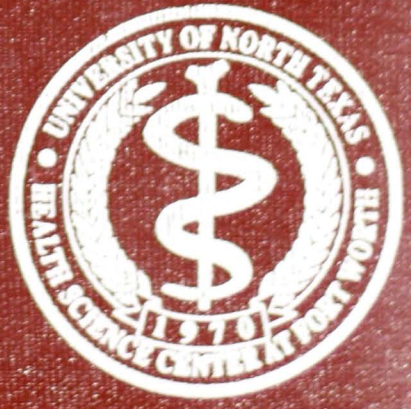
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The purpose of these studies was to determine if vitamin E supplementation, a well-studied antioxidant, could improve the cognitive functions of old mice either by preventing age-dependent impairments or reversing age-related dysfunction. Cellular oxidative stress is believed to be a causal factor in scenescence, and the brain appears to be particularly susceptible to oxidative damage because of a relatively high rate of reactive oxygen species generation without commensurate levels of antioxidant defenses. If oxidative stress indeed plays a role in age-related brain dysfunction, then it can be predicted that experimental interventions capable of lowering oxidative stress would either prevent or restore function.

This was tested using apolipoprotein E-deficient mice, which have an increased susceptibility to neuronal oxidative damage, maintained on 3 different doses (2 mg/kg, 20 mg/kg, or 200 mg/kg /day) of dl-α-tocopheryl acetate (vitamin E) via supplemented food pellets from 8 weeks of age throughout behavioral testing when 6 or 18 mo of age. A separate experiment used wild type mice 24 months of age to examine whether or not a combination of vitamin E (123 mg/kg/day) with coenzyme Q_{10}, (200 mg/kg/day) which leads to higher tissue levels of vitamin E, could improve brain functions in old mice. Mice were tested on multiple behavioral tasks that required utilization of various components of memory and learning, as well as sensorimotor testing. The highest dose of vitamin E prevented the decline of spatial memory in old apolipoprotein E-deficient

mice, but did not prevent age-related impairments in learning and memory for discriminated escape. When old wild type mice were treated with the combined vitamin E and coenzyme Q_{10}, the mice learned and remembered to avoid a preemptive shock significantly more than old mice treated with vitamin E or coenzyme Q_{10} alone. A followup experiment with higher doses of coenzyme Q_{10} alone (250 or 500 mg/kg/day) resulted in no cognitive improvements. No treatments improved sensorimotor performance.

LIFELONG vs. LATE LIFE TOCOPHEROL

ON LEARNING AND MEMORY

IN MICE

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LIFELONG vs. LATE LIFE TOCOPHEROL ON LEARNING AND MEMORY IN MICE

DISSERTATION

Presented to the Graduate Council of the

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In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Shelley R. McDonald, B.A., M.C.G.

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CHAPTER I

INTRODUCTION

Theories of Aging

Aging can be viewed as a stage of the lifespan in which universal, progressive, deleterious changes over time lead to functional impairment and ultimately death. Decrements of advanced age occur at the molecular, cellular, and tissue/organ levels, as well as with the integrated functions of the whole organism, such that growth and normal homeostasis cease. However, the rates of aging and apparent declines of function occur independently within systems, and the range of performance among animals increases greatly among older animals. This suggests that aging is complex and governed by many factors, unlike disease states, which more often have identifiable pathogens and predictable courses. To study diseases associated with aging presents a unique challenge because of the difficulty separating the two processes. It is imperative to keep in mind the theoretical concepts that govern research in the field. Two basic theories have been proposed to explain aging: (i) an intrinsic genetically programmed decline of function following growth and reproduction or (ii) an accumulation of random damage to molecules that leads to loss of functional integrity with advanced age [4].

The Intrinsic Theory is supported by the observation that each species has a set maximum lifespan potential (MLSP). Even within cells lines there are a limited number of replications [19]. Manipulation of genes from yeast to mice has been shown to alter the average and maximum lifespans [55]. Purported mechanisms for these observations may be due in part to specific longevity genes, telomere length, apoptotic regulation, control of the hypothalamic-pituitary-adrenal (HPA) axis or even immunolgical failure [55]. One particular gene associated with longevity in humans, apolipoprotein E, codes for a protein involved in cholesterol transport. Three isoforms of this protein are found in humans of which the E4 isoform is inversely related to longevity due to both increased prevalence of Alzheimer's disease and coronary artery disease, while E2 is more represented among centenarians [43].

Pure environmental insults were speculated to cause aging based on early observations that ionizing radiation shortened lifespan. This was the origin of the Stochastic Theory of Aging that proposed reduced longevity was due to an accumulation of molecular damage leading to loss of function. The source of the damage does not necessarily have to be from exogenous sources, but can be the toxic by-products of normal aerobic metabolism, known collectively as reactive oxygen species (ROS) [15, 46]. The overproduction of reactive oxygen species and/or decreased antioxidant defenses causing cellular and molecular damage over time is the oxidative stress theory of aging [18]. During aging, the rates of mitochondrial $O_2^{-\bullet}$ and H_2O_2 generation are inversely related to the MLSP of mammals [46] suggesting that age-associated molecular damage causing loss of function operates within a defined genetic framework. Even with

the observations implicating genetic components as a causal factor in aging, certain environmental factors are known to alter the expression of the genetic baseline. The oxidative stress theory of aging is a plausible explanation to reconcile the two basic theories of aging.

Alzheimer's disease

A prototypical disease of aging is Alzheimer's disease (AD), which is the most common cause of dementia in the elderly. Dementia is defined as a global deterioration of intellectual and cognitive functions. In AD this deterioration is progressive, irreversible, and results in total debilitation. Observational studies have identified many risk factors that associated are with an increased probability of developing AD, such as hypertension, diabetes, smaller head circumference, depression, paucity of leisure activity, higher intake of calories and fats and lower dietary antioxidant intake. Conversely a higher dietary intake of vitamins E and C is associated with decreased risk [32]

Impairment in oxidative regulation may have a significant role in the pathogenesis of neuronal death in AD, as evidenced by increased markers of oxidative stress [11, 26] and reduced serum superoxide dismutase, an endogenous antioxidant enzyme, observed in patients with AD [52]. This damage may be subject to modification based on the observation that vitamin E treatment (2000 IU / day) in humans may slow the progression of functional impairments associated with AD [42] which suggests that lipid peroxidation may be a critical factor in neuronal degeneration of AD.

Apolipoprotein E

At least 6 different genetic loci have been linked to the clinical signs, symptoms, and neuropathology of AD [40]. However, the gene encoding for apolipoprotein E on chromosome 19 is the only genetic factor identified thus far that is a known susceptibility gene for late-onset familial and sporadic AD [40, 49]. Miyata and Smith demonstrated the differential neuroprotective abilities of apoE isoforms when cultured B12 neuronal cells were exposed directly to β-amyloid or hydrogen peroxide[31]. All isoforms protected against hydrogen peroxide toxicity significantly more than cells without any apoE; apoE2 > apoE3 > apoE4 > control. Thus, the mechanism whereby apoE4 confers increased AD risk may involve a reduced capability to protect against oxidative stress. ApoE appears to be an important genetic factor that could be part of a proposed 'deleterious network' in which multiple factors come together in a final common pathway that results in AD [58].

ApoE has an essential role for lipid homeostasis in the brain; it serves as a ligand for the low-density lipoprotein receptor pathway that internalizes lipid particles into central nervous system (CNS) neurons. Although the mechanisms involved are not clear, evidence also suggests apoE may be important for CNS repair following injury [36], compensatory synaptogenesis in patients with AD [41], and differential neurite extension and branching [2].

Apolipoprotein E-deficient mice

ApoE-deficient mice are an essential tool to elucidate the specific functions and mechanisms of apoE in the CNS in an *in vivo* system. Earlier studies in apoE-deficient

mice, from ages 2 to 7 months, identified increased serum low-density lipoprotein oxidation and aortic atherosclerotic lesions [34]. The atherosclerosis was reduced by 36% in apoE-deficient mice fed a diet supplemented with the antioxidant N,N' -diphenyl 1,4-phenylenediamine (DPPD). This particular study also reported that treatments with DPPD reduced susceptibility of isolated lipoproteins to copper-induced oxidation and produced a 2-fold delay in the formation of conjugated dienes after addition of $CuSO_4$ to the lipoproteins [51]. Furthermore, apoE-deficient mice exhibit a pattern of neuropathology that parallels certain neurological changes in AD. Brain choline acetyltransferase is reported to be markedly lower in the hippocampus and frontal cortex, and the microtubule associated protein tau appears to be hyperphosphorylated in apoE-deficient mice [10, 14]. An age-dependent decrease in the percentage of neuropil area that reacts with synaptophysin and microtubule associated protein-2 in neocortex and hippocampus of apoE-deficient mice suggests synaptic damage, neurodegeneration, and disruption of the cytoskeleton [28]. Learning and memory deficits correlate with the neuropathological changes in apoE-deficient mice. Impairments in working memory and spatial learning and memory have been reported as measured by the Morris water maze task [14, 29, 33]. Nevertheless, the results of cognitive studies of apoE-deficient mice in the literature vary widely according to age, gender, genetic background and testing protocol [1, 12-14, 16, 17, 24, 29, 33, 37-39, 56].

Preliminary Studies

The data from our laboratory evaluating the cognitive capacities of the apoE-deficient mice clearly supports the hypothesis that apoE is essential for maintaining

cognitive functioning in older mice. We performed a cross-sectional study of spatial learning and memory in apoE deficient mice using a modified Morris water maze task [7] (see chapter II for methods). Two groups of mice were behaviorally tested at 5 or 18 mo of age. The young age group consisted of 7 apoE-deficient mice and 13 controls and the old age group had 8 and 10 respectively. The mice were obtained through collaboration with Dr. Jonathan Smith from The Rockefeller University, New York, NY. Fig. 1 shows the effect of apoE deficiency on acquisition, retention, and reversal components of the water maze task. The path length of the 18 mo. apoE-deficient mice decreases less during acquisition and reversal phases as a function of session than in the wild type controls, thus indicating an age-associated impairment in the ability of apoE deficient animals to learn. The proportional increase in path length from session 8 to 9 (a 60 h no test interval) is greater in the older apoE-deficient mice suggesting an age-associated memory loss. The apoE-deficient mice demonstrated similar age-associated impairments during variable interval probe trial performance, in which the platform was lowered to the bottom of the tank and raised after a period of time (15, 20, 30, or 40 s) on different sessions. A battery of motorskill tests in the same groups of mice revealed no differences in sensorimotor performance, which suggests the observed differences cannot be attributed any to sensorimotor decline. ApoE-deficient mice may thus serve as an animal model to study enhanced risk for AD, by virtue of the apparently accelerated decline of memory and learning capacity.

Vitamin E

Vitamin E has been studied extensively in regard to what role its antioxidative function may play in preventing neurological dysfunction. Numerous reports support its function in attenuating molecular damage as well as mitigating functional deficits in animal studies. For example, when rats were pretreated with vitamin E before hypoxia-induced neuronal injury, the number of conjugated dienes in the hippocampus was dramatically reduced [20]. Place learning deficits and impaired hippocampal high-affinity choline uptake caused by intracerebroventricular injection of cholinotoxin can be reversed in rats pretreated with vitamin E [57]. Aged rats treated for 5 mo with an antioxidant cocktail that included vitamin E showed significantly improved water maze performance, suggesting that neurodegeneration associated with aging may involve oxidative damage [44].

Vitamin E is the generic term used to describe a group of lipid soluble compounds that exhibit the biological activity of α-tocopherol. Eight compounds in nature have vitamin E activity: d-α-, d-β-, d-γ-, and d-δ-tocopherol; and d-α-, d-β-, d-γ-, and d-δ-tocotrienol. These compounds differ in the number and position of the methyl groups on the chroman ring and saturation of the phytyl tail. (Fig 2.) Vitamin E is produced in nature by plants which synthesize predominately the d-β-, d-γ-, and d-δ-tocopherol forms. Synthetic vitamin E is a mixture of 8 stereoisomers of α-tocopherol. The free alcohol forms of vitamin E can be oxidized when exposed to air, thus lose biopotency, so acetate and succinate esters of vitamin E are used in supplements and fortified foods. The acetate

and succinate derivatives, however, are not biologically active unless the free tocopherol is regenerated by esterases [6].

The molecule functions as a lipid soluble chain breaking antioxidant [6]. Lipids, especially membrane phospholipids and plasma lipoproteins, are protected against oxidative damage when the phenolic hydroxyl group of tocopherol reacts with organic peroxyl radicals. The resulting organic hydroperoxides can be detoxified via nonradical reactions, and tocopheroxyl radicals can be reduced with hydrogen donors back to tocopherol [54].

$$\text{toc-OH} + \text{LOO}\cdot \rightarrow \text{toc-O}\cdot + \text{LOOH}$$

$$\text{toc-O}\cdot + \text{CoQH}_2 \rightarrow \text{toc-OH} + \text{CoQH}\cdot$$

Vitamin E is clearly involved in neuronal metabolism and maintenance, partially through antioxidative functions. In rats maintained on a vitamin E deficient diet, brain tissue was more susceptible to *in vitro* oxidative stress than muscle, spinal cord, or peripheral nervous tissues [25]. In humans a rare neuropathy is associated with an inborn error of lipoprotein metabolism for which high doses of vitamin E are able to prevent and delay the associated neurological dysfunction [47]. Vitamin E may also be able or prevent or delay the neurodegeneration and dysfunction that is evident in apolipoprotein E deficient mice. If apoE has a significant antioxidant function in CNS neuronal maintenance, then supplementation with vitamin E should significantly reduce the levels of oxidative stress in brains of apoE-deficient mice and should prevent or delay occurrence of learning and memory deficits.

Brain levels with vitamin E supplementation

The apoE-deficient mice are characterized by marked hypercholesterolemia primarily due to elevated levels of very low-density lipoproteins and intermediate-density lipoproteins, both of which transport vitamin E in the circulation [35, 53]. The liver preferentially incorporates the α-tocopherol form of vitamin E into nascent very low-density lipoproteins, which eventually deliver α-tocopherol to peripheral tissues via low-density lipoproteins [54]. When lipoprotein concentrations are raised, the vitamin E concentrations are also raised [6]. Pharmacologic doses of vitamin E in hypercholesterolemic mice such as the apoE-deficient mice may provide a means to facilitate a higher utilization rate of antioxidants in the CNS, particularly the presynaptic nerve terminals. Vitamin E levels have been shown to be significantly increased in the brain of rodents following dietary supplementation. In fact, levels in the brain have been shown to increase in a dose-dependent manner until a ceiling effect occurred at doses of 60 mg/kg dl-α-tocopherol per kg of diet and greater [27]. Our lab observed an increase consistent with the reported maximal brain concentration in the literature for the cerebral cortex concentrations of α-tocopherol following supplementation with 1650 mg per dl-α-tocopheryl acetate per kg of food in both young and old mice after 12 weeks of dietary fortification [50].

Late life initiation of antioxidant treatment

Despite increased brain concentration of vitamin E, results from our lab show that supplementation with dietary vitamin E in aged mice failed to reverse either oxidative

damage or functional deficits in behavior [50]. However, supplemental antioxidants or antioxidant-rich foods, when given to old mice or rats for relatively short periods of time, have been shown to be effective in ameliorating age-related deficits in cognitive and/or psychomotor function, thus supporting the premise that some aspects of age-related dysfunction are reversible [3, 5, 45]. The hypothesis that oxidative stress is a reversible mechanism in age-related cognitive impairment is further supported by studies using dietary restriction. Restriction of dietary intake for as little as 3 weeks in old rodents, has been shown to lower protein oxidation in the brain and reverse some aspects of age-related behavioral dysfunction [8, 9, 30].

Combination antioxidant treatment

Theoretically combinations of antioxidant treatments designed to augment the functions of one another may allow for more effective biological activity by modulation of the redox state. Coenzyme Q (CoQ) and vitamin E have an apparent interaction in respiring mitochondria. CoQ, itself a potent antioxidant, is thought to have a sparing effect on α-tocopherol, a membrane antioxidant that in mitochondria, plays a significant role in modulating oxidant production [23, 48]. When α-tocopherol is oxidized to a tocopheroxyl radical by peroxyl or superoxide anion radicals, reduced CoQ in respiring mitochondria can regenerate α-tocopherol [21, 23, 48]. That such an interaction occurs in vivo is supported by studies indicating that supplementation with CoQ elevates levels of α-tocopherol in mitochondria of many tissues of aged mice [22]. The concurrent intake of vitamin E and CoQ may have an additive effect, thus leading to greater antioxidant

potential and more efficient modulation of oxidant production in mitochondria of aged mice.

Goals of the current research

Collectively the goal of these studies was to support oxidative stress as the fundamental mechanism of age-dependent declines in learning and memory by administering therapies with known antioxidant actions to aging and aged mice, which have age-dependent cognitive declines analogous to humans. Additionally, by replicating the findings of the pilot study and further characterizing multiple cognitive processes in apoE-deficient mice, we hope to show that apoE-deficient mice are a model of more rapid brain aging. This is based on intrinsic elevated susceptibility to neuronal oxidative damage with age due to lack of the protein apoE. Therefore, the goal of the first study was to determine if the activity of apoE protects against age-associated susceptibility to neuronal oxidative damage, and if lifelong experimental augmentation of oxidative defenses in animals lacking apoE prevented any consequential age-dependent decline in neuronal function from 6 to 18 months of age.

The goal of the second study was to determine if the brain functions of aged mice could be improved. Enhancement of mitochondrial function and thus improvement of oxidation-sensitive brain functions were the basis for targeted combinations of dietary antioxidants. If oxidative stress indeed plays a role in age-related brain dysfunction, then it is predicted that experimental interventions capable of lowering oxidative stress and decreasing steady-state concentrations of oxidized macromolecules, would be sufficient

to restore molecular functioning, and thus to reverse age-related losses of cognitive or motor performance.

Fig 1. Effect of apoE-deficiency on spatial learning and memory as measured by the mean path length (cm ± SEM) traveled as a function of session for acquisition, retention and reversal components of the water maze task. Separate age groups of apoE-deficient mice and wild type controls were tested when 5 or 18 mo of age. The top panel shows the age-associated changes in wild type controls. The bottom panel shows the age-associated changes in the apoE-deficient mice.

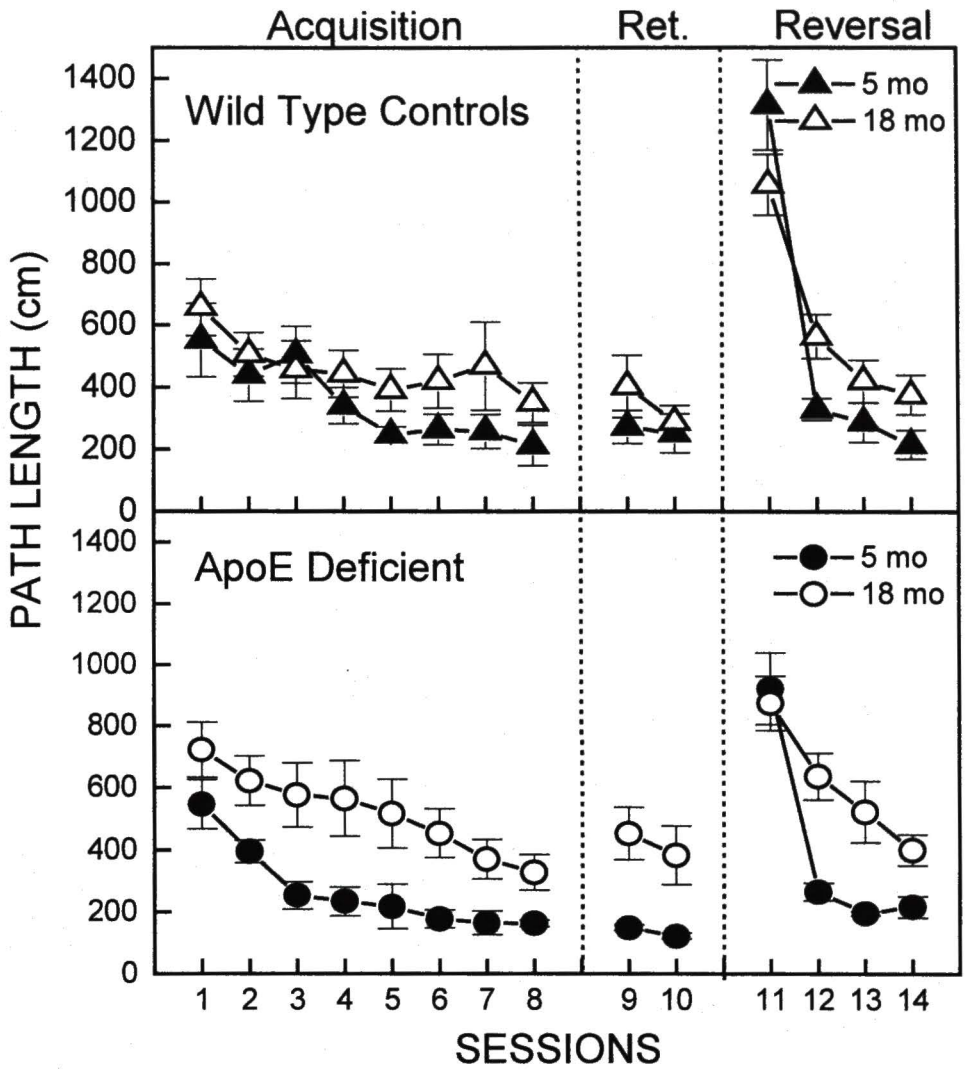
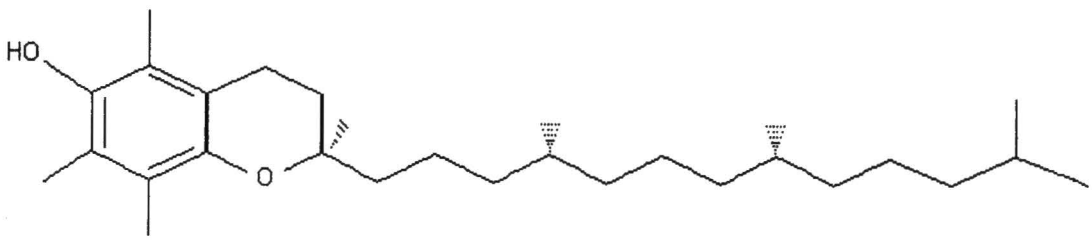

Acquisition
Ret.
Reversal
Wild Type Controls
5 mo
18 mo
ApoE Deficient
5 mo
18 mo
PATH LENGTH (cm)
0
200
400
600
800
1000
1200
1400
1 2 3 4 5 6 7 8
9 10
11 12 13 14
SESSIONS

Fig 2. The structure of d-α-tocopherol.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LIFELONG VITAMIN E TREATMENT AMELIORATES SPATIAL MEMORY DEFICITS IN OLD MICE

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Running title: Vitamin E in apoE-deficient mice

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CHAPTER II

LIFELONG VITAMIN E TREATMENT AMELIORATES SPATIAL MEMORY DEFICITS IN OLD MICE

SUMMARY

The potential for lifelong vitamin E supplementation to protect against loss of cognitive decline in mice susceptible to age-associated neuronal oxidative damage was tested in apoE-deficient and wild type C57BL/6 mice maintained on 2.0 mg/kg, 20.0 mg/kg, or 200.0 mg/kg body weight of dl-α-tocopheryl acetate via supplemented food pellets from 8 weeks of age throughout behavioral testing when 6 or 18 mo of age. Cognitive functioning was assessed using swim maze and discriminated avoidance testing. Medium and high doses of vitamin E supplementation eliminated the age-associated impairment in spatial accuracy and memory for wild type mice as compared to controls in the water maze task, whereas only the high dose was able to prevent the decline in spatial accuracy and memory apparent in older apoE-deficient mice. Equivalent age-related impairments in learning and memory for discriminated escape were observed, and no vitamin E dose had any effect on performance for either genotype at any age. These results suggest that differential utilization of lifelong dietary

antioxidants may be dependent on underlying susceptibility to age-dependent neuronal dysfunction.

1. Introduction

Alzheimer's disease (AD) affects an estimated 4.5 million [20] with advanced age being the single most significant risk factor for developing AD. The symptomatic alterations in memory and cognitive function characteristic of late-onset, sporadic Alzheimer's disease begin after the age of 55 years with the mean age of onset at 70 years of age [42] and increase exponentially into very old age [22]. Even though this seems to be a problem encountered late in life, it is a costly one, with the estimated annual national direct and indirect costs estimated to be at least $100 billion [10]. This burden to future generations could be dramatically lowered based on the estimation that the number of individuals with Alzheimer's disease would be reduced almost 50 percent after 50 years if the onset of the disease could be delayed by 5 years [2].

Apolipoprotein E (apoE) genotype is the most well-studied genetic loci associated not only with late-onset, sporadic AD but with an earlier progression of usual age-associated cognitive slowing [7, 8, 33, 36, 48]. This proposed susceptibility gene is found in three allelic variants: apoE2, apoE3 and apoE4 [37]. Inheritance of the apoE4 allele confers a dose effect for an increased risk of clinical expression and earlier onset of AD [8, 38, 43] while apoE2 is associated with later onset [6]. However, some of the oldest-old may remain cognitively intact even in the presence of a homozygous E4 genotype [21] suggesting that genetic components known to be associated with an increased risk of developing AD appear to be modulated by other factors, such as age-associated oxidative

damage. With advanced age, the brain becomes especially vulnerable to the toxic by-products of normal metabolism due to increased generation of reactive oxygen species (ROS) along with concomitant decreases in antioxidant defenses which results in an accumulation of oxidized macromolecules and decrement of neuronal function or pathological neurodegeneration in susceptible individuals [41]. If the activity of apoE protects against age-associated susceptibility to neuronal oxidative damage, then experimental augmentation of oxidative defenses in animals lacking apoE should prevent consequential decline in neuronal function as the animals age.

This functional activity of the apoE gene product to protect against age-associated oxidative damage has been shown both *in vitro* [29] and *in vivo*. Mice targeted for genetic deletion of apolipoprotein E (apoE-deficient mice) when compared to wild type mice exhibit age-dependent synaptic and microtubule neuropathologies, as well as reduced antioxidant levels, increased oxidative damage to lipids and proteins and age-associated cognitive deficits [3, 14, 17, 25-27, 30, 32, 34, 35, 39]. The apoE-deficient mice used in this study were identified to have age-associated damage to specific oxidation-sensitive proteins at younger ages than in wild type mice [4]. Transgenic mice expressing human apoE4 exhibited the same age-dependent neurodegeneration as apoE-deficient mice, whereas transgenic mice expressing human apoE3 closely resembled wild-type mice [3] suggesting that apoE-deficient mice are a sufficient model to study therapies directed at delaying or preventing the functional consequences of accelerated oxidative damage.

A small study with female apoE-deficient mice treated with a 1% vitamin E fortified diet for 12 months, showed decreased CNS lipid peroxidation along with preserved

dendritic structure and more rapid spatial learning [47]. Vitamin E maintains membrane fluidity and functional integrity by protecting membrane phospholipids and plasma lipoproteins against oxidative damage [5]. Our lab has shown that when vitamin E is given to old mice late in life after learning impairments are evident, dietary supplementation fails to reverse the age-associated cognitive impairments [45]. It is not known to what extent cognitive functions may be protected by life-long dietary fortification or the optimal doses needed to achieve a redox shift in order to prevent age-related cognitive dysfunction.

The goals of present study were to more fully characterize the decline of cognitive loss by exploring multiple cognitive domains in apoE-deficient mice, which are known to be more vulnerable to age-associated neuronal oxidative damage, and to evaluate whether or not lifelong dietary supplementation with vitamin E will prevent learning and memory impairments of aging in a dose-dependent response in either wild type or apoE-deficient mice.

2. Materials and methods

2.1 Animals

The study began with 147 male, 4 week-old C57BL/6J mice and 162 homozygous for the $Apoe^{tm1Unc}$ mutation (apoE-deficient mice) backcrossed 10 times to C57BL/6J mice, obtained from The Jackson Laboratory [31]. The Jackson Laboratory estimated that

the N10 generation contained 99.91% of the genome from the C57BL/6J. Controls were the C57Bl/6J inbred strain. Upon arrival at the University of North Texas Health Science Center (UNTHSC) vivarium, the mice were housed individually in 28 x 19 x 12.5 cm solid bottom polycarbonate cages with wire tops, modified into two mouse units by insertion of a stainless steel divider. The colony room was maintained at 23°±1°C/40% humidity, under a 12-h light dark cycle with lights on at 0700 h. The mice had access to food and water, *ad libitum* at all times except during testing periods. Sentinels from the colony room tested serum negative for common pathogens during routine screening performed at 3-month intervals by Charles River Laboratories.

2.2 Vitamin E supplementation

Three weeks following their arrival in the UNTHSC vivarium, separate groups of 48-54 mice of each genotype, approximately 8 weeks of age, were assigned to receive the control diet (NIH-31 open formula) or one of two dl-α-tocopheryl acetate (Vitamin E) supplemented diets to either 10- (10X) or 100- (100X) times the concentration of dl-α-tocopheryl acetate added to the control diet. The adjusted concentrations of the three diets compounded by Harlan Teklad (Madison, WI) equaled 16.5- (control), 165- or 1650 mg dl-α-tocopheryl acetate per kg of food. This acetate ester was used to prevent oxidation during storage. The estimated daily doses of dl-α-tocopheryl acetate were based on food intake per kg body weight. Plasma and tissue concentrations of α-tocopherol following 100x supplementation with this test diet increased ~3 to 5 –fold in plasma and 50 to 60 %

in the cerebral cortex of both young and old groups of C57BL6 mice after 12 weeks of dietary fortification [44, 45]. The supplementation was maintained throughout the experiment.

2.3 Assessment of cognitive capacities

Each mouse received approximately 6 weeks of behavioral testing, 2 weeks for spatial discrimination in a swim maze followed by approximately 4 weeks of discriminated escape testing using a T-shaped maze, beginning when they were either 6 or 18 months of age. Twenty-four mice from each of the 6 genotype/supplementation groups were tested when 6 months of age (4 months following assignment) whereas the remainders were maintained in the colony until 18 months, at which point the survivors received the same test battery.

2.3a Spatial swim task

The apparatus used for this study consisted of a tank (110cm dia x 60 cm deep, filled to a depth of 34 cm with 24°± 2° C tap water, colored opaque white) [11]. A 10 cm square platform was placed in the tank 1 cm below the surface of the water so that it could not be seen by the mouse. The test consisted of a pretraining phase in which the mice learned the simple response components of swimming and standing on the hidden platform and a place discrimination acquisition phase in which the mice learned and

Performance was recorded via a computerized tracking system (San Diego instruments model # SA-3).

During the pretraining phase, a clear acrylic corridor (76 x 11 x 41 cm) leading directly to the platform was placed in the tank and a black curtain was draped around the tank to remove any visual cues. The mouse was placed at one end of the acrylic alley and allowed to swim to the end with the hidden platform. The mouse remained on the platform for 10 s and then was placed into a holding cage for an inter-trial interval (ITI) of 3 min. This phase consisted of 4 sessions of 5 trials delivered in 2 days. The latency to reach the platform in sec was recorded for each trial.

In the acquisition phase, each mouse was placed into the open tank (curtain and alley removed) from one of four starting positions and allowed to swim until it found the hidden platform up to a maximum time of 90 s. After 10 s on the platform, the mouse was removed and placed into a holding cage for an ITI of 10 min. Each acquisition session consisted of 5 platform trials with the raised platform in the same location, placed about 40 cm from the edge of the tank. This phase consisted of 8 total sessions, with 2 sessions per day, separated by at least 3 h. Distance traveled and latency to locate the platform were the primary measures of place discrimination.

A sixth probe trial was performed during acquisition sessions 4 and 7 in which the platform was lowered at the start of the trial and then raised after a period of 40 s. The performance criteria measured was the proportion of time spent within a 20-cm annulus from where the target had been lowered. This measure was interpreted as the strength and accuracy of the spatial memory.

2.3b Discriminated avoidance task

This test involved a comprehensive paradigm, which assessed learning for avoidance and simple spatial discrimination. The apparatus consisted of an acrylic T-shaped maze as described by [12, 13] with compartments in the stem and each goal arm separated by doors. The maze rested on a grid floor wired to deliver 0.27 mA scrambled shock, which was controlled manually by a footswitch.

Each mouse was trained to leave the start box and run to a designated correct goal arm of the T-maze within 5 sec following the opening of the start door. The correct goal arm was designated as the one opposite from the mouse's first choice point turn. After the start door opened, shock was initiated 5 sec later if the mouse had not entered the correct goal arm, or immediately if the mouse entered the incorrect goal arm. Shock was continued for a maximum of 60 s or until the correct goal arm was entered. A correct avoidance trial was recorded when the mouse entered the correct goal arm within 5 sec of the opening of the start door. After 10 sec in the correct goal arm, the mouse was removed and placed into a holding cage for an ITI of 1 min. Each session continued until the mouse made a correct avoidance on 4 out of 5 consecutive trials for a maximum of 25 trials. This demonstrated that the mice learned to use information presented on a single trial (the first trial of each daily session) to actively avoid shock on subsequent trials. One session was conducted per day. Training continued until each mouse acquired a learning set of discriminated avoidance consisting of 4 consecutive sessions with a correct

avoidance on 4 of the first 5 trials of each session. Mice that did not acquire the learning set by 30 sessions were discontinued from behavioral training. Recent memory was assessed by interposing a 7 m delay between the first and second trials on the session following acquisition of the learning set.

2.5 Data analysis

Factorial analyses of variance were used to evaluate group differences with Genotype, Vitamin E Dose, and Age as between groups factors for each behavioral measure. Repeated measures analyses of variance were used whenever possible. A chi-squared statistic was obtained for categorical data involving single measurements. Planned individual comparisons were performed by individual F tests using the error term from the overall analysis.

3. Results

3.1 Weight

Weekly body weight measurements (Fig. 1) of each animal indicated apoE-deficient mice weighed significantly less than C57BL/6J control mice at all ages, $F(1,300)=68.545$, $p < 0.001$. Furthermore, a within subjects analysis of repeated body weight measurement revealed a significant interaction with genotype $F(1,133)=91.288$, p

< 0.001 indicating C57BL/6J control mice have a greater rate of weight gain. The safety of vitamin E supplementation is well studied [9] and was not expected to have any effect on body weight. Vitamin E supplemented food pellets had no effect on body weight at any dose in either genotype of mice $F(2,300) = 1.973$, $p = 0.141$ indicating no diet-induced alterations in each mouse's ability to maintain a stable body weight. The onset of progressive age-related weight loss occurred 3 months earlier in the apoE-deficient mice at 13 months of age vs. 16 months of age in the control mice.

3.2 Food Intake

Resultant dosage levels of tocopheryl acetate were based on food intake. Throughout the duration of the study, tocopheryl acetate intake levels for each genotype remained proportionately 10X and 100X of the control diet as indicated in Fig. 2. It is important to note that the apoE-deficient mice consumed significantly more tocopherol acetate per body weight at all age points except at age 5 months ($p < 0.001$) but proportionally the doses remained essentially 10X and 100X of the control diet.

3.3 Mortality

The relationship between the vitamin E dose and the survival curve of each genotype is depicted in Fig. 3. ApoE-deficient mice had significantly higher mortality than wild-type controls per Kaplan-Meier survival analysis, $\chi^2(1, n = 308) = 13.664$, $p < 0.001$. Vitamin E supplemented diets had no effect on survival for wild-type controls or

apoE-deficient mice. Although, there was no statistically significant difference of supplementation on mortality during the study, there may be the beginning of an effect on survival at older ages for apoE-deficient mice maintained on the 100X supplemented vitamin E diet. There was a trend toward a decelerated death rate in the 100X treatment group as compared to the control and 10X apoE-deficient groups.

3.4 Water maze

The ability of the mice to learn a spatial discrimination task was measured by the distance to locate a hidden platform over sessions 1-4. Performance remained stable from session 5 to 8 indicating the maximum accuracy of the mice to locate the platform. Place discrimination acquisition (Fig. 4) showed that all mice when 6 mo of age, regardless of genotype or diet, equivocally learned the place location of the hidden platform. Overall, there were significant main effects of age, genotype and age X genotype. Spatial learning as measured by acquisition sessions 1-4 demonstrated significant between subject age effects for both wild type controls $F(1,138) = 27.084$, $p < 0.001$ and apoE-deficient mice $F(1,136) = 45.675$, $p < 0.001$, indicating an age-associated impairment in the ability to learn the location of a hidden platform. Repeated measures analyses reveal an overall significant session X age X diet interaction $F(6,822) = 2.743$, $p = 0.012$. When analyzed by genotype, this interaction remained only in the apoE-deficient mice $F(6,408) = 2.619$, $p = 0.017$ suggesting that lifelong vitamin E supplementation reduces the magnitude of age-related declines in spatial learning ability in mice lacking apolipoprotein E.

The maximum accuracy for spatial learning was best illustrated during acquisition sessions 5-8. Significant age effects were evident overall and for each genotype when analyzed by analysis of variance: $F(1,138) = 9.700$, $p = 0.002$ for wild type controls and $F(1,136) = 4.776$, $p < 0.001$ for apoE-deficient mice. When an analysis of variance was performed for each diet, the age effect was significant for mice receiving the control diet $F(1,92) = 13.988$, $p < 0.001$ and the 10X diet $F(1,89) = 10.351$, $p = 0.002$. However, no significant age differences were evident for mice supplemented with the 100X vitamin E diet indicating that lifelong vitamin E supplementation may preserve the ability of older mice to accurately locate a spatial location.

Accuracy for spatial memory as assessed by temporarily lowering the platform is shown in Fig. 5 for wild-type controls and Fig. 6 for apoE-deficient mice. All mice were naive to the lowered platform during session 4. No significant effects of age, genotype or diet were observed on the proportion of time spent within the 20 cm annuli from where the target had been lowered during session 4. When the second probe trial, which was performed on session 7, was compared to the first probe trial, all young wild-type mice significantly ($p < 0.025$, using paired samples t-test) increased the proportion of time spent in the annuli during session 7 independent of dietary treatment, suggesting the mice accurately remembered enough information to learn the location of the platform. The old wild-type mice maintained on the control diet showed a significant decrease ($p = 0.017$, using paired samples t-test) in probe trial time spent within the target annuli upon their second exposure to the probe procedure, indicating an age-related decrease in the accuracy for spatial memory, whereas the control mice maintained on supplemented diets

had no change in either direction for both sessions (p >0.424). When session 7 was analyzed with an age X diet ANOVA for wild-type mice, a significant main effect of age, $F(1,138) = 11.653$, $p = 0.001$ was evident. Further analysis with planned comparisons by individual F tests using the error term identified an age-associated decrement in accuracy for spatial memory in mice maintained on the control diet ($p = 0.025$) as opposed to the wild-type mice on fortified (10X & 100X) diets, which showed no significant age differences in the proportion of probe trial time during session 7 ($p > 0.130$).

Upon initial exposure to the probe trial during session 4, (Fig. 6) the proportion of time spent within the 20 cm annuli by the apoE-deficient mice was not significantly affected by dietary supplementation or age. During the second probe trial all dietary groups of young apoE-deficient mice tended to spend a greater proportion of time within the 20 cm annuli than during session 4. However, this was only a significant improvement for the mice on the 10X diet (paired t-test, $p = 0.008$); control apoE-deficient mice (paired t-test, $p = 0.051$) and 100X diet (paired t-test, $p = 0.184$). An analysis of variance of session 7 revealed a significant main age effect $F(1,136) = 15.004$, $p < 0.001$ and a significant age X diet interaction $F(2,136) = 3.462$, $p = 0.034$. Planned comparisons indicated significant age-associated declines in the proportional probe trial times for both apoE-deficient mice maintained on the control diet ($p < 0.001$) and the apoE-deficient mice maintained on the 10X diet ($p = 0.031$) indicating an impairment in spatial memory in older apoE-deficient mice. No significant age difference was apparent for the 100X fortified treatment group ($p = 0.682$). More interestingly, the pattern of performance of the 18 month apoE-deficient 100X group was the only group of old mice

in which performance improved from session 4 to session 7, which was the same pattern of response seen in all the young groups of mice.

3.5 T-maze

The first training session of the active avoidance paradigm as shown in panel A of Fig. 7, measured learning for correct avoidance behavior, which requires both learning for simple discrimination as well as avoidance. Two-way analyses of variance indicated a significant main effect of age for both wild-type mice $F(1, 138) = 21.274, p < 0.001$ and apoE-deficient mice $F(1,131) = 7.47, p = 0.007$ There was also a significant overall decline $F(1,1467) = 72.284, p < 0.001$ in the abilities of the older mice to reach the discriminated avoidance criterion as compared to the young mice when the task required a reversal of the correct goal arm from what had been initially learned during the first session (Panel B of Fig. 7). The number of sessions required for the mice to maintain a consistent level of performance was measured based on a learning set acquisition as shown in panel C of Fig.7. This demonstrated significant age-related decline in active avoidance behavior for both wild-type mice $F(1, 138) = 42.889, p < 0.001$ and apoE-deficient mice $F(1,131) = 28.513, p < 0.001$. Thus, discriminated active avoidance appeared to be an age-sensitive component of cognitive functioning, however genotype or diet status had no effects on this type of learning.

Short-term memory for discriminated active avoidance was measured after each mouse achieved a stable baseline of performance as previously indicated. Panel D of Fig. 7 shows the number of sessions required to successfully complete the delay procedure in

which a 7 min wait was incorporated between the first and second trials in the session following acquisition of the learning set. If the mouse failed to make a correct avoidance at that time, the mouse was be retrained according to the acquisition criterion. Consistent with the learning set acquisition, short-term memory also revealed significant decline with age for both wild-type mice $F(1, 138) = 31.030, p < 0.001$ and apoE-deficient mice $F(1,131) = 40.494, p < 0.001$. There was no significant effect of genotype on short-term memory for discriminated active avoidance, and vitamin E supplementation failed to alter this age-associated decline in performance.

4. Summary and conclusions

The main findings of this study are: (i) apoE-deficient mice had significantly higher mortality and an earlier age-associated decline in weight, (ii) apoE-deficient mice had a selective age-dependent decline in acquisition for spatial navigation, (iii) lifelong dietary supplementation with vitamin E ameliorated the age-associated deficits in spatial memory, and (iv) age-associated decline in working memory was not accelerated in apoE-deficient mice nor was protected by lifelong dietary vitamin E supplementation. Overall these results suggest that apoE-deficient mice are an apparent model of more rapid but selective brain aging.

The observed increase in mortality may be related to severe atherosclerosis known to occur in apoE-deficient mice, which could also affect the observed changes in cognitive performance secondary to vascular disease in the brain or perhaps deficits of

motor function from peripheral vascular disease. The significantly increased mortality could also represent accelerated aging of organ systems independent of cognitive abilities, leading one to reasonably question whether or not the impairments in learning and memory are a result of psychomotor deficits.

Impaired sensory or motor skills could have detrimental effects on cognitive performance. This explanation is unlikely based on observations from our pilot study, which found no greater age-associated decrements in measures for maximum running speed, balance, coordination, strength, anxiety, shock sensitivity or locomotor activity in apoE-deficient mice as compared to wild type controls [28]. Swim speeds measured in the present study were not affected by genotype or diet. The behavioral data for apoE-deficient mice in the literature is varied according to age, gender, genetic background and testing protocol [1, 15-19, 23, 26, 30, 32-34, 47]. Overall, there is nothing to indicate any unusual sensory or motor deficits.

The developing pattern of the apparent deficits in apoE-deficient mice is that of an age-dependent slowing in learning for spatial navigation. One strength of the present study was to study mice beyond the ages currently reported in the literature, which could explain the lack of age effects in some of the previous results. The observed impairment of learning in the apoE-deficient mice appears to be confined to a particular cognitive domain as evidenced by only impairment of spatial abilities. This is further reinforced when considering that apoE-deficient mice had equivalent performance when compared to wild type controls in measures of working memory. Working memory was sensitive to

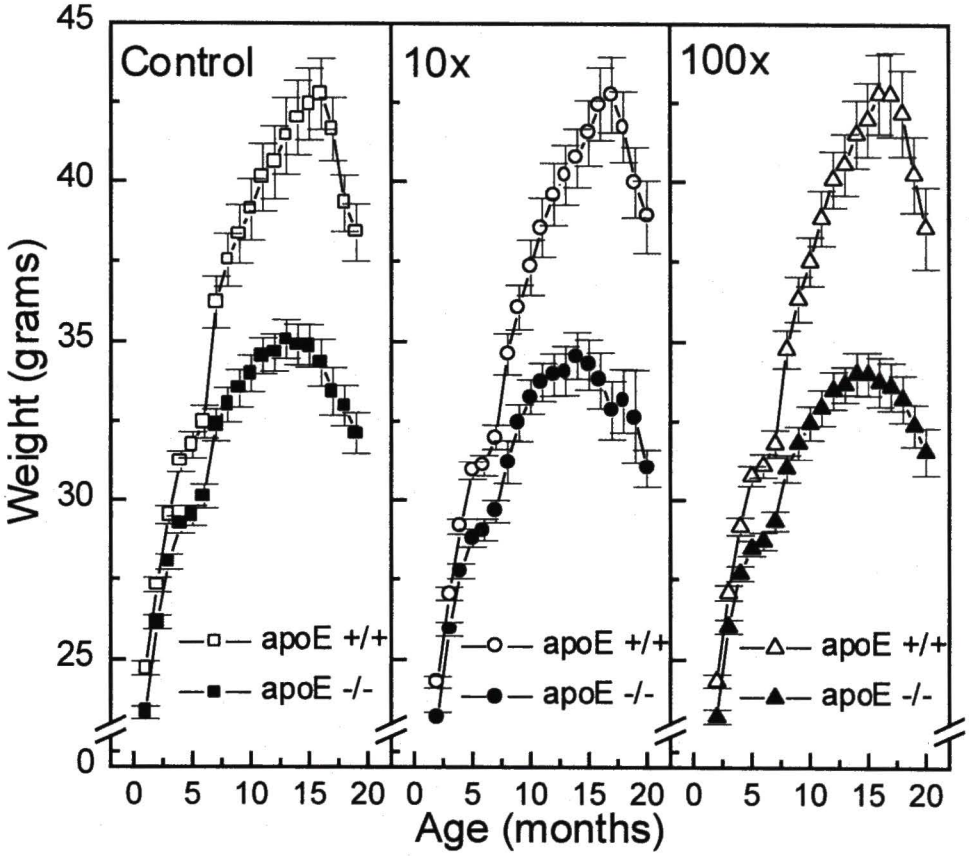
impairments with age, but there was no protection of the age-dependent loss with lifelong vitamin E as observed for spatial abilities.

Spatial memory appears to be selectively sensitive to oxidative stress, as evidenced by the region-specific, protein-specific oxidative damage found predominately in the hippocampus. Choi et al reported a 2-fold increase in total protein oxidation in the hippocampus of young apoE-deficient mice, comparable to that of both old wild type and apoE-deficient mice. Moreover, 2 of the proteins, creatine kinase and dihydropyramidase-related protein 2 have recently been implicated as protein targets of oxidative damage in patients with AD [4]. Oxidative stress is proposed to be the inciting event for the observed amyloid-β deposits and neurofibrillary tangles seen in AD [40]. It seems likely that apoE is the primary genetic risk factor for increased susceptibility to oxidative stress which facilitates the sequence of neurodegeneration.

The effect of lifelong supplementation with vitamin E on these specific targets of oxidation damage in apoE-deficient mice is unknown. However, the observed amelioration of functional decline in spatial memory with lifelong dietary vitamin E supplementation suggest a mechanism related to the prevention of damage from oxidative insults. It is interesting to note that both the 10X and 100X diets eliminated the age-dependent impairments for spatial memory in wild type mice, but only the 100X dose eliminated the age impairments in apoE-deficient mice. It is reasonable to assume adequate levels of vitamin E were achieved in the brains of both genotypes, because Martin et al showed no further increase in CNS tissue concentration of vitamin E for doses greater than 60 mg/kg of dietary intake of vitamin E in rats [24].

It appears that a dynamic process of vitamin E utilization may be involved on the premise that the apoE-deficient mice have a higher lifelong steady-state level of oxidative stress in neuronal tissues secondary to a more advanced state of aging. On the other hand, perhaps entirely different mechanisms for the effects of vitamin E are responsible for the findings, such as modulation of inflammation, gene regulation or cell signaling [46]. In conclusion, the use of apoE-deficient mice appears to be a useful tool to study the fundamental mechanisms of brain aging on select cognitive processes.

FIG. 1. Effect of apolipoprotein E status and vitamin E supplementation on body weight of mice as a function of age. Weekly body weights are shown as the weight averaged over 4-week periods. Each value represents the mean ± SEM of 23-54 mice. Analysis indicated a significant main effect of genotype, but supplementation had no effect at any dose on body weight. The inflection in the curves between 6 and 8 months occurred due to behavioral testing and subsequent euthanasia in the young cohort.


Control
10x
100x
Weight (grams)
Age (months)
apoE +/+
apoE -/-
apoE +/+
apoE -/-
apoE +/+
apoE -/-
0
25
30
35
40
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0 5 10 15 20
0 5 10 15 20
0 5 10 15 20

FIG. 2. Dosages of dl-α-tocopheryl acetate per Kg body weight per day were calculated as a function of dietary intake of supplemented food pellets. The dietary intake of all young mice was measured at the same time to get a cross-sectional analysis of intake between the ages of 2 to 5 months (n = 11-14). The dietary intake at 12 and 18 months-of-age (n = 22-29) was assessed longitudinally on all remaining mice. Each value represents the mean ± SEM for the average of a 3-week period at each age.

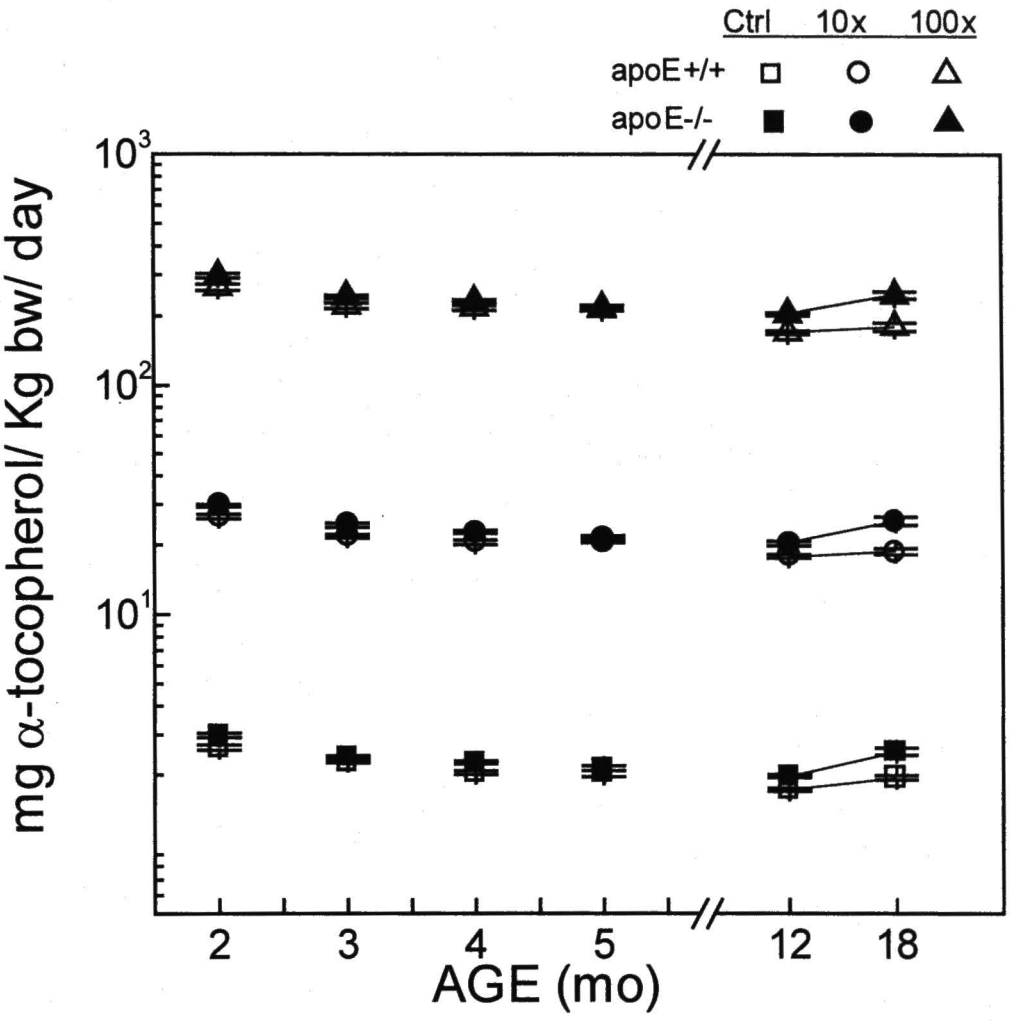

Ctrl
10x
100x
apoE+/+
apoE-/-
mg α-tocopherol/ Kg bw/ day
10^3
10^2
10^1
2
3
4
5
12
18
AGE (mo)

FIG. 3. Effect of apolipoprotein E genotype and vitamin E supplementation on survivor function. The absence of apoE resulted in a significantly greater mortality when compared with wild type controls, furthermore the addition of lifelong dietary supplementation with vitamin E did not significantly alter the mortality within either genotype.

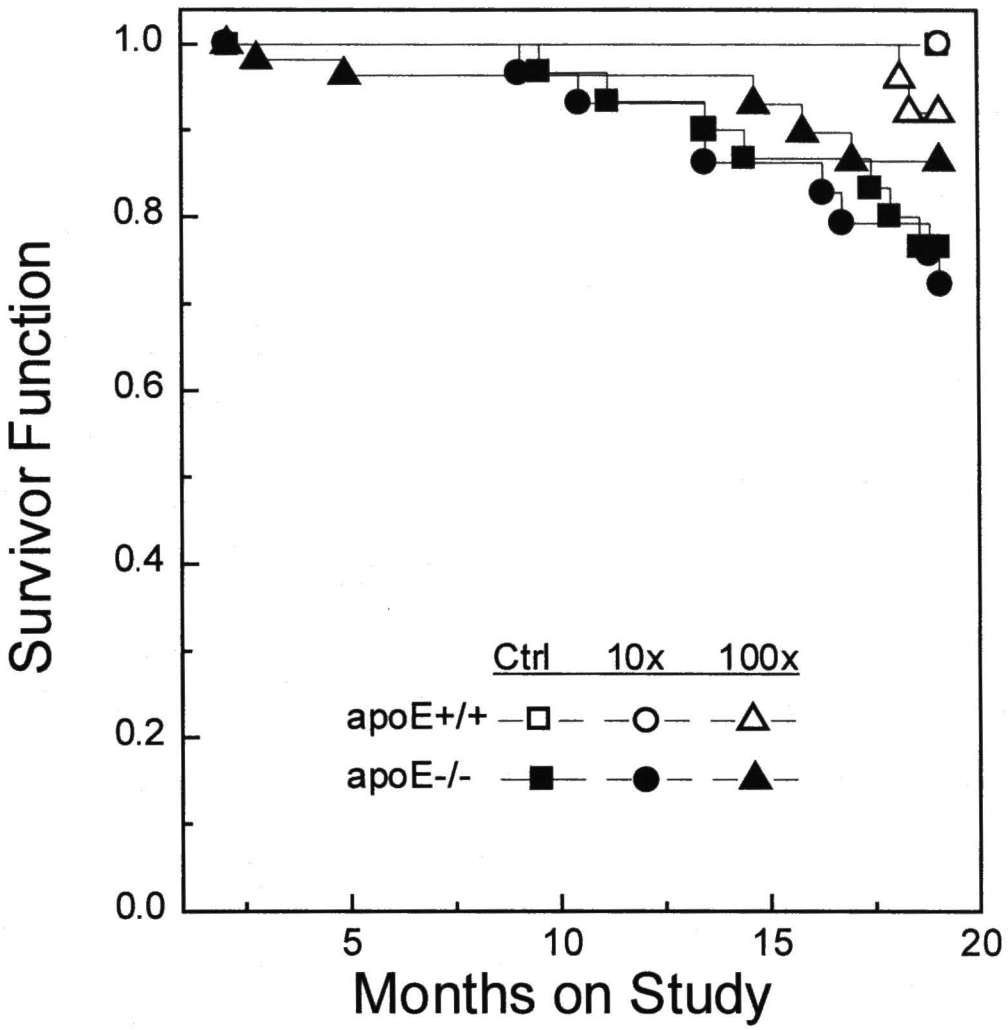

Survivor Function
1.0
0.8
0.6
0.4
0.2
0.0
5
10
15
20
Months on Study
Ctrl
10x
100x
apoE+/+
apoE-/-

FIG. 4. Acquisition (sessions1-4) and accuracy (sessions 5-8) of place discrimination as measured by cm traveled to reach a hidden platform within a swim maze for wild type control (left panel) and apoE-deficient (right panel) mice. Both genotypes swam significantly longer path lengths during acquisition to locate the platform when 18 months of age as compared to mice aged 6 months. The quality of the improvement in performance over sessions 1-4 in the old apoE-deficient mice maintained on the 100X vitamin E diet, more closely resembled that of wild type mice. Accuracy for platform location during sessions 5-8 also was improved in the apoE-deficient mice maintained on the 100X vitamin E diet.

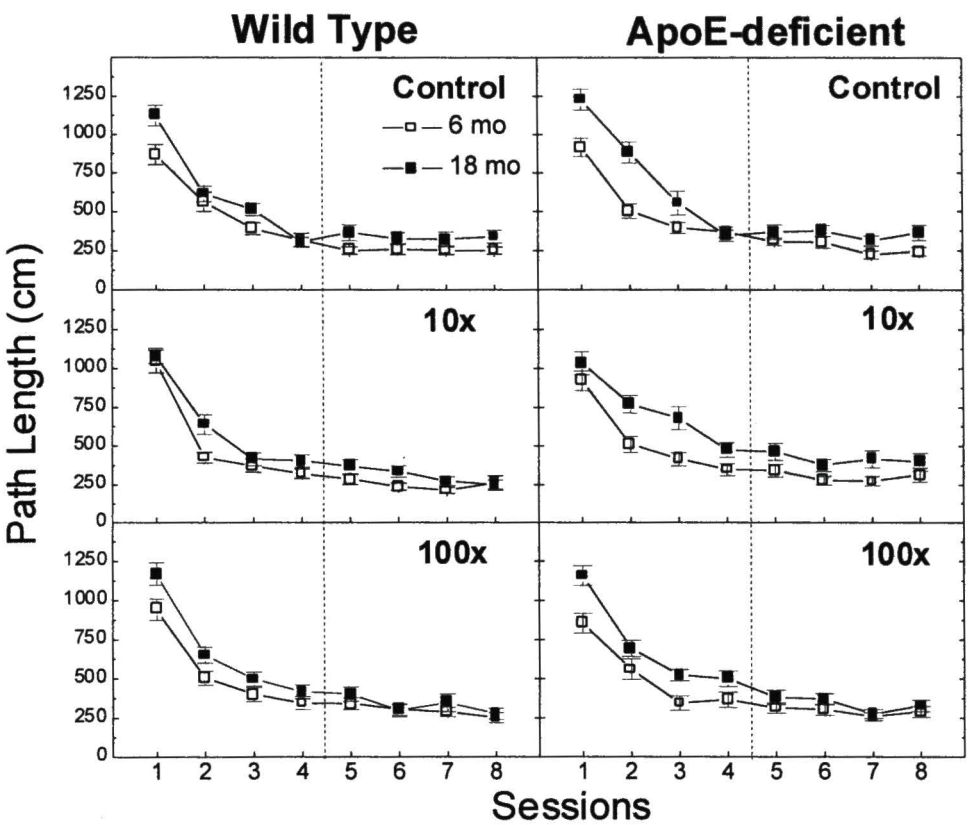

Wild Type
ApoE-deficient
Control
6 mo
18 mo
10x
100x
Path Length (cm)
Sessions
0
250
500
750
1000
1250
1
2
3
4
5
6
7
8

FIG. 5. Probe trial performance for wild-type control mice fed a standard diet or lifelong fortification with 10X or 100X tocopheryl acetate per kg of food. The probe trial was performed as the last trial during sessions 4 and 7 in which the platform was lowered at the start of the trial and then raised after a period of 40 s. The values indicate the mean ± SEM for the proportion of time spent in the 20-cm annuli from where the target had been lowered; (n = 23-25 for each group); * indicates a significant age effect (p = 0.025).

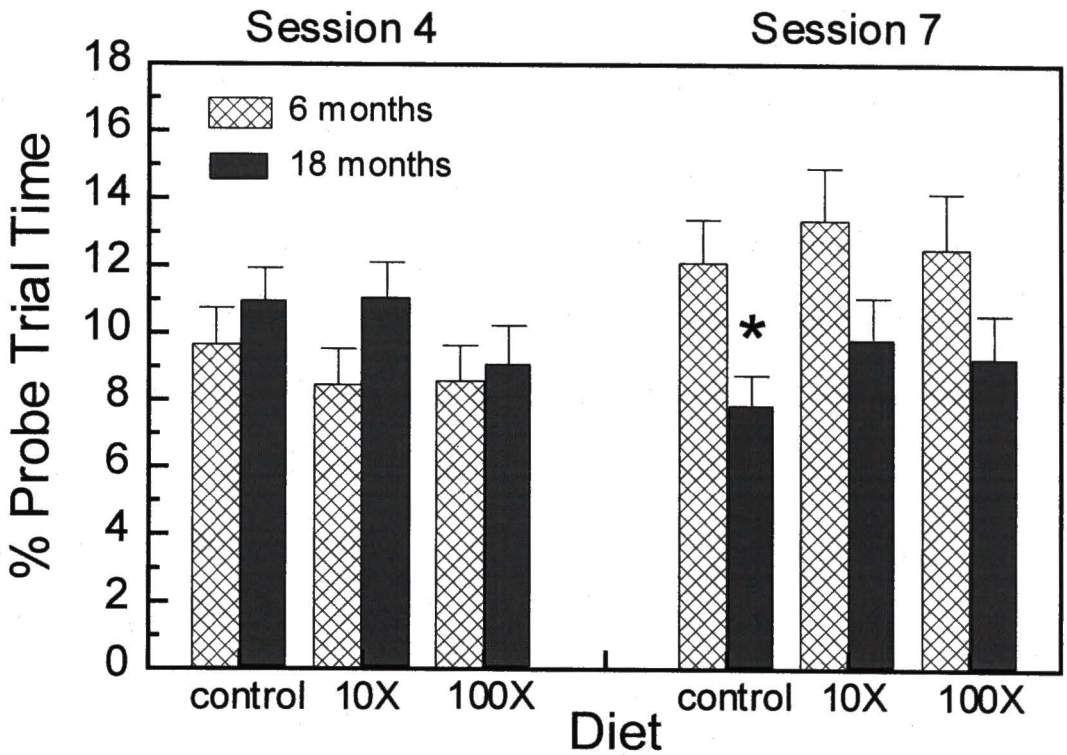

Session 4
Session 7
6 months
18 months
% Probe Trial Time
18
16
14
12
10
8
6
4
2
0
*
control
10X
100X
control
10X
100X
Diet

FIG. 6. Probe trial performance for apoE-deficient mice maintained on a standard diet or lifelong fortification with 10X or 100X tocopheryl acetate per kg of food. Mice were naïve to the probe trial during session 4. The second probe trial was performed during session 7. The values indicate the mean ± SEM; (n = 20-26 for each group); * indicates a significant age effect ($p < 0.032$).

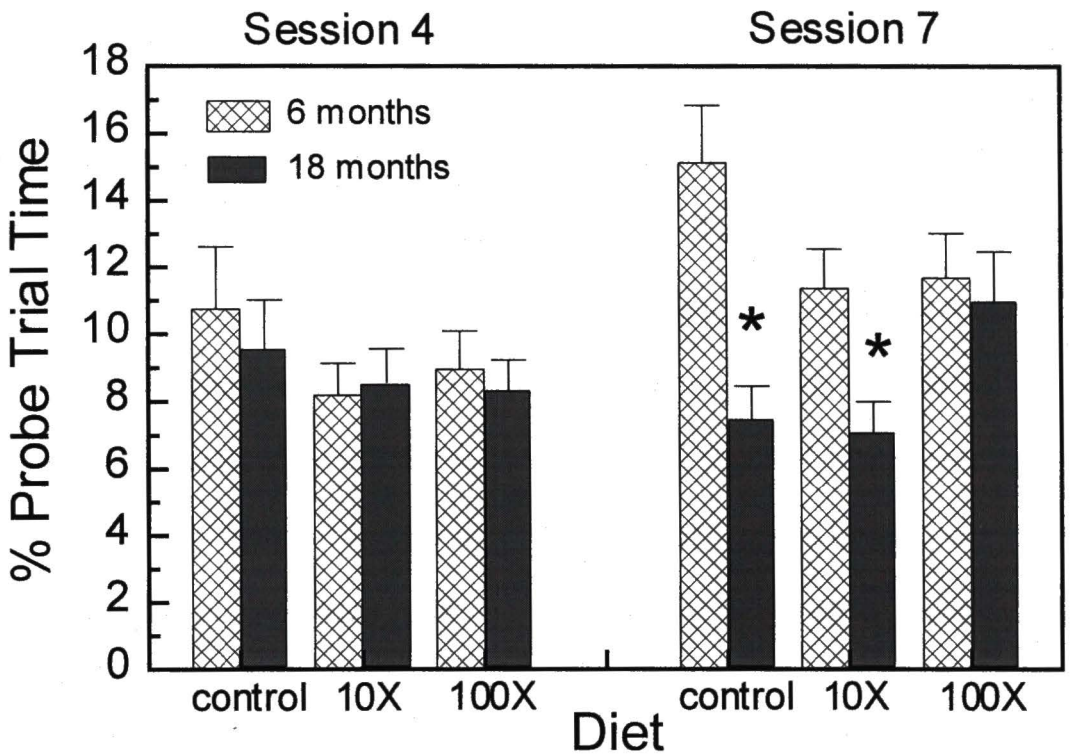
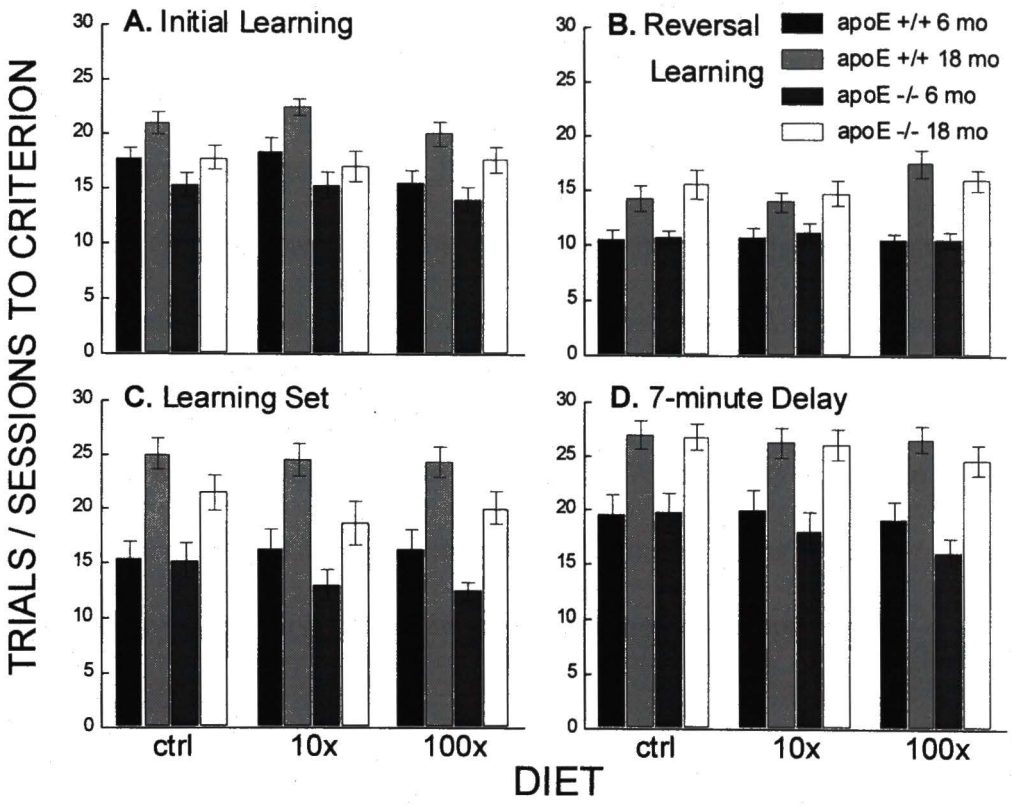

Session 4
Session 7
6 months
18 months
% Probe Trial Time
0
2
4
6
8
10
12
14
16
18
*
*
control
10X
100X
control
10X
100X
Diet

FIG. 7. Effect of age on performance components of the discriminated avoidance task. The performance criterion shown in panels A and B was determined as the number of trials to make a correct turn with a latency of less than 5 s on 4 out of 5 consecutive trials. Older mice had slower learning for discriminated escape as measured for session 1 (panel A), as well as during the first reversal as identified during session 2 (panel B). The criterion for the performance of the learning set (panel C) was 4 consecutive sessions with a correct avoidance on 4 of the first 5 trials of each session. Panel D represents the session on which the mice made a correct discriminated escape on the test trial following a 7-min delay after the information trial. If mice failed the 7-min delay they had to successfully complete the learning set before the next delay trial. Older mice were significantly slower to acquire both the learning set and a correct avoidance after the delay, however neither genotype nor supplementation with lifelong vitamin E affected performance.


A. Initial Learning
B. Reversal Learning
C. Learning Set
D. 7-minute Delay
apoE +/+ 6 mo
apoE +/+ 18 mo
apoE -/- 6 mo
apoE -/- 18 mo
TRIALS / SESSIONS TO CRITERION
0
5
10
15
20
25
30
ctrl
10x
100x
DIET

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TRANSITION REMARKS

In the previous chapter (chapter II), lifelong supplementation with vitamin E appeared to prevent age-associated loss of neural function. Spatial learning and memory abilities were selectively protected in both wild type control mice as well as apoE-deficient mice, which were shown to have a greater age-dependent deficit in spatial learning. Thus antioxidant treatment may be able to protect neurons from accumulated age-related insults, even though the effect appeared limited to hippocampal neuronal function only.

There is evidence to suggest that age-dependent dysfunction may be able to be reversed with dietary antioxidants, as well. Chapter III focuses on whether or not oral antioxidant supplementation will have any effect on impaired performance in mice that becomes evident in old age. The main question was whether or not age-dependent loss of function could be reversed. We were postulating that the molecular functioning of neural tissues would have to be improved in old mice in order to ameliorate any age-dependent impairment.

CONCURRENT COENZYME Q_{10} AND α-TOCOPHEROL ADMINISTRATION IMPROVES LEARNING IN AGED MICE

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CHAPTER III

CONCURRENT COENZYME Q_{10} AND α-TOCOPHEROL ADMINISTRATION IMPROVES LEARNING IN AGED MICE

SUMMARY

The purpose of this study was to determine if intake of coenzyme Q_{10} (CoQ), either alone or concurrently with α-tocopherol, could improve brain function of aged mice, as reflected in their cognitive or psychomotor performance. Separate groups of aged mice (24 months) received either CoQ (123 mg/kg/day), α-tocopherol acetate (200 mg/kg/day), a combination of these treatments, or the vehicle (soybean oil) via gavage for a period of 14 weeks. Three weeks following initiation of the treatments, the mice received a battery of age-sensitive behavioral tests for assessment of learning, recent memory and psychomotor function. In a test that required the mice to rapidly identify and remember the correct arm of a T-maze, and to respond preemptively in order to avoid shock, the combination of α-tocopherol and CoQ resulted in more rapid learning when compared with the control group. Learning was not significantly improved in the groups receiving either treatment alone. None of the treatments resulted in significant improvement of psychomotor performance in the old mice. In a second experiment,

chronic treatment with higher doses of CoQ alone (250 or 500 mg/kg/day) failed to yield effects similar to the combination of α-tocopherol and CoQ. The apparent interaction of the CoQ with the α-tocopherol treatments is consistent with the previous suggestion that coenzyme Q has a sparing effect on α-tocopherol *in vitro* and *in vivo*. Overall, the findings suggest that as a potential treatment for age-related learning deficits, concurrent supplementation of α-tocopherol with CoQ is more likely to be effective than supplementation with CoQ alone.

INTRODUCTION

Coenzyme Q_{10} (CoQ), or ubiquinone, is a lipophilic substance present in cellular membranes that has several important functions. In addition to the well-known functions as an electron carrier and proton translocator within the mitochondrial electron transport chain [1], CoQ is also thought to act both as an antioxidant directly and as a regenerator of α-tocopherol [2-4]. Genetic CoQ deficiency appears to have deleterious effects on muscle and nervous system functions [5], conversely CoQ supplementation is associated with improvement in muscular [6, 7] and nervous system [8] pathology. In particular, CoQ supplementation has been found to have neuroprotective effects in experimental models of Parkinsonism [9, 10] and to improve function in patients with this disease [11].

The antioxidant properties of CoQ and its effect in age-related neurodegenerative disease suggest that CoQ supplementation could be a beneficial treatment for age-associated cognitive or psychomotor dysfunctions, which are believed to involve accumulation of oxidative damage and alteration of oxidation-sensitive brain functions [12-15]. Although several antioxidant interventions have been reported to improve age-related brain dysfunction [16-18], the potential for CoQ to produce such an effect is unknown. Likewise, it is not known if supplementation with CoQ might cause deleterious alterations of brain function, based on its potential to act as a pro-oxidant as well as an antioxidant [19]. Such information would be especially relevant because a significant number of humans, regardless of age, use CoQ as a dietary supplement in anticipation of

various health benefits.

In this context, the purpose of the present study was to evaluate the potential for CoQ to affect the impaired cognitive and motor performance of old mice. The effect of CoQ was studied when supplemented by itself or when combined with α-tocopherol supplementation. Based on the apparent interaction of these antioxidants in respiring mitochondria, in which CoQ has a sparing effect on mitochondrial α-tocopherol [2-4], it was believed that concurrent intake of these antioxidants could have a synergistic effect.

Separate groups of aged (24-month-old) mice were administered the CoQ, α-tocopherol or combination treatment for a period of 14 weeks during which they were tested on an array of age-sensitive tests for learning, memory and psychomotor functions. A discriminated avoidance test [20] was used that required the mice to rapidly identify and remember the correct arm of a T-maze, and to respond preemptively in order to avoid shock. Previous investigations established that aged mice learn this task more slowly than younger mice, and that pharmacological interventions can ameliorate the age-related deficit [21, 22]. A second set of tests was used to evaluate different dimensions psychomotor performance of the same animals, including coordinated running (rotorod), balance (bridge-walking) and muscle strength (wire hanging).

MATERIALS AND METHODS

Animals

The first experiment began with a total of 49 male C57BL/6JNia mice obtained from the National Institute on Aging. The mice were 24 months of age at the initiation of

treatment. An additional experiment involved 31 male C57BL/6JNia mice aged 21 months at the initiation of treatment. Upon arrival at the University of North Texas Health Science Center (UNTHSC) vivarium, the mice were housed individually in 28 x 19 x 12.5 cm clear polycarbonate cages with wire tops, modified into two mouse units by insertion of a stainless steel divider. The colony room was maintained at 23°±1°C/40% humidity, under a 12-h light dark cycle with lights on at 0600 h. The mice had access to food and water, *ad libitum,* at all times except during testing periods. The mice were weighed at weekly intervals throughout the treatment period.

Chronic Treatment

Three weeks after arrival at UNTHSC, separate groups of the mice were assigned at random to receive oral daily treatment of either CoQ (123 mg/kg body wt) alone or (+)-α-tocopherol acetate (200 mg/kg body wt) (Sigma Chemical) alone, or both CoQ and α-tocopherol, or the soybean oil vehicle (control). In the follow-up experiments, separate groups of mice received daily treatment of oral CoQ in doses of 250 mg/kg body wt, 500 mg/kg body wt, or soybean oil alone. All treatments were administered to the mice via gavage in a volume of 0.1 ml once daily between 1500 and 1700 h. Treatment began 3 weeks prior to the beginning of behavioral testing and was maintained for 14 weeks thereafter.

Discriminated Avoidance Testing

Mice were tested for learning and recent memory performance using a discriminated avoidance test described previously [20], The apparatus was an acrylic T-maze with compartments in the stem and goal arms, each demarcated by a removable door. The

width of the stem and goal arms was 6.4 cm and the clear ceiling was 5 cm high. The stem was 20 cm long (from base to choice point) and included a start-box demarcated by a manual guillotine-type, acrylic door 10.3 cm from the stem base. Each arm of the maze could be closed by an opaque, sliding door moving flush to each outside wall of the stem. The distance from the outside wall of the stem to the end of each arm was 14.5 cm. The maze rested on a grid floor composed of 3-mm diameter stainless steel rods spaced 7 mm center to center and wired for 0.27 mA scrambled shock from a Coulbourn Instruments (Allentown, PA) shock source (Model E13-08). The rods were oriented parallel to the stem and perpendicular to the arms of the maze.

In series of daily training sessions, the mice learned to use information presented on a single trial (the first trial of each session) to successfully avoid shock from the grid floor on all subsequent trials of that session. Each session consisted of an information trial followed by discriminated avoidance training to a performance criterion. The discriminated avoidance training consisted of discrete trials in which shock to the grid floor was initiated 5 sec following opening of the start door if the mouse had not entered the correct goal arm. Shock was initiated immediately following entry into the incorrect arm. After initiation, the shock continued (up to a maximum of 60 sec) until the correct arm was entered, after which the mouse was removed and placed in a holding cage for one minute. On the *information trial*, the same contingencies were in effect except that the arm first entered was always "incorrect", and avoidance of shock on the next trial (the *test* trial), and on all subsequent trials of a given day, required the mice to remember which goal was correct on the information trial, and to employ a strategy of "reversal"

from the arm choice that was incorrect. Previous studies indicated that older mice require significantly more training than young ones to learn this reversal strategy [20].

The discrimination and avoidance components of the task were scored separately. A stem avoidance (an error-independent measure of avoidance) was recorded if the mouse entered either one of the goal arms within 5 seconds of the start door opening. A correct turn was scored when the first arm selected on a given trial was correct, regardless of the response latency. Avoidance training continued on each day until the mouse had made a correct avoidance (i.e, a correct turn as well as a stem avoidance response) on four of the last 5 consecutive trials (with a correct avoidance on the last 2 trials). This was the "day-to-day" avoidance performance criterion. The daily training sessions were discontinued when a mouse could perform the avoidance and recent memory components of the task to a criterion of accuracy and stability. To fulfill the "recent memory performance" criterion, the following conditions had to be met: (i) The mouse made a correct avoidance on the test trial in each of four consecutive sessions and, (ii) during the last two sessions, the mouse had to also meet the day-to-day discriminated avoidance criterion within 5 trials. The mice received the daily testing sessions until they had met both requirements or a maximum of 30 sessions had been conducted.

Measurement of motor skills

Wire suspension. The mouse was allowed to grip, with its front paws, a horizontal wire suspended 33 cm above a foam pad, and the latency to fall was recorded and averaged over 4 consecutive daily sessions.

Bridge-walking. Each mouse was tested for the latency to fall after being placed on

one of 4 bridges, mounted 33 cm above a padded surface. The bridges differed in diameter (small vs. large) and shape (rounded or square) providing 4 levels of difficulty. Each bridge was presented 3 times and the measure of performance was the mean latency to fall (up to a maximum of 60 s) averaged for all bridges.

Coordinated running. Motor learning and maximum running performance were measured using an accelerating rotorod test described previously [23]. The apparatus was a motor-driven treadmill (Omnitech Electronics, Omnirotor treadmill, Model # RRF) that consisted of a 3.2-cm diameter nylon cylinder mounted horizontally at a height of 35.5 cm above a padded surface. In 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 considered by administering intermittent training sessions (2 per day), each consisting of 4 trials. The training sessions continued until the running performance (the average latency to fall from the cylinder) failed to increase for three consecutive sessions. The treatment groups were compared for their average latency to fall on the first session and for the final session (on which a given mouse had reached the maximum level of performance).

Statistical analysis of data

The data from the learning studies were subjected to 2-way analyses of variance, with Treatment as a between groups factor and testing Session as a within-groups factor. Psychomotor performance data were considered in 1-way analyses. Planned individual comparisons between each supplemented group with the control group were made using single-degree of freedom F tests within the Treatment main effect. For analysis of

survival data, Kaplan-Meier survival distributions were calculated and a log-rank X^2 statistic was used to compare the treatments.

RESULTS

In the first experiment, mice aged 24 months were supplemented with CoQ, α-tocopherol, or the combination and tested for their ability to learn the discriminated avoidance task and to perform various tests of motor skills, relative to their age-matched, non-supplemented controls. The results of that experiment suggested that only the combination treatment had additive effects on learning ability of the old mice. Accordingly, in a second experiment, higher doses of CoQ alone were given to different groups of mice of similar age (21 months), to determine if incrementing the dose of CoQ alone could produce an effect equivalent to that of the combined treatment.

Body weight and survival

All mice were weighed upon receipt at UNTHSC and subsequently divided into weight-matched treatment groups. The body weights of the mice in all groups were recorded weekly until the completion of the study. A 2-way analysis of variance on the body weights (Fig 1, left panel) indicated a significant effect of time, reflecting the overall decline in body weights throughout the first experiment ($p < 0.001$). However, there was no significant effect of either α-tocopherol, CoQ, or their combination, as suggested by the lack of a main effect or interaction involving the treatment ($p > 0.38$).

The second experiment (not shown) resulted in similar significant decline in body weight over time ($p = 0.009$) but no effects of Treatment ($p > 0.41$).

Thirteen of the initial 49 mice in the first experiment died before completion of the study. The relationship between survival and treatment group is shown in Fig. 1 (right panel). A log-rank test failed to indicate a significant difference in survival among the treatment groups [$\chi^2 = 1.2, p = 0.74$]. The follow-up study (not shown) also failed to suggest any effect of treatment with CoQ 250 mg/kg or CoQ 500 mg/kg on survival [$\chi^2 = 0.66, p = 0.72$].

Psychomotor function

The results from the tests of psychomotor function are summarized in Table 1. None of the treatments resulted in any overall difference from the control group in the tests for balance (bridge-walking), or muscle strength (wire grip) when analyzed using 1-way analyses of variance ($p > 0.57$). In the test for coordinated running, all treatment groups showed significant improvement in performance with training as determined by a significant main effect in a 2-way analysis of variance ($p < 0.001$). There was no apparent effect of the treatments on the maximum performance achieved after training ($p = 0.608$), although the α-tocopherol alone and α-tocopherol/CoQ groups performed more poorly than the control group during the first session ($p < 0.05$, for planned comparisons).

Discriminated avoidance learning and memory

Effect of α-tocopherol and CoQ alone and in combination. A total of 5 of the original

49 mice died before they completed testing. Additionally, a total of 10 of the surviving mice failed to reach the recent memory performance criterion within 30 testing sessions. The failure rate was greatest in the control (36%), CoQ (36%), and α-tocopherol groups (22%), whereas no failures occurred in the α-tocopherol/CoQ combination group. The latter rate of failure differed significantly from that of the control group ($\chi^2 = 4.9, p < 0.027$). There was a similar trend in the mean number of sessions required to reach the recent memory performance criterion by the groups, although analysis of variance did not indicate a significant difference ($p > 0.168$).

Analysis of the session-to-session performance of the mice suggested that the treatment groups differed in the rate at which they learned the discriminated avoidance response. Figure 2 shows the effect of the treatments on the two different components of the learning task, learning to respond rapidly to avoid punishment (avoidance responses, upper panels) and learning to turn into the appropriate arm of the maze (correct turns, lower panels). The left panels of Fig. 2 show the average performance over the first 6 sessions of the training, before any of the mice had reached the recent memory criterion. Analysis of variance revealed a significant effect of treatment group on the number of avoidances during the learning phase ($p = 0.017$), a result that was mostly attributable to the performance of the α-tocopherol/CoQ group. This group made significantly more avoidance responses than vehicle controls ($p = 0.002$, planned individual comparison), whereas the α-tocopherol and CoQ alone groups did not ($p > 0.084$).

A similar trend was evident among the treatment groups for the number of correct

turns during the learning phase, although analysis of these data did not indicate a significant difference ($p = 0.063$). For both avoidances and correct turns, all of the groups showed nearly equivalent performance on the final session of their training (Fig. 2, right panels) on which they had reached the performance criterion (or had been trained for 30 sessions) ($p > 0.574$) for correct turns and avoidances.

Effect of CoQ dose. Four of the 31 mice used in the dose-response study of CoQ died before completion of the study. There was no indication of a difference in the frequency of mice reaching the recent memory performance criterion or in the average number of trials required to reach criterion ($p > 0.132$). As suggested in Fig. 3., 250 and 500 mg/kg/d of CoQ, when administered to old mice for 14 weeks, failed to improve performance during the learning phase of the discriminated avoidance task. While there was a trend toward impaired avoidance performance in the mice treated with 500 mg/kg/d CoQ during the learning phase, as well as on the final session, none of the analyses performed on the number of correct turns or avoidances suggested a significant main effect of CoQ dose, or a difference from control in planned comparisons ($p > 0.208$).

DISCUSSION

The main findings of this study are: (i) CoQ administered concurrently with α-tocopherol for up to 14 weeks improved learning and memory performance of old mice; (ii) CoQ by itself failed to have a similar effect, even when administered in higher doses up to 500 mg/kg/d; and (iii) neither CoQ, α-tocopherol, nor the combination improved

psychomotor performance of the mice.

Previous studies have reported that supplemental antioxidants or antioxidant-rich foods, when given to old mice or rats for relatively short periods of time, may be effective in ameliorating age-related deficits in cognitive function. [16-18, 24]. The current studies represent the only report addressing the potential for CoQ to produce such an effect. When administered in combination with alpha-tocopherol, CoQ improved the ability of the old mice to learn and remember a preemptive response to avoid shock, but affected their ability to learn or remember a specific choice to a much lesser extent. Previous studies in this laboratory have suggested that learning and memory for both the avoidance and choice components is impaired with age [20, 25], and thus the current findings suggest that the beneficial effect of CoQ/α-tocopherol supplementation may not generalize to all dimensions of age-related cognitive impairment. The retention of the active avoidance response over sessions, a working memory, by the old mice could involve an effect on brain processes mediating associative fear conditioning, which is involved in learning of the avoidance response and reported to decline with age [26]. However, additional studies, involving different behavioral tests, will be needed to fully characterize the nature and extent of the performance improvement following CoQ/α-tocopherol supplementation.

Although a significant improvement in avoidance learning was evident following concurrent CoQ and a-tocopherol supplementation, supplementation with either compound alone in the same dosages failed to result in significant effects. The trends evident in the single compound alone groups (Fig. 2.) were suggestive that the resultant

effect of the combined antioxidant compounds was through an additive process. Nevertheless, additional groups of old mice that were treated with larger doses of CoQ, for an equivalent period of time, did not show comparable does-dependent improvement in their learning ability. Thus, supplementation CoQ alone is apparently not sufficient to produce a facilitation of learning in the aged mice. Moreover, a trend present in the results suggested that the relatively high dose of 500 mg/kg/d may impair performance of the discriminated avoidance task.

The current studies did not address the possibility that a higher level of α-tocopherol supplementation, without concurrent CoQ treatment, could have improved learning of the mice. Such an effect seems unlikely given the relatively large dose of α-tocopherol (200 mg/kg/d) administered to the mice in the α-tocopherol alone group. This dose, which yielded a 30% increase in whole brain synaptosomal α-tocopherol [4], was nearly twice as high as that needed to achieve maximal increases in brain α-tocopherol content, and to modify brain function, in a dose response study [27]. A recent study, employing an equivalent level of α-tocopherol supplementation, reported no improvement in age-related deficits of mice in several tests of cognitive or psychomotor function and, instead, indicated impairment on some behavioral tests [28]. These findings confirm the lack of an effect of α-tocopherol treatment in the current study.

While the combination of CoQ and α-tocopherol tended to improve learning ability of the old mice, the experimental treatments failed to yield improvements in the tests for balance (bridge-walking), muscle strength (wire grip) and coordinated running (Table 1). These findings are in general accordance with other reports suggesting that

supplementation with vitamin E, as well as a variety of other antioxidant treatments and antioxidant-rich foods, fails to result in improvements of psychomotor performance when administered to mice at ages following the onset of the age-related decline [28, 29]. It is noteworthy that supplementation of α-tocopherol, alone or in combination with CoQ, was associated with a modest impairment of coordinated running performance during the initial testing session. A similar negative effect was reported in previously in old, but not relatively young mice that had been maintained under vitamin E supplementation [28].

Because treatment with the antioxidants in these studies was initiated in relatively old mice, the current results do not directly address the issue of whether or not any of them can provide protection against the age-related losses of cognitive or psychomotor performance. Previous investigations have suggested that vitamin E supplementation has beneficial effects on cognitive and motor performance when initiated in young or middle-aged rodents and maintained until old age [30, 31], whereas no benefits are associated with supplementation delayed until at older ages [28]. Thus, while CoQ alone was without effect in the current studies, these results do not rule out the possibility that CoQ supplementation could provide protection against age-related losses of cognitive or motor dysfunction. A related caveat applies to the beneficial effect observed following concurrent CoQ and tocopherol administration in these studies, since it is not yet known how such treatment would affect the performance younger mice, or the subsequent decline in their performance as a function of age. These issues need to be considered in additional studies.

The effect of concurrent antioxidant supplementation in the facilitation of learning in

the present experiment is consistent with the hypothesis that age-related brain dysfunction, and related deficits in learning and memory of mice, are associated with the accrual of oxidative damage and the related perturbation of oxidation-sensitive brain functions [12, 32, 33]. It is clear from previous work that vitamin E intake alone, when initiated in old mice, is insufficient to lower oxidative damage in both the brain and various peripheral tissues [28, 34], and does not improve behavioral performance. Following administration of CoQ alone, Kwong et al., [35] reported a decrease in the protein carbonyl content of skeletal muscle mitochondria, as well as a reductive shift in plasma aminothiol status. On the other hand, supplementation of CoQ alone did not affect activity of antioxidative defense enzymes or the rate of hydrogen peroxide generation in brain mitochondria [35].

It is not yet known whether or not the combination of CoQ and α-tocopherol results in a reduction of oxidative stress in the brains of the aged animals, yet there is reason to speculate that a cooperative effect of these compounds could lead to a relatively greater antioxidant potential and more efficient modulation of oxidant production. The generation of $O_2^{-\cdot}$ by mitochondria appears to be regulated by the level of α-tocopherol [3, 36], which is oxidized to a tocopheroxyl radical by peroxyl or superoxide anion radicals. Reduced CoQ in respiring mitochondria can regenerate α-tocopherol [2, 37, 38]. That such an interaction occurs *in vivo* is supported by studies indicating that supplementation with CoQ alone increases the level of α-tocopherol in mitochondria of many tissues of aged mice [4, 39]. Accumulation of both alpha-tocopherol and CoQ in brain tissue appears to be limited, relative to that which occurs in the peripheral tissues,

and it is noteworthy that significant increases in α-tocopherol of brain mitochondria could only be detected in mice receiving the combination of α-tocopherol and CoQ [4]. Increases in mitochondrial tocopherol could not be detected following either treatment alone.

To conclude, the results of these studies indicated that α-tocopherol and CoQ supplementation were, by themselves, ineffective in improving learning or motor performance of aged mice. When supplemented concurrently, however, these antioxidants resulted in improved performance in an age-sensitive behavioral test involving learning and memory for a preemptive response. These results are consistent with previous studies suggesting a cooperative interaction of α-tocopherol and CoQ in the modulation of oxidative stress in mitochondria, and provide additional rationale for supplementation involving antioxidant combinations.

Fig. 1. Effect of chronic treatment with either α-tocopherol acetate (200 mg/kg body wt) alone, CoQ (123 mg/kg body wt) alone, both CoQ and α-tocopherol acetate, or the soybean oil vehicle (control) on body weight and survival of male C57BL/6 mice aged 24 months at the initiation of treatment. The left panel shows mean body weight ± SE, averaged within 4-week periods. The right panel shows the Kaplan-Meier probability of survival for the same mice. There was no significant effect of the chronic treatments on body weight [$F(3,37) = 0.609, p = 0.613$] or survivorship [$\chi^2 = 1.2, p = 0.74$].

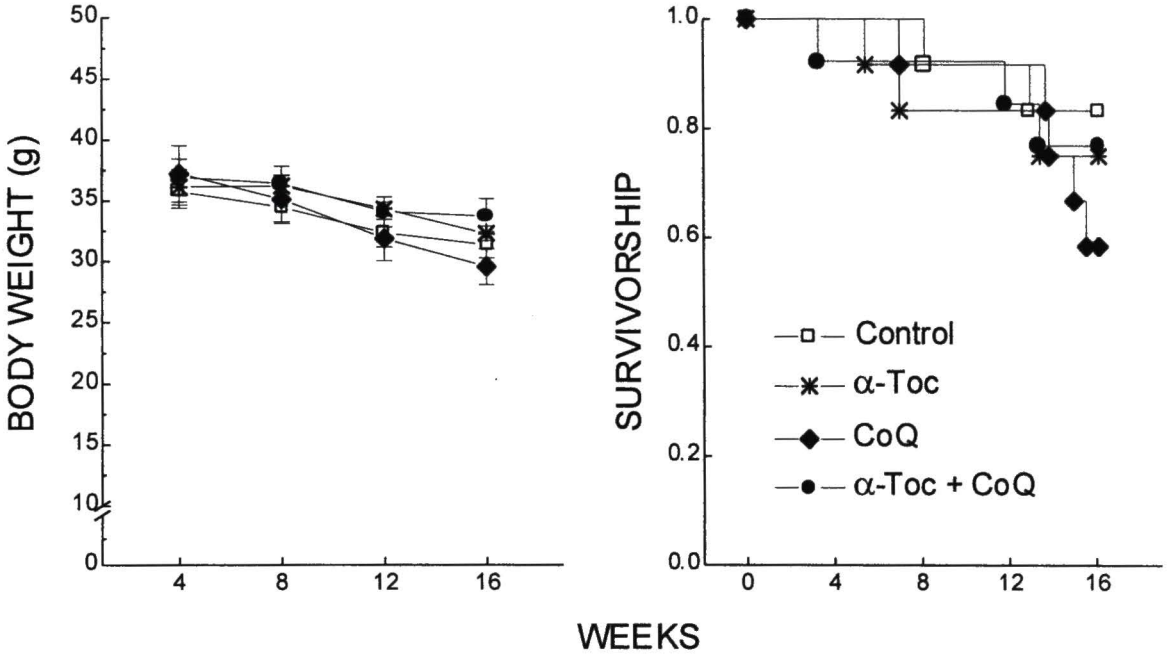
BODY WEIGHT (g)
50
45
40
35
30
25
20
15
10
0
4
8
12
16
SURVIVORSHIP
1.0
0.8
0.6
0.4
0.2
0.0
0
4
8
12
16
Control
α-Toc
CoQ
α-Toc + CoQ
WEEKS

Fig. 2. Effect of chronic treatments in aged mice on their avoidance behavior and ability to identify the correct goal during discriminated avoidance training. The results are expressed as the mean number (± SE) of stem avoidance responses (upper panels) or correct choice-point turns (lower panels) within the first 5 trials of each session. The left panels show the mean number per session over the first 6 sessions (LEARNING PHASE) whereas the right panels show the mean for the session on which the criterion of reversal performance was met (FINAL SESSION). ANOVA indicated a significant effect of supplementation on avoidance ($p = 0.017$), but not correct turns ($p = 0.301$) during the learning phase. No effect of treatment was evident on the final session (all $p > 0.208$). (* indicates $p < 0.05$ for individual comparison with control).

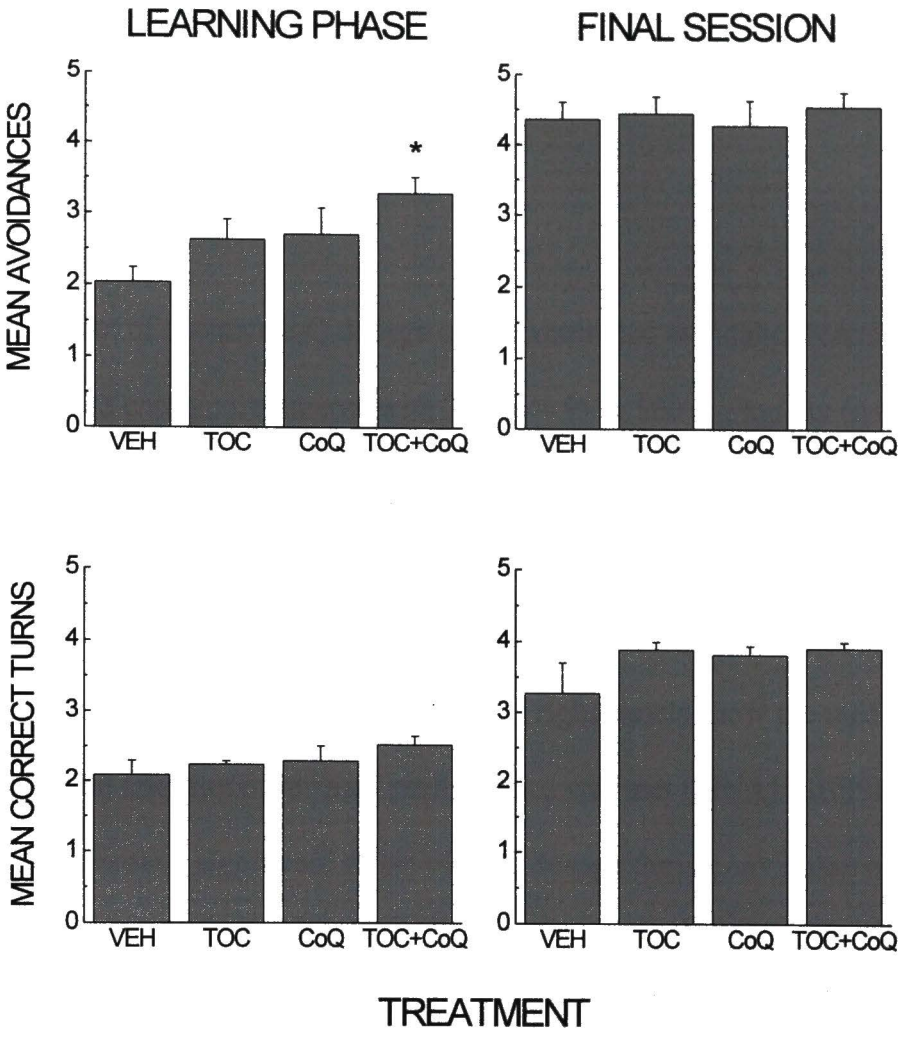
LEARNING PHASE
FINAL SESSION
MEAN AVOIDANCES
MEAN CORRECT TURNS
0
1
2
3
4
5
*
VEH
TOC
CoQ
TOC+CoQ
TREATMENT

Fig. 3. Effect of coenzyme Q dosage on discriminated avoidance learning of old mice. The received chronic treatments with 250 (n = 9) or 500 mg/kg/day (n= 11) CoQ, or the vehicle (n= 8). The results are expressed as the mean number (± SE) of stem avoidance responses (upper panels) or correct choice-point turns (lower panels) within the first 5 trials of each session. The left panels show the mean number per session over the first 6 sessions (LEARNING PHASE) whereas the right panels show the mean for the session on which the criterion of reversal performance was met (FINAL SESSION). ANOVA failed to indicate a significant effect of CoQ dosage during any phase of the testing (all $p > 0.325$).

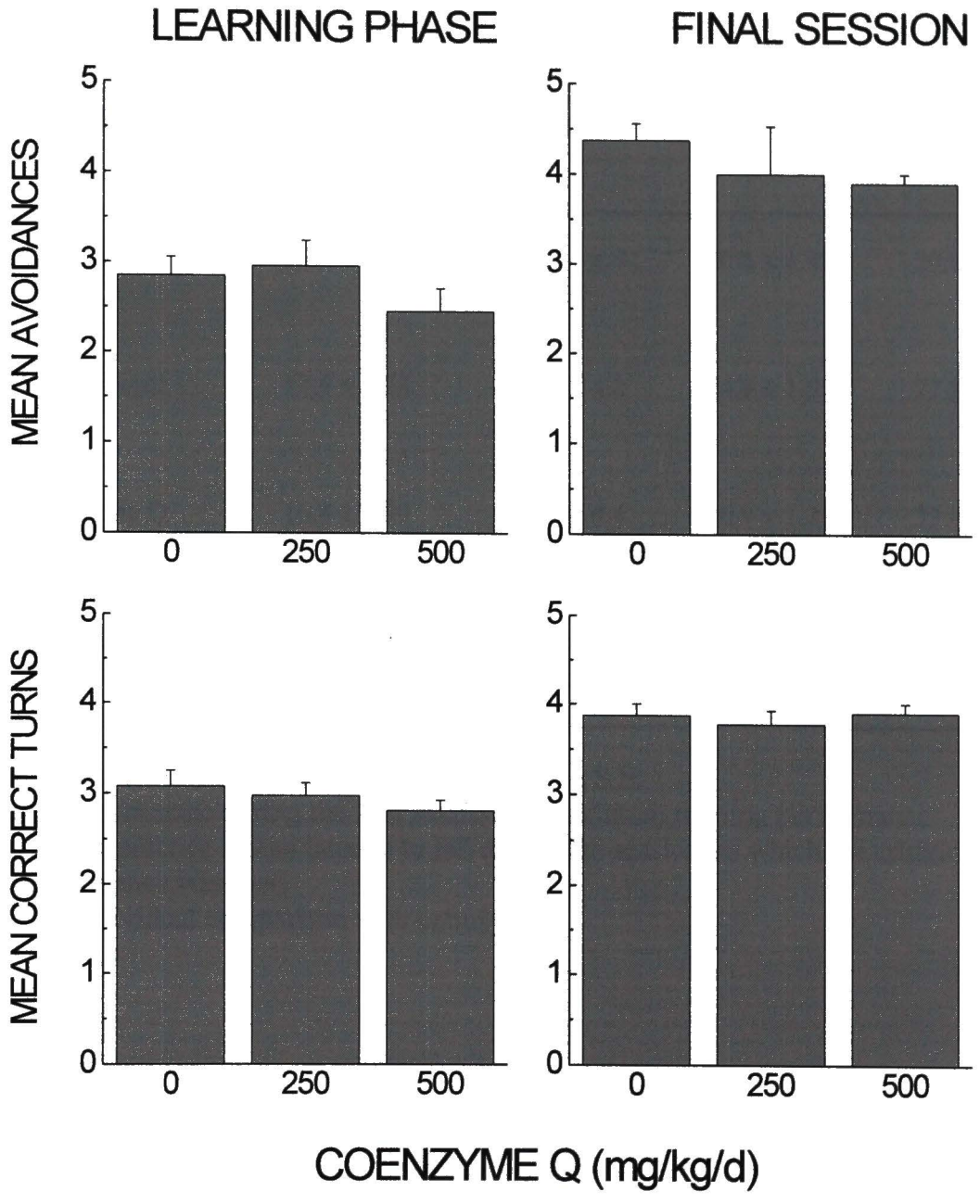
LEARNING PHASE
FINAL SESSION
MEAN AVOIDANCES
MEAN CORRECT TURNS
0
1
2
3
4
5
0
250
500
COENZYME Q (mg/kg/d)

Table 1: Effects of Coenzyme Q and α-Tocopherol on Psychomotor Performance[1]

GROUP	Bridge walking	Wire grip	Coordinated running[2]	
			first session	maximum
Control	13.7 ± 1.60	41.0 ± 4.4	20.6 ± 1.48	29.6 ± 3.22
α-tocopherol	17.6 ± 3.24	45.4 ± 4.2	14.9 ± 1.55 *	27.8 ± 2.76
Coenzyme Q	16.8 ± 2.99	36.3 ± 4.4	16.9 ± 1.21	33.8 ± 4.26
α-tocopherol + Coenzyme Q	14.2 ± 1.38	34.8 ± 4.1	15.8 ± 2.28 *	32.2 ± 2.8

[1]All values are the session mean latency in seconds ± S.E.
[2]Running performance during the first session vs maximum running performance achieved after practice (mean latency to fall during the session on which the criterion of stable performance was met)
*$p<.05$ for individual comparison with control.

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CHAPTER IV

BIOCHEMICAL ANALYSIS

INTRODUCTION

Additional support for the oxidative stress hypothesis of aging would be to determine if dietary supplementation could lower the levels of either oxidative damage or decrease the generation of reactive oxygen species in the brains of treated mice. The levels of oxidatively modified macromolecules would be an indirect measure of the lifelong redox balance. Therefore susceptibility to oxidative stress was to be inferred from the level of oxidative damage, specifically lipids and proteins in regional homogenates of brain tissue. ApoE-deficient mice are known to be particularly susceptible to increased peripheral lipid peroxidation due to elevated circulating lipoprotein levels [9]. Some of the markers of oxidative damage in the brains of apoE-deficient mice include increases in arachadonic acid (AA) oxidation [10] and whole brain lipid peroxidation [11]. Concentrations of 3-nitrotyrosine, a marker of nitration of proteins produced by peroxynitrite, are increased as well [7]. Increased microglial nitric oxide production has also been recorded in apoE4 transgenic mice [2]. The expectation was to find region specific oxidative damage that would correlate with the corresponding behavioral measures as well as level of impairment. This is particularly important

because of the increased variation of physiological decline as animals age. The levels of protein oxidative damage in the brains of old mice are known to be directly correlated with the level of functional impairment [3]. This could be due to age-related increased rates of mitochondrial $O_2^{-\bullet}$ generation. For the mice in the lifelong vitamin E study in apoE-deficient and wild-type mice, both lipid peroxidation and protein carbonyl content were determined on subsets of the mice. Collaborative investigators tested another subset of mice from this study for total protein oxidation in the cortex and hippocampus and two-dimensional electrophoresis (2-DE) coupled with immunostaining to identify specific proteins, of which the results are in press [1]. The results have been published for the mice aged 24 months treated with individual or combination antioxidants for tissue and mitochondrial levels of vitamin E and coenzyme Q [5]. Further analysis has been published on the rate of $O_2^{-\bullet}$ generation by submitochondrial particles [4].

METHODS

Lipid peroxidation

Lipid peroxidation was determined using the thiobarbituric acid reactive substances assay (TBARS) based on the method by Ohkawa [8]. Cortex and hippocampus were thawed on ice and 10% (wt/vol) whole region tissue homgenates were prepared in a 50 mM phosphate homogenization buffer (pH = 7.5) with 4 mM butylated hydroxytoluene (BHT) and 10% sodium dodecyl sulfate (SDS) using a teflon on glass homogenizer. A 100 μL aliquot of each homogenate was treated with 0.75 mL 20% acetic acid at pH =

3.5 and 0.75 mL 0.8% thobarbituric acid (TBA). Each mixture was vortexed and placed in a heat bath at 95 °C for 60 min. After cooling to room temperature, each mixture was centrifuged at 1300 x g for 10 min. Content of the TBARS in each sample was measured in 3 mL cuvettes using a Perkin-Elmer LS-5B flurometer (Perkin –Elmer, Norwalk, CT, USA) with an excitation at 525 nm and emission at 548 nm. Concentrations were calculated using a standard curve, and the results were expressed as nmol TBARS/ mg protein. Protein content of the samples was determined using a modified Lowry method.

Protein carbonyl content

Protein carbonyl content was measured in mouse brain homogenates using the 2,4-dinitrophenylhydrazine (DNPH) procedure [6]. A 20% (wt/ vol) tissue homogenate of cortex was prepared in a 5 mM phosphate buffer (pH = 7.5) containing 0.1% Triton X-100, 1mM diethylenetriaminepentaacetic acid (DPTA), 5mM ethylenediaminetetraacetic acid (EDTA), 5 mM BHT and protease inhibitors leupeptin, (0.7 μg/ml) aprotinin (0.5μg/ml) and papstatin (0.7 μg/ml), using a teflon to glass homogenizer. The homogenates were centrifuged at 5000 x g for 10 min. Two hundred μl aliquots of supernatant were treated with 10 mM DNPH dissolved in 2M HCL or with only 2M HCL in the controls, then incubated for 30 min in the dark at room temperature and stirred every 10 min. The samples were then precipitated with cold 10% trichloroacetic acid (final concentration), left on ice for 10 min and then centrifuged at 6000 x g for 10 min to collect the precipitate. The pellet was washed 3 times with 1.0 ml ethanol/ethyl acetate (1:1; v/v) and each time the pellet was resuspended and centrifuged at 6000 x g for 10

min. The final pellet was resuspended overnight in a 100 mM phosphate buffer (pH = 6.7) containing 3% SDS. The samples were centrifuged at 12,000 x g to remove insoluble materials. The difference in absorbance between the DNPH-treated and the HCL-treated samples was determined at 360 nm, and the results were expressed as nmoles of carbonyl groups per milligram of protein using the extinction coefficient 22.0 mM^{-1} cm^{-1}.

RESULTS AND DISCUSSION

Lifelong dietary supplementation with vitamin E had no effect on lipid peroxidation in the cortex of 6 and 18 mo apoE-deficient mice as compared to wild type controls (Fig. 1). No dose of supplementation had any effect on the levels of lipid peroxidation for either genotype at either age. In fact there is an absolute level of higher lipid peroxidation in the younger mice for both apoE-deficient and the wild type controls indicating a possible autooxidation during storage. This is a confound that could not be circumvented due to the confounds on behavioral measures that would have resulted from testing mice of differing cohorts, as opposed to measuring the age differences in the same cohort.

Cortex carbonyl concentration was not affected by lifelong dietary supplementation with vitamin E in 6 or 18 mo mice of either genotype as measured by the Levine method (Fig. 2). Similar results for the cortex were obtained when analyzed in another laboratory. However, total protein oxidation in the hippocampus was 2-fold greater in the young apoE-deficient mice as compared to the young wild-type mice, which corresponded to the levels of hippocampal protein oxidation in the 18 mo mice of both genotypes. Moreover,

2-DE revealed the protein carbonylation limited to 6 specific proteins. Two of the these proteins, creatine kinase and dihydropyramidase-related protein 2, have been found to be oxidized in the brains of human Alzheimer's patients [1]. The initial lack of differences in measurements of oxidatively modified macromolecules could have been due to the region differences in oxidative damage as well as the sensitivity of the testing methods.

Lass at Southern Methodist University performed and published all the biochemical analysis on the 24 mo mice maintained on oral supplementation with vitamin E or CoQ. Fourteen weeks of administration of combination treatment elevated levels of vitamin E content in nearly all tissues and mitochondria. In the brain, only the combination treatment increased vitamin E levels in the mitochondria. No increase was found with combination treatment in the whole brain homogenate. This supports the notion that CoQ has a sparing effect on vitamin E in the mitochondria [5]. Mitochondrial analysis was performed on whole brain samples, so it is unknown whether or not this may have a specificity for any particular region. The elevated mitochondrial vitamin E appears to mechanistically govern the rates of superoxide anion radical generation as evidenced by an inverse relationship between vitamin E content and $O_2^{-\bullet}$ production in submitochondrial particles [4]. This study provided a nice demonstration of direct redox effect, but all measures were performed in peripheral tissues, nevertheless behavioral results support the notion of a cooperative effect of combination treatment in brain function.

Fig 1. The effect of lifelong dietary supplementation with vitamin E on the MDA product of lipid peroxidation in the cortex of 6 and 18 mo apoE-deficient mice as compared to wild type controls. No dose of supplementation had any effect on the levels of lipid peroxidation for either genotype at either age.

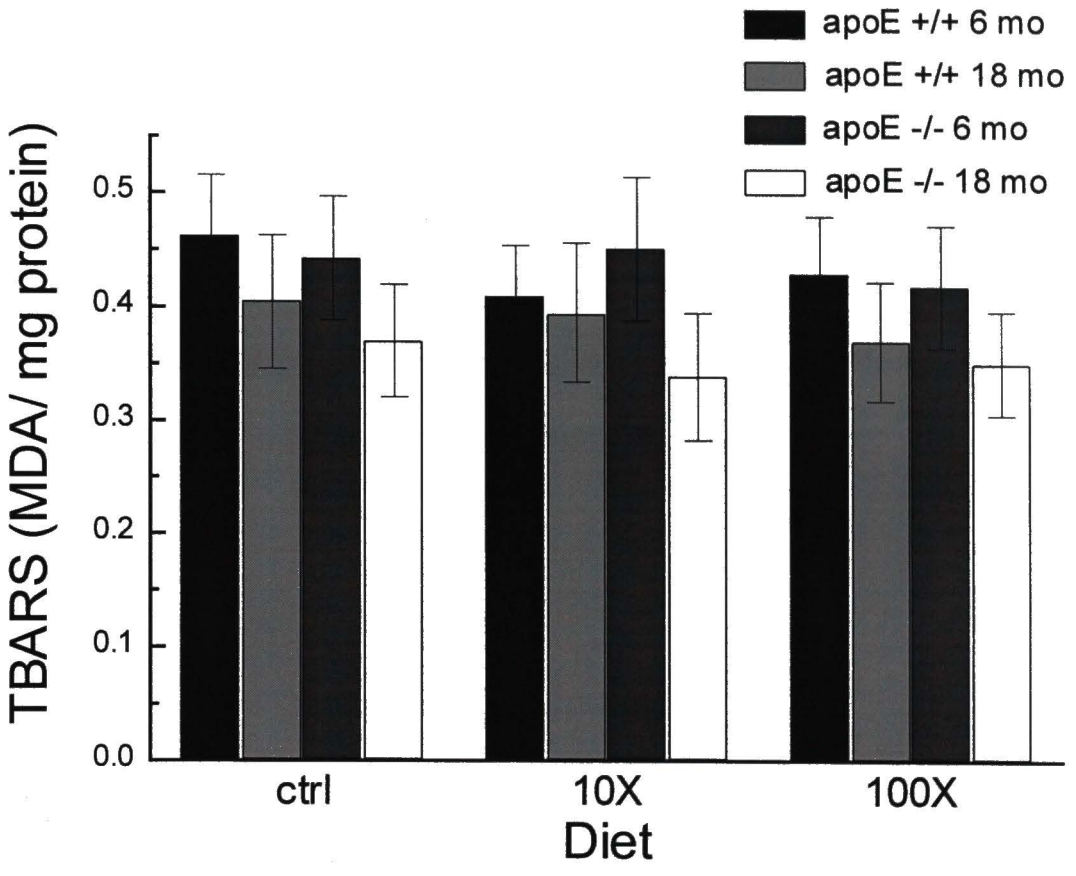
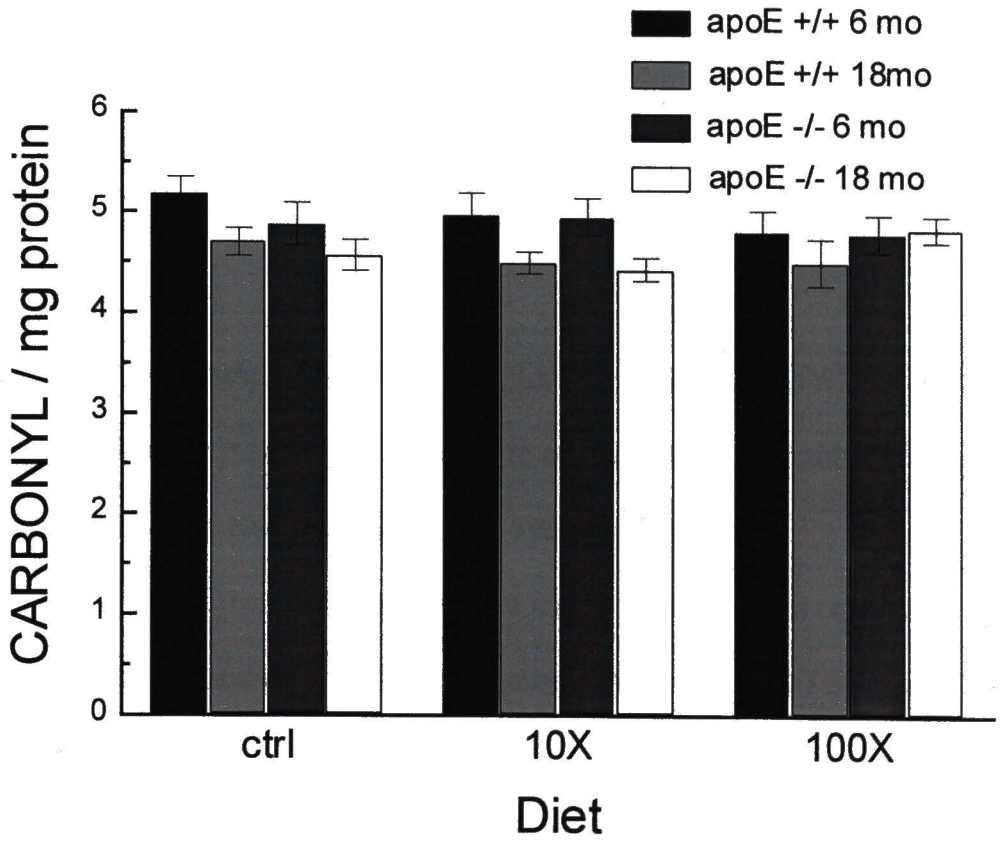

apoE +/+ 6 mo
apoE +/+ 18 mo
apoE -/- 6 mo
apoE -/- 18 mo
TBARS (MDA/ mg protein)
0.0
0.1
0.2
0.3
0.4
0.5
ctrl
10X
100X
Diet

Fig 2. The effect of lifelong dietary supplementation with vitamin E on cortex carbonyl concentration expressed per mg protein in 6 and 18 mo apoE-deficient mice as compared to wild type controls. No dose of supplementation had any effect on the levels of protein oxidative damage for either genotype at either age.


apoE +/+ 6 mo
apoE +/+ 18mo
apoE -/- 6 mo
apoE -/- 18 mo
CARBONYL / mg protein
0
1
2
3
4
5
6
ctrl
10X
100X
Diet

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CHAPTER V

DISCUSSION

The hypothesis that an imbalance between prooxidants and antioxidants as a causal factor in senescence is supported by the observations that the rate of mitochondrial oxidant generation increases with age [15, 16] as well as age-related increases in the steady-state concentrations of oxidized lipids, proteins and DNA [2, 17]. Furthermore, caloric restriction, which is known to increase the life span, is also associated with less molecular oxidative damage [14]. The role of oxidative stress in neural functions associated with brain aging was identified in mice when level of protein oxidative damage was correlated with the degree of age-related impairments in cognitive and motor functions [4].

Experimental intervention to prevent, delay or reverse age-related dysfunction by supplementing animals with compounds with well-delineated molecular antioxidant activity is a valid means to test the oxidative stress hypothesis of aging. ApoE is a known susceptibility factor for AD presumably due to oxidatively induced neural dysfunction. The results in chapter II support the hypothesis that apoE is important for maintaining cognitive functions with advancing age. Albeit, only for spatial learning and memory, but this type of cognition is highly dependent on hippocampal function, which deteriorates

with AD. The highest dose of lifelong dietary vitamin E completely prevented any impairment in spatial memory. The observation that the both medium and high doses prevented age-related impairments in wild type mice does not exclude apoE-deficient mice as a model of accelerated age-associated oxidative stress because the impaired spatial memory is an age-dependent dysfunction. The difference in the dose response suggests a differential utilization of vitamin E within the CNS of apoE-deficient mice. Perhaps molecular functioning is altered, either directly or indirectly through an altered cellular redox environment.

Altered stress response could be a potential source of both observed behavioral differences and any biochemical measurements in apoE-deficient mice due to a reported impaired regulation of the hypothalamic-pituitary-adrenal (HPA) axis. ApoE-deficient mice have been shown to have an increased baseline corticosterone level and greater elevation after restraint stress when 6 mo of age [13]. The apoE-deficient mice in our study showed no overt signs of distress in either study. The anxiety measures during the pilot studies were equivalent for genotype. The difference could perhaps be attributed to the housing of the mice. Group housing was used in the HPA study, whereas the mice in Chapter II were individually housed.

Differences in behavioral measures of performance when using targeted mutant knockout mice have been shown to be the result of the progenitor strain upon which the knockout was created [12], so this could also be a source of the reported genotype differences. Strain differences are also known to affect water maze performance, which is the most consistent behavioral deficit reported in the literature for apoE-deficient mice

[10]. The mice in Chapter II are estimated to contain 99.91% of their genome from the C57BL/6 strain, indicating a small likelihood that the background genotype may have influenced the reported measures.

There is no indication for most of the literature measuring the behavioral effects of apoE-deficiency in mice as to the number of backcrossings for the mice used, which could contribute to the discrepancies in the literature. The results of Chapter II reconcile some of reported differences. When the present study began, there was some concern that the lack of apoE produced such a severe impairment that the mice were unable to learn a spatial task [9]. Our results clearly show that in a very large sample both 6 and 18 mo apoE-deficient mice were able to learn. These results were consistent for 4 different blocks of mice tested in a staggered schedule, in effect the results were repeated 4 times. Other reports in the literature found no impairments in spatial learning [1]. This result was reported for mice aged 8 mo, so in comparison with the present results, the mice were probably too young for the age-associated deficits in spatial learning to be apparent. The majority of the literature shows modest deficits in limited measures of behavior, of which many are comparing small samples of very young mice to those 3 or 6 mo of age [5, 6]. The testing procedures vary widely among investigators as well. This study is probably the oldest age in which the apoE-deficient mice can be tested which may account for our age-dependent effect that is more robust than any currently reported. Globally, the results of the present study for the apoE-deficient mice support their role as a valid model of accelerated but selective impairment of age-associated cognitive decline. This is important because of the need for models to study the molecular mechanisms of

age-dependent dysfunction for which the effectiveness of therapies can then be evaluated in a whole animal system.

The beneficial effects of vitamin E appear to be consistent with its known antioxidant function, but very little is known as to how vitamin E is transported and utilized within neurons. Collectively, Chapters II and III suggests multiple mechanisms may be present: (i) that lifelong vitamin E prevents functional neuronal impairment and (ii) that late life use of antioxidants can also reverse some aspects of neuronal dysfunction. Biochemical measures need to be performed to identify neuronal processing of lipid soluble antioxidants and to establish a causal link with functional measures. The combined treatment used in chapter III was administered specifically based on its ability to augment antioxidant molecular activity with resultant selective improvement in working memory of old mice, a more cortical function. Overall, each intervention either prevented or improved selective cognitive domains.

ApoE-deficient mice appear to be more susceptible to age-associated oxidative stress and thus be a potential model to study the influences of environmental factors on genetic risk. Nutritional intervention with fruits and vegetables in old rats has been shown to improve cognitive function [7] and therefore dietary supplementation may be a conceivable therapeutic option for individuals who have genetic risks for neurodegenerative disorders.

Clearly only the most motivated individuals would partake of a lifelong dietary supplementation strategy and many would not have any identifiable risks to warrant the concern. However, AD has a unique pattern of extensive oxidative damage to selective

neurons, unlike other neurodegenerative diseases [11], which may allow the process to be altered or modified with intensive antioxidant therapy. The exponentially increasing incidence of AD with advanced age is another feature that intractably binds the neuropathology of AD with aging. Perhaps conditions so closely related to aging could be potentially reduced by increasing the USDA's Recommended Dietary Allowance to reflect an intake level that would optimize molecular function [8]. This could realistically lower the number of individuals with AD to the estimated 50 % reduction after 50 years if the onset of the disease could be delayed by 5 years [3].

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