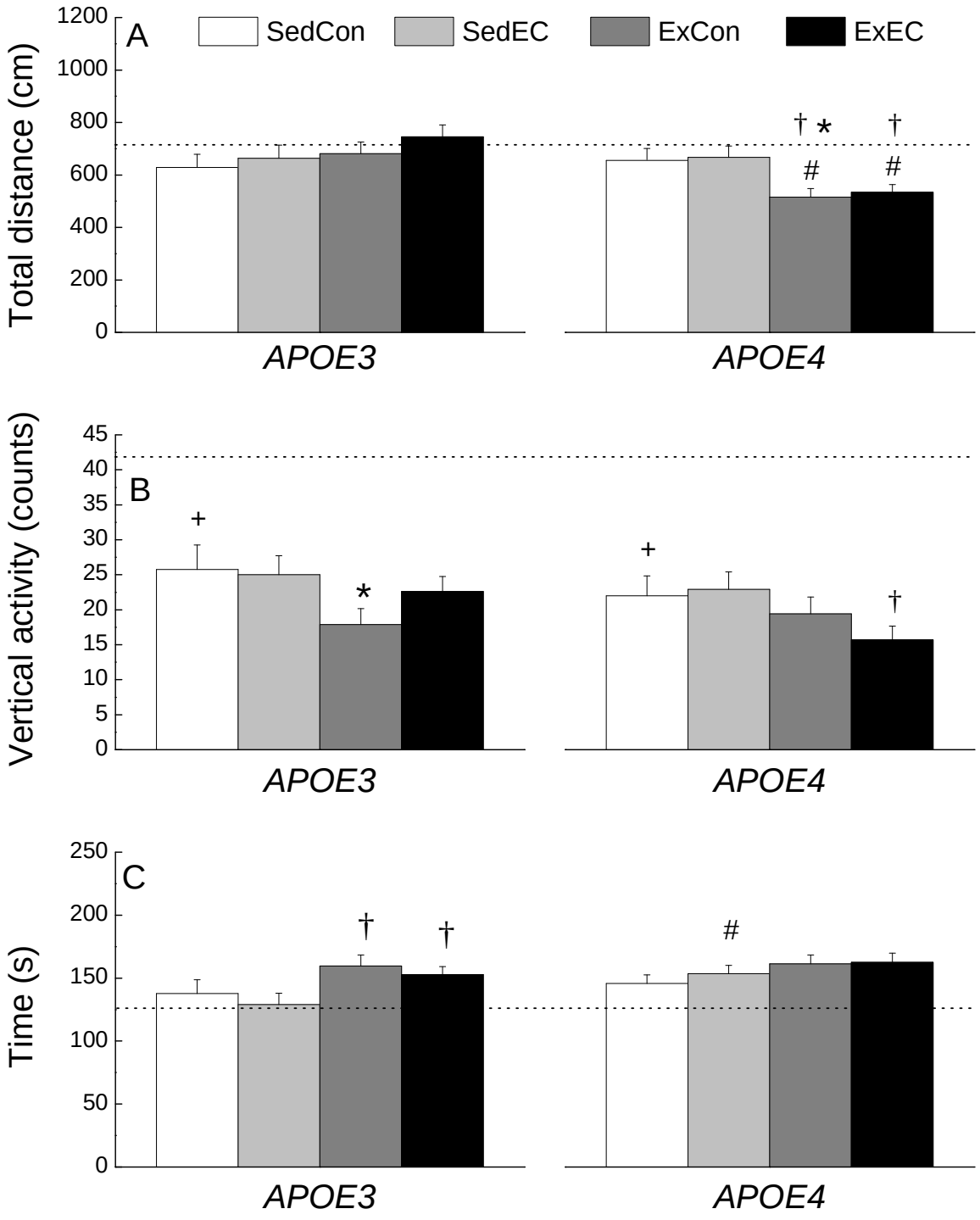

A
SedCon
SedEC
ExCon
ExEC
Total distance (cm)
1200
1000
800
600
400
200
0
† *
†
#
#
APOE3
APOE4
B
Vertical activity (counts)
45
40
35
30
25
20
15
10
5
0
+
*
+
†
APOE3
APOE4
C
Time (s)
250
200
150
100
50
0
†
†
#
APOE3
APOE4

Figure 3: Effect of exercise and/or antioxidant regimen on coordinated running performance as measured by latency to fall from accelerated rotorod across sessions (A), learning phase (B) and when reaching a criterion of plateau performance (C) in adult GFAP-*APOE*3 (left panel), GFAP-*APOE*4 (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=19-21. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE3;*$*p<0.05$ compared to SedCon; $+p<0.05$ compared to WT Sedcon.

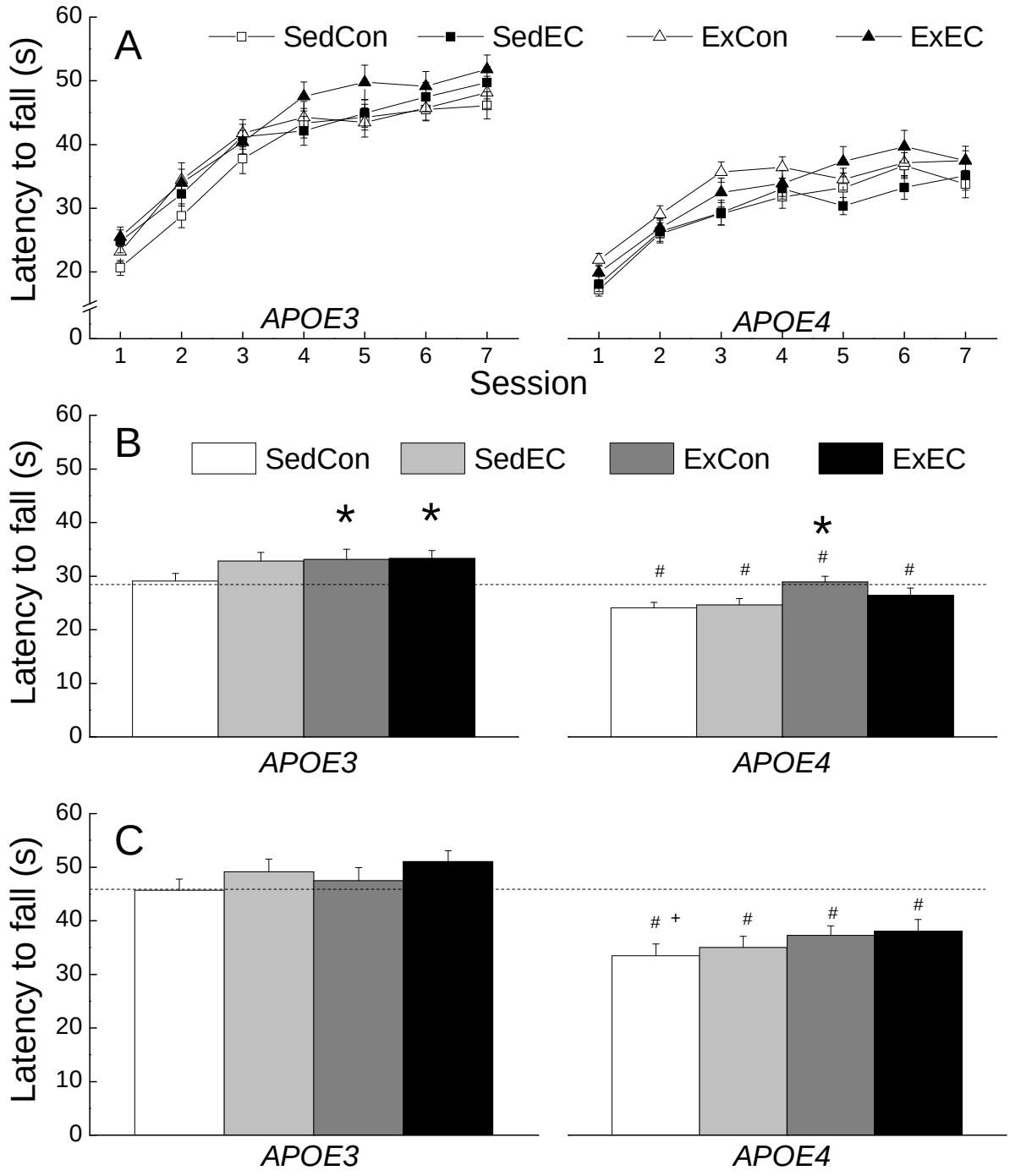

A
SedCon
SedEC
ExCon
ExEC
Latency to fall (s)
APOE3
APOE4
Session
B
C
*
#
+

Figure 4: Effect of exercise and/or antioxidant regimen on musculoskeletal reflex responses measured as latency to initiate walking (A), to turn in a dead-end alley (B), and to turn 180 degrees (C) in adult GFAP-*APOE*3 (left panel), GFAP-*APOE*4 (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=19-21. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE3;*$p<0.05$ compared to SedCon; +$p<0.05$ compared to WT Sedcon; †$p<0.05$ compared to SedEC.

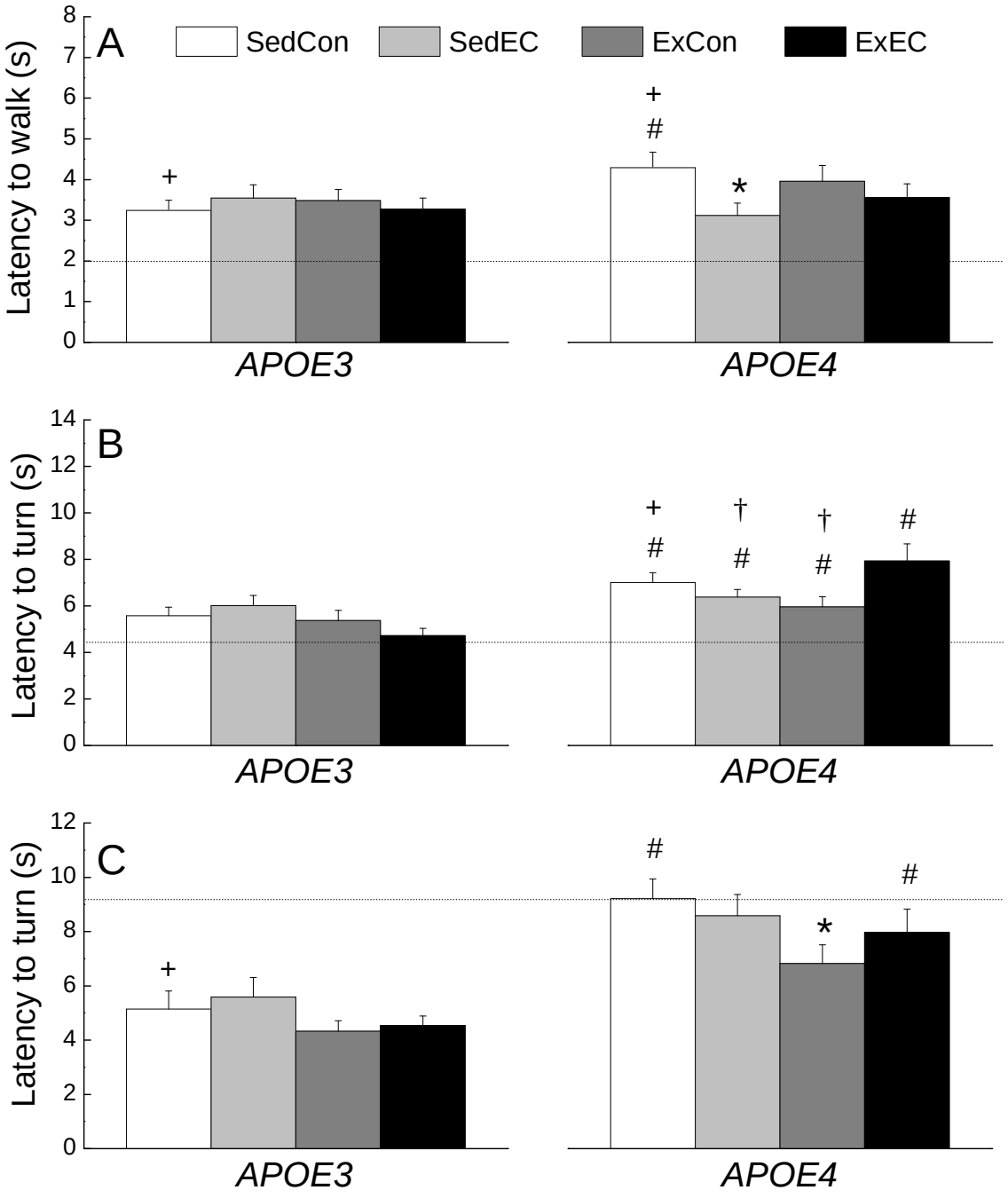

A
SedCon
SedEC
ExCon
ExEC
Latency to walk (s)
APOE3
APOE4
B
Latency to turn (s)
APOE3
APOE4
C
Latency to turn (s)
APOE3
APOE4

Figure 5: Effect of exercise and/or antioxidant regimen on reflex measured as latency to tread (A) and fall (B) from n a wire in adult GFAP-*APOE*3 (left panel), GFAP-*APOE*4 (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=19-21. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE3;**$p<0.05$ compared to SedCon; +$p<0.05$ compared to WT Sedcon; †$p<0.05$ compared to SedEC.

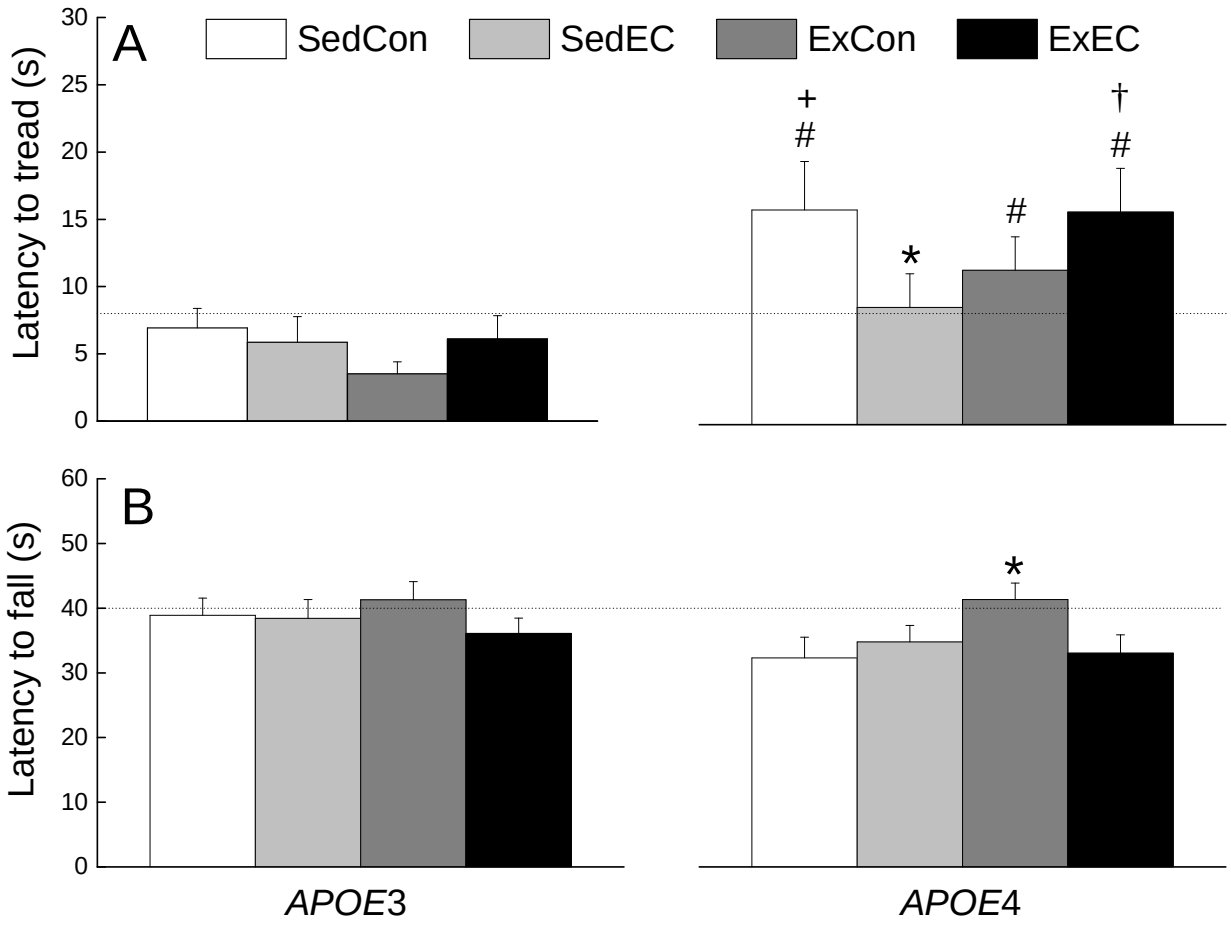
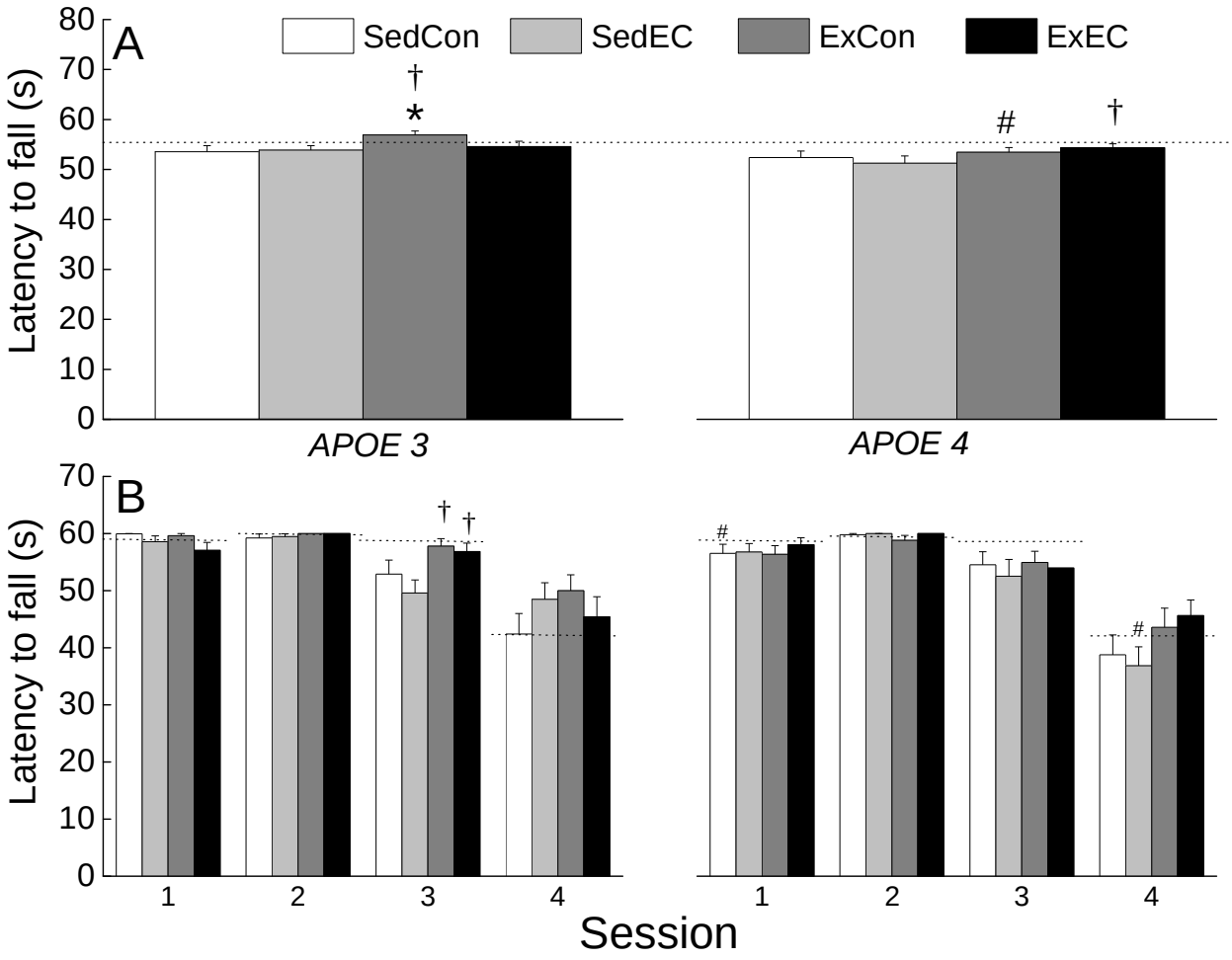

A
SedCon
SedEC
ExCon
ExEC
Latency to tread (s)
30
25
20
15
10
5
0
+
#
*
#
†
#
B
Latency to fall (s)
60
50
40
30
20
10
0
*
APOE3
APOE4

Figure 6: Effect of exercise and/or antioxidant regimen on balance as measured by latency to fall (in seconds) from elevated bridge in adult GFAP-*APOE*3 (left panel) and GFAP-*APOE*4 (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=18-21. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. #$p<0.05$ compared to treatment matched *APOE3;*$p<0.05$ compared to SedCon; +$p<0.05$ compared to WT Sedcon; †$p<0.05$ compared to SedEC.


A
SedCon
SedEC
ExCon
ExEC
Latency to fall (s)
80
70
60
50
40
30
20
10
0
†
*
#
†
APOE 3
APOE 4
B
Latency to fall (s)
70
60
50
40
30
20
10
0
† †
#
#
1
2
3
4
1
2
3
4
Session

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CHAPTER 4

GENOTYPE-DEPENDENT COGNITIVE RESPONSE OF EXERCISE TRAINING AND ANTIOXIDANT SUPPLEM IN GFAP-*APOE* MICE

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Running title: Lifestyle modification improve brain function in GFAP-APOE mice

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ABSTRACT

This study determined the effect of antioxidant supplementation and or moderate exercise on enhancing the cognitive and motor performance in middle age mice in the context of apolipoprotein E (*APOE*) genotype.

Starting at the age of 12 months, separate groups of male and female mice expressing the human E3 (GFAP-*APOE*3) or E4 (GFAP-*APOE*3) were fed either a control diet or diet supplemented with vitamins E and C. The mice were further separated into a sedentary group or a group that followed a daily exercise regimen. After 8 weeks on the treatments, the mice were administered a battery of functional tests including tests to measure reflex and motor, cognitive, and affective function. Subsequently, brain regions were biochemically assessed for catalase activity, and expression of BDNF, TrkA, Erk5, Erk1/2, and pErk1/2.

The middle age mice expressed genotype effect with poor spatial learning and memory in APOE4 mice reversed with exercise and combination of treatments. Though there is no overall genotype effect observed in the discriminated avoidance task, treatments improved the performance in both genotypes for the discrimination as well as avoidance component. Overall, exercise containing treatments improved performance of *APOE*3 mice on various aspects of the active avoidance task. Antioxidant supplementation improved performance only in the *APOE*4 mice. *APOE*4 has slower reflexes, weaker muscle strength and poor coordinated running performance. None of these deficits were reversed with treatments. While, only *APOE*3 mice benefitted from treatments in a balance function test. Exercise was the most effective treatment at improving cognitive function in both genotypes, while antioxidants seemed to be effective only in the *APOE*4. In middle age mice only spatial and non-spatial cognitive function were improved. The combination of the two treatments yielded further improvement in cognition only

on the active avoidance test, and there was no antagonistic action of the antioxidant supplementation on the beneficial effects of exercise.

Keywords: APOE, exercise, antioxidants, cognition, motor, vitamin E, vitamin C

INTRODUCTION

In human, apolipoprotein E (*APOE*) exists in three major isoforms E2, E3 and E4. Of the three isoforms, *APOE4* allele is the most prevalent and well established genetic risk factor for development of late-onset sporadic Alzheimer's disease (AD) (1-3). Especially, in the middle-aged and old aged individuals, *APOE4* has been associated with cognitive deterioration and memory loss (4,5) and exacerbated cognitive declines in non-AD dementia (6-8). This is supported by deficits in spatial learning and memory - a function of hippocampus (9), profound working memory impairments - a function of cortex and hippocampus (10) as well as increased anxiety – a function of amygdala (11,12) in mice expressing human *APOE4*.

Apart from cognitive decline, motor function disability and inability to learn new motor skills is substantiated in AD patients (13-16). Interestingly, similar to the acceleration in the cognitive decline, the presence of *APOE*4 allele was associated with a two-fold increase in the rate of global motor function decline with comparable age, sex and education (17). The motor control systems situated in multiple interconnected cortical and subcortical motor regions, namely motor cortex, basal ganglia, and cerebellum (18-21) regulates the initiation, planning and execution of motor function. Pathological changes occurring in these brain regions (motor cortex, striatum, substantia nigra) might be responsible for motor function decline in AD (22-24). Further, Purkinje cells in cerebellum are involved in AD with loss of distal dendritic segments and decreased total number of dendritic spines (25,26). This could explain the coordination, balance dysfunction and injurious fall tendency in initial stages of AD. Risk factors predisposing and aggravating AD might also exacerbate these motor symptoms along with accelerated cognitive pathology.

Oxidative stress is a major contributor to AD pathology. The brain of AD patient is more vulnerable to accumulation of oxidative stress with outnumbered antioxidant defenses as evidenced in animal models and humans (27,28). *APOE*4 is present in approximately 36% cases of AD, and there is evidence of *APOE4* associated aggravation of AD pathophysiology through an increased oxidative stress (29,30). Hence, AD symptomology, especially, in the presence of *APOE*4 allele would respond to oxidative stress neutralizing therapy as observed with improved cognition with vitamin E supplementation alone (31,32) or with combination of antioxidants (33,34).

Apart from antioxidants, another life style modification therapy such as physical activity / exercise reduced the risk of AD (35-37), delayed onset (38), and improved AD symptoms (39). Further, there is a potential beneficial interaction between *APOE4* genotype and exercise in the context of aging and cognition (40,41). Exercise training also lowered oxidative stress (42-45) and improved cognition (46,47). In addition to cognition, exercise reduced anxiety in elderly (75 years age) (48) and in rats by lowering oxidative stress (49). Interestingly, various exercise regimes improve motor function in cognitively impaired geriatric population (50-52). The rate of injurious fall has been dramatically reduced among such AD patients with motor training (53-55). With these evidences and the probable common mechanism of action, it can be hypothesized that combining antioxidant with exercise training will lead to a synergistic or additive beneficial effect (56-59), an approach often employed by health conscious individuals and healthcare professionals and a target for further research.

However, the occurrences of such plausible benefits and their underlying mechanisms have not been fully explored. Previous studies have indicated age and exercise intensity as predictors in outcome of the interactive effects of the combination of exercise and antioxidant

supplementation. Antioxidants lowers high intensity forced exercise induced oxidative stress (60). Further, the possibility of genetic make-up influencing the outcome measures of such combinational interventions as an important deciding factor in promoting such therapeutic approach has not been evaluated. *APOE* ε4 allele carriers, with higher oxidative stress levels might be the major benefactor of such therapy.

Despite previous studies on antioxidant intake and exercise training, there are no data available evaluating the effect of these factors in two genotypes in middle aged mice within the same study. The goals of the current study were to determine 1) cognitive, motor and anxiety phenotypes of the middle aged GFAP-*APOE*3 and E4 mice; 2) whether antioxidant intake and exercise training led to beneficial improvements; 3) whether the combination of antioxidant and exercise yielded additive beneficial effect; 4) the involvement of catalase, BDNF and Erk pathway in the potential improvements.

The outcomes are important in deciding the need for antioxidant supplementation in exercising individuals, and a guiding parameter in genotype based requirement.

MATERIAL AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at the UNT Texas Health Science Center at Fort Worth. Separate groups of male and female GFAP-*APOE**3 (B6.Cg-Tg(GFAP-*APOE**3)37Hol *APOE*tm1Unc/J) and GFAP-*APOE**4 (B6.Cg-Tg(GFAP-*APOE**4)1Hol *APOE*tm1Unc/J) mice were obtained from Jackson Laboratories (catalog numbers 004633 and 004631; total n of 180) at the age of 2 months and subsequently maintained in the UNT Health Science Center vivarium. The mice were housed in groups of 3 or 4 in standard polycarbonate cages (28 × 17 × 12.5 cm) with corncob bedding and ad libitum access to food and water, and were maintained at ambient temperature (23 ± 1 °C), under a 12-h light/dark cycle starting at 06:00. The mice were weighed weekly, and survival was monitored throughout the study. The mice were aged until they reached 12 months of age, and were then assigned to an experimental treatment group. A group of male and female C57BL/6 mice (wild-type, n=12/ group) was also aged to 12 months and used as a control to compare the *APOE*3 and *E*4 controls to determine whether the behavioral differences between *APOE*3 and *E*4 were due to an altered phenotype of the transgenic mice.

Treatment

Upon arrival, the mice were randomly assigned to one of four experimental groups: (1) sedentary fed the control diet (SedCon), (2) sedentary fed the vitamins E and C supplemented diet (SedEC), (3) forced exercise fed the control diet (ExCon), (4) forced exercise fed the vitamins E and C supplemented diet (ExEC). Each experimental group was balanced for sex of the mice.

The mice were fed, ad libitum, either a control diet (LabDiet® R&M 5LG6 4F, cat #: 5S84) or the control diet supplemented with vitamins E and C (modified 5LG6 with1.65 mg/g diet of ascorbic acid and 1.12 IU/g diet of α-tocopheryl acetate, cat#: 5SH0). Furthermore, the mice were either sedentary or following a moderate exercise regimen. The moderate exercise regimen was introduced progressively using treadmills (AccuPacer Treadmill; Omnitech Electronics Inc., Columbus, OH, USA). Over a 12-day period, the training was gradually incremented in time and speed to reach a maximal exercise of 1 h (6, 8, 10, and 12 m/min for 5 min each, and then at 14 m/min for 40 min). The training protocol used was a modification of previously published exercise protocols. Forced exercise was implemented via transient 0.29 mA electric foot shock to the feet. Each exercise mouse was paired with a control which received the same number of shock for each training day.

Food intake

Food intake was monitored three times during the course of the study: during the first week of treatment; a week prior to the start of the behavioral testing and after the completion of the behavioral testing. Each time point was an average of food consumption during five consecutive days measured at the same time of day to control for any diurnal variation. The data was presented as the averaged over the 3 time points.

Functional Testing

The mice were on their respective treatments for 8 weeks prior to and throughout behavioral assessments for a total of 16 weeks. The mice received a series of behavioral tests in the following order: elevated plus maze, spontaneous activity, coordinated running, reflexes, wire suspension, bridge walking, Morris water maze, active avoidance. The mice were 14 months of age when behavioral testing started, and 16 months when they were euthanized.

Elevated plus maze

To measure anxiety, an elevated plus maze test was conducted using a plus maze elevated three feet above the floor in a dimly lit test room (60 W) consisting of two arms opened to the room and two arms enclosed such that the floor is not visible. A computerized tracking system was used to monitor the position of the mice in the maze (Any-maze). The mice were positioned in the center of the plus facing an open arm and were given 5 min to explore the maze. The amount of time spent in the closed vs. open arms was recorded.

Locomotor activity

Spontaneous locomotor activity was measured using a Digiscan apparatus (Omnitech Electronics, model RXYZCM-16), as described previously (61). Each mouse was placed in a clear acrylic test cage (40.5×40.5×30.5 cm) that was surrounded by a metal frame lined with photocells. The test cage was enclosed in a dimly lit, sound-attenuating chamber equipped with a fan that provided background noise (80 dB). Each mouse was placed in a chamber for 4 consecutive sessions, each 4 minutes in duration for a total of 16 minutes. During a 16-min period, movements in the horizontal plane, as well as a vertical plane 7.6 cm above the floor, were detected by the photocells and processed by a software program to yield different variables describing distance, vertical, and spatial components of spontaneous activity in the apparatus.

Coordinated running

Motor learning and maximum running performance were measured using an accelerating rotorod test described previously (62). The apparatus was a motor-driven treadmill (Omnitech Electronics, Model # AIO411RRT525M) that consisted of a 3-cm diameter nylon cylinder mounted horizontally at a height of 35 cm above a padded surface. On a given trial, the mouse was placed on the cylinder, which then began rotating with increasing speed until the animal fell

to a well-padded surface. Ability of the mice to improve running performance was assessed in a series of training sessions (two per day), each consisting of four trials at 10-min intervals. The training sessions continued until the running performance (the average latency to fall from the cylinder) failed to show improvement over three consecutive sessions. The treatment groups were compared for their average latency to fall on the first seven sessions and for the final session on which each mouse had reached its maximum stable level of performance.

Reflexive musculoskeletal responses

Over four consecutive daily sessions, the mice were administered three simple reflex tests. The first test consisted of placing the mouse on a flat smooth surface and recording the latency to move one body length (walk initiation). The second test measured the latency to reverse direction when the mouse was placed in a 3.5-cm wide, 14-cm long, dead-end alley (alley turning). For the third test, the mouse was placed facing downward on a flat surface that was tilted 45°, and the latency to turn 180° in either direction was measured (negative geotaxis). The mouse was allowed to grip a horizontal wire with the front paws when suspended 27 cm above a padded surface (wire suspension). The latency to tread (reach the wire with their hind legs) and the latency to fall were recorded and averaged over four consecutive daily sessions (two trials /day).

Bridge walking

Each mouse was tested for the latency to fall or reach a safe platform after being placed on one of four acrylic bridges, each mounted 50 cm above a padded surface. The bridges differed in diameter (small or large) and shape (round or square), providing four levels of difficulty. Each bridge was presented three times, and the principal measure was the latency to fall (maximum of 60s), either examined as the average latency to fall (of three trials) for each bridge individually, or a single overall mean representing the average latency to fall from all four types of bridges.

Morris water maze (MWM)

Spatial learning and memory were measured using an MWM test slightly modified from described previously (55). On a given trial, the mouse was allowed to swim in a tank filled with opacified water and maintained at 24 ± 1 °C. The mice were able to escape the water by means of a hidden platform (1.5 cm below the surface of the water). A computerized tracking system recorded various measures such as path length and swimming speed (Any-maze; Stoelting Co., Wood Dale, IL, USA).

The test consisted of four phases: (1) pre-training phase: the tank was covered by a black curtain to hide surrounding visual cues. The mice learned the components of swimming and climbing onto a platform using a straight alley that had a platform at one end. The mice were allowed to swim until they reached the platform or a maximum of 60 s had elapsed. The mice received two sessions consisting of five trials with an intertrial interval of 5 min; (2) acquisition phase: the black curtain was removed and the mice were tested for their ability to locate a hidden platform using spatial cues around the room. Each daily session consisted of five trials, at 2-min intervals, during which the mouse had to swim to the platform from one of four different starting points in the tank. The mice were allowed to swim until they reached the platform or a maximum of 90 s had elapsed. Testing was conducted over nine sessions (Tuesday-Friday and Monday–Friday). On sessions 2, 4, 5, 7, and 9, a probe trial was conducted as the fifth trial during which the platform was submerged to a depth that prevented the mice from climbing onto it. The platform was raised after 30 s, and the trial was ended when the mouse successfully located it; (3) retention phase: one 60-s probe trial session was conducted 1 week after the ninth session of the previous phase; (4) visible platform phase: the mice were given a total of eight sessions (2/day separated by 2 h), each consisting of five trials with a 10-min inter-trial interval. The

platform was identified by a triangular flag that was raised above the surface of the water. On each trial the mouse had to swim to the platform from a different starting point and the platform was moved to a different location before each trial. Thus, the mouse had to learn to associate the location of the flag with location of the platform.

Path length (distance taken to reach the platform) and latency (time taken to reach the platform) over sessions were used as the primary measure of performance. The path-independent swim speed was calculated by dividing distance by the latency to reach the platform. On probe trial, spatial bias for the platform location was evaluated in terms of the percentage of time spent within a 40-cm diameter annulus surrounding the platform location.

Discriminated avoidance

A T-maze constructed of acrylic (black for the sides and clear for the top) was utilized for the discriminated avoidance task. The maze was divided into three compartments: a start box (10 × 6.3 × 6 cm), a stem (17.5 × 6.3 × 6 cm), and two goal arms (14.5 × 6.3 × 6 cm), each separated by clear acrylic doors. The maze rested on a grid floor wired to deliver 0.69-mA scrambled shock to the feet. The test consisted of three sessions separated by 1 h. On each training trial, the mouse was placed in the start box, and the start door was removed to signal the beginning of the trial. On the first trial of the first session (information trial), the mouse received shock in the first arm entered (preference arm) and was permitted to escape shock by running to the opposite arm, which was then designated the correct arm for the remainder of the session. On subsequent trials, shock was initiated 5 s after the opening of the start door if the mouse had not entered the correct goal arm or immediately upon entry into the incorrect arm. In either case, the shock continued until the correct goal arm was entered or a maximum of 60 s had elapsed. Upon the mouse's entry into the correct arm, the door was closed (to prevent departure), and, after 10 s, the mouse

was removed (by detaching the goal arm) and allowed to enter a holding cage for 1 min. Training in this fashion continued at 1-min intervals until the mouse had met the criterion of a correct avoidance (defined as running directly to the correct arm within 5 s) on four of the last five training trials of which the last two must be within 5 s. The second session of avoidance training was a reversal such that the mice were required to run to the goal arm opposite that to which they had been trained on the previous session. Two measures were considered to show the ability of the mice to learn the discrimination and avoidance components of the task. Their ability to learn was considered inversely proportional to the number of trials required to reach the avoidance criterion aforementioned and the number of trials required to reach the discrimination criterion (4 out 5 correct turns regardless of the time taken).

Biochemical measurements

Catalase Activity

The activity of the antioxidative enzyme, catalase, was evaluated using the Cayman Chemical Catalase Assay Kit (chemical Item Number 707002). Methanol reacted with catalase in the presence of optimal concentration of H_2O_2. The production of the resulting formaldehyde was measured colorimetrically with Purpald (4-amino-3-hydrazino- 5-mercapto-1,2,4-triazole) at 540 nm (63,64).

Western Blot

Each mouse brain was dissected into six regions: cerebral cortex, cerebellum, midbrain, brain stem, striatum, and hippocampus. Each brain region was homogenized in phosphate buffer (50 mM potassium phosphate, pH 7.0, containing 1mM EDTA) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) and 0.1% Triton-X 100. Homogenates were spun at 10,000 x g for 15 min; the pellets were discarded and the supernatant was used. After a BCA

protein assay, the homogenates were resolved by SDS-PAGE (Mini-PROTEAN® TGX ™ Gels, 4-20%w/v Bio-Rad, Richmond, CA) followed by electrophoretic gel transfer to nitrocellulose membranes (Pierce, Rockford, IL) with a Mini-Trans-Blot electrophoretic transfer cell (Bio-Rad, Richmond, CA). Transfer onto membrane was carried out at 100 V for 100min at 4°C in a buffer containing 25 mM Tris, 192 mM glycine, 10% methanol (v/v), pH 8.3. The blots were incubated in 5% nonfat dried milk (w/v) at room temperature for 2 h, followed by 10 min washing, with Tris-buffered saline that contained 0.1% Tween-20 (TBST). Blots were then incubated overnight at 4°C with either anti-BDNF antibodies (1:200, sc-546, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Erk1/2 antibodies (1:200, sc-93 and sc-154, Santa Cruz Biotechnology, Santa Cruz, CA), or anti- Erk5 antibodies (1:1000, #3372, Cell Signaling) or anti-TrkA antibodies (1:1000, #2505, Cell Signaling) or anti-pErk1/2 antibodies (1:1000, #9106, Cell Signaling) in TBST containing 0.1% nonfact dried milk (w/v). The primary antibody was removed and the blots were washed three times with TBST. The blots were then incubated in either horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, sc-2004, Santa Cruz Biotechnology, Santa Cruz, CA) or horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000, sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA) in TBST containing 0.1% nonfat dried milk for at least 2 h at room temperature. After washing the blots with TBST five times (7 min each), proteins were visualized with an enhanced chemiluminescence kit (Amersham™, ECL™ Western Blotting Detection Reagents, GE Healthcare, Buckinghamshire, UK). The blots were stripped by incubating the blots with stripping buffer (100 mM 2-Mercaptoethanol, 2% SDS, 62.5 mM Tri-HCL pH 6.7) at RT for 20 min. After washing the blots with TBST thrice (10 min each), the blots were blocked and probed in the same manner as stated above. Anti-actin (1:1000, sc-1616 Santa Cruz Biotechnology, Santa Cruz, CA) was used as primary antibody and horseradish

peroxidase-conjugated bovine anti-goat IgG (1: 5000, sc-2350, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the secondary antibody. All immunoblot images were scanned by Bio Spectrum Imaging System, (^{U}UVP - Ultra-Violet Products Ltd, Cambridge, UK) and densitometric quantifications were performed using VisionWorksLS Image Acquisition and Analysis Software). Actin intensity was used to normalize protein intensity.

Statistical analysis

Functional performance of the mice on the behavioral tests as well as the biochemical measurements were assessed using two-way analyses of variance (ANOVA) with Genotype and Treatment as between-group factors. Planned individual comparisons between different genotype groups (*E*3 vs. *E*4) and treatment groups (SedCon vs. SedEC vs. ExCon vs. ExEC) were performed using a single degree-of-freedom F tests involving the error term from the overall ANOVA. Some behavioral performances, catalase and BDNF were also considered in three-way with Session or Brain Region as the repeated measure. The effects of strain within the SedCon groups were analyzed using a one-way ANOVA with Strain (wild-type vs. *E*3 vs. *E*4) as a factor. Planned individual comparisons were performed using a single degree-of-freedom F tests involving the error term from the overall ANOVA. Pooling male and female data was not responsible for driving any of the main results. The α level was set at 0.05 for all analyses. The software used for the analyses was Systat 13 (Systat Software Inc., San Jose, CA, USA).

RESULTS

Body weight and food intake

Weekly body weights and average food intake are presented in Figure 1. Overall, the *APOE*3 and *APOE*4 mice weighed the same, supported by no main effect of Strain (p=0.245). However, each strain responded differently to the treatments, supported by a significant interaction between Strain and Treatment (p=0.029). In the *APOE*3 mice, the ExCon and ExEC mice seemed to weigh less than the controls once behavioral testing started (week 7). In the *APOE*4 group, the SedEC and ExEC mice weighed more than the other groups; however this difference was also present on week 1. Food intake was slightly higher in the SeEC *APOE*3 mice compared to the SedCon *APOE*3 ones, and the ExCon *APOE*4 mice ate less that their genotype-matched SedCon. These individual differences were not reflected by main effects as only an effect of Strain approached significance (p=0.086).

Elevated plus maze

The effect of Strain and Treatment were analyzed in terms of percent time spent in the closed and open arms of the plus maze as well as the distance travelled by the mice during the test (Figure 2). Overall, the *APOE*3 and *APOE*4 mice spent relatively the same amount of time in the closed and open arms, supported by a lack of a significant main effect of Strain (all ps>0.535). There were no significant effect of the treatments on these measures, supported by a lack of a significant effect of Treatment (all ps>0.185). It is noteworthy that interactions between Strain and Treatment approached significance (p=0.079 for open arms and p=0.063 for closed arms). The *APOE*3 mice receiving antioxidant and exercise treatments together spent less time in the open arms (p=0.091) leading to a significant genotype effect in this treatment group (p=0.043). The *APOE*4 mice being exercised only spent more time in the closed arms than their

controls (p=0.071). When compared to SedCon wild-type mice, the *APOE*4 mice spent 68% more time in the open arm (p=0.013) and consequently less time in the closed arms (p=0.02). The *APOE*3 mice did spend more time in the open arms but it did not reach significance (p=0.055).

Overall, the *APOE*3 mice moved around the plus maze more than the *APOE*4 mice, and treatments seem to affect that activity. These observations were supported by a two-way ANOVA revealing main effects of Strain and Treatment (all ps<0.01) without an interaction between Strain and Treatment (p=0.499). While all treatment groups containing exercise seemed to move around more, it was only significant in the *APOE*4 mice when comparing the ExEC and SedCon groups (p=0.004). When the data for time spent in open and closed arms were reanalyzed with distance as a covariate, the analysis for time spent is closed arm did not change, ruling out the involvement of motor activity on anxiety measure. There was no difference in activity when comparing the SedCon *APOE*3 and *E*4 mice to the SedCon wild-type ones (all ps>0.179).

Spontaneous locomotor activity

Horizontal distance (cm) and rearing (counts) were selected as measures of spontaneous locomotor activity (Figure 3). Overall, *APOE*4 mice had 20% lower rearing activity and 41% less distance travelled when compared to the *APOE*3 groups. There were no effects of treatment, antioxidant or exercise, on these measures. A two-way ANOVA resulted in significant main effects of Strain for both measures (p<0.05), and failed to indicated significant effects of Treatment or interactions of Strain and Treatment (all ps > 0.18). While the *APOE*3 mice travelled 25% longer distance than the wild-type, the *APOE*4 were 29% less active (all

*p*s<0.029). There was no difference between the *APOE* genotypes and the wild-type in their rearing counts (all *p*s >0.349).

Coordinated running performance

The effects of strain and treatment on the ability of the mice to learn coordinated running task and reach a criterion of stable running performance are indicated in Figure 4. There was an improvement of all groups of mice over a period of 7 sessions ($p<0.001$); however the *APOE*4 mice performed worse than the *APOE*3 mice. The *APOE*4 mice did not learn as well as the *APOE*3 over the 7 training sessions , which was supported by a significant Session by Strain interaction ($p<0.001$), and a main effect of Strain ($p<0.001$). There was no effect of the treatments on the performance of the mice either in *APOE*3 or *APOE*4 groups (all *p*s>0.134). When the mice reached criterion (Figure 4B), the latency to fall of the *APOE*4 mice from the rotating rod was reduced by 30% when compared to the *APOE*3 mice. Supplementation with antioxidants and/or exercise training failed to improve performance in the *APOE*3 or *APOE*4 mice. A two-way ANOVA indicated significant main effect of Strain ($p<0.001$), but not a significant main effect of Treatment or interaction between the two factors (All *p*s > 0.846). There was no significant difference between the performance of the *APOE* mice and wild types (all *p*s >0.078).

Reflexive musculoskeletal responses

Performance was measured daily and averaged over 4 sessions, and is presented in Table 1. The initiation of walking was affected by genotype as *APOE*4 mice took slightly longer latencies, which was supported by a main effect of Strain ($p<0.05$). This effect of *APOE* was mainly driven by a difference in the exercise group as the treatment seemed to reduce latency to walk in the *APOE*3 mice and increase it in the *APOE*4 mice. The latency to turn in a dead-end

alley was affected differently by treatments depending on the strain, as supported by a significant interaction between Strain and Treatment (p=0.024). The mice under antioxidants and/or exercise exhibited reduced latencies to turn in the *APOE*3 groups though it did not reach significance (all ps >0.059), however SedEC mice took 25% longer latencies than any other treatment groups in the *APOE*4 mice. The initiation of negative geotaxis was marginally affected by Strain (p=0.01) and mainly due to a reduction in the latency in the SedEC *APOE*3 group compared to the SedCon *APOE*3 and the SedEC *APOE*4. The performance of the *APOE* mice was only different on the walk initiation test between the wild-type and the *APOE*3 mice (p=0.016).

When suspended from the wire, the latency to tread (reflex) and the latency to fall were measured and are presented in Table 1. There was no effect of strain or treatment observed on the latency to fall from the wire, which was supported by a lack of main effects and interaction from a two-way ANOVA (all ps >0.259). The latency to tread seemed to be reduced with exercise by 25-40% in the *APOE*3 mice, and increased by 22-25% in the *APOE*4 ones, which lead to a significant main effect of Strain (p<0.01).

Bridge walking

The latency to fall from a high bridge were averaged over the 4 sessions and analyzed, but also analyzed across increasing level of difficulty (Figure 5). The mice on the antioxidants and/or exercise had 15-22% longer latencies to fall compared to the SedCon within the *APOE*3 group, while there was no effect of the treatments in the *APOE*4 groups. This was supported by two-way ANOVA yielding a significant interaction between Strain and Treatment (p=0.047). We further analyzed the data across sessions to determine if this interaction was present at all levels of difficulty of the bridge walking test (Figure 5B). All mice took shorter latencies as the

difficulty of the bridges increased, supported by a significant effect of Sessions (p<0.01). Most of the effects of treatment were observed during sessions 3 and 4 (large and small rods). Treatment effects were only observed in the *APOE*3 group, even though an interaction between Session, Strain and Treatment or a Strain by Treatment interaction did not reach significance (all ps>0.086). While all treatment increased the latency to fall during session 3, only the treatments with exercise improved performance in session 4 for the *APOE*3 mice.

Morris water maze

The performance of the mice as measured by latency, path length and swimming speed is presented in Figure 5. All mice learn the location of the platform as latency and path length decrease over sessions (Figures 6A and B), supported by main effects of Sessions (all ps<0.01). While, it seems that the SedCon *APOE*4 mice took longer path lengths and latencies to reach the platform, an interaction between Sessions and Strain did not reach significance for either measure (all ps>0.082). The *APOE*4 mice under treatments exhibited shorter path length and latencies to reach the platform, which was supported by a significant main effect of Treatment for the latency measure (p=0.004) and close to significance for the path length measure (p=0.056).

Overall, the *APOE4* mice swam faster than the *APOE3* ones, and there was no effect of the treatments on swimming speed in either strain. A two-way ANOVA yielded a significant main effect of Strain (p<0.01), but no main effect of Treatment or an interaction between Strain and Treatment (all ps>0.237).

To further decipher the effect of strain and treatment on the performance of the mice on the Morris water maze, a learning index (average of path length for sessions 2, 3 and 4) and maximum performance (average path length for sessions 6 through 9) were calculated and

analyzed (Figure 7). There was no difference between the learning indices of *APOE3* and *APOE4* strains ($p = 0.151$). In *APOE*4, ExCon and ExEC significantly improved learning index, but failed to do so in *APOE3* mice. A two-way ANOVA revealed no main effects or interaction between Strain and Treatment (all ps>0.089). While there was no main effect of Strain on the maximum performance (p=0.258), individual comparisons revealed that *APOE*4 SedCon took 32% longer path length compared to SedCon *APOE*3. The treatments affected only the *APOE*4 mice even though an ANOVA did not yield a significant interaction between Strain and Treatment (p=0.134). All treatment groups had 25-30% shorter path length to reach the platform when compared to SedCon *APOE*4, supported by significant individual comparisons and a main effect close to significance (p=0.066).

Retention of spatial memory was tested 1 week after the last session in a single probe trial (session 10). All the mice retained the previously learned information well. In comparing genotypes within the SedCon groups, there was no effect of Strain ($p = 0.328$) on the performance of the mice. There was no effect of Treatments on the E3 and E4 mice (all $p >$ 0.221).

The last phase of Morris water maze is the visible platform to determine whether vision had a role in their learning of the water maze paradigm (Figure 8). All the mice learned the task (Figure 8A) as observed with a decreased path length taken to reach the platform across sessions (p<0.01), but there was no effect of Strain or Treatment on the performance on the mice (all ps>0.283). Swimming speed was also analyzed (Figure 8B) and revealed main effects of Strain and Treatment, and a Session x Strain x Treatment interaction (p=0.015). Overall the *APOE*4 mice swam faster than the *APOE*3 mice across all sessions. While there was no effect of

treatment in the *APOE*4 mice, there was an increase in the speed of the ExCon and EXEC mice over sessions.

Discriminated avoidance test

Components of the discriminated avoidance learning were considered for effects of Strain and Treatment during the acquisition and reversal sessions. Learning of the discriminative component is shown in Figure 9A, whereas the avoidance component is shown in Figure 9B. Perusal of the discriminative component (Figure 9A) during acquisition and reversal revealed strain and treatment effects on the performance of the mice. Interestingly, *APOE*4 mice learned the discriminative component during acquisition with fewer trials than *APOE*3mice, but there was no difference during reversal. During the acquisition phase for the *APOE*3, ExCon and ExEC took 24-26% fewer trials than the SedCon, while the treatments did not affect performance in the *APOE*4 group. A two-way ANOVA yielded main effects of Strain and Treatment (all *p*s <0.05), but did not reveal a significant interaction between Strain and Treatment ($p = 0.121$). In the reversal session, all treated *APOE*3 mice took 14%-25% fewer trials than the SedCon mice while in the *APOE*4 group, only ExCon and ExEC mice took 25% fewer trials than the SedCon ones. An analysis of the data during session 2 indicated significant main effect of Treatment ($p < 0.01$), but no main effect of Strain or an interaction between the two factors (all *p*s>0.23)
For the avoidance component of the task during acquisition and reversal, there were no differences between the two strains; however treatment effects were observed (Figure 9B). During acquisition, the ExCon and ExEC *APOE*3 mice took 15-18% fewer trials to reach the avoidance criterion compared to the SedCon *APOE*3 mice. In *APOE4* mice, only the ExEC mice took significantly fewer trials than the SedCon group. Analysis of the trials to avoidance criterion for session 1 yielded a significant main effect of Treatment ($p < 0.01$) and no effect of

Strain or an interaction (all *p*s>0.268). During reversal all *APOE*3 mice receiving antioxidant or exercise took fewer trials to reach criterion (17-33%) when compared to the SedCon group. In the *APOE*4, even though the SedEC and ExEC group took 19% fewer trials, it did not reach significance. Analysis of the data from session 2 indicated only a significant main effect of Strain and Treatment (all *p*s < 0.01), and did not yield a significant Strain × Treatment interaction ($p = 0.334$).

Catalase

Catalase activity was measured in the cortex, hippocampus, cerebellum and midbrain and presented in Figure 10. The cortex had lower catalase activity than the other regions, supported by a main effect of Region ($p<0.01$). There was no difference between *APOE*3 and *APOE*4 mice in any of the regions, however overall treatments, especially exercise seemed to be associated with decreased catalase activity. An ANOVA yielded a significant main effect of Treatment ($p=0.03$), and no main effect of Strain or an interaction between the two factors (all *p*s> 0.362). Antioxidant supplementation had no effect on catalase activity in any of the regions in either genotype. The ExCon and ExEC group had lower catalase acitivities in most brain regions and in both genotypes, though it was most pronounced in the hippocampus and midbrain of *APOE*4 mice. This was supported by main effects of Treatments for both regions (all *p*s <0.01).

BDNF

BDNF levels were measured in the cortex, hippocampus, cerebellum and midbrain and presented in Figure 11. Overall, there was no effect of strain or treatment on BDNF levels in any of the regions studied (all *p*s>0.126).

Erk pathway

Levels of TrkA, Erk5, Erk1/2 and phosphorylated Erk1/2 (pErk1/2) were measured and presented by brain regions: cerebellum (Figure 12), midbrain (Figure 13), cortex (Figure 14) and hippocampus (Figure 15).

There were no major differences with genotype or treatment on the levels of each measured proteins in the cerebellum (Figure 12). However noticeable trends are noteworthy: SedEC and ExEC seemed to lower Erk5 in *APOE*3 mice, and all treatments lowered pErk1/2 in both strains, though most pronounced in the *APOE*4 mice. In the midbrain (Figure 13), there were no major differences observed between the two strains or with treatments. The ExEC group had lower levels of all proteins in the *APOE*4 mice; however individual differences were not significant. In the cortex (Figure 14), TrkA levels were slightly higher in the *APOE*4 groups than in the *APOE*3 ones (p=0.072). Levels of Erk5 and pErk1/2 were lower in the *APOE*4 groups, most noticeably in the ExEC group. In the hippocampus (Figure 15), there seems to be lower levels of all proteins in *APOE*4 compared to *APOE*3 but the only significant effect was found for pErk1/2 (p<0.05). All treatments seemed to increase TrkA in *APOE*4 mice, and lower pERk1/2 in *APOE*3 mice, however none of the individual differences were significant.

While there were noticeable trends that treatments lowered the levels of some proteins dependent upon the genotype and the brain regions, ANOVAs did not reveal any main effects of Strain, Treatment or an interaction between the two factors (all ps >0.05).

DISCUSSION

The main findings of this study were (1) *APOE*4 mice had lower motor activity, reflexes and decreased motor learning compared to *APOE*3 mice, but no differences were found in strength, balance; (2) *APOE*4 SedCon mice exhibited poorer spatial learning and memory, and were not different than the *APOE*3 on non-spatial task; (3) all treatments improved balance in *APOE*3 mice only; (4) supplementation with vitamins E and C improved spatial learning in APOE4 mice only, and cognitive flexibility in *APOE*3 mice only, independent of motor reflexes and anxiety; (5) exercise training improved cognitive function in both genotypes and increased ability to balance in *APOE*3 genotype, independent of motor reflexes, strength or anxiety measures; lastly, (7) additive effect observed on cognitive flexibility in *APOE*3 mice only.

Cognitive decline is often the major concern associated with AD; however there are non-cognitive components to AD that include increased anxiety and decreased motor coordination as well as poor muscular reflexes. As *APOE*4 is a well-known major genetic risk factor that is prevalent among the largest set of AD population, this study determined the occurrence of cognitive as well as non-cognitive functional deficits in association with *APOE*4 genotype. Further this study also evaluated the ability of the non-conventional therapeutic approach using antioxidants supplementation alone or in combination with forced exercise regimens to reverse motor and cognitive declines associated with *APOE*4.

Anxiety is the most common non-cognitive symptom reported in nearly 70% cases of AD (65). Anxiety symptoms are significantly correlated with impaired daily activities (66). Furthermore, the pharmacological management of anxiety with benzodiazepines might lead to further cognitive and motor function decline (67,68) in AD. Hence identifying non-pharmacotherapeutic agents like antioxidants and exercise on anxiety might have an overall

positive approach towards managing anxiety symptoms in AD. In young adult mice (4 months), APOE4 mice were less anxious than the *APOE*3 ones (69), yet in older mice (14 months) the difference has subsided. This may indicate a pleiotropic effect of the *APOE* genotype on anxiety levels (70), as has been described with cognitive function (69,71,72). These data are in contradiction with a previous study by Raber *et.al.*. (11) that reported higher anxiety among adult (6-8 months) GFAP-*APOE*4 mice compared to *APOE*3 mice. Neither the antioxidant or exercise treatment had any effect on the anxiety level of the mice. In our previous study of young mice, antioxidants were observed to increase the anxiety of the young *APOE*4 mice (69). Interestingly, *APOE*3 mice were more active in this test than the *APOE*4 mice; and the combination of exercise and antioxidant seemed to reverse this decrease. However, even though the APOE3 mice were also more active during the test for spontaneous locomotor activity than the APOE4 ones, there was no effect of the treatments. This difference in response may be due to a short-term effect of the treatment on activity as the plus maze test only last 5 min while the locomotor activity is followed over 16 min. This lower activity in APOE4 mice has been previously reported by Bour et.al.(5), in a transgenic mouse model expressing human *APOE*. Interestingly, *APOE*3 travelled 25% longer distance than wild-type mice, also observed previously by Bour et.al.(5).

During early phases of dementia, AD is often associated with motor function impairments, gait deformity, inability to learn new skills, decline in gait and/or speed of walking, and reduced mobility. We supported the findings of Meer *et.al.*(73), that the *APOE*4 genotype performed poorly during coordinated running task training. Exercise and or antioxidants had no effect on the performance of the mice in *APOE*3 or and the treatments failed to revert the performance deficit in *APOE*4 groups (all *p*s>0.134). At younger ages, exercise did reverse *APOE*4-associated decline in performance (69). Even though it does not seem as though the

performance of the *APOE*3 or *APOE*4 mice declined with age, their response to treatment was inexistent at an advanced age, indicative of a therapeutic window of treatment.

While there was a genotype difference on coordination, there was no such effect on balance as measure by bridge walking. This is indicative that each test measures a different aspect of motor function. Interestingly, treatments improved balance only in the *APOE*3, more specifically on the most difficult bridges. This genotype-dependent effect was previously observed in younger mice (69) though the effects were not as large as the younger mice performed better and ceiling effects may have limit the effects of the treatments. On the most difficult bridge, the mice that received the exercise regimen performed better than the control or antioxidant-supplemented ones, indicating that exercise may be a more promising therapeutics. However, these data in the *APOE*4 mice revealed that neither treatment are responsive and may not be effective in reversing *APOE*4-associated declines in motor function if implemented too late.

Spatial learning and memory is the most commonly used tool to test cognitive function in various transgenic rodent models for AD. Mice expressing human *APOE*4 either in targeted replacement mouse model or GFAP-*APOE* or NSE-*APOE* mouse model exhibited poor spatial learning and memory (5,71,73-76). Our data also suggest a significant decline in spatial learning and memory in *APOE*4 compared to *APOE*3 mice, a difference that was not observed in younger mice (69). While exercise was effective at improving the learning index as well as the maximum performance of the mice, antioxidants were only significantly effective at improving the maximum performance. This difference may prove that exercise might be most effective therapies, and that improvements in learning index and maximum performance might involve slightly different pathways. Improvements with exercise have been shown before by Nichol

et.al. (2009)(77). It is noteworthy that adding exercise and antioxidant did not lead to additive effects but also no antagonistic effects, as seen in motor function (69).

Vision could be an important confounding parameter in spatial cognitive test (78). Treatment and strain did not affect the performance of the mice and they all learned to navigate the maze and associate the presence of the flag with the location of the safe platform. Hence, the treatment effects on spatial learning and memory were not confounded by effects on vision.

AD symptoms start with loss of non-spatial short term / working memory (sometimes referred as immediate memory) leading to the inability to use, manipulate and apply such immediate information as observed in people with mild cognitive impairment (MCI) (79,80). While we found strain differences in the spatial task, *APOE*3 and *APOE*4 performed similarly in the non-spatial task used in this study: active avoidance. This is in contrast with previous studies that have identified genotype differences in working memory, which was deteriorating in an *E*4 allele dose dependent manner (81). However, it is important to note that the test utilized were different and may be measuring different aspects of cognitive function. During acquisition, the discrimination component requires less trial to learn than the avoidance component of the task. Exercise improved performance on both components in the *APOE*3 mice, while it reached significance in the *APOE*4 mice only for the avoidance one. The improvement in discrimination performance associated with the treatments in the *APOE*4 mice may not have been significance due to reaching a ceiling effect, as the mice may not be able to improve further. Interestingly, an additive effect was observed on the avoidance performance of the *APOE*4 mice, where the combination of the two treatments improved the mice further than each treatment alone. During reversal measuring cognitive flexibility, antioxidant and exercise were successful at improving performance of the *APOE*3 mice, and it seems that the combination was even more successful

than each treatment alone. In the *APOE*4 mice, exercise improved the discrimination performance but failed to reach significance in the avoidance performance. Exercise has been previously shown to improve short term / working memory task performance (82,83). These effects were similar to the effects seen in the younger mice, where performance was better though no genotype differences were observed either (69). While spatial learning and memory test was only affected in the older mice and the active avoidance could be improved regardless of the age of the mice, the therapeutic window of treatment might be different dependent on the type of cognition involved. This becomes important when implementing therapies, as spatial impairments seem to be subject to a smaller therapeutic window than working memory. Furthermore, the differential responses to the treatment support the notion that these two aspects of cognition are independent and are supported by different neural pathways.

Contrary to what was expected, catalase activity was reduced in all the brain regions from mice that were exercised, especially the ones combined with antioxidants. There are conflicting reports on physical activity or exercise such as in some studies there was no effect on catalase activity in brain (84,85), while others showed catalase activity up-regulation as a result of the oxidative bursts associated with acute exercise (86). In our study, it is likely that the mice under moderate long term exercise regimen have attained a new level of homeostasis that does not required catalase activation, Furthermore, the extra presence of antioxidants in the supplements probably leads to either sparing of catalase or a feedback down-regulation of catalase.

BDNF has been largely studied as a marker of neurogenesis and synaptic plasticity (87). Exercise has been known to improve cognition via neurogenesis and neuroplasticity by up-regulating BDNF synthesis in GFAP-*APOE* mice (77). Interestingly, in this study, the cognitive benefits could not be reflected at the molecular levels as BDNF expression remained unaltered

with either exercise or combination treatment. Although exercise has been shown to increase BDNF protein levels in the hippocampus (more precisely in CA1 region) regardless of age, older animals maintained these elevated levels for a short duration (88). It is likely that in our study due to the length of the treatment, the short duration of BDNF up-regulation was missed at the time of euthanasia. The beneficial effects could also be explained as BDNF-independent mechanism such as vascular endothelial growth factor (89,90) or insulin like growth factor (91).

Erk5 activation leads to enhanced adult neurogenesis and improved hippocampus dependent long-term potentiation (92). Activation of Erk5 sends a retrograde neurotrophic signal (93), and therefore decreased Erk5 could be important player in neuronal and behavioral impairments (94). In contrast to this notion, *APOE*4 mice with cognitive improvements after ExEC treatments exhibited a decline in Erk5.

Neurotrophins are growth factors involved in survival, proliferation, differentiation and synaptic plasticity via TrkA receptor (95). Cognitive impairments have been correlated with quantitative and qualitative loss of TrkA receptor protein (96), which can be reversed with the selective activation of TrkA receptor (97). The spatial cognitive benefits observed in our study could be related to this marginal increased expression of TrkA with the treatments.

In conclusion, *APOE*4 middle age mice had motor and spatial cognitive deficit compared to *APOE*3. Exercise was the most effective treatment and improved spatial and non-spatial cognitive performance, but not the motor decline in middle age mice. The benefits associated with antioxidant supplementation seemed to be genotype dependent while exercise was not. In these middle age mice, combination of antioxidants and exercise had additive beneficial effect on non-spatial cognition task but not on spatial leaning and memory.

FIGURES AND LEGENDS

Figure 1: Effect of exercise and/or antioxidant regimen on weekly body weights (A) and closed (B) food intake (C) in middle age GFAP-*APOE*3 (left panel) and GFAP-*APOE*4 mice (right panel).

Each value represents mean ± SEM, n=18-26 for body weights, and n=8-11 for food intake. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment-matched *APOE3*. * $p<0.05$ compared to genotype-matched control.+ $p<0.05$ compared to SedCon wild-type.

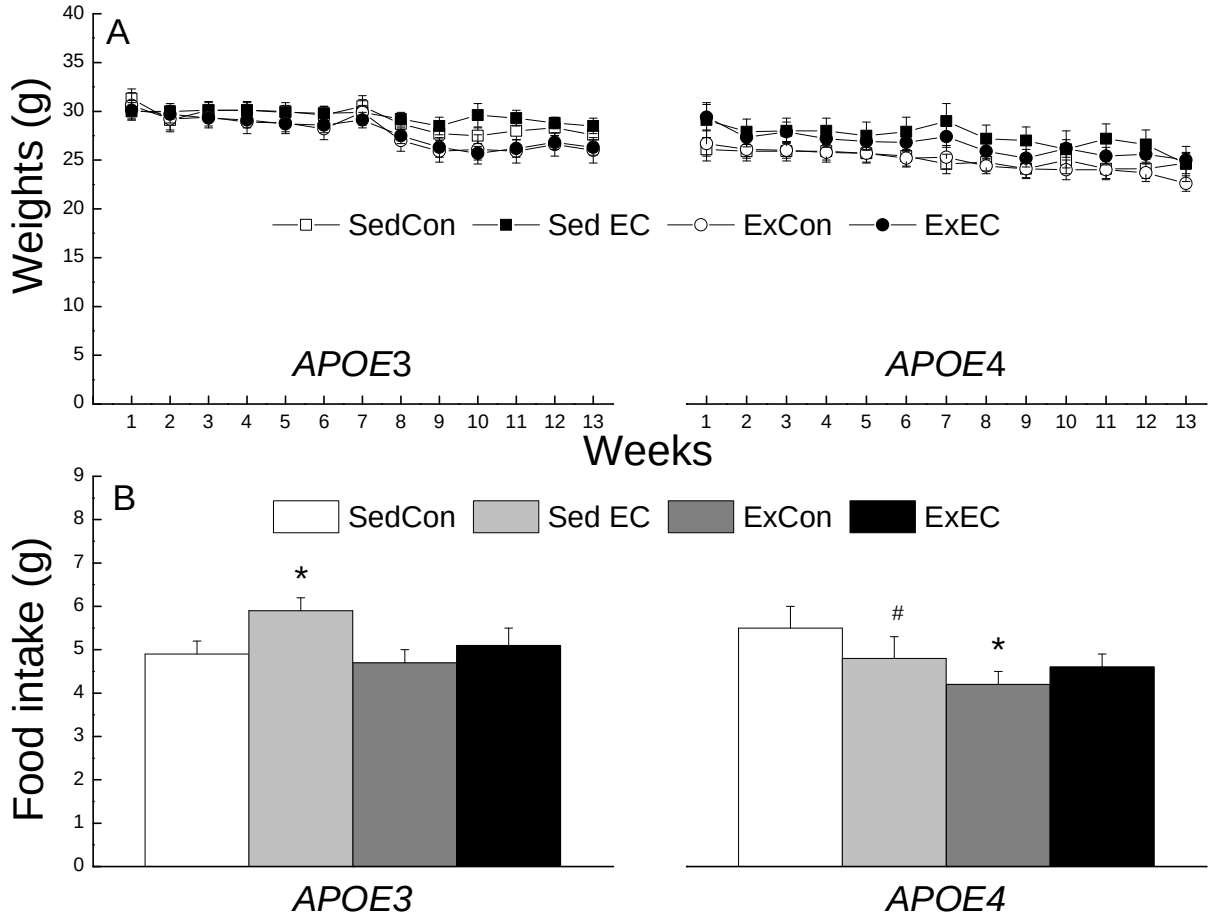
A
Weights (g)
SedCon
Sed EC
ExCon
ExEC
APOE3
APOE4
Weeks
B
Food intake (g)
SedCon
Sed EC
ExCon
ExEC
*
#
*
APOE3
APOE4

Figure 2: Effect of exercise and/or antioxidant regimen on anxiety measured as percent time spent in the open (A) and closed (B) arms of elevated plus maze and distance travelled (C) in middle age GFAP-*APOE*3 (left panel), GFAP-*APOE*4 mice (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=18-28. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment-matched *APOE3*. * $p<0.05$ compared to genotype-matched control.+ $p<0.05$ compared to SedCon wild-type.

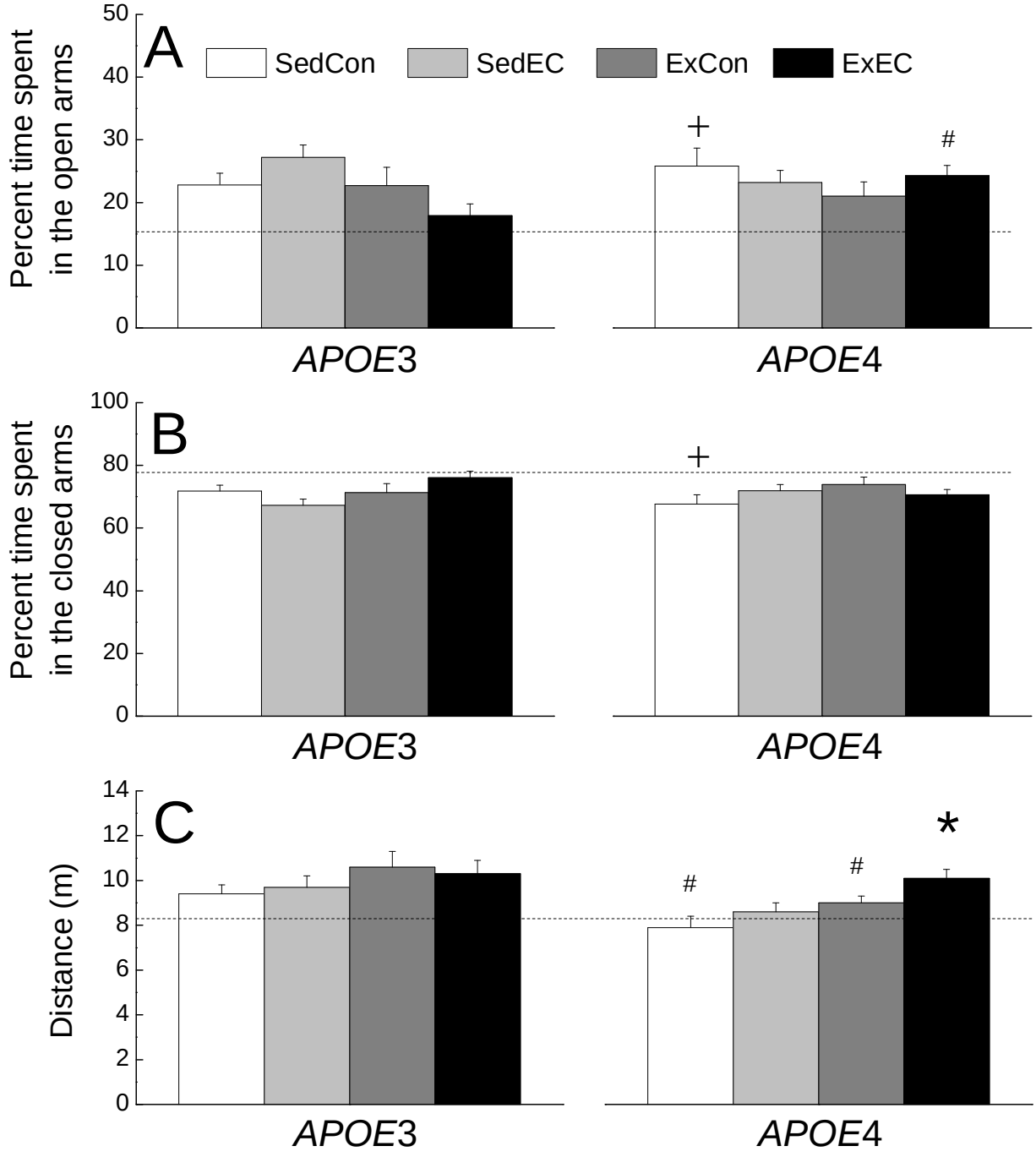
A
SedCon
SedEC
ExCon
ExEC
Percent time spent in the open arms
50
40
30
20
10
0
+
#
APOE3
APOE4
B
Percent time spent in the closed arms
100
80
60
40
20
0
+
APOE3
APOE4
C
Distance (m)
14
12
10
8
6
4
2
0
#
#
*
APOE3
APOE4

Figure 3: Effect of exercise and/or antioxidant regimen on locomotor activity as measured by total distance travelled (cm) (A) and vertical activity counts (B) in middle age GFAP-*APOE*3 (left panels) and GFAP-*APOE*4 (right panels) and C57BL/6 wild-type mice (dashed line).

Each value represents mean ± SEM, n=19-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE3*.+ $p<0.05$ compared to SedCon wild-type.

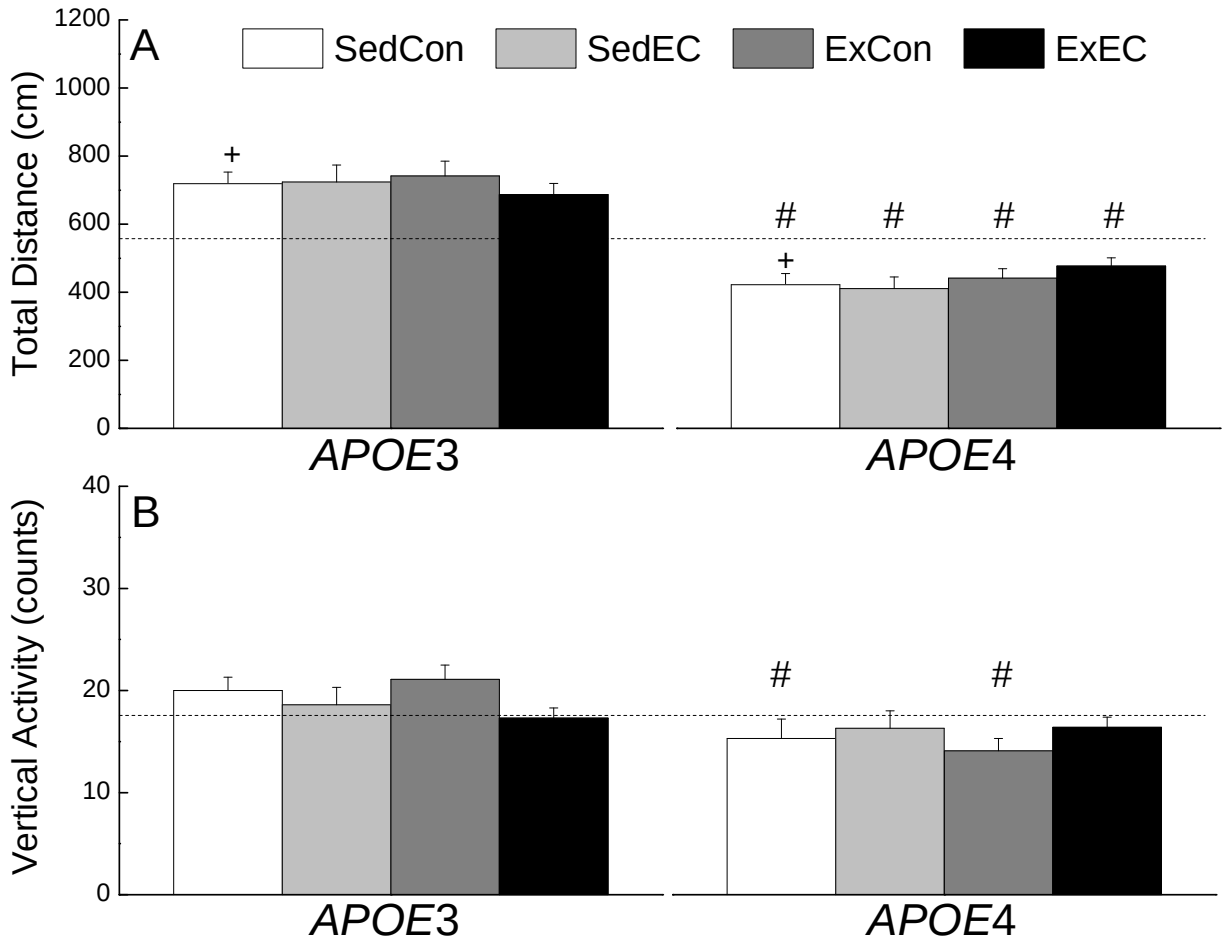
A
SedCon
SedEC
ExCon
ExEC
Total Distance (cm)
1200
1000
800
600
400
200
0
+
+
# # # #
APOE3
APOE4
B
Vertical Activity (counts)
40
30
20
10
0
# #
APOE3
APOE4

Figure 4: Effect of exercise and/or antioxidant regimen on coordinated running performance as measured by latency to fall (in seconds) from rotating rod across seven sessions (A) and when reaching a criterion of stable performance (B), middle age GFAP-*APOE*3 (left panels) and GFAP-*APOE*4 (right panels) and C57BL/6 wild-type mice (dashed line).

Each value represents mean ± SEM, n=18-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ when compared with treatment-matched *APOE*3.

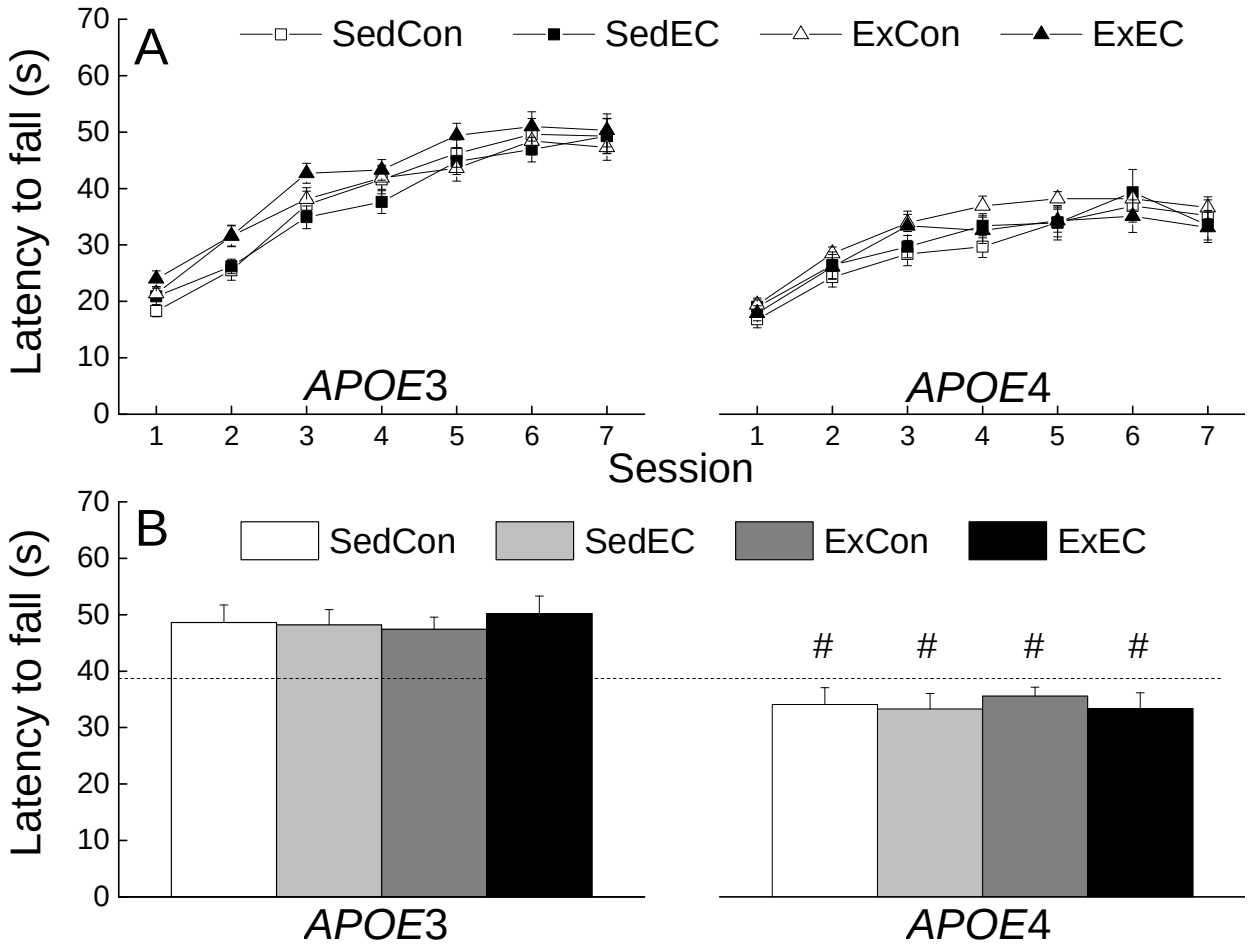
A
SedCon
SedEC
ExCon
ExEC
Latency to fall (s)
APOE3
APOE4
Session
B
SedCon
SedEC
ExCon
ExEC
Latency to fall (s)
#
#
#
#
APOE3
APOE4

Figure 5: Effect of exercise and/or antioxidant regimen on balance as measured by latency to fall from elevated bridge average across all sessions (A) and individual session (B) in middle age GFAP-*APOE*3 (left panel), GFAP-*APOE*4 mice (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=19-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C.

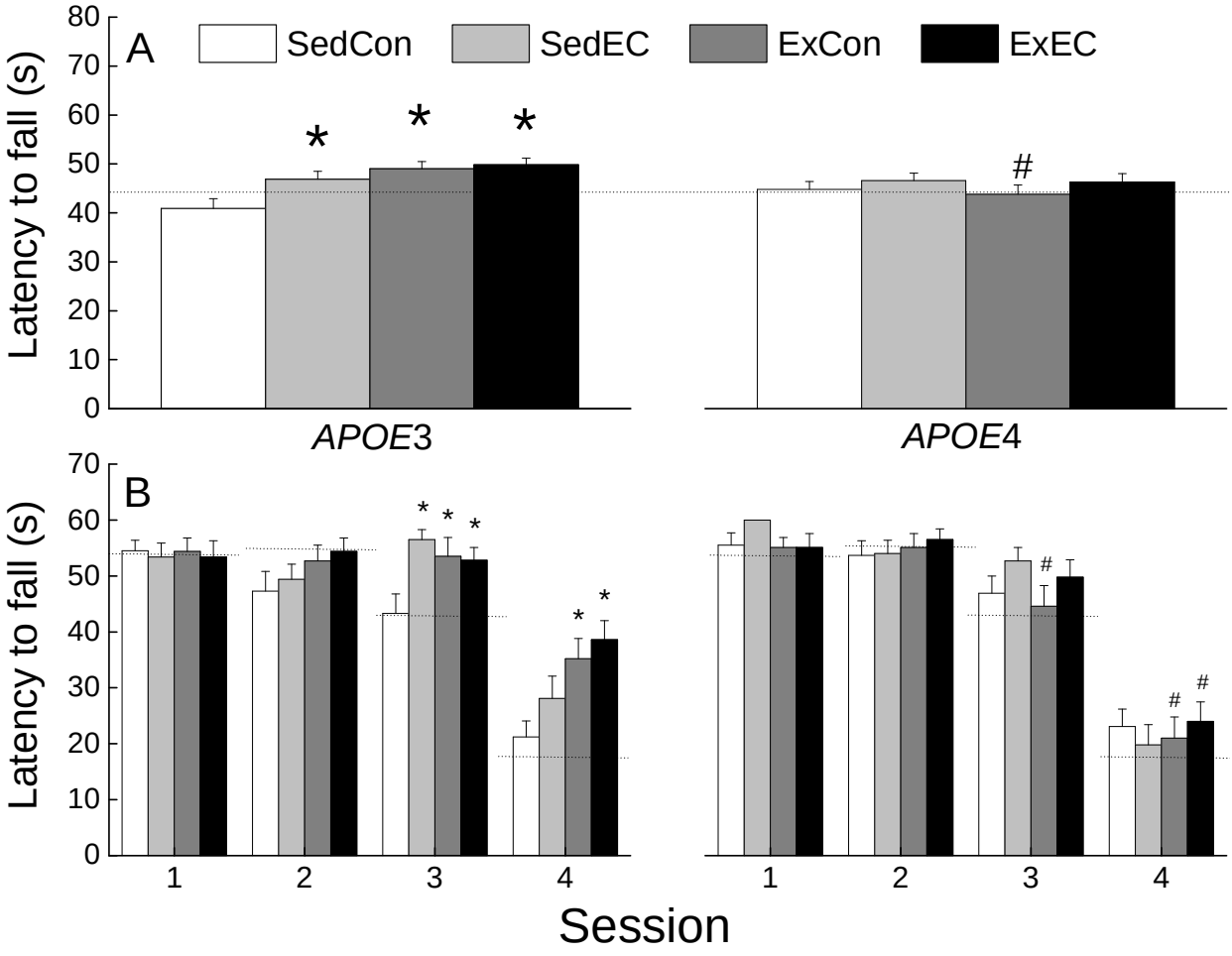
A
SedCon
SedEC
ExCon
ExEC
Latency to fall (s)
80
70
60
50
40
30
20
10
0
*
*
*
#
APOE3
APOE4
B
Latency to fall (s)
70
60
50
40
30
20
10
0
*
*
*
*
*
#
#
#
1
2
3
4
1
2
3
4
Session

Figure 6: Effect of exercise and/or antioxidant regimen on Morris water maze performance as measured by latency (s) (A) path length (cm) (B) and swimming speed (cm/s) (C) in middle age GFAP-*APOE*3 (left panel) and GFAP-*APOE*4 (right panel).

Each value represents mean ± SEM, n=13-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C.

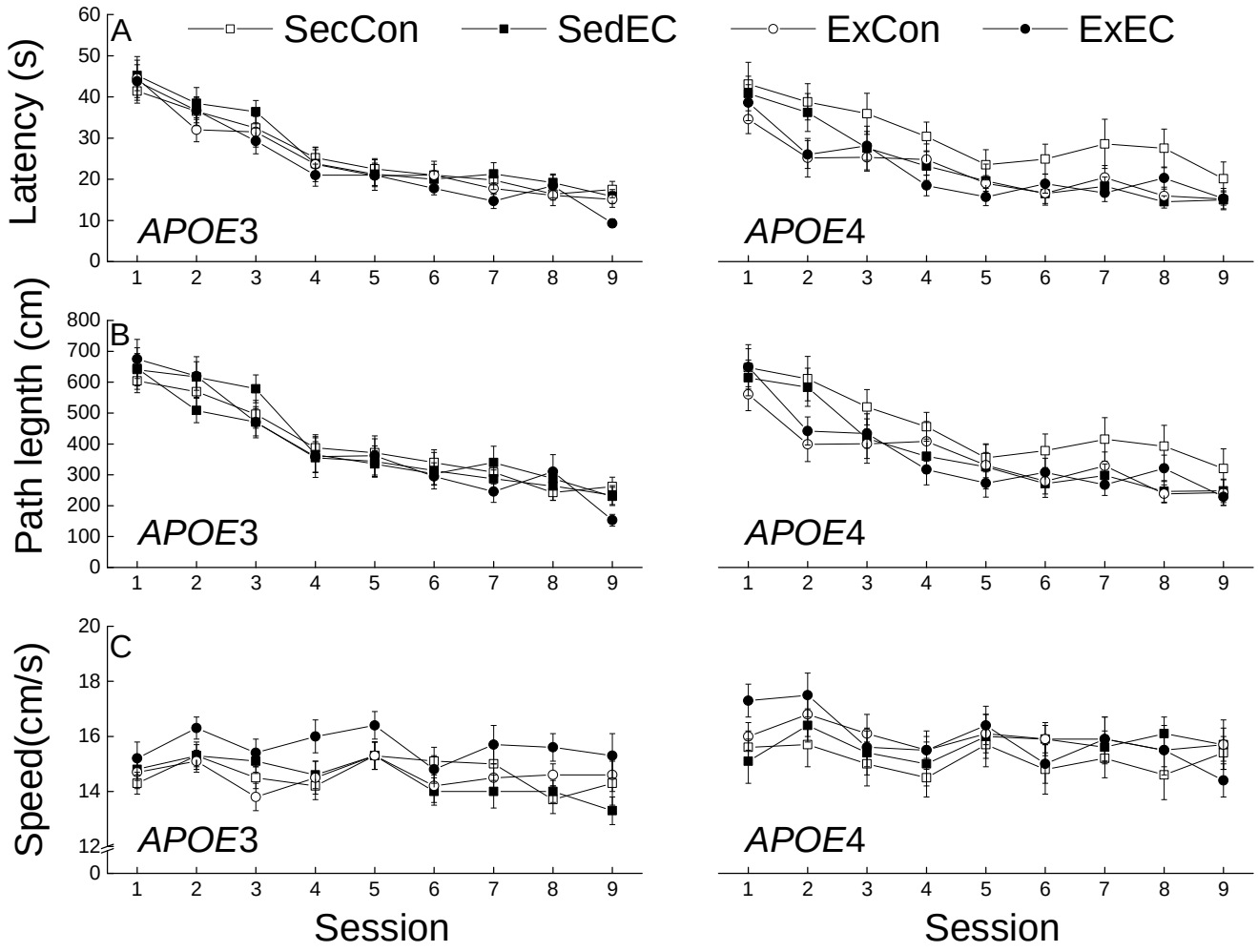
A
SecCon
SedEC
ExCon
ExEC
Latency (s)
APOE3
APOE4
B
Path legnth (cm)
C
Speed(cm/s)
Session
Session

Figure 7: Effect of exercise and/or antioxidant regimen on Morris water maze performance as measured by learning index (A) maximum performance (B) in middle age GFAP-*APOE*3 (left panel), GFAP-*APOE*4 (right panel) and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=13-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3;*$p<0.05$ compared to SedCon.

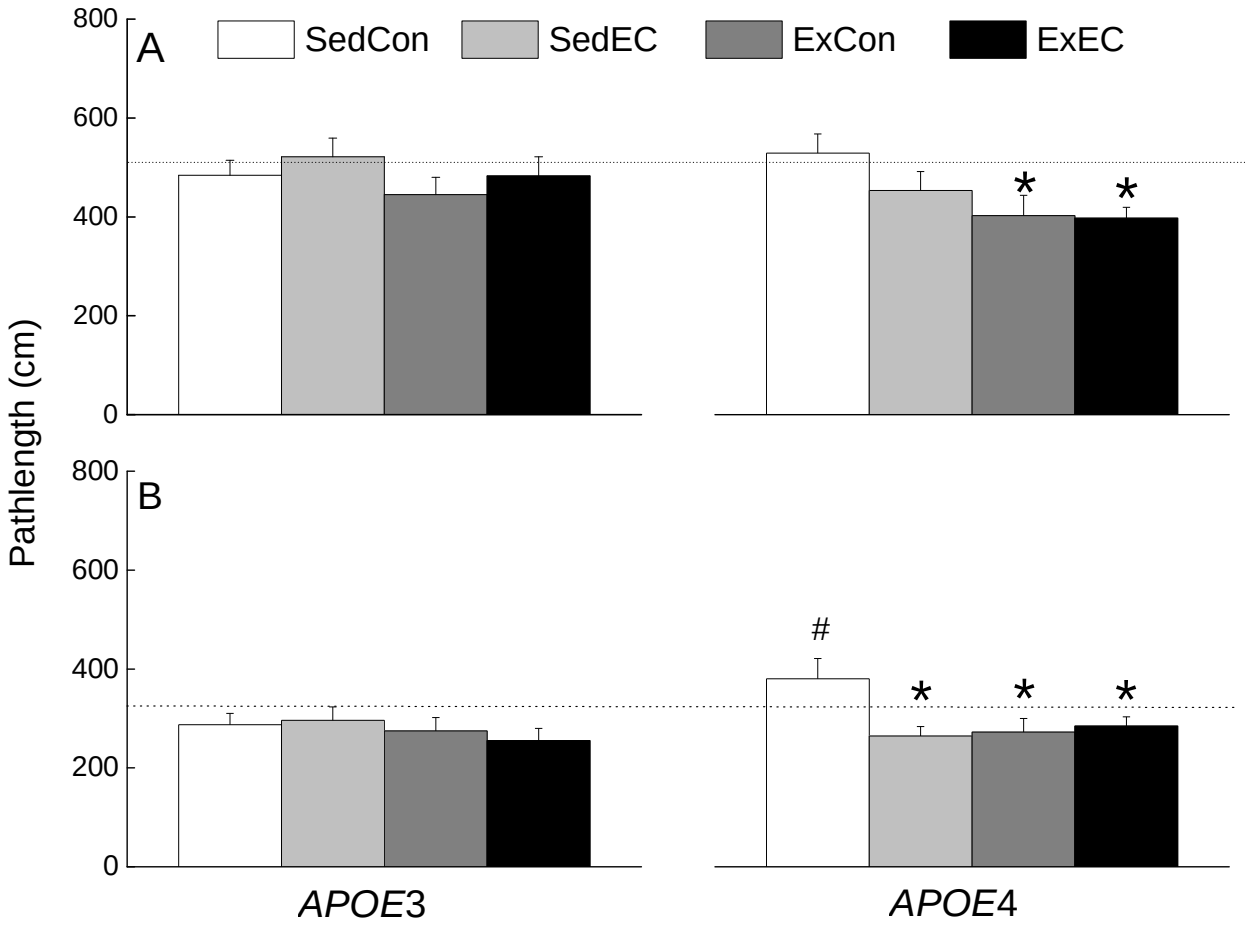
A
SedCon
SedEC
ExCon
ExEC
800
600
400
200
0
B
Pathlength (cm)
#
*
APOE3
APOE4

Figure 8: Effect of exercise and/or antioxidant regimen on visible platform performance as measured by path length (cm) (A) and swimming speed (cm/s) (B) in middle age GFAP-*APOE*3 (left panel) and GFAP- *APOE4* (right panel) mice.

Each value represents mean ± SEM, n=13-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C.

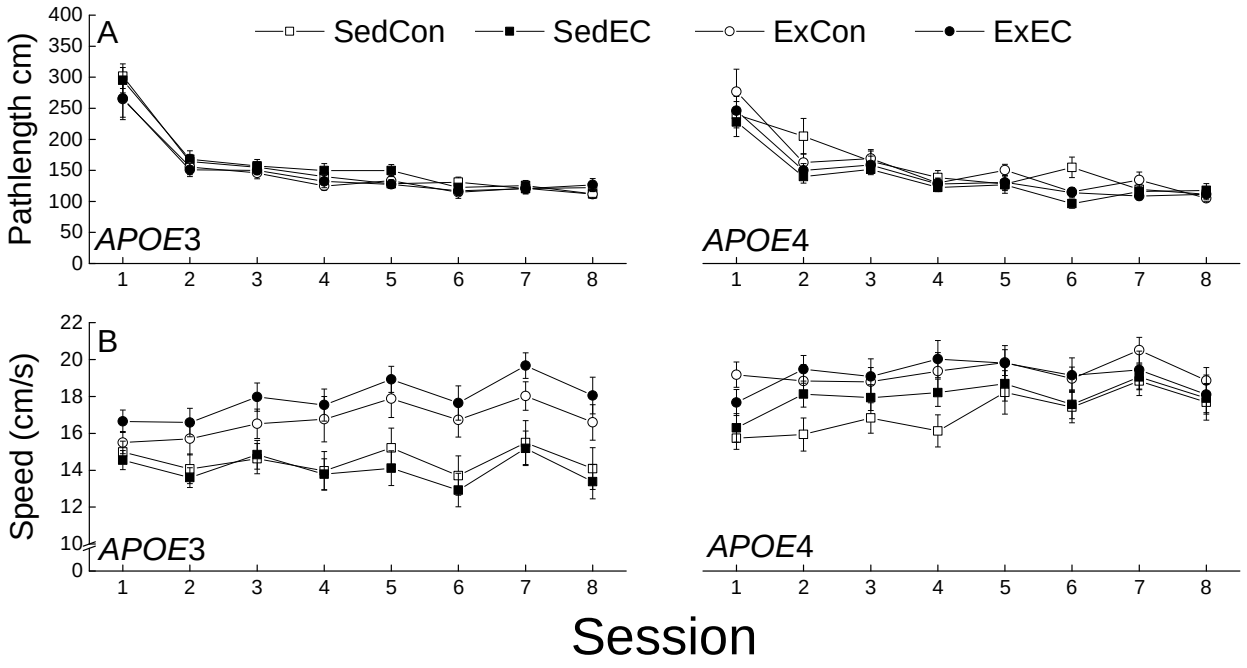
A
SedCon
SedEC
ExCon
ExEC
Pathlength cm)
APOE3
APOE4
B
Speed (cm/s)
APOE3
APOE4
Session

Figure 9: Effect of exercise and/or antioxidant regimen on discriminated avoidance performance as measured by the number of total trial taken to reach discrimination criterion (A) and avoidance criteria (B) during acquisition and reversal sessions in middle age GFAP-*APOE*3, GFAP-*APOE*4 and C57BL/6 (wild-type) mice (dashed line).

Each value represents mean ± SEM, n=13-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3;*$p<0.05$ compared to SedCon.

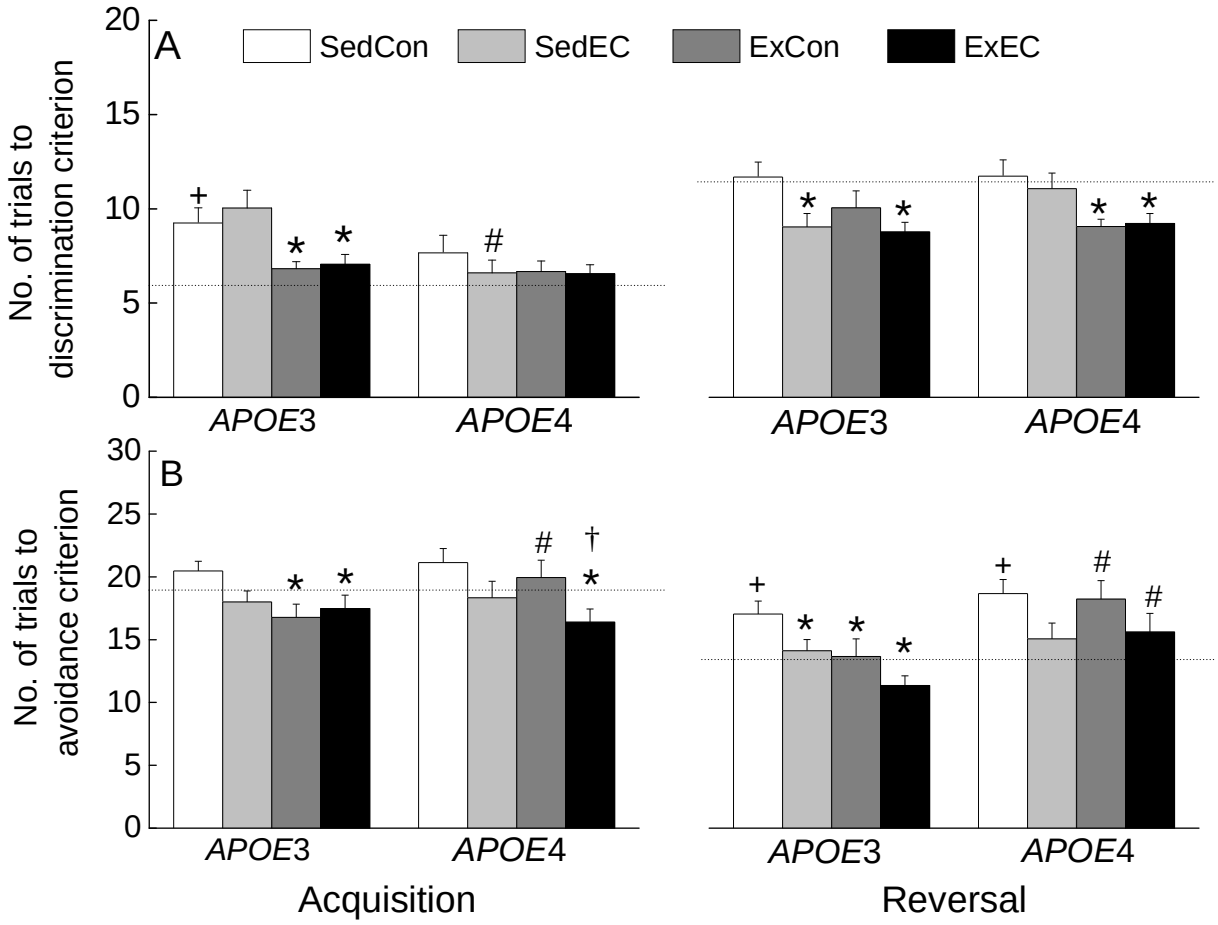
A
SedCon
SedEC
ExCon
ExEC
No. of trials to discrimination criterion
20
15
10
5
0
+
*
*
#
*
*
*
*
APOE3
APOE4
APOE3
APOE4
B
No. of trials to avoidance criterion
30
25
20
15
10
5
0
*
*
#
†
*
+
*
*
*
+
#
#
APOE3
APOE4
APOE3
APOE4
Acquisition
Reversal

Figure 10: Effect of exercise and/or antioxidant regimen on catalase activity in Cortex (A), Hippocampus (B), Cerebellum (C) and Midbrain (D) in middle age GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=7-8. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3; *$p<0.05$ compared to genotype-matched SedCon; †$p<0.05$ compared to genotype-matched SedEC.

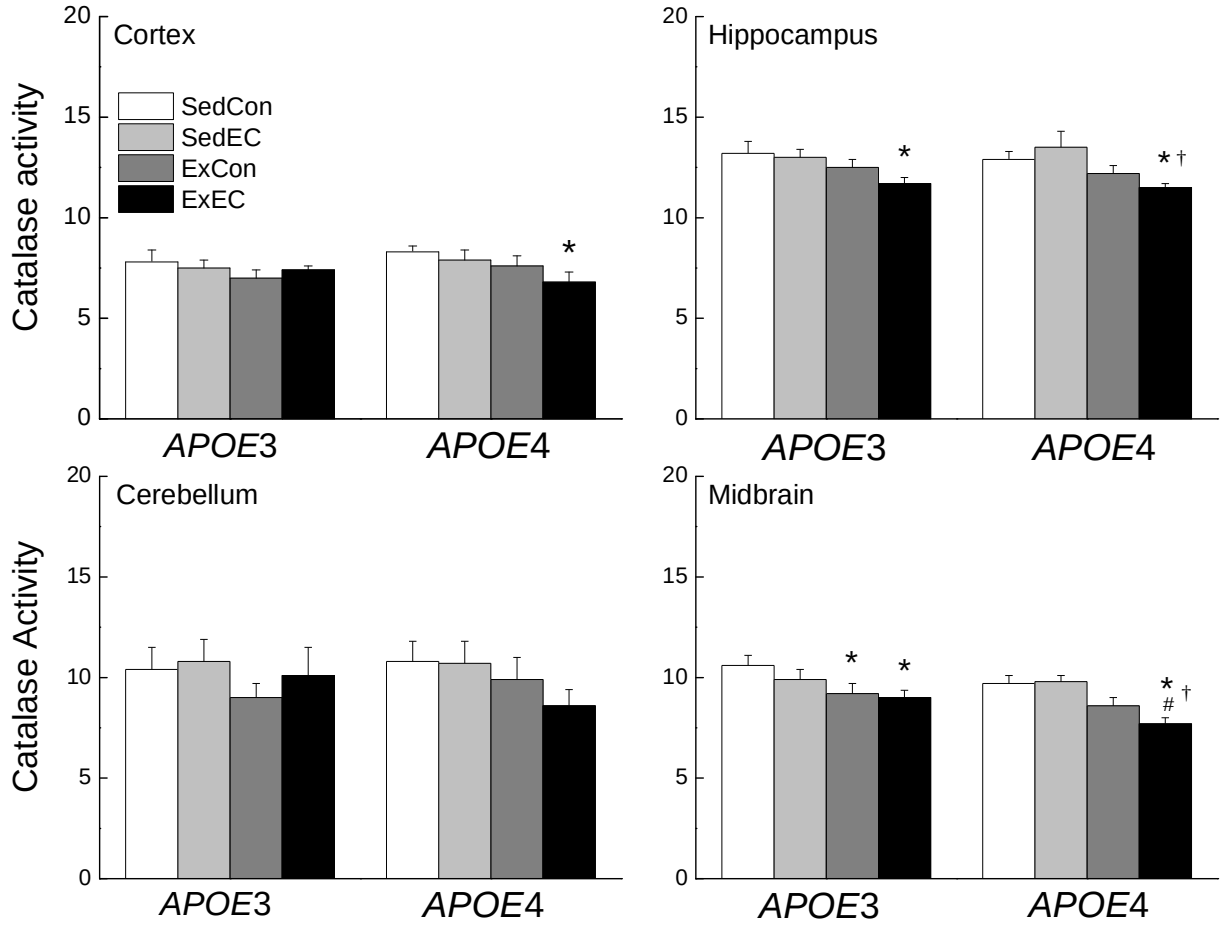
Cortex
SedCon
SedEC
ExCon
ExEC
Catalase activity
20
15
10
5
0
*
APOE3
APOE4
Hippocampus
*
*†
Cerebellum
Catalase Activity
Midbrain
*
*
*
#
†

Figure 11: Effect of exercise and/or antioxidant regimen on BDNF expression in Cortex (A), Hippocampus (B), Cerebellum (C) and Midbrain (D) in middle age GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=7-8. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3; *$p<0.05$ compared to genotype-matched SedCon; †$p<0.05$ compared to genotype-matched SedEC.

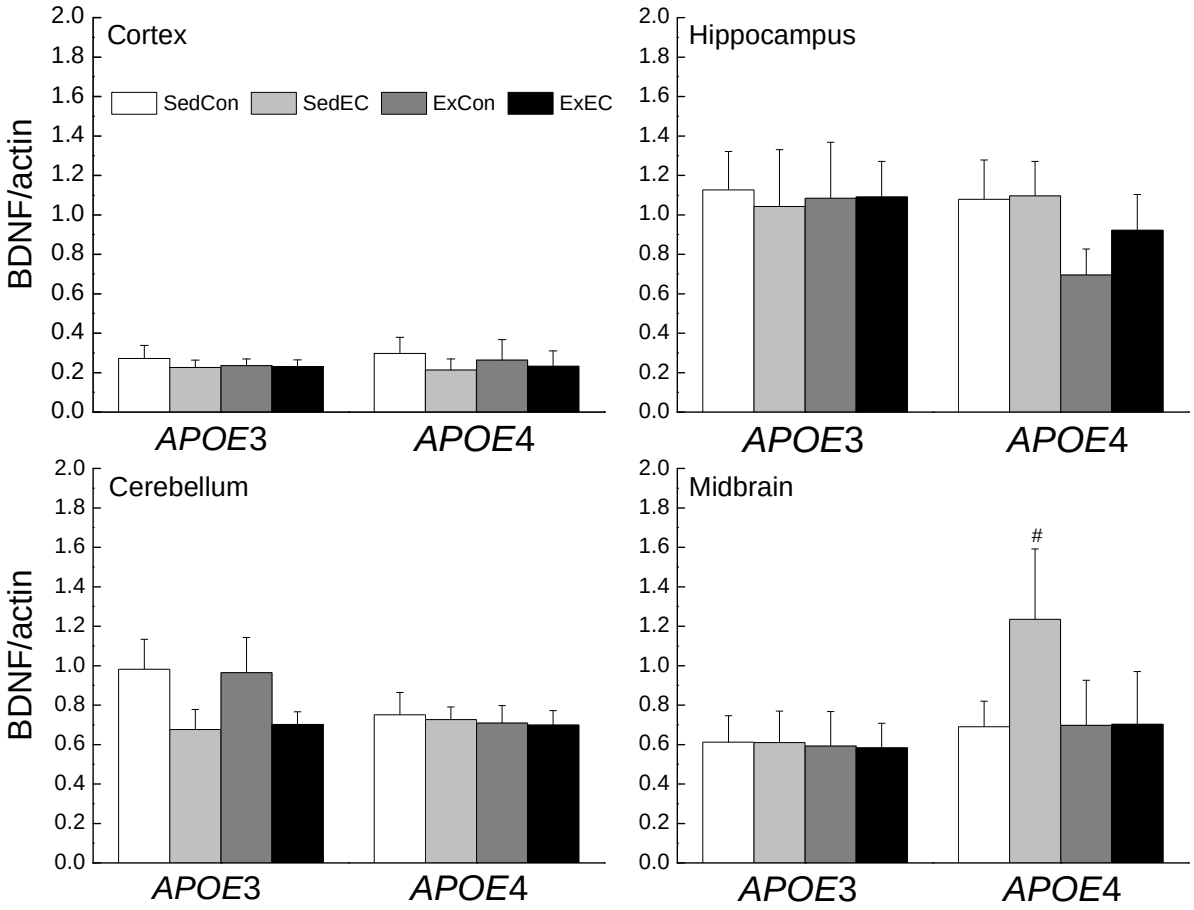
Cortex
SedCon
SedEC
ExCon
ExEC
BDNF/actin
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
APOE3
APOE4
Hippocampus
Cerebellum
Midbrain
#

Figure 12: Effect of exercise and/or antioxidant regimen on the expression of Trk A (A), Erk5 (B), and pErk1/2 / Erk1/2 ratio (C) in the cerebellum in middle age GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=8. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C.

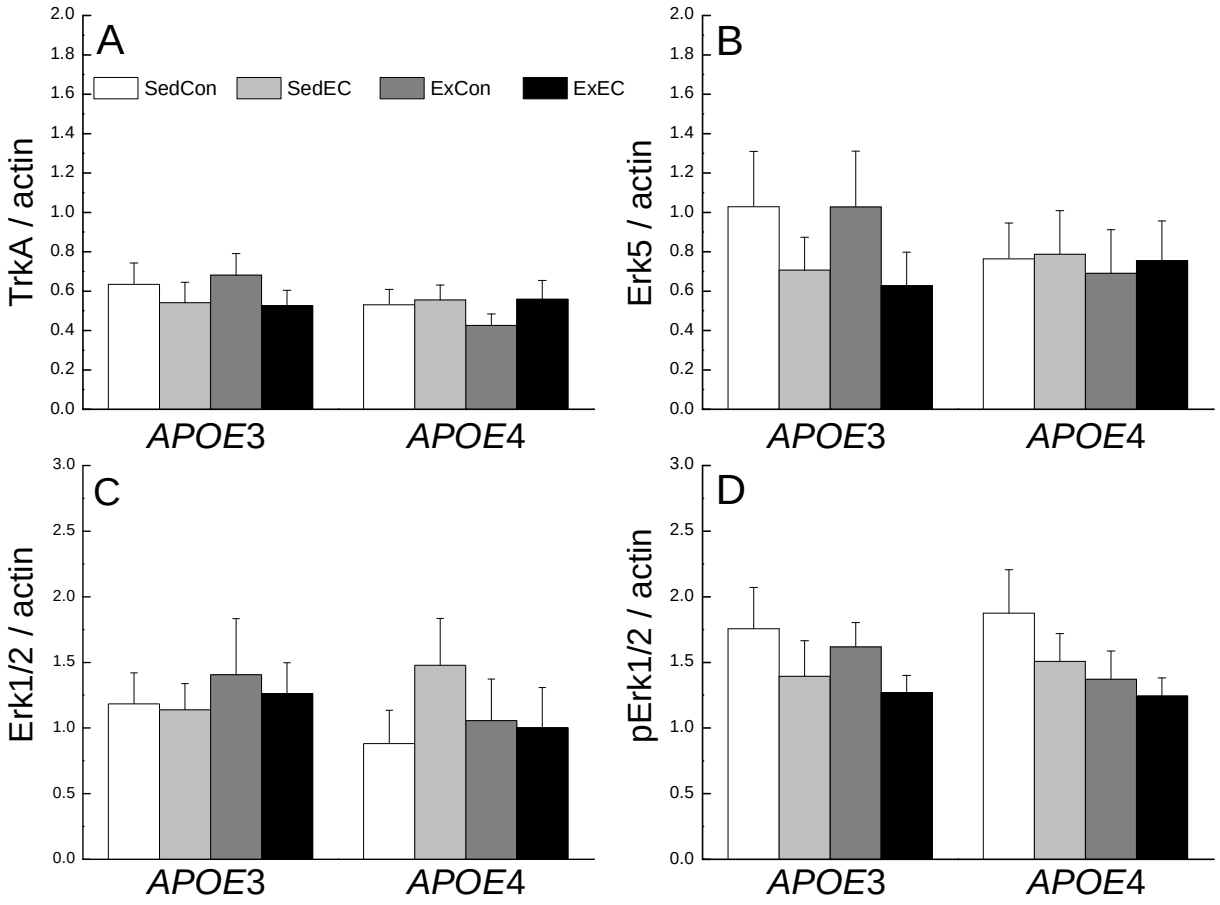
A
SedCon
SedEC
ExCon
ExEC
TrkA / actin
APOE3
APOE4
B
Erk5 / actin
C
Erk1/2 / actin
D
pErk1/2 / actin

Figure 13: Effect of exercise and/or antioxidant regimen on the expression of Trk A (A), Erk5 (B), and pErk1/2 / Erk1/2 ratio (C) in the midbrain in middle age GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=8. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C.

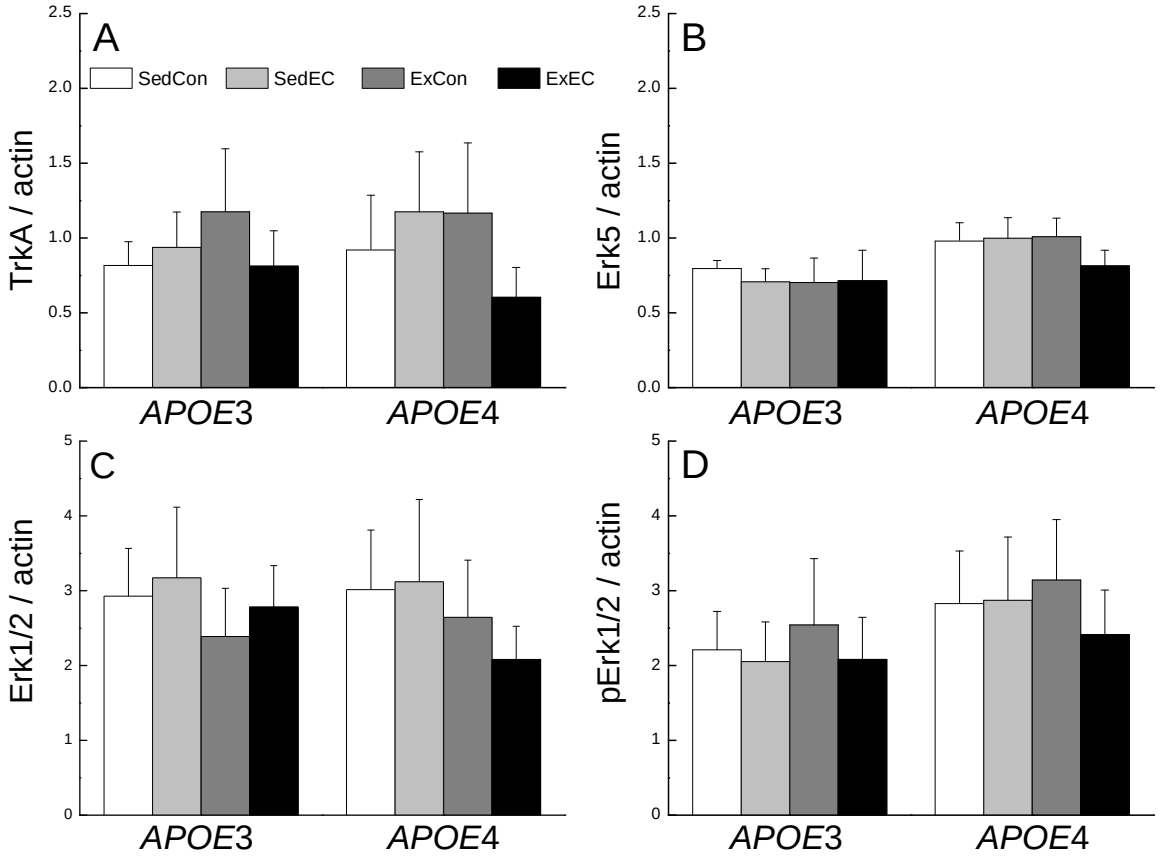
A
SedCon
SedEC
ExCon
ExEC
TrkA / actin
B
Erk5 / actin
C
Erk1/2 / actin
D
pErk1/2 / actin
APOE3
APOE4

Figure 14: Effect of exercise and/or antioxidant regimen on the expression of Trk A (A), Erk5 (B), and pErk1/2 / Erk1/2 ratio (C) in the cortex in middle age GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=8. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3.

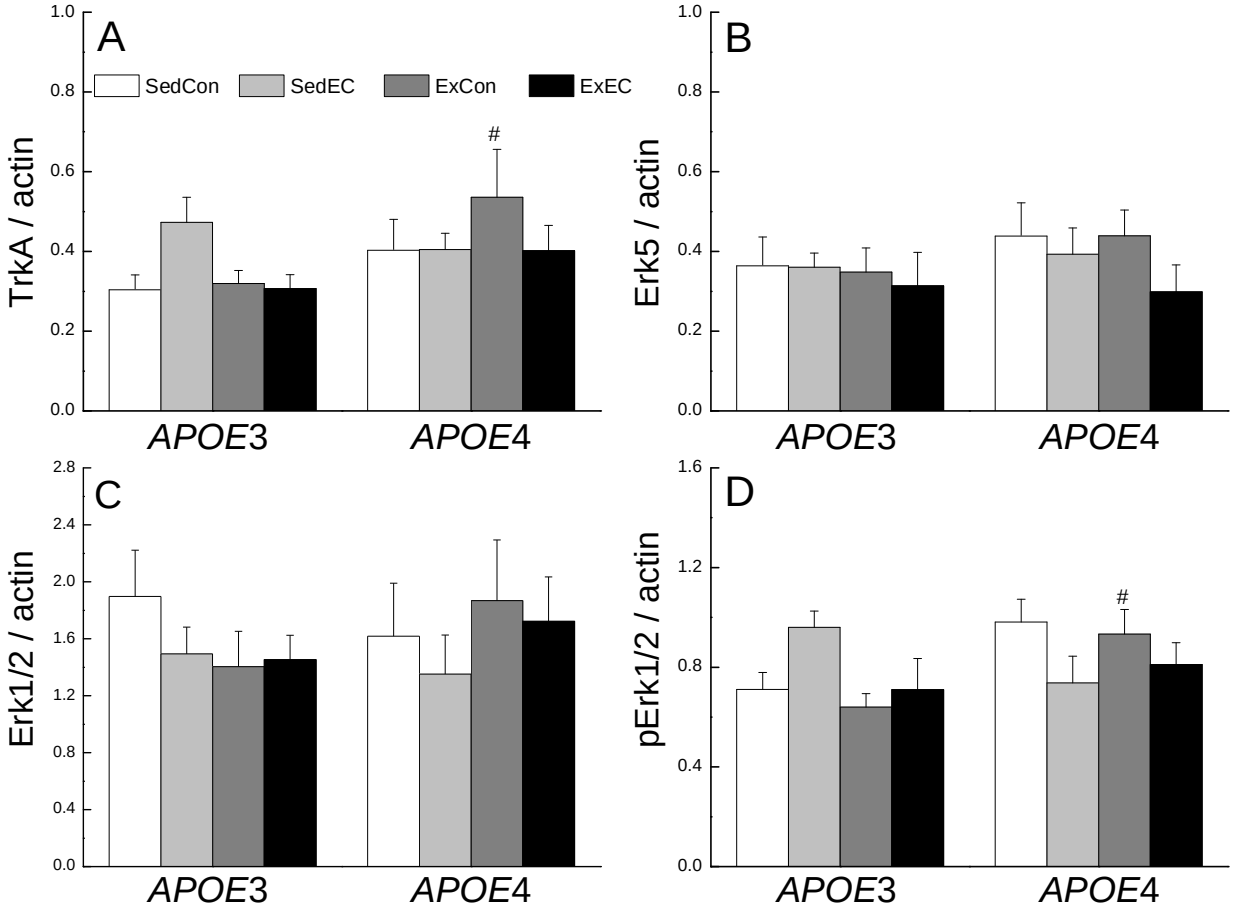
A
SedCon
SedEC
ExCon
ExEC
TrkA / actin
#
APOE3
APOE4
B
Erk5 / actin
C
Erk1/2 / actin
D
pErk1/2 / actin

Figure 15: Effect of exercise and/or antioxidant regimen on the expression of Trk A (A), Erk5 (B), and pErk1/2 / Erk1/2 ratio (C) in the hippocampus in middle age GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=8. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3.

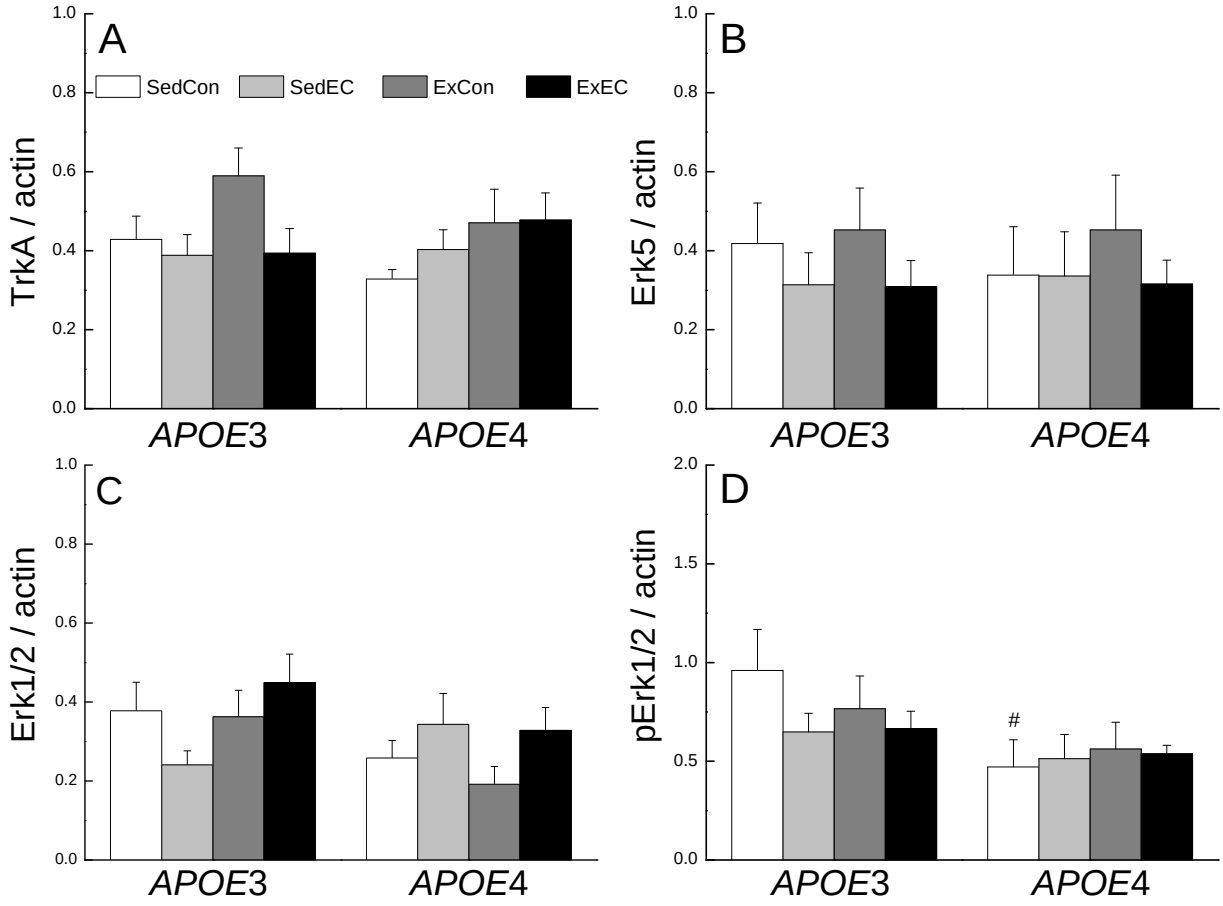
A
SedCon
SedEC
ExCon
ExEC
TrkA / actin
1.0
0.8
0.6
0.4
0.2
0.0
APOE3
APOE4
B
Erk5 / actin
C
Erk1/2 / actin
D
pErk1/2 / actin
2.0
1.5
1.0
0.5
#

Table 1: Effect of exercise and/or antioxidant regimen on reflex and muscle strength measured as latency (in seconds) in middle age (12-16months) GFAP-*APOE*3 and GFAP-*APOE*4 mice.

Each value represents mean ± SEM, n=18-26. SedCon, sedentary with control diet; SedEC, sedentary with diet rich in vitamin E and vitamin C; ExCon, exercise with control diet and ExEC, exercise with diet rich in vitamin E and vitamin C. # $p<0.05$ compared to treatment matched *APOE*3; *$p<0.05$ compared to genotype-matched SedCon; †$p<0.05$ compared to genotype-matched SedEC

Behavior Test	*APOE*3				*APOE*4			
	SedCon	SedEC	ExCon	ExEC	SedCon	SedEC	ExCon	ExEC
Walking Initiation (s)	3.34 ± 0.2	3.49 ± 0.33	2.83 ± 0.22	3.22 ± 0.36	3.60 ± 0.35	3.36 ± 0.26	3.92 ± 0.25$^{\#}$	3.85 ± 0.22
Alley Turn (s)	7.29 ± 0.41	6.17 ± 0.37	5.90 ± 0.38	5.76 ± 0.37	8.83 ± 0.71	11.08 ± 1.27$^{\#,}$	8.82 ± 0.58$^{\#,\dagger}$	7.63 ± 0.49$^{\#,\dagger}$
(-) geotaxis 180 (s)	5.10 ± 0.39	3.80 ± 0.23*	5.13 ± 0.36	4.32 ± 0.35	5.66 ± 0.53	5.78 ± 0.65$^{\#}$	4.90 ± 0.41	5.22 ± 0.55
Wire Tread (s)	17.40 ± 3.40	16.38 ± 3.35	13.14 ± 2.69	10.45 ± 2.31	20.15 ± 3.61	19.59 ± 3.23	25.12 ± 3.66$^{\#}$	24.64 ± 4.18$^{\#}$
Wire Fall (s)	27.16 ± 2.58	26.24 ± 2.57	28.45 ± 2.51	30.30 ± 2.64	25.14 ± 2.10	30.10 ± 2.36	24.96 ± 3.07	23.95 ± 2.79

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CHAPTER 5

General Discussion

Apolipoprotein E4 (*APOE*4) is a major genetic risk factor for development of late-onset sporadic Alzheimer's disease (AD) (1-3). While in middle age individuals, *APOE*4 has been associated with cognitive deterioration and memory loss (4,5), there has been inconsistent reports within a younger population (6-9). In young individuals, studies have reported conflicting findings with a lack of difference between *APOE*4 carriers and non-carriers (8,9), a worsening associated with *APOE*4 (7), and even an improved cognitive performance with the presence of *APOE*4 (10-13). Such discrepancies amongst studies warrant more detailed evaluation of such outcomes. As such antagonistic pleiotropy may also impact the outcome of interventions. Furthermore, apart from cognitive decline, motor function impairment has been substantiated in AD patients (14-17), as well as a two-fold increase in motor dysfunction in the presence of *APOE*4 allele (18). Pathological changes occurring in motor control system (motor cortex, striatum, substantia nigra) might be responsible for this motor function decline in AD (19-21). In addition, the involvement of cerebellum (22,23) could explain the coordination, balance, gait dysfunction leading to injurious falls in initial stages of AD.

Oxidative stress is a major contributor to AD pathology (24,25) and *APOE*4-associated exacerbation of AD pathophysiology might occur through increased oxidative stress (26,27). Hence, antioxidant therapy might be even more beneficial in the presence of *APOE*4 allele (28-

31). Another life style modification, exercise has been shown to improve symptoms and delay the progression of AD (32-36). Exercise benefits were more pronounced in the presence of *APOE4* genotype in the context of aging and cognition (37,38). Furthermore, exercise training seems to improve cognition (39,40) and reduce anxiety (41) by lowering oxidative stress (42). Interestingly, various exercise regimens have also improved motor function in cognitively-impaired geriatric population (43-45).

Our study was set out to study the effect of antioxidant and exercise on motor and cognitive function, and to determine whether *APOE* genotype would influence the outcome. Our study is the first to thoroughly look at all aspects of behavioral profile of the GFAP-*APOE* mice (young and old). Furthermore, the study was set to also determine whether antioxidant and exercise would improve motor and cognitive dysfunction associated with *APOE*4. The central hypothesis was that *APOE*4-associated brain dysfunction would be reversed in mice receiving a combination of antioxidant supplementation and exercise training, even more so than in mice receiving each treatment alone.

The main findings of this study are illustrated in Table 1 (young adult GFAP-*APOE* mice) and Table 2 (middle age GFAP-*APOE* mice). *APOE* genotypes showed differential behavioral phenotype depending on the brain function, the age and the treatments.

One very interesting finding was the antagonistic pleiotropy associated with the *APOE* genotype. In humans, *APOE*4 was associated better performance in young individuals which then shifts to a negative outcome in older individuals (11-13). This antagonistic pleiotropy has not been well studied and has remained elusive. In our study, we observed that in young mice *APOE*4 was associated with better performance on a non-spatial task such as active avoidance and no difference on the spatial task. However, in the middle age group, *APOE*4 mice performed

worse on the spatial task and identical on the non-spatial one. It is noteworthy than when comparing young adult with middle age, the rate of performance deterioration was higher in the *APOE*4 mice than *APOE*3. Our data were supportive on the antagonistic pleiotropic aspect of *APOE*4. Our mice were about 5-7 months and 14-16 months when tested for cognitive function, and it is possible that the *APOE* genotype effect would have been larger with a younger age group. Mice expressing human *APOE*4 either in targeted replacement mouse model or GFAP-*APOE* or NSE-*APOE* mouse model have been shown to have poor spatial learning and memory at later age. Even though we are also reporting impaired spatial learning and memory, the effects observed in our study are not as large as others have reported. These differences in effect size can be due to several factors such as the age of the mice, the mouse model being used, and the way behavioral tests were conducted (5,10,46-49).

Interestingly, both young adult and middle age *APOE*4 mice swam faster in water maze task. Faster swimming can be considered as a sign of higher motivation; however it did not translate to an improvement in spatial learning and memory in any group. Another reason for fast swimming could be a higher level of anxiety, which we tested using the elevated plus maze paradigm. However, while in the young adult groups the *APOE*4 mice spent more time in the open arms than the *APOE*3 mice, there was no difference between the two strains at middle age. Therefore, the higher swimming speed was not related to changes in anxiety levels and was also not related to spontaneous activity as there was no major differences in that test either Furthermore, in comparison with wild-type mice, *APOE*3 and E4 seemed to exhibit less anxiety which is in contrast with previous studies (50).

While other studies in humans have reported hyperactivity being associated with the presence of *APOE*4 (51), rodent studies have reported decreased locomotor activity in *APOE*4-

TR mice (52). The transgenic mice in our study did not exhibit increased or decreased locomotion or exploration during open field test or elevated plus maze at younger age but at the middle age the rearing counts were low in *APOE*4 compared to *APOE*3 and WT similar to previous report using NSE-*APOE* mouse model (47) and GFAP-*APOE* mouse model (50). This lower spontaneous activity could be related to lesser anxiety among these mice as reported previously (53).

An interesting finding of these studies was the unexpected effects of *APOE*4 on motor function. While most studies focus on the cognitive aspects, it is important to also consider motor function as AD is often associated with motor function deficits, gait issues, slower learning of new motor skills, and slower speed of walking with reduced overall mobility. Furthermore, motor function assessment in terms of muscular reflex, plantar responses and muscle tone deficiencies have been studied as cognition independent neurologic symptoms in AD (54). In our study, we also observed motor deficiencies associated with *APOE*4 in both age groups, which were consistent with previous studies by Meer *et.al.*(46), with poor performance in coordinated running task in presence of *APOE*4 genotype.

After understanding the genotype variation in cognitive and motor functions, we further assessed the impact of antioxidants and /or exercise behavioral as a function of genotype and age. Only in the *APOE*4 young adult mice, antioxidants containing treatments increased anxiety. This was in contrast with previous reports relating that vitamin E depletion increased anxiety (55) and decreased with vitamin E and vitamin C supplementation (56). No such effect was observed in the middle age mice. This effect on anxiety was not reflected in the outcome of spatial cognition.

On spatial learning and memory, all treatments were effective in improving performance in the middle age *APOE*4 mice while no effects were observed in the younger mice or *APOE*3 mice. There seems to be a genotype-dependent pathway underlying the improvements in spatial learning. On non-spatial task, improvements in performance were observed regardless of the genotype or age of the mice. These differing behavioral outcomes also support different time windows for treatment depending on the cognitive aspects being studied. Based on these data, the window to improve spatial learning might be narrower than the window to improve non-spatial cognitive function. It could also signify that the underlying pathways for non-spatial task are independent of *APOE* genotype, and therefore interventions can improve performance regardless of an association with exacerbated impairments via *APOE*4. Physical activity is known to improve the cognitive function and to have a positive impact on functional plasticity (32) especially in individuals carrying *APOE*4 (57,58) supported by transgenic mouse model (59). In this study, exercise improved spatial cognition in middle age mice only in the *APOE*4 mice. This effect also supports a genotype-dependent pathway regarding spatial learning and memory that is independent of the pathway for non-spatial tasks. Visual dysfunction was discarded as a potential confounding factor; as neither genotype nor treatments affected the performance of the mice on the visible platform task.

While *APOE*4 had diminished motor function capacities, the effects of the treatments were genotype and age dependent. The treatments were more effective in improving function in the younger mice, while the effects were marginal in the older groups. Furthermore, exercise was more effective at improving motor function than antioxidants. Exercise improved coordination, reflexes and muscle strength in *APOE*4 mice, while antioxidants only improved

reflexes in the younger mice. In the older mice, interventions only improved balance and solely in the *APOE*3 mice.

Studies on combination of antioxidant and exercise have led to conflicting results with an additive effect in aged rodents, (60,61) while an antagonistic effect in young mice (62). Since, *APOE*4 has been associated with lower antioxidant activity (63), decreased capacity to remove by-products of oxidative stress (64) and increased oxidative stress (65); a combination of antioxidants to lower oxidative stress and exercise to boost antioxidant defenses should lead to a further improvement than each intervention independently. Our study revealed no such beneficial additive / synergistic interaction; in fact most effects observed on cognition, coordination in both age groups and motor function in middle age mice with the combination treatment mimicked the effects seen with exercise. The lack of an additive/synergistic effect on cognitive function may have been due to reaching a maximum ceiling of performance.

Interestingly, in young adult mice, it was evident that the combination of exercise and antioxidants had an antagonistic effect across all of the motor function tests. Among *APOE*3 mice, exercise induced decreased rearing activity and improved latency to fall on high bridge walk test were nullified with (the addition antioxidants) ExEC treatments. Similarly, among *APOE*4 mice, exercise led lowered spontaneous locomotion, improved learning phase latency in coordinated running performance task, faster reflexes achieved in negative geotaxis test and lastly improved muscle strength in wire suspension test became insignificant with ExEC treatment. Further, antioxidant treatment directed improved treading reflex and shorter spontaneous walk initiation were cancelled with combination of antioxidants and exercise.

In conclusion, this study indicated that the GFAP-*APOE* mice may not be the best model for cognitive dysfunction as the differences were relatively minor on spatial and non-spatial

tasks. However, this mouse model could become of interest to study the motor dysfunction associated with *APOE*4, especially since they appear early in the life of the animals. While both exercise and antioxidants improved different aspects of motor and cognitive function, exercise seemed to be the most promising as it improved more aspects in both genotypes. However, in cases when individuals cannot exercise, it is important to know that antioxidant supplementation can lead to the same effects. Lastly, there were no additive effect of the combination of antioxidants with exercise, and in some case antagonistic action of the antioxidant on the beneficial effect of exercise was observed in the young mice. Therefore, the age of the individual as well as their genotype might be of the upmost importance to know prior to starting any interventional strategies.

Table 1: Adult GFAP-*APOE* mice (5-7 months)

Function	***APOE*3 Vs *APOE*4**	**SedEC**	**ExCon**	**ExEC**
Spatial Learning	E4↔ E3	↔	↔	↑ E4
Discriminated avoidance	E4 > E3	↑ E4	↑ E3, E4	↑ E3, E4
Swim Speed	E4 > E3	↔	↔	↔
Anxiety	E4 < E3	↑ E4	↔	↑ E4
Locomotor activity	E4↔ E3	↔	↓ E3, E4	↔
Coordinated running	E4 < E3	↔	↑ E3, E4	↑ E3
Reflexes	E4 < E3	↑ E4	↑ E4	↔
Muscle strength	E4 < E3	↔	↑ E4	↔
Balance	E4 < E3	↔	↑ E3	↔

Table 2: Middle age GFAP-*APOE* mice (14-16 months)

Function	***APOE*3 Vs *APOE4***	**SedEC**	**ExCon**	**ExEC**
Spatial Learning	E4 < E3	↑ E4	↑ E4	↑ E4
Discriminated avoidance	E4 ↔ E3	↑ E3	↑ E3, E4	↑ E3, E4
Swim Speed	E4 > E3	↔	↔	↔
Anxiety	E4 ↔ E3	↔	↔	↔
Locomotor activity	E4↔ E3	↔	↔	↔
Coordinated running	E4 < E3	↔	↔	↔
Reflexes	E4 < E3	↔	↔	↔
Muscle strength	E4 < E3	↔	↑E4	↔
Balance	E4 ↔ E3	↑ E3	↑ E3	↑ E3

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