EGCG AND VOLUNTARY EXERCISE: AN EXAMINATION OF TREATMENT
EFFECTS USING THE TGCRRND8 MOUSE MODEL OF ALZHEIMER'S DISEASE

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EGCG AND VOLUNTARY EXERCISE: AN EXAMINATION OF TREATMENT
EFFECTS USING THE TGCRND8 MOUSE MODEL OF ALZHEIMER'S DISEASE

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LIST OF ABBREVIATIONS

Alzheimer's disease – AD

Amyloid-beta - A β

Amyloid precursor protein – APP

Analysis of variance – ANOVA

Area under the curve - AuC

Barnes maze – BM

(-)-Epigallocatechin-3-gallate - EGCG

Morris water-maze - MWM

Pittsburgh Blue Positron Emission Technology - PiB PET

Radial-arm water maze – RAWM

Reactive oxygen species – ROS

Transgenic - Tg

Wildtype or Non-transgenic – Wt; nTg

Untreated transgenic – Tg-U

ABSTRACT

Previous work with the TgCRND8 mouse model of Alzheimer's disease has shown that voluntary exercise implemented for long time periods (5-months) and starting at very young ages (1-month), prior to the development of disease pathology, reduces cognitive impairments; however, approximately 1-month of exercise started at 80-days failed to provide cognitive improvements for male mice. Additionally, research has shown that EGCG, a botanical polyphenol, can reduce amyloid-beta levels, mitigate oxidative stress and reduce some of the cognitive impairments associated with Alzheimer's disease in the Tg2576 murine model; however, oral administration of EGCG had not been yet evaluated in the TgCRND8 strain. The present study investigated the effects of 4-months of exercise treatment, implemented at the beginning of pathology development (2-months of age), in conjunction with the effects of EGCG treatment on: 1) behavioral measures: learning and memory performance in the Barnes maze, nest building, the open-field, anxiety in the light-dark box, and 2) soluble, amyloid-beta levels in the cortex and hippocampus. Untreated Tg mice showed deficits in nest building behaviors, as well as poor spatial learning in the Barnes maze. Four-months of EGCG and exercise treatment reversed nest building and spatial learning deficits, and lowered soluble, A β 1-42 levels in the cortex of Tg animals. This research was supported by NIH grant funding (2P01 AG18357).

INTRODUCTION

Alzheimer's disease (AD) is a progressively degenerative and debilitating brain disease whose name is sadly salient to an ever-growing and aging population. The worldwide prevalence is estimated to be 35 million individuals (5.5 million in the US alone), and this number is expected to more than double in the next 40 years (Querfurth & LaFerla, 2010). There are two types of the disease: familial and sporadic. Familial AD has an early-onset (before age 65) and is genetically inherited (via an autosomal dominant gene mutation). It is by far the less common form of the disease, accounting for roughly 4-5% of all cases. The more common form, sporadic Alzheimer's, occurs after age 65 (late-onset); it has also been associated with certain genetic risk factors (e.g. inheritance of the Apolipoprotein E ϵ 4 allele; Blennow et al., 2006).

Characteristics of the Disease

The disorder has four stages (pre, early, moderate, and advanced dementia) and is characterized by progressive cognitive and functional impairment in areas like problem solving, orientation/spatial navigation, attention, language, perceptual skills and memory. The severity of the dementia progresses over an average course of 7-10 years before the disease culminates in death (see Holtzman et al., 2011 for a review). The hallmark pathological features are dense cerebral amyloid-beta ($A\beta$) plaques and neurofibrillary tangles (composed of tau protein) in the medial-temporal lobe. Other key features include: oxidative stress and damage, neuron and white matter loss, and inflammation

(see Querfurth & LaFerla, 2010 for a review). While clinical diagnostic criteria are available to assist physicians in making probable diagnoses, the disease currently can only be definitively confirmed after death by pathological examination of the brain (Blennow et al., 2006); however, recent medical research developments, like the PiB PET (*in vivo* imaging of amyloid-beta using a tracer which binds to plaques), may open up future diagnostic possibilities for those still living (Ikonomovic et al., 2008). In addition, several studies have provided evidence for early diagnosis using biomarkers from the cerebrospinal fluid (Blennow et al., 2010).

Amyloid-beta and APP

Amyloid-beta ($A\beta$) is a 36-43 amino acid peptide created by the proteolysis of amyloid precursor protein (APP). APP is an integral membrane protein that varies in size from 695-770 amino acids; APP695 is the most common form found in the brain. $A\beta$ is produced through the sequential cleavage of APP by β -secretase (at the N-terminus) and γ -secretase (at the C-terminus); production of $A\beta$ can be precluded by the cleavage of APP by α -secretase (for review, see Mattson, 2004). Genes responsible for the production of APP in humans are located on Chromosome 21, and differences in these genes have been linked to increased risk of developing AD and dementia (Blennow et al., 2006). Additionally, it has been found that the oligomeric $A\beta_{1-42}$ peptide (soluble, both intracellular and extracellular) inhibits synaptic transmission via a variety of molecular mechanisms, and may be responsible for the early symptoms of AD's pathophysiology

(Mattson, 2004). This is supported by research showing that oligomer levels, and not total plaque burden, are correlated with the severity of cognitive impairments observed in AD (Lue et al., 1999).

The functional biological roles of APP and amyloid-beta are not well understood. Research has implicated the involvement of APP processing on long-term potentiation, neural plasticity, synapse formation and repair, cell signaling and cell adhesion (Priller et al., 2006; Turner et al., 2003; Zheng & Koo, 2006). Interestingly, in animal models, both APP-knockout mice and APP-upregulated mice show impairments in learning and memory (Phinney et al., 1999; Matsuyama et al., 2007).

Transgenic mouse models of AD

Several murine strains have been developed which overproduce A β 1-42 and/or A β 1-40 and mimic many of the behavioral and physiological aspects of AD. Transgenic AD mice develop plaques that are structurally similar to those observed in humans; they start as diffuse plaques comprised of A β 1-42, which then develop dense cores. Later, these plaques will incorporate A β 1-40 and other non-A β components. A β 1-42 is more neurotoxic and prone to aggregation compared to the A β 1-40 form. (see Morrisette et al., 2009 for a review).

Transgenic animal models have helped to produce a better understanding of the etiology and treatment of the disorder. For example, studies using the Tg2576 strain highlighted the relationship between elevated levels of the A β 1-42 peptide and accelerated plaque formation. Tg AD mice show cognitive deficits of learning and

memory in a variety of behavioral tasks, yet they do not exhibit significant neuronal loss (as is seen in human AD). Additionally, it has been observed that the cognitive deficits displayed by Tg AD mice occur before the development of extracellular plaques – this led to further research indicating that cognitive decline is proportionally related to levels of soluble oligomeric A β 1-species, and not plaque burden. It has also been shown that intraneuronal A β 1-42 and A β 1-40 accumulation impairs long-term potentiation in an age-dependent fashion, and this synaptic dysfunction is also positively correlated with levels of the soluble oligomeric A β -species (see Morrisette et al., 2009 for a review).

Use of TgCRND8 mice to examine the disease

The TgCRND8 murine model of AD was developed by Dr. Westaway and his colleagues (University of Toronto), and exhibits the early-onset, familial form of the disease. TgCRND8 mice are created by inserting a human APP695 transgene [with the Swedish (K670N/M671L) and Indiana (V717F) mutations, specifically], into a hybrid C3H/HeJ-C57BL/6 (i.e., wildtype) strain background (Chishti et al., 2001). This strain has recently been used to better understand AD characteristics and to develop possible treatments. TgCRND8 mice lack neurofibrillary tangles, but portray significant cerebral amyloid-beta plaque deposition by 3 months of age, high levels of the A β 1-42 peptide, as well as spatial memory and learning deficits (Chishti et al., 2001). This early age of plaque onset is unique in this transgenic line, as other Tg lines (e.g., Tg2576, PDAPP) do not display plaques until at least 6-9 months of age; this is a benefit to researchers who wish to conduct studies in shorter time frames (Chishti et al., 2001). Additionally, this

AD model shows adrenocortical hyperactivity (Touma et al., 2004) and progressive neuroinflammation, including activated microglia found predominantly around A β accumulations, by 3-months of age (Dudal et al., 2004).

TgCRND8 mice also exhibit altered activity patterns and spontaneous stereotypic behavior, such as jumping and circling (Ambrée et al., 2006; Richter et al., 2008). These behaviors are thought to mimic the noncognitive deficits seen in humans with AD, like sundowning (a syndrome involving the occurrence/increase of abnormal behaviors during late afternoon through night-time, which may be related to a disturbed circadian rhythm), restlessness and wandering. Research concerning working memory and spatial learning in these mice has revealed deficits (compared to wildtype littermates) in tasks such as: the Morris-Water Maze (Janus, 2004), the six-arm radial water maze (Lovasic et al., 2005), and the Barnes maze (Ambrée et al., 2009; Görtz et al., 2008; Richter et al., 2008), as demonstrated by longer escape latencies and higher error rates.

Oxidative Stress & Neuroinflammation

As previously mentioned, key features of AD include oxidative stress and neuroinflammation. During normal cellular respiration, reactive oxygen species (ROS), also known as free radicals, are produced in the mitochondria and cytoplasm, and under normal homeostatic conditions, play a role in cellular proliferation and host defense (i.e. they are crucial to the destruction of bacteria within phagocytes). ROS levels are regulated by their metabolism through enzymatic (e.g. catalase) and nonenzymatic (e.g. Vitamins A, C, and E) antioxidant defense systems. Oxidative stress represents an

imbalance in the cell, such that ROS are overproduced and/or not being metabolized in sufficient amounts, and this can damage mitochondrial DNA, leading to impaired function, and thereby propagating further ROS overproduction. The brain is especially susceptible to damage from oxidative stress, due in part to its poor antioxidant systems relative to other parts of the body. Furthermore, recent research suggests that cellular responses to acute oxidative stress may decrease with age (see Finkel & Holbrook, 2000 for a review), and increases in oxidative stress are associated with the early development of AD (see Butterfield et al., 2002 for a review).

The plaques seen in AD are abnormal aggregations of extracellular, fibrillar amyloid-beta, which is associated with the activation of microglia cells (the brain's primary immune cells) and astrocytes. These cells are involved in normal, beneficial, neuroinflammatory responses; however, their *uncontrolled and sustained activation* in AD can disturb calcium regulation and is associated with the production of nitric oxide, glutamate, and pro-inflammatory cytokines (e.g., TNF-alpha, IL-1b), which can further increase ROS-levels and oxidative stress, and can lead to secondary neuronal injury and death. Neuronal insult and cell death can *also* activate inflammatory processes, which in turn increase oxidative stress through the overproduction of reactive-oxygen species (ROS), which can then cause damage to mitochondrial DNA, resulting in even further overproduction of ROS, oxidative stress, and apoptosis in a positive feedback loop fashion (see Heneka & O'Banion, 2006 for a review).

As previously mentioned, specific intracellular signaling pathways regulate these oxidative events and neuroinflammatory processes. A growing body of research,

including experimental, pre-clinical and clinical studies (see Singh et al., 2008; Kim et al., 2010; Pocernich et al., 2011 for review) indicate that dietary supplementation rich in antioxidants may reduce the risk of developing AD. Additionally, dietary intake of plant-based polyphenols (i.e., *(-)-epigallocatechin-3-gallate*: He et al, 2011; Sun et al., 2008; *garlic*: Chauhan & Sandoval, 2007; *ginseng*: Chen et al., 2006; *pomegranate*: Hartman et al., 2006; *curcumin*: Ishrat et al., 2009) exert neuroprotective effects in AD through the reduction of oxidative events, and thus, inflammatory cascades, primarily by regulating the intracellular signaling pathways, but also by scavenging ROS.

EGCG: Antioxidative & Anti-Inflammatory Properties

(-)-Epigallocatechin-3-gallate (EGCG) is a polyphenol found in green tea that shows antioxidative, anti-inflammatory, and anti-amyloidogenic properties (He et al., 2011; Sun et al., 2008). Many studies have shown that EGCG reduces amyloid-beta levels (Li et al., 2009; Lee et al., 2009a; Lee et al., 2009b), and in so doing, can decrease A β -induced mitochondrial dysfunction (Dragicevic et al., 2011) and memory impairments in tasks such as passive avoidance and the MWM (Lee et al., 2009a). EGCG has been investigated in the Tg2576 AD mouse model (Rezai-Zadeh et al., 2005), where it was shown to decrease A β -levels by promoting increased α -secretase cleavage activity through its activation of ADAM10, an α -secretase enzyme (Obregon et al., 2006). Additionally, oral administration of EGCG (50mg/kg/daily for 6-months) in these mice resulted in decreased levels of soluble and insoluble oligomeric A β -species, as well as amelioration of spatial learning deficits, as shown by improved acquisition in the radial-

arm water maze (RAWM; Rezai-Zadeh et al., 2008).

Based on recent molecular research (see Kim et al., 2010 for a review), EGCG may offer *increased* neuroprotective effects compared to other polyphenols, such as curcumin. For example, both of these polyphenols (curcumin & EGCG) are able to cross the blood-brain barrier and have been shown to protect cells against A β -induced toxicity, decrease A β -levels, -fibrils, and plaques, as well as reducing cognitive impairment in animal models; however, only EGCG (but not curcumin) has been shown to increase α -secretase activity, decrease β -secretase activity, reduce cortico-hippocampal ROS, and ameliorate A β -induced neuronal cell death. Additionally, a recent in-vitro study showed that pretreatment of cortical neurons with EGCG protected against A β -induced production of ROS, mitochondrial dysfunction, and impairment of NMDA-receptor mediated calcium influx (He et al., 2011).

Lifestyle Factors: Physical Activity

It is well known that regular physical activity can reduce an individual's risk for cardiovascular disease, diabetes, cancer and depression, and can even improve cognition in healthy, older adults (see Dishman et al., 2006 for a review). Recent research suggests that exercise may also reduce the risk of certain neurological conditions, such as brain/spinal cord injury, stroke (Endres et al., 2003), and Alzheimer's disease (Larson et al., 2006; Laurin et al., 2001). Additionally, long-term exercise can attenuate some negative effects of acute stress exposure, such as learned helplessness, presumably through alteration of the serotonin and norepinephrine systems in the brain stem and

cortex (Dishman et al., 2006).

Aged, male C57/BL6 mice provided with running wheels for 45 days showed increased neurogenesis in the hippocampus, as well as improved reference memory in the MWM (Van Praag et al., 2005). With regard to murine models of AD, Adlard and colleagues (2005) showed that 5-months of unlimited access to a home-cage running wheel (beginning prior to the onset of disease pathology; 1-month old) improved the cognitive performance of TgCRND8 mice in the MWM task, compared to Tg animals in standard housing. This study also found that long-term exercise decreased extracellular amyloid burden in the cortex and hippocampus, and decreased levels of soluble oligomeric A β -species (A β 1-40 and A β 1-42). In contrast, another study (Richter et al., 2008) found no cognitive benefit of exercise, as measured by object recognition and Barnes maze tasks, when the running wheels were provided to male TgCRND8 mice at 80 days of age (mice had access up to 5-months of age; however, behavioral testing started after only 40 days of running wheel access). Interestingly, running distance was negatively correlated with stereotypic behaviors in the Tg group.

Additionally, research involving a different AD mouse model, Tg2576, showed that 3-week access to running wheels provided to aged Tg mice (after development of pathology) still offered some cognitive benefits; exercised mice showed better performance in both working- and reference-memory measures of the radial-arm water maze compared to sedentary Tg mice (Nichol et al., 2007). Exercise alone, however, did not completely eradicate cognitive impairments; and interestingly, Tg mice provided with a locked/immobile running-wheel also showed some improvements in long-term memory

performance. Other research involving the Tg2576 strain found larger hippocampal volumes in 4-month voluntary- and forced-exercise groups, compared to sedentary animals (Yuede et al., 2009). This latter study was also unique in finding that forced-exercise was not as beneficial as voluntary exercise, and this discrepancy could not be explained by differences in distance traveled, since running velocity was held constant between groups. The authors suggest that the stress induced by forced running mitigated the beneficial effect of the exercise. Another study, which used male 3xTg-AD mice (Garcia-Mesa et al., 2011), found that 6-months of voluntary wheel-running significantly reduced soluble, oligomeric A β -levels and cerebral oxidative stress, in addition to improving cognitive and behavioral impairments (i.e. anxiety, emotionality, lack of exploration).

Possible mechanisms that have been suggested to explain how physical activity exerts its cognitive enhancing effects include: by mediating the release of neurotrophic growth factors, such as BDNF, which is important for neurogenesis and synaptic plasticity in the hippocampus (Adlard et al., 2005b; Vaynman et al., 2004), and shows reduced expression in the TgCRND8 model, both in aged (Peng et al., 2009) and very young mice (Francis et al., 2010); by reducing oxidative stress, and by maintaining neuronal plasticity.

Present Study

Rationale

Previous work with the TgCRND8 strain (Adlard et al., 2005a) has shown that voluntary exercise implemented for long periods (5-months) and starting at very young

ages (1-month), prior to the development of disease pathology, reduces cognitive impairments; however, roughly 1-month of exercise started at 80-days failed to provide cognitive improvements in male mice. Therefore, this study aimed to investigate the effects of an intermediate time period of exercise treatment (4-months), implemented at the beginning of pathology development (2-months of age), on learning and memory performance, as well as behavioral disturbances (i.e. anxiety, nest construction, circadian rhythms).

Additionally, research has shown that EGCG can decrease amyloid-beta levels, oxidative stress, and neuroinflammation, as well as ameliorating some of the cognitive impairments associated with Alzheimer's disease; however, EGCG treatment had not been yet evaluated in the TgCRND8 mouse strain. The TgCRND8 strain may be a more suitable model compared to Tg2576 for testing treatment effects, due to their high levels of oxidative stress and inflammation (Dudal et al., 2004; Herring et al., 2009) and age-dependent changes in metabolic processes (Salek, et al., 2010), which more closely resemble the pathology of AD in human patients.

Materials, Methods, & Procedures

Animals

Both male and female mice were used. Forty-one TgCRND8 (Tg) mice and twelve wildtype (Wt) littermates bred by the Cell, Molecular and Animal Core Facility of the Alzheimer's Disease Program Project, University of Missouri, Columbia (<http://muadppg.missouri.edu>), survived to the end of the study and were included in the

analyses. We observed a high mortality rate (~35%) in our Tg mice from 2-months to 6-months of age, with 22 deaths (11 female, 11 male) out of 63 total Tg mice. The breeders were obtained from Dr. David Westaway (University of Toronto), and the mice were maintained on a hybrid genetic background (C57/BL6//C3H/HeJ). At 1-month of age, animals were weaned, then genotyped via DNA extraction from tail samples and PCR analysis. Mice were group-housed until 2-months of age, then separated into individual housing.

Animals were maintained on a 12-h light/dark cycle (lights on at 7:00 AM) and given unrestricted access to food and water. Body weight was measured weekly. Each mouse received a cotton pad and an environmental enrichment toy (small plastic house), and was observed for good health before, during and after testing. Behavioral testing occurred during the first half of the light-cycle. All procedures were carried out in accordance with the University of Missouri Animal Care and Use Committee, in accordance with NIH guidelines.

Treatments

Treatments began at 2 months of age. Transgenic animals were assigned to one of four groups (see Table 1): 1) voluntary exercise (n=11) – homecage running wheel, 2) EGCG treatment (n=10) – standard housing and EGCG (>95% purity; herbs-tech.com) dissolved in water (0.2mg EGCG/ml), 3) Combination (Combo) treatment (n=11) – homecage running wheel and EGCG water, or 4) Untreated (Tg-U; n=11) – normal water, standard housing. In addition, a control group (normal water, standard housing) of wildtype littermates (n=12) (Wt/nTg) was used.

Table 1. Housing conditions, gender and samples sizes by group (4 Tg, 1 Wt). All mice were individually housed starting at 60 days of age.

Group	Housing conditions	Sample Size & Gender
Wildtype Control (Wt)	Standard housing	6 F / 6 M
Untreated Tg (Tg-U)	Standard housing	7 F / 4 M
Exercise (Tg-Ex)	Homecage running wheel	6 F / 5 M
EGCG (Tg-EGCG)	0.2 mg/ml EGCG water	5 F / 5 M
Combo (Tg-Combo)	Homecage running wheel + 0.2 mg/ml EGCG water	7 F / 4 M
		<i>N</i> = 55

Wheel-Running

Mice in the voluntary exercise and combination treatment groups had unlimited access to a running wheel (*ENV—44; Med Associates, Inc.; 10.9 cm l x 7.62 cm w x 10.9 cm h*) in their home cage until the end of the experiment (~4-month duration). Data from each wheel were transmitted wirelessly every 30 seconds to a HUB (DIG-804; Med Associates, Inc.), which was set up with a computer in the animal colony room. Raw data were analyzed using Wheel Manager software (Med Associates, Inc.; Windows XP OS). Wheels were cleaned and batteries were checked regularly to ensure proper functioning.

EGCG

Mice in the EGCG and combination treatment groups were provided with a drinking tube containing 0.2 mg/ml EGCG/water solution. Since mice typically drink 4-5 ml water daily, this concentration was chosen to ensure each animal would receive an EGCG dose of at least 50mg/kg body weight daily, an oral administration dose which previous research (Rezai-Zadeh et al., 2008) has shown to be effective at reducing A β -plaque

burden, and both soluble and insoluble forms of A β 1-40 and A β 1-42, as well as reducing cognitive impairments, in the Tg2576 AD model. Water consumption was measured daily, and fresh EGCG water was prepared every day. Additionally, the average weekly dosage of EGCG (mg/kg) consumed was calculated based on consumption values and body weights.

Behavioral Assessment

All mice were tested in four tasks: nest building, the open-field, the light/dark box, and the Barnes maze (see Figure 1). With the exception of nest building, which was measured in each mouse's homecage in the colony room just prior to lights-off, mice were acclimated to the testing room for 30 min prior to the start of each behavioral test, and testing occurred between 9:00am – 1:00pm (first half of the light cycle). Mice were handled for 7 days prior to the start of maze testing.

Figure 1. Procedure by age (days) of mice

Nest Building

Nest construction by mice is important for survival in three ways: 1) conservation of body heat, 2) shelter from predators/environment, and 3) reproduction; this natural behavior of mice has been used to test changes in emotional states, such as apathy and social withdrawal (Boissonneault et al., 2009). Murine models of AD (APPswe/PS1: Filali et al., 2009 & Boissonneault et al., 2009; Tg2676: Deacon et al., 2008), as well as mice with hippocampal lesions, show much poorer nest construction compared to wildtype littermates. It has also been argued that nest-building behaviors may be indicative of executive functioning and behavioral organization in mice (Filali et al., 2009). To my knowledge, this task has not been evaluated in the TgCNRD8 model of AD.

Nest building was assessed at 2 time points: 2-months old (24-hrs after transfer to individual housing) and 6-months old (24-hrs prior to sacrifice). Animals were provided with nesting material in the form of two cotton pads (5 cm x 5 cm each; 5 g total weight). Twenty-four hours later, each animal's nest was video-recorded in the colony room and later scored on a scale of 1-5 (for full details, see Deacon, 2006) by at least two trained, independent coders. Any unshredded nesting material was weighed and recorded for each mouse.

Open-Field

The open-field is a test of exploratory and locomotor activity. The apparatus was made of clear acrylic plastic, and the area measured 40cm x 40cm. A clear, acrylic plastic

wall (40 cm high) surrounded the area. Exploratory activity was measured over one 10 min trial. The apparatus was cleaned before and after each trial with a 20% ethanol solution.

Light-Dark Box

The light-dark box is a behavioral measure of both novelty-seeking and anxiety-like behaviors in mice; specifically, it measures the conflict between two natural tendencies of mice: 1) exploring novel environments, and 2) avoiding aversive properties of brightly lit, open areas (Crawley, 2000).

The apparatus was modified based on the previously described open-field apparatus (40cm x 40cm); specifically, half of the open-area was replaced with a dark, covered compartment. A small opening allowed mice to travel freely between compartments. Testing occurred using one trial. Each mouse was removed from its home cage, placed into the light side of the box, and allowed to move freely for 5 min (1 trial). The maze was cleaned after every trial with a 20% ethanol solution. Each trial was video-recorded (Logitech digital webcam) and analyzed using *Any-maze* software (v.4.72, Stoelting Co.; Windows XP OS). After trial completion, each mouse was returned to its home cage. Four measures were calculated for each mouse: 1) latency to first entry into the dark area, 2) number of entries to the dark area, 3) time spent in the light, and 4) total distance traveled.

Barnes Maze

The Barnes maze is a hippocampal-dependent, spatial learning task, where rodents learn to navigate their way off a brightly lit circular platform into a dark escape box, using stable visual cues located around the maze.

The maze consisted of a brightly lit, grey circular platform 74.9 cm in diameter. A black wall (27.9 cm in height) surrounded the platform to prevent mice from jumping. The platform was elevated 56.5 cm above the floor by a stand. Twenty holes measuring 5.3 cm in diameter were evenly spaced around the perimeter. A triangular black escape box (17.8 cm length x 5.1-10.2 cm wide x 7 cm high) containing a small ramp could be slid into place beneath any hole. The floor beneath the stand was covered with black fabric, and curtains of the same color were hung above the maze 150 cm high from floor level. These curtains surround the apparatus and prevent the mice from using distal visual cues located outside of the maze, as research has indicated that mice show a strong preference for using these cues, even when proximal cues are present (Harrison et al., 2006). Four visual cues consisting of various shapes (triangle, square, circle, vertical bar) were placed at evenly spaced intervals on the inside of the maze wall. Two 150-W lights (encased in aluminum shells) were hung above the platform to create a potentially adverse environment and motivate the photophobic mice to escape from the brightly lit, open surface, in favor of the dark environment of the box. While previous studies (i.e. Prut et al., 2007) have used a buzzer in addition to bright lighting as an aversive stimulus to motivate escape from the platform, the present study did not use such a procedure since research has indicated that TgCRND8 mice show increased auditory startle

responses (McCool et al., 2003).

Each mouse was assigned an escape hole number; assigned hole numbers were alternated by 90 degrees (i.e. 5, 10, 15, 20) to eliminate odor cues for consecutively tested mice. The escape box location remained constant for any individual mouse across test trials. Testing consisted of two shaping trials on the first day, and 8 evaluation trials with two trials per day over a period of four days.

Each day, the animals were transferred from their cage-room to the testing room 30 min prior to the start of testing; this was done to ensure acclimation to the new room and reduce the unnecessary stress of being moved to a new room just before maze training. A trial began by placing the mouse under a black, opaque start box positioned in the center of the platform. After 60 sec, the box was lifted and the mouse had a maximum of 5 min (300 sec) to find and enter the escape box. Latency (time it will take for the mouse to find the escape box) and total errors (nose-pokes into non-escape holes) were recorded. If the mouse did not enter the escape box within 5 min, it was gently guided there by the experimenter's hand. After 30 sec, the mouse was removed from the escape box and returned to their home cage. The platform and escape box were cleaned after every trial with a 20% ethanol solution. Mice were allowed to rest in their home cage for 30 min before starting their second daily trial. All sessions were recorded with a Logitech digital webcam and data were analyzed using *Any-maze* software (v.4.72, Stoelting Co.; Windows XP OS).

Amyloid-Beta Levels

After the cessation of behavioral testing, animals were sacrificed, and their brains were removed and dissected to obtain the cerebral cortex, hippocampus and cerebellum. Brain samples were frozen/stored at -80° C until ELISA testing started.. Cortex and hippocampus samples were homogenized using 0.4% diethylamine solution to extract soluble amyloid-beta protein. Levels of soluble, oligomeric A β 1-42 in the cortex and hippocampus were determined using an ELISA kit, according to the manufacturer's instruction (Invitrogen; Carlsbad, CA). Further details concerning these procedures can be found in Appendix 1.

Results

Raw data for each response measure was checked for normality and equal variances between groups. Variables that violated these statistical assumptions were analyzed using nonparametric statistical tests.

Weekly EGCG dosage intake (mg/kg)

EGCG-water intake (ml) was measured daily, and a weekly average (ml drank) was calculated for each mouse. The intake (ml) was multiplied by the concentration (0.2 mg/ml EGCG), then divided by weekly body weight (kg) to obtain average weekly dosage intake (mg/kg) for each mouse in the EGCG and Combo treatment groups. These data were analyzed using a repeated-measures ANOVA with group as a factor. Results showed no differences in EGCG-intake across weeks [$F(5, 15) = 1.06, p=.518$] or between groups [$F(1, 19) = 0.05, p=.825$] (see Figure 2). Both the EGCG and Combo groups had an average EGCG daily dosage of 50/mg/kg across all weeks of the study.

Figure 2. Average daily EGCG dosage intake (mg/kg) by group and week.

Additionally, univariate tests examining each week separately showed no differences between groups in EGCG dosage intake. Since EGCG dosage did not differ across weeks, all weeks were averaged together to obtain a single measure of EGCG daily dosage for each mouse over the entire course of the study, and this variable was used for the correlations with other measures.

For EGCG-treated mice (*without* access to a running wheel), a significant positive

relationship was found between the average daily EGCG dosage and the total distance traveled during the open-field test ($r=.545$, $n=10$, $p=.05$), but no such relationship was found in the combination Tg group ($r=.269$, $n=11$, $p=.212$). Additionally, there was no relationship between EGCG dosage and running-wheel activity (average daily distance traveled on wheels) for combination Tg mice ($r=.237$, $n=11$, $p=.242$). Taken together, this suggests a possible stimulatory effect of EGCG in Tg mice without access to a running wheel.

No significant relationships were found between EGCG dosage and any of the variables from the Light-Dark Box test (i.e. distance traveled, number of dark entries, latency to first light zone exit, percent time spent in light zone). Additionally, no relationship was found between EGCG dosage and latencies in the Barnes maze task (AuC score). No relationship was found between EGCG dosage and nest building score at 6-months of age.

EGCG daily dosage was negatively correlated with cortical levels of soluble A β 1-42 in the EGCG treatment group ($r=-.575$, $n=9$, $p=.05$), such that mice who drank higher average dosages of EGCG daily showed lower levels of soluble A β 1-42 in the cortex. No such relationship was found for the combination treatment group ($r=.243$, $n=10$, $p=.249$).

Wheel Running

Daily distance traveled (m) was calculated for each mouse with access to a running wheel (exercise & combination treatment groups). These distances were then averaged across each week of the study, for a total of 16 weeks. Daily average running

distances for each week were analyzed using a repeated-measures ANOVA, with week, group and gender as factors. No group differences ($F(1, 18) = .074, p=.79$) or gender differences [$F(1, 18) = .555, p=.47$] were found. Additionally, no effect of week was found [$F(15, 270) = .905, p = 0.56$] (see Figure 3), and no interactions (i.e., group x gender, group x week, gender x week, group x gender x week) were observed. Data for each week was re-analyzed using univariate ANOVAs. There were slightly higher daily distances traveled in the combo group ($M=5023.07$ m/day), compared to the exercise group ($M=3886.45$ m/day), during Week 1 of the study ($F(1, 20) = 2.376, p=.139$), but this was not significant, and no group differences were observed during any subsequent week (see Figure 3).

Figure 3. Average daily distance traveled (m) by week (1-16) and group (Exercise, Combo).

Mice of either treatment group did not significantly increase or decrease their running distances across the 16 weeks of study; males and females did not differ in average daily running distance. Since running wheel activity did not differ across weeks, all weeks were averaged together to obtain a single measure of daily wheel activity (i.e. distance traveled in meters) for each mouse over the entire course of the study, and this variable was used for the correlations with other measures.

Correlational analyses were conducted for the average daily wheel activity and other behavioral measures (A β 1-42 in cortex, total distance in open-field, latencies in the BM, and nest building score). Only two significant relationships were found: Within the exercise-only group, mice who were more active (i.e. traveled greater daily distances on average) tended to have reduced levels of cortical A β 1-42 ($r = -.580$, $p = .031$; $n = 11$). Additionally, for all wheel-running mice, there was a negative correlation between average daily distance traveled and total distance traveled in the open-field ($r = -.447$, $p = .018$; $n = 22$), such that more active mice showed reduced locomotion in the open-field.

Nest Building

Due to unequal group variances, results were analyzed using nonparametric tests (Mann-Whitney U and Kruskal-Wallis) and pairwise comparisons.

At 2-months of age, there was a significant effect of genotype [$U = 36.0$, $p < .001$], such that Tg mice showed poor nest construction compared to wildtype littermates (see *Figure 4a*), as shown by lower nest building scores. At 6-months of age, untreated Tg mice still showed poor nest construction compared to Wt littermates ($U = 9.50$, $p < .001$),

while mice in all three treatment groups showed nest construction comparable to Wt mice (see Figure 4b).

Figure 4a. Nest building score (mean) at 2-months of age by genotype (wildtype=Wt, transgenic=Tg). Results showed poor nest construction in Tg mice compared to Wt littermates.

Figure 4b. Nest building score (mean) at 6-months of age by group (nTg=Wt). Results showed poor nest construction by untreated Tg (Tg) mice compared to wildtype (nTg) littermates. All three Tg treatment groups (EGCG, Exercise, Combo) showed improvements in nest construction compared to untreated Tgs.

In order to further examine any differences between Tg-U and the three Tg treatment groups at 6-months of age, the analysis was re-run, with the Wt control group data excluded. Results showed significant overall differences between Tg treatment groups ($H=14.70$, $df=3$, $p<.002$)(see Figure 4c). Pairwise comparisons revealed that EGCG-treated ($U=68.0$, $p=.065$), exercise-treated ($U=82.0$, $p=.01$) and combination-treated ($U=80.5$, $p=.003$) Tg mice all showed improved nest construction compared to untreated Tg mice. Exercise- and combination-treated mice did not differ from each other in nest score ($U=54.0$, $p=.942$). Exercise-treated mice showed better nest construction compared to EGCG-treated mice ($U=89.0$, $p<.02$). Combination-treated mice also showed better nest construction compared to EGCG-treated mice ($U=82.0$, $p=.01$).

Figure 4c. Nest building score (median) at 6-months of age by Tg treatment group (Tg-U, EGCG, Exercise, Combo). Whiskers represent ± 1 SE.

Nest building scores at 6-months of age were also re-analyzed using a 2 x 2 (EGCG x Exercise) ANOVA, with the wildtype group excluded. Results showed a main effect of EGCG [$F(1, 39) = 7.06, p=.012$], such that all EGCG-treated mice (EGCG & Combo; $M=4.10$) better nest construction compare to mice who did not receive EGCG (Tg-U & Exercise; $M=3.49$). There was also a main effect of exercise [$F(1, 39) = 22.22, p<.001$], such that mice who had access to running wheels (Exercise & Combo; $M=4.34$) showed much better nest construction compared to mice in standard housing without access to a running wheel (Tg-U & EGCG; $M=3.25$). Additionally, an EGCG (exposure or no exposure) x Exercise (exposure or no exposure) interaction was found [$F(1, 39) = 3.61, p=.066$], such that EGCG-treated mice showed better nest construction ($M=3.78$) compared to Tg-U ($M=2.72$), however, mice in both the exercise ($M=4.25$) and combo ($M=4.43$) groups showed better nest construction than the Tg-U or EGCG groups (see Figure 4d).

Figure 4d. Nest building score (mean) at 6-months of age by Tg treatment group. (Tg-U, EGCG, Exercise, Combo). The blue line/points represent the two groups that received normal drinking water, while the green line/points represent the two groups that received EGCG water (.2 mg/ml). The presence or absence of a homecage running wheel is indicated on x-axis, with the standard housing groups (Tg-U & EGCG) on the left, and the running wheel groups (Exercise & Combo) on the right.

Open-Field

Data totals (10 min; 1 trial) were analyzed using nonparametric Kruskal-Wallis tests and Mann-Whitney U for pairwise comparisons.

Totals: Between-groups differences were found for total distance traveled ($H=18.05$, $df=4$, $p=.001$) and number of center area entries ($H=12.04$, $df=4$, $p=.017$). No

differences were found for percentage time spent in the center ($p=.141$).

Pairwise comparisons showed that EGCG-treated Tg mice were hyperactive ($p=.001$) compared to wildtype controls. Untreated Tg mice were also hyperactive compared to Wt littermates, as measured by distance traveled ($p<.01$). No differences in locomotion were observed between the EGCG and Tg-U groups. Mice in the combination group showed reduced locomotion compared to the Tg-U group ($p=.05$) and the EGCG group ($p<.01$), and did not differ from Wt littermates or the exercise group. Mice in the exercise group showed reduced locomotion compared to the EGCG group ($p<.05$), but did not differ from the combination, Tg-U, or Wt groups (see Figure 5a).

Figure 5a. Open-field: Total distance traveled (m/10min) by group

Additionally, mice in the exercise group ($p=.033$), EGCG group ($p=.02$), and combination group ($p=.014$) made more entries into the center area compared to wildtype

mice (data not shown). The Tg-U group also made more entries to the center area compared to Wt mice ($p=.04$), and did not differ from any of the three Tg treatment groups. So, all Tg groups made more center entries compared to Wt littermates, and did not differ from each other. Correlational analyses showed a strong positive relationship between distance traveled and the number of center entries ($r=.752$, $p<.001$) in Tg mice, but no relationship was found in Wt mice ($r=.253$, $p=.383$); this finding makes it difficult to interpret the number of center entries in terms of strictly measuring anxiety, since the higher numbers of center entries may be related to increased locomotor activity. Percent time spent in center may be a better measure of anxiety in these mice, since it showed no relationship to distance traveled for any group (Wt or Tg). Since percent center-time did not significantly differ across groups, there is little evidence for group differences for Tg groups in anxiety-like behavior in the open-field.

Data for total distance traveled was re-analyzed using a 2 x 2 (EGCG x Exercise) ANOVA to examine treatment effects in Tg mice compared to untreated Tgs (Wt group was excluded). Results showed a main effect of EGCG [$F(1, 39) = 4.93$, $p=.032$], such that all EGCG-treated mice (EGCG & Combo; $M=60.45$) were hyperactive compared to mice who did not receive EGCG (Tg-U & Exercise; $M=43.3$). There was also a main effect of exercise [$F(1, 39) = 5.62$, $p=.023$], such that mice who had access to running wheels (Exercise & Combo; $M=42.73$) showed reduced locomotion compared to mice in standard housing without a running wheel (Tg-U & EGCG; $M=61.03$). Additionally, an EGCG x Exercise interaction was found [$F(1, 39) = 3.19$, $p=.082$], such that EGCG mice

without running wheel access showed hyperactivity ($M=76.50$) compared to untreated Tgs ($M=45.56$), as well as compared to the exercise ($M=41.04$) and combo ($M=44.41$) treatment groups (see Figure 5b).

Figure 5b. Open-field: Total distance traveled (m/10min) by Tg treatment group (Tg-U, EGCG, Exercise, Combo). The blue line/points represent the two groups that received normal drinking water, while the green line/points represent the two groups that received EGCG water (.2 mg/ml). The presence or absence of a homecage running wheel is indicated on x-axis, with the standard housing groups (Tg-U & EGCG) on the left, and the running wheel groups (Exercise & Combo) on the right. Results showed that EGCG mice without access to a running wheel were hyperactive compared to all other Tg groups ($p=.08$).

Five-min blocks: Distance traveled was analyzed across 2 time blocks, using a repeated-measures ANOVA with treatment group, sex, and block as factors. There was no main effect of block [$F(1,45) = .301, p=.586$], and no main effect of sex [$F(1, 45) = .083, p=.775$]; however, there was a significant main effect of group [$F(4, 45) = 5.94, p=.001$], a block x group interaction [$F(4, 45) = 2.92, p=.031$] (see Figures 5c-d), as well as a block x sex interaction [$F(1, 45) = 8.11, p=.007$] (see Figures 5e-f). Both male and female wildtype mice showed decreased locomotion across blocks; sex differences across blocks were only obvious in the Tg groups: male Tg mice tended to decrease locomotion (habituated) across blocks, but female Tg mice tended to increase locomotion across blocks.

Figure 5c. Open-field: Distance traveled (m) over time (5 min blocks) by group. Results showed that untreated Tgs (Tg-U) were hyperactive compared to wildtype (Wt) littermates; additionally, EGCG-treated Tg mice were hyperactive compared to untreated Tgs, and compared to Tg mice housed with a homecage running wheel (Exercise, Combo).

Figure 5d. Open-field: Distance traveled (m) over time (5 min blocks) by group (males only)

Figure 5e. Open-field: Distance traveled (m) over time (5 min blocks) by group (females only).

Figure 5f. Open-field: Distance traveled (m) over time (5 min blocks) by gender. The blue line represents male mice, while the green line represents female mice. Results showed that males tended to decrease locomotor activity, while females tended to increase it, across blocks.

Light-Dark Box

Data were analyzed using one-way ANOVAs, with treatment group as a factor and the following as dependent measures: 1) total distance traveled, 2) average speed, 3) latency to first exit from the light zone, 4) percentage time spent in the light zone, and 5) the number of entries to the dark zone. Bonferonni post-hoc tests were used for pairwise comparisons. Mice who never entered the dark zone ($n=2$ females; 1 exercise, 1 combo) were excluded from the analyses.

No between-groups differences were found for distance traveled [$F(4,47) = 0.40$, $p=.807$], average speed [$F(4, 47)=0.97$, $p=.433$], or percentage time spent in the light zone [$F(4, 46) = 0.81$, $p=.543$]. Overall, there was a trend towards group differences in latency to first exit from the light zone [$F(4, 46) = 2.00$, $p=.110$] (data not shown).

Pairwise comparisons showed that the Wt group did not differ from the Tg-U or EGCG groups in latencies. However, the exercise group showed increased latencies compared to Wt mice ($p=.026$), and the combination group also showed increased latencies compared to Wt mice ($p=.012$). Additionally, the Tg-U group did not differ from the EGCG group or the exercise group, but there was a trend towards increased latencies in the combination group compared to the Tg-U group ($p=.091$).

A significant between-groups difference was observed for the number of entries into the dark zone [$F(4, 49) = 2.70$, $p=.041$] (see Figure 6a). Pairwise comparisons showed that Tg mice in both the exercise ($p=.011$) and combo ($p=.018$) treatment groups made fewer dark zone entries compared to Wt littermates, but did not differ from the Tg-U group or each other. The EGCG group did not differ from the Wt or Tg-U groups, but

did make more dark zone entries compared to the exercise ($p=.042$) and combination ($p=.068$) groups. The Wt and Tg-U groups did not show differences in anxiety-like behavior, as measured by dark zone entries.

Figure 6a. Light-Dark Box: Mean number of entries to the dark zone by group (nTg=Wt, Tg=Tg-U, EGCG, Ex=Exercise, Combo). Whiskers represent +1 SE. Results showed that both the exercise and combo groups made fewer entries to the dark zone, compared to the Wt, Tg-U and EGCG groups.

Data for the 1) number of dark zone entries, and 2) latency to first exit from the light zone, were re-analyzed for only the Tg groups (Tg-U, EGCG, Exercise, Combo), using nonparametric Kruskal-Wallis tests and Mann-Whitney U tests for pairwise comparisons. These results were similar to the ANOVA results.

For number of dark zone entries, there was a trend towards Tg group differences

overall ($H=5.23$, $df=3$, $p=.15$) (see Figure 6b).

Figure 6b. Light-Dark Box: Boxplot of median number of dark zone entries by Tg group (Tg-U, EGCG, Exercise, Combo). Stars and circles represent outliers. Results showed that Tg mice with access to running wheels (Exercise, Combo) made fewer entries to the dark zone, indicating reduced anxiety-like behavior, compared to the untreated Tg and EGCG groups.

Pairwise comparisons showed that untreated Tg mice demonstrated increased anxiety-like behaviors by making many more entries to the dark zone compared to the exercise ($U=15.0$, $p=.013$) and combo groups ($U=21.0$, $p=.046$); the Tg-U group did not differ from the EGCG group ($U=46.5$, $p=.902$). The EGCG group showed increased anxiety-like behavior by making more dark zone entries compared to the exercise ($U=26.5$, $p=.072$) and combo ($U=29.0$, $p=.11$) groups. No differences were found between the exercise and combo groups ($U=61.5$, $p=.372$). So, mice with access to a running wheel (Exercise & Combo) showed reduced anxiety-like behavior, as measured

by fewer dark entries, compared to Tg mice in standard housing (Tg-U & EGCG).

For latency to first exit from the light zone (another measure of anxiety-like behavior), there was a significant difference overall between Tg groups ($H=10.72$, $df=3$, $p=.013$) (see Figure 6c).

Figure 6c. Light-Dark Box: Boxplot of latency to the first exit from the light zone (median) by Tg group (Tg-U, EGCG, Exercise, Combo). Stars and circles represent outliers.

Pairwise comparisons showed that untreated Tg mice showed increased anxiety-like behavior compared to Tg mice in the exercise ($U=80.0$, $p=.004$) and combo groups ($U=81.0$, $p=.003$), as measured by decreased latencies to first light zone exit. The EGCG group also showed decreased latencies compared to the exercise ($U=71.0$, $p=.11$) and combo ($U=77.0$, $p=.04$) groups, but showed comparable latencies to the Tg-U group ($U=58.0$, $p=.315$). The exercise and combo groups did not differ from each other in

latencies ($U=59.5$, $p=.473$). So, mice with access to a running wheel (Exercise & Combo) showed reduced anxiety-like behavior, as measured by longer latencies to first exit from the light zone, compared to Tg mice in standard housing (Tg-U & EGCG).

Barnes Maze

Mice who jumped from the platform were excluded from the analyses. Results were analyzed using a repeated-measures ANOVA with day and group as factors, and *latency* and *errors* as dependent measures. Huynh-Feldt corrections were used for violations of sphericity. Bonferonni post-hoc tests were used for pairwise comparisons.

Results showed a significant effect of day, such that all mice decreased their escape latencies [$F(2.82, 129.91) = 30.99$, $p<.001$] and errors [$F(2.43, 111.77) = 15.82$, $p<.001$] across test days. Additionally, there was a significant effect of group for both latency [$F(4, 46) = 3.17$, $p=.022$] and errors [$F(4, 46) = 4.47$, $p=.004$]. (see *Figures 7a & 7b*).

Figure 7a. Barnes maze. Mean latency (s) across days by group

Figure 7b. Barnes maze. Mean number of errors across days by group

Pairwise comparisons between groups showed that untreated Tg mice demonstrated poor spatial learning, as measured by increased latencies ($p=.046$) and errors ($p=.003$) compared to wildtype mice. No significant differences were found between the wildtype group and the exercise, EGCG, or combination treatment groups. However, it is worth noting that there was a nonsignificant trend ($p=.111$) towards increased errors in the EGCG group compared to wildtype mice.

For the purpose of correlating performance in this task with other behavioral measures, as well as amyloid-beta levels, the area under the curve (AuC) was calculated for each mouse, using their latency data across test days.

Amyloid-Beta levels

Cortex: Data were analyzed using a one-way ANOVA with group as a factor and Bonferonni post-hoc tests for pairwise comparisons. Results showed a significant effect of group [$F(4, 48) = 104.19, p < .001$]. Pairwise comparisons indicated that wildtype mice showed much lower levels of soluble A β 1-42 (ng/mg protein) compared to all Tg groups (all $ps < .001$). Untreated Tg mice showed much higher levels of amyloid-beta compared to all three Tg treatment groups (all $ps < .05$), and none of the treatment groups differed significantly from each other (see Figure 8).

Figure 8. Mean levels of soluble A β 1-42 (ng/mg protein) in the cortex by group

In order to further compare the four Tg groups (Wt excluded), data were re-analyzed using nonparametric Kruskal-Wallis tests, with Mann-Whitney U pairwise comparisons. Results showed a significant overall difference between Tg groups ($H=7.66$, $df=3$, $p=.05$). Pairwise comparisons indicated untreated Tg mice (Tg-U) showed higher cortical levels of soluble A β 1-42 compared to the EGCG ($U=25.0$, $p=.063$), exercise ($U=22.0$, $p=.011$), and combo ($U=30.0$, $p=.078$) treatment groups. The combination treatment group did not differ from the EGCG-only group ($U=44.0$, $p=.935$), or the exercise group ($U=67.0$, $p=.395$). The EGCG and exercise groups also did not differ from each other ($U=37.0$, $p=.342$).

Correlational analyses showed a negative relationship between soluble amyloid-beta levels and nest building score in Tg mice ($r=-.411$, $p=.002$), indicating that mice with higher levels of soluble amyloid-beta showed poorer nest building scores. However, no relationship was found in Tg mice between soluble A β 1-42 levels in the cortex and latency performance in the Barnes maze task, as measured by AuC score ($r=-.085$, $p=.303$).

Hippocampus: Results were analyzed using a one-way ANOVA with group as a factor. Results showed a significant difference in soluble A β 1-42 levels between wildtype mice ($M=3.31$) and all groups of Tg mice ($F(4, 44) = 37.82$, $p<.001$). Additionally, all 3 Tg treatment groups (EGCG: $M=344.59$; Exercise: $M=373.33$; Combo: $M=307.76$) showed reduced soluble A β 1-42 levels in the hippocampus compared to untreated Tg mice ($M=490.73$), though this only reached significance in the combination treatment

group ($p=.033$)(see Figure 9).

Figure 9. Mean levels of soluble A β 1-42 in the hippocampus by group.

Discussion

EGCG intake

Previous research using the Tg2576 murine strain of AD found that oral administration of EGCG at a dosage of 50 mg/kg daily improved spatial learning in the RAWM (Rezai-Zadeh et al., 2008). In line with this research, the present study found that the same daily dosage (50 mg/kg) of EGCG improved spatial learning (reduced both latencies and errors) in TgCRND8 AD mice, using the Barnes maze task.

Nest Building

Nest-building behaviors in mice are important to their survival, and are thought to mimic daily living activities in humans. In accordance with previous findings using both single- and double-mutant APP mouse models of AD (Filali et al., 2009, Deacon et al., 2008), we found that untreated Tg mice showed poor nest construction compared to wildtype littermates, at both 2-months (see Figure 4a) and 6-months of age (see Figure 4b). Additionally, poor nest construction at 6-months of age was associated with increased levels of soluble A β 1-42 in the cortex of Tg mice, indicating that this behavioral task may indeed be sensitive to changes in executive functioning in mice, as other authors (Filali, et al., 2009) have argued.

Four-months of unlimited access to a running wheel reversed nest building deficits in Tg mice. Mice supplemented with EGCG (but without access to a running wheel) also showed some improvements in nest building behaviors, but the effect was not as robust in this group compared to Tg mice who had access to running wheels (see Figure 4d). These findings indicate that daily intake of EGCG (at least 50 mg/kg) and daily physical activity can help ameliorate dysfunctions in daily living activities (i.e. nest building) in the TgCRND8 strain; however, regular physical activity may be more effective, compared to daily EGCG intake alone.

Locomotor Activity

EGCG-intake was associated with open-field hyperactivity in mice that did not have access to a running wheel, as shown by increased locomotion compared to all other

groups in the open-field (Figure 5a). Additionally, there was a positive relationship between the average daily EGCG dosage intake and distance traveled in the open-field, but only for EGCG-treated mice that did not have access to a running wheel. No relationship was found between EGCG dosage and locomotor activity in the Light-Dark box for EGCG-only mice. There was a trend towards higher daily distances traveled on the homecage running wheels for mice in the combination treatment group compared to exercise-only mice, but only at the very beginning of the treatment period (Figure 3). For combination-treated Tg mice, no relationship was found between daily EGCG dosage intake and 1) the average daily distance traveled on running wheels, 2) distance traveled in the open-field, or 3) distance traveled in the light-dark box. Taken together, these results provide limited evidence that EGCG may have a somewhat stimulatory effect in Tg mice without access to a running wheel. However, we did not examine EGCG treatment in wildtype mice, nor did we measure baseline locomotor activity prior to EGCG treatment; future research should examine locomotion in Wt and Tg mice before and after EGCG administration.

Anxiety

There are discrepancies in the literature regarding whether there are anxiety differences between untreated TgCRND8 and wildtype mice (see Walker et al., 2011 for a discussion). There is some evidence that EGCG has anxiolytic properties (Vignes et al., 2006), but the study used wildtype CD1 mice, and the elevated-plus maze task. Exercise has been shown to decrease measures of anxiety in a different transgenic AD mouse

model (3x-Tg-AD; García-Mesa et al., 2011). We did not find any differences in anxiety between Tg groups in the open-field test, as measured by percentage of time spent in the center area.

There was a trend towards decreased anxiety in all Tg treatment groups (compared to Wt and Tg-U groups) in the light-dark box test, as measured by increased latency to first exit from the light zone. Additionally, mice with access to running wheels showed reduced anxiety-like behavior compared to untreated Tg mice, as measured by fewer entries to the dark zone, but this effect was not found for EGCG-only mice. Taken together, these findings provide some evidence for exercise reducing anxiety-like behavior in TgCRND8 mice, and very limited evidence for EGCG reducing anxiety-like behavior (increased latency to first light zone exit) in these mice. Given our moderate sample sizes for each group, it is possible that more robust effects would have been found if we had more mice in each group; and if this is the case, it would indicate a weak anxiolytic effect of both voluntary exercise and EGCG treatment, which is in line with other research (García-Mesa et al., 2011; Vignes et al., 2006). Additionally, it is possible that the light-dark box is not as sensitive to detecting changes in anxiety compared to other tests (i.e. elevated-plus maze).

Spatial Learning

In line with our previous work (Walker et al., 2011), untreated TgCRND8 mice demonstrated poor spatial learning performance in the Barnes maze task compared to wildtype littermates, as shown by longer escape latencies and more errors.

We expected the EGCG- and exercise-alone treated Tg groups to show some cognitive improvements (e.g. shorter latencies, fewer errors), based on previous research (Rezai-Zadeh et al., 2008; Adlard et al., 2005a); however, these published studies used longer treatment periods for exercise (5-6 months), and in the case of EGCG-treatment, a different transgenic AD mouse model (i.e. Tg2576). In line with this previous research using longer treatment durations, we found that a shorter treatment duration (4 months) still improved spatial learning latencies in Tg mice, compared to untreated Tg controls. However, in contrast to other research using a combination antioxidant/exercise treatment (3xTg; García-Mesa et al., 2011), no additive effects were found in our combination treatment group; in general, our combination treatment group did not show improved cognitive performance compared to either the exercise-only or EGCG-only treatment groups.

Possible Mechanisms

As mentioned previously, levels of soluble, oligomeric A β 1-42 have been shown to positively correlate with some of the cognitive impairments seen both human and mouse models of AD. High levels of soluble, oligomeric A β -species, particularly A β 1-42, can cause neurotoxicity and mitochondrial dysfunction, and increase oxidative stress and neuroinflammation. With regards to EGCG, research has shown that this polyphenol can reduce amyloid-beta levels by increasing α -secretase activity and decreasing β -secretase activity. The mechanisms by which exercise decreases amyloid-beta levels are less clear, and likely complex. Adlard and colleagues (2005) did not find any differences

steady-state levels of APP, C-terminal fragments, or in secretase activity, suggesting that exercise did not *directly* affect APP production or processing. Additionally, there is some evidence that exercise may be enhancing APP metabolism via activation of the PKC and MAPK pathways, as well as by increasing cholinergic activity (see Turner et al., 2003 & Cotman, et al., 2002 for reviews). Given these research findings, coupled with research showing that exercise can increase neurogenesis in the hippocampus, as well as regulating gene products, and promoting neuroplasticity, it is likely that exercise is affecting APP metabolism through multiple, complex pathways.

Given that the single treatment groups and the combination treatment group were equally effective in reducing soluble A β 1-42 levels in the cortex of TgCRND8 mice, as well as reducing behavioral and cognitive impairments, it is possible that both EGCG and exercise exert much of their beneficial effects on cognition and behavior primarily by reducing soluble amyloid-beta levels. Since soluble A β 1-42 is highly prone to aggregation, reducing its levels in the brain would also be associated with a reduction in extracellular, insoluble A β plaques (as shown in Adlard et al., 2005), and thereby reduce levels of oxidative stress and neuroinflammatory responses in a secondary fashion. However, it is also possible that exercise and/or EGCG interfered with inflammation and oxidative stress on a primary level; research has shown that voluntary exercise can reduce oxidative damage in the brains of aged animals without AD (Cui et al., 2007).

The present study showed that 4 months of EGCG dietary supplementation (~50 mg/kg daily) and homecage access to a running wheel (both alone and in-combination),

beginning at the *onset* of disease pathology, improved nest building behaviors, improved spatial learning in the Barnes maze, and reduced levels of soluble A β 1-42 in the cortex of TgCRND8 mice (~25% decrease); additionally, high A β 1-42 levels were associated with poorer nest construction in Tg mice. These findings fit well with previous research using this dosage of EGCG in the Tg2576 strain (Rezai-Zadeh et al., 2008), as well as research using voluntary exercise treatment in the TgCRND8 strain (Adlard et al., 2005). Exercise did seem to be more effective than EGCG-alone in improving nest building behaviors, as well as reducing locomotor hyperactivity and anxiety-like behaviors.

Conclusion

Oral administration of EGCG, as well as a moderate treatment duration of voluntary exercise can ameliorate some of behavioral manifestations and cognitive impairments of Alzheimer's disease as modeled by the TgCRND8 strain. Previous research using the TgCRND8 strain (Adlard et al., 2005) found that voluntary exercise reduced soluble A β 1-42 levels in the cortex and improved spatial learning in the MWM, when treatment began *before* the development of disease pathology; the present study shows that regular physical activity, as well as daily EGCG dietary supplementation, can exert beneficial effects on cognition and lower amyloid burden in the cortex of TgCRND8 mice, when treatment is started at the *onset* of disease pathology.

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Appendix 1. Examples of nests by score (1-5). Nests were scored on the following scale (Deacon, 2008): 1) Nestlet untouched, 2) Nestlet partially shredded (10-25% shredded). 3) Nestlet mostly shredded, but no clear/organized nesting site, 4) Nestlet mostly shredded, clear nesting site, flat nest shape, 5) Nestlet mostly shredded, clear nesting site, nest includes walls.

1

2

3

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VITA

My research interests are in the field of neuroscience, specifically the processes underlying learning and behavior and the expression of pathology. I am currently engaged in behavioral assessment of animal models of disease, including: Alzheimer's disease, stroke, and traumatic brain injury. I have a specific interest in evaluating the impact of potential behavioral and pharmacotherapies on learning and memory in animal models of disease. Recently, I have conducted research involving TgCRND8 mice as a murine model of Alzheimer's disease; and I am currently conducting research examining the effects of voluntary exercise and EGCG on cognitive and non-cognitive behaviors in this line of mice.