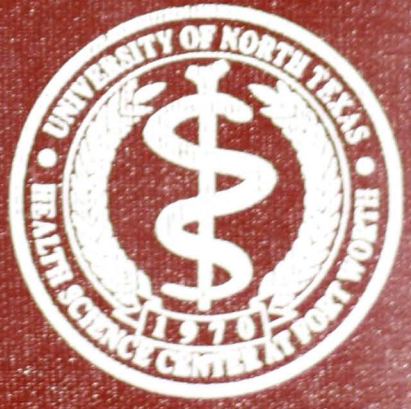
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Purpose: Accrued oxidative damage to brain tissue is a proposed mechanism of cognitive deficits observed with aging. In mammalian tissue, it is hypothesized that a balance normally exists between pro-oxidants (reactive oxygen/nitrogen species) and endogenous antioxidant enzymes that are able to inhibit the activity of reactive oxygen/nitrogen species. As long as this balance is maintained, oxidative damage is moderated, but if the production of pro-oxidants becomes excessive or if the activity of antioxidants lags, oxidative stress and ultimately oxidative damage to tissues may result. It is the hypothesis of this project that exercise training is able to prevent decreased antioxidant activity in brain tissue, produce a favorable shift in the pro-oxidant/antioxidant balance, and thus moderate oxidative damage in the aging mice brain. **Methods:** 3 and 20 month old C57BL/6 mice were either subjected to 8 weeks of treadmill exercise followed by 3 weeks of concurrent exercise and behavior testing, or else they were age-matched, non-exercised controls. Mice were tested on multiple behavioral tasks that tested sensorimotor learning as well as tasks that required utilization of various components of cognitive learning. After exercise and behavior testing regimens were completed, biochemistry assays for protein oxidative damage as well as for antioxidant enzyme activity were performed on several brain regions. **Results:** It is a finding of the study that moderate, short-term exercise initiated in aged C57BL/6 mice

resulted in increased fitness in the aged mice to the same degree as observed in young mice, improved some psychomotor skills, including bridge-walking and reaction time, and improved age-impaired spatial memory performance. Moreover, exercise training showed a lack of effect on oxidative damage in all brain regions, increased activity of glutathione peroxidase in the cerebellum and striatum of young, but not aged mice, and it increased the activity of catalase in the cortex of aged mice. **Conclusions:** The data presented in this project shows that exercise does moderate some age associated cognitive deficits, and the findings do not preclude the possibility that exercise produces this effect by reducing accrued oxidative damage that occurs with aging.

THE EFFECT OF EXERCISE TRAINING ON BEHAVIOR

AND OXIDATIVE STRESS IN

AGING MICE

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CHAPTER I

INTRODUCTION

Aging and Cognitive Deficits

During the course of aging, humans often experience a progressive decline in overall cognitive function, which can range from mild cognitive impairment to severe pathological dementia. Certainly age is a risk factor in neurodegenerative diseases such as Alzheimer's and Parkinson's that are characterized by significant declines in memory and/or cognitive function. Although there has been considerable research to suggest that severe cognitive decline is not necessarily a consequence of aging for the majority of elderly people, nonetheless mild to moderate cognitive deficits often manifest with normal aging (1, 2). In spite of substantial investigation, the mechanism involved in cognitive deficits seen with aging remains unclear. Some proposed hypotheses of cognitive aging include hypometabolism of brain cells (3), altered neuronal morphology (e.g., loss of synapses)(4), the decline in levels of key hormones and/or growth factors in aged individuals (5, 6), reduced oxygen availability to brain cells (primarily due to atherosclerosis or other types of heart disease)(7), dysfunction of DNA synthesis, protein synthesis, and/or post-translational modification of proteins (8) and the cumulative effect of oxidative stress in the brain over many years (9, 10, 11).

The differential contributions that specific brain areas make to cognitive processes must also be considered in the examination of aging and cognitive decline. Although cognitive performance is the result of myriad circuitry involving all of the brain areas, specific brain areas make distinct contributions to cognition. Moreover, specific brain regions appear to be more vulnerable to the effects of aging than others, suggesting that that aging has a differential effect on cognitive ability as a function of brain region (11, 12).

Oxidative Stress

In the 1950's Denham Harman introduced the free radical theory of aging (13). Briefly, the premise of the theory is that aging is the result cellular damage caused by destructive reactive oxygen species formed during the normal process of aerobic respiration. As can be seen in Figure 1, a sequence of events involving reactive oxygen species (charged oxygen molecules with an unpaired electron that can accept electrons from other radicals) results in the formation of an extremely unstable hydroxyl radical ($OH^{\cdot}$). The hydroxyl radical is ultimately responsible for oxidative damage to tissues, as it scavenges electrons from nearby biomolecules to fill its outermost orbital. In mammals, aerobic respiration is a major source of the formation of reactive oxygen species. Briefly, at various points in the mitochondrial electron transport chain, insufficient mitochondrial enzymes result in the premature transfer of electrons to O_2, reducing it first to superoxide anion ($O_2^{\cdot -}$), and then to hydrogen peroxide (H_2O_2). These initial intermediates in the formation of $OH^{\cdot}$ are known as pro-oxidants or oxidants (14). There is another reactive oxygen species to

be considered in the free radical theory of aging. Nitric oxide (NO) is a tiny gaseous molecule recognized as a signaling molecule involved with many physiological functions. However, NO is able to react with $O_2^{\cdot-}$ to form an oxidant, peroxynitrite ($OONO^-$) that is capable of damaging nearby biomolecules (15). Fortunately cells are able to suppress free radicals by means of antioxidant defense systems. There are two aspects of this defense system - one consisting of small molecule antioxidant nutrients that are consumed in the diet and the other comprised of endogenous antioxidant enzymes synthesized by the cell itself to neutralize damaging reactive oxygen species (15, 16). The major antioxidant enzyme defense system consists of the enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and catalase; reactive oxygen intermediates formed as a result of aerobic respiration may convert to dangerous and highly reactive radicals, or they may be neutralized to oxygen and water (O_2 and H_2O) when catalyzed by GSH-px and/or catalase (15, 16). The reduction of H_2O_2 to H_2O catalyzed by GSH-px is accompanied by concurrent oxidation of glutathione (GSH) to oxidized glutathione (GSSG). GSSG in turn is reduced to GSH by another antioxidant enzyme, glutathione reductase. The GSH/GSSG ratio is a common biomarker of oxidative stress, a high ratio indicates little oxidative stress as GSH scavenges reactive oxygen species and GSSG interferes with mitochondrial function by interfering with the function of NADPH dehydrogenase (14). It is hypothesized that a balance is maintained between pro-oxidants (reactive oxygen/nitrogen species) and antioxidant enzymes. Formation of pro-oxidants may stimulate increased production of antioxidant enzymes (16), but if the production of pro-oxidants becomes excessive, or if the activity or

synthesis of antioxidants lags, oxidative stress and ultimately oxidative damage to tissues may result. The $OH^{\cdot}$ anion formed as a result of oxidative stress is not discriminatory, it attacks nearby molecules such as DNA, lipids, and proteins to obtain electrons, thereby triggering free radical chain reactions that culminate in oxidative damage to the cell (16, 17).

All 3 major biological macromolecules are vulnerable to attack by reactive oxygen species: lipids, DNA and proteins. Peroxidation of the fatty acyl groups of phospholipids in cell membranes by reactive oxygen species results in damaged lipids that change membrane fluidity, thus compromising membrane function (16). Lipid peroxidation can promote protein damage which in turn has far ranging effects on cell metabolism including altered channel, transporter, and signaling function, altered enzymatic function, and altered structural components of the cell, all of which may contribute to aging (16, 17). Nucleic acids (DNA) are another biological macromolecule vulnerable to attack by reactive oxygen species. Oxidative DNA damage produces several DNA modifications including base and sugar modifications (adducts), strand breaks, cross-links to other nucleic acids or proteins, and base deletions and insertions (16, 17). Proteins are the third biological macromolecule that undergoe oxidative damage during aging. Proteins are involved with nearly every aspect of cellular activity so it is clearly understandable that damage to proteins results in substantial losses of cell integrity and optimal functioning. Oxidative damage to proteins can take many forms, including peptide bond cleavage, generation of carbonyls by oxidation of amino acid side chains, and cross-linking between proteins (16,17).

Oxidative Stress and Cognitive Deficits

Oxidative stress imposed on cells of the central nervous system leads to oxidative damage that could eventually result in degenerative senescence. Oxidative damage can reasonably be hypothesized to be involved in age related cognitive deficits as the brain utilizes a considerable portion (about 20%) of the body's oxygen and thus could be a major site of production of reactive oxygen species. During aging, brain tissue has been shown to manifest increases in reactive oxygen species and changes in antioxidant defenses, upsetting the balance between the two (18). Presumably this disordered equilibrium contributes to the increases in oxidative damage observed in the brains of aged individuals. It should also be noted that with some apparent exceptions (e.g. olfactory and hippocampal neurons) most of the neurons of the brain are amitotic and do not regenerate; consequently (in humans) neurons are exposed to insults from reactive oxygen species over a course of decades. Such long term exposure to the damaging effects of harmful reactive substances very likely results in cumulative damage to brain tissue. Studies have shown that cognitive deficits (19) and biochemical markers of oxidative stress in the brain (20) can be reversed with antioxidant treatment. Age related loss of ability in learning and memory-specific tasks have been shown to correlate with protein oxidative damage in aging rodents (11, 22, 23). Other researchers have shown a relationship between oxidative stress as measured by lipid and/or DNA damage and cognitive deficits as well. For example, the *klotho* gene is a recently characterized gene that is hypothesized to provide protection against aging. Accumulations of oxidatively damaged lipids and DNA have been observed in the hippocampi of *klotho* gene null

mutant mice concomitant with impaired recognition and associative memory in these mice (24). Learning and memory deficits as well as oxidatively damaged proteins and lipids in the brains of C57BL/6 mice have been reversed with the administration of synthetic catalytic scavengers of reactive oxygen species (25). These findings lend support to the hypothesis that oxidative stress is a cause of cognitive deficits seen in aging animals.

The Effect of Exercise Training on Cognitive Deficits

Many intervention strategies such as diet and nutritional supplements have been employed to minimize the effects of aging on physical as well as cognitive functioning. Exercise, or more specifically fitness training is another intervention strategy that has been employed successfully to delay cognitive deterioration. Studies performed on humans and animals have established beneficial effects of exercise training by demonstrating that exercise training may act as a modulator of aging, help prevent pathological diseases associated with aging, and prolong life span (26, 27, 28). More recently, researchers have directed their attention to the potential benefits of exercise training on cognitive function in aging subjects (29, 30, 31, 32).

Aerobic fitness appears to be a vital component of improved cognitive function as there are numerous studies showing that individuals displaying significantly better cardiovascular function (as measured by the volume of oxygen consumed while exercising at maximum capacity - $VO_2max.$) also show better performances in tests of cognitive function. Similarly tested individuals whose exercise training programs

consisted of toning and stretching or similarly tested sedentary individuals failed to exhibit either higher levels of cardiovascular fitness or superior cognitive ability (32, 33, 34, 35). Studies in rodents have also shown that increased physical activity is correlated with better cognitive functioning (22, 36, 37). Thus it appears there is a distinct relationship between greater fitness levels and better cognitive functioning in both human and rodent models and very likely exercise training is important in its ability to enhance cognitive function.

The Effect of Exercise Training on Oxidative Stress

The beneficial relationship between exercise and cognitive function in both humans and rodents appears to be well established. What remains unclear is the mechanism by which exercise might exert its beneficial effects. One possible mechanism is that exercise modulates the damaging effects of oxidative stress on cells, which in turn would result in diminished cognitive deficits. It is interesting to speculate what the effects of exercise on endogenously produced reactive oxygen species might be. Since endogenous reactive oxygen species are the product of normal metabolic processes, it stands to reason that increasing oxygen consumption (as occurs during exercise) would increase the production of reactive oxygen species, at least for the short term (21). On the other hand good correlation between improved health and exercise makes it reasonable to hypothesize that exercise induced increases in reactive oxygen species must be not only counterbalanced but perhaps overcompensated for in some way, at least in chronic exercisers. The extent of oxidative damage sustained by tissues during exercise is

dependent on two parameters, the amount of free radical generation and the effectiveness of cellular antioxidant systems. An increase in reactive oxygen species (ROS) generated with exercise would not necessarily be harmful as long as compensatory antioxidant mechanisms are intensified as well. Because the benefits of exercise paradigms appear to be maximized by exercise training regimens that involve fitness training and because compensatory mechanisms are the result of prolonged exercise, this project focused on the effect of chronic aerobic exercise training on antioxidant defense systems and oxidative stress as a function of age.

Fitness in C56BL/6 mice

In humans, aerobic fitness is measured by the total amount of oxygen that is consumed and utilized by an exercising individual. As exercise intensity increases so does the uptake and consumption of oxygen in order to provide sufficient energy to maintain the exercise training intensity. When the consumption of oxygen cannot increase despite an increase in exercise intensity, this is considered to be the maximum amount of oxygen that can be taken in and utilized (VO_2max).

It is possible to measure the intensity of exercise training in terms of VO_2 max, measured in ml/kg/h. Maximum heart rate (MHR) is the age-corrected number of beats per minute of the heart when working at its maximum, it usually is estimated as 220 minus one's age. The American College of Sports Medicine (ACSM) defines moderate exercise intensity as 60 - 79 % of MHR. The ACSM defines "heavy" exercise at 80 - 89 % MHR, and "very heavy" at 90 % and above, with 100 % being maximal exertion (40).

A relationship exists between MHR and VO_2 max such that: 95% MHR = VO_2 max. Therefore VO_2 max can be measured during an exercise training period and the intensity of the workout can be determined. There are technical limitations involved in accurately accessing MHR and VO_2max in small exercising animals, and most rodent studies that examine the effect of exercise training on cognitive deficits lack a definition of the "aerobic fitness" of the exercising animals. Based on previous investigations an exercise training protocol was designed to produce a moderate level of aerobic fitness in C57BL/6 mice (38, 39). In the literature it is not clear whether the same exercise training protocol leads to a similar level of aerobic fitness if implemented in young versus aged rodents (mice). In the current study, the level of aerobic fitness was determined in young and aged mice after they were subjected to a protocol of exercise training over the course of several weeks. Aerobic fitness of the mice was inferred from activity of citrate synthase, an enzyme that is found in the mitochondrial matrix as as part of the Kreb's cycle. Citrate synthase is also found in the mitochondrial inner membrane as part of the electron transport chain. Skeletal muscle responds to exercise training with both structural and biochemical adaptations which include increases in mitochondrial number. Thus, the activity of citrate synthase from skeletal muscle is a useful quantitative biomarker of increases in fitness as a result of an exercise training protocol (41).

Goals of the Current Research

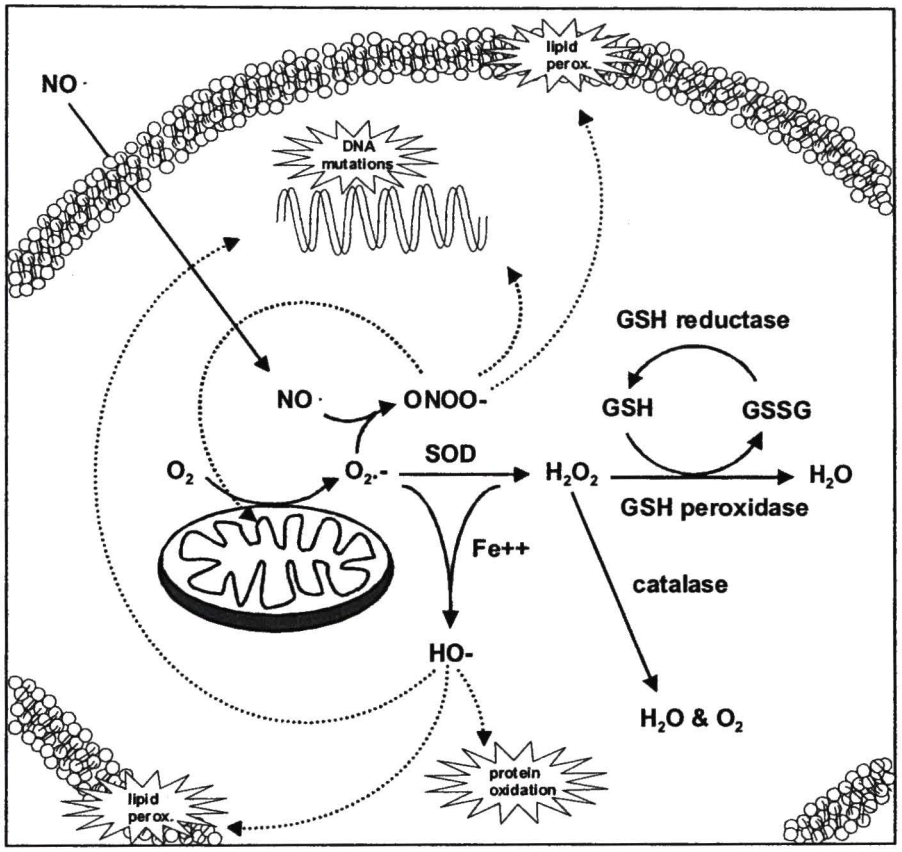
Wide variations in cognitive decline are seen in normal aging, doubtless due to variability in individual abilities to compensate for aging mechanisms. The oxidative

stress theory of aging proposes that cognitive deficits may be due to the accrual of oxidatively damaged cellular components that result in damage to neurons. Oxidative damage to neurons as a causative factor in brain aging is a likely hypothesis as the products of oxidative damage to cellular components in the brain have been correlated with cognitive deficits in aging subjects (25).

Exercise training is associated with a prolonged life span and diminished pathologies observed with aging, including cognitive deficits. Individuals displaying high levels of aerobic fitness show greater abilities in their performance of memory tasks, especially those tasks involving complex thought and memory. There have been numerous studies linking exercise training or aerobic fitness with improved cognitive function in both humans and rodents but there has been very little research addressing the issue of the ameliorating effects that exercise training may have on cognitive deficits resulting from accrued oxidative damage in mammals. Furthermore, there have been no studies addressing the issue of the effect of exercise training on oxidative stress in specific brain regions. Because different brain regions make distinct contributions to cognitive processes, it is worthwhile to examine such an effect. The purpose of the current project was to determine if exercise training could indeed moderate cognitive deficits that appear with aging, and to determine if a reduction in age-associated oxidative damage is a potential mechanism of such moderation. By examining markers of oxidative stress in separate brain regions in conjunction with assessment of behavioral capabilities in mice on an exercise training regimen, as compared to age-matched non-exercised controls, it

was possible to address the issue of exercise training as a moderator of the adverse effects of oxidative stress on cognitive performance that occurs with normal aging

Fig. 1. Generation of oxygen free radicals ($O_2^{\cdot}$- and $OH^{\cdot}$) in cells, potential sites of oxidative damage (cell proteins, lipid membranes and cellular DNA), and potential sites of antioxidant (SOD, GSH-px, and catalase) action in mammalian cells.


NO·
lipid perox.
DNA mutations
GSH reductase
NO·
ONOO-
GSH
GSSG
O_2
O_2·-
SOD
H_2O_2
H_2O
GSH peroxidase
Fe++
catalase
HO-
H_2O & O_2
protein oxidation
lipid perox.

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EXERCISE TRAINING IMPROVES COGNITIVE AND PSYCHOMOTOR DEFICITS IN AGING C57BL/6 MICE

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Running title: Exercise training in Aged Mice

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CHAPTER II

EXERCISE TRAINING IMPROVES COGNITIVE AND PSYCHOMOTOR DEFICITS IN AGING C57BL/6 MICE

SUMMARY

The ability of forced exercise training to ameliorate age-associated cognitive and psychomotor deficits was tested in aging C57BL/6 mice. Beginning at 3 or 20 months of age C56BLl/6 mice were either subjected to an 11-week exercise training protocol designed to be of moderate intensity but rigorous enough to produce significant increases in fitness, or they were part of an age-matched, non-exercised control group. Cognitive and psychomotor performances of the mice were assessed with a battery of behavioral tests including locomotor activity to test exploratory behavior in mice, various motor skills tests designed to elucidate musculoskeletal as well as neural processing, and a test of cognitive functioning – the spatial swim maze task. All of the behavioral tests were administered 8 weeks after the initiation of exercise, 3 weeks prior to the end of the exercise training protocol. To evaluate fitness in the mice subjected to exercise, a citrate synthase assay was used. Citrate synthase is a mitochondrial enzyme and skeletal muscle responds to exercise training by increasing its mitorchondrial fraction so that citrate

synthase levels can be used as a quantitative marker for increased fitness in exercised mice. As evidenced by increased citrate synthase activity, the exercise training protocol did indeed produce a significant and nearly equal degree of fitness in both the young and aged mice. Exercise training improved the ability of mice to acquire new psychomotor skills in C57BL/6 mice as a function of age, with the performance of the aged, exercised mice reaching significant levels on some but not all of the behavioral tasks. Most notably, although non-exercised mice displayed significant age-associated deficits on the elevated path (bridge walking) task, the aged, exercised mice showed recovered function on this task. The elevated path test is a motor skills test that is used to determine the animal's muscular coordination and balance. These motor skills are heavily influenced by the cerebellum, so improved performance on the bridgwalking task could reflect moderated age-associated cerebellar disfunction. A similar age-associated deficit in non-exercised mice was seen in the latency to startle test. Aged, non-exercised mice had significantly longer latencies to a startle reaction than did exercised mice. In the latency to startle, there was a recovery of startle response with exercise training in the aged mice. The startle test is a measure of the animal's reflexive response to shock stimulation, so decreased response times suggest improved sensory motor response. In addition to improving some age-associated psychomotor skills, exercise training apparently was able to moderate some age-associated cognitive deficits as demonstrated by the improved performance of aged, exercised mice in some aspects of the spatial swim task. Aged, exercised mice were able to remember the previous location of a hidden platform after a 24-hour delay in testing with significantly greater accuracy than non-exercised control

mice. Because their improved performance occurred 24 hours after they had acquired the knowledge of the original platform location, the results suggest that exercise training is able to ameliorate age-associated deficits in memory retention. Because these different psychomotor and cognitive function tasks are most directly under the control of different brain regions, these data are consistent with the hypothesis that exercise training may have differential effects on specific brain areas.

1. Introduction

The life expectancy of the human population has increased over the past several decades. Barring pathological dementia, as most individuals age they will experience a loss in cognitive function that will range in severity from mild to moderate (1). The memory loss experienced by individuals suffering from diseases of dementia such as Alzheimer's disease is certainly more extreme and debilitating than the impairments seen with normal aging, but nevertheless advancing age is characterized by an often troublesome loss of cognitive function. These decrements are especially noticeable with tasks involving executive control, planning, scheduling, coordination, inhibition & working memory (1, 2).

Since diminution of mental aptitude has a profound effect on the quality of life, there has been considerable interest in discovering intervention strategies designed to prevent cognitive deficits seen with aging. For example, the effect that diet and nutritional supplements have on the preservation of cognitive abilities has been extensively researched and widely employed as a medium through which cognitive deficiencies may be ameliorated (3). Likewise, environmental enrichment (mental stimulation) has been a successful means of minimizing the effects of aging on cognitive functioning (4). Exercise, or more specifically fitness training is another intervention strategy that has been employed to delay cognitive deficits. Numerous investigators have examined the effect of exercise training on cognitive deficits in both human and animal models. Clearly a strong relationship exists between exercise and better performance on cognitive

tasks. In human studies, several researchers have established that exercise benefits cognition when the exercise protocol involves aerobic training (5, 6, 7). Indeed beneficial effects of aerobic exercise training on cognition are seen in aerobically exercised individuals but not in individuals who participate in non-aerobic regimens that involve toning and stretching (8, 9). Thus aerobic fitness appears to be a vital component of the improved cognition seen in conjunction with an exercise training regimen.

It is possible to measure the intensity of exercise training in terms of maximum heart rate and the maximum amount of oxygen that can be taken in and utilized (VO_2 max)(10, 11). However there are technical limitations involved in accurately accessing MHR and VO_2 max in small exercising animals, and most rodent studies that have examined the effect of exercise training on cognitive deficits lack a definition of the "aerobic fitness" of the exercising animals. Based on previous investigations, an exercise training protocol was designed to produce a moderate level of aerobic fitness in C57BL/6 mice (13, 14). In the literature it is not clear whether or not the same exercise training protocol leads to a similar level of aerobic fitness if implemented in young versus aged rodents (mice). In the current study, the level of aerobic fitness was determined in young and aged mice after they were subjected to a regimen of exercise training over the course of several weeks. Aerobic fitness of the mice was inferred from activity of citrate synthase, an enzyme that can be found in the mitochondrial inner membrane as part of the electron transport chain. Skeletal muscle responds to exercise training with both structural and biochemical adaptations that include increases in mitochondrial number. Thus, the

activity of citrate synthase from skeletal muscle is a useful quantitative biomarker of increases in fitness as a result of an exercise training protocol (15).

The goal of this study was to test an exercise training regimen for its ability to produce aerobically fit young and aged mice, and to determine the effects of this exercise training regimen on psychomotor and cognitive performance in aging aerobically fit C57BL/6 mice.

2. Materials and Methods

Animals

A group of 62 young (3 months) and a group of 62 aged (20 months) C57BL/6J mice were obtained from the National Institute on Aging for testing. Upon arrival at the University of North Texs Health Science Center (UNTHSC) vivarium, the mice were group housed (4-7 to a cage) in 28 x 19 x 12.5 cm solid bottom polycarbonate cages with wire grid tops. The vivarium was maintained at 23 ± 1^0 C/40% humidity under a 12 hour light/dark cycle with lights on at 0700. The mice had *ad libitum* access to water and standard NIH-31 chow at all times throughout the project.

Effect of exercise training on citrate synthase activity

After a 1 week period of adjustment, 7 young and 7 aged mice were exercised daily on an OmniPacer treadmill Model LC4/M (Accuscan Instruments Inc., Columbus OH) for a period of 8 weeks. Variability in exercise training capacity of different inbred

mouse strains has been observed (12), so to achieve a moderate exercise training level in C57BL/6J mice the following exercise training protocol was used (13, 14). Mice were acclimated to exercise training over a 3 week period as follows. On day 1, mice were placed on a stationary treadmill with an 8^0 incline for 5 min, followed by a warm up period of running at 3 m/min for 5 min On day 2, the exercise training speed was 4 m/min and the length of the exercise training period was 10 min Thereafter on each succeeding day, the speed of the exercise training period was increased by 1 m/min, and the length of the exercise training period was increased by 5 min, until a maximum speed of 14 m/min and a total running time of 60 min was achieved. As the mice achieved running speeds of 6, 8, 10, and 12 m/min, the total running time included 5 min of running at each of those speeds with the remainder of the exercise training period devoted to running at the maximum speed designated for that day. This approach resulted in a final exercise training protocol consisting of a warm up period of running at 6 m/min for 5 min, 8 m/min for 5 min, 10 m/min for 5 min, 12 m/min for 5 min, and a final speed of 14 m/min for the duration of the 60 min exercise training period. The final exercise training protocol was reached after 3 weeks of acclimatization and continued for 5 weeks. Electric shock grids located behind the treadmill belts delivered 0.29 mA electric shock to the mice when not running. The number of shocks delivered to all exercising mice were tallied during each exercise training period. Non-exercised control mice were assigned as shock stimulus controls to the exercised mice, so that each non-exercised control received the same amount of shock stimulation as its exercising counterpart. To effect this, 7 young and 7 aged, non-exercised control mice were placed on a stationary

treadmill (belt not active) and retained on an inactive electric grid by means of a blocking device. The grids were temporarily activated and approximately 1 sec of 0.29 mA shock was administered to each mouse. The number of shock stimuli that each mouse received was based on the number of shock stimuli that its exercising counterpart received. An intershock interval of 30 seconds was maintained. After each mouse received their quota of shock stimuli, the blocking apparatus was removed and the mouse was allowed access to the stationary belt for the remainder of the exercise training period. Exercising mice that exceeded 30 shocks per hour for 3 consecutive days were eliminated from the study along with their non-exercised control partners. All mice were weighed at the beginning of the exercise training regime and twice a week thereafter. Mice in this study were euthanized and muscle taken for citrate synthase determination.

Citrate Synthase Assay

All mice rested in their home cages for 1 day before being euthanized. After euthanasia, the *rectus femoris* muscles were removed from the hindlegs and the red fibers were isolated before being flash frozen in liquid nitrogen in antioxidant buffer (50 mM phosphate buffer (pH 7.4), 1 mM butylated hydroxytoluene (BHT)(Sigma, St. Louis, MO), 100 μM diethylenetriaminepentaacetic acid (DTPA)(Sigma) prior to further preparation by grinding. Muscle tissue was ground in a mortar filled with liquid nitrogen, removing elastic tissue until the remaining muscle tissue was fine in texture. Muscles remained frozen at -80^0 C until the citrate synthase assay (15) was performed. Briefly, a 5% homogenate of the muscle tissue was prepared in a buffer containing 100 mM

potassium phosphate, monobasic (KPO_4)(Sigma), 5 mM ethylinediaminetetraacetic acid (EDTA)(Sigma) and 5 mM ethyleneglycol-bis (β-amino-ethylether)-N,N,N',N'-tetraacetic acid (EGTA)(Sigma). A secondary homogenate was made by further diluting the primary homogenate 1:20 with the buffer. Commercial 3 mM acetyl CoA (Sigma), 5 mM oxaloacetic acid (Sigma), and 1 mM DTNB (all in 100 mM Trizma base (TRIS)(Sigma)) were incubated in 1.5 ml acrylic cuvettes at 30^0 C for 3 min before adding the secondary tissue homogenate to start the reaction. The reaction mixture was read on a (Shumadzu UV-2401 PC, Columbia, MD) spectrophotometer at a wavelength of 412 nm. Citrate synthase activity of muscle tissue was followed by measuring the absorbancy of thionitrobenzoic acid (TNB) formed by the coupling of an enzymatic reaction catalyzed by citrate synthase (eq.1) to an irreversible chemical reaction (eq. 2).

$$\text{Acetyl CoA} + \text{oxaloacetate} + H_2O \xrightarrow{\textit{citrate synthase}} \text{citrate} + \text{CoA-SH} \qquad \text{(eq.1)}$$

$$\text{CoA-SH} + \text{DNTB} \longrightarrow \text{TNB} + \text{CoA-S-S-TNB} \qquad \text{(eq. 2)}$$

Citrate synthase activity was determined as μmol substrate transformed to product/min /gram of tissue wet mass, expressed as mmol/min/gram wet weight of the muscle.

Effect of exercise training on cognitive and psychomotor function

After a 1 week period of adjustment, 24 young and 24 aged mice were exercised daily on an OmniPacer treadmill Model LC4/M (Accuscan Instruments Inc., Columbus OH) for a period of 8 weeks using an exercise training protocol identical to that used for the mice in the citrate synthase study. After 8 weeks of exercise, behavior testing was begun on all mice and continued for the next 3 weeks. Mice continued to exercise daily, using the same protocol as during the first 8 weeks, throughout the behavior testing.

Behavioral Testing

All mice in this study began behavioral testing for cognitive function after 8 weeks of exercise, however as behavioral testing required 3 weeks to complete, the exercise training regimen continued throughout behavior testing so to maintain the effect of exercise. The battery of behavioral tests were administered in order as follows: Locomotor Activity, 1st session (LMA), Reflexive and psychomotor tests, Spatial swim maze task, Startle Response Test, and Locomotor activity, 2nd session. Behavior testing was conducted early each morning and exercise training was administered immediately following the behavior testing. This schedule was followed in order to allow the mice as long a rest period as possible after exercising before the next behavior testing period in order to minimize acute effects of exercise.

Reflexive and Psychomotor tests

Spontanous Locomotor Activity

The locomotor activity of mice was measured using a Digiscan apparatus (Omnitech model RXYZCM (16)) connected to a microcomputer. Individual mice were placed in clear acrylic chambers (40.5 X40.5 X30.5 cm) and spontaneous locomotion was measured during 4 consecutive 4-minute periods. A 7.5 W incandescent light provided dim illumination above each chamber. Fans provided an 80-dB ambient noise level. To measure horizontal activity of the mice, a panel of 16 infrared beams and photodetectors were spaced 2 cm apart along the sides of the chambers at a height of 2.4 cm. To measure vertical activity, a panel of 16 beams and photodetectors were located at a height of 7.6 cm. Mouse activity interrupted the detection of photobeams, and the activity was recorded by a computer. The LMA test was administered in one daily session at the beginning of the behavior test battery and then again at the end of the battery.

Motor Skills

A battery of five tests was used to measure simple reflexes, muscle strength, coordination and balance. The battery included: Walking Initiation, Alley Turning, Negative Geotaxis, Wire Suspension, and Elevated Path (Bridge). Each test was given in one daily session on four consecutive days. The animals had 60 seconds to complete each trial for each individual test. Mice not completing the test in 60 seconds received a maximum score of "60" for the test.

Walking Initiation. For the Walking Initiation test, the mouse was placed on a flat surface and the time (in seconds) for it to travel one body length was recorded. Body length was defined as the distance from the tip of the nose to the base of the tail. Walk Initiation was conducted once in a session.

Alley Turn. The alleyway was a black acrylic structure (14 X 3 X 14 cm) that was closed in the back and open in the front. The mouse was placed inside the alleyway facing the back wall. The time (in seconds) for the mouse to execute a 180^0 turn and face the open end of the alleyway was recorded. Alley Turning was conducted once in a session.

Negative Geotaxis. The Negative Geotaxis apparatus consisted of a hinged acrylic sheet (53 X 30 cm) that was covered with a fine black nylon wire mesh (0.10 X 0.10 cm). A restraining wire insures that the maximum incline of the apparatus was 45^0. A mouse was placed on the Geotaxis apparatus so that its head pointed towards the top of the apparatus. The time (in seconds) for the mouse to turn 90^0 and then 180^0 from its starting position was recorded. Negative Geotaxis was recorded once in a session.

Elevated Path (Bridge). For the Elevated path test, a clear acrylic bridge (60 cm in length) was suspended horizontally between two safe platforms at a height of 35.5 cm above a 2.5 cm padded surface. Four different bridges of increasing difficulty were utilized: on day one the bridge had a square shape of 2 cm, on day two the bridge was a 1 cm square, on day three the bridge was circular with a 2 cm diameter and on day four, a round bridge with a diameter of 1 cm was used. The mouse was placed on the left safe platform for a few seconds and then was gently dragged to the center of the bridge. The

time (in sec) for the mouse to return to either the left or the right safe platform, or to fall from the bridge, as well as any time spent not moving ("resting") was recorded. The Elevated Path test consisted of three trials in a session.

Wire Suspension. A horizontal steel wire (0.2 X 70 cm) was suspended 33 cm above a 2.5 cm foam pad. A mouse was suspended by its front paws and the time (in seconds) for it to return at least one hind paw to the wire, as well as the time required for it to fall from the wire was recorded. The Wire Suspension test consisted of two trials in a session. Each trial was separated by an Elevated Path test trial.

Startle Response Test.

The Startle Response Test measured the unconditioned, reflexive response of mice to shock stimuli. The startle response apparatus consisted of a startle chamber, an isolation cabinet and the control unit. The startle chamber was an acrylic tube (13.0 X 4.0 cm diameter) attached to an acrylic plate (20.0 X 13.0 X 5.0 cm) covering an electromagnetic force transducer. The transducer converted animal movements into data including a starting baseline, reaction time, maximum response amplitude and average response amplitude. The startle chamber was fitted with a shock grid consisting of 7 stainless steel bars (0.1 cm diameter) separated by 0.5 cm on center, designed to deliver a series of electric shocks. The shock grid and the startle chamber were housed in an isolation cabinet (39.0 X 38 X 59 cm) to minimize the confounding effects of extraneous room noise and vibrations. Computer software controlled the presentation of stimuli and collected data about animal responses to stimuli. Nine shock intensities (0.0, 0.2, 0.4,

0.8, 0.12, 0.16, 0.24, 0.32, and 0.64 mA) were presented randomly, in 5 independent series for a total of 45 trials. Each trial consisted of a 100 millisecond presentation of shock separated by a 30 second ITI between each presentation. Peak responses to all shock intensities and the latency to startle at .64 mA were the parameters of interest.

Assessment of cognitive capacities

Spatial Swim Maze Task.

A 1.12 m (diameter) by 60 cm (height) steel tank was filled with water made opaque with powdered white paint and maintained at 24^0 C. The water level was sufficient to cover a 10 by 10 cm square platform by 1 cm. A computerized tracking system (San Diego Instruments, San Diego, CA) was used to record data. Swim maze testing divided into 4 phases: pre-training , acquisition, retention, and reversal.

A *Pre-training phase* was conducted first, in which mice were trained with a straight alley apparatus to acquaint them with swimming and climbing onto a hidden platform. The straight alley apparatus consisted of 2 transparent acrylic sheets which guided the mice to the hidden platform. Mice were prevented from using visible cues in the room by use of a black curtain around the tank. Mice were given 5 consecutive trials in which to find the platform at the end of the straight alley after being placed at the opposite end. After finding the platform, the mice were allowed to remain there for 10 seconds before being removed to a holding cage for an intertrial interval (ITI) of 5 minutes until the next trial. The pre-training phase consisted of 4 sessions, 2 per day, separated by at least 3

hours. Sessions 1 and 2 were administered on the 1st day of testing, sessions 3 and 4 were administered on the 2nd day of testing after an interval of 60 hours.

The *place discrimination acquisition phase* followed the straight alley pre-training phase. In this phase the mice were tested for their ability to locate a hidden platform based on external cues in the room. Over a four day acquisition period, mice were required to locate a hidden platform in a fixed position five separate times starting from four different starting points. The acquisition phase consisted of 8 sessions administered 2 per day separated by at least 3 hours. The path length and swim speed as recorded by the tracking system were the performance measures of interest. The *learning index* is an average of certain sessions in the water maze task. The average path length of each group of mice was determined for sessions 2, 3, and 4 (during acquisition phase) and sessions 12, 13, 14 (during reversal phase) to determine how quickly each group was able to learn the water maze. At the end of session 8 a *probe trial* was performed. On the probe trial, the platform was lowered well below the surface of the water at the start of the trial and the mice were allowed to search for the platform for 30 seconds. After 30 seconds the platform was raised to its usual height of 1 cm below the surface of the water and the mice were allowed an additional 60 seconds to locate it. The performance measure was the amount of time that the mouse spent in the target quadrant (quadrant containing the platform), the 40 cm diameter annulus and the 20 cm diameter annulus surrounding the target, as well as the number of times that the mouse swam directly over the target. Time spent in the various zones represents the strength and the accuracy of spatial memory.

Retention phase. The acquisition phase of the spatial swim maze task test was followed by the retention phase. The retention phase consisted of sessions 9 and 10 administered on the same day, separated by at least 3 hours. In this phase, mice were tested to determine if they were able to remember the location of the hidden platform after 72 hours had elapsed since their last acquisition test session.

Reversal phase. Following the retention phase, during a 2 day reversal phase, 4 additional sessions (sessions 11-14) were used to test the mice for their ability to find the hidden platform in a new location. During the reversal phase, the hidden platform was moved to the opposite side of the tank, so that mice were required to unlearn the previous position of the platform and learn the new position. Path length and swim speed were used as performance measures. Additionally data for the 1st trial of session 11 were considered as a probe to determine the degree bias for the previous platform position.

Data Analysis

Statistical analysis was done by analysis of variance and individual comparisons using Systat Version 7.0 statistical software package. 3-way factorial analyses of variance were used to evaluate group differences with age, treatment (exercise), and session as between groups factors for each behavioral test if appropriate. Planned individual comparisons between age matched trained versus control groups, and between training-matched young versus aged groups were performed by individual F tests using the error term from the overall analysis.

3. Results

Body Weight

The body weights of the 4 groups of mice in the behavior study were recorded each week during the entire 11-week exercise training protocol (Fig. 1). Analysis by 3-way ANOVA with age, treatment, and weeks as the factors showed that there was a significant main effect of age, with the aged mice weighing more than the young throughout the 11-week period (both $p < .050$). The main effect of training was not significant, as were the two and three-way interactions confirming the observation that exercise training had little effect on the weight of either the young or the aged mice ($p = .643$).

Citrate Synthase

Individual comparisons of the citrate synthase results (Fig. 2) showed that the activity of citrate synthase in the *m. rectus femoris* was significantly increased in both the 3-month-old and the 20-month-old exercise-trained mice as compared to the age-matched non-trained controls ($p < .050$). Although there was more citrate synthase activity in the aged versus the young groups, this difference was not significant for either the control or the trained groups (*both* $p > .200$). The magnitude of the increase of citrate synthase activity in young trained versus non-trained controls was 1.55 fold, and the magnitude of the increase in aged exercised versus non-exercised control mice was nearly identical at 1.59 fold.

Spontaneous Locomotor Activity

Spontaneous locomotor activity was analyzed by two-way ANOVA with age and treatment as the factors, separate ANOVAs were done on the 1st and 2nd LMA sessions. In the 1st session of locomotor activity (Fig. 3.), there were no significant effects of either age, exercise training, or their interaction on forward movement in C57BL/6 mice (*all* $p > .100$). In the 2nd session, exercise training had no effect on forward movement ($p = .592$), but there was a significant main effect of age ($p < .050$). Planned individual comparisons done within the age by training interaction revealed that aged control mice displayed significantly less horizontal movement than the young controls ($p < .050$), whereas aged, trained mice did not differ from young, trained mice.

A similar analysis of the vertical activity of mice did not indicate any effect of age, training, or their interaction (all $p > .400$).

Walking Initiation, Alley Turning, Negative Geotaxis

Performance on the walking initiation, alley turning, and the two negative geotaxis tasks (90^0 and 180^0 turn) (Fig. 4.) were all analyzed by 2-way ANOVA with age and training as the factors. In the walking initiation task there was a main effect of treatment ($p < .050$). Planned individual comparisons revealed that this was the result of trained mice initiating walking movements significantly later than non-trained control mice. In the alley turning task there was a main effect of treatment ($p < .050$). There were no training by age interactions in either of these tasks. Planned individual comparisons between age-matched treatment groups indicated that the effect of training existed in the

young mice only, with young exercised mice taking longer to complete the alley turning task than the control mice. There was no main effect of age for either the walking initiation or the alley turning task (both $p > .800$). Analysis of the performance of the mice on the 90^0 negative geotaxis and the 180^0 negative geotaxis tasks revealed that there were no significant main effects of either age or training on these tasks ($p > .300$)

Elevated Path (Bridge)

For the bridge walking (elevated platform) test (Fig. 5.), analysis by 2-way ANOVA revealed a significant main effect of age as well as a main effect of exercise training on the ability of the mice to remain balanced on the bridges (*both* $p < .050$). These outcomes were the result of significantly reduced ability of aged mice to remain balanced on the bridges as compared to young mice, and the greater ability of aged, trained mice to remain on the bridge significantly longer than age-matched, non-trained control mice. These effects were confirmed by a planned individual comparison within the age by treatment interaction ($p < .050$).

Wire Suspension

The test for the ability of the mice to grasp a wire with their hind legs (latency to tread) and their ability to remain on the wire (latency to fall), was analyzed by two-way ANOVA that revealed that there was a significant main effect of age on both tasks (Fig. 6.)($p < .050$). Planned individual comparisons within the age by treatment interaction showed that aged mice in both treatment groups took significantly longer to tread than the

young controls ($p < .050$). Aged mice in both training groups also showed significantly less ability to remain on the wire than the young control mice ($p < .050$). However, there was no main effect of exercise training on either of the two tasks (*both* $p > .400$).

Startle

Analysis of the response to shock stimulation failed to reveal significant two or three-way interactions of age, treatment, and shock intensity but instead indicated a main effect of age. This outcome was primarily a reflection of age differences at the higher intensities (.24, .32, and .64 mA) of shock (Fig. 7) ($p > .100$). Planned individual comparisons revealed that the young non-trained control mice exhibited significantly more startle response to the shock stimulus at .24, .32, and .64 mA than did the young trained mice or either of the aged groups ($p < .050$). Planned individual comparisons revealed that none of the other 3 groups (young exercised, aged control, or aged exercised) had significantly different responses from each other to any of the shock stimuli (all $p > .100$).

Latency of response to the shock stimulation was considered at .64 mA of shock stimulus by two-way ANOVA, and disclosed a main effect of age (Fig 8). Planned individual comparisons revealed that the aged control group was significantly slower to respond to the shock stimulus than young control mice ($p< .050$). These analyses also showed that exercise training in aged mice resulted in significantly shorter startle latency compared with the non-trained controls ($p< .050$)

Spatial Swim Maze Task

The ability of the mice to learn a spatial discrimination task was determined by measuring the path length for mice to find the hidden platform as a function of sessions in the spatial swim maze test (Fig. 9, top). Both the young and the aged groups were able to locate the platform relatively quickly by the 8th session of the acquisition phase of the test. Evaluation by 3-way ANOVA with age, treatment and sessions as the factors showed that there was a significant overall effect of age on the path length required by different groups of mice to find the platform, with the young mice requiring a shorter path length to find the platform than aged mice ($p < .050$). The same type of analysis showed that there was a main effect of training during the acquisition phase ($p < .050$). Planned individual comparisons within the treatment by sessions interaction indicated that during the 4th and 6th session of acquisition, the aged, exercised mice had significantly shorter path lengths than their aged non-trained counterparts.

In the retention phase of spatial swim maze testing, a three-way analysis of variance showed that again there was a significant main effect of age ($p < .05$) on the ability of the mice to remember the location of the hidden platform after 66 hours had elapsed since the final trial of the acquisition phase of the test. This effect was due to young mice exhibiting significantly shorter path lengths to the platform. For this phase of the test, there was no main effect of treatment on performance in either age group ($p = .178$).

During the final phase of spatial swim maze testing (the reversal phase) a three-way analysis of variance showed that there was not a significant main effect of age nor was there a significant main effect of exercise training on the ability of the mice to learn a

new location of the hidden platform (both $p > .100$). There was no age by training interaction for path length in any of the 3 phases of the spatial swim task.

In each of the 3 phases of spatial swim maze testing, the speed of the mice was analyzed by three-way ANOVA (Fig. 9, bottom). The only significant differences observed were exhibited by the young mice during the reversal phase, in which young control mice showed a significant age by treatment effect ($p < .050$). Planned individual comparisons revealed that this was due to the young, non-exercised control group swimming quite slowly during the 1st two sessions of the reversal testing.

The learning index for water maze testing is shown in Fig. 10. Analysis by two-way ANOVA showed a significant effect of age on the ability of mice to learn the maze, with aged mice requiring a longer path length to find the platform ($p < .050$). Although there was not an effect of training, there was a trend for the exercise-trained mice to learn the location of the platform more quickly than the control mice ($p = .057$).

The strength and accuracy for spatial memory was assessed with a probe trial (Fig. 11). Separate two-way ANOVAs (with age and training as the factors) conducted on time spent in the 20-cm and 40-cm annulus, as well as the number of target entries, each revealed significant main effects of age ($p < .050$). This outcome reflected the generally shorter times and fewer entries of the aged when compared with young mice. Planned individual comparisons between treatment-matched age groups, conducted within the age by training interaction indicated that this decrease was significant for both trained and control groups for the number of target entries. The age difference was significant for both trained and control groups for the number of target entries. However, the age

difference was significant only for trained mice ($p < .050$) for the 40- and 20-cm annuli. A two-way analysis for time spent in the target quadrant failed to indicate a significant main effect of age. Exercise training did not affect the time spent in the target zone, annuli, or the number of target entries (main effect, interaction, all $p > .700$).

Session 11 of the water maze task was the first session of the reversal phase of the test. The number of entries into the previous location of the platform (its location during the acquisition phase) was analyzed with data acquired during session 11 (Fig. 12). Analysis by two-way ANOVA with age and treatment as factors indicated main effects of age and treatment ($p < .050$). Planned individual comparisons revealed that aged, control mice spent significantly less time in the target quadrant, 40 cm annulus, and 20 cm annulus, and they made significantly fewer target entries than did the young mice ($p < .050$). Planned individual comparisons showed that aged, trained mice spent more time in the target quadrant and 20 cm annulus, and made more target entries than non-trained control mice ($p < 0.50$). There was a strong trend for the aged, trained mice to spend more time in the 40 cm annulus compared to young controls ($p = .058$).

4. Discussion

The main findings of this study are that when moderate, short-term exercise training is initiated in aged C57BL/6 mice, it (a) results in increased fitness in the aged mice to the same degree as observed in young mice, (b) improves some psychomotor skills, including bridge-walking and reaction time, and (c) improves age-impaired spatial memory performance.

Most investigations of the effect of exercise training on cognitive deficits do not address the twin issues of (1) are the animals exercised to a significant level of fitness over the non-exercised controls? and (2) is the exercise training discontinued during extensive behavioral testing – resulting in behavioral data that is obtained from animals that are no longer benefiting from exercise? In this study the exercise training protocol was designed to result in a significant increase in fitness in both the young and the aged groups, and the exercise training regimen was continued throughout the behavioral testing. Fitness in the two groups of mice was evaluated with a citrate synthase assay that showed significant and nearly equal magnitudes of increased fitness in both the young and the aged groups, establishing that a significant and similar level of fitness was established in the young and aged mice in this study. The question of biological relevance of the increase in citrate synthase activity observed in this study should be addressed. The magnitudes of increases that were observed in this study were as great or greater than of others work in which citrate synthase activity was taken to be a measure of fitness (10, 11). In the current study, the exercise training protocol was kept in place during the entire 3-week period of behavior testing to ensure that the fitness achieved by exercised mice did not decline during behavior testing. Under these conditions, it cannot be determined if the beneficial effects of exercise training on cognitive or psychomotor function are the result of long-lasting improvements in brain, cardiovascular or muscoloskeletal function or if the benefits derive from acute effects of exercise. To minimize likelihood of the latter, the exercise training was administered immediately after behavior testing to ensure that each behavior test occurred at least 18 hours after the

exercise. Nevertheless, it will be necessary to assess the time-dependent effects of exercise training in order to fully understand the mechanism for improved cognitive and psychomotor performance.

Mice in the control groups of this study clearly demonstrated age-related declines in cognitive and psychomotor function in accordance with several previous studies, including those done in our laboratory (16, 17, 18, 19). However, the 11-week moderate exercise training protocol administered to the mice in this study tended to improve performance in some, but not all of the age-sensitive psychomotor and cognitive tests that were administered during the final 3 weeks of exercise. Exercise training reduced age-related deficits in bridge walking, startle latency, and it produced an improvement in age-related inability to learn and retain a spatial discrimination

The motor activity test is designed to study exploratory behavior in animals. The test measures a range of behaviors displayed by animals placed in a novel environment (20). It was expected that aged animals would not exhibit as much locomotor activity as young animals, as locomotor activity is a biomarker of aging. The motor activity of aged control mice was reduced compared to young mice, significantly so in the 2^{nd} session and there was a trend for this effect in the 1^{st} session as well.

Some of the motor skills tests did not reveal an age effect – walking initiation, alley turning and both of the negative geotaxis tasks. However, all of these tasks are simple tests of motor competence and reflexive behavior (21), and the lack of an age effect in these tests most likely indicates that the aged mice did not exhibit gross motor or reflexive deficits. In two of the tests (walking initiation and alley turning), there was a

significant effect of exercise, with trained animals requiring more time to complete the test than their non-trained controls. These data support the results of the startle test. In the startle test, trained animals had reduced responses to shock stimulation; this parallels the effect seen in walking initiation and alley turning. As with the startle response, these results could be due to the anxiolytic effect of exercise training (24). Examination of the amount of time spent in the center of the locomotor activity chamber indicated that trained mice spent more time in the center of the boxes than did the non-trained controls (data not shown), which supports the hypothesis that exercise training is anxiolytic for C57BL/6 mice. Exercise training resulted in no significant changes in the ability of the animals to turn either 90^0 or 180^0 in the negative geotaxis tasks. The negative geotaxis task does not depend on somatosensory input. The stimulus to turn during the negative geotaxis test is stimulation of the labyrinth organs of the inner ear. No effect of age reveals that at 20 months the mice are experiencing no deficits in their labyrinth organs and exercise training does not exert modulating influence on these organs.

The elevated path test is used to access motor coordination, muscular strength and balance (20, 21). The performance of the mice was significantly affected by both age and training with the aged groups unable to remain on the bridge as long as the young groups and the aged, trained groups displaying significantly improved performance over the age-matched, non-exercised controls. Balance and muscle coordination are generally considered to be under cerebellar control, thus improvement by exercise training on this motor skills task suggests that exercise training could ameliorate cerebellar deficits that occur with aging (22,23). Interestingly, although the cerebellum processes input from the

motor cortex, brainstem nuclei and sensory modalities to provide output to skeletal muscles that will result in smooth coordinated movements, more recently its function appears to include contribution to cognitive functions including spatial learning (23).

Aged control mice had longer latencies to tread and shorter latencies to fall on the wire suspension test, revealing deficits in muscular strength and coordination (both well-established biomarkers of aging) when compared to young mice. Exercise training had no effect in the performance of this task in either the young or the aged animals. Since the ability of the animal to perform the wire suspension task is dependent on digital strength (grasping) of both the front and hind limbs, it is a reasonable finding that running on a treadmill did not have a profound effect on performance of this task.

There was an age effect as well as a training effect on the response to shock stimulation with young, non-exercised mice responding significantly more vigorously to shock stimulation of .24, .32 and .64 mA. than any of the other groups. The mice in the exercised groups and the aged non-trained controls had lower and very similar responses to electrical stimulation. The significantly lower responses of the aged non-trained mice compared to the young non-trained mice is the expected result in this test. Apparently exercise training reduced the startle response of the young, exercised mice so that it was not significantly different from the response of the aged, exercised mice. The reduced responses of the young, exercised mice to shock stimulation to levels displayed by the both aged controls suggests that exercise training has an aging effect on mice, but this response might be due to the anxiolytic properties of exercise training (24). As

mentioned earlier, the results of startle test and the results of walking initiation and alley turning support each other.

The results of the latency to startle showed a clear age difference with aged control animals responding significantly more slowly to shock stimulation. As observed in previous studies (31), an age-associated increase in reaction time, measured as the latency to peak startle response following maximally effective shock stimuli (0.64 mA), resulted in improved performance (decreased reaction time) in aged mice. This aspect of shock startle performance reflects the time required for both neural processing and musculoskeletal implementation of the startle reflex. Thus, the improved reaction time of aged, exercised mice could involve either of these aspects or both of them.

The ability of mice in this study to learn a spatial discrimination task was determined by their performance on the spatial swim maze task. Performance on the spatial swim maze test is known to depend on function of the hippocampus as well as it's cortical inputs (26). Young mice clearly showed better ability to learn the location of a hidden platform using visual room cues as compared to aged mice during the acquisition phase of the spatial swim task. The young mice retained this superiority over aged mice during the retention phase of the test. During the reversal phase of water maze testing, the mice were required to unlearn the location of the platform during the acquisition phase and to learn a new location of the platform. During the reversal phase, young mice did not perform significantly better than the old mice. This phenomenon is likely due to the greater ability of young mice to remember the previous location of the platform as

compared to aged mice, rather than a disability to "unlearn" the original platform location (27).

The effect of exercise training on aging mice becomes especially interesting when the ability of mice to retain acquisition of recently learned spatial discrimination is examined with the probe trial and by analyzing the behavior of trained mice on the 1st day of reversal testing as if the target was still its original location. In the probe trial, there was a significant effect of age for the exercised mice only. Aged, exercise-trained mice spent significantly less time in the 40 cm and 20 cm annuli, and made significantly fewer target entries than young trained mice, but this effect of age was not observed in non-trained mice, suggesting that aged, exercise-trained mice did not retain the knowledge of platform location immediately after acquisition. However, data from the 11th session (the 1st session of reversal) was analyzed with a template of the acquisition maze to determine how often mice returned to the original platform location during their search for the new platform location. The young mice showed a marked preference to search for the platform in its original location, displaying strong and accurate memory for its position after a long delay between sessions. The aged non-exercise-trained mice spend significantly less time in each of the 4 target areas, revealing their lack of bias for the original platform location. The aged, trained mice resembled the young mice in their preference for the original platform location, suggesting that exercise training may improve their spatial memory. Apparently this effect does not exist immediately after acquisition, but after a 24 hour interval between training sessions, the effect becomes readily apparent. In contrast to the aged, trained mice, the aged control mice did not

forget the platform location immediately after acquisition, but were not able to retain their memory of its location after a prolonged interval. The ability to remember the original platform location is likely due to differences in consolidation by aged non-trained versus aged, trained mice. The hippocampus serves as a temporary site where information in working memory is held until it can be consolidated into a more permanent form in cortical storage site (28, 29). Previous work has established that the period of consolidation varies in relation to the size of the brain. Consolidation has been reported to occur in rodents rather quickly – possibly in a matter of hours (29), which could explain the differential memory displayed by the two groups of aged mice in the probe trial administered immediately after acquisition and the probe trial administered 24 hours later. Evidently aged, exercised mice (and both groups of young mice) have mechanisms (perhaps cellular (30)) that allow for consolidation, and these mechanisms are lost in the aged, non-exercise-trained mice.

The current results suggest that exercise training regimens, initiated late in life, may produce long-lasting beneficial effects on age-impaired brain functions that result in improved cognitive and pscyhomotor function of aged mice. While the results do not completely rule out the possibility that improved performance of the old mice involves enhanced musculoskeletal function, several results do not support that interpretation. Whereas the spatial swim maze task involves some physical challenge for its successful performance, the measurements related to cognitive performance, path length and spatial bias (probe trial), are independent of physical ability. Moreover, no effect of exercise training was observed on pure measures of physical performance such as swim speed.

Similarly, the bridge-walking test of balance was designed to have minimal requirements of strength and endurance and was affected by exercise, whereas the more physically challenging tests of wire grip and treading did not differentiate aged, exercise-trained and control groups. In light of this pattern of results, it seems unlikely that improved musculoskeletal function could fully account for the apparent improvements in cognitive and psychomotor function.

To summarize, exercise training appears to have beneficial effects in the acquisition of knowledge in aged mice in some but not all psychomotor and cognitive tasks and exercise training may have differential beneficial effects on various brain areas. The mechanism by which exercise training imparts its beneficial effects is an area that requires further investigation.

FIG. 1. Effect of exercise training on body weight of C57BL/6 mice. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from young mice.

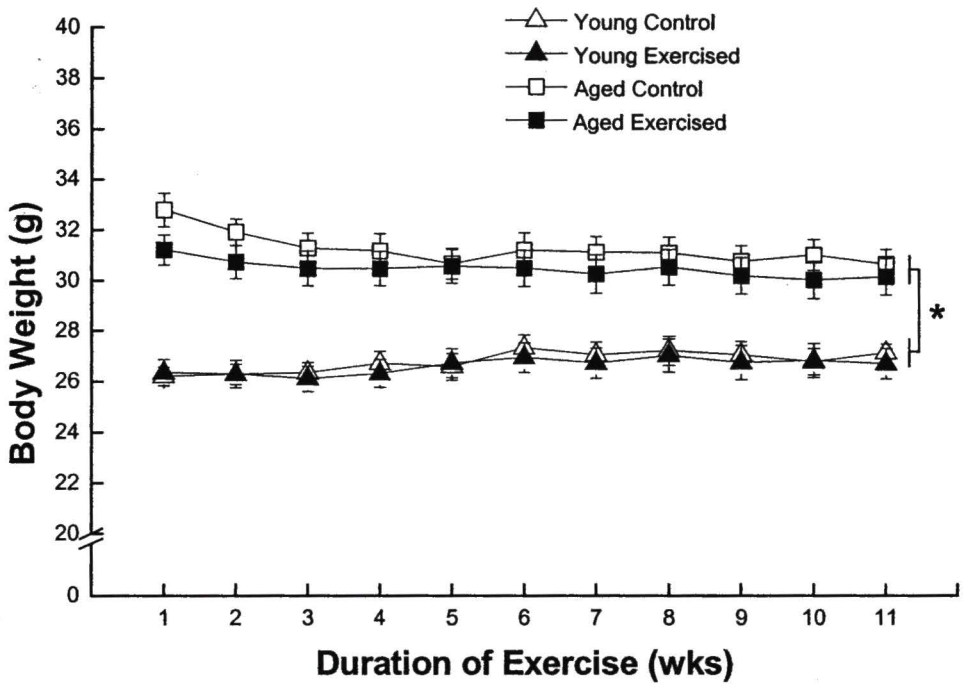

Young Control
Young Exercised
Aged Control
Aged Exercised
Body Weight (g)
40
38
36
34
32
30
28
26
24
22
20
0
1
2
3
4
5
6
7
8
9
10
11
Duration of Exercise (wks)
*

FIG. 2. Effect of exercise training on citrate synthase activity in the rectus femoris muscle in young and old mice. Each value represents the mean ± SE of 6-7 mice. * = significant ($p < .050$) difference from non-exercised control.

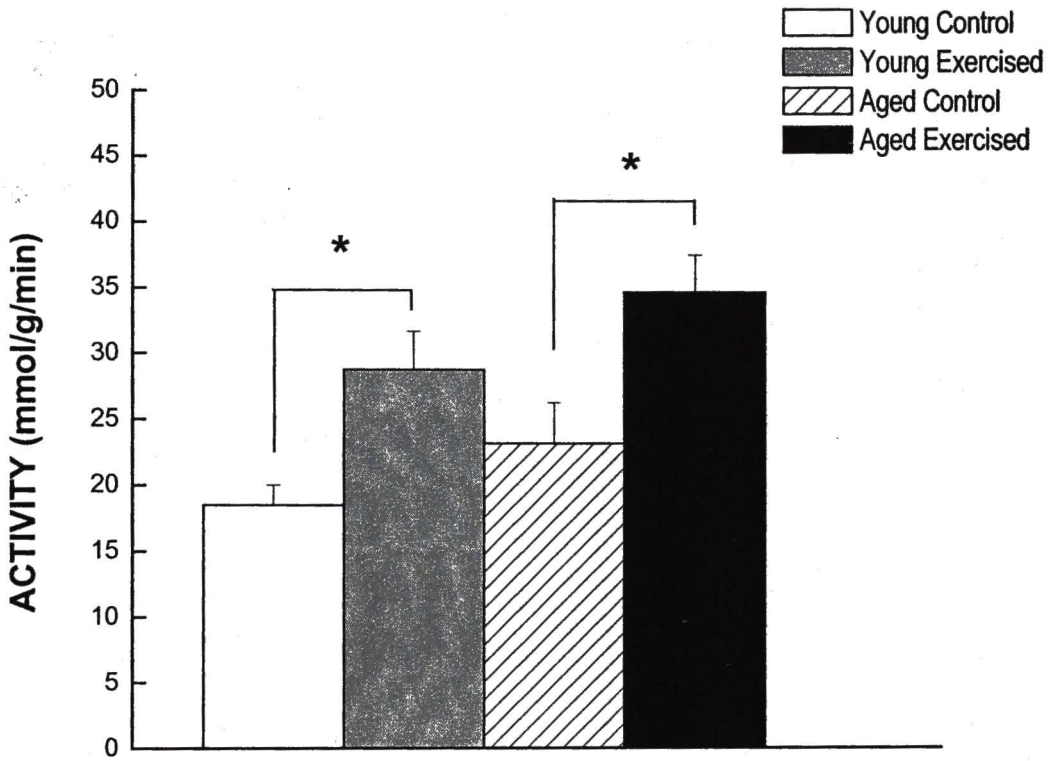

Young Control
Young Exercised
Aged Control
Aged Exercised
ACTIVITY (mmol/g/min)
50
45
40
35
30
25
20
15
10
5
0
*
*

FIG. 3. The effect of exercise training on spontaneous locomotor activity in young and aged C57BL/6 mice. Top: Mean forward movement of mice in sessions 1 and 2 measured in cm ± SE. Bottom: Rearing behavior in sessions 1 and 2 measured by interruptions in infrared photobeams (counts) ± SE in activity chamber. Each value represents the mean (±) SE of 17-21 mice. * = significant ($p < .050$) difference from young control.

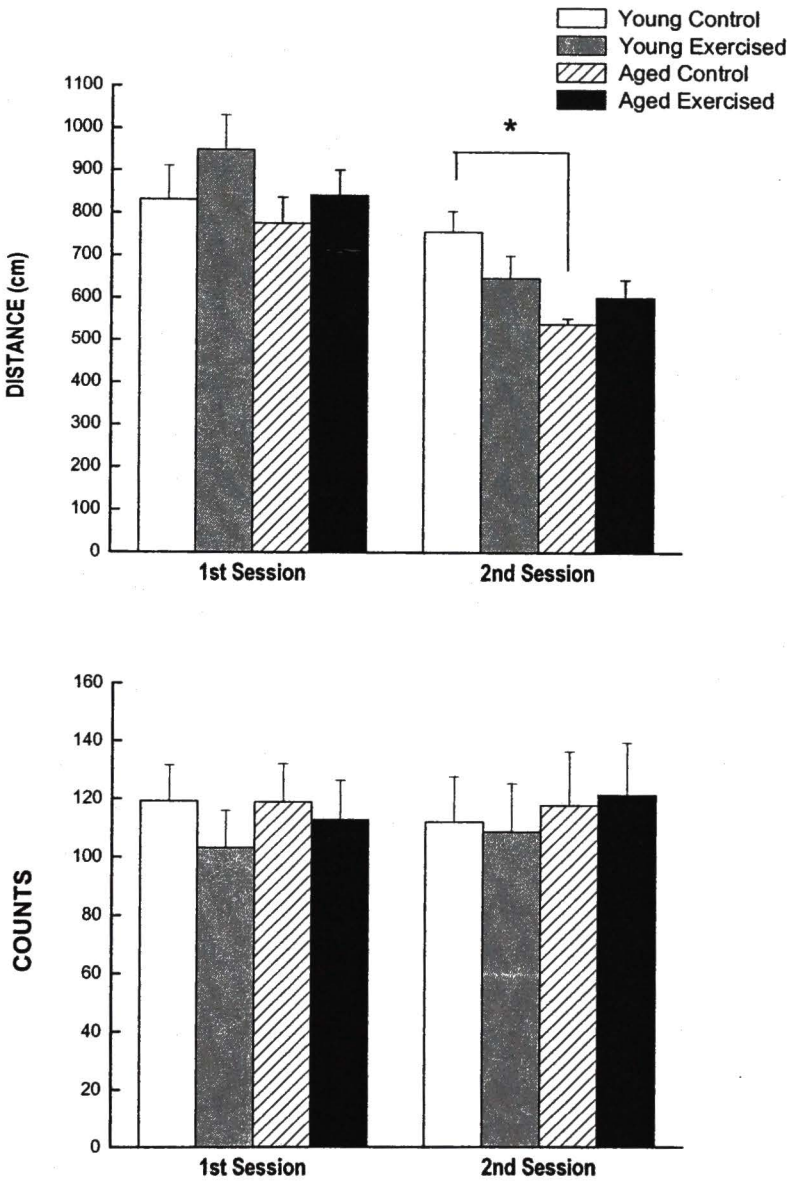

Young Control
Young Exercised
Aged Control
Aged Exercised
DISTANCE (cm)
1100
1000
900
800
700
600
500
400
300
200
100
0
*
1st Session
2nd Session
COUNTS
160
140
120
100
80
60
40
20
0
1st Session
2nd Session

FIG. 4. Performance by C57BL/6 mice on motor skills tasks as a function of age and exercise. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from age-matched control.

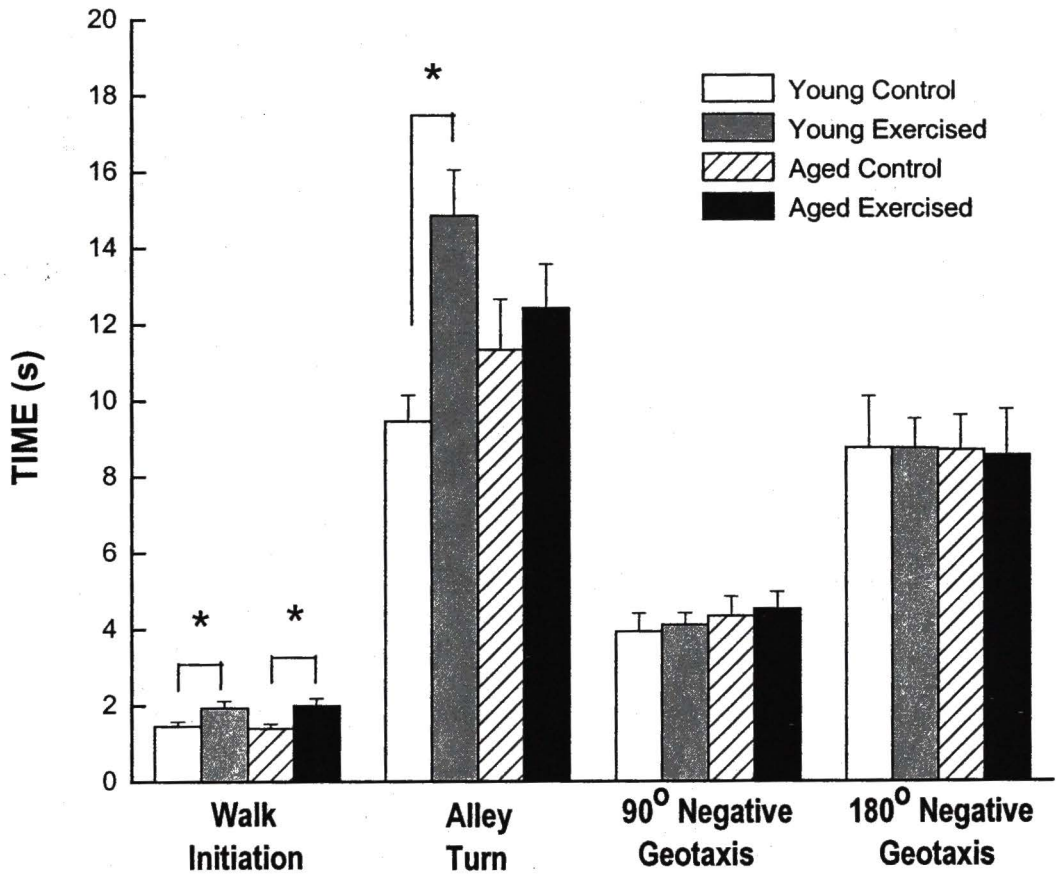

TIME (s)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
Young Control
Young Exercised
Aged Control
Aged Exercised
Walk Initiation
Alley Turn
90° Negative Geotaxis
180° Negative Geotaxis

FIG. 5. The effect of exercise training on bridge walking in C57BL/6 mice as a function of age. Columns represent average latency to fall among the 4 groups of mice. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from young mice. † = significant ($p < .050$) difference from non-exercised control.

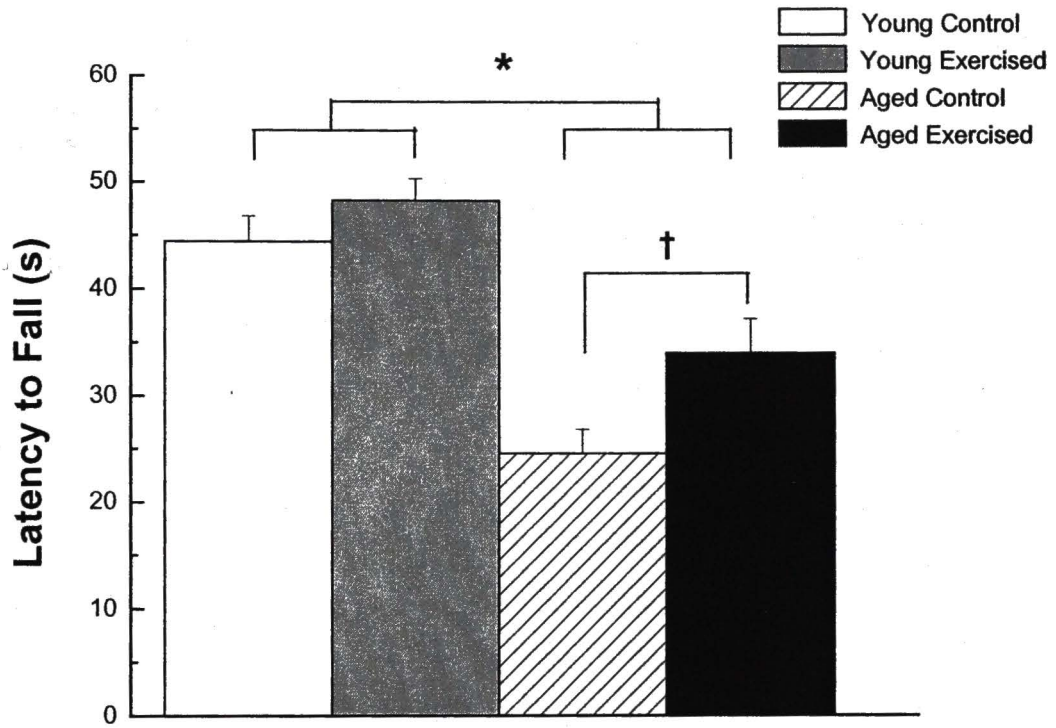

Young Control
Young Exercised
Aged Control
Aged Exercised
*
†
Latency to Fall (s)
60
50
40
30
20
10
0

FIG. 6. Performance on wire suspension as a function of age and exercise. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from age-matched control.

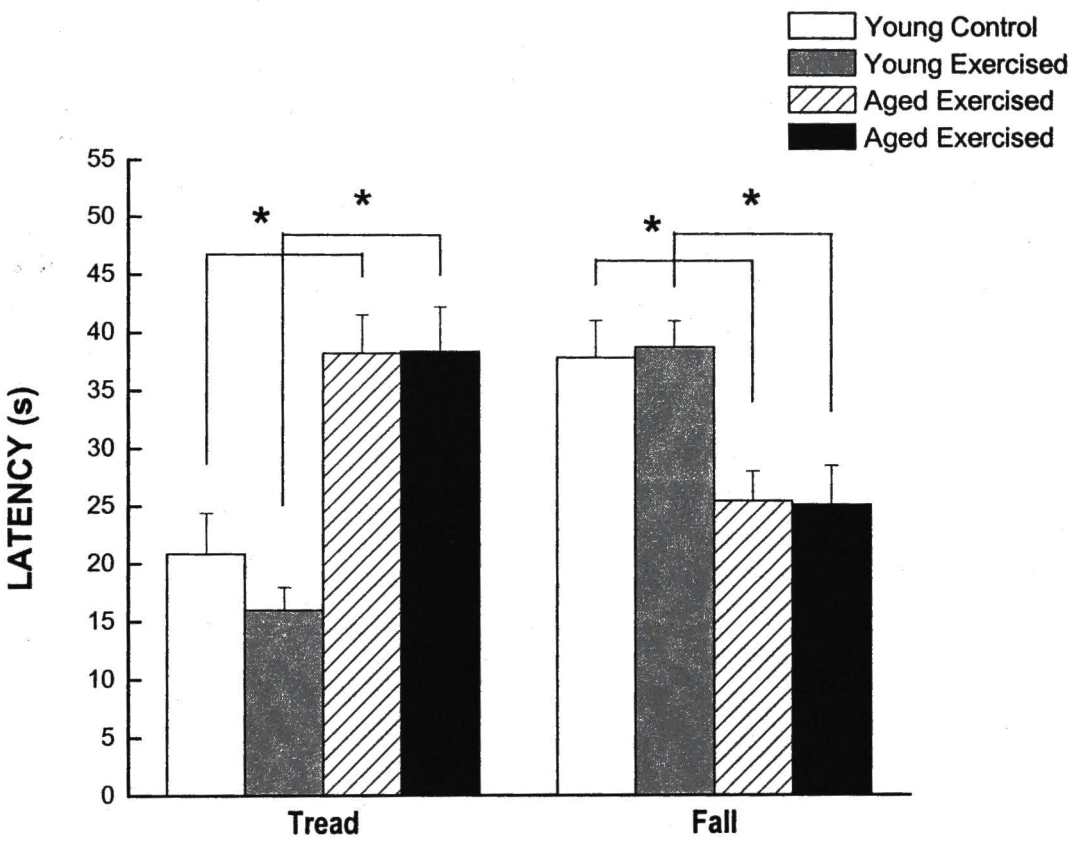

Young Control
Young Exercised
Aged Exercised
Aged Exercised
LATENCY (s)
55
50
45
40
35
30
25
20
15
10
5
0
*
*
*
*
Tread
Fall

FIG. 7. Shock (startle) peak performance by exercised C57BL/6 mice as a function of age Each value represents the mean peak startle response ± SE of 17-21 mice. * = significant ($p < .050$) difference from all other groups.

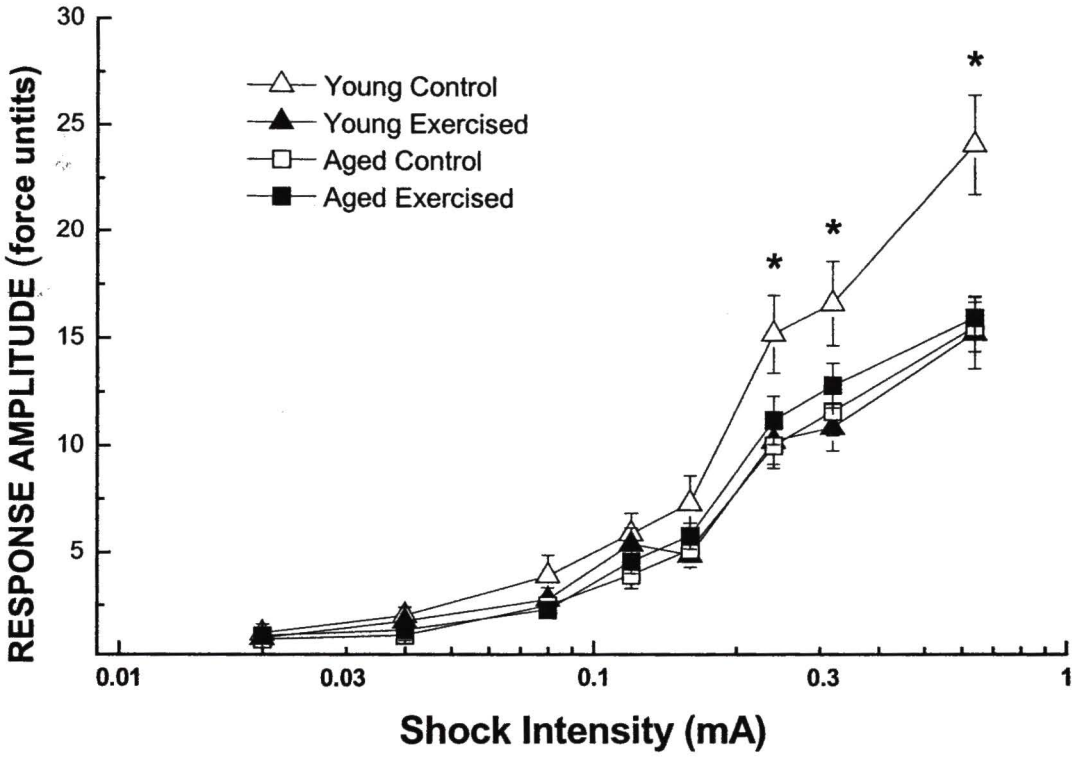

RESPONSE AMPLITUDE (force untits)
30
25
20
15
10
5
Young Control
Young Exercised
Aged Control
Aged Exercised
*
*
*
0.01
0.03
0.1
0.3
1
Shock Intensity (mA)

FIG. 8. Shock (startle) latency performance by exercised C57BL/6 mice as a function of age. Columns represent the mean latency to response at .64 mA ± SE of 17-21 mice. * = significant ($p < .050$) difference from young control. † = significant ($p < .050$) difference from non-exercised control.

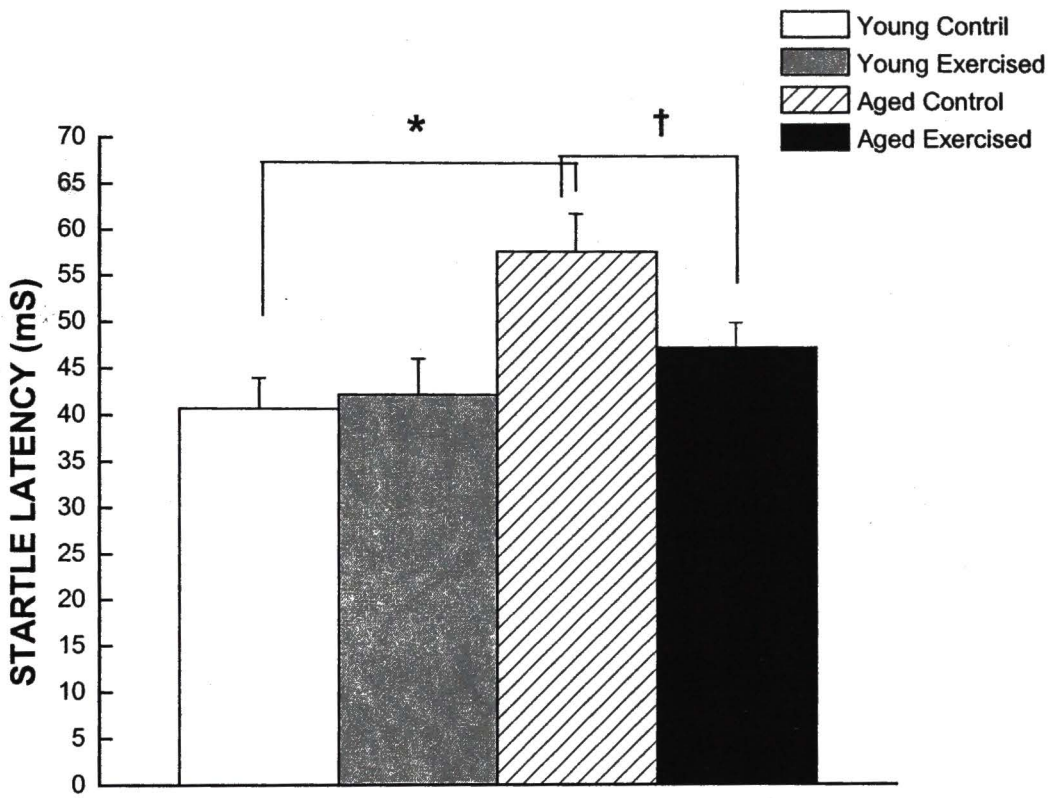

Young Contril
Young Exercised
Aged Control
Aged Exercised
*
†
STARTLE LATENCY (mS)
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0

FIG. 9. Effects of exercise training on swim maze performance as a function of age in C57BL/6 mice. Top: Path length (cm $\pm$ SE). Bottom: Path-independent swim speed (cm/s $\pm$ SE). * = significant ($p < .050$) difference from age-matched control.

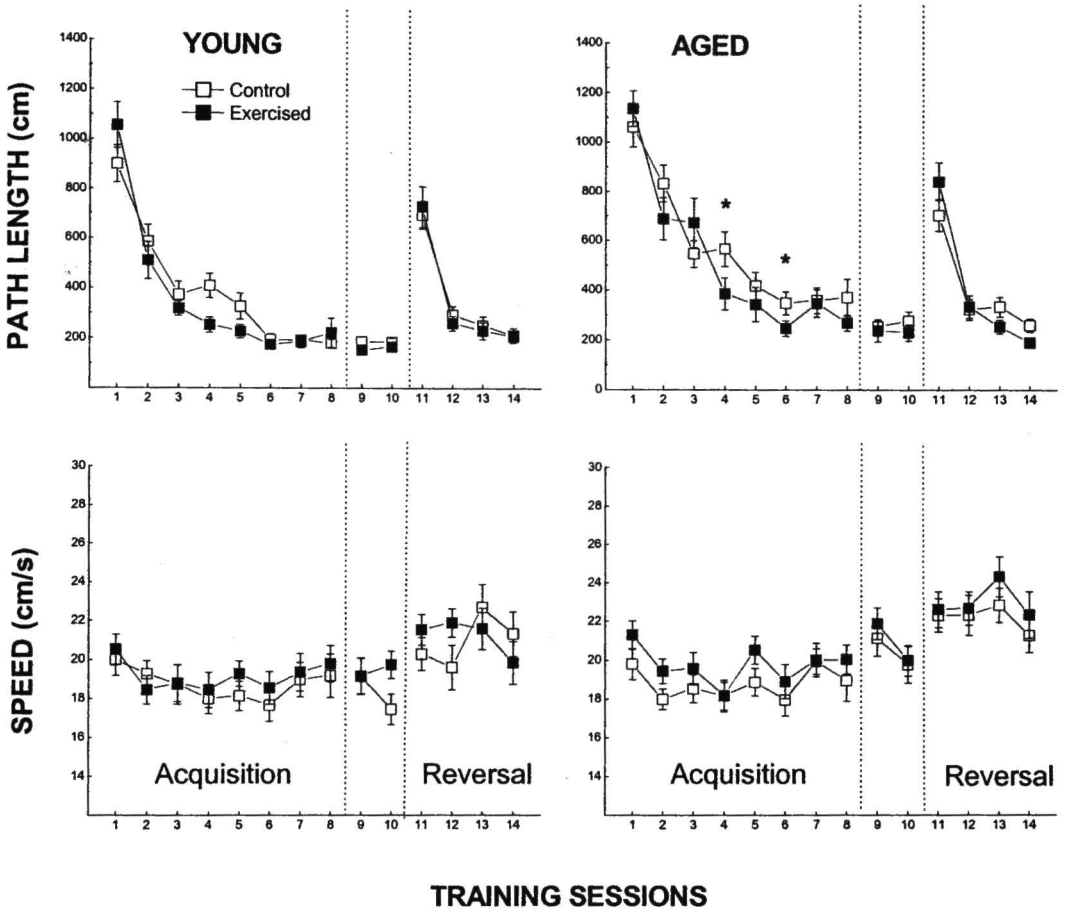

YOUNG
AGED
Control
Exercised
PATH LENGTH (cm)
SPEED (cm/s)
Acquisition
Reversal
Acquisition
Reversal
TRAINING SESSIONS

FIG. 10. Average learning index of the mice over sessions 2-4 of swim maze acquisition and sessions 12-14 of swim maze reversal. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from young group.

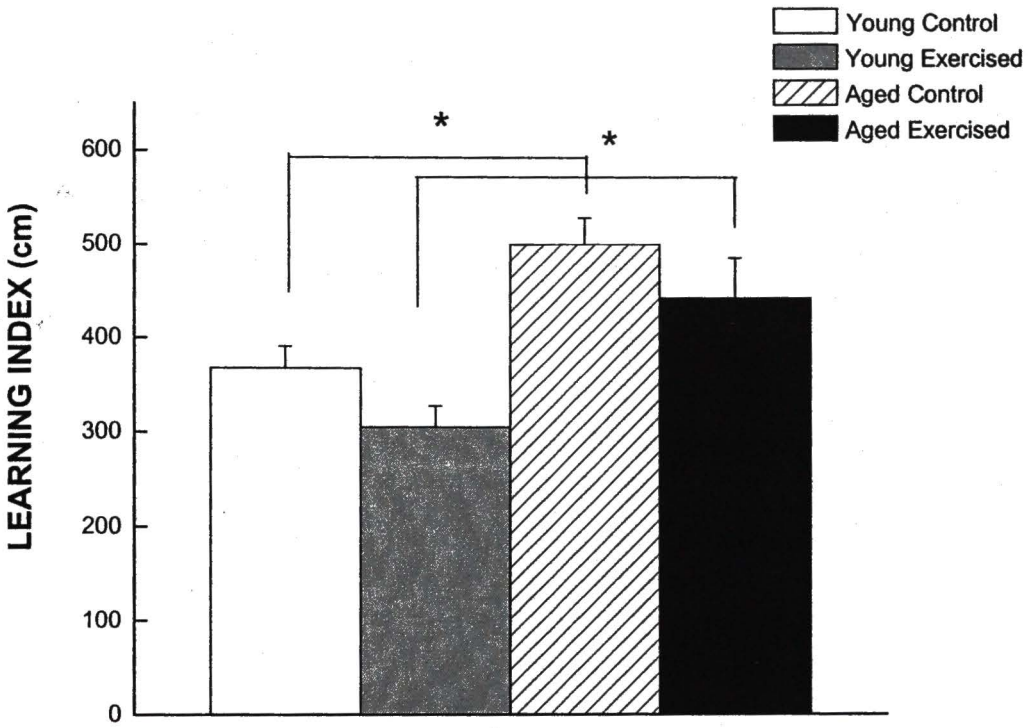

Young Control
Young Exercised
Aged Control
Aged Exercised
LEARNING INDEX (cm)
600
500
400
300
200
100
0
*
*

FIG. 11. Probe trial performance by exercised C57BL/6 mice as a function of age. Left: Columns represent the amount of time spent in a target area. Right: Columns represent number of entries made to target area. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from young group.

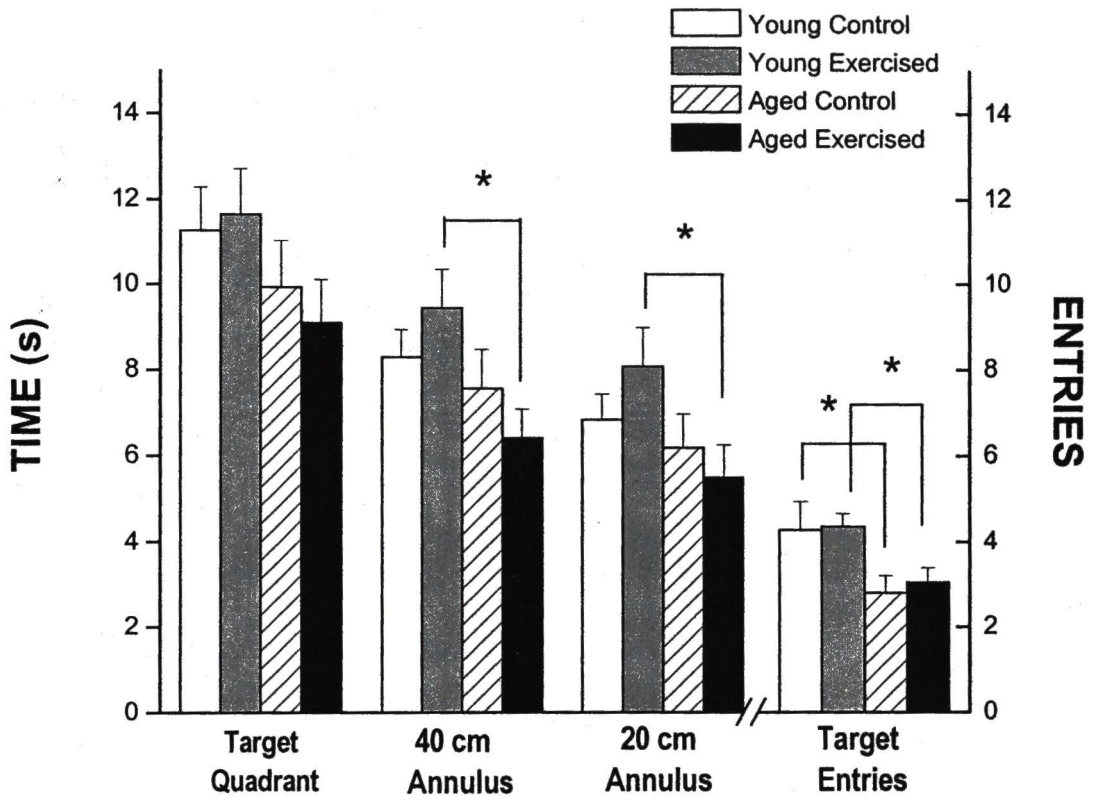
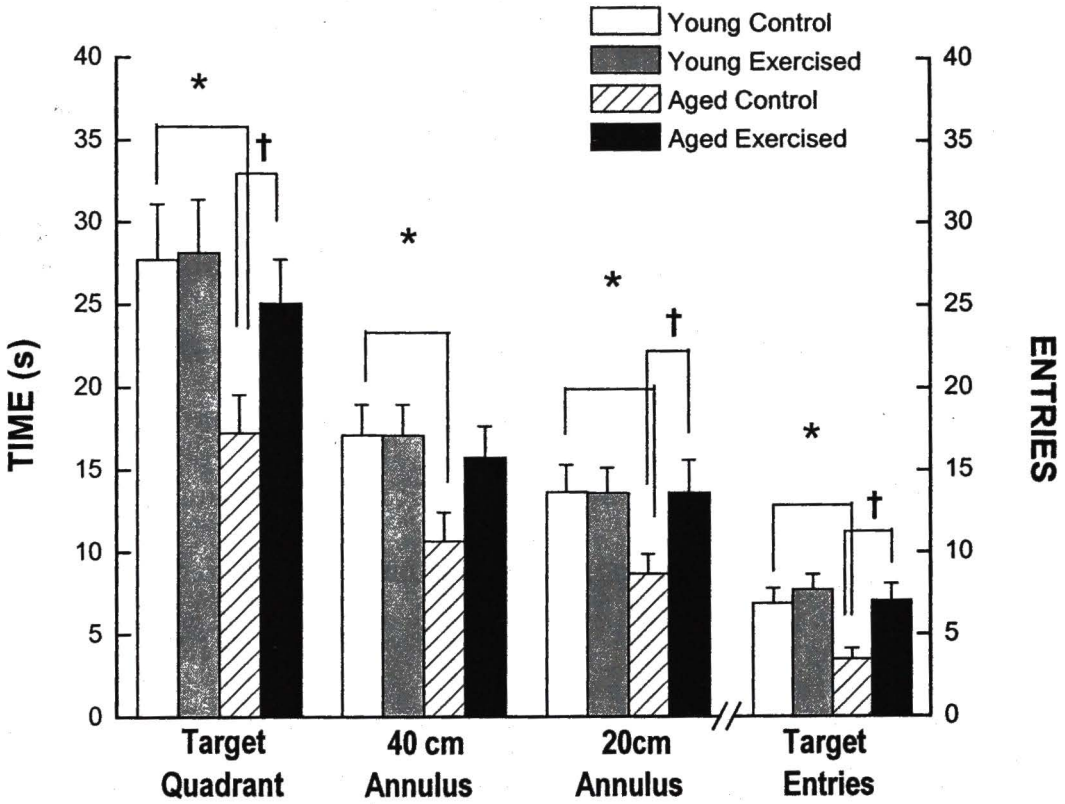

Young Control
Young Exercised
Aged Control
Aged Exercised
TIME (s)
ENTRIES
0
2
4
6
8
10
12
14
*
*
*
*
Target Quadrant
40 cm Annulus
20 cm Annulus
Target Entries

FIG. 12. Trial performance by exercised C57BL/6 mice as a function of age after a 24 hour delay in testing. Left: Columns represent the amount of time spent in a target area. Right: Columns represent number of entries made to target area. Each value represents the mean ± SE of 17-21 mice. * = significant ($p < .050$) difference from young group. † = significant (($p < .050$) difference from non-exercised control.


Young Control
Young Exercised
Aged Control
Aged Exercised
TIME (s)
ENTRIES
0
5
10
15
20
25
30
35
40
*
†
*
*
†
*
†
Target Quadrant
40 cm Annulus
20cm Annulus
Target Entries

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TRANSITION REMARKS

In Chapter II, the ability of exercise training to ameliorate age-associated cognitive deficits was tested in aging C57BL/6 mice. It appears that when a moderate short-term exercise training is initiated in aged C57BL/6 mice, there was a trend for exercise training to improve the acquisition of new psychomotor and cognitive skills in C57BL/6 mice as a function of age, with the performance of the aged, exercise-trainedmice reaching significant levels on some but not all of the behavioral tasks.

There is evidence to suggest that age-dependent cognitive deficits are caused by cumulative oxidative damage in the brain. Normally there is a delicate balance between molecules responsible for causing oxidative damage and endogenous antioxidant enzymes and oxidative damage is presumed to result from a shift in the balance between pro-oxidants and antioxidants. While much of the research examining the effects of exercise training as a function of age on the pro-oxidant and antioxidant status of the brain, most of the research has been aimed at examination of the whole brain. Since different brain regions contribute differentially to cognitive and psychomotor performance, Chapter III deals with the effect of exercise training on the oxidant status of various brain regions in aging C57BL/6 mice.

THE EFFECT OF EXERCISE TRAINING ON OXIDATIVE STRESS IN AGING MOUSE BRAIN

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Running title: Oxidative stress in aged, exercised mice

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CHAPTER III

THE EFFECT OF EXERCISE TRAINING ON OXIDATIVE STRESS IN AGING MOUSE BRAIN

SUMMARY

The ability of exercise training to ameliorate age-associated changes in oxidative damage and antioxidant enzymes was tested in aging C57BL/6 mice. Beginning at 3 or 20 months of age C56BLl/6 mice were either subjected to an 11-week exercise training protocol designed to be of moderate intensity but rigorous enough to produce significant increases in fitness, or they were part of an age-matched, non-exercised control group. To evaluate fitness in the mice subjected to exercise, a citrate synthase assay was used. As evidenced by increased citrate synthase activity, the exercise training protocol did indeed produce a significant and nearly equal degree of fitness in both the young and aged mice. At the conclusion of the exercise training regimen, the brains were removed, dissected into 6 regions (cerebellum, cortex, hindbrain, hippocampus, midbrain, and striatum) and analyzed for protein oxidative damage and antioxidant enzyme activity. Despite a general lack of effect, it is a finding of the study that exercise training increased the activity of some antioxidant enzymes in some but not all brain areas as a function of

age. Although superoxide dismutase levels did not change in response to exercise training as a function of age in any of the brain regions, glutathione peroxidase levels increased in the young mice in some regions (the cerebellum and the striatum) in response to exercise, and there were trends for this effect in the aged, exercised mice as well. Catalase activity showed a significant response to exercise training in aged mice in the cortex region. Exercise training did not able to ameliorate oxidative damage seen in aging C57BL/6 mice in any of the brain regions that were examined.

These data suggest that exercise training may have differential effects on antioxidant activity in specific brain areas as a function of age.

1. Introduction

As they age, many individuals begin to display symptoms of neurocognitive degeneration that presents with a range in severity from mild to moderate. There has been considerable research aimed at discovering the mechanisms responsible for the neurocognitive deficits that occur with normal aging and several hypotheses have been advanced to explain the phenomenon. One such theory to explain cognitive deficits seen with aging is the cumulative effect of oxidative damage in aging brain tissue (1, 2, 3). The hypothesis that oxidative damage causes aging is based on the premise that reactive oxygen species contribute to tissue damage that eventually results in senescence. The amount of damage that reactive oxygen species can inflict on tissue is influenced by the balance that is maintained between pro-oxidants such as free radicals, and endogenous antioxidant enzymes (4, 5). It remains unclear whether or not oxidative damage that accrues with aging is the result of increased pro-oxidants or if it is the consequence of declines in antioxidant enzyme defense systems (5, 6).

It is worthwhile to consider the individual regions of the brain when examining the effects of aging. Different brain regions make distinctive contributions to cognitive processes (7, 8, 9) and exhibit differential susceptibility to age-related damage (7). Although there is a paucity of literature addressing the antioxidant status in diverse brain regions as individuals age, a few researchers have found that changes in antioxidant status with aging varies among brain regions (10, 11). It is reasonable to assume that individual brain regions might adapt to exercise training in terms of oxidative stress/antioxidant defenses (27), or that the individual regions might adapt to exercise

training in old age that could reduce the deleterious effects of aging. an issue that has not as yet been addressed.

There has been considerable interest in attenuating cumulative tissue damage that is observed in aged individuals. Exercise, or more specifically fitness training , is a putative intervention strategy that has been employed to moderate oxidative damage seen in aging individuals. While it is true that strenuous aerobic exercise training is associated with increased production of reactive oxygen species in peripheral tissues, the effects of exercise training may present a challenge to the antioxidant system which then responds with increased production of antioxidant enzymes in order to maintain the balance between pro-oxidants and antioxidant defenses. Chronic exercise training has clearly been shown to increase antioxidant enzymes in skeletal muscle (12, 13, 14), heart (12, 14), and liver (12). Moreover, researchers have recently shown increases in antioxidant enzymes in whole brain tissues of exercised mice and rats, in conjunction with decreased biomarkers of oxidative damage (15, 16).

It was the goal of this research to elucidate the differential effects of age and exercise training on oxidative damage and antioxidant defenses in various brain regions.

2. Materials and Methods

Animals

A group of 48 young (3 months) and a group of 48 aged (20 months) C57BL/6J mice were obtained from the National Institute on Aging for testing. Upon arrival at the University of North Texs Health Science Center (UNTHSC) vivarium, the mice were

group housed (4-7 to a cage) in 28 x 19 x 12.5 cm solid bottom polycarbonate cages with wire grid tops. The vivarium was maintained at 23 ± 1^0 C/40% humidity under a 12 hour light/dark cycle with lights on at 0700. The mice had *ad libitum* access to water and standard NIH-31 chow at all times throughout the project.

Effect of exercise training on citrate synthase activity

After a 1 week period of adjustment, 24 young and 24 aged mice were exercised daily on an OmniPacer treadmill Model LC4/M (Accuscan Instruments Inc., Columbus OH) for a period of 8 weeks using a protocol identical to the one used during the first 8 weeks of determination of the effect of exercise training on oxidative damage and antioxidant defenses. After the completion of the exercise training protocol, mice in this study were euthanized and skeletal muscle taken for determination of citrate synthase activity.

Citrate Synthase Assay

All mice rested in their home cages for 1 day before being euthanized by cervical dislocation. After euthanasia, the *rectus femoris* muscles were removed from the hindlegs for determination of citrate synthase activity as a marker of fitness (data not published).

Effect of exercise training on oxidative damage and antioxidant defenses

After a 1 week period of adjustment, 24 young and 24 aged mice were exercised daily on an OmniPacer treadmill Model LC4/M (Accuscan Instruments Inc., Columbus OH) for a period of 8 weeks after which behavior testing was begun on all mice and continued for the next 3 weeks to obtain data for another study. Mice continued to exercise training daily, using the same protocol as during the first 8 weeks, throughout the behavior testing. Variability in exercise training capacity of different inbred mouse strains has been observed (17), so to achieve a moderate exercise training level in C57BL/6J mice the following exercise training protocol was used (18, 19). Mice were acclimated to exercise training over a 3 week period as follows. On day 1, mice were placed on a stationary treadmill with an 8^0 incline for 5 min, followed by a warm up period of running at 3 m/min for 5 min On day 2, the exercise training speed was 4 m/min and the length of the exercise training period was 10 min Thereafter on each succeeding day, the speed of the exercise training period was increased by 1 m/min, and the length of the exercise training period was increased by 5 min, until a maximum speed of 14 m/min and a total running time of 60 min was achieved. As the mice achieved running speeds of 6, 8, 10, and 12 m/min, the total running time included 5 min of running at each of those speeds with the remainder of the exercise training period devoted to running at the maximum speed designated for that day. This approach resulted in a final exercise training protocol consisting of a warm up period of running at 6 m/min for 5 min, 8 m/min for 5 min, 10 m/min for 5 min, 12 m/min for 5 min, and a final speed of 14 m/min for the duration of the 60 min exercise training period. The final exercise training

protocol was reached after 3 weeks of acclimatization and continued for 5 weeks. Electric shock grids located behind the treadmill belts delivered 0.29 mA electric shock to the mice when not running. The number of shocks delivered to all exercising mice was tallied during each exercise training period. Non-exercised control mice were assigned as shock stimulus controls to the exercised mice, so that each non-exercised control received the same amount of shock stimulation as its exercising counterpart. To effect this, 24 young and 24 aged, non-exercised control mice were placed on a stationary treadmill (belt not active) and retained on an inactive electric grid by means of a blocking device. The grids were temporarily activated and approximately 1 sec of 0.29 mA shock was administered to each mouse. The number of shock stimuli that each mouse received was based on the number of shock stimuli that its exercising counterpart received. An intershock interval of 30 seconds was maintained. After each mouse received its quota of shock stimuli, the blocking apparatus was removed and the mouse was allowed access to the stationary belt for the remainder of the exercise training period. Exercising mice that exceeded 30 shocks per hour for 3 consecutive days were eliminated from the study along with their non-exercised control partners. All mice were weighed at the beginning of the exercise training regime and twice a week thereafter. Mice in this study were euthanized and brain parts taken for determination of oxidative damage and antioxidant enzymes.

Isolation of tissues

All mice rested in their home cages for 1 day before being euthanized by cervical dislocation. After euthanasia, various brain regions (cerebellum, cortex, hindbrain,

hippocampus, midbrain, and striatum) were prepared to obtain homogenates for biochemical assays. The ventral and caudal borders of the midbrain were defined as the caudal edge of the mammillary bodies and the rostral border of the pons, respectively. The 6 brain areas were dissected and flash frozen by liquid nitrogen in antioxidant buffer (50 mM phosphate buffer (pH 7.4), 1 mM BHT, 100 μM DTPA) prior to storage at – 80^0C until assayed for protein quantitation, and measured for protein oxidation and antioxidant status. To prepare homogenates from frozen brain regions, a 1% w/v suspension was prepared in a homogenizing buffer (50 mM phosphate buffer (pH 7.4), 1 mM BHT, 100 μM DTPA and .1% Triton X-100 (Sigma). Protease activity was inhibited with protease inhibitor cocktail tablets (Roche, Germany). The homogenate was centrifuged at 200 x g for 5 min at 4^0 C to remove nucleic acids and cell debris. Measures of carbonyl concentrations, superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and catalase assays and were performed on the supernatant immediately.

Carbonyl Assay

Protein oxidation was assessed by measuring the carbonyl content of the brain region homogenates. Carbonyls were analyzed by a derivatization assay (21) with 2,4 dinitrophenylhydrazine (DNPH). All samples were incubated with 10 mM DNPH in 2N HCl or with 2N HCl (1:1) for 1 h at RT in the dark. The reaction was stopped and the proteins precipitated by addition of trichloroacetic acid (TCA)(Acros Organics, Geel, Belguim) (10% final concentration) to the samples which were then kept on ice for 10

min Excess DNPH was removed by a series of ethanol:ethylacetate resuspensions/centrifugations at 1000 x g for 5 min The resulting pellets were dissolved in denaturing buffer (150 mM sodium phophate buffer-pH 6.8, and 3% sodium dodecyl sulfate (SDS)(EMD Chemicals, Gibbstown, NJ). The difference in absorbance between the DNPH-treated and the HCl treated samples was determined by reading on a spectrophotometer (Beckman DU 640, Fullerton, CA) at a wavelength of 360 nm. An extinction coefficient of 22,000 M^{-1} cm^{-1} for DNP-labeled carbonyl groups was used for calculation. The results were expressed as nmol carbonyl groups/mg protein.

Superoxide Dismutase Assay

To assay for total SOD activity, a method that indirectly measures activity by measuring decreases in the maximal reaction rate was utilized (22). Varying amounts of tissue sample and 800 µl of total SOD working solution (13.2 ml 1.33 M DTPA + bovine serum albumin (BSA)(Sigma), 500 µl 40 units/mg catalase (Sigma), 500 µl 2.24 M nitro blue tetrazolium (NBT)(Sigma), 1700 µl 1.8 mM xanthine (Sigma), 100 µl 10 mM bathocuproinedisulfuric acid (BCS)(Sigma), were incubated at 30^0 C for 5 min and then added to a 1.5 ml acrylic cuvette along with 100 µl xanthine oxidase (Sigma) (diluted so that the rate was .02 - .025/min) and run on a spectrophotometer (Beckman DU 640, Fullerton, CA) at a 560 nm wavelength for 8 min In order to find the rate of maximal inhibition, several cuvettes were prepared with a range of protein concentration from tissue homogenate up to 50 mg/cuvette. The amount of inhibition levels off at concentrations less than the maximal amount of protein. The amount of SOD activity in

the sample was calculated by slope on Excel, with the inverse protein concentration plotted on the *x* axis and the inverse percentage of inhibition plotted on the *y* axis. One unit of SOD activity is defined as the amount of protein necessary to decrease the reaction rate to 50% of the maximum rate, activity expressed as U/mg protein.

Glutathione Peroxidase Assay

GSH-px activity was determined by following the oxidation of β-nicotinamide adenine dinucleotide phosphate (reduced NADPH) spectrophotometrically (23, 24). Brain homogenate sample was added to a working solution containing reaction buffer (100 µl 50 mM KPO_4, 15 µl 0.1M L-glutathione, reduced (Sigma), 20 µl 0.2 M EDTA, 100 µl 10 U/ml glutathione reductase (Sigma), 10 µl 0.4 M Na Azide (Sigma), 100 µl 2.5 mM NADPH (Sigma), 640 µl dH_2O) and incubated at 30^0 C for 5 min. The sample and working solution were transferred to a 1.5 ml methacrylate cuvette before adding 100 µl of 2.2 mM hydrogen peroxide (H_2O_2) to begin the reaction before reading on a spectrophotometer (Beckman DU 800, Fullerton, CA) at a 340 nm wavelength for 4-6 min. To calculate the glutathione peroxidase activity the rate of a blank (lacking sample) was subtracted from the rate of a sample taken at 4 min in order to determine true enzyme activity. 1 unit of activity is defined as 1 µmole/ NADPH oxidized/min, activity expressed as U/mg protein.

Catalase Assay

Catalase determinations were made by a method described previously (25) in which the disappearance of substrate H_2O_2 was measured spectrophotometrically at 240 nm. Brain homogenate was added to a quartz cuvette containing 3 ml of pre-warmed (30^0 C) H_2O_2 buffer (0.0125 M H_2O_2 in 0.0667 M Phosphate buffer) and mixed by inversion. The samples were read on a spectrophotomer (Beckman DU 800, Fullerton, CA) at a 240 nm wavelength for 60 seconds. One unit of catalase decomposes 1 mmole of H_2O_2 / min, activity expressed as U/mg protein.

Protein Quantitation

Protein concentrations of the brain tissue samples were measured so that biochemical activity could be determined per mg of protein. Protein concentrations were quantified with spectrophotometry using the Pierce BCA-200 protein assay kit (Pierce, Rockford, IL).

Data Analysis

Statistical analysis was done by analysis of variance and individual comparisons using Systat Version 7.0 statistical software package. 3-way factorial analyses of variance were used to evaluate group differences with age, treatment (exercise) and brain region as between groups factors for each biochemical assay. When appropriate, planned individual comparisons were performed by individual F tests using the error term from the overall analysis.

3. Results

Citrate Synthase Assay

The analysis of the citrate synthase results by two-way ANOVA using age and treatment as the factors showed that the activity of citrate synthase in the *m. rectus femoris* was significantly increased in both the 3-month-old and the 20-month-old exercise-trained mice as compared to the age-matched non-exercised controls, and that the magnitude of the increase was not different in the two age groups (data not shown).

Protein Oxidation

Carbonyl content of the different regions (Fig. 1) was analyzed by 3-way analysis of variance with age, exercise training and region as factors revealed a main effect of age ($p < .050$). Separate analysis of variance by region indicated a main effect of age for the cerebellum and cortex, with aged mice showing significantly higher concentrations of carbonyl groups as compared to young mice ($p < .050$). Although the results were not significant, there was a trend for this effect in all other regions with the exception of the midbrain (*all* $p > .070$). There was no effect of exercise training in any of the 6 regions for carbonyl content (*all* $p > .050$)

Antioxidant Enzyme Assays

For the superoxide dismutase assay, a 3-way analysis of variance with age, exercise training and region as factors revealed a significant main effect of region ($p < .050$) (Fig. 2). Individual comparisons within separate ANOVA indicated that the cortex displayed significantly lower enzyme activity than any of the other brain regions (*all* $p < .050$).

Planned individual comparisons failed to indicate differences in superoxide dismutase activity as a function of age or exercise training in any of the 6 regions of the brain. However there was a trend for increased SOD activity in all of the brain regions (except midbrain) with age, as well as a trend for exercise training to lower SOD activity in all of the regions (except striatum) in aged animals.

A 3-way ANOVA on glutathione peroxidase activity with age, exercise, and region as factors indicated significant main effects of exercise training and region ($p < .050$) (Fig. 3). Individual comparisons within separate analysis of variance by region confirmed that exercise training resulted in increased GSH-px activity in young mice within the cerebellum and striatum ($p < .050$). Although a similar trend was evident for aged mice within these two regions, these effects only approached significance (*both* $p > .100$). Similar to superoxide dismutase activity, the ANOVA revealed that there was a main effect of region, with lower enzyme activity in the cortex than in any of the other brain regions ($p < .050$).

Catalase enzyme assays (Fig. 4) analyzed by 3-way ANOVA with age, exercise training and region as factors showed a significant main effect of region due to markedly decreased catalase activity in the striatum ($p < .050$). Planned individual comparisons within separate analysis of variance by region disclosed a significant effect of exercise training in aged mice within the cortex with catalase activity in aged, exercised animals significantly increased compared to non-exercised controls ($p < .050$).

4. Discussion

The main findings of this study are that when moderate, short-term exercise training is initiated in aged C57BL/6 mice, it (a) shows a lack of effect on oxidative damage in all brain regions, (b) increases activity of GSH-px in cerebellum and striatum of young, but not aged mice, and (d) increases catalase activity in the cortex of aged mice.

A significant finding of this study was that the exercise training protocol used was indeed capable of producing significantly more fit mice in both age groups. This was evident based on previous findings that citrate synthase activity increased in both aged and young exercised mice as compared to the control mice, and that the magnitude of the increase in both age groups was nearly identical (data not published).

Determination of protein carbonyls by separate regions suggested that the cerebellum and the cortex had significantly more oxidatively damaged proteins as a function of age in accordance with previous work in which carbonyls were increased in these brain regions in aged as compared to adult mice (26). However exercise training showed no ability to lower the amount of oxidative damage in any of the brain regions in either age group. To our knowledge, the effect of exercise training on protein oxidative damage as a function of age has not been previously examined in specific brain regions, but this finding is at variance with previous work done by others in which chronic exercise training was shown to lower carbonyl levels in the whole brain (15, 16).

Exercise training had no effect on superoxide dismutase activity as a function of age across all brain regions, but this was not true for glutathione peroxidase and catalase. The activity of glutathione peroxidase was increased in the cerebellum and striatum of

young exercised animals compared to controls. And, although it did not reach significant levels, there was a strong trend for this effect in these regions in aged animals as well. In the cortex, catalase was significantly increased in aged, exercised animals compared to the age-matched control animals. These findings are interesting as some researchers (10, 11) have suggested that it is the ratio of superoxide dismutase to glutathione peroxidase activity rather than the absolute activity of these enzymes that correlate with cellular damage. Specifically these researchers have shown that relative increases in glutathione peroxidase as compared to superoxide dismutase are correlated with decreased cellular damage as assessed by products of lipid peroxidation. Like glutathione peroxidase, catalase is an enzyme that reduces potentially reactive H_2O_2 to harmless substances, so presumably relative increases in catalase as compared to superoxide dismutase would have similar kinds of beneficial effects as relative increases in glutathione peroxidase.

While it is true that the biomarker of cellular damage (carbonyl content) in our study did not decrease in the brain areas in which there were increases in glutathione peroxidase, still increased glutathione peroxidase levels in some brain areas was an interesting finding. Glutathione peroxidase exhibited a very similar pattern of activity in three brain areas, the cerebellum, the cortex and the striatum. In all three areas, glutathione peroxidase was increased in exercised animals of both age groups, although the increase did not always approach significance. Since various brain areas have differential effects on learning and memory, a pattern of increased glutathione peroxidase and/or catalase in conjunction with unchanged superoxide dismutase levels could result in ameliorating effects of age related cognitive deficits by moderation of cellular damage

(oxidative stress) in these areas. The fact that significant effects of exercise training on glutathione peroxidase levels were only seen in the young animals (in some areas) is still a positive effect of treatment. Additionally, catalase was increased in aged, exercised animals in the cortex, a region of the brain that makes countless contributions to cognitive function and movement.

The question of biological relevance of the changes seen in oxidative damage and antioxidant defenses should be addressed. In this project, two areas of the brain displayed significant increases in carbonyl content with age, the cerebellum and the cortex. The increases that were observed were as great or greater than increases in carbonyl content that were observed in conjunction with behavior deficits by others (15). The biological relevance of the antioxidant enzymes is a more difficult issue to address. The literature is equivocal concerning changes in antioxidant defense enzymes in conjunction with pathological, physiological, and cognitive changes. However, the magnitude of the increases seen with exercise training in this study in the areas of the brain where such changes were observed were as great or greater than changes (concomitant with behavioral changes) observed by others (15, 35).

Before exploring putative reasons for the failure of exercise training to reduce carbonyl levels and to consistently increase the relative amounts of antioxidant enzymes in aged brain regions, it should be pointed out that the literature is divided on the effect of aging and the effect of exercise training on oxidative damage and on antioxidant enzymes in different brain regions (27, 28, 29, 30). In this study, significantly more fit mice were produced with our exercise training protocol, but there were many variables in the

exercise training protocol that could influence the results. For instance, exercise training initiated earlier in the mouse's life may have produced more profound effects, especially given the finding that exercise training in young mice produced significant increases in glutathione peroxidase levels. Increases in glutathione peroxidase initiated early in life may persist in the aged animal, with coincident reductions in oxidative damage. It is possible that the exercise training protocol, although chosen so that it would be of moderate intensity, could have been optimized in terms of intensity. It has been shown in the rat brain that differential exercise training protocols have very different effects on biomarkers of oxidative stress, endogenous antioxidants as well as different effects on cognitive performance (31, 32). The type of exercise training administered may be a confounding variable – perhaps the mice would have shown more significant effects of exercise training if a different type of exercise training had been administered. It has not been investigated whether or not forced versus voluntary exercise training produces different effects on biomarkers of oxidative stress, endogenous antioxidants or even cognitive performance. However researchers investigating the effects of exercise training on some of these parameters have obtained conflicting results that might be due to the variable nature of the exercise training protocols utilized (15, 16, 30, 32).

Carbonyl modification of protein has been used extensively as a biomarker of oxidative stress because all reactive oxygen species examined thus far, including reactive NO species, give rise to carbonyl modification of proteins. It should be pointed out that there are other measurements of oxidative damage besides the one (protein oxidative damage) utilized in this project. Damage to DNA and products of lipid peroxidation are

commonly employed biomarkers of oxidative damage. It is quite possible that evaluation by a different biomarker of oxidative damage would have revealed different estimations of oxidative damage. Even within the parameter of carbonyl differences as a function oxidative damage there are confounding aspects to be considered. Many researchers examine carbonyl levels as a biomarker of oxidative damage but there remains a paucity of literature concerning the specificy of oxidatively damaged proteins in the brain. It is quite possible that specific proteins subject to oxidative damage could be vital to aging and yet be present in small quantities (33, 34). The general nature of the carbonyl assay in this case might obscure important differences in specific protein oxidative damage.

In summary, the results of this study suggest that exercise training does have a region-dependent effect on the endogenous antioxidant defense system in the young and aging mouse brain. The fact that many of the effects failed to make significance could be due to a variety of confounding variables within the experimental design, such a different exercise training protocol or examination of different biomarkers of oxidative stress, but the possibility of more favorable pro-oxidant and antioxidant outcomes as an effect of exercise training as a function of aging is certainly a viable outcome.

Fig. 1. The effect of exercise training on oxidative damage to proteins in various brain regions as a function of age. Values represent the mean ± SE of 6 samples. CB = cerebellum, CX = cortex, HB = hindbrain, HP = hippocampus, MB = midbrain, ST = striatum.

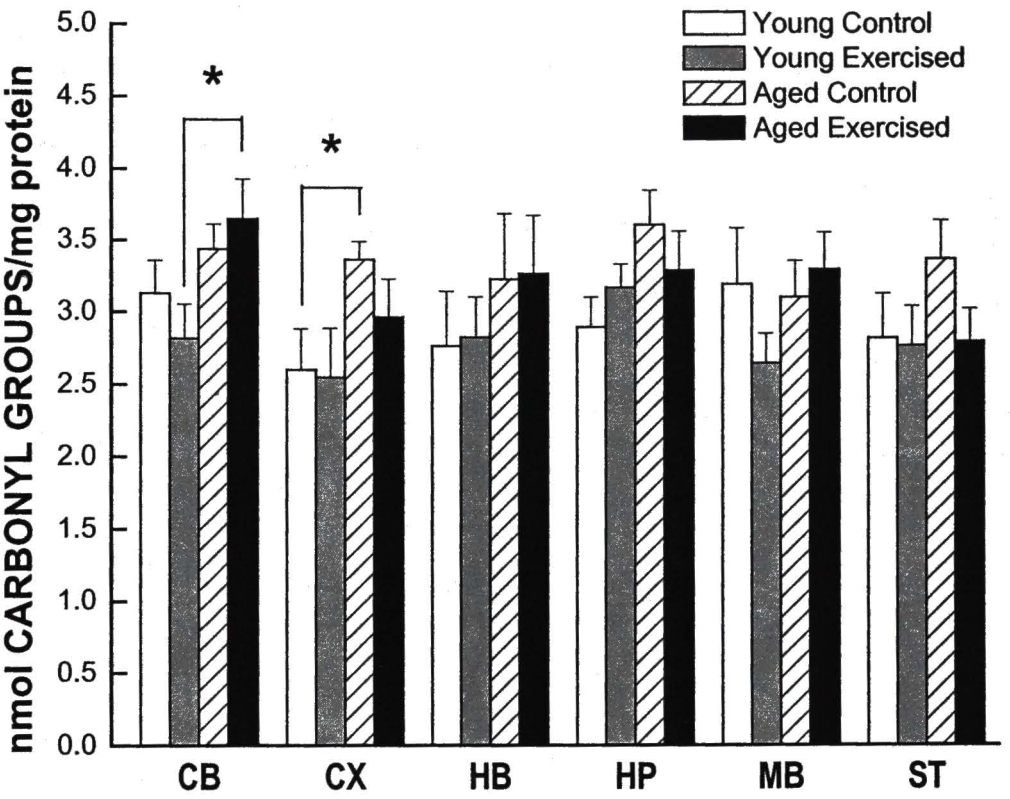

Young Control
Young Exercised
Aged Control
Aged Exercised
nmol CARBONYL GROUPS/mg protein
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
CB
CX
HB
HP
MB
ST

Fig. 2. Superoxide dismutase activity in various brain regions as a function of age and exercise. Values represent the mean ± SE of 6 samples. . CB = cerebellum, CX = cortex, HB = hindbrain, HP = hippocampus, MB = midbrain, ST = striatum.

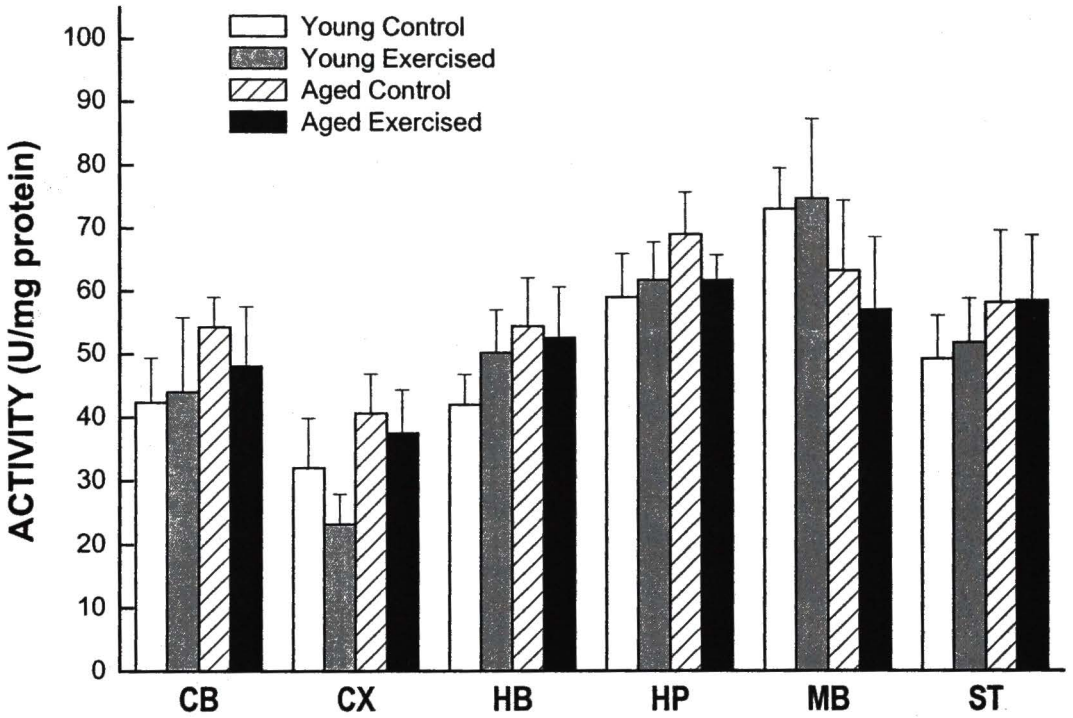

Young Control
Young Exercised
Aged Control
Aged Exercised
ACTIVITY (U/mg protein)
100
90
80
70
60
50
40
30
20
10
0
CB
CX
HB
HP
MB
ST

Fig. 3. The effect of exercise training on glutathione peroxidase activity in various brain regions as a function of age. Values represent the mean ± SE of 6 samples. * = significant ($p < .050$) difference from age-matched controls. CB = cerebellum, CX = cortex, HB = hindbrain, HP = hippocampus, MB = midbrain, ST = striatum.

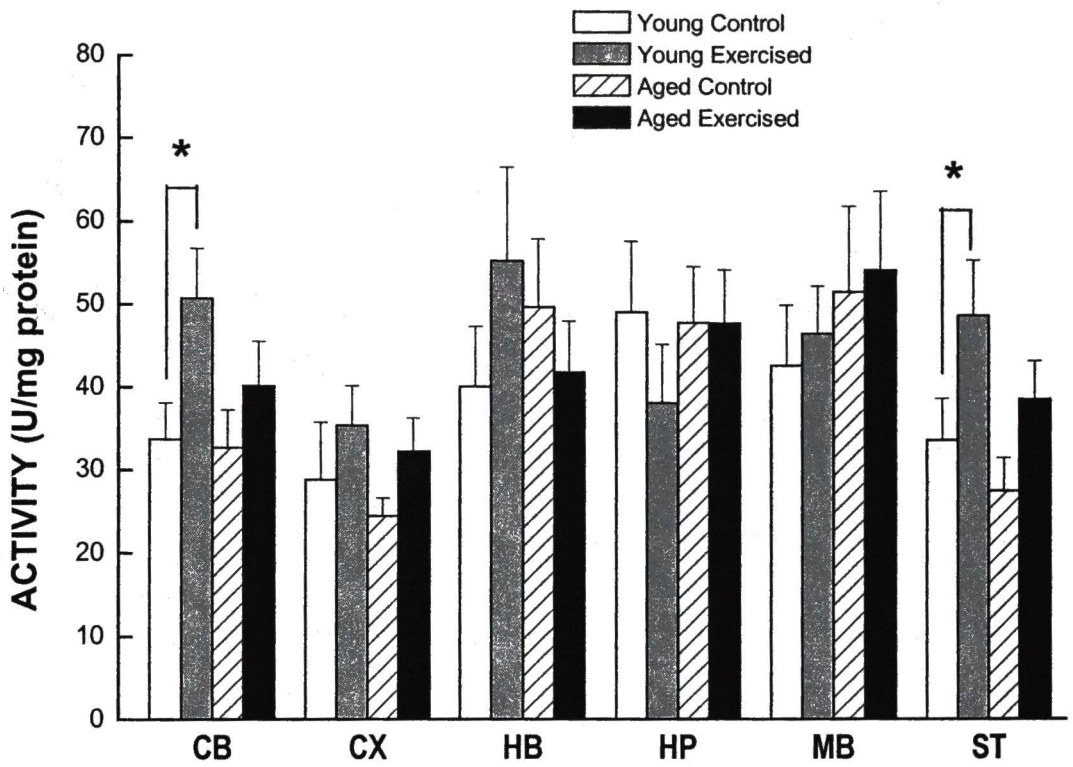
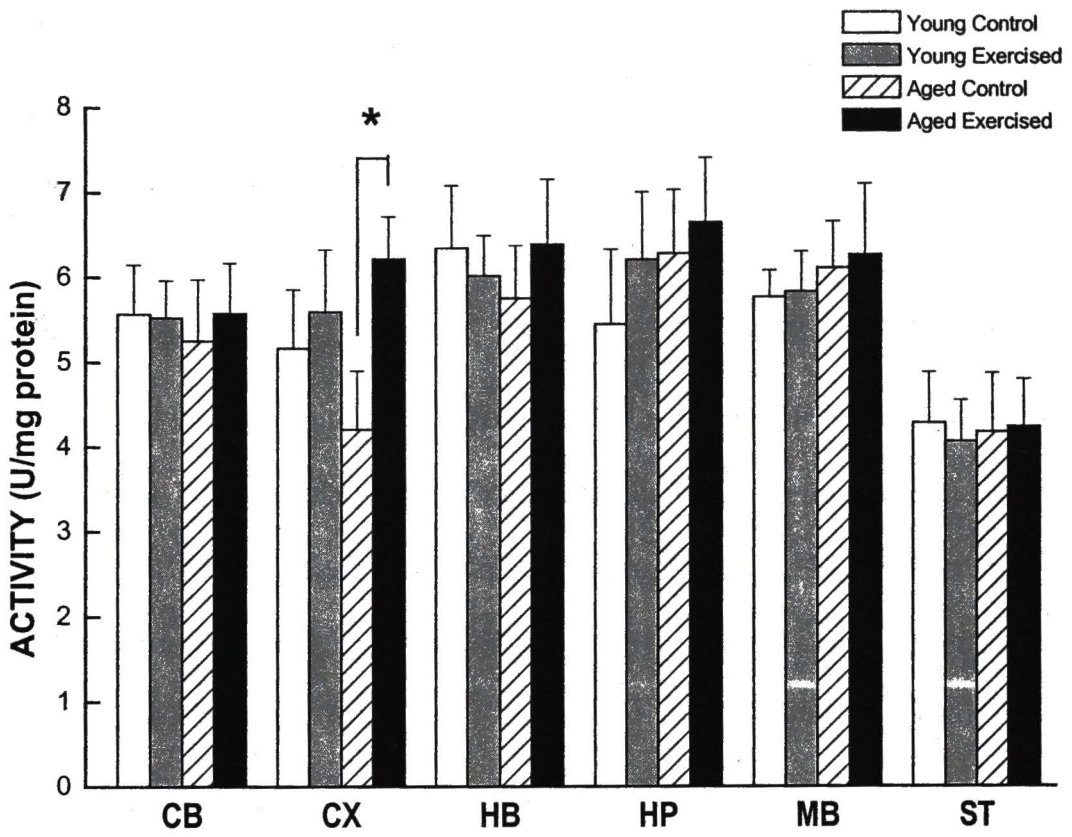

Young Control
Young Exercised
Aged Control
Aged Exercised
ACTIVITY (U/mg protein)
80
70
60
50
40
30
20
10
0
*
*
CB
CX
HB
HP
MB
ST

Fig. 4. Catalase activity in various brain regions as a function of age and exercise. Values represent the mean ± SE of 6 samples. * = significant ($p < .050$) difference from age-matched controls. CB = cerebellum, CX = cortex, HB = hindbrain, HP = hippocampus, MB = midbrain, ST = striatum.


Young Control
Young Exercised
Aged Control
Aged Exercised
ACTIVITY (U/mg protein)
0
1
2
3
4
5
6
7
8
*
CB
CX
HB
HP
MB
ST

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CHAPTER IV

DISCUSSION

The free radical theory of aging is one of the theories that has been put forward to explain cognitive deficits seen with aging. A salient point in the free radical theory of aging is that aerobic cells produce pro-oxidants that form molecular species capable of exerting oxidative stress on the cell itself (1). Such a challenge is a normal result of metabolism, but prolonged accrual could eventually lead to degeneration, especially in cells that are amitotic such many of those in the CNS. Cells also have endogenous antioxidant enzymes capable of reducing pro-oxidants to harmless substances, but what effect antioxidant enzymes have on aging and cognitive deficits remains unclear (2, 3). To answer this question we designed the current research project to include a mechanism (exercise training) that we hypothesized would ameliorate oxidative stress associated with aging, thereby moderating cognitive deficits. To elucidate the role of antioxidant enzymes in the diminution of cognitive abilities as a function of age, we examined the cognitive and psychomotor responses as well as biochemical analysis of antioxidant enzyme activity in aged C57BL/6 mice participating in an exercise training regimen to age-matched, non-exercised controls. Specific brain regions make unique contributions to cognitive processes, so we examined the antioxidant enzyme activity in various brain

regions to determine if age-related cognitive deficits could be the result of differential levels of oxidative stress/stress responses in various brain regions (4, 5, 6).

The main findings of the behavioral aspects of this project (chapter II) are that when moderate, exercise training was initiated in aged C57BL/6 mice, it resulted in increased fitness in the aged mice to the same degree as observed in young mice, it improved some psychomotor skills, including bridge-walking and reaction time, and it improved age-impaired spatial memory performance. Biochemical analysis of oxidative damage and the antioxidant status of separate brain regions (Chapter III) suggested that oxidative damage increased with age in all brain regions, and that exercise did not reduce oxidative damage in any of the brain regions. The biochemistry data also suggests that changes in antioxidant enzyme activity is not a causative factor in senescence, however some antioxidant enzyme activities did increase with exercise.

The results of biochemical analysis of antioxidant enzymes in various brain regions of aged, exercise-trained mice showed that superoxide dismutase activity did not change significantly with age in any of the brain regions studied. Glutathione peroxidase exhibited a similar trend of increased activity in exercised as compared to non-exercised mice in 3 brain regions - the cerebellum, cortex and striatum however these trends failed to make significance in the aged mice. Catalase was significantly higher in the cortex of aged, exercised mice as compared to non-exercised controls. Exercise training did not decrease the carbonyl groups in any of the brain regions of aged mice to a significant degree, including the cortex where catalase activity was significantly increased. Some researchers feel that the relative activities of SOD to GSH-px + catalase activity are more

important than the absolute activities of these antioxidant enzymes in causing cell damage (7, 8). Our data revealed favorable trends (reaching significance for catalase activity in the cortex) for the relative activities of these antioxidant enzymes in aged, exercised animals, but the unchanged carbonyl levels lead us to conclude that our hypothesis that exercise training can lower oxidative damage by enhancing antioxidant protection is not supported. However, the data does not completely rule out the hypothesis for several reasons. The experimental design of the study could be optimized in ways that might produce very different outcomes. The type of exercise training utilized for the protocol, the time of exercise training initiation, the duration, and the intensity of the exercise training may have all contributed to generation of results that did not support the hypothesis. Furthermore other measurements of oxidative damage besides evaluation of protein damage may have revealed that oxidative damage can be reduced with exercise. Evaluation of damage to DNA or of products of lipid peroxidation may have revealed different estimations of oxidative damage. The specificity of the proteins that sustain oxidative damage has not been determined. It is quite possible that specific oxidatively damaged proteins present in small quantities constitute the proteins that are vital to aging (9). The general nature of the carbonyl assay in this case might have obscured important differences in specific protein oxidative damage. Finally, not all of the antioxidant enzymes were examined in this study. We chose 3 commonly studied enzymes but there are many others as well. Glutathione S-transferase, glutathione reductase, glutathione synthetic enzymes, glucose-6-phophate dehydrogenase, and endonuclease IV are all enzymes with considerable antioxidant roles

(10, 11, 12). It's quite possible that these enzymes display a more robust response to exercise training than the enzymes chosen for this project. It must be conceded that in any case carbonyl levels were not moderated, but again if the exercise training protocol were optimized and different markers of oxidative damage assessed, the resulting data might have supported the hypothesis.

The behavioral aspects of the study reveal that exercise training appears to improve the performance of aged mice in some reflexive and psychomotor tests including the bridgewalking task and latency to startle stimulus. Bridgewalking is a motor skill that involves implicit memory. As such, it has a musculosketetal component that is most directly under cerebellar control, but as its performance is also dependent upon procedural memory, very likely it is influenced by the striatum (27, 28). The biochemical data revealed a trend for favorable antioxidant status in the cerebellum of aged, exercised mice but the oxidative damage to proteins was not ameliorated in these mice. There was a favorable trend in antioxidant activity and for decreased carbonyl groups in the striatum of aged, exercise-trained mice as compared to controls. The lack of significant decreases in oxidative damage in these two brain regions does not support our hypothesis that exercise training is able to ameliorate oxidative damage, but the potential of exercise training to moderate unfavorable antioxidant enzyme status in the aging brain is not ruled out. A notable cognitive improvement was seen in the ability of aged, exercised mice to recall a platform location after 24 hours had elapsed since they had fully acquired the knowledge of its placement. This latter finding suggests that exercise training improves age-associated deficits in the ability of mice to consolidate memories. Memory storage in

mammals is believed to be reorganized in the brain following acquisition. Sensory perceptions destined to become factual or declarative memory are first formed in several association areas of the cortex which integrates visual, auditory and somatic information. Integrated information is sent first to the parahippocampal and perirhinal cortices, and then to the entorhinal cortex. Information then enters the hippocampus where it is processed in the the dentate gyrus, CA3 and CA1 regions, and the subiculum. After processing by the hippocampus, information leaves the hippocampus, exiting to the entorhinal cortex where it returns to the association areas of the cortex via the parahippocampal and perirhinal cortices. It is somewhere in this loop that explicit memories are formed and stored in the association cortices (16, 29). Recently acquired knowledge is believed to be stored in parallel in the hippocampus and cortical networks and then hippocampal-cortical reactivation gradually strengthens cortical-cortical connections until new memories become independent of the hippocampus. Some theorists maintain that memory retrieval becomes hippocampus independent while others propose a permanent role for the hippocampus in retrieval, but there is agreement that newly acquired memories become integrated into pre-existing memories in the cortex (14, 15). There was a trend for favorable glutathione peroxidase activity and significantly improved catalase activity in concert with a trend for moderated protein damage in the cortex of aged, exercised mice. Since aged, exercised mice exhibited markedly improved consolidation compared with the non-exercised controls, it is possible that exercise training could moderate oxidative stress in brain areas associated with consolidation.

Exercise-trained mice have improved motor function compared to non-exercised controls, and their improved physical abilities must be considered when analyzing the results of their psychomotor behavior. All of the behavior tasks chosen for this project have a motor component so this caveat cannot be overlooked. It is possible that improved bridgewalking performance displayed by trained mice could be the result of their improved physical abilities since bridgewalking is dependent on muscular strength in addition to balance and coordination. However, if moderated cerebellar and striatal dysfunction did not contribute to improved bridgewalking performance (e.g., if the improvement was a function of improved muscular strength alone) it is likely that trained mice would have shown improvements in many of the other tests as well.

Trained mice displayed exercise training associated improvements in one aspect of water maze testing – persistence in returning to the original location of the platform after a delay. It could be argued that the trained mice were better swimmers and had the strength and endurance to keep searching for the platform longer than did the control mice. However, because the cognitive improvement seen during the swim maze task was confined to an improvement in searching for the platform in its original location only after a 24 hour delay, it isn't likely that improved motor function is driving this behavior. If this were the case, no doubt improvements in initial acquisition, retention and reversal as well as exercise training driven improvements in the proximate probe trial would have been noted. Instead, the improved performance in a probe trial administered after a delay appears to be due to moderation of age associated cognitive deficits associated with consolidation/retrieval (16).

The data presented in this project shows clearly that exercise training does moderate some age associated cognitive deficits, but it is possible that the mechanism is not by moderating oxidative stress. There has been much speculation about the mechanisms of cognitive aging; such conjecture has lead to hypotheses of mechanisms that can be positively altered by exercise. Such putative mechanisms include hypometabolism of brain cells in aged individuals (17, 18), altered neuronal morphology and growth factors (19, 20), the decline in levels of key hormones, peptides, and neurotransmitters in aged individuals (21, 22, 23), reduced oxygen availability to brain cells (24, 25), and the dysfunction of DNA synthesis, protein synthesis, and/or post-translational modification of proteins (20, 21, 26). Interestingly none of these proposed mechanisms of brain aging rule out the possibility of oxidative damage as a primary mechanism. It is entirely possible that initial oxidative damage is an upstream instigator for any of the proposed mechanisms.

In conclusion, the findings in this study do not preclude the plausibility of our hypothesis that exercise training can moderate cognitive deficits seen in aging by reducing the accrued oxidative damage concomitant with aging. Future directions should include examination of oxidative damage as an upstream regulator of multiple factors involved with cognitive deficits seen in aging individuals. Undoubtedly a key component of future research is to elucidate the specific nature of molecules altered by oxidative damage.

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