INFLUENCES OF DIET, EXERCISE, AND STRESS ON HIPPOCAMPAL HEALTH IN DEPRESSION AND ALZHEIMER'S DISEASE

INFLUENCES OF DIET, EXERCISE, AND STRESS ON HIPPOCAMPAL HEALTH IN DEPRESSION AND ALZHEIMER'S DISEASE

By CRAIG PARKER HUTTON, Hons.B.Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy (Ph.D.)

Descriptive Note

McMaster University DOCTOR OF PHILOSOPHY (2018)

Hamilton, Ontario, Canada (Psychology, Neuroscience, and Behaviour)

TITLE: Influences of Diet, Exercise, and Stress on Hippocampal Health in Depression and Alzheimer's Disease

AUTHOR: Craig Parker Hutton

SUPERVISOR: Professor Suzanna Becker

NUMBER OF PAGES: xii, 180

For my father, Stan, and grandfather, Lloyd.

Abstract

Chronic stress and Alzheimer's disease (AD) both lead to degenerative changes in the hippocampus, a brain structure involved in episodic memory and regulation of the stress response. Mechanisms of aging (inflammation, oxidative stress, membrane damage, mitochondrial dysfunction, and insulin resistance) and a loss of brain-derived neurotrophic factor (BDNF), occur in cases of both stress-related depression and AD. Three studies were conducted using mouse models to determine whether exercise or treatment with an anti-aging multi-ingredient supplement (MDS) designed to counteract these aging mechanisms could protect the hippocampus, and associated behavioural functions, from either stress or AD. The first experiment revealed that the upregulation of neurogenesis by aerobic exercise in c57Bl/6 male mice does not occur after stress exposure. The MDS and exercise, but neither intervention alone, alleviated anhedonia, upregulated BDNF and increased neurogenesis.

The other two experiments evaluated whether the MDS could counteract a range of AD behavioural and biological manifestations in both sexes of the 3xTg-AD mouse model. At 3-4 months of age, 2 months of MDS-supplementation protected 3xTg-AD mice from developing deficits in working memory and spatial learning seen in vehicle-treated transgenic mice. The MDS continued to benefit 3xTg-AD females, but not males, on tests of 24-h recall under conditions of high interference until 11-12 months of age, along with upregulating hippocampal BDNF. The MDS also attenuated the splenomegaly seen in 3xTg-AD mice and normalized the previously undiscovered aberrant recruitment of CA1 and CA3 neurons by 3xTg-AD males during spatial encoding.

This work supports the use of diet and exercise to buffer against major depressive disorder (MDD) and AD in part by acting upon the hippocampus. It also recommends the use of lifestyle-based interventions to promote functional improvements in MDD or AD, and further elucidates the potential of BDNF and neurogenesis as therapeutic targets in counteracting these debilitating conditions.

Acknowledgments

First and foremost is my deepest gratitude to my supervisor, Sue Becker, for her tremendous support and seemingly endless supply of academic wisdom over the years. Her advice will undoubtedly continue to benefit my research, writing, and teaching throughout my career. I would also like to express my appreciation to my supervisory committee members, Margaret Fahnestock, Dave Rollo, and Bruce Milliken. Margaret has been a great mentor in the complex world of molecular biology, who taught me more than anyone the necessity of precision in science. Her expertise in neurotrophin and Alzheimer's disease biology has also been an invaluable intellectual resource during my studies. Whenever I converse with Dave I am always impressed by his ability to distill a complex array of information into a coherent and interesting story. I would likely still be struggling to make sense of the mountain of mouse data we collected without his insight and advice. I would like Bruce to know how much I appreciate the time he took to show me how to effectively communicate my research. His influence plays a big part in the extent to which my writing and presentations are accessible to anyone without an advanced degree in behavioural neuroscience. Thanks as well to the external examiner for taking the time to assist with the defense of this thesis.

As the supervisor of my immunohistochemical work and an expert in adult hippocampal neurogenesis, Martin Wojtowicz (at the University of Toronto) has been an especially important role model for me. Without his support and guidance, I would not have obtained some of my most interesting results. The time I've spent in his lab also fueled my fascination with neural stem cells and surely contributed to my desire to continue studying them as a postdoctoral fellow and independent investigator in the future. My other collaborators: Olga Shevtsova, Jennifer Lemon, Doug Boreham, Minesh Kapadia and Boris Sakic, also deserve special recognition. When reading the list of authors in chapters 2-4, I urge you to recall my sincere appreciation of their contribution to this work, which was very much a team effort. Moreover, I happily share the credit for my research achievements with Dave Rollo, Jennifer Lemon, and Doug Boreham, who originally invented the multi-ingredient dietary supplement.

I thank friends and fellow lab members, Kiret Dhindsa, Saurabh Shaw, Nadia Wong, Nick Déry, Laura Keeting, Aaron Goldstein, and Ranya Amirthamanoharan for their helpful feedback on my lab meeting presentations and interesting conversations. I also can't forget the many undergraduate students who have been extremely helpful in bringing my studies to completion, particularly with respect to behavioural testing of the mice, feeding the dietary supplement to the mice, and video scoring. Thanks Kyle Fitzgibbon, Andrew LoGuidice, Damian Frederick, Patrick Te, Samantha Pasternak, Grace Lee, Norhan Elsaadawy, Daniela Chok, Hamnah Shahid, MacKenzie Campbell, Zain Patel, Lauren Ribeiro, Ledor Babatinca, Judith Tran, Gurleen Kaur, Kripa Thomas, Nicole Ramseyer, Braeden Terpou, Allison Mizzi, Miriam Kryzewska, Melanie Lysenko-Martin, Marija Radenovic, Imasha Perera, Andrea Alves, Andrenne Lavoie, Anisha Khosla, Dayle Parker, Shireen Fikree, and Emily Kaunismaa. In

addition, the assistance of Vadim Aksenov in the Rollo lab during tissue sample collection is really appreciated.

Without the support of my parents, the pursuit of this degree would have been much harder. Thanks Stan Hutton, Barb Hutton, and Suzanne Smith. Paul Ouellette, my closest friend since elementary school, has been one of my greatest supporters over the years, and some of my conversations with him initially sparked my interests in neuroscience and psychology. I would also like to express my appreciation to Blair Ellis, my closest friend from McMaster, for commiserating with me when grad school was tough, and helping me think through some difficult problems. Last, but not least, is my appreciation for my girlfriend, Melanie. She inspires me to improve every day and made one of the most challenging periods of my life one of the best. I can't wait for our lives to move forward together.

Contents

Declaration of Academic Achievement

This thesis includes three published or submitted manuscripts which address using lifestyle-based interventions to improve hippocampal function in mouse models of major depressive disorder and Alzheimer's disease. A list of these papers and my contributions to each are specified below.

Chapter 1:

Author: Craig P. Hutton

Chapter 2:

Manuscript:

Hutton, C. P., Déry, N., Rosa, E., Lemon, J. A., Rollo, C. D., Boreham, D. R., Fahnestock, M., deCatanzaro, D., Wojtowicz, J.M., & Becker, S. (2015). Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice. *Neuroscience*, *308*, 180-193.

Comments:

This project was initially started by a former PhD student in Dr. Becker's lab, N.D. The experiment was designed by N.D., D.d., M.F., and S.B. When N.D left the project and I took the lead on it in 2014, the in vivo portion of the study had been finished (i.e. the experimental design, administration of the stress, diet, and exercise interventions), but the data analysis, writing, and most of the bioassay work remained to be completed. I collected the majority of the biomarker data. I also analyzed all the data and took the lead on writing the manuscript, which was edited by S.B., M.F., C.D.R, and D.d.).

Chapter 3:

Manuscript:

Hutton, C. P., Lemon, J. A., Sakic, B., Rollo, C. D., Boreham, D. R., Fahnestock, M., Wojtowicz, J.M., & Becker, S. (2018). Early intervention with a multi-ingredient dietary supplement improves mood and spatial memory in a triple transgenic mouse model of Alzheimer's disease. *Journal of Alzheimer's Disease*, *64*(3), 835-857.

Comments:

I collaboratively designed the experiment with S.B., B.S., M.F. and J.M.W (I defined the original research question). B.S., J.A.L., and I established the 3xTg-AD and wild-type mouse breeding colonies. I developed the behavioural test battery with S.B., B.S., and one of B.S.'s former PhD students, Minesh Kapadia. I prepared the multi-ingredient dietary supplement (MDS) under the supervision of J.A.L. and C.D.R. I administered the MDS with the assistance

of undergraduate students. The data were collected by J.A.L., myself, and numerous undergraduate students (acknowledged in the manuscript). I analyzed the data. I also took the lead on writing the manuscript, which was edited by S.B., J.A.L., C.D.R, and M.F.

Chapter 4:

Manuscript:

Hutton, C. P., Kaunismaa, E., Lemon, J. A., Shevtsova, O., Kapadia, M., Sakic, B., Rollo, C. D., Boreham, D. R., Fahnestock, M., Wojtowicz, J. M., and Becker, S. (Submitted). Sexually dimorphic effects of a multi-ingredient dietary supplement on physical, cognitive and immunological function in a triple transgenic mouse model of Alzheimer's disease. 1-55.

Comments:

I collaboratively designed the experiment with S.B., B.S., M.F. and J.M.W (I defined the original research question). B.S., J.A.L., and I established the 3xTg-AD and wild-type mouse breeding colonies. I developed the behavioural test battery with S.B., B.S., and K.M. I prepared the multi-ingredient dietary supplement (MDS) under the supervision of J.A.L. and C.D.R. J.A.L. and I administered the MDS with the assistance of undergraduate students. The data were collected by J.A.L., myself, and numerous undergraduate students (acknowledged in the manuscript). I analyzed the data. I also took the lead on writing the manuscript in collaboration with E.K., S.B., C.D.R, and M.F.

Chapter 5:

Author: Craig P. Hutton

Preface

Dear reader,

This document has been prepared in a "sandwich" format. This means that the chapters have been written in the style of academic journal articles and that each chapter is a self-contained unit which assumes no knowledge of the other chapters. Since each chapter already provides a detailed introduction to, and discussion of, the relevant background literature, the main introductory section of the thesis (Chapter 1) has been limited to more general information aimed at facilitating comprehension of the other chapters.

To facilitate the reading process, all figures, references and appendices pertaining to a chapter are included at the end of that chapter rather than placing all visual and supplementary information at the end of the manuscript in a separate section. This should simplify the reading process by minimizing interruptions to the reading flow.

Chapters 2 and 3 are reproduced *verbatim* from published articles. Copyright information for these chapters is also included on the first page of them. It is worth noting that the dietary supplement used in the experiments described in Chapters 2-4 is referred to as the "complex dietary supplement (CDS)" in Chapter 2 but was then re-named upon the recommendation of Dr. Rollo to the "multi-ingredient dietary supplement (MDS)" in Chapters 3 and 4. Since Chapters 2 and 3 have both been published with these two different labels, this same supplement is only referred to as the CDS in Chapter 2, while it is labelled as the MDS elsewhere in the thesis.

1. An introduction to the Hippocampus, Depression, and Alzheimer's Disease

1.1. Depression, stress and the aging hippocampus

Major depressive disorder (MDD) is a devastating mood disorder and a leading cause of disability worldwide (World Health Organization; http://www.who.int/), affecting approximately 350 million people. MDD is characterized by prolonged sadness combined with a mosaic of psychological symptoms such as apathy, anhedonia, irritability and insomnia, often disrupting work and personal relationships and potentially leading to suicide (Belmaker & Agam, 2008). MDD also contributes to age-related mental and physical decline and increases the risk of death by 2-3x (Fiske et al., 2009). MDD affects 5.8% of Canadians aged 15-64 (Public Health Agency of Canada; http://www.phac-aspc.gc.ca/), and while the prevalence of major depression is lower in community dwelling adults over 65 years of age (~1.7%), the prevalence of depressive symptoms is significantly higher in this group than in younger adults (~15%; Fiske et al., 2009). Over half of geriatric patients with depression experience their first episode after the age of 60 (i.e. late-onset), demonstrating the high probability that the risk factors for MDD in older adults differ to some extent from those which affect younger adults. The economic toll of depression is substantial and rapidly increasing, growing by 21.5% to $210 billion between 2005 and 2010 in the United States (Greenberg et al., 2015). In 2013, the cost of depression in Ontario alone was also substantial, at approximately $276 million (Chiu et al., 2017). Thus, there is a tremendous need to investigate lifestyle factors that promote resilience against depression and healthy aging for the benefit of both individuals and society.

Chronic stress has a toxic impact on the brain and is widely agreed to be a major causal factor in the pathogenesis of MDD and other mood disorders (McEwen, 2003). In the elderly, a meta-analysis of 20 studies identified life stress due to bereavement as the greatest risk factor for MDD (Cole & Dendukuri, 2003), increasing the risk by 3.3x. Work in rodents (Sapolsky, 1985) and primates (Sapolski et al., 1990) has shown that stress is particularly damaging for the hippocampus, a region of the brain that also atrophies with age and is critically involved in episodic and declarative memory (Tulving & Markowitsch, 1998) as well as regulation of the stress response (Surget et al., 2011). Moreover, the degree of hippocampal volume loss in humans with MDD is highly related to the number of past episodes (MacQueen et al., 2003) and lifetime illness duration (Sheline et al., 1999). Evidence from animal models and humans suggests that inflammation and oxidative stress may be mediating factors in psychological stress-induced depression. In rodents, chronic mild stress leads to oxidative damage (Fontella et al., 2005) and inflammation (Zhang et al., 2016) in the hippocampus, as well as depressive-like behaviour and impaired performance on tests of hippocampal-dependent memory such as the water maze, effects that were prevented by anti-oxidant treatment (Nagata et al., 2009; Zhang et al., 2016). In humans, psychological stress and major depression are associated with increases in serum and cerebrospinal fluid (CSF) markers of inflammation and oxidative stress, both of which are alleviated by anti-depressant medication (Bilici et al., 2001; Tuglu et al., 2003).

Roles for oxidative stress and inflammation in the aging process are also well-established (Harman, 1956; Harman,1972; Liochev et al., 2013; Walford, 1964; Caruso et al., 2004). With respect to oxidative stress, the rate of oxygen radical production by mitochondria is inversely correlated with lifespan in numerous mammalian species (Ku et al., 1993). The lifespan of the fly *Drosophila melanogaster* is also extended by ~33% among flies which have been genetically modified to overexpress antioxidative enzymes (Orr & Sohal, 1994), while caloric restriction also reduces oxidative damage and extends lifespan in rodents (Ross, 1966; Sohal et al., 1994). With respect to the involvement of chronic inflammation in aging, white blood cells isolated from the blood of older adults produce more pro-inflammatory cytokines than those from younger adults (Fagiolo et al., 1993). In addition, plasma levels of the pro-inflammatory cytokine interleukin-6 are negatively associated with lifespan (Bonafè et al., 2001; Wassel et al., 2010), while genetic alleles associated with elevated levels of the anti-inflammatory cytokine interleukin-10 (IL-10) are more common among centenarians than younger adults, and polymorphisms resulting in low IL-10 production are associated with cardiovascular disease (Lio et al., 2004).

Dietary and exercise-based interventions show great potential as means of minimizing oxidative stress and inflammation. Aerobic exercise elevates neuroplasticity and memory and reduces symptoms of anxiety and depression in mice (Radecki et al., 2003; Marais et al., 2009). Evidence from animal models suggests that these behavioural effects are driven by the impact of exercise on brain-derived neurotrophic factor (BDNF; Neeper et al., 1995; Vaynman et al., 2004; Gomez-Pinilla et al., 2011), a critical moderator of neuron survival, growth and plasticity (Fahnestock, 2011). Chronic stress increases glucocorticoid levels (Smith et al., 1995) and decreases BDNF expression in the hippocampus of rats (Marmigère et al., 2003). Moreover, experimentally increasing BDNF levels protects against the development of learning and memory impairments and depressive-like behaviour in stress-exposed animals (Radecki et al., 2003; Marais et al., 2009). Further research has shown that BDNF upregulates endogenous antioxidants, which may underlie its neuroprotective effect against oxidative stress (Spina et al., 1992) and inflammation (Wu et al., 2011; Lee et al., 2012). Thus, by increasing BDNF, exercise may be a means by which individuals can reduce their risk of MDD.

Some of the cognitive alterations seen in depression are remarkably like those induced by head irradiation exposure or anti-mitotic chemotherapeutic drugs (see Becker & Wojtowicz, 2007, for a review) both of which are highly toxic to newly born neurons in the adult dentate gyrus of rodents (DG; Snyder et al., 2001; Laack & Brown, 2004; Winocur et al., 2006; Winocur et al., 2012; Winocur et al., 2014). In fact, the behavioural efficacy of serotonergic antidepressant medications depends in part upon upregulation of neurogenesis (Malberg et al., 2000; Santarelli et al., 2003), implicating neurogenesis as another excellent therapeutic target for stress-related disorders. In terms of behaviour, a vast and expanding literature has identified that these adult-born neurons, first discovered by Altman and Das (1965; 1967), are required for at least a few related cognitive abilities: the reduction of memory interference during encoding of

similar items (Leutgeb et al., 2007; Winocur et al., 2012; Luu et al., 2012), successful adaptation to novel or enriched environments (Kempermann et al., 1997; 1998; Kempermann 2002; 2008) and possibly in episodic memory encoding (Aimone et al., 2006; Rangel et al., 2014). In particular, the roles of neurogenesis in mitigating interference, as originally predicted by Becker's computational model (2005), and adapting to novelty, as proposed by Kempermann (2002), are now supported by a great deal of evidence from challenging discrimination tasks (Creer et al., 2010; Sahay et al., 2011; Yassa et al., 2010; Niibori et al., 2012), tests of reversal learning or cognitive flexibility (Garthe et al., 2009; Winocur et al., 2012; Luu et al., 2012; Burghardt et al., 2012), and novel object (Jessberger et al., 2009) or context discrimination (Tronel et al., 2012). The heightened plasticity of these newborn neurons (Ge et al., 2006; 2007) also renders them susceptible to the influence of a wide range of environmental factors (e.g. dietary antioxidants, Valente et al., 2009; or exercise, Van Praag et al., 1999, 2005), consequently affecting behaviour.

Nutrition is at least as relevant to MDD and brain aging as exercise. Although overall dietary patterns are more complicated to evaluate than an individual's level of physical activity there is evidence that both individual nutrients (e.g. omega-3 fatty acids, Sinn et al., 2012) and certain dietary patterns (e.g. Mediterranean diet; Sánchez-Villegas et al., 2015) can reduce the risk of age-related cognitive decline and improve depressive symptoms. For example, a 9-year longitudinal study found an association between adherence to a Mediterranean-style diet (high in fish, olive oil, nuts, fruits and vegetables, while low in dairy and red meat) and reduced age-related decline in episodic memory (Pelletier et al., 2015). In contrast, malnutrition or adherence to a western style diet (high in saturated fats, red meat, poultry and starches, low in fruits, fish and vegetables) is associated with an increased risk of MDD (Cabrera et al., 2007; Akbaraly et al., 2009) and accelerated age-related cognitive decline (Kanoski et al., 2011; Saka et al., 2010).

The evidence reviewed above suggests that a combination of dietary factors and aerobic exercise may reduce oxidative stress and inflammation and increase BDNF and neurogenesis, thereby imparting a degree of resilience to the brain against depression and memory loss.

1.2. The Hippocampus and Alzheimer's disease

Alzheimer's disease (AD) is a devastating neurodegenerative condition that remains refractory to treatment. Since the initial report by Alois Alzheimer (see Stelzman et al., 1995, for an English translation) over a century ago, most research into AD pathogenesis has focused on counteracting the accumulation of misfolded proteins amyloid-β (Aβ) and hyperphosphorylated tau (p-tau) into the hallmark plaques and tangles that are observed throughout the brains of AD patients at autopsy. The most well-established theory of AD pathogenesis, the amyloid cascade hypothesis (Hardy & Higgins, 1992; Selkoe & Hardy, 2016), posits that AD is primarily caused by either an overproduction of Aβ or a deficit in the clearance of Aβ. Briefly, Aβ is produced

biochemically from the proteolytic cleavage of the integral membrane protein amyloid precursor protein (APP) by β- and γ-secretase enzymes, a process which generates several products including Aβ40 and Aβ42. Genetic mutations in either APP or the active site portion of the γ-secretase enzyme, called presenilin 1 or presenilin 2 (PS1/2), cause early-onset AD in humans. These Aβ fragments then aggregate into larger and larger forms from small toxic oligomeric species up to less toxic fibrillary species of amyloid that give rise to the characteristic plaques seen in AD brains (Shankar et al., 2008). As implied by the "cascade hypothesis", Aβ40 and Aβ42 have multiple downstream neurotoxic effects, including the hyperphosphorylation of tau, followed by the formation of neurofibrillary tangles (the other hallmark lesion of AD), dissociation of axonal microtubules, synaptic atrophy and cell death (Alonso et al., 1996; Jin et al., 2011).

While the amyloid hypothesis has dominated clinical research over the past 25 years, it has substantial limitations, most notably that nearly all human clinical trials aimed at reducing Aβ directly have failed, and more importantly that genetic mutations affecting either the biochemical pathways leading to Aβ production (e.g. APP or PS1) or clearance (e.g. ApoE4) only represent the minority of AD cases (for a review see Herrup, 2015). Which factors then, in addition to Aβ production or clearance, are altered in most AD cases? Evidence in the literature is accumulating that late-onset (or sporadic) AD may represent a form of accelerated brain aging (Gatta et al., 2014), in that AD model mice begin to show age-related changes in gene expression (mitochondrial functioning, inflammation, synaptic plasticity, etc.) much earlier than healthy animals. More convincingly, despite the well-known absence of a naturally developing AD-like pathology in rodents under standard laboratory conditions (Bilkei-Gorzo, 2014), a couple of reports suggest that animal and/or cell culture models (without mutations directly in the Aβ or p-tau pathways) can exhibit AD-like pathology following manipulations involving systemic immune activation (Krstic et al., 2012) or mitochondrial challenge associated with oxidative stress (Leuner et al., 2007). Furthermore, mitochondrial dysfunction (Mosconi et al., 2008), oxidative stress (Lovell et al., 2011), and inflammation (Tarkowski et al., 2003) predict the conversion from mild cognitive impairment to AD in humans.

The cognitive deficits and biochemical alterations (e.g. inflammation and oxidative stress) observed in AD (Mattson, 2004) have been associated with reductions in brain-derived neurotrophic factor (BDNF; in humans; Peng et al., 2005) and neurogenesis (in rodents; Rodríguez et al., 2008). Elevated BDNF levels increase neurogenesis in rat brain (Pencea et al., 2001). In rodent models of AD, cognitive deficits are ameliorated by upregulation of BDNF (Nagahara et al., 2009) and neurogenesis (Blanchard et al., 2010). Moreover, antioxidants elevate levels of neurogenesis (Casadesus et al., 2004; Valente et al., 2009) and BDNF (Williams et al., 2008; Fahnestock et al., 2012) in non-human animals. Aerobic exercise is also a well-known inducer of both neurogenesis (van Praag et al., 1999, 2005; Creer et al., 2010; Winocur et al., 2014) and BDNF (Neeper et al., 1995; Erickson et al., 2011; Gomez-Pinilla et al., 2011). Recent evidence from animal studies suggests that multi-intervention approaches may be much more

effective at counteracting age-related degeneration than single factor interventions (Wu et al., 2008; Fahnestock et al., 2012). Thus, anti-inflammatory and antioxidant nutrients with, or without, exercise may also prevent or delay AD.

1.3. Overview of This Thesis

Motivated by the literature described above, the overarching goal of this work was to determine whether lifestyle-based interventions such as aerobic exercise or complex dietary supplementation could protect a highly plastic region of the brain, the hippocampus, and its associated behavioural functions, from the neurotoxic effects of chronic psychological stress or Alzheimer's disease. To achieve this goal, we followed a different approach (to single-compound pharmacological intervention) based on the emerging literature implicating physiological mechanisms of aging and a loss of neurotrophic support (e.g. BDNF) in depression, cognitive decline and AD (see sections 1.1 and 1.2). In contrast to nearly all other studies on animal models of depression (e.g. Surget et al., 2008) and AD (e.g. Parachikova et al., 2010; Wolf et al., 2012), we also chose to focus on prevention rather than treatment. Experimental results from studies in mice are presented here from a basic research perspective with an emphasis on translation to humans in the future. Although there is extensive discussion of differences that were observed in the hippocampus and other biomarkers, the main emphasis is upon functional outcomes at the symptomatic (behavioural) level, which have the greatest clinical relevance. Findings presented in this thesis have important implications for the treatment and understanding of depression, brain aging, Alzheimer's disease, and other conditions such as anxiety disorders or frontotemporal dementia. They strengthen the case for the great potential of using diet and exercise to maintain brain health throughout the lifespan. A more detailed overview of each chapter is provided below.

In **Chapter 2**, using male c57BL/6 mice we examined the effects of a 4-week-long unpredictable series of stressors (compared to mice which had not been disturbed) on sucrose preference, a measure of anhedonia (a core symptom of depression; Belmaker & Agam, 2008), and a number of hippocampal biomarkers including BDNF expression, neurogenesis and the average cross-sectional (coronal) area of the DG and CA1. With the future translatability of our work to humans in mind, we also examined serum protein levels of BDNF, vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1). Mice were also assigned to receive access to running wheels (exercise condition) or not, daily supplementation with a complex dietary supplement or vehicle control, or the combination of both for the duration of the experiment. This study served to address the depression component of the overall thesis goal, in that we sought to evaluate whether dietary supplementation alone, exercise alone, or the combination of diet and exercise could protect mice from exhibiting persistent depressive-like behaviour or hippocampal degeneration under conditions of chronic stress.

Chapter 3 describes the first half of a longitudinal experiment we conducted on the multiple-ingredient dietary supplement (MDS) using the 3xTg-AD mouse model of Alzheimer's disease. We administered the MDS (which is the same supplement as the CDS in Chapter 2), or a vehicle treatment, to both sexes of triple transgenic (3xTg-AD) mice and B6129SF2/J wild-type mice for 2 months from 2-4 months of age. Mice were tested in a battery of behavioural tests at 1-2 (prior to treatment) and 3-4 months of age (after treatment). Since it has become clear that there are important sex differences in the 3xTg-AD model (e.g. Stover et al., 2015), we decided to include both sexes of mice in this study. Although we would have also preferred to examine a combination of dietary supplementation and exercise in this study (and Chapter 4) as well, limited resources, capacity to house and test animals in the behavioural battery, and timing constraints prevented us from including exercise as a separate or synergistic intervention in the experiment. The MDS, rather than exercise, was chosen as the intervention for these experiments because the aging mechanisms targeted by the MDS are more strongly implicated in the pathogenesis of AD than MDD (particularly inflammation and oxidative stress, as described above). Age is, after all, still the single greatest risk factor for AD (Lindsay et al., 2002; Prince et al., 2013). This chapter therefore addresses a portion of the AD component of the thesis goal, to evaluate whether a complex dietary supplement can delay the onset of early behavioural changes in AD using the 3xTg-AD model mouse system.

Chapter 4 extends the work of Chapter 3 by describing two experiments which sought to determine whether an extended period of MDS supplementation (6 months in Experiment 1 and 10 months in Experiment 2) would have long-lasting benefits on behaviour and a variety of biomarkers including hippocampal neurogenesis, the pattern of neuronal activity across hippocampal subfields following spatial learning, and BDNF levels. In the first experiment, both sexes of 3xTg-AD mice were fed the MDS or a vehicle control treatment from 1-7 months of age. From 6-7 months of age, half of the animals from each group were tested in a behavioural battery followed by post-mortem biomarker assays. By comparing levels of adult neurogenesis between mice which had been tested in the behavioural battery to those which had not, we also addressed the potential confound that the experience of training on a ~month long battery of behavioural tests might act as a source of environmental enrichment, which might affect neurogenesis on its own (e.g. Kempermann et al., 1998).

In the second experiment, we continued supplementing the male and female 3xTg-AD mice and WT mice used in Chapter 3 until they reached 12 months of age and re-tested them in the behavioural battery from 11-12 months of age. After the completion of behavioural testing, an expanded set of biomarkers was examined, including: brain and spleen size, TBS-soluble amyloid-β (Aβ) in the cortex, hippocampal BDNF mRNA, subgranular zone (SGZ) neurogenesis, DG and CA1 volume, and upregulation of the immediate early gene c-Fos after water maze reversal learning. Together, these experiments sought to determine if a complex dietary supplement could protect against AD-mediated changes in hippocampal structure and

function (e.g. memory encoding, test performance) using the 3xTg-AD mouse as a model system.

Chapter 5 consists of a summary of the main results of the empirical chapters (2-4), followed by a general discussion of the limitations of the work and some promising avenues of future research. There is also a description of some preliminary work that I have been doing in collaboration with Sue Becker, Melanie-Lysenko Martin, Parminder Raina, Lauren Griffith, and Anne Gilsing to examine relationships between nutrition, exercise, mood and cognition in over 30,000 Canadians between 45-86 years old using data from the Canadian Longitudinal Study on Aging.

References

Aimone, J. B., Wiles, J., & Gage, F. H. (2006). Potential role for adult neurogenesis in the encoding of time in new memories. *Nature neuroscience*, *9*(6), 723.

Akbaraly, T. N., Brunner, E. J., Ferrie, J. E., Marmot, M. G., Kivimaki, M., & Singh-Manoux, A. (2009). Dietary pattern and depressive symptoms in middle age. *The British Journal of Psychiatry*, *195*(5), 408-413.

Alonso, A. D. C., Grundke-Iqbal, I., & Iqbal, K. (1996). Alzheimer's disease hyperphosphorylated tau sequesters normal tau into tangles of filaments and disassembles microtubules. *Nature medicine*, *2*(7), 783-787.

Altman, J., & Das, G. D. (1965). Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *Journal of Comparative Neurology*, *124*(3), 319-335.

Altman, J., & Das, G. D. (1967). Postnatal neurogenesis in the guinea-pig. *Nature*, *214*(5093), 1098.

Becker, S. (2005). A computational principle for hippocampal learning and neurogenesis. *Hippocampus*, *15*(6), 722-738.

Becker, S., & Wojtowicz, J. M. (2007). A model of hippocampal neurogenesis in memory and mood disorders. *Trends in cognitive sciences*, *11*(2), 70-76.

Belmaker, R. H., & Agam, G. (2008). Major depressive disorder. *New England Journal of Medicine*, *358*(1), 55-68.

Bilkei-Gorzo, A. (2014). Genetic mouse models of brain ageing and Alzheimer's disease. *Pharmacology & therapeutics*, *142*(2), 244-257.

Blanchard, J., Wanka, L., Tung, Y. C., del Carmen Cárdenas-Aguayo, M., LaFerla, F. M., Iqbal, K., & Grundke-Iqbal, I. (2010). Pharmacologic reversal of neurogenic and neuroplastic abnormalities and cognitive impairments without affecting Aβ and tau pathologies in 3xTg-AD mice. *Acta neuropathologica*, *120*(5), 605-621.

Bonafè, M., Olivieri, F., Cavallone, L., Giovagnetti, S., Marchegiani, F., Cardelli, M., ... & Rizzo, M. R. (2001). A gender–dependent genetic predisposition to produce high levels of IL-6 is detrimental for longevity. *European journal of immunology*, *31*(8), 2357-2361.

Burghardt, N. S., Park, E. H., Hen, R., & Fenton, A. A. (2012). Adult-born hippocampal neurons promote cognitive flexibility in mice. *Hippocampus*, *22*(9), 1795-1808.

Cabrera, M. A. S., Mesas, A. E., Garcia, A. R. L., & de Andrade, S. M. (2007). Malnutrition and depression among community-dwelling elderly people. *Journal of the American Medical Directors Association*, *8*(9), 582-584.

Caruso, C., Lio, D., Cavallone, L., & Franceschi, C. (2004). Aging, longevity, inflammation, and cancer. *Annals of the New York Academy of Sciences*, *1028*, 1–13.

Casadesus, G., Shukitt-Hale, B., Stellwagen, H. M., Zhu, X., Lee, H. G., Smith, M. A., & Joseph, J. A. (2004). Modulation of hippocampal plasticity and cognitive behavior by short-term blueberry supplementation in aged rats. *Nutritional Neuroscience*, *7*(5-6), 309-316.

Chiu, M., Lebenbaum, M., Cheng, J., de Oliveira, C., & Kurdyak, P. (2017). The direct healthcare costs associated with psychological distress and major depression: A population-based cohort study in Ontario, Canada. *PloS one*, *12*(9), e0184268.

Cole, M. G., & Dendukuri, N. (2003). Risk factors for depression among elderly community subjects: a systematic review and meta-analysis. *American Journal of Psychiatry*, *160*(6), 1147-1156.

Creer, D. J., Romberg, C., Saksida, L. M., van Praag, H., & Bussey, T. J. (2010). Running enhances spatial pattern separation in mice. *Proceedings of the national academy of sciences*, *107*(5), 2367-2372.

Erickson, K. I., Voss, M. W., Prakash, R. S., Basak, C., Szabo, A., Chaddock, L., Kim, J.S., Heo, S., Alves, H., White, S.M., Wojicicki, T.R., Mailey, E., Vieira, V.J., Martin, S.A., Pence, B.D., Woods, J.A., McAuley, E., & Kramer, A. F. (2011). Exercise training increases size of hippocampus and improves memory. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(7), 3017–22.

Fahnestock, M. (2011). Brain-derived neurotrophic factor: the link between amyloid-β and memory loss. *Future Neurology*, *6*(5), 627-639.

Fahnestock, M., Marchese, M., Head, E., Pop, V., Michalski, B., Milgram, W. N., & Cotman, C. W. (2012). BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. *Neurobiology of Aging*, *33*(3), 546–54.

Fiske, A., Wetherell, J. L., & Gatz, M. (2009). Depression in older adults. *Annual Review of Clinical Psychology*, 5, 363.

Fontella, F. U., Siqueira, I. R., Vasconcellos, A. P. S., Tabajara, A. S., Netto, C. A., & Dalmaz, C. (2005). Repeated restraint stress induces oxidative damage in rat hippocampus. *Neurochemical research*, 30(1), 105-111.

Garthe, A., Behr, J., & Kempermann, G. (2009). Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. *PloS one*, *4*(5), e5464.

Gatta, V., D'Aurora, M., Granzotto, A., Stuppia, L., & Sensi, S. L. (2014). Early and sustained altered expression of aging-related genes in young 3xTg-AD mice. *Cell Death & Disease*, 5(2014), e1054.

Ge, S., Goh, E. L., Sailor, K. A., Kitabatake, Y., Ming, G. L., & Song, H. (2006). GABA regulates synaptic integration of newly generated neurons in the adult brain. *Nature*, *439*(7076), 589.

Ge, S., Yang, C. H., Hsu, K. S., Ming, G. L., & Song, H. (2007). A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. *Neuron*, *54*(4), 559-566.

Gomez-Pinilla, F., Zhuang, Y., Feng, J., Ying, Z., & Fan, G. (2011). Exercise impacts brain derived neurotrophic factor plasticity by engaging mechanisms of epigenetic regulation. *The European Journal of Neuroscience*, *33*, 383–90.

Greenberg, P. E., Fournier, A. A., Sisitsky, T., Pike, C. T., & Kessler, R. C. (2015). The economic burden of adults with major depressive disorder in the United States (2005 and 2010). *The Journal of clinical psychiatry*, *76*(2), 155-162.

Hardy, J. A., & Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science*, *256*(5054), 184–185.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, *11*, 298–230.

Harman, D. (1972). The biologic clock: the mitochondria? *Journal of the American Geriatrics Society*, *20*(4), 145-147.

Herrup, K. (2015). The case for rejecting the amyloid cascade hypothesis. *Nat Neurosci*, *18*(6), 794–799.

Jessberger, S., Clark, R. E., Broadbent, N. J., Clemenson, G. D., Consiglio, A., Lie, D. C., ... & Gage, F. H. (2009). Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learning & memory*, *16*(2), 147-154.

Jin, M., Shepardson, N., Yang, T., Chen, G., Walsh, D., & Selkoe, D. J. (2011). Soluble amyloid β-protein dimers isolated from Alzheimer cortex directly induce tau hyperphosphorylation and neuritic degeneration. *Proceedings of the National Academy of Sciences*, *108*(14), 5819-5824.

Kanoski, S. E., & Davidson, T. L. (2011). Western diet consumption and cognitive impairment: links to hippocampal dysfunction and obesity.*Physiology & behavior*, *103*(1), 59-68.

Kempermann, G. (2002). Why new neurons? Possible functions for adult hippocampal neurogenesis. *Journal of neuroscience*, *22*(3), 635-638.

Kempermann, G. (2008). The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for?. *Trends in neurosciences*, *31*(4), 163-169.

Kempermann, G., Brandon, E. P., & Gage, F. H. (1998). Environmental stimulation of 129/SvJ mice causes increased cell proliferation and neurogenesis in the adult dentate gyrus. *Current Biology*, *8*(16), 939-944.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature*, *386*(6624), 493.

Krstic, D., Madhusudan, A., Doehner, J., Vogel, P., Notter, T., Imhof, C., ... & Riether, C. (2012). Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. *Journal of neuroinflammation*, *9*(1), 1.

Ku, H. H., Brunk, U. T., & Sohal, R. S. (1993). Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radical Biology and Medicine*, *15*(6), 621-627.

Laack, N. N., & Brown, P. D. (2004, October). Cognitive sequelae of brain radiation in adults. In *Seminars in oncology* (Vol. 31, No. 5, pp. 702-713). WB Saunders.

Lee, D. H., Geyer, E., Flach, A. C., Jung, K., Gold, R., Flügel, A., ... & Lühder, F. (2012). Central nervous system rather than immune cell-derived BDNF mediates axonal protective effects early in autoimmune demyelination. *Acta neuropathologica*, *123*(2), 247-258.

Leutgeb, J. K., Leutgeb, S., Moser, M. B., & Moser, E. I. (2007). Pattern separation in the dentate gyrus and CA3 of the hippocampus. *Science*, *315*(5814), 961-966.

Lindsay, J., Laurin, D., Verreault, R., Hébert, R., Helliwell, B., Hill, G. B., & McDowell, I. (2002). Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. *American journal of epidemiology*, *156*(5), 445-453.

Lio, D., Candore, G., Crivello, A., Scola, L., Colonna-Romano, G., Cavallone, L., ... & Branzi, A. (2004). Opposite effects of interleukin 10 common gene polymorphisms in cardiovascular diseases and in successful ageing: genetic background of male centenarians is protective against coronary heart disease. *Journal of medical genetics*, *41*(10), 790-794.

Liochev, S. I. (2013). Reactive oxygen species and the free radical theory of aging. *Free Radical Biology and Medicine*, *60*, 1–4.

Lovell, M. A., Soman, S., & Bradley, M. A. (2011). Oxidatively modified nucleic acids in preclinical Alzheimer's disease (PCAD) brain. *Mechanisms of ageing and development*, *132*(8), 443-448.

Luu, P., Sill, O. C., Gao, L., Becker, S., Wojtowicz, J. M., & Smith, D. M. (2012). The role of adult hippocampal neurogenesis in reducing interference. *Behavioral neuroscience*, *126*(3), 381.

MacQueen, G. M., Campbell, S., McEwen, B. S., Macdonald, K., Amano, S., Joffe, R. T., ... & Young, L. T. (2003). Course of illness, hippocampal function, and hippocampal volume in major depression. *Proceedings of the National Academy of Sciences*, *100*(3), 1387 1392.

Malberg, J. E., Eisch, A. J., Nestler, E. J., & Duman, R. S. (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *Journal of Neuroscience*, *20*(24), 9104-9110.

Marais, L., Stein, D. J., & Daniels, W. M. (2009). Exercise increases BDNF levels in the striatum and decreases depressive-like behavior in chronically stressed rats. *Metabolic brain disease*, *24*(4), 587-597.

Marmigère, F., Givalois, L., Rage, F., Arancibia, S., & Tapia-Arancibia, L. (2003). Rapid induction of BDNF expression in the hippocampus during immobilization stress challenge in adult rats. *Hippocampus*, *13*(5), 646-655.

Mattson, M. P. (2004). Pathways towards and away from Alzheimer's disease. *Nature*, *430*(7000), 631–639.

McEwen, B. S. (2003). Mood disorders and allostatic load. *Biological psychiatry*, *54*(3), 200 207.

Mosconi, L., Pupi, A., & De Leon, M. J. (2008). Brain glucose hypometabolism and oxidative stress in preclinical Alzheimer's disease. *Annals of the New York Academy of Sciences*, *1147*(4), 180–195.

Nagahara, A. H., Merrill, D. A., Coppola, G., Tsukada, S., Schroeder, B. E., Shaked, G. M., ... & Rockenstein, E. (2009). Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nature medicine*, *15*(3), 331.

Nagata, K., Nakashima-Kamimura, N., Mikami, T., Ohsawa, I., & Ohta, S. (2009). Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus dependent learning tasks during chronic physical restraint in mice. *Neuropsychopharmacology*, *34*(2), 501-508.

Neeper, S. A., Gomez-Pinilla, F., Choi, J., & Cotman, C. (1995). Exercise and brain neurotrophins. *Nature*, *373*, 109.

Niibori, Y., Yu, T. S., Epp, J. R., Akers, K. G., Josselyn, S. A., & Frankland, P. W. (2012). Suppression of adult neurogenesis impairs population coding of similar contexts in hippocampal CA3 region. *Nature communications*, *3*, 1253.

Orr, W. C., & Sohal, R. S. (1994). Extension of life-span by overexpression of superoxide dismutase and catalase in Drosophila melanogaster. *Science*, *263*(5150), 1128-1130.

Parachikova, A., Green, K. N., Hendrix, C., & LaFerla, F. M. (2010). Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. *PloS one*, *5*(11), e14015.

Pelletier A, Barul C, Féart C, Helmer C, Bernard C, Periot O, Dilharreguy B, Dartigues J-F, Allard M, Barberger-Gateau P, Catheline G, Samieri C (2015) Mediterranean diet and preserved brain structural connectivity in older subjects. *Alzheimer's Dement* 11:1023 1031.

Pencea, V., Bingaman, K. D., Wiegand, S. J., & Luskin, M. B. (2001). Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *Journal of Neuroscience*, *21*(17), 6706-6717.

Peng, S., Wuu, J., Mufson, E. J., & Fahnestock, M. (2005). Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. *Journal of Neurochemistry*, *93*(6), 1412–1421.

Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., & Ferri, C. P. (2013). The global prevalence of dementia: a systematic review and metaanalysis. *Alzheimer's & Dementia*, *9*(1), 63-75.

Radecki, D. T., Brown, L. M., Martinez, J., & Teyler, T. J. (2005). BDNF protects against stress induced impairments in spatial learning and memory and LTP. *Hippocampus*, *15*(2), 246 253.

Rangel, L. M., Alexander, A. S., Aimone, J. B., Wiles, J., Gage, F. H., Chiba, A. A., & Quinn, L. K. (2014). Temporally selective contextual encoding in the dentate gyrus of the hippocampus. *Nature communications*, *5*, 3181.

Rodríguez, J. J., Jones, V. C., Tabuchi, M., Allan, S. M., Knight, E. M., LaFerla, F. M., … & Verkhratsky, A. (2008). Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. *PloS One*, *3*(8), e2935.

Ross, M. H. (1966). Life expectancy modification by change in dietary regimen of the mature rat. *Proc. VII Int. Congr. Nutr.* 5: 35–38.

Sahay, A., Scobie, K. N., Hill, A. S., O'carroll, C. M., Kheirbek, M. A., Burghardt, N. S., ... & Hen, R. (2011). Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. *Nature*, *472*(7344), 466.

Saka, B., Kaya, O., Ozturk, G. B., Erten, N., & Karan, M. A. (2010). Malnutrition in the elderly and its relationship with other geriatric syndromes.*Clinical Nutrition*, *29*(6), 745-748.

Sánchez-Villegas, A., Henríquez-Sánchez, P., Ruiz-Canela, M., Lahortiga, F., Molero, P., Toledo, E., & Martínez-González, M. A. (2015). A longitudinal analysis of diet quality scores and the risk of incident depression in the SUN Project. *BMC medicine*, *13*(1), 1.

Santarelli, L., Saxe, M., Gross, C., Surget, A., Battaglia, F., Dulawa, S., ... & Belzung, C. (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *science*, *301*(5634), 805-809.

Sapolsky, R. M. (1985). A mechanism for glucocorticoid toxicity in the hippocampus: increased neuronal vulnerability to metabolic insults. *The Journal of Neuroscience*, *5*(5), 1228 1232.

Sapolsky, R. M., Uno, H., Rebert, C. S., & Finch, C. E. (1990). Hippocampal damage associated with prolonged glucocorticoid exposure in primates. *The Journal of Neuroscience*, *10*(9), 2897-2902.

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*, (e201606210), 1–14.

Sheline, Y. I., Sanghavi, M., Mintun, M. A., & Gado, M. H. (1999). Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. *The Journal of neuroscience*, *19*(12), 5034-5043.

Sinn, N., Milte, C. M., Street, S. J., Buckley, J. D., Coates, A. M., Petkov, J., & Howe, P. R. (2012). Effects of n-3 fatty acids, EPA v. DHA, on depressive symptoms, quality of life, memory and executive function in older adults with mild cognitive impairment: a 6 month randomised controlled trial.*British Journal of Nutrition*, *107*(11), 1682-1693.

Smith, M. A., Makino, S., Kvetnansky, R., & Post, R. M. (1995). Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 messenger-RNAs in the Hippocampus. *Journal of Neuroscience*, *15*, 1768–1777.

Sohal, R. S., Ku, H. H., Agarwal, S., Forster, M. J., & Lal, H. (1994). Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mechanisms of ageing and development*, *74*(1-2), 121 133.

Spina, M. B., Squinto, S. P., Miller, J., Lindsay, R. M., & Hyman, C. (1992). Brain-derived neurotrophic factor protects dopamine neurons against 6-hydroxydopamine and N methyl-4-phenylpyridinium ion toxicity: involvement of the glutathione system. *Journal of neurochemistry*, *59*(1), 99-106.

Snyder, J. S., Kee, N., & Wojtowicz, J. M. (2001). Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. *Journal of neurophysiology*, *85*(6), 2423-2431.

Stelzmann, R. A., Schnitzlein, H. N., & Murtagh, F. R. (1995). An English translation of Alzheimer's 1907 paper, "uber eine eigenartige erkankung der hirnrinde." *Clinical Anatomy*, *8*(6), 429–431.

Stover, K. R., Campbell, M. A., Van Winssen, C. M., & Brown, R. E. (2015). Analysis of motor function in 6-month-old male and female 3xTg-AD mice. *Behavioural brain research*, *281*, 16-23.

Surget, A., Saxe, M., Leman, S., Ibarguen-Vargas, Y., Chalon, S., Griebel, G., ... & Belzung, C. (2008). Drug-dependent requirement of hippocampal neurogenesis in a model of depression and of antidepressant reversal. *Biological psychiatry*, *64*(4), 293-301.

Surget, A., Tanti, A., Leonardo, E. D., Laugeray, A., Rainer, Q., Touma, C., ... & Belzung, C. (2011). Antidepressants recruit new neurons to improve stress response regulation. *Molecular psychiatry*, *16*(12), 1177.

Tarkowski, E., Andreasen, N., Tarkowski, A., & Blennow, K. (2003). Intrathecal inflammation precedes development of Alzheimer's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *74*(9), 1200–5.

Tronel, S., Belnoue, L., Grosjean, N., Revest, J. M., Piazza, P. V., Koehl, M., & Abrous, D. N. (2012). Adult-born neurons are necessary for extended contextual discrimination. *Hippocampus*, *22*(2), 292-298.

Tuglu, C., Kara, S. H., Caliyurt, O., Vardar, E., & Abay, E. (2003). Increased serum tumor necrosis factor-alpha levels and treatment response in major depressive disorder. *Psychopharmacology*, *170*(4), 429-433.

Tulving, E., & Markowitsch, H. J. (1998). Episodic and declarative memory: role of the hippocampus. *Hippocampus*, *8*(3), 198-204.

Valente, T., Hidalgo, J., Bolea, I., Ramirez, B., Anglés, N., Reguant, J., ... & Unzeta, M. (2009). A diet enriched in polyphenols and polyunsaturated fatty acids, LMN diet, induces neurogenesis in the subventricular zone and hippocampus of adult mouse brain. *Journal of Alzheimer's disease*, *18*(4), 849-865.

Van Praag, H., Kempermann, G., & Gage, F. H. (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nature neuroscience*, *2*(3), 266.

Van Praag, H., Shubert, T., Zhao, C., & Gage, F. H. (2005). Exercise enhances learning and hippocampal neurogenesis in aged mice. *Journal of Neuroscience*, *25*(38), 8680-8685.

Vaynman, S., Ying, Z., & Gomez-Pinilla, F. (2004). Hippocampal BDNF mediates the efficacy of exercise on synaptic plasticity and cognition. *European Journal of Neuroscience*, *20*, 2580–2590.

Walford, R. L. (1964). The immunologic theory of aging. *The Gerontologist*,*4*(4), 195-197.

Wassel, C. L., Barrett-Connor, E., & Laughlin, G. A. (2010). Association of circulating C reactive protein and interleukin-6 with longevity into the 80s and 90s: The Rancho Bernardo Study. *The Journal of Clinical Endocrinology & Metabolism*, *95*(10), 4748 4755.

Williams, C. M., El Mohsen, M. A., Vauzour, D., Rendeiro, C., Butler, L. T., Ellis, J. A., ... & Spencer, J. P. (2008). Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. *Free Radical Biology and Medicine*, *45*(3), 295-305.

Winocur, G., Becker, S., Luu, P., Rosenzweig, S., & Wojtowicz, J. M. (2012). Adult hippocampal neurogenesis and memory interference. *Behavioural brain research*, *227*(2), 464-469.

Winocur, G., Wojtowicz, J. M., Huang, J., & Tannock, I. F. (2014). Physical exercise prevents suppression of hippocampal neurogenesis and reduces cognitive impairment in chemotherapy-treated rats. *Psychopharmacology*, *231*(11), 2311-2320.

Winocur, G., Wojtowicz, J. M., Sekeres, M., Snyder, J. S., & Wang, S. (2006). Inhibition of neurogenesis interferes with hippocampus-dependent memory function. *Hippocampus*, *16*(3), 296-304.

Wolf, A.B., Braden, B.B., Bimonte-Nelson, H., Kusne, Y., Young, N., Engler-Chiurazzi, E., Garcia, A.N., Walker, D.G., Moses, G.S., Tran, H. and LaFerla, F., 2012. Broad-based nutritional supplementation in 3xTg mice corrects mitochondrial function and indicates sex-specificity in response to Alzheimer's disease intervention. *Journal of Alzheimer's Disease*, *32*(1), pp.217-232.

Wu, S. Y., Wang, T. F., Yu, L., Jen, C. J., Chuang, J. I., Wu, F. S., ... & Kuo, Y. M. (2011). Running exercise protects the substantia nigra dopaminergic neurons against inflammation-induced degeneration via the activation of BDNF signaling pathway. *Brain, behavior, and immunity*, *25*(1), 135-146.

Wu, A., Ying, Z., & Gomez-Pinilla, F. (2008). Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. *Neuroscience*, *155*(3), 751-759.

Yassa, M. A., Stark, S. M., Bakker, A., Albert, M. S., Gallagher, M., & Stark, C. E. (2010). High-resolution structural and functional MRI of hippocampal CA3 and dentate gyrus in patients with amnestic mild cognitive impairment. *Neuroimage*, *51*(3), 1242-1252.

Zhang, Y., Su, W. J., Chen, Y., Wu, T. Y., Gong, H., Shen, X. L., ... & Jiang, C. L. (2016). Effects of hydrogen-rich water on depressive-like behavior in mice. *Scientific reports*, *6*: 23742.

2. Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice

Hutton, C. P., Déry, N., Rosa, E., Lemon, J. A., Rollo, C. D., Boreham, D. R., Fahnestock, M., deCatanzaro, D., Wojtowicz, J.M., & Becker, S. (2015). Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice. *Neuroscience*, *308*, 180-193.

Neuroscience 308 (2015) 180–193

SYNERGISTIC EFFECTS OF DIET AND EXERCISE ON HIPPOCAMPAL FUNCTION IN CHRONICALLY STRESSED MICE

C. P. HUTTON,[a†] N. DÉRY,[a†] E. ROSA,[b] J. A. LEMON,[c] C. D. ROLLO,[d] D. R. BOREHAM,[c] M. FAHNESTOCK,[b] D. DECATANZARO,[a] J. M. WOJTOWICZ[e] AND S. BECKER[a*]

[a] *Department of Psychology, Neuroscience and Behaviour, McMaster University, Hamilton, ON L8S 4K1, Canada*

[b] *Department of Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, ON L8S 4K1, Canada*

[c] *Department of Medical Physics and Applied Radiation Sciences, McMaster University, Hamilton, ON L8S 4K1, Canada*

[d] *Department of Biology, McMaster University, Hamilton, ON L8S 4K1, Canada*

[e] *Department of Physiology, University of Toronto, Toronto, ON M5S 1A8, Canada*

Abstract—Severe chronic stress can have a profoundly negative impact on the brain, affecting plasticity, neurogenesis, memory and mood. On the other hand, there are factors that upregulate neurogenesis, which include dietary antioxidants and physical activity. These factors are associated with biochemical processes that are also altered in age-related cognitive decline and dementia, such as neurotrophin expression, oxidative stress and inflammation. We exposed mice to an unpredictable series of stressors or left them undisturbed (controls). Subsets of stressed and control mice were concurrently given (1) no additional treatment, (2) a complex dietary supplement (CDS) designed to ameliorate inflammation, oxidative stress, mitochondrial dysfunction, insulin resistance and membrane integrity, (3) a running wheel in each of their home cages that permitted them to exercise, or (4) both the CDS and the running wheel for exercise. Four weeks of unpredictable stress reduced the animals' preference for saccharin, increased their adrenal weights and abolished the exercise-induced upregulation of neurogenesis that was observed in non-stressed animals. Unexpectedly, stress did not reduce hippocampal size, brain-derived neurotrophic factor (BDNF), or neurogenesis. The combination of dietary supplementation and exercise had multiple beneficial effects, as reflected in the number of doublecortin (DCX)-positive immature neurons in the dentate gyrus (DG), the sectional area of the DG and hippocampal CA1, as well as increased hippocampal BDNF messenger ribonucleic acid (mRNA) and serum vascular endothelial growth factor (VEGF) levels. In contrast, these benefits were not observed in chronically stressed animals exposed to either dietary supplementation or exercise alone. These findings could have important clinical implications for those suffering from chronic stress-related disorders such as major depression. © **2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: stress, neurogenesis, hippocampus, exercise, dietary supplements, psychological depression.

INTRODUCTION

Chronic stress can have a profoundly negative impact on the brain and contributes to a number of psychological disorders including major depression (McEwen, 2003; Miller and Hen, 2015). Evidence from rodents and primates links severe and prolonged elevation of glucocorticoids (corticosterone, cortisol) to hippocampal damage (Sapolsky, 1985; Sapolsky et al., 1995). This damage includes synaptic atrophy (Watanabe et al., 1992; Magariños et al., 1997) and reduced neurogenesis (Watanabe et al., 1992; Gould et al., 1992, 1997, 1998). Glucocorticoids bind extensively in the healthy hippocampus to both glucocorticoid and mineralocorticoid receptors (Reul and De Kloet, 1985; Aronsson et al., 1988), and chronic stress can reduce the number of hippocampal mineralocorticoid receptors (López et al., 1998). Normally, the hypothalamic–pituitary–adrenal (HPA) axis shows habituation to repeated exposure to a stressor, such that glucocorticoid elevation following the stressor diminishes, but the reduction in hippocampal mineralocorticoid receptors can lead to impairment of this habituation (Cole et al., 2000).

These detrimental effects on the hippocampus correlate with behavioral signs of major depressive disorder. Evidence indicates that the effects of serotonergic antidepressant medications rely upon intact adult hippocampal neurogenesis (Malberg et al., 2000; Santarelli et al., 2003; Sahay et al., 2011). For example, social isolation stress induced anhedonia and depression-like behavior in monkeys; this was alleviated by fluoxetine treatment, which also upregulated neurogenesis (Perera et al., 2011). However fluoxetine's behavioral antidepressant effect was abolished by focal hippocampal X-irradiation (Perera et al., 2011), which is highly toxic to immature neurons (Snyder et al., 2001; Winocur et al., 2006). Similarly, chronic unpredictable mild stress induced

*Corresponding author. Tel: +1-905-525-9140x23020; fax: +1-905-529-6225.

E-mail address: becker@mcmaster.ca (S. Becker).

† These authors contributed equally to this work.

Abbreviations: BDNF, brain-derived neurotrophic factor; CDS, complex dietary supplement; CUS, chronic unpredictable stress; DCX, doublecortin; DG, dentate gyrus; HPA, hypothalamic–pituitary–adrenal; IGF-1, insulin-like growth factor-1; mRNA, messenger ribonucleic acid; VEGF, vascular endothelial growth factor.

http://dx.doi.org/10.1016/j.neuroscience.2015.09.005
0306-4522/

anhedonic behavior in mice, accompanied by a 30% reduction in neurogenesis and habituation of the hippocampal inhibitory influence on the HPA axis (Surget et al., 2011). This habituation was evident in a reduction in the number of newly born neurons in the dentate gyrus (DG) activated by dexamethasone, a synthetic glucocorticoid. Moreover, disruption of neurogenesis by irradiation impaired the ability of fluoxetine to restore hippocampal modulation of HPA activity during chronic stress, suggesting that this modulation may depend upon neurogenesis. Although it is challenging to study human neurogenesis, a number of studies have associated the number and duration of depressive episodes with loss of hippocampal volume and memory function (see e.g. Sheline et al., 1999; MacQueen et al., 2003).

Two factors that can enhance neurogenesis and offset stress and depression are dietary antioxidants (Lau et al., 2005; Valente et al., 2009) and long-term aerobic exercise (van Praag et al., 1999, 2005; Creer et al., 2010; Déry et al., 2013; Winocur et al., 2014). Voluntary aerobic exercise enhanced neurogenesis in rodents for up to 9 months (Merkley et al., 2014). In humans, exercise was found to increase serum brain-derived neurotrophic factor (BDNF; Erickson et al., 2011), DG blood volume (indicative of angiogenesis; Pereira et al., 2007) and memory scores on a behavioral test of pattern separation (Déry et al., 2013). Diet and exercise affect biochemical processes and signaling pathways that are also altered in age-related cognitive decline. These include neurotrophin expression (Fahnestock et al., 2012), cellular oxidative stress (Valente et al., 2009), inflammation (Goshen et al., 2008) and mTOR regulation (Ota et al., 2014). In rodents, a complex dietary supplement (CDS) greatly ameliorated age-related physiological and cognitive decline in transgenic growth hormone mice (a model of accelerated aging) and aged wild-type controls (Lemon et al., 2003). When aged mice received the same supplement from weaning onward, they performed as well as young mice on the hidden platform version of the Morris water maze (Aksenov et al., 2013) – a test on which younger mice typically outperform older ones. Aged mice who received the CDS also had larger brains than age-matched non-supplemented controls. The same CDS also protected mice from radiation-induced DNA damage and immunological apoptosis (Lemon et al., 2008a,b).

The CDS (Table 1) was designed to target five major mechanisms associated with aging: inflammation, oxidative stress, mitochondrial dysfunction, insulin resistance and membrane integrity. Although this approach may not identify contributions of any one ingredient, mounting evidence supports the potent neuroprotective effects of CDSs exhibiting some overlap in ingredients or physiological targets (Milgram et al., 2002; Parachikova et al., 2010). Broad-spectrum, antioxidant-rich micronutrient supplementation also shows promise in treatment of mood disorders, while single nutrient supplements generally produce weak results (Rucklidge and Kaplan, 2013; Popper, 2014). For example, pre-partum micronutrient supplementation lessens the risk and severity of postpartum depression (Leung et al., 2013).

Table 1. Ingredients included in the complex dietary supplement

Ingredient	Daily dose for a 35 g mouse
Acetyl-L-Carnitine	14.4 mg
Acetylsalicylic Acid	2.5 mg
Alpha-Lipoic Acid	0.72 mg
β-Carotene	50 IU
Bioflavonoids	4.32 mg
Chromium picolinate	1.44 μg
Cod Liver Oil	5.04 IU
Coenzyme Q10	0.44 mg
DHEA	0.15 mg
Flax Seed oil	21.6 mg
Folic Acid	0.01 mg
Garlic	26.6 μg
Ginger	7.2 mg
Gingko Biloba	1.44 mg
Ginseng	8.64 mg
Green Tea Extract	7.2 mg
L-Glutathione	0.36 mg
Magnesium	0.72 mg
Melatonin	0.01 mg
N-Acetyl Cysteine	7.2 mg
Potassium	0.36 mg
Rutin	0.72 mg
Selenium	1.08 μg
Vitamin B1	0.72 mg
Vitamin B3	0.72 mg
Vitamin B6	0.72 mg
Vitamin B12	0.72 μg
Vitamin C	3.6 mg
Vitamin D	2.5 IU
Vitamin E	1.44 IU
Zinc	0.14 mg

Although both exercise and nutraceuticals can enhance hippocampal volume (Erickson et al., 2011) and neurogenesis (Lau et al., 2005), the two together may produce greater effects. The combination of an antioxidant-fortified diet and environmental enrichment reduced age-related cognitive impairment and increased BDNF levels in dogs more than did either treatment alone (Fahnestock et al., 2012). Beneficial interactions between combinations of dietary supplementation and environmental enrichment or exercise have also been observed in investigations of Alzheimer's disease (Pop et al., 2010) and synaptic plasticity (Wu et al., 2008). Although environmental enrichment and exercise affect neurogenesis via distinct pathways (neuronal survival and proliferation respectively; Olson et al., 2006), they are difficult to dissociate experimentally because environmental enrichment protocols typically have an exercise component. Conversely, exercise protocols enrich the animal's environment by affording it access to novel complex objects with which it can interact meaningfully.

We exposed mice to a complex series of unpredictable stressors while concurrently giving some of them the CDS, a running wheel in the home cage, or both. We predicted that dietary supplementation and aerobic exercise would synergistically mitigate the impact of chronic stress on the hippocampus. Specifically, we hypothesized that the combination of CDS and exercise would prevent depression-like

behavior and normalize hippocampal volume, neurogenesis and BDNF levels.

EXPERIMENTAL PROCEDURES

Subjects

The subjects were 154 male C57BL/6 mice (Charles River, St. Constant, Quebec, Canada), initially aged 6 weeks and weighing 20–25 g. Upon arrival, animals were divided into one of eight treatment groups, but no experimental procedures were conducted until animals had acclimated to the facility for approximately two weeks. However, the CDS or plain bagel chip was provided to the animals during this time. Animals were individually housed with a reversed 12:12-h light/dark cycle (lights off during the day, lights on at night) and constant temperature (~22 °C ±0.5 °C) and humidity. Mice were provided Harlan™ Teklad 22/5 Rodent Diet chow and water *ad libitum* except as required for some of the stress manipulations described below. Animals were also given the CDS and saccharin-flavored water (0.1%) depending on which experimental group they had been assigned to. Following the two-week acclimation period, animals were divided into and left in their experimental cages for 4 weeks. We employed a 3-factor 2 × 2 × 2 design: exercise (wheel running or not), diet (CDS or not) and stress (chronic unpredictable stressors or not). Because mice exercise by running on the cage walls and ceiling in wire cages we prevented this via Plexiglas barriers. All mice were caged with woodchip bedding and provided with standard ABS plastic tubes in which to hide/sleep.

As described below, half of the animals were designated for immunohistochemical assays (n = 77), while the other half were designated for hippocampal BDNF assays (n = 77). All mice were delivered isoflurane via nosecone and it was confirmed they were in a plane of anesthesia via toe pinch. Blood was then collected via cardiac puncture of the right atrium for later analysis of serum BDNF, insulin-like growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF). Thus, all animals, whether destined for immunohistochemical or neurotrophic assays, were euthanized via exsanguination. The mice designated for immunohistochemistry were administered intracardiac perfusion using paraformaldehyde (PFA) and brains were extracted. For those mice that were not perfused, cerebral cortex and hippocampi were immediately dissected and flash frozen using liquid nitrogen and then adrenal glands were dissected and subsequently weighed. All procedures were conducted in accordance with Canadian Council for Animal Care and the McMaster University Animal Research Ethics Board guidelines.

CDS

The dosages, preparation and administration of the CDS were previously described (Lemon et al., 2003). Briefly, ingredients were included based on human tolerability and doses were based on human recommendations with adjustments for differences in body size and metabolic rate. The CDS was prepared in aqueous solution, absorbed onto a small piece of bagel and left to dry. These bagel chips were given daily to mice in the supplemented group halfway through the photoperiod (at the beginning of their awake cycle) and were usually consumed within minutes. All animals who were not assigned to a supplemented group were provided with similarly sized bagel pieces that did not contain the CDS. The dietary supplement was provided to animals during the two-week acclimation period. Thus, there was a brief pre-load phase with the CDS prior to the onset of stress. See Table 1 for CDS ingredients.

Chronic unpredictable stress (CUS)

This procedure involved a novel combination of stressors, each validated in other studies (Harkin et al., 2002; Elizalde et al., 2008; Strekalova and Steinbusch, 2010). Stressors were selected based on their likelihood of increasing circulating stress hormones without compromising the animals' health in other ways. During the four-week protocol, animals were pseudo-randomly exposed to 3 of 13 of the following stressors on each day (see Table 2):

Elevated pillar. This consisted of a small, cloth-covered platform approximately 10.1 cm wide by 20.3 cm long and approximately 1.2 m off the ground (Thorpe et al., 2014). When on this platform a mouse had to be vigilant to avoid falling off. The elevated pillar stress lasted 1 h and was administered during the animal's normal awake period (i.e., dark phase).

Lights off/cage tilt overnight. During the animal's sleep cycle, its cage was placed on a 45° tilt for a period of 12 h with lights turned off. This can disturb the circadian rhythm of the mouse.

Cage tilt overnight. During the animal's sleep period, its cage was placed on a 45° tilt for a period of 12 h.

Lights on during the day. Mice are most active in the dark so turning the lights on for 6 h during the waking period can disrupt the circadian rhythm, sleep cycle and associated hormones and neurotransmitters (Harkin et al., 2002; Elizalde et al., 2008).

Strobe light during the day. Similar to the "lights on during the day" stressor, a bright, blinking strobe during the animal's waking (dark) period can impact sleep (Harkin et al., 2002; Elizalde et al., 2008; Schmidt and Duman, 2010) and also represents a direct stress. The strobe light was always delivered concurrently with one of predator odor or ultrasonic noise.

Predator odor. Mice were exposed to cat hair during their normal awake cycle (lights off) (McEuen et al., 2008; Schmidt and Duman, 2010; d'Audiffret et al., 2010), in combination with a strobe light turned on (during the animals' awake period).

Ultrasonic noise. Sound was emitted in the 32–62-kHz frequency range from small electrical outlet-mounted speakers. This frequency range is outside the audible

Table 2. The chronic unpredictable stress (CUS) paradigm used to simulate the chronic stressors and sleep disturbances thought to underlie major depression in humans

Day #	Day	Morning	Afternoon	Overnight
1	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
2	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
3	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
4	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
5	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
6	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
7	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)
8	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
9	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
10	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
11	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
12	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
13	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
14	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)
15	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
16	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
17	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
18	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
19	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
20	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
21	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)
22	Monday	water deprivation (3 h)	lights on (6 h)	lights off/cage tilt (12 h)
23	Tuesday	restraint (30 min)	predator odor/strobe (6 h)	ultrasonic noise (12 h)
24	Wednesday	handling (10 min)	predator exposure (3 h)	cage tilt (12 h)
25	Thursday	elevated pillar (1 h)	ultrasonic noise (6 h)	wire cage (12 h)
26	Friday	ultrasonic noise/strobe (6 h)	restraint (30 min)	wire cage (12 h)
27	Saturday	predator exposure (6 h)	lights on (6 h)	food dep./cage tilt (12 h)
28	Sunday	water deprivation (3 h)	elevated pillar (1 h)	cage tilt (12 h)

bandwidth for human hearing but is detectable to rodents. High-frequency sound waves were emitted at a rate of 80 oscillations per second, which is claimed to be irritating to rodents who will actively try to escape the sound. Since our mice were kept in cages that would render the sound inescapable, these high-frequency sound waves should activate the animal's stress response while not causing any other sort of physiological damage. The ultrasonic emitters are commercially available and were purchased from Victor®, a pest control company. The Victor® ultrasonic rodent repeller was only turned on periodically and for no longer than 12 h, alone or in combination with a strobe light turned on (during the animal's awake cycle).

Water deprivation. Mice were deprived of water for periods of no longer than 3 h. The anticipation of a prolonged water deprivation event is stressful and also impacts hydration and feeding (Mantella et al., 2005; Elizalde et al., 2008). Water deprivation did not occur on the same day as, and did not immediately precede, saccharin preference testing.

Food deprivation/cage tilt. Mice were deprived for 12 h during their normal sleep period, in combination with cage tilt, which may have made it slightly more difficult for them to regulate body weight (Harkin et al., 2002; Mantella et al., 2005; Schmidt and Duman, 2010).

Handling. During the mouse's awake period (lights off), the experimenter placed the mouse on a table and held it by the base of the tail for 10 min. The mouse was then returned to its home cage. Restraint is a commonly applied stress for rodents (e.g. Magariños et al., 1997).

Restraint. Restraint is a stressor that is commonly implemented in the rodent literature (e.g. Snyder et al., 2011). The restraint system that we used was DecapiCone (Braintree Scientific, Braintree, MA, USA). These restraint cones are made from a tapered plastic film. One must simply hold the plastic cone with one hand and place the mouse into the cone using the other hand. Once the mouse has been placed inside of the DecapiCone, with their head through one end to allow for breathing and the other end tied off to create a snug fit, they are rendered immobile. Otherwise, the mice are completely unharmed. Each animal was not restrained for more than 30 min per session.

Predator exposure. Mice in our stressed group were wheeled into an adjacent room that houses rats. Each mouse was then placed into one side of a specially constructed cage that has been partitioned into two halves, while a rat was placed into the other half. The partition is a metal wire screen with small openings, which prevented the animals from being able to harm one another. However, this type of screening would still allow for enough contact between the two animals to effectively initiate the mouse's stress response. Mice and rats were left in these cages for either 3 or 6 h periods, with 2 or 3 days between each exposure. Following each exposure, mice were returned back to their original cages [and their holding room].

Deprived housing. For durations of 12 h, stressed mice were placed into specially constructed cages with wire mesh floors and no bedding material. However, they still had free access to food and water. The lack of bedding material or enrichment devices made for a type of impoverished housing that was mildly stressful for the animals, but did not otherwise cause any type of bodily harm. Following each deprivation period, mice were placed back into their original cages with adequate bedding material.

Aerobic exercise

Animals in the exercise condition were provided with a running wheel in their home cage. Daily observations and an ergometer attached to a subset of running wheels (Mouse Igloo Fast-Trac, Bioserv) verified that, in most cases, mice repeatedly used the running wheels. For sedentary groups Plexiglas liners minimized physical activity.

Behavioral measures

Saccharin preference during the waking period was used as a non-invasive behavioral measure of anxiety and stress (Willner et al., 1992). Reduced preference for saccharin is a sign of anhedonia, a core symptom of major depression in humans. Saccharin preference was measured by the proportion of saccharin-flavored water consumed relative to unflavored water. Saccharin is an artificial sweetener that was used in place of sucrose to avoid any physiological changes associated with the increased intake of sucrose. Mice were always provided with two water bottles fixed to their cages. On two days of the week one of these contained saccharin-flavored water, while the other contained regular, unflavored water. Every other day both water bottles contained unflavored water, to maintain the hedonic value of saccharin over time. The position of the saccharin and unflavored water bottles was swapped upon each administration, so that the animal could not learn the position of, and become biased toward drinking from the bottle containing saccharin-flavored water. Each test began 1 h before the animals' awake period (7:00 h) and ended 1 h after initiation of the sleep period (21:00 h). Body weight was measured at the start of the experiment, once a week during CUS and the day before sacrifice.

Immunohistochemistry

Animals were anaesthetized with isoflurane and euthanized via transcardial perfusion with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA). Following perfusion, whole brains were extracted and post-fixed in PFA for 24 h, after which they were transferred to a 0.1% sodium azide (in PBS) solution and stored at 4 °C until sectioning. The right hemisphere of each brain was dissected and sectioned from anterior to posterior in the coronal plane in a PBS-filled well on a Leica VT1000S Vibratome (Heidelberg, Germany) into 40-μm slices that were transferred to a 48-well plate (four sections per well) filled with 0.1% sodium azide solution at 4 °C. Nine sections containing the DG were sampled from each animal using immunohistochemistry methods described previously (Wojtowicz and Kee, 2006).

Doublecortin (DCX) was assayed as a marker of neurogenesis. DCX is a microtubule binding protein expressed transiently in proliferating neural precursor cells and migrating neuroblasts, but not in mature neurons. This is a recognized biomarker of immature, adult-born neurons (Brown et al., 2003; McDonald and Wojtowicz, 2005). All sections were first incubated with anti-DCX antibody ab18723 (Abcam, Toronto, ON, Canada) and then Alexa Fluor 568 Donkey Anti-Rabbit IgG antibody (Life Technology). Antibodies were suspended in a phosphate-buffered saline solution containing 0.3% Triton X-100 detergent, which enabled them to penetrate cell membranes. Sections were washed (on a rotomixer) in PBS 3 times (5 min each) before and after each antibody incubation period. After the final wash, sections were mounted on labeled glass slides using a paintbrush and distilled water, permitted to air dry in a shaded area (to limit light exposure), coated with PermaFluor™ aqueous mounting medium (Thermo Scientific, Mississauga, ON, Canada), covered with a glass coverslip, and then stored in a slide folder at 4 °C before being transferred to a slide box for storage.

Cell counts and hippocampal substructure area measurements

Immunolabeled cells in the subgranular zone (SGZ) of the DG were counted using a 40× objective lens (Nikon, OPTIPHOT-2 fluorescence microscope). Cells were counted exhaustively throughout the entire 40-μm thickness of each section by gradually adjusting the focus of the microscope but excluding the upper and lower edges of the sections. The counting procedure was verified by at least two independent investigators who were blinded with respect to the animal's group assignment. The SGZ was defined as a two-cell diameter wide (or approximately 20 μm) zone beneath the granule cell layer. Black and white images of each section, captured using a Sensicam CCD camera and SensiControl v4.02 software at 4× magnification, were then used to obtain length measurements (mm) of the SGZ on the upper and lower blades of the DG using Image J software (http://rsb.info.nih.gov/ij/). Cell counts and length measurements were then used to calculate the number of DCX + cells per mm SGZ, averaged across sections in each region (dorsal, medial and ventral) and throughout the DG. Additional 4× magnification images of the same sections were also used with Image J software to obtain surface area measurements of the DG and CA1 as indicators of hippocampal size.

Quantification of hippocampal BDNF messenger ribonucleic acid (mRNA) using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Following sacrifice of animals by exsanguination under isoflurane anesthesia, the hippocampi were dissected

and immersed in liquid nitrogen, then stored at −80 °C until use. The methods employed for the subsequent isolation of RNA, reverse transcription and quantification of BDNF using qRT-PCR followed protocols similar to those reported previously (Fahnestock et al., 2012). Briefly, hippocampal tissue samples were first weighed then sonicated for 3–5 s in cold Trizol™ solution (Life Technologies, Inc., Gaithersburg, MD, USA). RNA was isolated from the homogenate by centrifugation and extraction using a 70% ethanol solution, RNeasy™ spin column (Qiagen, Mississauga, ON, Canada), DNase treatment and elution as per the manufacturer's instructions.

RNA concentrations and integrity were determined by spectrophotometry and agarose gel electrophoresis, respectively. Only samples with A260/A280 ratios greater than 1.7 were processed further. One microgram of each RNA sample was then reverse transcribed and 50 ng of the resulting cDNA was amplified in the Stratagene MX3000p machine as previously described (Fahnestock et al., 2012) using qPCR SuperMix (Invitrogen), forward and reverse primers [BDNF: 5′ GCG-GCA-GAT-AAA-AAG-ACT-GC 3′ (forward) and 5′ CTT-ATG-AAT-CGC-CAG-CCA-AT 3′ (reverse); β-actin: 5′ AGC-CAT-GTA-CGT-AGC-CAT-CC 3′ (forward) and 5′ CTC-TCA-GCT-GTG-GTG-GTG-AA 3′ (reverse)], ROX reference dye (Invitrogen), and cDNA or reference standard for absolute quantification. A "no template" control lacking cDNA was included. Only those qRT-PCR runs with efficiencies greater than 90% and R^2 greater than 0.99 were included in subsequent statistical comparisons. BDNF copy numbers for each animal determined with MXP Pro v3.0 software were normalized to copy numbers of β-actin (Fahnestock et al., 2012).

Enzyme-linked Immmunosorbent Assays (ELISA)

Animals were anaesthetized with isoflurane, blood was collected via cardiac puncture and then immediately placed on ice in labeled 1.5 mL microfuge tubes. After remaining on ice for 1–1.5 h to permit coagulation, blood samples were centrifuged for 15 min at 3000 rpm at 4 °C. The supernatant (serum) was then transferred to 1.5 mL cryotubes and stored at −80 °C until use. Serum was analyzed using a sandwich BDNF ELISA kit (BDNF ELISA kit: Human, Rat, Mouse; Biosensis) according to the manufacturer's instructions. Each sample was tested in duplicate on separate plates, and the measurements from the two plates were averaged prior to the statistical analyses described below. VEGF and IGF-1 were analyzed using the same procedures with kits from Biosensis®.

Statistical analyses

Correlation analyses were performed on all measures, in addition to an omnibus 2 × 2 × 2 (stress, exercise and diet) factorial ANOVA. All measures were also tested using a linear contrast (Fox, 2008) for the a priori hypothesis that the combination of diet and exercise, but neither intervention alone, would reveal benefits in animals exposed to the CUS paradigm (stress = stress × diet = stress × exercise < stress × exercise × diet, hereafter referred to as the (S = S × D = S × E) < (S × D × E) contrast. An alpha level of 0.05 was used to determine significance on all statistical tests.

RESULTS

Saccharin preference and adrenal weight

To determine the extent to which animals in stress groups were affected by CUS exposure, we recorded the ratio of saccharin-flavored water to regular water consumed by the animals twice per week during acclimation as well as each of the four weeks of the experiment. The ratio of the adrenal gland to body weight at the time of euthanasia was also calculated. Saccharin preference is a measure of anhedonia, while adrenal weight is a physiological indicator of allostatic load. Separate 2 × 2 × 2 ANOVAs applied to the saccharin preference data revealed a reduced saccharin preference among stress groups beginning in the first week of stress exposure ($F_{1,61}$ = 44.6, p < 0.0001) that continued for the duration of the experiment (see Fig. 1).

The 2 × 2 × 2 ANOVA results for the post mortem adrenal weight measurements revealed significantly enlarged adrenal glands in the stress groups (corrected to body weight, main effect of stress, $F_{1,68}$ = 90.74, p < 0.0001; Fig. 2). There was also a significant negative correlation between saccharin preference (any of weeks 1–4) and adrenal weight/body weight (r = −0.498, p = 0.002). We verified that there were no group differences in saccharin preference prior to stress exposure (week 0 or baseline, $F_{7,61}$ = 1.5, p = 0.18). In addition to the main effect of stress, there was also an interaction effect between exercise and adrenal mass ($F_{1,68}$ = 14.001, p = 0.0004). Evaluation of our a priori hypothesis that the combination of diet and exercise would counteract the effects of CUS, using the (S = S × D = S × E) < (S × D × E) contrast, revealed that by the 4th week of CUS exposure the combination of diet and exercise (but neither alone) did partially restore saccharin preference ($t_{1,61}$ = 5.1, p = 0.0001, not significant for data from weeks 0–3), but did not affect adrenal gland size. These data show that mice were strongly impacted by stress manipulations but this impact was partially reversed with a combination of the CDS and exercise (at least for hedonic status).

DG and CA1 size

To determine the effects of stress, exercise and diet on the overall structure of the hippocampus, we measured the area (mm^2) of both the DG and CA1, which were clearly visible in photographs of the sampled DCX-immunolabeled sections. These were also used for quantification of adult born neurons in the DG (e.g., Fig. 3A). Area measurements are shown in Table 3. ANOVAs for the DG area measurements revealed diet by stress ($F_{1,64}$ = 5.169, p = 0.026) and exercise × stress ($F_{1,64}$ = 4.406, p = 0.0398) interactions. The (S = S × D = S × E < S × E × D) contrast was also significant ($t_{1,64}$ = 3.218, p = 0.002). These effects were also observed for the sectional CA1 area data

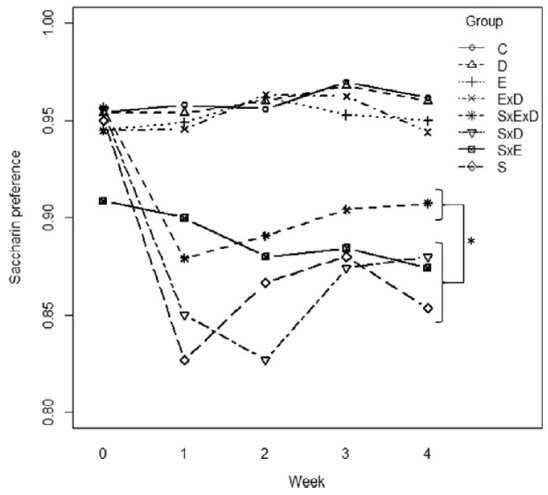


Fig. 1. Saccharin preference (proportion of saccharin to plain water consumed) over time. Chronically stressed animals showed a persistent reduction in their preference for saccharin flavored water over unsweetened water ($2 \times 2 \times 2$ ANOVA, $p < .0001$). By the fourth week of CUS exposure, only those animals which had also exercised and received dietary supplementation exhibited an amelioration of this anhedonic behavior ($S = S \times D = S \times E$) < ($S \times D \times E$) planned contrast; $^{*}p = 0.0001$). (C = Control; S = Stress; D = Diet; E = Exercise).

(diet × stress, $F_{1,64} = 6.23$, $p = 0.015$; $F_{1,64} = 6.71$, $p = 0.012$; ($S = S \times D = S \times E < S \times E \times D$) contrast: $t_{1,64} = 2.806$, $p = 0.007$). ANOVAs of the CA1 area data revealed a strong trend (significant at the 0.10 level) suggesting a negative effect of stress ($F_{1,64} = 3.8897$, $p = 0.053$). Comparison between stress groups for both measures can be seen in Fig. 3. The similarity in results between DG and CA1 measures suggests an overall hippocampal structural modification by the combination of exercise and the CDS.

Neurogenesis

Estimates of neurogenesis were obtained by measuring the number of DCX-positive immunolabeled cells per 40-μm coronal section of DG (sectional quantity) averaged across nine sampled sections (Fig. 4A, B). A $2 \times 2 \times 2$ ANOVA showed a robust main effect of exercise on neurogenesis ($F_{1,64} = 7.272$, $p = 0.009$) and a diet by stress by exercise interaction ($F_{1,64} = 4.156$, $p = 0.0456$). The ($S = S \times D = S \times E < S \times E \times D$) contrast was also significant (groups $t_{1,64} = 2.887$, $p = 0.0053$), suggesting that the main effect of exercise was driven by differences in animals that had not been exposed to chronic stress, while also showing the effectiveness of the diet × exercise combination in increasing neurogenesis in animals exposed to chronic stress. Considering that we observed similar effects in the DG and CA1 sectional area data, we also applied the same analysis to the DCX-positive cell counts after first correcting for the length (mm) of the subgranular zone (SGZ), to obtain neurogenesis density measurements. This correction for SGZ length attenuated the effect of diet and exercise in stress groups (diet × exercise × stress interaction no longer significant, $F_{1,64} = 2.28$, $p = 0.135$; ($S = S \times D = S \times E$) < ($S \times E \times D$) contrast, $t_{1,64} = 1.837$, $p = 0.07$), but not that of exercise alone ($F_{1,64} = 9.756$, $p = 0.003$), although it revealed a trend toward an effect of stress in reducing the density of adult born neurons ($F_{1,64} = 3.39$, $p = 0.07$). This effect of stress was marginally significant when examined using a directional *t*-test (controls > stress) based on the a priori prediction of a negative effect of stress on neurogenesis ($t_{1,64} = 1.837$, $p = 0.054$).

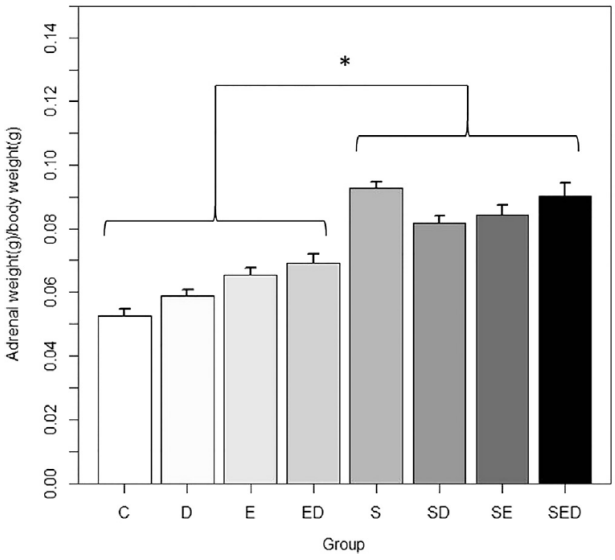


Fig. 2. Adrenal weight (corrected to body weight) in stressed vs. non-stressed animals. Chronically stressed animals showed enlarged adrenal glands ($2 \times 2 \times 2$ ANOVA, $^{*}p < .0001$). Mice in exercise groups also showed increased adrenal mass ($2 \times 2 \times 2$ ANOVA, $p = 0.0004$), demonstrating that both chronic stress and exercise can induce adrenal gland growth. (C = Control; S = Stress; D = Diet; E = Exercise).

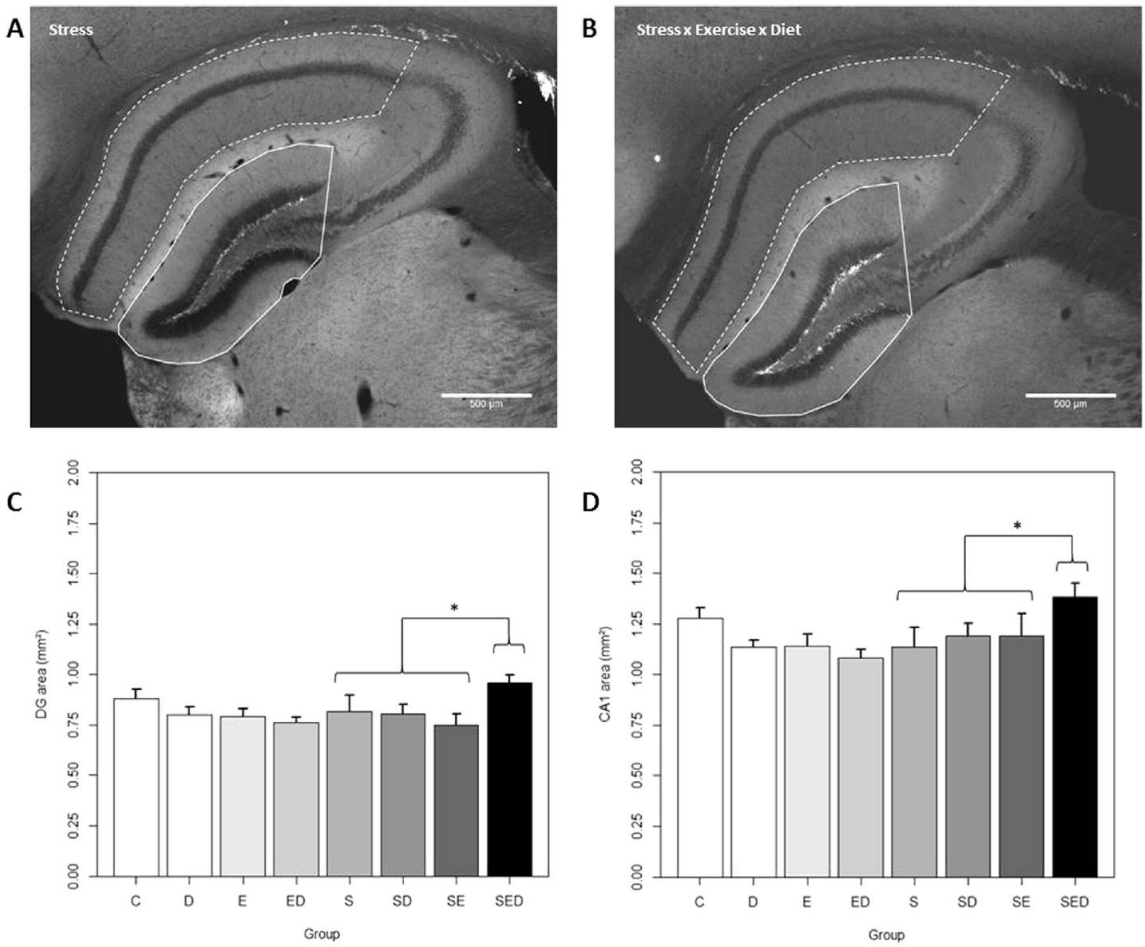


Fig. 3. Effect of diet and exercise on hippocampal area in stressed mice. (A, B) Fluorescence micrographs (4× magnification) showing the surface area of the DG (solid outline) and CA1 (dashed outline) in samples from the stress-only (A) and stress × diet × exercise (B) groups. (C, D) Surface area measurements (mean ± SE, mm^2) of DG (**C**) and CA1 (D) per 40-µm section of the hippocampus for each group reveal that the among chronically stressed animals in the current study, hypertrophy of the hippocampus is only induced by the use of diet and exercise in combination (S = S × D = S × E < S × E × D contrast; $^*p = 0.002$ for DG; $^*p = 0.007$ for CA1). (C = Control; S = Stress; D = Diet; E = Exercise).

Table 3. Average area measurements (mm^2) for each group ± standard error

Group	Structure	
	DG	CA1
Control	0.88 ± 0.03	1.28 ± 0.03
Diet	0.80 ± 0.02	1.13 ± 0.02
Exercise	0.79 ± 0.03	1.14 ± 0.04
Diet × Exercise	0.76 ± 0.02	1.08 ± 0.03
Stress	0.82 ± 0.06	1.14 ± 0.07
Stress × Diet	0.80 ± 0.03	1.21 ± 0.05
Stress × Exercise	0.77 ± 0.04	1.12 ± 0.08
Stress × Exercise × Diet	0.96 ± 0.03*	1.39 ± 0.05*

* Diet × exercise interaction effect, significantly different from the stress-only group.

Hippocampal BDNF expression

We evaluated alterations due to stress, exercise or diet in the expression of the key neurotrophic factor, BDNF (which regulates changes in synaptic density, neurogenesis and hippocampal volume). We used qRT-PCR to quantify BDNF mRNA (normalized to the level of β-actin, a housekeeping gene) in hippocampal tissue homogenates from animals that had not been used for immunohistochemistry (Fig. 5). The $2 \times 2 \times 2$ ANOVA revealed a strong trend toward a main effect of stress: $F_{1,57} = 3.049$, $p = 0.0862$, while the (S = S × D = S × E < S × E × D) contrast indicated that the combination of exercise and diet significantly increased hippocampal BDNF levels in chronically stressed animals ($t_{1,57} = 2.226$, $p = 0.03$).

Serum BDNF, IGF-1 and VEGF

Levels of BDNF, IGF-1 and VEGF in the peripheral circulation were quantified using ELISA in serum samples collected from all animals, to determine if the changes observed in the CNS extended to peripheral factors. Application of the same statistical methods as used above, a $2 \times 2 \times 2$ ANOVA and planned (S = S × E = S × D < S × E × D) contrast, revealed

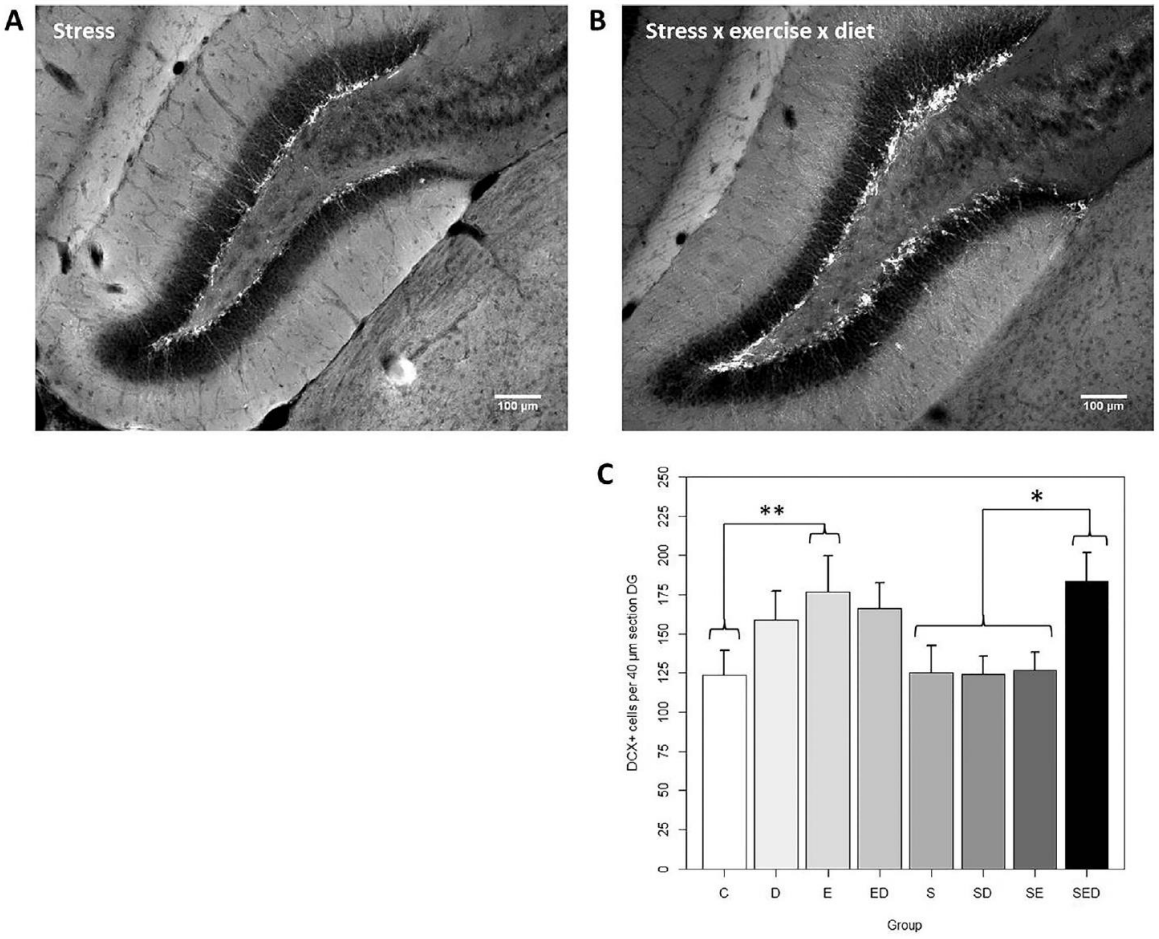


Fig. 4. Effect of stress, exercise and diet on neurogenesis. (A, B) Fluorescence micrographs (10× magnification) showing doublecortin (DCX) labeled adult born neurons in the DG of subjects from the stress-only (A) and stress × exercise × diet (B) groups. (C) Quantity of DCX-positive cells (mean ± SE) per 40-µm section of the DG for each group demonstrating that exercise (but not diet) is sufficient to enhance neurogenesis in non-stressed animals ($2 \times 2 \times 2$ ANOVA, $^{**}p = 0.009$). Among stressed animals neither exercise nor diet alone increases neurogenesis, but the combination does (S = S × D = S × E < S × E × D contrast, $^{*}p = 0.0053$), demonstrating a synergistic effect of diet and exercise under conditions of chronic stress. (C = Control; S = Stress; D = Diet; E = Exercise).

no significant changes in serum BDNF or IGF-1 across groups. However, in the case of VEGF, the omnibus ANOVA revealed a main effect of stress ($F_{1,42} = 2.125$, $p = 0.029$; Fig. 6) and a trend toward a diet × stress interaction ($F_{1,42} = 3.03$, $p = 0.089$), suggesting that the CDS affected VEGF differently in stress group animals compared to controls.

DISCUSSION

These results demonstrate that a combination of aerobic exercise and supplementation with a complex nutraceutical formulation exerted potent neurotrophic effects in stressed rodents evident across diverse biomarkers relevant to hippocampal physiology. Although mice in all stressed groups were strongly affected in measures of depressive-like behavior (saccharin preference) and allostatic burden (adrenal gland size), by the final week of testing some recovery of saccharin preference was seen in stressed animals that exercised and received the CDS. The adrenal glands were also enlarged in exercising but otherwise non-stressed animals. Such an effect of exercise alone has been previously reported (Song et al., 1973; Droste et al., 2003), but this effect does not result in increased corticosteroid binding in the hippocampus (Droste et al., 2003). While others have noted a larger effect of chronic stress on hippocampal structure and plasticity than reported here (Watanabe et al., 1992; Gould et al., 1997, 1998), we did consistently observe trends toward atrophic effects of stress across a wide variety of measures including hippocampal CA1 and DG size, neurogenesis and BDNF mRNA. The fact that these results did not reach significance may have been due to the specific stress protocol used here. In the literature, chronic stress has widely varying effects, ranging from mild to severe pathological changes in the hippocampus. This may reflect the wide range of stress paradigms used in these studies. A study comparing CUS to chronic immobilization stress (CIS) found that the former more robustly impacted

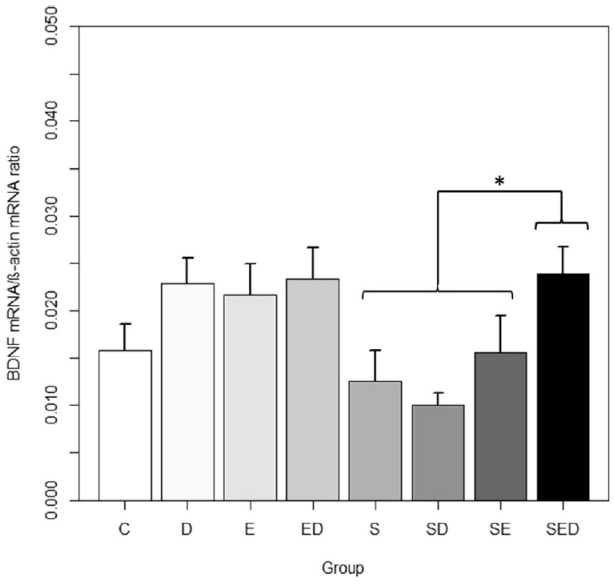


Fig. 5. Effect of stress, exercise and diet on hippocampal BDNF mRNA. qRT-PCR measurements of BDNF mRNA copy numbers normalized to β-actin copy numbers in hippocampal tissue. Under conditions of chronic stress, BDNF expression in the hippocampus is upregulated by a combination of diet and exercise (S = S × D = S × E < S × E × D contrast, $^{*}p = 0.03$), but not by exercise or diet alone. (C = Control; S = Stress; D = Diet; E = Exercise).

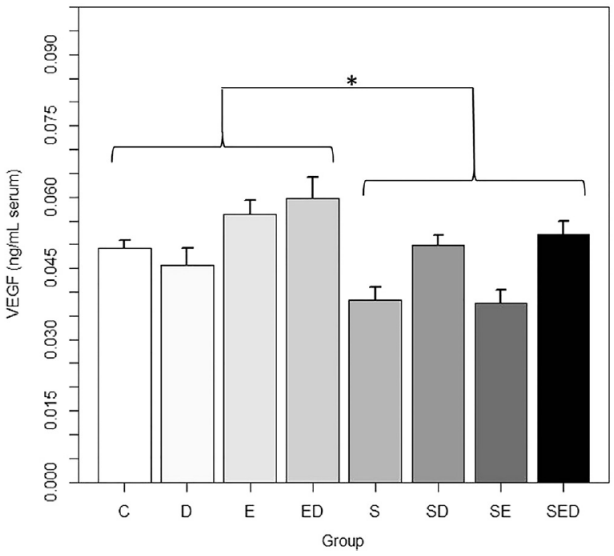


Fig. 6. Influence of stress, exercise and diet on VEGF in peripheral circulation (serum). ELISA measurements of serum VEGF revealed reduced levels in mice exposed to chronic stress (2 × 2 × 2 ANOVA, $^{*}p = 0.029$). A trend toward a diet × stress interaction (2 × 2 × 2 ANOVA, $p = 0.089$) was also detected, suggesting that the effect of the CDS on VEGF may differ in stress group animals compared to controls (Fig. 6). (C = Control; S = Stress; D = Diet; E = Exercise).

the amygdala, while the latter was more detrimental to the hippocampus and behavioral measures of anxiety (elevated plus maze; Vyas et al., 2002). Thus, the use of CIS may be more effective in future studies on hippocampal physiology. Furthermore, the disparity between the behavioral and neurological results in the present study suggests that the former are more overtly altered by CUS.

We also found a potent enhancement of neurogenesis by a prolonged period of voluntary aerobic exercise in non-stressed control groups, which was abolished by chronic stress. The positive effect of exercise on neurogenesis is consistent with previous studies (van Praag et al., 1999, 2005). In contrast to the benefits of exercise, we did not observe a statistically significant benefit of diet in healthy animals. The dietary supplement

might not be expected to have an impact in healthy, young adult control animals given that they might not exhibit markedly elevated levels of oxidative stress, inflammation or other mechanisms targeted by the CDS. The dietary supplement alone also failed to affect most of our measures significantly in stressed groups, consistent with the work of Fahnestock et al. (2012) examining the effects of diet and environmental enrichment in a canine model of age-related cognitive decline. The supplement was originally designed to ameliorate aging (Lemon et al., 2003). Thus, supplementation over longer periods of time or in older animals, as in previous studies (Aksenov et al., 2013), would likely produce much more robust effects in both healthy animals and those that are chronically stressed (which can accelerate cognitive decline, see Lupien et al., 2009).

The finding that the combination of diet and exercise (but neither one alone) partially restored hedonic behavior and exerted multiple trophic effects on the hippocampus underscores the potential for potent synergistic effects between the CDS and aerobic exercise in the treatment of stress-related psychiatric disorders in humans. To our knowledge this is the first report of a successful attempt at disrupting the trophic effects of exercise on neurogenesis using chronic stress and then reinstating it through dietary supplementation. Future research could examine whether similar synergistic interactions are observed when combining other interventions known to affect hippocampus-dependent cognitive functions, such as environmental enrichment (Kempermann et al., 1997; Fahnestock et al., 2012) or cognitive training (Madore et al., 2014), with a similar complex nutraceutical supplement.

It is remarkable that benefits of the combined intervention were observed across nearly all measures included in this study: anhedonia, hippocampal BDNF, DG and CA1 volume, neurogenesis and serum VEGF, suggesting that the effects of diet and exercise in mitigating stress were not specific to a single physiological process or biochemical pathway. It is also noteworthy that the neurogenesis and hippocampal area measurements were conducted in a separate cohort of animals from the hippocampal BDNF assays, yet parallel effects of exercise and diet were observed in both groups. Moreover, the amelioration of anhedonia was observed in both groups of stressed animals receiving diet and exercise. Future studies could investigate whether other measures of neuroplasticity such as dendritic spine density, arborization and synapse morphology would be similarly affected, in light of the changes we observed in the CA1 and DG sectional areas, and previous evidence suggesting that stress-induced hippocampal volume loss is due to synaptic degeneration rather than cellular apoptosis (Watanabe et al., 1992; Magariños et al., 1997; Lucassen et al., 2001). In support of this it has been found that, with the exception of newly born neurons in the subgranular and subventricular zones (Linnarsson et al., 2000), BDNF is not a survival factor for postnatal CNS neurons but rather regulates dendritic complexity and synaptic plasticity (Rauskolb et al., 2010). This insight in particular hints at the possibility that the neurotoxic effects of chronic stress, when observed, may be reversible given the correct combination of nutraceutical and exercise based interventions. Moreover, comparable or even greater effects of diet and exercise in animals exposed to stress may be seen in other brain structures, such as the dorsolateral and dorsomedial prefrontal cortices, which are implicated in stress-induced depression (Larrieu et al., 2014).

While it is impossible to determine the mechanism by which diet and exercise exerted their neuroprotective benefits based on the design of the current study and the complexity of the dietary supplement, the data did provide some clues. Notably, the large effect of exercise, but not diet, on neurogenesis in non-stress groups hints at the possibility that chronic upregulation of the HPA axis interferes with the agonistic action of exercise on neurogenesis, which is known to depend upon the intact signaling of neurotrophic factors, including BDNF (Li et al., 2008), VEGF (Fabel et al., 2003) and IGF-1 (Vivar et al., 2013).

While BDNF features most prominently in the neurophysiological influence of exercise on neurogenesis, our results for VEGF protein levels in serum are especially informative. Given that we observed a trend toward a selective effect of the CDS, but not exercise on serum VEGF (diet $\times$ stress interaction) in animals exposed to chronic stress, it is conceivable that the potential restoration of exercise-mediated neurotrophic effects are driven by VEGF. This is supported by research implicating glucocorticoid receptor signal transduction in the suppression of VEGF and BDNF expression (Smith et al., 1995; Koedam et al., 2002; Kawashima et al., 2010). Alternatively, given the established effects of the CDS on mitochondrial activity (Aksenov et al., 2013), perhaps chronic stress exposure reduced the amount of running in the animals who exercised but did not receive the CDS. On the other hand, physical activity may have been restored by the metabolic effects of the diet. These alternatives could easily be examined in future studies by inhibiting VEGF signaling and/or recording the amount of running of exercise group animals. Lack of detailed records on the amount, duration and speed of running in exercising animals is a limitation in the current study. Regardless, if these hypotheses prove to be correct, it seems reasonable to conclude that the synergistic effects of exercise and diet depend, at least in part, on the upregulation of BDNF and/or VEGF.

A limitation of this study is the exclusive use of male mice, as is common in studies that investigate neurogenesis because female circulating hormones impact neurogenesis levels (Ormerod and Galea, 2001). Nonetheless, considering the relatively high incidence of depression and anxiety disorders in women (Altemus, 2006), it is critically important for future work to include both sexes. Overall, our findings have important clinical implications for those suffering chronic stress-related psychiatric disorders such as major depression. Our results clearly demonstrate the potential of combining CDSs and exercise as an alternative to the pharmacological treatment of mood and anxiety disorders associated with

chronic stress. In addition, the ineffectiveness of either exercise or dietary supplementation alone suggests that their effects are synergistic and recommends against future attempts at monotherapeutic treatment.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

Acknowledgments—We recognize the substantial contributions of A. LoGuidice in assistance with the qRT-PCR analysis of BDNF mRNA from hippocampal tissue samples and B. Michalski for technical support with neurotrophic assays. We also thank M.E. Cybulski and L. Stoa for help with preparation and administration of the dietary supplement and stressor administration, and J. Thorpe for her assistance in pilot testing and stressor administration. Finally, we thank S. Alikanzadeh, C. Leung and Y. Zhou for their assistance with the DCX immunochemistry and Y.F. Tan for her technical support with the immunohistochemical assays. This work was supported by NSERC discovery grants to S. Becker, D.R. Boreham, D. deCatanzaro and J.M. Wojtowicz, a CIHR operating grant (MOP: 119271) to J.M. Wojtowicz, and a grant from the Alzheimer's Society of Canada to M. Fahnestock.

REFERENCES

Aksenov V, Long J, Liu J, Szechtman H, Khanna P, Matravadia S, Rollo CD (2013) A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. Age (Dordr) 35:23–33.

Altemus M (2006) Sex differences in depression and anxiety disorders: potential biological determinants. Horm Behav 50:534–538.

Aronsson M, Fuxe K, Dong Y, Agnati LF, Okret S, Gustafsson JA (1988) Localization of glucocorticoid receptor mRNA in the male rat brain by in situ hybridization. Proc Natl Acad Sci U S A 85:9331–9335.

Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1–10.

Cole MA, Kalman BA, Pace TWW, Topczewski F, Lowrey MJ, Spencer RL (2000) Selective blockade of the mineralocorticoid receptor impairs hypothalamic–pituitary–adrenal axis expression of habituation. J Neuroendocrinol 12:1034–1042.

Creer DJ, Romberg C, Saksida LM, van Praag H, Bussey TJ (2010) Running enhances spatial pattern separation in mice. Proc Natl Acad Sci U S A 107:2367–2372.

d'Audiffret AC, Frisbee SJ, Stapleton PA, Goodwill AG, Isingrini E, Frisbee JC (2010) Depressive behavior and vascular dysfunction: a link between clinical depression and vascular disease? J Appl Physiol 108:1041–1051.

Déry N, Pilgrim M, Gibala M, Gillen J, Wojtowicz JM, Macqueen G, Becker S (2013) Adult hippocampal neurogenesis reduces memory interference in humans: opposing effects of aerobic exercise and depression. Front Neurosci 7:66.

Droste SK, Gesing A, Ulbricht S, Müller MB, Linthorst ACE, Reul JMHM (2003) Effects of long-term voluntary exercise on the mouse hypothalamic–pituitary–adrenocortical axis. Endocrinology 144:3012–3023.

Elizalde N, Gil-Bea FJ, Ramírez MJ, Aisa B, Lasheras B, Del Rio J, Tordera RM (2008) Long-lasting behavioral effects and recognition memory deficit induced by chronic mild stress in mice: effect of antidepressant treatment. Psychopharmacology 199:1–14.

Erickson KI, Voss MW, Prakash RS, Basak C, Szabo A, Chaddock L, Kim JS, Heo S, Alves H, White SM, Wojcicki TR, Mailey E, Vieira VJ, Martin SA, Pence BD, Woods JA, McAuley E, Kramer AF (2011) Exercise training increases size of hippocampus and improves memory. Proc Natl Acad Sci U S A 108:3017–3022.

Fabel K, Fabel K, Tam B, Kaufer D, Baiker A, Simmons N, Kuo CJ, Palmer TD (2003) VEGF is necessary for exercise-induced adult hippocampal neurogenesis. Eur J Neurosci 18:2803–2812.

Fahnestock M, Marchese M, Head E, Pop V, Michalski B, Milgram WN, Cotman CW (2012) BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. Neurobiol Aging 33:546–554.

Fox J (2008) Applied regression analysis and generalized linear models, second ed., pp 176–186. Thousand Oaks, CA: Sage Publications.

Goshen I, Kreisel T, Ben-Menachem-Zidon O, Licht T, Weidenfeld J, Ben-Hur T, Yirmiya R (2008) Brain interleukin-1 mediates chronic stress-induced depression in mice via adrenocortical activation and hippocampal neurogenesis suppression. Mol Psychiatry 13:717–728.

Gould E, Cameron HA, Daniels DC, Woolley CS, McEwen BS (1992) Adrenal hormones suppress cell division in the adult rat dentate gyrus. J Neurosci 12:3642–3650.

Gould E, McEwen BS, Tanapat P, Galea LA, Fuchs E (1997) Neurogenesis in the dentate gyrus of the adult tree shrew is regulated by psychosocial stress and NMDA receptor activation. J Neurosci 17:2492–2498.

Gould E, Tanapat P, McEwen BS, Flügge G, Fuchs E (1998) Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A 95:3168–3171.

Harkin A, Houlihan DD, Kelly JP (2002) Reduction in preference for saccharin by repeated unpredictable stress in mice and its prevention by imipramine. J Psychopharmacol 16:115–123.

Kawashima H, Numakawa T, Kumamaru E, Adachi N, Mizuno H, Ninomiya M, Kunugi H, Hashido K (2010) Glucocorticoid attenuates brain-derived neurotrophic factor-dependent upregulation of glutamate receptors via the suppression of microRNA-132 expression. Neuroscience 165:1301–1311.

Kempermann G, Kuhn HG, Gage FH (1997) More hippocampal neurons in adult mice living in an enriched environment. Nature 386:493–495.

Koedam JA, Smink JJ, van Buul-Offers SC (2002) Glucocorticoids inhibit vascular endothelial growth factor expression in growth plate chondrocytes. Mol Cell Endocrinol 197:35–44.

Larrieu T, Hilal LM, Fourrier C, De Smedt-Peyrusse V, Sans N, Capuron L, Layé S (2014) Nutritional omega-3 modulates neuronal morphology in the prefrontal cortex along with depression-related behaviour through corticosterone secretion. Transl Psychiatry 4:e437.

Lau FC, Shukitt-Hale B, Joseph JA (2005) The beneficial effects of fruit polyphenols on brain aging. Neurobiol Aging 26(Suppl 1):128–132.

Lemon JA, Boreham DR, Rollo CD (2003) A dietary supplement abolishes age-related cognitive decline in transgenic mice expressing elevated free radical processes. Exp Biol Med 228:800–810.

Lemon JA, Rollo CD, Boreham DR (2008a) Elevated DNA damage in a mouse model of oxidative stress: impacts of ionizing radiation and a protective dietary supplement. Mutagenesis 23:473–482.

Lemon JA, Rollo CD, McFarlane NM, Boreham DR (2008b) Radiation-induced apoptosis in mouse lymphocytes is modified by a complex dietary supplement: the effect of genotype and gender. Mutagenesis 23:465–472.

Leung BMY, Kaplan BJ, Field CJ, Tough S, Eliasziw M, Gomez MF, McCargar LJ, Gagnon L (2013) Prenatal micronutrient supplementation and postpartum depressive symptoms in a pregnancy cohort. BMC Pregnancy Childbirth 13:2.

Li Y, Luikart BW, Birnbaum S, Chen J, Kwon CH, Kernie SG, Bassel-Duby R, Parada LF (2008) TrkB regulates hippocampal neurogenesis and governs sensitivity to antidepressive treatment. Neuron 59:399–412.

López JF, Chalmers DT, Little KY, Watson SJ (1998) Regulation of serotonin(1A), glucocorticoid, and mineralocorticoid receptor in rat and human hippocampus: implications for the neurobiology of depression. Biol Psychiatry 43:547–573.

Linnarsson S, Willson CA, Ernfors P (2000) Cell death in regenerating populations of neurons in BDNF mutant mice. Mol Brain Res 75:61–69.

Lucassen PJ, Vollmann-Honsdorf GK, Gleisberg M, Czéh B, De Kloet ER, Fuchs E (2001) Chronic psychosocial stress differentially affects apoptosis in hippocampal subregions and cortex of the adult tree shrew. Eur J Neurosci 14:161–166.

Lupien SJ, McEwen BS, Gunnar MR, Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat Rev Neurosci 10:434–445.

MacQueen GM, Campbell S, McEwen BS, Macdonald K, Amano S, Joffe RT, Nahmias C, Young LT (2003) Course of illness, hippocampal function, and hippocampal volume in major depression. Proc Natl Acad Sci U S A 100:1387–1392.

Madore KP, Gaesser B, Schacter DL (2014) Constructive episodic simulation: dissociable effects of a specificity induction on remembering, imagining, and describing in young and older adults. J Exp Psychol Learn Mem Cogn 40:609–622.

Magariños AM, Verdugo JM, McEwen BS (1997) Chronic stress alters synaptic terminal structure in hippocampus. Proc Natl Acad Sci U S A 94:14002–14008.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. J Neurosci 20:9104–9110.

Mantella RC, Vollmer RR, Amico JA (2005) Corticosterone release is heightened in food or water deprived oxytocin deficient male mice. Brain Res 1058:56–61.

McEwen BS (2003) Mood disorders and allostatic load. Biol Psychiatry 54:200–207.

McEuen JG, Beck SG, Bale TL (2008) Failure to mount adaptive responses to stress results in dysregulation and cell death in the midbrain raphe. J Neurosci 28:8169–8177.

McDonald HY, Wojtowicz JM (2005) Dynamics of neurogenesis in the dentate gyrus of adult rats. Neurosci Lett 385:70–75.

Merkley CM, Jian C, Mosa A, Tan YF, Wojtowicz MM (2014) Homeostatic regulation of adult hippocampal neurogenesis in aging rats: long-term effects of early exercise. Front Neurosci 8:1–12.

Milgram NW, Zicker SC, Head E, Muggenburg BA, Murphey H, Ikeda-Douglas CJ, Cotman CW (2002) Dietary enrichment counteracts age-associated cognitive dysfunction in canines. Neurobiol Aging 23:737–745.

Miller BR, Hen R (2015) The current state of the neurogenic theory of depression and anxiety. Curr Opin Neurobiol 30:51–58.

Olson AK, Eadie BD, Ernst C, Christie BR (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. Hippocampus 16:250–260.

Ormerod BK, Galea LAM (2001) Reproductive status influences cell proliferation and cell survival in the dentate gyrus of adult female meadow voles: a possible regulatory role for estradiol. Neuroscience 102:369–379.

Ota KT, Liu R, Voleti B, Maldonado-Aviles JG, Duric V, Iwata M, Dutheil S, Duman C, Boikess S, Lewis DA, Stockmeier CA, DiLeone RJ, Rex C, Aghajanian GK, Duman RS (2014) REDD1 is essential for stress-induced synaptic loss and depressive behavior. Nat Med 20:531–535.

Parachikova A, Green KN, Hendrix C, LaFerla FM (2010) Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. PLoS One 5:e14015.

Perera TD, Dwork AJ, Keegan KA, Thirumangalakudi L, Lipira CM, Joyce N, Lange C, Higley JD, Rosoklija G, Hen R, Sackeim HA, Coplan JD (2011) Necessity of hippocampal neurogenesis for the therapeutic action of antidepressants in adult Nonhuman primates. PLoS One 6:1–13.

Pereira AC, Huddleston DE, Brickman AM, Sosunov AA, Hen R, McKhann GM, Sloan R, Gage FH, Brown TR, Small SA (2007) An in vivo correlate of exercise-induced neurogenesis in the adult dentate gyrus. Proc Natl Acad Sci U S A 104:5638–5643.

Pop V, Head E, Hill M-A, Gillen D, Berchtold NC, Muggenburg BA, Milgram NW, Murphy MP, Cotman CW (2010) Synergistic effects of long-term antioxidant diet and behavioral enrichment on beta-amyloid load and non-amyloidogenic processing in aged canines. J Neurosci 30:9831–9839.

Popper CW (2014) Single-micronutrient and broad-spectrum micronutrient approaches for treating mood disorders in youth and adults. Child Adolesc Psychiatr Clin N Am 23:591–672.

Rauskolb S, Zagrebelsky M, Dreznjak A, Deogracias R, Matsumoto T, Wiese S, Erne B, Sendtner M, Schaeren-Wiemers N, Korte M, Barde Y-A (2010) Global deprivation of brain-derived neurotrophic factor in the CNS reveals an area-specific requirement for dendritic growth. J Neurosci 30:1739–1749.

Reul JMHM, De Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117:2505–2511.

Rucklidge JJ, Kaplan BJ (2013) Broad-spectrum micronutrient formulas for the treatment of psychiatric symptoms: a systematic review. Expert Rev Neurother 13:49–73.

Sahay A, Scobie KN, Hill AS, O'Carroll CM, Kheirbek MA, Burghardt NS, Fenton AA, Dranovsky A, Hen R (2011) Increasing adult hippocampal neurogenesis is sufficient to improve pattern separation. Nature 472:466–470.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S, Weisstaub N, Lee J, Duman R, Arancio O, Belzung C, Hen R (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. Science 301:805–809.

Sapolsky R (1985) A possible mechanism for glucocorticoid toxicity in the hippocampus: increased vulnerability of neurons to metabolic insults. J Neurosci 5:1228–1232.

Sapolsky RM, Uno H, Rebert CS, Finch CE (1995) Hippocampal damage associated with prolonged glucocorticoid exposure in primates. J Neurosci 10:2897–2902.

Schmidt HD, Duman RS (2010) Peripheral BDNF produces antidepressant-like effects in cellular and behavioral models. Neuropsychopharmacology 35:2378–2391.

Sheline YI, Sanghavi M, Mintun MA, Gado MH (1999) Depression duration but not age predicts hippocampal volume loss in medically healthy women with recurrent major depression. J Neurosci 19:5034–5043.

Smith MA, Makino S, Kvetnansky R, Post RM (1995) Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus. J Neurosci 15:1768–1777.

Snyder JS, Kee N, Wojtowicz JM (2001) Effects of adult neurogenesis on synaptic plasticity in the rat dentate gyrus. J Neurophysiol 85:2423–2431.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. Nature 476:458–461.

Song MK, Ianuzzo CD, Saubert IV CW, Gollnick PD (1973) The mode of adrenal gland enlargement in the rat in response to exercise training. Pflugers Arch Eur J Physiol 339:59–68.

Strekalova T, Steinbusch HWM (2010) Measuring behavior in mice with chronic stress depression paradigm. Prog Neuro-Psychopharmacology Biol Psychiatry 34:348–361.

Surget A, Tanti A, Leonardo ED, Laugeray A, Rainer Q, Touma C, Palme R, Griebel G, Ibarguen-Vargas Y, Hen R, Belzung C (2011) Antidepressants recruit new neurons to improve stress response regulation. Mol Psychiatry 16:1177–1188.

Thorpe JB, Gould KE, Borman ED, deCatanzaro D (2014) Circulating and urinary adrenal corticosterone, progesterone, and estradiol in response to acute stress in female mice (*Mus musculus*). Horm Metab Res 46:211–218.

Valente T, Hidalgo J, Bolea I, Ramirez B, Anglés N, Reguant J, Morelló JR, Gutiérrez C, Boada M, Unzeta M (2009) A diet enriched in polyphenols and polyunsaturated fatty acids, LMN

diet, induces neurogenesis in the subventricular zone and hippocampus of adult mouse brain. J Alzheimers Dis 18:849–865.
van Praag H, Kempermann G, Gage FH (1999) Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci 2:266–270.
van Praag H, Shubert T, Zhao C, Gage FH (2005) Exercise enhances learning and hippocampal neurogenesis in aged mice. J Neurosci 25:8680–8685.
Vivar C, Potter MC, van Praag H (2013) All about running: synaptic plasticity, growth factors and adult hippocampal neurogenesis Carmen. Curr Top Behav Neurosci 15:289–320.
Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002) Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci 22:6810–6818.
Watanabe Y, Gould E, McEwen BS (1992) Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res 588:341–345.
Willner P, Muscat R, Papp M (1992) Chronic mild stress-induced anhedonia: a realistic animal model of depression. Neurosci Biobehav Rev 16:525–534.
Winocur G, Wojtowicz JM, Huang J, Tannock IF (2014) Physical exercise prevents suppression of hippocampal neurogenesis and reduces cognitive impairment in chemotherapy-treated rats. Psychopharmacology 231:2311–2320.
Winocur G, Wojtowicz JM, Sekeres M, Snyder JS, Wang S (2006) Inhibition of neurogenesis interferes with hippocampus-dependent memory function. Hippocampus 16:296–304.
Wojtowicz JM, Kee N (2006) BrdU assay for neurogenesis in rodents. Nat Protoc 1:1399–1405.
Wu A, Ying Z, Gomez-Pinilla F (2008) Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. Neuroscience 155:751–759.

(Accepted 2 September 2015)
(Available online 7 September 2015)

3. Early intervention with a multi-ingredient dietary supplement improves mood and spatial memory in a triple transgenic mouse model of Alzheimer's disease.

Hutton, C. P., Lemon, J. A., Sakic, B., Rollo, C. D., Boreham, D. R., Fahnestock, M., Wojtowicz, J.M., & Becker, S. (2018). Early intervention with a multi-ingredient dietary supplement improves mood and spatial memory in a triple transgenic mouse model of Alzheimer's disease. *Journal of Alzheimer's Disease, 64*(3), 835-857.

Journal of Alzheimer's Disease xx (20xx) x–xx
DOI 10.3233/JAD-170921
IOS Press

Early Intervention with a Multi-Ingredient Dietary Supplement Improves Mood and Spatial Memory in a Triple Transgenic Mouse Model of Alzheimer's Disease

Craig P. Hutton[a], Jennifer A. Lemon[b], Boris Sakic[c], C. David Rollo[d], Douglas R. Boreham[b], Margaret Fahnestock[c], J. Martin Wojtowicz[d] and Suzanna Becker[a,*]
[a]*Department of Psychology, Neuroscience & Behavior, McMaster University, Hamilton, ON, Canada*
[b]*Department of Biology, McMaster University, Hamilton, ON, Canada*
[c]*Department of Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, ON, Canada*
[d]*Department of Physiology, University of Toronto, Toronto, ON, Canada*

Handling Associate Editor: Isabelle Aubert

Accepted 9 May 2018

Abstract. The increasing global burden of Alzheimer's disease (AD) and failure of conventional treatments to stop neurodegeneration necessitates an alternative approach. Evidence of inflammation, mitochondrial dysfunction, and oxidative stress prior to the accumulation of amyloid-β in the prodromal stage of AD (mild cognitive impairment; MCI) suggests that early interventions which counteract these features, such as dietary supplements, may ameliorate the onset of MCI-like behavioral symptoms. We administered a polyphenol-containing multiple ingredient dietary supplement (MDS), or vehicle, to both sexes of triple transgenic (3xTg-AD) mice and wildtype mice for 2 months from 2–4 months of age. We hypothesized that the MDS would preserve spatial learning, which is known to be impaired in untreated 3xTg-AD mice by 4 months of age. Behavioral phenotyping of animals was done at 1-2 and 3-4 months of age using a comprehensive battery of tests. As previously reported in males, both sexes of 3xTg-AD mice exhibited increased anxiety-like behavior at 1-2 months of age, prior to deficits in learning and memory, which did not appear until 3-4 months of age. The MDS did not reduce this anxiety or prevent impairments in novel object recognition (both sexes) or on the water maze probe trial (females only). Strikingly, the MDS specifically prevented 3xTg-AD mice (both sexes) from developing impairments (exhibited by untreated 3xTg-AD controls) in working memory and spatial learning. The MDS also increased sucrose preference, an indicator of hedonic tone. These data show that the MDS can prevent some, but not all, psychopathology in an AD model.

Keywords: Alzheimer's disease, anhedonia, anxiety, dietary supplements, learning, memory, mice, mild cognitive impairment, reversal learning, transgenic, working memory

INTRODUCTION

Alzheimer's disease (AD) afflicts over 33 million people worldwide (http://www.who.int) at a cost of US$ 604 billion and is projected to reach 135 million cases by 2050. Conventional treatments, such as cholinergic drugs, fail to stop progression, highlighting the need for new treatments [1]. In AD, the rapid

*Correspondence to: Suzanna Becker, Department of Psychology, Neuroscience & Behaviour, Psychology Building, Room 312, McMaster University, 1280 Main St. W., Hamilton, Ontario, L8S 4K1, Canada. Tel.: +1 905 525 9140/Ext. 23020, Fax: +1 905 529 6225, E-mail: becker@mcmaster.ca.

decline in cognitive ability reflects damage to brain regions important in learning, memory and mood regulation, notably the hippocampus and prefrontal cortex [2–4]. Thus, AD is also associated with high levels of depression (43.6%) and anxiety (25.4%) far exceeding those in age-matched controls [5].

Most research into AD pathogenesis has focused on counteracting the accumulation of misfolded proteins amyloid-β (Aβ) and hyperphosphorylated tau that contribute to plaques and tangles throughout the brains of AD patients. This work has led to the prominent "amyloid cascade hypothesis" of AD [6, 7]. Briefly, the cascade hypothesis proposes that excessive production or impaired clearance of Aβ leads to its subsequent accumulation, which triggers a neurotoxic cascade resulting in tau hyperphosphorylation and aggregation, synaptic atrophy, inflammation and oxidative damage, Ca^{2+} imbalance and ultimately neuronal death.

The amyloid cascade hypothesis is well supported by evidence from *in vitro* and animal studies [7–9]. However, it appears to have some serious limitations, most notably the fact that all anti-amyloidogenic drugs have thus far failed in human clinical trials [10, 11]. Furthermore, downstream effectors such as synapse loss [12] and reduced levels of brain-derived neurotrophic factor (BDNF) [13, 14], rather than plaque or tangle burden, are better predictors of cognitive symptoms in AD patients. Moreover, an emerging literature implicates exacerbation of age-related physiological changes in cognitive decline and AD. Specifically, the preclinical stage of AD, mild cognitive impairment (MCI) [15], has been linked to elevated levels of inflammation [16], insulin resistance [17, 18] and oxidative stress [19], and reduced levels of BDNF [13] and brain glucose metabolism [20]. Therefore, in contrast to most other studies on AD model animals [21, 22], we chose to focus on prevention rather than treatment.

Lifestyle based approaches to MCI and AD prevention, such as nutritional supplements, are emerging as alternatives to pharmaceutical compounds [1, 11]. For example, 6 months of supplementation with the omega-3 fatty acid docosahexaenoic acid (1.16 g/d DHA) in healthy adults was found to improve episodic memory, working memory, and attention [23]. Further, in a 6–8 year longitudinal study, the risk of AD was 60% lower in those who drank polyphenol-containing fruit or vegetable juices at least 3x per week [24].

Such findings suggest that a combination of polyphenols and unsaturated fatty acids may protect against MCI and consequently AD. We propose that a particularly promising approach is to use a broad-based multiple ingredient dietary supplement (MDS; Table 1) developed to target age-related alterations in inflammation, oxidative stress, mitochondrial dysfunction, insulin resistance and membrane integrity [25–29], all of which are also implicated in AD disease progression as discussed above. This MDS has been shown to reduce age-related declines in spatial learning, brain volume, neuronal atrophy, neuronal death and DNA damage in aged mice [25, 26].

The current study administered the MDS to both sexes of 3xTg-AD mice [30] and wildtype (WT) mice and compared their behavior across a wide range of measures (Table 2) to vehicle-treated 3xTg-AD and WT controls. This battery of tests was designed to clarify which behavioral alterations appear in this mouse model at the earliest stages; most previous studies examined behavioral outcomes at 6 months of age or later (e.g., [31–33]), reported conflicting sex differences (e.g., [34, 35]), used only a single behavioral measure starting at 2–4 months of age [34, 36], or evaluated only one sex (e.g., [37, 38]).

The importance of including both sexes is becoming increasingly appreciated, as male and female 3xTg-AD mice exhibit different trajectories of Aβ accumulation [39] and behavioral abnormalities [33, 35]. Behavioral alterations, progressing from anxiety to learning and memory impairments, emerge in males at 2–4 months of age [30, 36, 38, 40, 41]), while females develop similar symptoms at 4–6 months of age [33, 42, 43]. After initial presentation, it is unclear whether memory deteriorates more rapidly in males or females, as different behavioral tests have yielded conflicting results (e.g., [34, 35]). Behavioral deficits are followed by the emergence of amyloid and tau accumulation starting at 3–6 months of age, with more rapid accumulation in females [44–47]. Plaques appear later, at 8–14 months of age in females and 16–18 months of age in males [38, 39, 44, 45, 48]. Still later, tangles emerge at 16–18 months of age in females [44, 48] and 21–26 months of age in males [46, 49].

Given the rate of disease progression described above, we anticipated that by 3-4 months of age 3xTg-AD mice would exhibit anxiety-like behavior (males only; [38]) and selective deficits on the Morris water maze (MWM; both sexes [36]). Moreover, we predicted especially pronounced deficits in the MWM for the high interference reversal trials, during which the mouse is required to neglect the original location of the submerged platform and learn a new location.

Table 1
Ingredients included in the multiple ingredient dietary supplement

Ingredient	Daily Dose (mg)	Ingredient	Daily Dose (mg)
Acetyl L-Carnitine	14.4	L-Glutathione	0.36
Acetylsalicylic Acid	2.5	Magnesium	0.72
Ascorbic Acid (Vit. C)	3.6	Melatonin	0.01
Bioflavinoids	4.32	N-Acetyl Cystein	7.2
Cholecalciferol (Vit. D)	0.0000625	Niacin (Vit. B3)	0.72
Chromium Picolinate	0.00144	Potassium Pyridoxine	0.36
Cobalamin (Vit. B12)	0.00072	Hydrochloride (Vit. B6)	0.72
Curcumin	1.8	Quercitin	0.9
D-a-tocopherol (Vit. E)	0.965	Rutin	0.72
Folic Acid	0.01	Selenium	0.00108
Garlic	0.0216	Thiamine (Vit. B1)	0.72
Ginger	7.2	Ubiquinone (CoQ10)	0.44
Gingko Biloba	1.44	Wild Fish Oil	21.6
Ginseng	8.64	a-Lipoic Acid	0.72
Green Tea	7.2	ß-Carotene	0.03

Table 2
Mouse behavioural test battery

Day(s)	Test	Measures
–4 to 0	Animal Handling	n/a
1	visual placing response, righting reflex, grasping reflex, hind limb placing response, postural reflex, negative geotaxis	basic motor and visual reflexes
	Rotarod	sensorimotor coordination
2	Hanging Basket	muscle strength
3–8	Olfactory Acuity and Discrimination	olfactory acuity and discrimination
9	Beam Walking	balance/motor coordination
10 - 11	Open Field	anxiety (agoraphobia/photophobia)
12	Elevated Plus Maze	anxiety (acrophobia/photophobia)
13–16	Novel Object Recognition	novelty detection
17–21	INBEST Monitoring	spontaneous activity, food and water intake, sucrose preference
22–27	Spontaneous Alternation	visuospatial working memory and navigation strategy
28–36	Morris Water Maze	visual acuity, Spatial learning/memory

High interference learning is markedly impaired in MCI patients [50] and has been shown previously to be dependent upon hippocampal neurogenesis in animal models [51, 52]. Moreover, adult neurogenesis and BDNF are among the earliest biomarkers depleted in rodent models of AD (by 2–4 months of age [53–56]). Given that the MDS (and similar supplements) upregulate neurogenesis [57, 58] and BDNF [57, 59–61], we hypothesized that the MDS would ameliorate deficits in the 3xTg-AD mice on water maze reversal trials.

MATERIALS AND METHODS

Animals

The subjects were 26 male and 20 female B6;129-*Psen1*^{tm1Mpm} Tg(APPSwe,tauP301L) 1Lfa/Mmjax (3xTg-AD) mice, plus 25 male and 26 female mice of the wildtype genetic background B6129SF2/J strain (WT). Fewer 3xTg-AD females and WT males were used due to a lack of availability from the breeding colony (see below). The 3xTg-AD mouse has been described in detail elsewhere [30]. It is a homozygous carrier of 3 human mutations for genes associated with early onset AD and frontotemporal dementia affecting amyloid and tau: APPswe, Psen1, and tauP301L. This model was chosen as one of the few to exhibit both amyloid and tau pathology concomitant with impaired memory and altered mood-related behaviors [62, 63]. Mice used in the study were obtained from an in-house breeding colony in the Psychology Department Animal Facility at McMaster University, which was established using breeders ordered from Jackson Laboratories (Bar Harbor, USA).

All animals, including breeders, were housed with a reversed 12:12 h light/dark cycle (lights off from 7:00–19:00) and stable temperature (~22°C ± 0.5°C) and humidity (~62%). Upon weaning, experimental mice were housed individually and provided with Harlan™ Teklad 22/5 Rodent Diet chow and water *ad libitum*. All mice were caged with woodchip bedding and provided with nestlets as a source of environmental enrichment. The mice were then tattooed on the ears under isoflurane anesthesia for identification purposes and subsequently handled for 5 days (~1 min each per day) prior to the start of baseline behavioral testing (at ~4.5–5 weeks of age) using the behavioral battery (Table 2). Animals were also given *ad libitum* access to sucrose-water (1%) for 10 h during INBEST testing. After baseline behavioral testing, mice of each genotype were randomly assigned to the treatment (MDS) or control (vehicle) condition (see below), yielding 8 groups: MDS 3xTg-AD females ($n = 10$), vehicle 3xTg-AD females ($n = 10$), MDS 3xTg-AD males ($n = 13$), vehicle 3xTg-AD males ($n = 13$), MDS WT females ($n = 13$), vehicle WT females ($n = 13$), MDS WT males ($n = 12$), vehicle WT males ($n = 13$). All procedures were approved by the McMaster University Animal Research Ethics Board.

Multiple ingredient dietary supplement

The multi-ingredient dietary supplement (MDS) was first developed to determine whether targeting multiple cellular processes with nutritional compounds might attenuate the processes associated with the premature aging phenotype of transgenic growth hormone mice. Specifically, increased oxidative stress, inflammation, impaired glucose metabolism, membrane deterioration, and mitochondrial dysregulation are processes common to normal brain aging and exacerbated in neuropathologies [64–69]. Ingredients were selected based on established efficacy in one or more of the above processes, leading to the combination of 30 ingredients (Table 1) that comprise the MDS. See Lemon et al. [28], for the processes targeted by each ingredient. The MDS has demonstrated significant protective effects on cognition, longevity and motor function in normal and TGM mice [25–29, 70].

The combination of ingredients, preparation method and doses of the MDS used were identical to those described previously [27]. These parameters were originally defined according to recommendations for human consumption adapted to mice, accounting for differences in body size and metabolic rate. Briefly, the 30 MDS ingredients were mixed into aqueous solution, pipetted onto a small piece of bagel and left to dry. These bagel chips were then provided to mice between 17:00–19:00, i.e., leading up to the sleep cycle (lights on), because of the presence of melatonin in the MDS. Mice assigned to the vehicle control condition were provided with plain bagel chips only (no MDS). All bagel chips were consumed within several minutes. Bagel chips were fed to the mice daily from 2 months of age for 2 months, until the end of behavioral testing at 4 months of age.

Behavioral test battery

The battery of tests (described below; Table 2) was used to assess a wide variety of motor, sensory, mood-related, learning and memory functions in mice that are known (except for reflexes) to be altered in 3xTg-AD mice at different ages. Animals were tested in the battery (Table 2) at 1-2 months of age (baseline), randomly assigned to receive the MDS or vehicle daily for 2 months and subsequently retested at 3-4 months of age. The 1-2 and 3-4 month testing ages were therefore chosen because they represent distinct stages of early disease progression in this specific strain (see Introduction). This combination of tests represents an expansion from a similar battery used previously [38], and is, to our knowledge, the most comprehensive battery of tests that has been administered to 3xTg-AD mice. This broad range of measures was designed to capture the spectrum of cognitive, behavioral, and psychiatric symptoms commonly exhibited by patients with MCI or AD. While it is not possible to evaluate some symptoms (such as hallucinations [71]) in mice, we were able to test many of the behavioral characteristics that are affected in MCI or AD such as motor co-ordination, anxiety, working memory, recognition memory, visuospatial learning and memory, olfactory function, and anhedonia [15, 50, 71–73]. Subjects were tested individually in random order (generated using R software) on each day of the battery (tests administered in fixed order) to ensure that no animals were tested earlier or later in the day on average relative to others. Note that an abridged description of each test is included below. Please see the Supplementary Materials for additional details.

Motor and visual reflexes

On the first day of testing, several reflexes were assessed to determine if any mouse was affected

by gross neurological deficits that would impair visual or motor function to a degree that would limit their ability to complete the other tests. Reflexes tested were: visual placing response, righting reflex, grasping reflex, hind-limb clasping reflex, postural reflex, and negative geotaxis. Testing procedures have been described elsewhere [74, 75]. Performance was scored only on a pass/fail basis because more detailed assessments were done in subsequent tests (e.g., beam walking and rotarod). Since no mice exhibited abnormal responses, this portion of the battery is not covered further in this report.

Motor coordination and muscle strength

Although motor coordination, strength and mobility are considered to be affected only later in the progression of AD (http://www.alz.org; [76], there is some evidence that fine/complex coordination might be impacted in MCI [72]. The rotarod [77] and hanging basket test [78] assess gross coordination and muscle strength, respectively. Since performance on these tests can be affected by body weight [33], we also recorded weight as a potential covariate.

The rotarod

The procedure and apparatus (MedAssociates, Inc., St. Albans, VT) used in the present experiment were described previously [38]. Mice were tested on 3 trials in a single session, and the average fall time was used as a measure of performance.

The hanging basket test

The mouse was placed on top of a wire mesh that made up the bottom surface of a transparent Plexiglas basket (29 cm × 19 cm × 16 cm). The basket was then turned upside down and rested on a padded table top. The time until falling (up to 10 min) was recorded by stopwatch, and then the mouse was returned to its home cage. Each mouse was tested on 2 trials.

Beam walking test

On this test the animals crossed a narrow beam from a moderately aversive location (brightly illuminated open space) to a less aversive location (enclosed shelter). The apparatus consisted of a translucent Plexiglas beam (70 cm × 1 cm^2) with a small rectangular (6 cm × 8 cm) Plexiglas platform on one end and a large circular platform (diameter = 28.5 cm) at the other. The protocol was comprised of 3 shaping trials and a single testing trial. In each shaping trial the mouse was placed at a different starting location on the beam, initially adjacent to the large platform and on successive trials, starting progressively farther along the beam toward the small rectangular platform. After the 3rd shaping trial, the mouse was placed on the small rectangular platform and observed as it crossed the beam. Mice were scored for time and number of foot slips.

Olfactory acuity and discrimination

Olfactory function is becoming increasingly recognized as a potential diagnostic tool in helping to discriminate between healthy older adults, those with MCI, and those with AD [79, 80].

Acuity testing

The olfactory acuity testing method used has been described previously [38]. 5 trials were run over 5 days (1/d) using a different concentration (wt/vol, 1%, 0.1%, 0.01%, 0.001% or 0%/control) of peanut butter each day. In each trial, 60 uL of the peanut butter (in mineral oil) solution was pipetted onto 3 cm^2 square pieces of filter paper that were left in the cage with the mouse for 3 min. Mice were scored for sniffing time.

Discrimination testing

The olfactory discrimination testing was done the day after acuity testing, using the same equipment. 0.001% cinnamon and 0.001% paprika (President's Choice, Loblaws Inc., Brampton, ON) dissolved in mineral oil were used as odorants, as they are considered to be appetitively neutral [81]. In a single session, mice were exposed to 4 consecutive trials lasting 2 min each using cinnamon as the odorant. Between trials, the testing cages were cleaned using 1.5% acetic acid in aqueous solution. Paprika was used as the odorant on the 5th trial. The difference in sniffing time between trial 4 (last cinnamon trial) and trial 5 (the paprika trial) was used as the measure of performance, where animals which exhibited an increase in sniffing time were considered able to discriminate between the two odorants.

Open field

Anxiety-like behavior was of great interest in the present study, as 83% of MCI patients who also suffer from anxiety disorders later convert to AD [73]. The open field is a common test of anxiety and exploratory behavior in rodents [82]. The apparatus used was an empty white polyethylene water maze

pool (height = 60 cm, diameter = 112 cm). Each animal was tested in a single 20-min trial initiated by releasing the subject facing the wall of the apparatus. Trials were recorded using an overhead camera. For tracking purposes, the open field was divided into 3 equal area zones (3284 cm^2): outside, intermediate and center, using the arena settings in Ethovision XT (Noldus, Toronto, ON). Live tracking data were subsequently processed to obtain measures including latency to approach, time spent in the center zone, total distance and average speed of ambulation, freezing time and frequency, and the incidence of defecation. Generally, more anxious mice are less willing to explore exposed areas, tend to freeze more often and leave more fecal boli than calmer mice [82, 83].

Elevated plus maze

The plus maze is another standard test of anxiety in rodents [82]. The apparatus consisted of a black Plexiglas plus shaped maze (4 arms each 56 cm × 12.5 cm) elevated 60 cm off the floor, with 2 closed arms (15.5 cm high walls) and 2 open arms (no walls). Mice were placed in the center of the maze facing an open arm and left to explore the maze for a single 10-min trial before being returned to their home cages. Mice were scored for time spent in the open arms.

Integrated behavioral station (INBEST)

The INBEST is a Plexiglas monitoring apparatus (39 cm L × 53 cm W × 50 cm H) designed by Sakic et al. [84] that enables simultaneous automated tracking of multiple behaviors over extended periods in a minimally-stressful environment. Mice were placed in an INBEST box for a single 10-h trial before being returned to their home cages. INBEST measures included in the present study were sucrose preference, locomotor activity and food consumption. Locomotor activity was recorded as total ambulatory distance and average speed calculated from Ethovision XT tracking data. Sucrose preference is a measure of anhedonia [85] and was calculated by comparing consumption of a 1% sucrose solution (w/v) to total consumption of sucrose-water plus water alone. This concentration of sucrose was chosen based on previous literature [86]. Sucrose preference was of interest in this study because symptoms of depression, including anhedonia/apathy, reportedly increase the risk of conversion from MCI to AD by a factor of 1.9 [73].

Novel object recognition test

Recognition memory, rather than episodic memory [37], was measured in this study, as both are impaired in MCI patients [15]. The novel object test is a widely used means of evaluating object recognition memory in rodents [87], which takes advantage of the natural inclination of rodents to interact preferentially with novel objects over familiar ones. The apparatus consisted of a white high-density polyethylene box (area = 60 cm^2 × wall height = 40 cm). An assortment of glass, plastic, polished rock and painted metal statues were used as objects.

We employed a single trial, non-matching-to-sample procedure similar to that reported by others [87]. On the first 3 days of testing, mice were placed in the apparatus for a 10-min habituation trial (per day) with no objects present. On the 4th day, mice were tested in 2 trials in which objects were also present in opposite corners of the box. The mouse was left to explore and interact with the objects for 5 min. It was then returned to its home cage for 5 min while the apparatus and objects were cleaned and one of the objects was switched with a different one. The mouse was then returned to the box for 3 min. Mice were scored for the percentage of time spent interacting with the novel object out of the total time spent interacting with both objects.

Spontaneous alternation

This test takes advantage of rodents' natural tendency to explore new environments and is generally considered a metric of spatial working memory [88], which is impaired in AD patients [15] and 3xTg-AD mice [34]. The apparatus and protocol used in the present study were described in detail previously [38]. Briefly, the apparatus was a plus-shaped maze with arms and sliding doors. Large objects in the testing room served as available visual cues for navigation. There were 2 stages per trial. Each stage was started by placing the mouse in a blocked section of a predetermined starting arm. During the first stage, 2 arms were blocked, and the mouse was restricted to the start arm and one additional arm. The mouse was released from the starting location, and when it entered the other arm it was locked inside the arm for 60 s. It was then returned to its home cage for 30 s while the maze was cleaned and an additional arm was made to be accessible.

In the second stage of trials on days 1–5, the mouse was returned to the same starting location as for stage

1. It was then released after 5 s and permitted to enter one of the two other open arms. The mouse's choice (alternation or perseveration) was recorded. Two trials were administered each day. On stage 2 of the trials on day 6, instead of returning the mice to the same starting location, they were started on the opposite side of the maze, enabling evaluation of which navigation strategy had been used during days 1–5.

Morris water maze

Impairments in visuospatial learning and memory are well-known symptoms of AD [15]. In addition, learning under conditions of high interference, of which water maze reversal trials are an example, is markedly impaired in MCI patients [50]. The Morris water maze is a standard test of spatial learning and memory in rodents [89]. The apparatus and procedure used have been described elsewhere [38]. Briefly, the apparatus was a white pool (height = 60 cm, diameter = 112 cm), partially filled with tap water (24–26°C) and surrounded by a variety of visual cues. Testing was conducted over 8 days at baseline. On the first day of 9 days of follow-up testing, a single 2 min probe trial, to assess residual memory for the former platform location, was administered. Four trials per day were administered on all subsequent days, consisting of cued acquisition trials in which a transparent platform (diameter = 15 cm) was placed in the center of a quadrant and made visible with a black rubber cork.

Sixteen acquisition trials were administered over the next 4 days, in which the platform was submerged. To assess memory for the platform location, the following day consisted of 4 probe trials in which the platform was removed from the pool. The next day consisted of cued reversal trials (i.e., platform visible again) with the platform relocated to the opposite quadrant. On the final day, 4 reversal trials were administered in which the platform was submerged again but in the 2nd location.

All trials were filmed using an overhead video camera, and Ethovision XT (Noldus, Toronto, ON) tracking data were scored for swim path length, average swimming speed, and latency to locate the platform. For probe trials only, the time spent in the target quadrant was used as a measure of memory. For other trials, there were differences in swimming speed between groups, so swim path length was used instead of latency and as a measure of learning. For the cued trials, swim path length was considered to be a measure of visual acuity, whereas on acquisition and reversal trials when the platform was hidden, it was considered to be a measure of spatial learning and memory.

Statistical analyses and graphics

Olfactory acuity and water maze data were analyzed using repeated-measures genotype × sex × diet × trial/session (or concentration) mixed effects ANOVAs. As they measure different cognitive processes, the cued acquisition/reversal, probe, acquisition and reversal trials of the water maze were analyzed separately. Performance on these measures was predicted *a priori* to improve linearly across trials for olfactory acuity, water maze acquisition, and water maze reversal (e.g., [38]), therefore, the linear change across trials (i.e., the first order polynomial contrast) was also evaluated for these ANOVAs. In cases where the sphericity assumption was violated (Mauchly's test), the corrected Greenhouse-Geisser p-values are reported instead of the uncorrected p-values. If significant interactions were found between two between-subjects factors, the simple main effects were similarly evaluated. If a significant interaction was detected, *post-hoc* t-tests were used, with Tukey's HSD correction for multiple comparisons applied to p-values. Since we hypothesized that the MDS would selectively preserve learning on the hidden platform reversal trials of the water maze in 3xTg-AD mice, an *a priori* contrast [90] [(3xTg-AD × MDS) > (3xTg-AD × vehicle = WT × MDS = WT × vehicle)] was also evaluated for the data from these water maze trials only.

All other measures were evaluated with univariate 2 × 2 × 2 (genotype × sex × diet) factorial ANOVAs unless either normality (Shapiro-Wilk test) or homoscedasticity (Levene's test) was violated, in which case an equivalent factorial permutation test (10,000,000 iterations) was used [91, 92]. Instead of comparing data from baseline to 3-4 month testing using repeated measures ANOVAs, difference scores from baseline were examined in separate factorial ANOVAs or permutation tests following the same logic described above. This was done to improve the power of the tests to detect effects of the MDS based on the experimental design.

T-tests were also used to assess whether the change in spontaneous alternation rate between days 1–5 and day 6 was significantly non-zero for each group at baseline or follow-up testing, to determine the navigation strategy that had likely been used by

the mice in that group during the previous 5 days. Statistical tests were conducted using the car [93], afex [94], lsmeans [95] and lmPerm [91] packages in R [96]. An α-level of 0.05 was used to determine statistical significance. Figures were generated using the ggplot2 package in R [97], and tables were created using Microsoft Excel 2010 (Microsoft Canada Inc., Mississauga, ON). All subjects were included in all analyses, therefore, as specified in the Animals section above, group sample sizes for statistical comparisons were: MDS 3xTg-AD females ($n = 10$), vehicle 3xTg-AD females ($n = 10$), MDS 3xTg-AD males ($n = 13$), vehicle 3xTg-AD males ($n = 13$), MDS WT females ($n = 13$), vehicle WT females ($n = 13$), MDS WT males ($n = 12$), vehicle WT males ($n = 13$). With respect to the results of the ANOVAs and factorial permutation tests, this is equivalent to: MDS ($n = 48$) versus vehicle ($n = 49$) for main effects of the MDS; males ($n = 51$) versus females ($n = 46$) for main effects of sex; and 3xTg-AD ($n = 46$) versus WT ($n = 51$) for main effects of genotype.

Partial eta-squared (η_p^2) was used as a measure of effect size for all effects and interactions reported for ANOVAs and permutation tests that modelled non-repeated-measures data [98]. Generalized eta-squared (η_g^2) was used as an effect size measure for repeated- measures data [99]. $r_{contrast}$-squared (r_c^2 [100, 101]) was used as an effect size measure for the *post-hoc* *t*-tests, first order (linear) polynomial contrasts (olfactory acuity and water maze data), as well as the *a priori* contrast [(3xTg-AD × MDS)<(3xTg-AD × vehicle = WT × MDS = WT × vehicle)] used in analyzing the water maze reversal trial data. For reference, Cohen's benchmarks for small (0.01), medium (0.06), and large (0.14) effects, are recommended for these measures [98, 101, 102]. Cohen's d [102] was also used as an effect size measure for the *post-hoc* one-sample *t*-test comparing the performance of 3xTg-AD females to chance performance (30 s in the target quadrant) on the first probe trial of the Morris water maze (for reference: small effect d = 0.2, medium effect = 0.5, large effect d = 0.8).

RESULTS

Motor tests

The rotarod, beam walking and hanging basket tests were used to evaluate motor co-ordination and muscle strength. Data from these tests (and many of the others in the battery) were non-normally distributed or heteroscedastic, so the equivalent factorial permutation tests were used for analyses instead of ANOVA in such cases (see Methods). A genotype × sex × diet factorial permutation test on the rotarod fall time (Supplementary Figure 1a) at baseline did not uncover any effects or interactions when body weight was included as a co-variate in the model (Supplementary Table 1). At 3-4 months of age (Supplementary Table 1), after 2 months of MDS supplementation, there were still no differences between groups on the rotarod using raw scores (Supplementary Figure 1b) or difference scores (Supplementary Figure 1c) from baseline ([3-4 month score]-[baseline score]). Equivalent permutation tests for beam walking crossing time and the number of foot slips exhibited a similar pattern (Supplementary Table 2), with no significant effects or interactions for genotype, sex, or diet at baseline or at 3-4 months of age (Supplementary Figure 2). Thus, motor co-ordination does not differ between genotypes or sexes and is not affected by 2 months of MDS supplementation from 2–4 months of age. At baseline, but not 3-4 months of age, female mice did, however, take longer to fall in the hanging basket test (Supplementary Figure 3; Supplementary Table 3) than males (main effect of sex, permutation $p = 0.002$, $\eta_p^2 = 0.104$), even when body weight was included as a covariate (main effect of sex, permutation $p = 0.002$).

Olfactory function

Olfactory acuity was evaluated with increasing concentrations of peanut butter dissolved in mineral oil. Total sniffing time was used as a measure of acuity. A genotype × sex × diet × concentration mixed effects ANOVA did not reveal any significant effects or interactions between groups at baseline (Supplementary Table 4). Since we anticipated that sniffing time would increase with concentration, we also examined *post hoc* linear contrasts across concentrations for each sex × genotype group (repeated measures one-way ANOVA for each group). Tukey HSD corrected comparisons indicated that all groups showed a linear increase in sniffing time except for the 3xTg-AD females ($t_{(1,374)} = 1.567$, $p = 0.118$, $r_c^2 = 0.007$; 3xTg-AD males, $t_{(1,374)} = 6.955$, $p < 0.001$, $r_c^2 = 0.115$; WT females, $t_{(1,374)} = 3.201$, $p = 0.002$, $r_c^2 = 0.027$; WT males, $t_{(1,374)} = 4.537$, $p < 0.001$, $r_c^2 = 0.052$; Supplementary Figure 4a-c), suggesting that olfactory

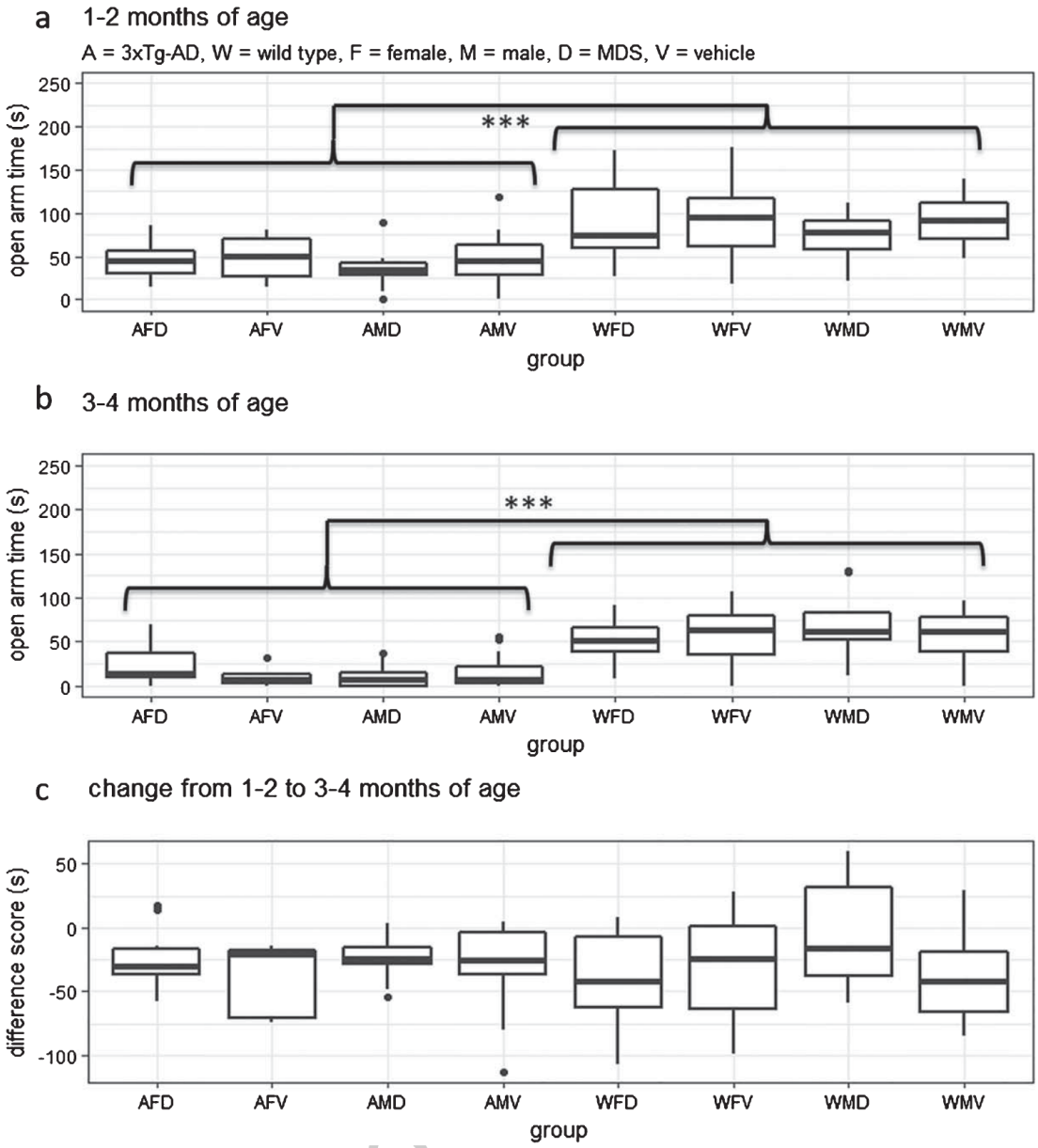


Fig. 1. Box plots of the time (s) spent in the open arms of the elevated plus maze at 1-2 months of age (a), 3-4 months of age (b), and the difference scores (c) for each group between 1-2 and 3-4 months of age. 3xTg-AD mice spent less time than WT mice in the open arms at both 1-2 and 3-4 months of age ($2 \times 2 \times 2$ ANOVA, $^{***}p < 0.001$). (AFD = MDS 3xTg-AD females [$n = 10$]; AFV = vehicle 3xTg-AD females [$n = 10$]; AMD = MDS 3xTg-AD males [$n = 13$]; AMV = vehicle 3xTg-AD males [$n = 13$]; WFD = MDS WT females [$n = 13$]; WFV = vehicle WT females [$n = 13$]; WMD = MDS WT males [$n = 12$]; WMV = vehicle WT males [$n = 13$]).

function may be impaired in 3xTg-AD females from 1-2 months of age (Supplementary Table 4).

After exposure to the MDS for 2 months and at 3-4 months of age, there was no longer a genotype × sex × concentration difference (Supplementary Table 4). Instead, based on a genotype × sex× diet × concentration mixed effects ANOVA, there was a main effect of sex ($F_{(1,89)} = 4.051$, $p = 0.047$, $\eta_g^2 = 0.021$), a sex x diet interaction ($F_{(1,89)} = 5.740$, $p = 0.019$, $\eta_g^2 = 0.029$), a sex × concentration interaction ($F_{(4,356)} =$

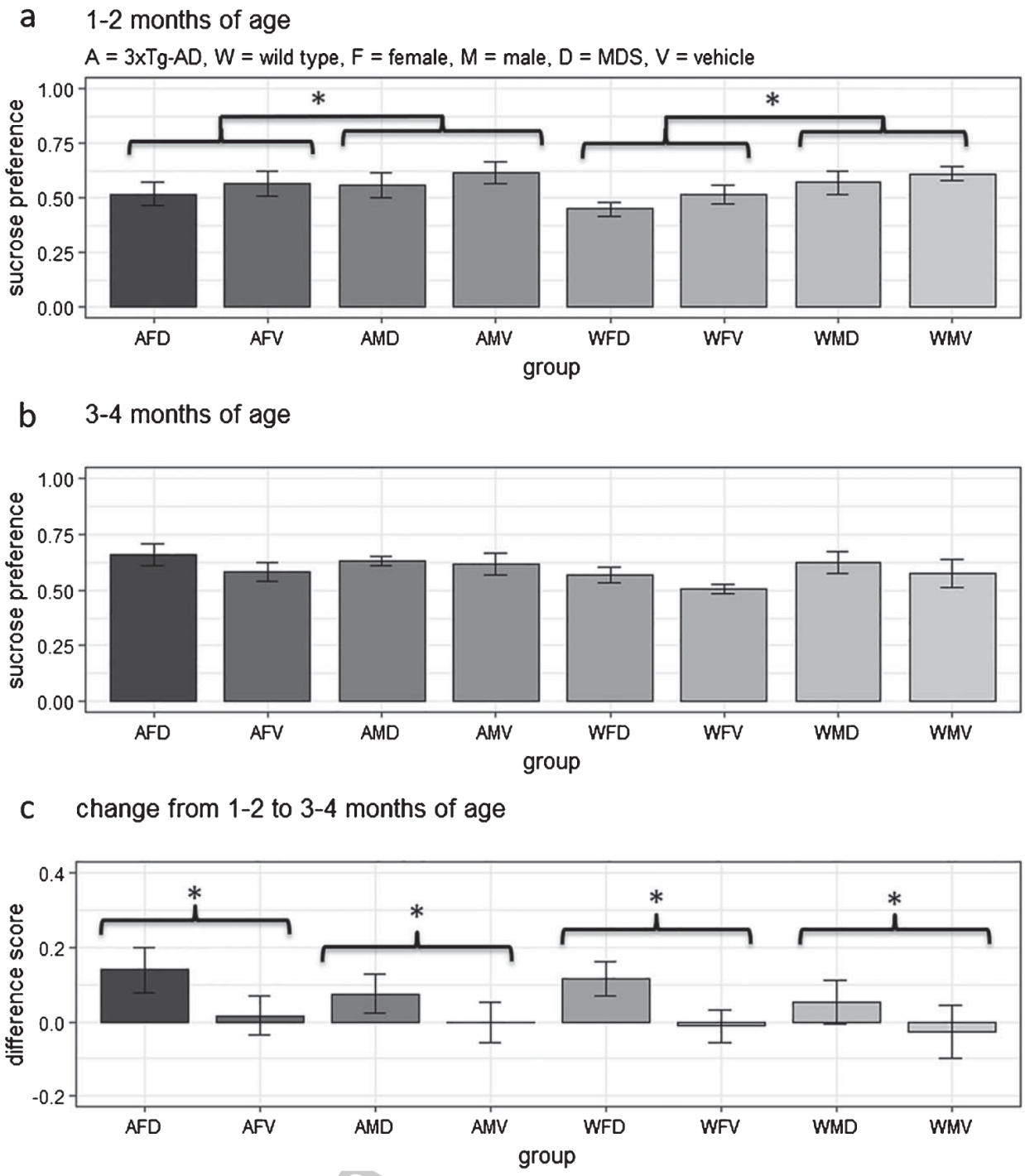


Fig. 2. Preference for 1% sucrose water over unsweetened water ([weight 1% sucrose water consumed] / [total liquid consumed]; mean ± SE) during INBEST testing at 1-2 months of age (a), 3-4 months of age (b), and the difference scores (c) between 1-2 and 3-4 months of age. At baseline (a), but not 3-4 months of age (b), sucrose preference was higher for males than females ($2 \times 2 \times 2$ ANOVA, main effect of sex, $^*p = 0.028$). c) Examination of the difference scores determined that there was a significant increase with MDS supplementation ($2 \times 2 \times 2$ ANOVA, main effect of diet, $^*p = 0.013$). (AFD = MDS 3xTg-AD females [$n = 10$]; AFV = vehicle 3xTg-AD females [$n = 10$]; AMD = MDS 3xTg-AD males [$n = 13$]; AMV = vehicle 3xTg-AD males [$n = 13$]; WFD = MDS WT females [$n = 13$]; WFV = vehicle WT females [$n = 13$]; WMD = MDS WT males [$n = 12$]; WMV = vehicle WT males [$n = 13$]).

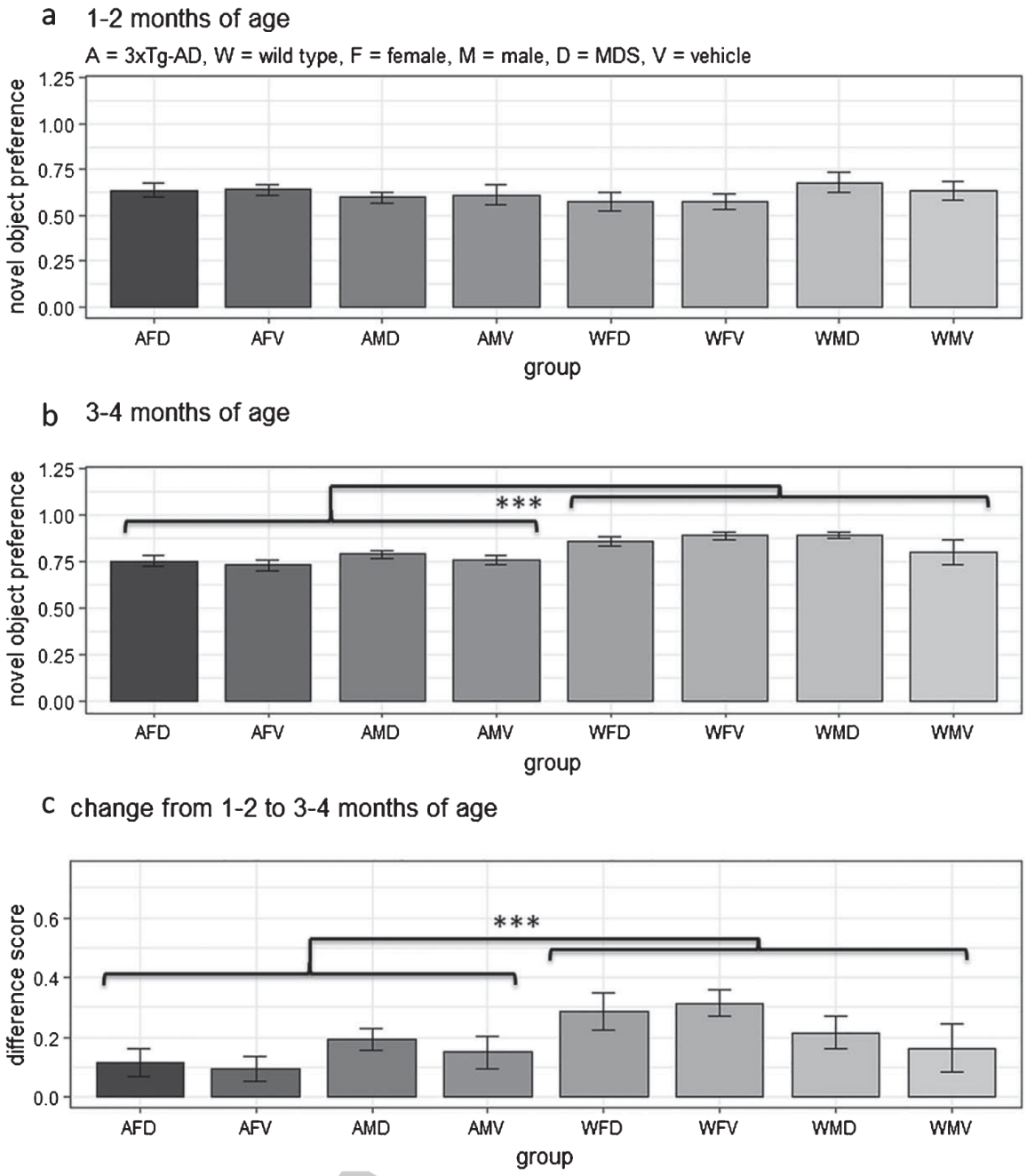


Fig. 3. Preference for the novel object ([time spent interacting with the novel object] / [total time spent interacting with both objects]; mean ± SE) during novel object testing at 1-2 months of age (a), 3-4 months of age (b), and the difference scores (c) for each group between 1-2 and 3-4 months of age. A) At baseline, there were no differences between groups. b) By 3-4 months of age WT mice spent proportionally more time interacting with the novel object compared to 3xTg-AD mice ($2 \times 2 \times 2$ ANOVA, main effect of genotype, $^{***}p < 0.001$). c) This represented a greater increase from baseline testing for WT mice compared to 3xTg-AD mice ($2 \times 2 \times 2$ ANOVA, main effect of genotype, $^{***}p < 0.001$). (AFD = MDS 3xTg-AD females [$n = 10$]; AFV = vehicle 3xTg-AD females [$n = 10$]; AMD = MDS 3xTg-AD males [$n = 13$]; AMV = vehicle 3xTg-AD males [$n = 13$]; WFD = MDS WT females [$n = 13$]; WFV = vehicle WT females [$n = 13$]; WMD = MDS WT males [$n = 12$]; WMV = vehicle WT males [$n = 13$]).

Table 3
Effects of the MDS on behavioral alterations in 3xTg-AD vs. WT mice prior to 4 months of age

Behavioral Domain	Test	Alterations in 3xTg-AD mice	Effect of MDS
	rotarod	none	null
motor (co-ordination, strength)	hanging basket	none	null
	beam walking	none	null
sensory (olfaction and vision)	olfactory acuity	decreased in females at 1-2 months of age	decreased in females of both genotypes
	olfactory discrimination	none	null
	water maze cued trials	none	null
anxiety-like behavior	open field	increased in both sexes at 1-2 months of age	null
(photophobia/agoraphobia)	elevated plus maze	increased in both sexes at 1-2 months and 3-4 months of age	null
depressive-like behavior (anhedonia)	sucrose preference	none	increased in both genotypes and sexes
recognition memory	novel object	impaired in both sexes at 3-4 months of age	null
working memory	spontaneous alternation	impaired in both sexes at 3-4 months of age	prevented deficits
long term memory	water maze probe trials	impaired in females only at 3-4 months of age	null
spatial learning and memory	water maze acquisition trials (low interference)	none	null
	water maze reversal trials (high interference)	impaired in both sexes at 3-4 months of age	prevented deficits

2.605, $p = 0.047$, $\eta_g^2 = 0.015$), and a sex x diet x concentration interaction ($F_{(4,356)} = 3.159$, $p = 0.022$, $\eta_g^2 = 0.019$; Supplementary Figure 4d-f). Follow-up Tukey HSD corrected comparisons for sex × diet groups (averaging across genotype: MDS supplemented females, MDS supplemented males, vehicle-control females and vehicle-control males) were also examined for each concentration. These revealed lower sniffing times in MDS-supplemented females than MDS-supplemented males for the 2nd lowest (0.01% PB; $t_{(1,308)} = -2.899$, $p = 0.021$, $r_c^2 = 0.027$) and highest (1% PB, $t_{(1,308)} = -2.875$, $p = 0.022$, $r_c^2 = 0.026$) peanut butter concentrations. Sniffing time for MDS supplemented females was also lower than the vehicle control females at the highest concentration (1% PB, $t_{(1,308)} = -3.546$, $p = 0.003$, $r_c^2 = 0.039$). Olfactory discrimination was assessed by comparing the difference in time spent sniffing the odorant between the last exposure of 0.001% cinnamon (trial 4/5) and 1st exposure of 0.001% paprika (trial 5/5), where animals that exhibited a dishabituation effect or an increase in sniffing time were considered able to discriminate between the two odorants. At baseline, the genotype × sex × diet permutation test did not yield any significant effects or interactions, and there were also no effects or interactions in the equivalent analysis of the data from follow-up testing (Supplementary Figure 5, Supplementary Table 5). There were therefore no effects of 2 months of MDS on olfactory discrimination ability or any differences between males and females of either 3xTg-AD or WT mouse strains at 3-4 months of age (Supplementary Table 5).

Anxiety-like behavior

A genotype × sex × diet factorial permutation test determined that at baseline, 3xTg-AD mice spent less time in the open arms of the plus maze than wild type mice, for both males and females (main effect of genotype, $p < 0.001$, $\eta_p^2 = 0.293$; Fig. 1a, Supplementary Table 6). By 3-4 months of age, 3xTg-AD mice still spent less time in the open arms than WT mice (ANOVA; main effect of genotype $F_{(1,86)} = 67.585$, $p < 0.001$, $\eta_p^2 = 0.440$; Fig. 1b; Supplementary Table 6). No additional effects or interactions were revealed using the difference scores from baseline (Fig. 1c).

Several potential anxiety-related behaviors were measured via video tracking in the open field test, specifically: total time spent in the center zone, latency to approach the center, total freezing time, and the number of fecal boli secreted. In contrast

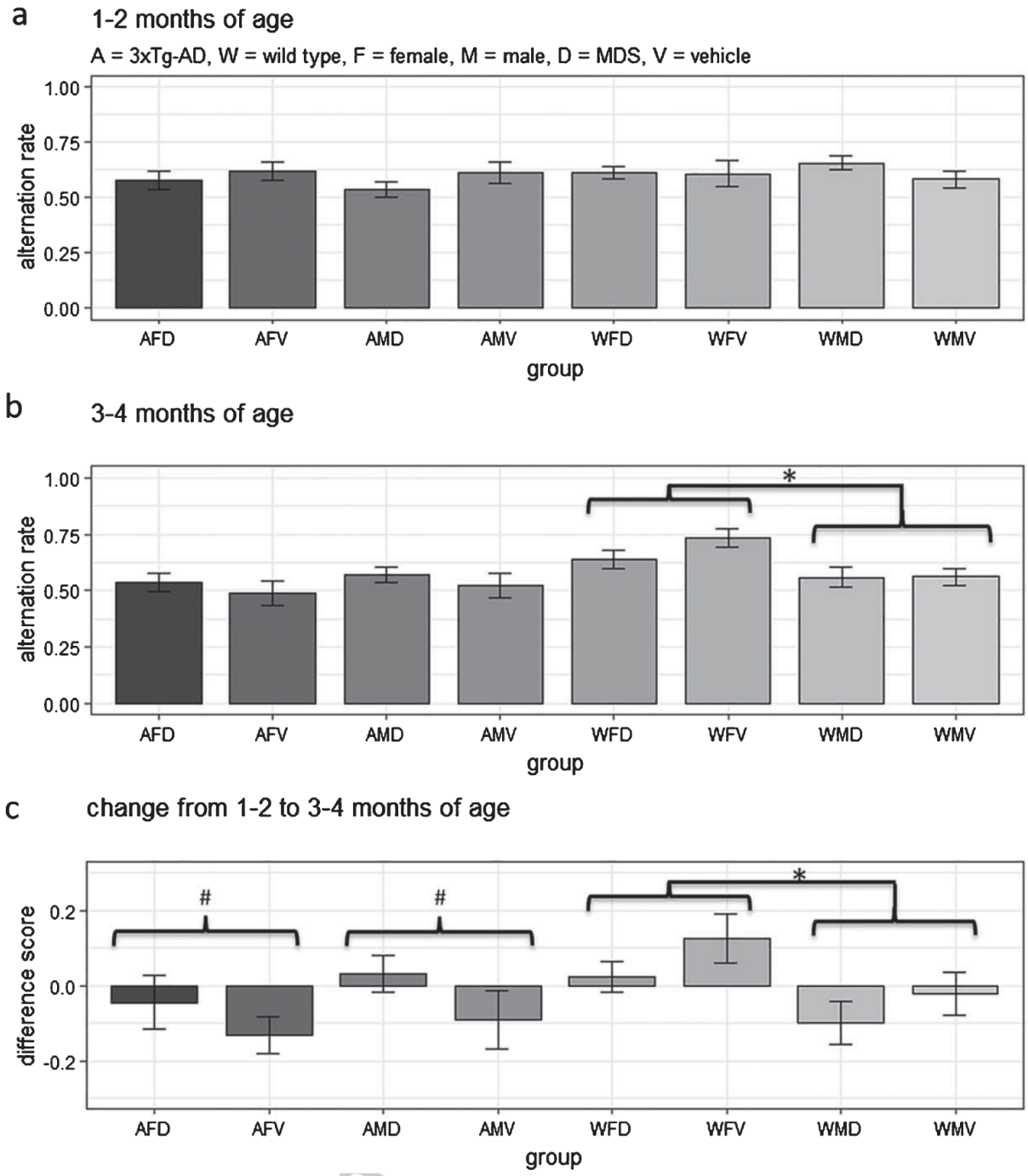


Fig. 4. Alternation rate (mean ± SE) during days 1–5 of spontaneous alternation testing at 1-2 months of age (a), 3-4 months of age (b), and the difference scores (c) for each group between 1-2 and 3-4 months of age. A) There were no differences between groups at baseline. b) At 3-4 months of age WT females alternated more frequently than WT males ($2 \times 2 \times 2$ ANOVA, genotype × sex interaction, $^{*}p = 0.012$). c) Comparison of the difference scores between baseline and follow-up testing ($2 \times 2 \times 2$ ANOVA) revealed a relative increase in alternation rates in WT females (genotype × sex interaction, $^{*}p = 0.025$) compared to WT males but also that alternation rates were decreased in vehicle-supplemented 3xTg-AD mice but not MDS-supplemented 3xTg-AD mice (genotype x diet interaction, $^{\#}p = 0.023$). (AFD = MDS 3xTg-AD females [$n = 10$]; AFV = vehicle 3xTg-AD females [$n = 10$]; AMD = MDS 3xTg-AD males [$n = 13$]; AMV = vehicle 3xTg-AD males [$n = 13$]; WFD = MDS WT females [$n = 13$]; WFV = vehicle WT females [$n = 13$]; WMD = MDS WT males [$n = 12$]; WMV = vehicle WT males [$n = 13$]).

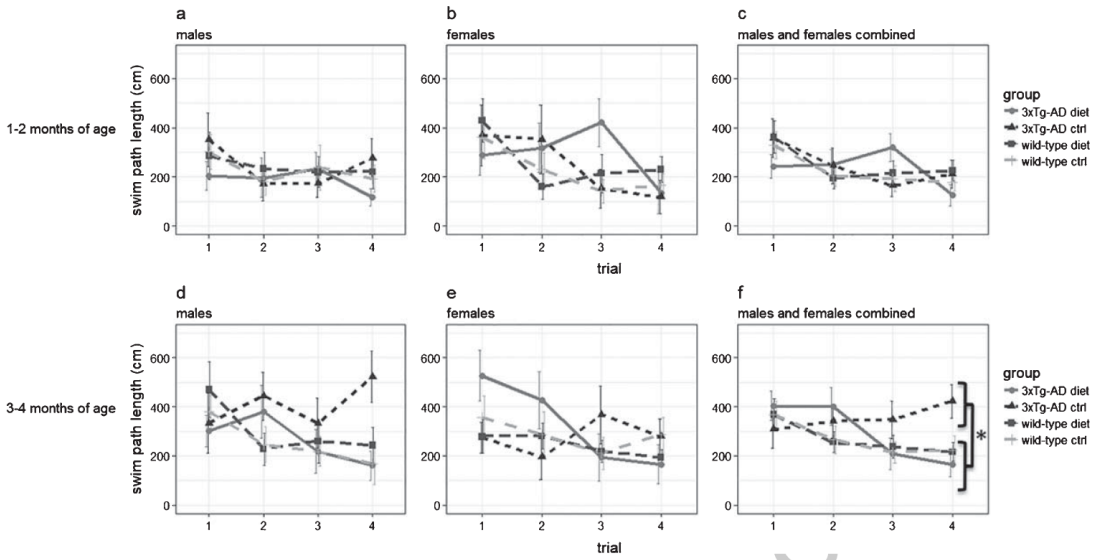


Fig. 5. Swim path length (cm, mean ± SE) on the reversal trials of the water maze (2nd hidden platform location) at 1-2 (a-c) and 3-4 months of age (d-f). There were no differences between groups at 1-2 months of age, and all groups improved linearly across trials (a-c; $2 \times 2 \times 2 \times 4$ ANOVA, linear contrast for trial, $p < 0.001$). f) At 3-4 months of age, only vehicle-supplemented 3xTg-AD mice failed to improve across trials ($2 \times 2 \times 2 \times 4$ ANOVA, interaction between a linear contrast for trial and the hypothesis contrast [vehicle-supplemented 3xTg-AD>MDS-supplemented 3xTg-AD = MDS-supplemented WT = vehicle-supplemented], $^*p = 0.011$).

to the plus maze results, 3xTg-AD females (not males) spent more time in the center of the open field than WT females at 1-2 months of age (permutation test; genotype × sex interaction, $p = 0.013$, $\eta_p^2 = 0.067$; Supplementary Figure 6a, Supplementary Table 7). Follow-up tests for the simple main effect of genotype for males and females separately showed that only 3xTg-AD females exhibited this behavior ($p < 0.001$, $\eta_p^2 = 0.214$, for effect of genotype in females; $p = 0.823$, $\eta_p^2 < 0.001$, for males).

The permutation test for latency to approach the center at baseline also yielded a significant genotype × sex interaction ($p = 0.039$, $\eta_p^2 = 0.048$; Supplementary Figure 7a, Supplementary Table 7). Follow-up tests for the simple main effect of genotype in each sex determined that only 3xTg-AD males took longer to approach the center (for females, $p = 0.367$, $\eta_p^2 = 0.029$; for males, $p = 0.013$, $\eta_p^2 = 0.090$; Supplementary Figure 8a). In addition, both sexes of 3xTg-AD mice spent more time frozen than WT mice (main effect of genotype, permutation $p = 0.004$, $\eta_p^2 = 0.135$; Supplementary Table 7). The ANOVA for the number of fecal boli left in the open field (Supplementary Figure 9a) yielded significant main effects of genotype ($F_{(1,89)} = 5.437$, $p = 0.026$, $\eta_p^2 = 0.055$) and sex ($F_{(1,89)} = 4.644$, $p = 0.041$, $\eta_p^2 = 0.048$), where more fecal boli were secreted respectively by 3xTg-AD mice than WT mice and by males more than females. By 3-4 months of age, there was no longer a genotype x sex interaction for time spent in the center of the open field, only a main effect of sex (females > males; permutation $p = 0.015$, $\eta_p^2 = 0.064$; Supplementary Figure 6b, Supplementary Table 7). There were no other significant effects or interactions detected, and a factorial permutation test on the center time difference scores also did not yield any significant effects or interactions (Supplementary Figure 6c, Supplementary Table 7).

For the open field center approach latency at follow-up, males took longer to approach the center than females ($2 \times 2 \times 2$ factorial permutation test; main effect of sex, $p = 0.022$, $\eta_p^2 = 0.054$; Supplementary Figure 7b, Supplementary Table 7), and there were no other significant main effects or interactions using the raw 3-4 month testing scores or difference scores (Supplementary Figure 7c). There were also no main effects or interactions from the equivalent permutation tests for freezing times at 3-4 months of age (or the change from baseline; Supplementary Figure 8, Supplementary Table 7). There was a main effect of sex according to the ANOVA for the number of fecal boli left in the open field at 3-4 months of age (males > females, $F_{(1,89)} = 11.301$, $p = 0.001$, $\eta_p^2 = 0.113$; Supplementary Figure 9b), and there were

no main effects or interactions detected using the difference scores (Supplementary Figure 9c, Supplementary Table 7). Taken together, although 3xTg-AD mice exhibit anxiety-like behavior on both the open field and elevated plus maze at 1-2 months of age, the difference is only observed on the plus maze by 3-4 months of age. Lastly, the MDS does not seem to affect anxiety-like behavior that is already present prior to the start of treatment (at 1-2 months of age), given that it did not alter performance on the plus maze (Fig. 1), or any of the open field measures (Supplementary Figures 6–9).

Anhedonia, activity, and appetitive behavior

The INBEST apparatus enabled us to monitor habitual motor and appetitive behavior while also affording an opportunity to evaluate sucrose preference over a 10-h session. Sucrose preference was found to be higher in males than females at baseline (ANOVA; main effect of sex, $F_{(1,89)} = 5.556$, $p = 0.028$, $\eta_p^2 = 0.058$; Fig. 2a) but not by 3-4 months of age (Fig. 2b, Supplementary Table 8). The effect of MDS supplementation, but not genotype, was significant when the difference scores were examined in an ANOVA (main effect of diet, $F_{(1,89)} = 6.370$, $p = 0.013$, $\eta_p^2 = 0.068$), indicating that MDS supplementation increases sucrose preference (Fig. 2c, Supplementary Table 8). The effect of the MDS was attenuated slightly but remained significant ($F_{(1,39)} = 5.378$, $p = 0.026$, $\eta_p^2 = 0.073$) when the difference scores for food eaten during INBEST testing were included in the analysis as a covariate. This suggests that there is a genuine increase in hedonic motivation with MDS supplementation that is independent of appetite.

Both the total ambulatory distance and average movement speed were used as measures of motor activity. At baseline, genotype × sex × diet factorial permutation tests did not uncover any differences in ambulatory distance (Supplementary Figure 10a, Supplementary Table 8), although 3xTg-AD mice moved faster than WT mice (main effect of genotype, $p = 0.004$, $\eta_p^2 = 0.088$; Supplementary Figure 11a). After supplementation with the MDS at follow-up testing, there were no differences between groups in terms of either ambulatory distance or speed (factorial permutation tests; Supplementary Figures 10b and 11b), indicating that the baseline genotype difference in speed did not persist with age. Mirroring this change between baseline and follow-up testing was the finding of more negative difference scores in speed for 3xTg-AD mice than for WT mice (factorial permutation test; main effect of genotype, $p = 0.011$, $\eta_p^2 = 0.069$; Supplementary Figure 11c, Supplementary Table 8).

Object recognition memory

Recognition memory was assessed based on the preference of mice to explore a novel object over a familiar one. Examination of the fraction of time spent interacting with the new object out of the total interaction time for both objects in the arena did not reveal any effects or interactions between genotypes, sexes, or pre-treatment differences between supplementation groups (MDS versus vehicle) at baseline ($2 \times 2 \times 2$ ANOVA; Fig. 3a, Supplementary Table 9). However, by 3-4 months of age 3xTg-AD mice spent less time interacting with the novel object than WT mice (main effect of genotype, $F_{(1,89)} = 18.107$, $p < 0.001$, $\eta_p^2 = 0.169$; Fig. 3b, Supplementary Table 9). This main effect was also the only significant one when the difference scores were analyzed ($F_{(1,89)} = 54.528$, $p < 0.001$, $\eta_p^2 = 0.336$; Fig. 3c, Supplementary Table 9). Thus, compared to WT mice, both sexes of 3xTg-AD mice exhibit an attenuation of the developmental improvement in object recognition memory between 1-2 and 3-4 months of age that is not affected by the MDS.

Working memory

Working memory was assessed here using the spontaneous alternation test. For the day 1–5 alternation rate at baseline, the $2 \times 2 \times 2$ ANOVA did not reveal any main effects or interactions (Fig. 4a, Supplementary Table 10). At 3-4 months of age, analysis of the raw scores revealed only a significant interaction between genotype and sex ($F_{(1,89)} = 6.555$, $p = 0.012$, $\eta_p^2 = 0.069$; Fig. 4b, Supplementary Table 10). When the difference scores from baseline to 3-4 month testing were compared, this interaction was still present ($F_{(1,89)} = 5.231$, $p = 0.025$, $\eta_p^2 = 0.056$), but there was also a genotype x diet interaction ($F_{(1,89)} = 5.359$, $p = 0.023$, $\eta_p^2 = 0.057$; Fig. 4c).

Follow-up ANOVAs for the simple main effects of these interactions determined that vehicle-control ($F_{(1,46)} = 5.64$, $p = 0.022$, $\eta_p^2 = 0.112$), but not MDS-supplemented ($F_{(1,47)} = 0.314$, $p = 0.578$, $\eta_p^2 = 0.007$), 3xTg-AD mice exhibited a decrease in spontaneous alternation rate from baseline relative to their WT counterparts (Fig. 4c). There was also a relative

increase in alternation rates for WT females compared to 3xTg-AD females ($F_{(1,44)} = 7.039$, $p = 0.011$, $\eta^2_p = 0.138$), but not for male WT mice compared to 3xTg-AD mice ($F_{(1,49)} = 0.226$, $p = 0.636$, $\eta^2_p = 0.005$). Thus, MDS supplementation maintains spontaneous alternation rates in 3xTg-AD mice, similar to the level of WT mice, at 3-4 months of age. Furthermore, WT female mice show an increase in alternation rate between 1-2 and 3-4 months of age (Supplementary Table 10). In terms of navigation strategy, separate *t*-tests for the difference scores between the day 1–5 average alternation rate and day 6 alternation rate of each group at both 1-2 and 3-4 months of age determined that no group exhibited a change in alternation rate at day 6 from their day 1–5 average (data not shown).

Visuospatial learning and memory

Visual acuity and visuospatial learning and memory under conditions of low and high interference were evaluated using different stages of the water maze. On the cued acquisition trials, mixed effects ANOVAs (genotype × sex × diet × trial) determined that there were differences between 3xTg-AD mice and WT mice in terms of swimming speed at baseline (main effect of genotype, $F_{(1,89)} = 18.12$, $p < 0.001$, $\eta^2_g = 0.081$; Supplementary Figure 12a-c) and at 3-4 months of age (main effect of genotype, $F_{(1,89)} = 33.49$, $p < 0.001$, $\eta^2_g = 0.087$; Supplementary Figure 12d-f, Supplementary Table 11). Therefore, swim path length, rather than the latency to locate the platform, was used as a measure of performance on trials in which there was either a visible or submerged platform. On probe trials, during which the platform was removed from the pool, the time spent in the target quadrant was used as an indicator of memory for the location of the first platform.

Analysis of the swim path length data on the cued acquisition and cued reversal trials (i.e., platform was visible) using mixed effects ANOVAs did not indicate a disparity in visual acuity between any groups at either testing age (Supplementary Figures 13 and 14; Supplementary Table 11). On the acquisition trials, during which the platform was submerged in the first testing location (low interference), the 2 × 2 × 2 × 16 (genotype × sex × diet × trial) mixed effects ANOVA did not detect any effects or interactions at baseline or at 3-4 months of age, aside from the expected improvement across trials for all groups (main effect of trial; $F_{(15,1335)} = 7.154$, $p < 0.001$, $\eta^2_g = 0.063$ at baseline; $F_{(10,855)} = 3.399$, $p < 0.001$, $\eta^2_g = 0.032$, at 3-4 months of age; Supplementary Figure 15, Supplementary Table 12). These results indicate that all groups were able to learn the first platform location equally well.

Examination of the time spent in the target quadrant in probe trials using a mixed effects ANOVA detected only a significant main effect of sex ($F_{(1,89)} = 6.5$, $p = 0.013$, $\eta^2_g = 0.042$; males higher than females) and trial ($F_{(3,267)} = 11.427$, $p < 0.001$, $\eta^2_g = 0.049$) at 1-2 months of age (Supplementary Figure 16a-c, Supplementary Table 13). Addition of a linear contrast to the ANOVA for the trial factor confirmed that, as expected, mice spent progressively less time in the target quadrant across trials when the platform was not in the pool (i.e., an extinction effect, $t_{(1,288)} = -4.940$, $p < 0.001$, $r^2_c = 0.078$). At 3-4 months of age, there was a genotype x sex x trial interaction ($F_{(3,267)} = 3.530$, $p = 0.020$, $\eta^2_g = 0.015$; Supplementary Figure 16b, Supplementary Table 13). Follow-up Tukey HSD-corrected comparisons of each genotype × sex group on each trial demonstrated that 3xTg-AD females spent less time in the target quadrant of the maze than WT females on trial 1 only ($t_{(1,207)} = -3.506$, $p = 0.003$, $r^2_c = 0.056$). Although this memory impairment in 3xTg-AD females was not ameliorated by the MDS (the genotype x sex x diet interaction in the ANOVA was not significant, Supplementary Table 13), a further *t*-test demonstrated that the time they spent in the target quadrant was significantly greater than chance ($t_{(19)} = 2.832$, $p = 0.011$, $d = 0.633$; Supplementary Figure 16b), suggesting that they could remember the trained platform location. On the probe trial used prior to the start of the water maze cued trials at 3-4 months of age (i.e., to test for residual memory for the former platform location after testing at 1-2 months of age), one tailed *t*-tests for each group confirmed that no group spent more than 30 s, or 25%, of the trial in the previously rewarded quadrant (all *p*-values >0.85, data not shown). This indicated that there was no residual memory of the platform location from the previous round of testing.

On the reversal trials, in which the platform was submerged in the second location, at 1-2 months of age, the genotype × sex × diet × trial ANOVA on the swim path length data confirmed only the expected linear improvement of all groups across trials (Fig. 5a-c; main effect of trial, $F_{(3,255)} = 4.009$, $p = 0.011$, $\eta^2_g = 0.063$; linear contrast for trial, $t_{(1,265)} = -2.967$, $p < 0.001$, $r^2_c = 0.023$; Supplementary Table 12). For the data from the follow-up

testing at 3-4 months of age, we examined the *a priori* hypothesis that only vehicle-control 3xTg-AD mice would fail to exhibit an improvement across reversal trials using: 1) a linear contrast for the trial factor and, 2) a planned contrast of 1, –1/3, –1/3, and –1/3 for the genotype × diet groups [(vehicle-control 3xTg-AD)>(MDS-supplemented 3xTg-AD = MDS-supplemented WT = vehicle-control)] within a $2 \times 4 \times 4$ (sex × [genotype × diet] × trial) mixed effects ANOVA. As predicted, this analysis revealed only a significant interaction for the two contrasts ($F_{(1,368)} = 6.468$, $p = 0.011$, $r_c^2 = 0.018$; Fig. 5f, Supplementary Table 12), indicating that only vehicle-control 3xTg-AD mice had difficulty learning the second platform location. A summary of this and the other effects of the MDS reported above are presented in Table 3.

DISCUSSION

While we have previously observed benefits of the MDS on the age-related decline in spatial learning and memory among wild-type mice following an extended period of supplementation (~1 year [25]), the present study demonstrates that similar benefits can be obtained in an AD mouse model with only 2 months of supplementation. That strong benefits can be observed after such a short intervention period is remarkable considering that most similar studies (e.g., [21, 22, 103]) report positive results after longer treatment periods (at least 3–6 months). Specifically, we show here that the MDS prevents the emergence of deficits in working memory and reversal learning that occur in 3xTg-AD by 4 months of age. The reversal learning deficit is expected as part of a human-like MCI phenotype (see Methods for a description), whereas the working memory impairment seems to emerge earlier in 3xTg-AD mice [34] than in humans [15]. Thus, the MDS may impart a degree of resilience against the deterioration of learning and memory in AD.

On the spontaneous alternation test, we also did not observe a decrease in performance when the starting viewpoint was shifted (i.e., alternation rate on days 1–5 versus day 6). This suggests the use of an allocentric rather than egocentric navigation strategy [104]. The particular strategy used was of interest, since (in humans) allocentric navigation preferentially engages the hippocampus, while egocentric navigation depends more on parietal association and striatal areas. Furthermore, there is a shift toward egocentric navigation with age [104] that may reflect changes in hippocampal volume that also occur with age and in AD [105]. Thus, the decreased alternation rate among 3-4-month-old control 3xTg-AD mice is not due to a change in navigation strategy (i.e., from allocentric to egocentric).

Evidence from the literature suggests that performance on the reversal trials of the water maze depends upon adult hippocampal neurogenesis [106–109] (although see [110]). More generally, adult neurogenesis seems to be important for hippocampal-dependent learning under conditions of high interference [51, 52, 111]. Similarly, in AD patents, deficits on a high-interference, picture-matching memory test are also correlated with Aβ levels [112]. Promoting neurogenesis may therefore impart resilience against Aβ toxicity. In addition, the benefit of the MDS on reversal learning shown here may be due to preserved levels of neurogenesis in MDS-supplemented 3xTg-AD mice, which merits further investigation.

Our wide-ranging battery of tests across different ages has also yielded a more detailed and comprehensive picture of the early behavioral trajectory of the 3xTg-AD mouse than has been available thus far. To our knowledge, this is the first study aimed at preventing the development of symptoms in an AD mouse model using a complex supplement that was administered starting at 2 months of age. The fact that not all our results were positive suggests that the use of supplements alone may not be sufficient to prevent AD onset. Similarly, at least two other recent studies used complex supplements (at least 5 ingredients) in AD model mice. In one [21], a combination of 11 ingredients, 10 of which are also in the MDS used here, was administered to Tg2576 mice for 6 months starting at 6 months of age (behavioral deficits begin at 3 months of age in this strain). Benefits of varying magnitude were observed for all 3 measures studied: the novel object test and water maze, and levels of Aβ oligomers in whole brain homogenates. However, without the use of a more comprehensive behavioral battery of tests, it is unknown if the treatment could impact other aspects of an AD-like phenotype such as sensory/motor function or emotionality (see below for more information). Another study [22] using 3xTg-AD mice reported sex-dependent benefits of 4 months of treatment starting at 7–10 months of age on mitochondrial function (cytochrome C oxidase activity; males only) and a delayed-match-to-position, short-term memory task (only a 30 s delay; males only),

using a 29-component supplement containing 5 similar ingredients to the MDS (turmeric/curcumin, green tea, ginger, fish oil, and vitamin D). However, the treatment did not improve memory deficits exhibited by the 3xTg-AD mice over longer periods (30 min or 24 h). Additionally, mitochondrial function was reduced in supplemented females, highlighting the importance of studying both sexes during pre-clinical experiments.

Our results suggest that, prior to 4 months of age, the 3xTg-AD mouse models the human behavioral phenotype of an MCI-like syndrome reasonably well, although not perfectly. For instance, the 3xTg-AD mouse did exhibit anxiety-like behavior (both sexes) and deficits in olfactory acuity (females only), but not motor co-ordination impairments, prior to 4 months of age. These changes in olfaction and anxiety level were the earliest behavioral symptoms to appear in these animals, developing prior to the spatial learning, working memory, or object recognition memory impairments at 3-4 months of age. Although symptoms of depression and anxiety are both common in patients with dementia [5], our results suggest the possibility that, among behavioral symptoms, anxiety or olfactory deficits may appear earlier than anhedonia or memory impairments in some older adults who later develop MCI. These findings are in agreement with those of Marchese et al. [38] who reported similar findings in male 3xTg-AD mice only. In that study, compared to WT males, 3xTg-AD males exhibited elevated anxiety-like behavior on the step-down test at 1.5 months of age and increased olfactory acuity starting at 6 months of age. Here, we confirm that the anxiety-like behavior is also present in 3xTg-AD females at 1-2 months of age, and we show that olfactory alterations (increased sensitivity in males, decreased sensitivity in females) are present in 3xTg-AD mice at 1-2, but not 3-4, months of age. Our finding of a beneficial effect of the MDS on sucrose preference also suggests that supplementation with the MDS may help protect older adults at risk for MCI from developing anhedonia or other depressive symptoms.

Regarding the observed anxiety, there is evidence of increased HPA axis activation in 3-4-month-old 3xTg-AD mice due to upregulation of glucocorticoid and mineralocorticoid receptors, but not corticosteroid levels, in CA3 and the DG of the hippocampus [113]. If this upregulation is also present at 1-2 months of age in this strain, then it might explain the increased anxiety in transgenic mice evident in our plus maze and open field data and would merit further research. Additionally, the stress response (increased corticosterone levels) of 3xTg-AD females, but not males, following water maze testing is increased over WT females between 6–15 months of age [35], emphasizing the relevance of comparing performance on tests of spatial memory to anxiety measures in both males and females when interpreting treatment effects.

The MDS did not reverse this pre-existing anxiety or prevent the appearance of deficits in novel object recognition (both males and females) or long-term recall on the water maze probe trials (females only) in 3xTg-AD mice by 3-4 months of age. Previous work with the MDS in 12-month-old C57BL6 mice demonstrated superior object recognition memory following 9+ months of supplementation, suggesting that longer treatment periods may have a positive impact on recognition memory in 3xTg-AD mice as well [114]. In fact, given the strength of some results with our relatively short treatment period suggests that some of the AD features that proved refractory to supplementation might still show some benefit by a longer treatment period. It also appears that the MDS negatively impacts olfactory acuity in 3-4-month-old females of both genotypes. This was surprising, since previous work on the MDS has found beneficial effects on olfactory function in older animals (9 months and older [26, 27]), suggesting that this side effect is age-dependent and is unlikely to be an issue if treatment is started at a later age in wild type animals. The sources of these deficits require further investigation.

Our recent work suggests that the MDS in combination with aerobic exercise [57] may yield greater benefits than either treatment alone, particularly when elevated levels of stress are involved in a disease phenotype, such as is the case for the 3xTg-AD mouse [35, 113]. Again, the benefit of supplementation (and perhaps the interaction with exercise) may have been improved with a longer supplementation period. With respect to the current water maze, if 3xTg-AD females experience a greater stress response to testing than males at 3-4 months of age, it could explain why they, but not 3xTg-AD males, exhibited a deficit in long term memory based on the water maze probe trials that was not responsive to the MDS alone. Given that impairment in 3xTg-AD females on the probe trials was relatively small (i.e., they could still remember the platform location), we recommend that future studies of young 3xTg-AD mice use converging evidence from additional performance measures, some of which (e.g., platform crossings or average

proximity to the platform) are more sensitive to group differences than the time spent in the target quadrant [115].

Our previous finding that both exercise and the MDS, but neither alone, were sufficient to improve anhedonia, hippocampal neurogenesis, BDNF, and hippocampal size in chronically stressed mice [57] suggests that the MDS and exercise together may prevent the deficit in probe trial performance observed here in 3xTg-AD females. This is consistent with other literature showing increased effects of combined diet and exercise [59, 116, 117].

Taken together, the results presented here suggest that as little as 2 months of supplementation with the MDS protects cognitive functions such as working spatial memory in juvenile 3xTg-AD mice. These functions are supported by highly plastic structures such as the prefrontal cortex and hippocampus that are affected most severely in AD [1]. However, such a short period of supplementation was insufficient to preserve recognition memory or reverse pre-existing anxiety, functions which depend more on other brain areas such as the perirhinal cortex (supports object recognition [118]) or amygdala (affects anxiety-like behavior [119]). Future studies are indicated to determine if longer periods of supplementation or combination with exercise can reverse these other impairments or impact AD-relevant biomarkers (e.g., amyloid-β or hyperphosphorylated tau or BDNF). Regardless, the present data suggest that dietary supplements can obtain remarkable results, at least in mice. Ours and other studies suggest that supplements may serve as a key part of a multi-pronged intervention program to protect against AD-related behavioral changes.

ACKNOWLEDGMENTS

We would like to thank Dr. M. Kapadia for advice on the olfactory testing protocol. We also recognize the substantial efforts of undergraduate students J. Tran, M. Lysenko-Martin, Z. Patel, N. Ramseyer, M. Kryzewska, G. Kaur, N. Elsaadawy, D. Chok, H. Shahid, M. Campbell, L. Ribeiro, L. Babatinca, K. Thomas, B. Terpou, A. Mizzi, M. Radenovic and I. Perera in assisting with the behavioral testing, the scoring of behavioral video recordings, and administration of the dietary supplement. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to S.B. (RGPIN-2014-03580), D.R.B. (RGPIN 238495-10), and J.M.W. (RGPIN 194616-11). It was also supported by a grant from the Canadian Institutes of Health Research to J.M.W. (MOP 11927), grants from the Alzheimer's Society of Canada (grant #17-04) and the Canadian Consortium on Neurodegeneration in Aging Team 2 to M.F., and an NSERC Canada Graduate Scholarship to C.H.

Authors' disclosures available online (https://www.j-alz.com/manuscript-disclosures/17-0921r2).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-170921.

REFERENCES

[1] Mattson MP (2004) Pathways towards and away from Alzheimer's disease. *Nature* **430**, 631-639.

[2] Burgess N, Maguire EA, O'Keefe J (2002) The human hippocampus and spatial and episodic memory. *Neuron* **35**, 625-641.

[3] Sekeres MJ, Bonasia K, St-Laurent M, Pishdadian S, Winocur G, Grady C, Moscovitch M (2016) Recovering and preventing loss of detailed memory: Differential rates of forgetting for detail types in episodic memory. *Learn Mem* **23**, 72-82.

[4] Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behavior. *Nature* **476**, 458-461.

[5] Lyketsos GC, Lopez O, Jones B, Fitzpatrick AL, Breitner J, DeKosky S (2013) Prevalence of neuropsychiatric symptoms results from the cardiovascular health study. *JAMA* **288**, 1475-1483.

[6] Hardy JA, Higgins GA (1992) Alzheimer's disease: The amyloid cascade hypothesis. *Science* **256**, 184-185.

[7] Selkoe DJ, Hardy J (2016) The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Mol Med* **8**, 1-14.

[8] Ma Q-LQ-L, Yang F, Rosario ER, Ubeda OJ, Beech W, Gant DJ, Chen PP, Hudspeth B, Chen C, Zhao Y, Vinters HV, Frautschy SA, Cole GM (2009) Beta-amyloid oligomers induce phosphorylation of tau and inactivation of insulin receptor substrate via c-Jun N-terminal kinase signaling: Suppression by omega-3 fatty acids and curcumin. *J Neurosci* **29**, 9078-9089.

[9] Jin M, Shepardson N, Yang T, Chen G, Walsh D, Selkoe DJ (2011) Soluble amyloid beta-protein dimers isolated from Alzheimer cortex directly induce Tau hyperphosphorylation and neuritic degeneration. *Proc Natl Acad Sci U S A* **108**, 5819-5824.

[10] Herrup K (2015) The case for rejecting the amyloid cascade hypothesis. *Nat Neurosci* **18**, 794-799.

[11] Mattson MP (2015) Late-onset dementia: A mosaic of prototypical pathologies modifiable by diet and lifestyle. *NPJ Aging Mech Dis* **1**, 15003.

[12] Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss

is the major correlate of cognitive impairment. *Ann Neurol* **30**, 572-580.

[13] Peng S, Wuu J, Mufson EJ, Fahnestock M (2005) Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the preclinical stages of Alzheimer's disease. *J Neurochem* **93**, 1412-1421.

[14] Michalski B, Corrada MM, Kawas CH, Fahnestock M (2015) Brain-derived neurotrophic factor and TrkB expression in the "oldest-old," the 90+ Study: Correlation with cognitive status and levels of soluble amyloid-beta. *Neurobiol Aging* **36**, 3130-3139.

[15] Morris JC, Storandt M, Miller JP, McKeel DW, Price JL, Rubin EH, Berg L (2001) Mild cognitive impairment represents early-stage Alzheimer disease. *Arch Neurol* **58**, 397-405.

[16] Tarkowski E, Andreasen N, Tarkowski A, Blennow K (2003) Intrathecal inflammation precedes development of Alzheimer's disease. *J Neurol Neurosurg Psychiatry* **74**, 1200-1205.

[17] Talbot K, Wang H, Kazi H, Han L, Bakshi KP, Stucky A, Fuino RL, Kawaguchi KR, Samoyedny AJ, Wilson RS, Arvanitakis Z, Schneider JA, Wolf BA, Bennett DA, Trojanowski JQ, Arnold SE (2012) Demonstrated brain insulin resistance in Alzheimer's disease patients is associated with IGF-1 resisitance, IRS-1 dysregulation, and cogntive decline. *J Clin Invest* **122**, 1316-1338.

[18] Willette AA, Bendlin BB, Starks EJ, Birdsill AC, Johnson SC, Christian BT, Okonkwo OC, La Rue A, Hermann BP, Koscik RL, Jonaitis EM, Sager MA, Asthana S (2015) Association of insulin resistance with cerebral glucose uptake in late middle–aged adults at risk for Alzheimer disease. *JAMA Neurol* **72**, 1013.

[19] Lovell MA, Soman S, Bradley MA (2011) Oxidatively modified nucleic acids in preclinical Alzheimer's disease (PCAD) brain. *Mech Ageing Dev* **132**, 443-448.

[20] Mosconi L, Pupi A, De Leon MJ (2008) Brain glucose hypometabolism and oxidative stress in preclinical Alzheimer's disease. *Ann N Y Acad Sci* **1147**, 180-195.

[21] Parachikova A, Green KN, Hendrix C, Laferla FM (2010) Formulation of a medical food cocktail for Alzheimer's disease: Beneficial effects on cognition and neuropathology in a mouse model of the disease. *PLoS One* **5**, e14015.

[22] Wolf AB, Braden BB, Bimonte-Nelson H, Kusne Y, Young N, Engler-Chiurazzi E, Garcia AN, Walker DG, Moses GSD, Tran H, LaFerla F, Lue L, Lombardo NE, Valla J (2012) Broad-based nutritional supplementation in 3xTg mice corrects mitochondrial function and indicates sex-specificity in response to Alzheimer's disease intervention. *J Alzheimers Dis* **32**, 217-232.

[23] Stonehouse W, Conlon CA, Podd J, Hill SR, Minihane AM, Haskell C, Kennedy D (2013) DHA supplementation improved both memory and reaction time in healthy young adults: A randomized controlled trial 1–3. *Am J Clin Nutr* **94**, 1134-1143.

[24] Dai Q, Borenstein AR, Wu Y, Jackson JC, Larson EB (2006) Fruit and vegetable juices and Alzheimer's disease: The Kame Project. *Am J Med* **119**, 751-759.

[25] Aksenov V, Long J, Liu J, Szechtman H, Khanna P, Matravadia S, Rollo CD (2013) A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. *Age (Dordr)* **35**, 23-33.

[26] Lemon JA, Aksenov V, Samigullina R, Aksenov S, Rodgers WH, Rollo CD, Boreham DR (2016) A multi-ingredient dietary supplement abolishes large-scale brain cell loss, improves sensory function, and prevents neuronal atrophy in aging mice. *Environ Mol Mutagen* **57**, 382-404.

[27] Lemon JA, Boreham DR, Rollo CD (2003) A dietary supplement abolishes age-related cognitive decline in transgenic mice expressing elevated free radical processes. *Exp Biol Med* **228**, 800-810.

[28] Lemon JA, Rollo CD, McFarlane NM, Boreham DR (2008) Radiation-induced apoptosis in mouse lymphocytes is modified by a complex dietary supplement: The effect of genotype and gender. *Mutagenesis* **23**, 465-472.

[29] Aksenov V, Long J, Lokuge S, Foster JA, Liu J, Rollo CD (2010) Dietary amelioration of locomotor, neurotransmitter and mitochondrial aging. *Exp Biol Med* **235**, 66-76.

[30] Oddo S, Caccamo A, Shepherd JD, Murphy MP, Golde TE, Kayed R, Metherate R, Mattson MP, Akbari Y, LaFerla FM (2003) Triple-transgenic model of Alzheimer's disease with plaques and tangles: Intracellular Aβ and synaptic dysfunction. *Neuron* **39**, 409-421.

[31] Sterniczuk R, Antle MC, Laferla FM, Dyck RH (2010) Characterization of the 3xTg-AD mouse model of Alzheimer's disease: Part 2. Behavioral and cognitive changes. *Brain Res* **1348**, 149-155.

[32] Filali M, Lalonde R, Theriault P, Julien C, Calon F, Planel E (2012) Cognitive and non-cognitive behaviors in the triple transgenic mouse model of Alzheimer's disease expressing mutated APP, PS1, and Mapt (3xTg-AD). *Behav Brain Res* **234**, 334-342.

[33] Stover KR, Campbell MA, Van Winssen CM, Brown RE (2015) Analysis of motor function in 6-month-old male and female 3xTg-AD mice. *Behav Brain Res* **281**, 16-23.

[34] Stevens LM, Brown RE (2015) Reference and working memory deficits in the 3xTg-AD mouse between 2 and 15-months of age: A cross-sectional study. *Behav Brain Res* **278**, 496-505.

[35] Clinton LK, Billings LM, Green KN, Caccamo A, Ngo J, Oddo S, McGaugh JL, LaFerla FM (2007) Age-dependent sexual dimorphism in cognition and stress response in the 3xTg-AD mice. *Neurobiol Dis* **28**, 76-82.

[36] Billings LM, Oddo S, Green KN, McGaugh JL, LaFerla FM (2005) Intraneuronal Aß causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron* **45**, 675-688.

[37] Davis KE, Easton A, Eacott MJ, Gigg J (2013) Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J Alzheimers Dis* **33**, 681-698.

[38] Marchese M, Cowan D, Head E, Ma D, Karimi K, Ashthorpe V, Kapadia M, Zhao H, Davis P, Sakic B (2014) Autoimmune manifestations in the 3xTg-AD model of Alzheimer's disease. *J Alzheimers Dis* **39**, 191-210.

[39] Carroll JC, Rosario ER, Kreimer S, Villamagna A, Gentzschein E, Stanczyk FZ, Pike CJ (2010) Sex differences in β-amyloid accumulation in 3xTg-AD mice: Role of neonatal sex steroid hormone exposure. *Brain Res* **1366**, 233-245.

[40] Davis KE, Burnett K, Gigg J (2017) Water and T-maze protocols are equally efficient methods to assess spatial memory in 3xTg Alzheimer's disease mice. *Behav Brain Res* **331**, 54-66.

[41] Attar A, Liu T, Chan W-TC, Hayes J, Nejad M, Lei K, Bitan G (2013) A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PLoS One* **8**, e80355.

[42] Pietropaolo S, Feldon J, Yee BK (2014) Environmental enrichment eliminates the anxiety phenotypes in a triple transgenic mouse model of Alzheimer's disease. *Cogn Affect Behav Neurosci* **14**, 996-1008.

[43] García-Mesa Y, López-Ramos JC, Giménez-Llort L, Revilla S, Guerra R, Gruart A, Laferla FM, Cristòfol R, Delgado-García JM, Sanfeliu C (2011) Physical exercise protects against Alzheimer's disease in 3xTg-AD mice. *J Alzheimers Dis* **24**, 421-454.

[44] Hirata-Fukae C, Li H-F, Hoe H-S, Gray AJ, Minami SS, Hamada K, Niikura T, Hua F, Tsukagoshi-Nagai H, Horikoshi-Sakuraba Y, Mughal M, Rebeck GW, LaFerla FM, Mattson MP, Iwata N, Saido TC, Klein WL, Duff KE, Aisen PS, Matsuoka Y (2008) Females exhibit more extensive amyloid, but not tau, pathology in an Alzheimer transgenic model. *Brain Res* **1216**, 92-103.

[45] Mastrangelo MA, Bowers WJ (2008) Detailed immunohistochemical characterization of temporal and spatial progression of Alzheimer's disease-related pathologies in male triple-transgenic mice. *BMC Neurosci* **9**, 81.

[46] España J, Giménez-Llort L, Valero J, Miñano A, Rábano A, Rodriguez-Alvarez J, LaFerla FM, Saura CA (2010) Intraneuronal β-amyloid accumulation in the amygdala enhances fear and anxiety in Alzheimer's disease transgenic mice. *Biol Psychiatry* **67**, 513-521.

[47] Sawmiller D, Habib A, Li S, Darlington D, Hou H, Tian J, Shytle RD, Smith A, Giunta B, Mori T, Tan J (2016) Diosmin reduces cerebral Aβ levels, tau hyperphosphorylation, neuroinflammation, and cognitive impairment in the 3xTg-AD mice. *J Neuroimmunol* **299**, 98-106.

[48] Walls KC, Ager RR, Vasilevko V, Cheng D, Medeiros R, LaFerla FM (2014) P-Tau immunotherapy reduces soluble and insoluble tau in aged 3xTg-AD mice. *Neurosci Lett* **575**, 96-100.

[49] Liu S, Breitbart A, Sun Y, Mehta PD, Boutajangout A, Scholtzova H, Wisniewski T (2014) Blocking the apolipoprotein E/amyloid β interaction in triple transgenic mice ameliorates Alzheimer's disease related amyloid β and tau pathology. *J Neurochem* **128**, 577-591.

[50] Ally BA, Hussey EP, Ko PC, Molitor RJ (2013) Pattern separation and pattern completion in Alzheimer's disease: Evidence of rapid forgetting in amnestic mild cognitive impairment. *Hippocampus* **23**, 1246-1258.

[51] Luu P, Sill OC, Gao L, Becker S, Wojtowicz JM, Smith DM (2012) The role of adult hippocampal neurogenesis in reducing interference. *Behav Neurosci* **126**, 381-391.

[52] Winocur G, Becker S, Luu P, Rosenzweig S, Wojtowicz JM (2012) Adult hippocampal neurogenesis and memory interference. *Behav Brain Res* **227**, 464-469.

[53] Francis BM, Kim J, Barakat ME, Fraenkl S, Yücel YH, Peng S, Michalski B, Fahnestock M, McLaurin J, Mount HT (2012) Object recognition memory and BDNF expression are reduced in young TgCRND8 mice. *Neurobiol Aging* **33,** 555-563.

[54] Rodríguez JJ, Jones VC, Tabuchi M, Allan SM, Knight EM, LaFerla FM, Oddo S, Verkhratsky A (2008) Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. *PLoS One* **3**, e2935.

[55] Demars M, Hu Y-S, Gadadhar A, Lazarov O (2010) Impaired neurogenesis is an early event in the etiology of familial Alzheimer's disease in transgenic mice. *J Neurosci Res* **88**, 2103-2117.

[56] Hamilton LK, Aumont A, Julien C, Vadnais A, Calon F, Fernandes KJL (2010) Widespread deficits in adult neurogenesis precede plaque and tangle formation in the 3xTg mouse model of Alzheimer's disease. *Eur J Neurosci* **32**, 905-920.

[57] Hutton CP, Déry N, Rosa E, Lemon JA, Rollo CD, Boreham DR, Fahnestock M, deCatanzaro D, Wojtowicz JM, Becker S (2015) Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice. *Neuroscience* **308**, 180-193.

[58] Valente T, Hidalgo J, Bolea I, Ramírez B, Anglés N, Reguant J, Morelló JR, Gutiérrez C, Boada M, Unzeta M (2009) A diet enriched in polyphenols and polyunsaturated fatty acids, LMN diet, induces neurogenesis in the subventricular zone and hippocampus of adult mouse brain. *J Alzheimers Dis* **18**, 849-865.

[59] Fahnestock M, Marchese M, Head E, Pop V, Michalski B, Milgram WN, Cotman CW (2012) BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. *Neurobiol Aging* **33**, 546-554.

[60] Rendeiro C, Vauzour D, Kean RJ, Butler LT, Rattray M, Spencer JPE, Williams CM (2012) Blueberry supplementation induces spatial memory improvements and region-specific regulation of hippocampal BDNF mRNA expression in young rats. *Psychopharmacology (Berl)* **223**, 319-330.

[61] Williams CM, El Mohsen MA, Vauzour D, Rendeiro C, Butler LT, Ellis JA, Whiteman M, Spencer JPE (2008) Blueberry-induced changes in spatial working memory correlate with changes in hippocampal CREB phosphorylation and brain-derived neurotrophic factor (BDNF) levels. *Free Radic Biol Med* **45**, 295-305.

[62] Hall AM, Roberson ED (2012) Mouse models of Alzheimer's disease. *Brain Res Bull* **88**, 3-12.

[63] Webster SJ, Bachstetter AD, Nelson PT, Schmitt FA, Van Eldik LJ (2014) Using mice to model Alzheimer's dementia: An overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front Genet* **5**, 1-23.

[64] Lyras L, Cairns NJ, Jenner A, Jenner P, Halliwell B (1997) An assessment of oxidative damage to proteins, lipids, and DNA in brain from patients with Alzheimer's disease. *J Neurochem* **68**, 2061-2069.

[65] Mattson MP, Pedersen WA, Duan W, Culmsee C, Camandola S (1999) Cellular and molecular mechanisms underlying perturbed energy metabolism and neuronal degeneration in Alzheimer's and Parkinson's diseases. *Ann N Y Acad Sci* **893**, 154-175.

[66] Butterfield DA, Drake J, Pocernich C, Castegna A (2001) Evidence of oxidative damage in Alzheimer's disease brain: Central role for amyloid beta-peptide. *Trends Mol Med* **12**, 548-554.

[67] Mutlu-Türkoğlu U, Ilhan E, Oztezcan S, Kuru A, Aykaç-Toker G, Uysal M (2003) Age-related increases in plasma malondialdehyde and protein carbonyl levels and lymphocyte DNA damage in elderly subjects. *Clin Biochem* **36**, 397-400.

[68] Sastre J, Pallardó FV, Viña J (2003) The role of mitochondrial oxidative stress in aging. *Free Radic Biol Med* **35**, 1-8.

[69] Antier D, Carswell HV, Brosnan MJ, Hamilton CA, Macrae IM, Groves S, Jardine E, Reid JL, Dominiczak AE (2004) Increased levels of superoxide in brains from old female rats. *Free Radic Res* **38**, 177-183.

[70] Long J, Aksenov V, Rollo CD, Liu J (2012) A complex dietary supplement modulates nitrative stress in normal

mice and in a new mouse model of nitrative stress and cognitive aging. *Mech Ageing Dev* **133**, 523-529.

[71] Mega MS, Cummings JL, Fiorello T, Gornbein J (1996) The spectrum of behavioral changes in Alzheimer's. *Neurology* **46**, 130-135.

[72] Kluger A, Gianutsos JG, Golomb J, Ferris SH, George a. E, Franssen E, Reisberg B (1997) Patterns of motor impairment in normal aging, mild cognitive decline, and early Alzheimer' disease. *Journals Gerontol Ser B Psychol Sci Soc Sci* **52B**, P28-P39.

[73] Palmer K, Berger AK, Monastero R, Winblad B, Bäckman L, Fratiglioni L (2007) Predictors of progression from mild cognitive impairment to Alzheimer disease. *Neurology* **68**, 1596-1602.

[74] Heyser CJ (2004) Assessment of developmental milestones in rodents. *Curr Protoc Neurosci* **Chapter 8**, Unit 8.18.

[75] Guyenet SJ, Furrer SA, Damian VM, Baughan TD, La Spada AR, Garden GA (2010) A simple composite phenotype scoring system for evaluating mouse models of cerebellar ataxia. *J Vis Exp*. doi: 10.3791/1787

[76] Pettersson AF, Olsson E, Wahlund LO (2005) Motor function in subjects with mild cognitive impairment and early Alzheimer's disease. *Dement Geriatr Cogn Disord* **19**, 299-304.

[77] Jones BJ, Roberts DJ (1968) The quantitative measurement of motor inco-ordination in naive mice using an accelerating rotarod. *J Pharm Pharmacol* **20**, 302-304.

[78] Klein SM, Vykoukal J, Lechler P, Zeitler K, Gehmert S, Schreml S, Alt E, Bogdahn U, Prantl L (2012) Noninvasive *in vivo* assessment of muscle impairment in the mdx mouse model - A comparison of two common wire hanging methods with two different results. *J Neurosci Methods* **203**, 292-297.

[79] Devanand DP, Michaels-Marston KS, Liu X, Pelton GH, Padilla M, Marder K, Bell K, Stern Y, Mayeux R (2000) Olfactory deficits in patients with mild cognitive impairment predict Alzheimer's disease at follow-up. *Am J Psychiatry* **157**, 1399-1405.

[80] Djordjevic J, Jones-Gotman M, De Sousa K, Chertkow H (2008) Olfaction in patients with mild cognitive impairment and Alzheimer's disease. *Neurobiol Aging* **29**, 693-706.

[81] Gheusi G, Cremer H, McLean H, Chazal G, Vincent JD, Lledo PM (2000) Importance of newly generated neurons in the adult olfactory bulb for odor discrimination. *Proc Natl Acad Sci U S A* **97**, 1823-1828.

[82] Carola V, D'Olimpio F, Brunamonti E, Mangia F, Renzi P (2002) Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviours in inbred mice. *Behav Brain Res* **134**, 49-57.

[83] Lister RG (1990) Ethologically-based animal models of anxiety disorders. *Pharmacol Ther* **46**, 321-340.

[84] Sakic B, Cooper MPA, Taylor SE, Stojanovic M, Zagorac B, Kapadia M (2015) Behavioral phenotyping of murine disease models with the integrated behavioral station (INBEST). *J Vis Exp* doi: 10.3791/51524

[85] Willner P, Muscat R, Papp M (1992) Chronic mild stress-induced anhedonia: A realistic animal model of depression. *Neurosci Biobehav Rev* **16**, 525-534.

[86] Strekalova T, Spanagel R, Bartsch D, Henn FA, Gass P (2004) Stress-induced anhedonia in mice is associated with deficits in forced swimming and exploration. *Neuropsychopharmacology* **29**, 2007-2017.

[87] Bevins RA, Besheer J (2006) Object recognition in rats and mice: A one-trial non-matching-to-sample learning task to study "recognition memory." *Nat Protoc* **1**, 1306-1311.

[88] Lalonde R (2002) The neurobiological basis of spontaneous alternation. *Neurosci Biobehav Rev* **26**, 91-104.

[89] Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. *J Neurosci Methods* **11**, 47-60.

[90] Fox J (2008) *Applied regression analysis and generalized linear models*, 2nd ed., Sage Publications, Thousand Oaks, CA.

[91] Anderson M, Braak CT (2003) Permutation tests for multi-factorial analysis of variance. *J Stat Comp Sim* **73**, 85-113.

[92] Wheeler RE, Torchiano M (2016) *lmPerm: Permutation tests for linear models in R*. The R project for statistical computing. URL: https://github.com/mtorchiano/lmPerm

[93] Fox J, Weisberg, S (2011) *An {R} companion to applied regression*, 2nd ed., Sage Publications, Thousand Oaks, CA.

[94] Singmann H, Bolker B, Westfall J, Aust F (2016) afex: Analysis of factorial experiments. *The R project for statistical computing*. URL: https://CRAN.R-project.org/package=afex

[95] Lenth RV (2016) Least-squares means: The R package lsmeans. *J Stat Softw* **69**, 1-33.

[96] R Core Team (2017) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. URL: https://www.R-project.org/

[97] Wickham H (2009) *ggplot2: Elegant graphics for data analysis*, Springer-Verlag, New York.

[98] Richardson JT (2011) Eta squared and partial eta squared as measures of effect size in educational research. *Educ Res Rev* **6**, 135-147.

[99] Bakeman R (2005) Recommended effect size statistics for repeated measures designs. *Behav Res Methods* **37**, 379-384.

[100] Rosnow RL, Rosenthal R (2003) Effect sizes for experimenting psychologists. *Can J Exp Psychol* **57**, 221.

[101] Furr RM (2004) Interpreting effect sizes in contrast analysis. *Understanding Stat* **3**, 1 25.

[102] Cohen J (1969) *Statistical power analysis for the behavioural sciences*. Academic Press, New York.

[103] Ye M, Chung HS, An YH, Lim S jin, Choi W, Yu AR, Kim JS, Kang M, Cho S, Shim I, Bae H (2016) Standardized herbal formula PM012 decreases cognitive impairment and promotes neurogenesis in the 3xTg-AD mouse model of Alzheimer's disease. *Mol Neurobiol* **58**, 5401-5412.

[104] Bohbot VD, McKenzie S, Konishi K, Fouquet C, Kurdi V, Schachar R, Boivin M, Robaey P (2012) Virtual navigation strategies from childhood to senescence: Evidence for changes across the life span. *Front Aging Neurosci* **4**, 28.

[105] Jack Jr. CR, Petersen RC, Xu Y, O'Brien PC, Smith GE, Ivnik RJ, Boeve BF, Tangalos EG, Kokmen E (2000) Rates of hippocampal atrophy correlate with change in clinical status in aging and AD. *Neurology* **55**, 484-489.

[106] Garthe A, Behr J, Kempermann G (2009) Adult-generated hippocampal neurons allow the flexible use of spatially precise learning strategies. *PLoS One* **4**, e5464.

[107] Garthe A, Huang Z, Kaczmarek L, Filipkowski RK, Kempermann G (2014) Not all water mazes are created equal: Cyclin D2 knockout mice with constitutively suppressed adult hippocampal neurogenesis do show specific spatial learning deficits. *Genes, Brain Behav* **13**, 357-364.

[108] Hörster H, Garthe A, Walker TL, Ichwan M, Steiner B, Khan MA, Lie DC, Nicola Z, Ramirez-Rodriguez G, Kempermann G (2017) p27kip1 is required for functionally relevant adult hippocampal neurogenesis in mice. *Stem Cells* **35**, 787-799.

[109] Zhang C-L, Zou Y, He W, Gage FH, Evans RM (2008) A role for adult TLX-positive neural stem cells in learning and behaviour. *Nature* **451**, 1004-1007.

[110] Brozka H, Pistikova A, Radostova D, Vales K, Svoboda J, Grzyb AN, Stuchlik A (2017) Adult neurogenesis reduction by a cytostatic treatment improves spatial reversal learning in rats. *Neurobiol Learn Mem* **141**, 93-100.

[111] Becker S (2005) A computational principle for hippocampal learning and neurogenesis. *Hippocampus* **15**, 722-738.

[112] Wesnes KA, Annas P, Basun H, Edgar C, Blennow K (2014) Performance on a pattern separation task by Alzheimer's patients shows possible links between disrupted dentate gyrus activity and apolipoprotein E ∈4 status and cerebrospinal fluid amyloid-β42 levels. *Alzheimers Res Ther* **6**, 20.

[113] Hebda-Bauer EK, Simmons TA, Sugg A, Ural E, Stewart JA, Beals JL, Wei Q, Watson SJ, Akil H (2013) 3xTg-AD mice exhibit an activated central stress axis during early-stage pathology. *J Alzheimers Dis* **33**, 407-422.

[114] Maei HR, Zaslavsky K, Teixeira CM, Frankland PW (2009). What is the most sensitive measure of water maze probe test performance? *Front Integr Neurosci* **3**, 4.

[115] Aksenov V (2014) *Complex dietary Interventions to Slow Rates of Aging*. (Doctoral dissertation) Retrieved from macsphere.mcmaster.ca. (http://hdl.handle.net/11375/15347; Identifier #5485838).

[116] Pop V, Head E, Hill M-A, Gillen D, Berchtold NC, Muggenburg BA, Milgram NW, Murphy MP, Cotman CW (2010) Synergistic effects of long-term antioxidant diet and behavioral enrichment on beta-amyloid load and non-amyloidogenic processing in aged canines. *J Neurosci* **30**, 9831-9839.

[117] Wu A, Ying Z, Gomez-Pinilla F (2008) Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. *Neuroscience* **155**, 751-759.

[118] Wan H, Aggleton JP, Brown MW (1999) Different contributions of the hippocampus and perirhinal cortex to recognition memory. *J Neurosci* **19**, 1142-1148.

[119] McHugh SB, Deacon RMJ, Rawlins JNP, Bannerman DM (2004) Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav Neurosci* **118**, 63-78.

4. Sexually dimorphic effects of a multi-ingredient dietary supplement on physical, cognitive and immunological function in a triple transgenic mouse model of Alzheimer's disease.

Hutton, C. P., Kaunismaa, E., Lemon, J. A., Shevtsova, O., Kapadia, M., Sakic, B., Rollo, C. D., Boreham, D. R., Fahnestock, M., Wojtowicz, J. M., and Becker, S. Sexually dimorphic effects of a multi-ingredient dietary supplement on physical, cognitive and immunological function in a triple transgenic mouse model of Alzheimer's disease. 1-55.

Sexually dimorphic effects of a multi-ingredient dietary supplement on physical, cognitive and immunological function in a triple transgenic mouse model of Alzheimer's disease.

Craig P. Hutton[1], Emily Kaunismaa[2], Jennifer A. Lemon[2], Olga Shevtsova[3], Minesh Kapadia[4], Boris Sakic[4], C. David Rollo[2], Douglas R. Boreham[2], Margaret Fahnestock[4], J. Martin Wojtowicz[5], and Suzanna Becker[1*]

[1]Department of Psychology, Neuroscience & Behaviour, McMaster University, Hamilton, ON, L8S 4K1, Canada

[2]Department of Biology, McMaster University, Hamilton, ON, L8S 4K1, Canada

[3]Division of Medical Sciences, University of Victoria, Victoria, BC, V8P 5C2, Canada

[4]Department of Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, ON, L8S 4K1, Canada

[5]Department of Physiology, University of Toronto, Toronto, ON, M5S 1A8, Canada

*Correspondence to: Suzanna Becker, Department of Psychology, Neuroscience & Behaviour, Psychology Building, Room 312, McMaster University, 1280 Main St. W., Hamilton, Ontario, Canada, L8S 4K1, Tel.: 905-525-9140 ext. 23020, Fax: 905-529-6225, Email: becker@mcmaster.ca

ABSTRACT

The amyloid cascade hypothesis of Alzheimer's disease (AD) has yielded a great deal of insight into the biological basis of the disorder. However, to date, this approach has failed to generate effective treatments for the disease. In the present study we took an alternative approach to determine whether prophylactic treatment with a multi-ingredient dietary supplement (MDS) designed to counteract mechanisms of aging such as inflammation and oxidative stress, which are exacerbated in AD, would delay the phenotype of the 3xTg-AD mouse model. Two experiments were conducted in which the effects of the MDS on a wide range of behavioural and biological measures were evaluated in both sexes of 3xTg-AD mice. In the first experiment, 24 male and 24 female 3xTg-AD mice were fed the MDS or a vehicle control treatment (plain bagel chips) from 1-7 months of age. From 6-7 months of age, half of the animals from each group were tested in a 17-test behavioural battery followed (in all animals) by post-mortem assays of brain and spleen size, neurogenesis in the subventricular zone (SVZ) and subgranular zone (SGZ), and BDNF mRNA expression in the hippocampus. In the second experiment, 20 male and 19 female 3xTg-AD mice, plus 22 male and 21 female WT control mice were fed either the MDS or vehicle control from 2-12 months of age, beginning after baseline behavioural testing at 1-2 months of age, and were retested on the battery from 3-4 and 11-12 months of age. After the completion of behavioural testing, brain and spleen size, TBS-soluble amyloid-β (Aβ) in the cortex, hippocampal BDNF mRNA, SGZ neurogenesis, DG and CA1 volume, and the upregulation of immediate early gene c-Fos after water maze reversal learning were examined.

Here we report that after 6-7 months of age 3xTg-AD males and females diverge, with males exhibiting larger impairments in spatial learning on reversal trials coupled with lower SGZ neurogenesis. In contrast, females showed higher levels of neurogenesis and impaired long-term spatial memory retention (delayed reversal probe trials in the Barnes maze) which was rescued by the diet supplementation. Importantly, behavioural testing at 6-7 months of age had no impact on the number of doublecortin-positive cells in either the SGZ or SVZ. At 6-7 months of age, the MDS also increased hippocampal BDNF expression in both sexes. At 11-12 months of age the performance of 3xTg-AD mice on the water maze and Barnes maze had deteriorated further. They also had smaller brains, larger spleens, shrunken hippocampi, elevated levels of soluble amyloid-β in the cortex, and reduced levels of neurogenesis in the SGZ compared to WT mice. The MDS attenuated the splenomegaly seen in both sexes of 3xTg-AD mice relative to WT controls, perhaps due to the anti-inflammatory actions of the MDS. Long term treatment with the MDS yielded sexually dimorphic impacts on several biomarkers and cognitive measures at 11-12 months. Supplemented 3xTg-AD females, but not males, showed improved hippocampal BDNF levels and superior learning and memory under conditions of high interference in reversal learning. Neural activation patterns across different hippocampal sub-regions (as revealed by c-Fos) following water maze reversal learning were also affected by the MDS. Vehicle-treated 3xTgAD-males showed an abnormal pattern of activation, with less c-Fos labelling in the dentate gyrus and more labelling in the CA1 and CA3 relative to wild type animals, potentially reflecting

use of a compensatory strategy for learning this task. The MDS prevented this abnormal pattern of activation seen in vehicle-control 3xTg-AD males following water maze reversal learning. Together, these results provide further evidence of important sex differences in the 3xTg-AD strain and indicate that the MDS delays cognitive decline in 3xTg-AD females for longer than males, perhaps by upregulating BDNF in the hippocampus.

GENERAL INTRODUCTION

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder and a leading cause of death (www.alz.org). The progressive cognitive decline of AD patients has been linked to damage in regions of the brain that are central to episodic memory (Scoville & Milner, 1957; Penfield & Milner, 1958; Sekeres et al., 2016), spatial navigation/memory (O'Keefe & Dostrovsky, 1971; Burgess et al., 2002), regulation of the stress response (Diorio et al., 1993; Snyder et al., 2011), and working memory (Kubota & Niki, 1971; Fuster & Alexander, 1971; Watanabe & Niki, 1985) such as the prefrontal cortex and hippocampus. To date, most research into AD pathogenesis has focused on counteracting the accumulation of misfolded proteins amyloid-β (Aβ) and hyperphosphorylated tau (p-tau) into the hallmark plaques and tangles that are observed throughout the brains of AD patients at autopsy (Stelzmann et al., 1995; Braak & Braak, 1991; Thal et al., 2002). This enormous body of work has yielded many key insights into AD, most notably the rise of the prominent "amyloid cascade hypothesis" of AD (Hardy & Higgins, 1992; Selkoe & Hardy, 2016). Briefly, the amyloid cascade hypothesis proposes that an accumulation of Aβ due to impaired clearance or overproduction triggers a cascade which (among other consequences) leads to a loss of trophic support, synaptic atrophy, and neuron death. More recently, several limitations of the cascade hypothesis have become apparent. For instance, anti-amyloidogenic drugs have thus far failed to stop the progression of AD in clinical trials (Herrup, 2015). Moreover, a large fraction of the population (up to 35%) in whom plaques and tangles are found do not show AD-like cognitive symptoms (Geddes et al., 1997; Aizenstein et al., 2008). Central injections of different species of Aβ (at various stages of aggregation) into the primate brain suggest that the soluble oligomeric form, rather than the insoluble plaques, is the most toxic form of amyloid (Shankar et al., 2008). There is also reason to believe that altered processes downstream of Aβ signalling, such as the downregulation of brain-derived neurotrophic factor (BDNF; Peng et al., 2005; Garzon & Fahnestock, 2007; Michalski et al., 2015; Rosa & Fahnestock, 2015) and synapse degeneration (Terry et al., 1991) are better predictors of cognitive symptoms in AD patients than the degree of plaque or tangle burden.

BDNF is critically important for the survival and growth of neurons which wither in multiple neurodegenerative diseases (Howells et al., 2000; Peng et al., 2005). BDNF also regulates synaptic plasticity during learning and memory consolidation (Fahnestock, 2011). In AD, these processes are perturbed, as Aβ alters the phosphorylation of cyclic adenosine monophosphate response element binding protein (CREB; DaRocha-Souto et al., 2012) and decreases its transcription (Rosa & Fahnestock, 2015). The potential for BDNF and neurogenesis as therapeutic targets in AD is apparent from animal studies. Increasing BDNF directly by intracerebroventricular infusion in rodent and primate models of AD reversed neuronal atrophy, prevented cell death and attenuated cognitive decline (Nagahara et al., 2009; Blurton-Jones et al., 2009).

In addition to its pleiotropic effects on developmentally generated neurons, BDNF is also involved in maintaining neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG) in

adult mammals (Linnarsson et al., 2000; Lee et al., 2002). Hippocampal neurogenesis is necessary for hippocampal-dependent learning under conditions of high interference in rodents (Luu et al., 2012; Winocur et al., 2012); similarly, in humans, high interference memory performance varies as a function of stress, ageing and exercise, all of which are known correlates of neurogenesis (Déry et al., 2013; Déry et al., 2015; Stark et al., 2015, Heisz et al., 2017). Similarly, pharmacological enhancement of adult neurogenesis restored cognition and neuroplasticity without affecting amyloid or tau levels in the 3xTg-AD mouse model of AD (Blanchard et al., 2010). That adult neurogenesis promotes hippocampal function in humans is also supported by a recent study (Briley et al., 2016) on post-mortem tissue from AD patients and non-demented controls with AD pathology which showed that cognitively normal individuals, but not AD patients, had preserved levels of neurogenesis in the SGZ. Additionally, deficits on a high-interference picture matching memory test are correlated with Aβ levels in AD patients (Wesnes et al., 2014). Adult neurogenesis declines with normal aging (Knoth et al., 2010) and is among the earliest biomarkers depleted in rodent models of AD (Rodríguez et al., 2008; Demars et al., 2010; Hamilton et al., 2010). For example, neurogenesis levels are reduced in the 3xTg-AD mouse beginning at 2-4 months of age (Rodríguez et al., 2008; Demars et al., 2010; Hamilton et al., 2010), prior to the accumulation of Aβ or phospho-tau. Thus, promoting neurogenesis may delay or prevent AD.

In support of this approach, we recently demonstrated (Hutton et al., 2018) that prophylactic treatment of 3xTg-AD mice between 2-4 months of age with a multi-ingredient dietary supplement (MDS; Table 1) prevents the early decline in spatial reversal learning on the water maze (a high interference condition in the water maze task) and working memory on the spontaneous alternation test that was observed in vehicle-treated transgenic animals. The MDS was designed to counteract age-related alterations in oxidative stress, inflammation, insulin resistance, membrane integrity, and mitochondrial dysfunction (Lemon et al., 2003). Effectiveness on these targets as well as other benefits (e.g., protection against radiation-induced DNA damage) have been reported elsewhere (Lemon et al., 2003; Lemon et al., 2008; Aksenov et al., 2010; Aksenov et al., 2013). We have previously shown that the MDS can ameliorate the age-related deterioration of spatial learning, brain volume, and neuron integrity seen in wild-type mice and in a model of accelerated aging (transgenic growth hormone mouse; Aksenov et al., 2013; Lemon et al., 2016). Moreover, in chronically stressed wild type mice, the MDS was found to synergistically increase hippocampal neurogenesis, BDNF expression, and the size of the DG and CA1 in combination with exercise (Hutton et al., 2015).

The present study extended this work in several ways. The primary aim of these experiments was to determine whether the MDS impacts hippocampal BDNF, neurogenesis, or other relevant biomarkers (e.g. soluble Aβ levels) in the 3xTg-AD mouse model. To evaluate the functional contribution of hippocampal neurons to performance on the high-interference reversal trials on the water maze, we also examined the expression of the immediate-early gene c-fos (Guzowski et al., 2001; Cahill et al., 2017) in the DG, CA1, and CA3 subfields of the

hippocampus 90 minutes after reversal learning trials. Given that both 3xTg-AD mice (Marchese et al., 2014; Kapadia et al., 2018) and AD patients (Tarkowski et al., 2003) exhibit autoimmune changes (e.g. chronic inflammation, splenomegaly), we additionally tested the hypothesis that the MDS would attenuate the dramatic (up to 10x) increase in spleen weight seen in 3xTg-AD mice. We examined these biomarkers and a variety of behavioural measures in older animals (at 6-7 months of age and 11-12 months of age), to determine if the behavioural benefits we observed at 3-4 months of age persisted into later stages of the 3xTg-AD mouse phenotype (i.e. after intracellular and soluble Aβ have started to accumulate), in both males and females. Finally, by comparing mice that underwent behavioural testing to those that did not, we evaluated whether the experience of being tested in a battery of behavioural tasks (Hutton et al., 2018; Table 2) itself affects hippocampal neurogenesis, since other forms of environmental enrichment are known to upregulate neurogenesis (Kempermann et al., 1997; Olson et al., 2006).

EXPERIMENT 1

Introduction

The first of the two experiments (see Figure 1 for a diagram of the design) conducted in this study aimed to determine whether daily supplementation with the MDS for 6 months would affect the performance of 6-7-month-old male and female 3xTg-AD mice on a variety of behavioural measures, increase the expression of BDNF in the hippocampus, and/or increase levels of neurogenesis (DCX+ cells) in the SGZ or the subventricular zone (SVZ). As a secondary measure, we also examined whether extensive behavioural testing (over 42 days) itself would affect the number of DCX+ cells. We predicted that the MDS, but not behavioural testing, would increase hippocampal BDNF as well as SGZ and SVZ neurogenesis. We also predicted that animals given the MDS would show improved performance on tests of learning and memory, specifically the spontaneous alternation test and the reversal portion of the water maze and Barnes maze, consistent with the behavioural effects of the MDS we observed in this strain at 3-4 months of age (Hutton et al., 2018). Spatial reversal learning, which is a challenging, high interference memory task, was specifically of interest, as reversal learning has been shown in wild type mice to depend upon hippocampal neurogenesis (Garthe et al., 2009), consistent with a role for neurogenesis in other high interference memory tasks (e.g. Luu et al., 2012; Winocur et al., 2012).

Methods

Animals

The experimental subjects were 24 male and 24 female B6;129-*Psen1tm1Mpm* Tg(APPSwe,tauP301L) 1Lfa/Mmjax (3xTg-AD) mice, which have been described by others (Oddo et al., 2003). Briefly, the 3xTg-AD strain carries 3 human transgenes for mutations which

affect amyloid and tau (APP_{swe}, PSen1 and tauP301L) and are associated with frontotemporal dementia and early onset AD. This rodent model was selected because the progression of amyloid/tau accumulation and behavioural deterioration mimics the sequence observed in human AD to a greater extent than most other models (Hall & Roberson, 2012; Webster et al., 2014). Mice included in this study were obtained from a breeding colony we established in the Psychology department animal facility at McMaster University using breeders from Jackson Laboratories (Bar Harbor, USA). Experimental mice and breeders were all individually housed on a reversed 12:12 h light/dark cycle (lights off from 7:00 – 19:00), in standard conditions as described previously (Hutton et al., 2018).

After weaning at 4 weeks of age, the mice were anesthetized using isoflurane and then tattooed on the ears for identification purposes. These male and female 3xTg-AD mice were then randomly assigned to treatment with either the MDS or the vehicle control for 6 months (see below). Half of the mice in each sex x treatment condition were then tested in the behavioural battery (Table 2), or not (i.e. untested controls) from 6-7 months of age, yielding a 2 x 2 x 2 (behavioural testing x sex x diet) design with 8 groups (n = 6 for each): MDS-supplemented females tested in the behavioural battery (BFD), vehicle-supplemented females tested in the battery (BFV), MDS-supplemented males tested in the battery (BMD), vehicle-supplemented males tested in the battery (BMV), MDS-supplemented female untested controls (CFD), vehicle-supplemented female untested controls (CFV), MDS-supplemented male untested controls (CMD), and vehicle-supplemented male untested controls (CMV). During INBEST testing (see Hutton et al., 2018, for details), animals were also all given *ad libitum* access to sucrose-water (1%) for 10 h to test for anhedonia.

Upon completion of cognitive assessments in the behavioural testing groups, all animals were euthanized via exsanguination while under isoflurane anesthesia. The correct plane of anesthesia was verified via toe pinch. Mice were weighed immediately prior to sacrifice and then blood was collected via cardiac puncture of the right atrium for later analysis of serum proteins. After blood collection, the spleen and brain were extracted and weighed. From each animal, one hemisphere of the brain (selected at random) was prepared for immunohistochemistry by immersion in a solution of 4% paraformaldehyde in 1x PBS for 36 hours, while the hippocampus and cerebral cortex of the other hemisphere (designated for BDNF, amyloid and tau measurements) were rapidly dissected and flash frozen using liquid nitrogen. To facilitate dissections, brain samples were cooled by light irrigation with 1xPBS (kept at 4˚C). All procedures employed in this study were approved by the McMaster University Animal Research Ethics Board.

Multiple Ingredient Dietary Supplement

The multi-ingredient dietary supplement (MDS) is a 30-ingredient nutritional compound (Table 1) that was designed to target multiple mechanisms associated with aging (see Lemon et al., 2008 for processes targeted by each ingredient). The MDS was prepared and administered as described previously (Hutton et al., 2018), from 1-7 months of age. As in previous experiments (e.g. Lemon et al., 2003; Hutton et al., 2015; Hutton et al., 2018), the MDS was prepared in liquid form, soaked on small pieces of bagel, and left to dry. Starting at 1 month of age, treated mice were fed an MDS bagel piece once per day between 17:00–19:00, receiving 140.15 mg of dry weight supplement. This dosage was calculated by considering amounts normally prescribed to humans, adjusting for the smaller body size and higher metabolic rate of mice (Lemon et al., 2003). Mice were monitored to ensure they consumed the entire bagel piece, and researchers estimated percent consumption when part of the bagel piece was left. In prior studies, the MDS was palatable and showed no negative side effects (Aksenov et al., 2013; Hutton et al., 2015; Lemon et al., 2016, 2003). The same protocol was used for control-diet (vehicle) mice except they were fed a plain bagel piece without the MDS. All mice were provided *ad libitum* access to regular mouse chow and water.

Behavioural battery

At 6 months of age, animals were habituated to handling for 5 days. They were then tested in an 18-item battery of behavioural tests designed to assess several motor, sensory, mood-related, learning, and memory functions that are known to be altered in 3xTg-AD mice at different ages. This age was chosen based on previous reports that 3xTg-AD mice exhibit some Aβ, but not tau, pathology (Mastrangelo & Bowers, 2008; Kapadia et al., 2018), reduced levels of neurogenesis (Demars et al., 2010), and behavioural alterations (Hutton et al., 2018; Marchese et al., 2014) which resemble early stage AD in humans (Mega et al., 1996; Morris et al., 2001; Ally et al., 2013). Prior to behavioural testing, mice were habituated to handling for 1 min/d for 5 d. During the battery, tests were performed in the following order: basic reflexes (visual placing response, righting reflex, hind limb placing response, postural reflex, negative geotaxis), rotarod, hanging basket, olfactory acuity, olfactory discrimination, beam walking, open field, elevated plus maze, novel object, INBEST monitoring, spontaneous alternation, Barnes maze, and water maze. These tests were administered exactly as previously described (Hutton et al., 2018) except for the Barnes maze (see description below), which was added because the water maze is a more stressful testing experience for mice than the Barnes maze (Harrison et al., 2009), and this stress response is exaggerated in 3xTg-AD female mice at 9, but not 15, months of age (Clinton et al., 2007). Our previous data (Hutton et al., 2018; Marchese et al., 2014) revealed that 3xTg-AD mice exhibit anxiety-like behaviour and memory impairments (relative to wild type controls) by 4 months of age. Thus, our current analyses focused on the measures of anxiety, learning, and memory in the battery (elevated plus maze, open field, novel object, spontaneous alternation,

water maze, and Barnes maze) to determine if a longer period of MDS supplementation (6 months vs. 2 months in Hutton et al., 2018) affected these behavioural domains in the 3xTg-AD model. See Experiment 2 Results for a comprehensive behavioural comparison between 3xTg-AD and WT controls at 11-12 months of age.

Barnes Maze

Deficits in spatial learning and memory are hallmark symptoms of AD (Morris et al., 2001). These deficits may be preceded by impairments in learning under conditions of high interference (Ally et al., 2013), of which the reversal trials are an example. The Barnes maze (1979) is a widely used test of general hippocampus-dependent learning and memory (phase 1), as well as a test of cognitive flexibility (phase 2), which are both impacted in the 3xTg-AD model (Oddo et al., 2003; Hutton et al., 2018).

Apparatus

The maze design was modelled after that used by Attar et al. (2013). It consisted of a white circular HDPE plastic platform, 122 cm in diameter, with 20 equally spaced circular holes (4.45 cm in diameter) around the circumference of the platform (2.54 cm from the edge) and elevated approximately 89 cm above the floor on 4 cylindrical table legs. One hole led to a black Plexiglas escape box that the mice could hide in (5 cm deep x 15.2 cm long x 5 cm wide), while the other 19 holes were occluded with false black Plexiglas boxes (1.3 cm deep x 5 cm long x 5 cm wide) that were too shallow to use as hiding places. The bottom of each box was lined with a thin layer of unsoiled woodchip bedding (the same as used in home cages) to encourage mice to investigate the boxes. The maze was surrounded by 4 visual cues that could serve as reference points during navigation, mounted on black curtains (hung from aluminum racks, covering ~45 cm to 182 cm above the floor) located at approximately 30 cm from the edge of the maze platform. No cues were placed directly behind the escape box, to minimize the potential for the use of hippocampus-independent visual association strategies (Schenk and Morris, 1985; Devan et al., 1996; O'Leary & Brown, 2015) by the mice during navigation. The curtains were used to ensure that the cues were located close enough to the maze that the 3xTg-AD mice could see them, as transgenic AD mice reportedly exhibit visual acuity impairments with age (Stover and Brown, 2012). The maze was brightly illuminated using a lamp attached to the top of a curtain rack in the west quadrant of the maze (as an additional visual cue), and while subjects were on it (i.e. until they escaped), white noise was played on speakers in the room, rendering the maze environment an unpleasant, exposed space from which mice are normally motivated to escape (Barnes, 1979).

Protocol

Phase one (days 1-4) of the protocol was based on that reported by Attar et al. (2013). Phase two (days 5- 7) nearly identical, except that it involved reversal learning. On Day 1, mice competed 2 habituation trials. Mice were first placed in the centre of the maze inside a transparent PVC cylinder (17.8 cm in diameter x 23 cm tall), while white noise (White Noise Generator software, Sobolsoft) was played through nearby computer speakers. After 30 seconds, the cylinder was removed, and the mice were given 2 minutes to enter the escape box independently. If they failed to do so, they were gently nudged into it using the cylinder. Once the mouse entered the escape box, the white noise was turned off and the mouse stayed in the box for 45 seconds before being removed and returned to its home cage.

On Days 2 and 3, mice completed three acquisition trials per day. At the start of each trial, white noise was played, and mice were placed in an opaque cylinder (25.4 cm tall x 17.8 cm in diameter) in the centre of the maze, so that they started the task facing a random direction. After 15 seconds in the opaque cylinder, mice were released to explore the maze and try to escape for up to 2 minutes, after which they would be guided to the escape box. Once the mouse escaped, the white noise was turned off and it was left in the escape box for 45 seconds before being returned to its home cage.

On Day 4, each mouse completed one probe trial. This trial was the same as previous acquisition trials, except that the escape box was also blocked. After 15 seconds in the opaque cylinder, mice were left to explore the maze for 2 minutes (but unable to escape) before being returned to their home cage.

In phase 2 (Days 5–7), the protocol was identical to days 2-4 of phase 1 except that the escape box was located on the opposite side of the maze. By relocating the escape location, we tested reversal learning. Similar to reversal trials of the Morris water maze (Garthe et al., 2009), the reversal learning trials in the Barnes maze were predicted to be neurogenesis-dependent. All trials were filmed from above via an autopole-mounted video camera, and the latency to locate the escape box (localization time), distance moved, and mean locomotor speed of the mice during testing was collected via Ethovision XT (Noldus, Toronto, ON). For acquisition and reversal trials, the localization time was used as a measure of performance. For the probe trials (Days 4 and 7), the time spent in the target quadrant was used to evaluate memory for the escape location used over the previous 2 days.

Quantification of hippocampal BDNF mRNA using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Following the sacrifice of animals by exsanguination under isoflurane anesthesia, the hippocampus from one hemisphere was dissected and immersed in liquid nitrogen, then stored at -80˚C until use. The methods employed for the subsequent isolation of RNA, reverse transcription and quantification of BDNF using RT-qPCR employed reagents and followed protocols identical to those reported previously (Hutton et al., 2015; Rosa et al., 2016). Briefly,

hippocampal tissue samples were first weighed and then sonicated in cold Trizol™ solution (Life Technologies, Inc., Gaithersburg, MD, USA). RNA was isolated from the homogenate by centrifugation and ethanol extraction, followed by DNase treatment on column and elution from an RNeasy™ spin column (Qiagen, Mississauga, ON, Canada), as per the manufacturer's instructions.

After extraction, RNA concentrations and purity were determined by spectrophotometry. RNA integrity was verified by agarose gel electrophoresis on a subset of samples selected at random. One microgram of each RNA sample was reverse transcribed, and 50 ng of the resulting cDNA was amplified as previously described (Rosa et al., 2016; Hutton et al., 2015) using forward and reverse primers [BDNF: 5' GCG-GCA-GAT-AAA-AAG-ACT-GC 3' (forward) and 5' CTT-ATG-AAT-CGC-CAG-CCA-AT 3' (reverse); β-actin: 5' AGC-CAT-GTA-CGT-AGC-CAT-CC 3' (forward) and 5' CTC-TCA-GCT-GTG-GTG-GTG-AA 3' (reverse)], ROX reference dye (Invitrogen), and cDNA or reference standard for absolute quantification. A "no template" control lacking cDNA was included. Only those RT-qPCR runs with efficiencies greater than 90% and R^2 greater than 0.99 were included in subsequent statistical comparisons. BDNF and β-actin were both measured in triplicate. BDNF and β-actin copy numbers for each animal were collected using MXP Pro v3.0 software (Fahnestock et al., 2012).

Immunohistochemistry

Following euthanasia, the right hemisphere of the brain from each animal was extracted and post-fixed by immersion in a solution of 4% paraformaldehyde in phosphate buffered saline (PBS) for 36 hours, after which the samples were transferred to a 0.1% sodium azide (in PBS) solution and stored at 4˚C until sectioning. The procedures used for sectioning, sampling and cell counting were identical to those reported previously (Hutton et al., 2015). Doublecortin (DCX) was assayed as a marker of neurogenesis. DCX is a microtubule-binding protein expressed transiently in proliferating neural precursor cells and migrating neuroblasts that is recognised as a biomarker of immature, adult-born neurons (Brown et al., 2003; McDonald and Wojtowicz, 2005). All sections were first incubated with anti-DCX goat polyclonal IgG antibody sc-8066 (Santa Cruz) and then Alexa 488 Donkey anti-goat IgG antibody A-11055 (Life Technologies). Antibodies were suspended in a PBS solution containing 0.3% Triton X-100, which facilitated cell membrane penetration. Sections were washed (on a rotomixer) in PBS 3 times (5 min each) before and after each antibody incubation period. After the final wash, sections were mounted on labelled glass slides using a paintbrush and distilled water, permitted to air dry in a shaded area (to limit light exposure), coated with PermaFluor™ aqueous mounting medium (Thermo Scientific), covered with a glass coverslip, and then stored in a slide folder at 4˚C overnight before being transferred to a slide box for storage.

Immunolabelled cells in the subgranular zone (SGZ) of the dentate gyrus were counted using a 40x objective lens (Nikon, OPTIPHOT-2 fluorescence microscope) as previously described (Hutton et al., 2015). The cell counts and length measurements were then used to calculate the number of DCX+ cells per mm DG, averaged across sections in each region (dorsal, medial, and ventral) and throughout the DG. This sectional average was then multiplied by the number of sections containing hippocampus (~45-50) to yield an estimate of the number of DCX+ cells per DG. The number of DCX+ cells in the subventricular zone (SVZ) were counted for sections which also contained the dorsal or medial regions of the hippocampus (the same

sections used for SGZ counts; the SVZ was not visible in the ventral sections), averaged across 14 sections per mouse. This average per section was used as a measure of SVZ neurogenesis.

Statistical Analyses and Graphics

The Barnes maze and water maze data were analyzed using repeated-measures 4-factor (behavioural testing x sex x diet x trial/session) mixed effects ANOVAs. Since they measure different cognitive processes, the cued acquisition/reversal (water maze only), probe, acquisition and reversal trials of these tests were analyzed separately. Given that the performance of mice on the acquisition and reversal components was predicted *a priori* to improve linearly across trials (e.g. Garthe et al., 2009; Attar et al., 2013; Marchese et al., 2014), we also evaluated the linear change across trials (i.e. the first order polynomial contrast) for these ANOVAs. In cases where the sphericity assumption was violated (Mauchly's test), the corrected Greenhouse-Geisser p-values are reported instead of the uncorrected p-values. If a significant interaction was detected, *post hoc t*-tests were used, with Tukey's (Benjamini & Braun, 2002) or Holm's (Holm, 1979) correction (depending on whether all possible [Tukey] or a subset [Holm] of the possible comparisons were tested) for multiple comparisons applied to p-values. Holm's (modification of the Bonferroni) p-value adjustment was used for *post hoc* tests instead of the classical Bonferroni adjustment because the former effectively controls the familywise alpha level and the latter has been shown to over-inflate the risk of type II errors (Perneger, 1998; Eichstaedt et al., 2013). Since we hypothesized that the MDS would selectively preserve working memory (spontaneous alternation rate) and learning on the reversal trials of the Barnes maze and water maze, Holm-corrected one-tailed *t*-tests for the effect of diet (MDS superior to vehicle) were evaluated separately for males and females on those measures only.

All non-repeated measures behavioural data were analyzed using 2 x 2 (sex x diet) factorial ANOVAs, unless either homoscedasticity (Levene's test) or normality (Shapiro-Wilk test) assumptions were violated, in which case an equivalent factorial permutation ANOVA (10,000,000 iterations) was used (Anderson & Braak, 2003; Wheeler & Torchiano, 2016). Biomarker data were examined using 2 x 2 x 2 (behavioural testing x sex x diet) ANOVAs or factorial permutation tests following the same logic. To evaluate whether MDS-related changes in behaviour were associated with changes in biomarkers (e.g. neurogenesis, spleen weight), Pearson correlations were also calculated for those measures.

Statistical tests were conducted using the car (Fox & Weisberg, 2011), afex (Singmann et al., 2016), emmeans (Lenth, 2018), lmPerm (Wheeler & Torchiano, 2016), lsr (Navarro, 2015), and psych (Revelle, 2017) packages in R (R Core Team, 2017). An α-level of 0.05 was used to determine statistical significance. Figures were generated using the ggplot2 package in R (Wickham, 2009), and tables were created using Microsoft Excel 2010 (Microsoft Canada Inc., Mississauga, ON). An alpha level of 0.05, effect size measures, and power calculations were used to interpret effects and interactions.

With respect to effect size metrics, partial eta-squared (η^2_p) was used for the results of ANOVAs and permutation tests on non-repeated-measures data (Richardson, 2011), while generalized eta-squared (η^2_g) was used for repeated-measures data (Bakeman, 2005). In addition, $r_{contrast}$-squared (r^2_c; Rosnow & Rosenthal, 2003) was employed for the first order (linear) polynomial contrasts (Barnes maze and water maze data). Cohen's benchmarks for

small (0.01), medium (0.06), and large (0.14) effects are recommended when interpreting these measures (Cohen, 1969; Furr, 2004; Richardson, 2011). Finally, Cohen's d was also used as an effect size measure for *post hoc t*-tests comparing the performance of 3xTg-AD females to chance performance (30 seconds in the target quadrant) on the first probe trial of the Morris water maze (for reference: small effect d = 0.2, medium effect = 0.5, large effect d = 0.8).

Results

Elevated Plus Maze and Open Field

Analysis of the time spent in the open arms of the plus maze (2 x 2 ANOVA) did not reveal any significant main effects or interactions between sexes of mice or in response to MDS treatment (data not shown). In the open field test, several anxiety-related behaviours were measured using video tracking software: total time spent in the center zone, latency to approach the center, total freezing time, and the number of fecal boli secreted. Statistical examination of these measures also did not yield any significant differences between treatment groups or sexes (data not shown). Thus, at 6-7 months of age, male and female 3xTg-AD mice do not seem to differ in levels of anxiety-like behaviour, and 6 months of MDS supplementation (from 1-7 months of age) does not have an anxiolytic effect in the 3xTg-AD mouse.

Novel Object Recognition

The preference of mice to explore a novel object over a familiar object was evaluated as a measure of recognition memory. Analysis of the proportion of time spent interacting with the new object out of the total interaction time for both objects in the testing arena did not reveal any effects of sex or diet (data not shown). The MDS therefore does not affect the object recognition memory of 3xTg-AD males or females, which perform similarly on this test at 6-7 months of age.

Working Memory

Spatial working memory was examined using the spontaneous alternation test. Although the 2 x 2 ANOVA for the day 1-5 alternation rate did not reveal any significant main effects or interactions (diet, $F_{(1, 20)} = 2.581$, $p = 0.124$, $\eta^2_p = 0.114$; sex, $F_{(1, 20)} = 0.692$, $p = 0.124$, $\eta^2_p = 0.008$, sex x diet interaction, $F(1, 20) = 1.290$, $p = 0.269$, $\eta^2_p = 0.061$), the planned one-tailed t-tests (see Methods) for the effect of diet (MDS > vehicle) in each sex did uncover a benefit of the MDS for 3xTg-AD females ($t_{(10)} = 1.865$, $p = 0.046$ [unadjusted] or 0.097 [with Holm-adjustment]; $d = 1.077$) but not 3xTg-AD males (treatment group means were identical: $t_{(10)} = 0$,

$p = 0.5$; $d = 0$; Figure 2). The marginal significance of this effect in females (depending on whether Holm p-adjustment was applied) in this sample was evidently due to low power, despite the large effect size (cohen's $d = 1.07$), as suggested by a follow-up power analysis (power = 0.73 for a one-tailed test, or 0.57 for a two-tailed test). The power analysis also determined that an additional 3 subjects per group (i.e. $n = 9$, as used in Experiment 2) would be needed to achieve a power level of 0.8 using two-tailed tests. Thus, MDS supplementation may improve working memory until at least 7 months of age in 3xTg-AD females, but not 3xTg-AD males.

Visuospatial Learning and Memory

Visual acuity and visuospatial learning and memory under conditions of low (1st platform location) and high interference (2nd platform location) were evaluated using different stages of learning in the water maze and Barnes maze.

Water Maze

On the cued water maze trials, a mixed effects ANOVA (sex x diet x trial) did not reveal any differences between males and females in terms of swimming speed (data not shown). Therefore, the latency to locate the platform (rather than swim path length) was used as a measure of performance on water maze trials in which there was either a visible or submerged platform. Analysis of the escape time data for these trials did not reveal any significant effects or interactions, indicating that the visual acuity of each group was similar (data not shown). Separate mixed effects ANOVAs of the same measure on the acquisition and reversal trials, during which the platform was submerged in the first and second testing locations, uncovered a main effect of trial only for acquisition ($F_{(6, 121)} = 2.828$, $p = 0.013$, $\eta^2_g = 0.116$), and a sex x trial interaction for reversal trials ($F_{(2, 41)} = 2.957$, $p = 0.048$, $\eta^2_g = 0.078$; Figure 3). The addition of a linear contrast to the trial factor in these ANOVAs confirmed that all groups improved linearly across trials when learning the 1st platform location ($t_{(1, 300)} = -3.716$, $p < 0.001$, $r^2_c = 0.045$), but only males improved linearly on the 2nd location (males, $t_{(1, 60)} = -2.942$, $p = 0.005$, $r^2_c = 0.126$; females, $t_{(1, 60)} = -0.054$, $p = 0.957$, $r^2_c < 0.001$). Subsequent Tukey-corrected pairwise *t*-tests for the effect of sex on each trial determined that this sex difference in the learning curve was due to males taking longer than females to locate the hidden platform on the first reversal trial only (trial 1, $t_{(69)} = 2.433$, $p = 0.017$, $d = 2.004$; trial 2, ($t_{(69)} = -0.356$, $p = 0.723$, $d = 0.043$; trial 3, $t_{(69)} = -1.009$, $p = 0.316$, $d = 0.121$; trial 4, $t_{(69)} = -0.011$, $p = 0.991$, $d = 0.001$). Thus, the MDS did not affect learning on the water maze, and while both sexes of 3xTg-AD mice were able to learn the 1st hidden platform location equally well, males had more difficulty learning the 2nd location.

With respect to long term spatial memory, a 2 x 2 ANOVA of the time spent in the target quadrant on the probe trial for the 1st platform location (when the platform was not in the pool) also did not detect any differences between treatment groups or sexes (data not shown). Although there were no differences between groups, Holm-corrected one-tailed *t*-tests comparing

each group to chance performance (target quadrant time > 30s or 25% of the 120s trial) confirmed that each group could remember the 1st platform location 24 hours after the last acquisition trial (MDS-supplemented females, $t_{(5)} = 3.466$, $p = 0.032$, $d = 1.415$; vehicle-control females, $t_{(5)} = 3.342$, $p = 0.040$, $d = 1.364$; MDS-supplemented males, $t_{(5)} = 4.927$, $p = 0.008$, $d = 2.016$; vehicle-control males, $t_{(5)} = 7.264$, $p = 0.001$, $d = 2.965$).

Barnes maze

Like the water maze, there were no differences between sex x diet groups in terms of average locomotor speed on the Barnes maze acquisition or reversal trials (2 x 2 ANOVA, data not shown). Localization time (s), rather than locomotor distance, was therefore used to examine learning of the mice during the acquisition and reversal trials, while the time spent in the target quadrant was used to evaluate memory for the former escape box location on probe trials. The repeated measures 2(diet) x 2(sex) x 6(trial) ANOVAs for the acquisition trials confirmed that all groups effectively learned the first escape box location, based on a reduced latency across trials in the acquisition phase (main effect of trial, $F_{(3, 50)} = 7.001$, $p = 0.001$, $\eta^2_g = 0.257$; $t_{(1, 85)} = -5.110$, $p < 0.001$, $r^2_c = 0.235$; data not shown). There were also no differences between groups in terms of the time spent in the target quadrant on the acquisition probe trial, and follow-up (Holm-corrected) one-tailed *t*-tests comparing each group to chance performance (30s/120s or 25%) confirmed that each group was able to recall the 1st platform location at least 24 hours after the last acquisition trial (MDS-supplemented females, $t_{(5)} = 4.090$, $p = 0.009$, $d = 2.004$; vehicle-control females, $t_{(5)} = 4.536$, $p = 0.012$, $d = 1.851$; MDS-supplemented males, $t_{(5)} = 5.244$, $p = 0.007$, $d = 2.141$; vehicle-control males, $t_{(5)} = 4.635$, $p = 0.011$, $d = 1.892$; data not shown).

On the reversal training trials (2nd escape box location), the repeated measures ANOVA revealed a trend towards a sex x trial interaction ($F_{(3, 53)} = 2.533$, $p = 0.065$, $\eta^2_g = 0.102$; data not shown). Subsequent Tukey-corrected pairwise *t*-tests for the effect of sex on each trial determined that males took longer than females to locate the escape box on reversal acquisition trials 1 ($t_{(98)} = 2.199$, $p = 0.030$, $d = 2.004$) and 2 ($t_{(98)} = 1.684$, $p = 0.095$, $d = 0.169$), but not trials 3-6 (trial 3, $t_{(98)} = 1.247$, $p = 0.215$, $d = 0.125$; trial 4, $t_{(98)} = 0.035$, $p = 0.972$, $d = 0.004$; trial 5; $t_{(98)} = 0.786$, $p = 0.434$, $d =$; trial 6, $t_{(98)} = 2.199$, $p = 0.030$, $d = 2.004$). Thus, male 3xTg-AD mice were slower to learn the second escape location than females on the Barnes maze as well as the water maze. On the reversal probe trial, at 24 hours after the last reversal acquisition trial, females spent significantly less time in the target quadrant than males (ANOVA; main effect of sex, $F_{(1, 19)} = 4.454$, $p = 0.048$, $\eta^2_p = 0.190$), indicating a long-term retention deficit in the reversal phase. However, Holm-corrected one-tailed *t*-tests comparing each group to chance performance revealed that only vehicle-control 3xTg-AD females failed to exceed chance levels of 25% of the time spent in the target quadrant on the probe trial (MDS-supplemented females, $t_{(5)} = 4.233$, $p = 0.016$, $d = 2.179$; vehicle-control females, $t_{(5)} = 1.156$, $p = 0.150$, $d = 1.851$; MDS-supplemented males, $t_{(5)} = 4.119$, $p = 0.016$, $d = 2.160$; vehicle-control males, $t_{(5)} = 4.872$, $p = 0.015$, $d = 2.180$; Figure 4). Finally, power analyses confirmed that the sample size was sufficient for these *post hoc t*-tests (n of 5 needed for power of 0.8). Thus, females learned the

second escape location more rapidly than males, while the memory for the 2nd platform location persisted for longer in MDS-females than vehicle-control females.

Spleen and Brain mass

A comparison of the spleen weights between diet, sex, and behavioural testing groups determined that 3xTg-AD males had larger spleens than 3xTg-AD females (factorial permutation test, main effect of sex, $p = 0.003$, $\eta^2_p = 0.178$; Supplementary Figure 1). This sex difference was not observed for the brain weight to body weight ratios, which also did not differ between treatment or behavioural testing conditions (data not shown).

Hippocampal BDNF mRNA

A 2 x 2 x 2 factorial ANOVA of the expression of the neurotrophic factor BDNF in hippocampal tissue revealed no main effects of diet, behavioural testing or sex and no interactions (data not shown). However, when the sex and behavioural testing factors were dropped from the model, hippocampal BDNF was found to be significantly higher in MDS-treated mice vs. vehicle controls ($F_{(1, 41)} = 4.081$, $p = 0.049$, $\eta^2_p = 0.091$; Supplementary Figure 2). Moreover, this diet effect was not seen for the expression of a housekeeping gene, β-actin (data not shown). However, this effect should be replicated in a larger sample, as the power of this test was low (power = 0.403; n = 44 required for power = 0.8). Hippocampal BDNF mRNA levels were also not correlated with performance on the spontaneous alternation test, the Barnes maze, or the water maze (data not shown), perhaps because of the small sample size.

Neurogenesis in the SGZ and SVZ

The level of neurogenesis in the SGZ was estimated by counting the number of DCX-positive immunolabelled cells per 40 µm coronal section of dentate gyrus (sectional quantity), averaged across 21 sampled sections. This average was then multiplied by the total number of sections containing hippocampus to yield an estimate per dentate gyrus. Based on this estimate, females had more DCX+ cells per DG than males (main effect of sex; ($F_{(1, 40)} = 5.390$, $p = 0.025$, $\eta^2_p = 0.119$; Figure 5). There were no effects of the MDS or behavioural testing on SGZ neurogenesis. Since there were many more cells per section in the SVZ than in the SGZ, the number of DCX+ cells in the SVZ were counted for coronal sections which also contained the dorsal or medial regions of the hippocampus (the same sections used for SGZ counts), averaged across 14 sections per mouse. A comparison of these SVZ section averages did not detect any effects of diet, sex, or behavioural testing on neurogenesis in the SVZ (Figure 5). Thus, neurogenesis in the SGZ, but not the SVZ, was higher in 3xTg-AD females than 3xTg-AD males, was increased by the MDS in male mice that were not tested in the battery and was not affected by behavioural testing itself.

SGZ neurogenesis was also negatively correlated with spleen weight ($r_{(48)}$ = -0.289, p = 0.046; Supplementary Figure 3) and positively correlated with brain weight to body weight ratio ($r_{(48)}$ = .403, p = 0.005; Supplementary Figure 4), suggesting that hippocampal neurogenesis is highest in (3xTg-AD) mice with the largest brains and smallest spleens. Lastly, to determine whether levels of SGZ neurogenesis were related to the performance of mice on the spontaneous alternation, Barnes maze or water maze tests, we examined correlations between the number of DCX+ cells per DG and several measures of interest based on the above results: spontaneous alternation rate, the escape time on the first hidden reversal trial of the water maze, localization time on the first reversal trial of the Barnes maze and the time spent in the target quadrant on the reversal probe trial of the Barnes maze. Of these measures, neurogenesis was only found to be (negatively) correlated with escape time on the first water maze reversal trial ($r_{(24)}$ = -.530, p = 0.008; Supplementary Figure 5), consistent with a role for neurogenesis in mitigating proactive memory interference during reversal learning. A summary of the results for Experiment 1 is presented in Table 3.

Discussion

This experiment underscores the potential merit of using dietary supplements to prevent or delay the cognitive decline that occurs in AD, especially as seen in female rodent models. We previously demonstrated that the MDS reduces spatial learning and working memory declines in both sexes of 3xTg-AD mice up to 4 months of age (Hutton et al., 2018). The present study extends these findings by showing that ongoing supplementation with the MDS improves working memory (spontaneous alternation) and long term spatial memory retention (on the delayed probe trial after reversal learning in the Barnes maze) in 3xTg-AD females, but not males, to at least 6-7 months of age. Additional studies with age-matched wild type controls should be conducted to determine the extent to which these benefits restore performance to wild-type levels. Importantly, the spontaneous alternation rates we observed here for MDS-supplemented 3xTg-AD females are similar to those we reported previously for 3-4-month-old WT mice (~60-65%, Hutton et al., 2018). This work also provides some evidence that prolonged supplementation with the MDS (6 months) may increase hippocampal BDNF levels, although this needs to be confirmed in additional trials with a larger sample.

Here we also observed several sex differences which were not present at 4 months of age. In contrast to earlier reports (Oddo et al., 2003; Rodriguez et al., 2008; Carroll et al., 2010), our results are consistent with more recent work (Mastrangelo and Bowers, 2008; Stevens and Brown, 2014; Stover et al., 2015) suggesting that male mice exhibit greater cognitive impairment than females on several measures of learning. We also observed a deficit in hippocampal neurogenesis in males relative to females, associated with greater difficulty in overcoming the interference with a previously acquired response and learning a new spatial location on both the water maze and Barnes maze, although the correlation was significant for the water maze only.

Thus, despite the use of a more challenging protocol on the Barnes maze than the water maze (there were no cued trials for the Barnes maze), this interference effect seems to be more pronounced on the water maze. This disparity may be due in part to the added stress of water maze testing compared to Barnes maze testing (Clinton et al., 2007), increasing the difficulty of overcoming the previously learned escape response in the water maze. Although the correlation between DCX+ cells in the SGZ and behaviour was only significant for reversal on the water maze, these data replicate previous reports (Garthe et al., 2009) of a role for adult hippocampal neurogenesis in mitigating interference (Luu et al., 2012; Winocur et al., 2012) during the encoding of new spatial memories (e.g. during reversal learning).

Interestingly, performance of the animals on the Barnes maze reversal probe trial followed an opposite trend (to that for trials 1-2 of reversal training), where males outperformed females. This suggests that females may have greater neuroplasticity and spatial reversal learning than males (alternatively males could be perseverating); but that the spatial memory of males is better than females once they have learned the second platform location. These seemingly contradictory effects of sex on learning and memory, which have not yet been reported by many others, are consistent with the hypothesis put forward by Frankland et al. (2014) that neurogenesis both facilitates the consolidation of new information during encoding but also the clearance of previously learned information (forgetting), as part of its role in reducing interference and as a component of hippocampal plasticity. The forgetting hypothesis has recently received some support from exercise-based manipulations of neurogenesis prior to or following fear conditioning (Akers et al., 2014), although to our knowledge the current data are the first to show a similar trend on the Barnes maze. An alternative interpretation is that the females, based on their higher levels of neurogenesis, were able to maintain more distinct, separate representations of the previously learned location that interfered less with the learning of the new location.

The negative correlation we found between hippocampal neurogenesis and splenomegaly is also a novel observation which strongly suggests that the reduction in neurogenesis that occurs in this strain (Demars et al., 2010; Hamilton et al., 2010) may be driven in part by autoimmune changes (Marchese et al., 2014). For example, in addition to splenomegaly, we recently found the number of CD3+ T-cells to be severely depleted in 3xTg-AD males, concomitant with increases in circulating autoantibodies (Kapadia et al., submitted; Kapadia et al., 2018). It would therefore be of great interest to determine whether such autoimmune changes or chronic inflammation directly impact neurogenesis levels, and consequently behaviour, in the 3xTg-AD model. This is one area we are actively exploring in an ongoing study. Lastly, the lack of a difference between animals tested using the behavioural battery and cage controls on neurogenesis or BDNF suggests that the test battery itself should not be considered a form of environmental enrichment (known to be a proneurogenic stimulus; Kempermann et al., 1997) and is therefore unlikely to be a confound for future studies which evaluate a combination of behavioural and biological measures in the 3xTg-AD mice.

EXPERIMENT 2

Introduction

The second experiment (see Figure 1) in this study was conducted to evaluate whether supplementation of both 3xTg-AD and B6129SF2/J (the wild-type genetic background strain) mice with the MDS from 2 months of age to 12 months of age would preserve the performance of 3xTg-AD mice on a comprehensive battery of cognitive, sensory and motor tests and normalize hippocampal neurogenesis, BDNF and soluble Aβ at a later stage (12 months) of the 3xTg-AD phenotype. Based on the results of Experiment 1 (above), we hypothesized that there would be a larger benefit of the MDS in female over male mice at 11-12 months of age. The induction of immediate early genes, such as c-Fos or arc, following learning has been shown to be important for the induction of synaptic plasticity during memory encoding in the hippocampus (Guzowski et al., 2001; Snyder et al., 2009). For example, optogenetically silencing CA3 engram cells labelled via arc-coupled channelrhodopsin expression in the hippocampus abolishes contextual fear memory (Niibori et al., 2012). Although hippocampal immediate early gene (c-Fos) expression is deceased in 3xTg-AD mice relative to wild-type controls (Chen et al., 2012; Rodriguez-Ortiz, 2014) in the absence of behavioural training, its expression in the 3xTg-AD mouse hippocampus in response to a relevant learning experience is unknown. Thus, to gain additional insight into whether the hippocampal encoding of spatial memories under conditions of high interference is altered in 3xTg-AD mice, we quantified the number of c-Fos+ cells (Guzowski et al., 2001; Snyder et al., 2009) in the CA1, CA3, and DG subfields of the hippocampus during encoding of the 2nd hidden platform location in the reversal phase of the Morris water maze. Based on evidence that impairments in reversal learning for rats are associated with a reduction in hippocampal c-Fos+ cells (Mendez et al., 2010), we hypothesized that similar deficits in reversal learning and DG c-Fos expression, ameliorated by the MDS, would be observed in 3xTg-AD mice at 11-12 months of age. Motor and olfactory function were also examined, since we expected (based on previous work in wild-type mice; Lemon et al., 2003; Lemon et al., 2016) the MDS to ameliorate age-related declines in these measures, and they are altered in both 3xTg-AD mice (Stover et al., 2015; Marchese et al., 2014; Hutton et al., 2018) and AD patients (Djordjevic et al., 2008; Pettersson et al., 2005). In humans, olfactory impairments also predict the conversion from mild cognitive impairment to AD (Devanand et al., 2000; 2015).

Methods

Animals, MDS supplementation, and behavioural testing

The subjects were 20 male and 19 female B6;129-*Psen1*^{tm1Mpm} Tg(APPSwe,tauP301L) 1Lfa/Mmjax (3xTg-AD) mice, plus 22 male and 21 female mice of the wildtype genetic background B6129SF2/J strain (WT). Mice used in the study were obtained from the same in-

house breeding colony as those used in Experiment 1. They were also housed, tattooed, and maintained as described for Experiment 1. After 5 days of handling (~ 1 min each per day) at approximately 5 weeks of age, mice were tested in a variety of measures of sensory acuity, motor strength/co-ordination, and cognition as well as fear/anxiety using a behavioural battery (Table 2). After this baseline testing, mice of each genotype were randomly assigned to receive the same MDS (Table 1) or vehicle control that were used in Experiment 1, daily from 2-12 months of age. They were then re-tested on the behavioural battery from 3-4 and 11-12 months of age (Figure 1b). The battery used at 11-12 months of age was identical to that used in Experiment 1, It differed from the battery used at 1-2 and 3-4 months of age (Hutton et al., 2018), in that it also included the Barnes maze (exactly as described for Experiment 1). The Barnes maze was used at the last testing age only to control for the potential effects (Billings et al., 2007) of repeated testing on water maze performance (the other spatial learning and memory test in the battery).

Briefly, tests from the battery which were analysed in Experiment 2 in addition to those in Experiment 1 used the following measures of performance: muscle strength was measured as the time to fall, averaged across 2 trials, on the hanging basket test; motor coordination was assessed via two measures, the average time to fall on the rotarod, and the time to cross an elevated narrow plastic beam on the beam walking test; olfactory acuity was measured as the time spent by the mice sniffing different concentrations of peanut butter (PB) in mineral oil solution (0%/control, 0.001%, 0.01%, 0.1%, and 1%); olfactory discrimination was quantified as the change in time spent by the mice from sniffing 0.001% cinnamon (after the fourth consecutive trial with cinnamon as the odorant) to the time spent sniffing filter paper soaked with 0.001% paprika; during INBEST testing, the preference for water sweetened with sucrose (1%) out of the total amount of liquid consumed (unsweetened and sweetened water) over a 10 h monitoring period was used as an indicator of anhedonia; the proportion of the trial time that mice spent interacting with the novel object over the total interaction time for both objects was used to quantify object recognition memory, while rate of alternation on the spontaneous alternation test was used as a measure of working memory; on the Morris water maze and Barnes maze, the locomotor or swimming distance of the mice to escape the maze on the acquisition and reversal training trials was considered a metric of learning a memory within a session (1 h delay between trials), while the time spent in the target quadrant on probe trials (platform or escape box removed) was used as an indicator of 24 h recall for the escape location which had been trained most recently; swim path length on the cued trials of the water maze were also considered to be a measure of visual acuity.

This study compared 8 groups: MDS 3xTg-AD females (AFD; n = 11), vehicle 3xTg-AD females (AFV; n = 9), MDS 3xTg-AD males (AMD; n = 10), vehicle 3xTg-AD males (AMV; n = 10), MDS WT females (WFD; n = 10), vehicle WT females (WFV; n = 11), MDS WT males (WMD; n = 10), vehicle WT males (WMV; n = 12). At the start of the study there were 13 animals per group in all groups except for the 3xTg-AD females (n = 11; due to lack of availability from the breeding colony). The current sample sizes reflect the premature death of 8 3xTg-AD and 9 WT mice at 9-11 months of age, primarily due to ulcerative dermatitis (a common condition in mice with a c57BL/6 background; Blackwell et al., 1995).

Upon completion of motor, sensory and cognitive testing at 12 months of age, all animals were euthanized via exsanguination while under isoflurane anesthesia, and tissue samples were collected and prepared for immunohistochemical and molecular assays as described for Experiment 1. To capture peak c-Fos expression levels associated with performance on the last

hidden platform reversal trial on the Morris water maze, animals were deprived of further visual sensory stimulation (their home cages were covered in blankets after testing) until euthanized 90 minutes (Snyder et al., 2009) following the completion of that trial. Although behavioural data were collected from animals in this experiment at 1-2 and 3-4 months of age, the results of those measures have already been reported elsewhere (Hutton et al., 2018) and therefore will only be briefly summarized here (see Discussion). All procedures employed in this study were approved by the McMaster University Animal Research Ethics Board.

RT-qPCR, ELISA, and immunohistochemistry

RT-qPCR was used to measure BDNF mRNA levels in hippocampal tissue using the same methods and primers as described for Experiment 1. Soluble Aβ levels in cortical tissue were examined using an enzyme-ligated immunosorbent assay (ELISA). Extraction of Aβ closely followed published methods (Michalski et al., 2015). Briefly, frozen cortical samples (~50 mg each) were sonicated in tris-buffered saline (TBS) with protease (cOmplete^{TM} ULTRA Tablets, Mini, EASYpack Protease Inhibitor Cocktail) and phosphatase (PhosSTOP EASYpack, phosphatase inhibitor tablets) inhibitors (Roche, Mississauga, ON, CAN), and then kept on ice for 5-10 minutes. These TBS homogenates were subsequently centrifuged for 20 min at 14,000×g at 4°C and supernatants were collected, aliquoted, and stored at −80°C until use. Aβ protein levels in these supernatant fractions were measured by Chemiluminescent BetaMark x-42 ELISA following the manufacturer's instructions (BioLegend, San Diego, CA, USA). Aβ concentrations were obtained using a MultiskanGO and SkanIt software (Thermo Scientific, Nepean, ON, CAN) at 620 nm. Values are presented as pg Aβ per mg of total protein.

Immunohistochemical assays for DCX in the SGZ were also the same as Experiment 1. In addition, instead of counting DCX+ cells in the SVZ, we also measured the engagement of the CA1, CA3, and DG subfields of the hippocampus during encoding of the 2nd hidden platform location in the reversal phase of the Morris water maze using the expression of the immediate early gene c-fos (Snyder et al., 2009). Immunolabelling of c-Fos was conducted at the same time as immunolabeling of DCX (i.e. sections were co-incubated with primary, then secondary antibodies), and thus slide preparation was identical except for the additional antibodies used (primary, Anti-c-Fos Rabbit-polyclonal Donkey [Millipore]; secondary; Alexa Fluor 568 Anti-Rabbit IgG (H+L) [Life Technologies]). Immunolabeled cells in DG, CA1, and CA3 of the hippocampus were counted using the 40× objective lens of a fluorescence microscope by an observer who was blinded to the experimental conditions of the samples. All c-Fos^{+} cells were counted in the entire 40 µm thick slice by gradually adjusting the focus of the microscope. The counting procedure was verified by at least one independent observer who was also blind to the animal's group assignment. We stained 18 random hippocampal sections per mouse, calculated an average number of c-Fos+ cells per section, then multiplied this average by the total number of HPC sections (there were about 50–60 sections per mouse). This allowed us to estimate the total number of c-fos+ cells in the DG, CA1, and CA3 per hippocampus of each mouse. In addition, black and white images of each immunolabelled section, captured using a Sensicam CCD camera and SensiControl v4.02 software at 4x magnification, were used for the measurement of CA1 and DG area (Image J software; http://rsb.info.nih.gov/ij/). These area measurements were then multiplied by the total number of sections containing hippocampus to yield estimates of CA1 and DG volume per hemisphere, to determine if the pattern of group differences for these hippocampal sub-regions differed from that observed for overall brain size.

Statistical Analyses and Graphics

All measures were analyzed similarly to Experiment 1, with the obvious exception that the factors which were compared in the 2 x 2 x 2 ANOVAs and factorial permutation tests were genotype x sex x diet, not sex x diet x behavioural testing. Since both 3xTg-AD and WT mice were included in the design, we also examined all the data from the behavioural battery (not just anxiety and learning/memory tests), to determine if there were any genotype-related differences in motor function or other measures at 11-12 months of age which were not apparent by 3-4 months of age (Hutton et al., 2018). Body weight was included as a covariate in the analysis of motor test data (beam walking, rotarod, and hanging basket test) since it has been previously found to influence performance on those tests (Stover et al., 2015; Hutton et al., 2018). An alpha level of 0.05, in combination with effect size measures, was used to interpret effects and interactions between groups.

Results

Muscle Strength and Motor Coordination

On the hanging basket test there was a main effect of sex (2 x 2 x 2 factorial permutation test; females > males; $p = 0.009$, $\eta^2_p = 0.080$) and a sex x diet interaction ($p = 0.021$; $\eta^2_p = 0.178$; Supplementary Figure 6). Tukey-corrected follow-up comparisons for the simple main effect of diet in males and females separately determined that MDS-supplemented females held on longer than the vehicle-control females, but there was no difference between treatment groups among males (MDS females vs. vehicle females, $t_{(81)} = 3.125$, $p = 0.002$, $d = 0.345$; MDS males vs. vehicle males, $t_{(81)} = -0.654$, $p = 0.515$, $d = 0.072$). On the rotarod, there were no differences between groups (data not shown), but females were found to take slightly longer than males to cross the beam on the beam walking test (factorial permutation test with body weight as a covariate; main effect of sex, $p = 0.030$, $\eta^2_p = 0.039$; data not shown). The paws of 3xTg-AD mice also slipped more than WT mice on the beam walking test (factorial permutation test, main effect of genotype, $p < 0.001$), suggesting an impairment in co-ordination, but this difference was no longer significant when body weight was included in the model (permutation $p = 0.139$; data not shown). At 11-12 months of age, female mice are therefore stronger but less coordinated than males, while the MDS improves strength in females only.

Olfactory Acuity and Discrimination

A repeated measures ANOVA of time spent sniffing different concentrations of peanut butter revealed a main effect of genotype (AD > WT, $F_{(1, 80)} = 7.276$, $p = 0.009$, $\eta^2_g = 0.035$) and a genotype x concentration interaction ($F_{(4, 307)} = 3.093$, $p = 0.018$, $\eta^2_g = 0.023$; Supplementary

Figure 7). Tukey-corrected pairwise comparisons for the simple main effect of genotype at each concentration level revealed that 3xTg-AD mice spent more time than WT mice interacting with the odorant-soaked filter paper at the lowest and highest concentrations only (0% PB/control, $t_{(324)} = 2.626$, $p = 0.009$, $d = 0.146$; 0.001% PB, $t_{(324)} = -0.117$, $p = 0.907$, $d = 0.006$; 0.01% PB, $t_{(324)} = 1.825$, $p = 0.069$, $d = 0.101$; 0.1% PB, $t_{(324)} = 0.490$, $p = 0.623$, $d = 0.027$; 1% PB, $t_{(324)} = 3.635$, $p < 0.001$, $d = 0.201$). Since there was no peanut butter present at the lowest concentration, we also examined the linear change across concentrations for each genotype and confirmed that sniffing time increased linearly with concentration for both genotypes (AD, $t_{(1, 320)} = 4.966$, $p < 0.001$, $r^2_c = 0.072$; WT, $t_{(1, 320)} = 3.522$, $p < 0.001$, $r^2_c = 0.037$). AD mice therefore expressed more interest in the filter paper than WT mice (especially when it contained 1% PB), while both genotypes spent progressively more time sniffing the filter paper as the concentration of PB increased. On the olfactory discrimination test, when the difference in sniffing time for cinnamon versus paprika was examined, there were no main effects or interactions, indicating 3xTg-AD mice of both sexes and in both treatment groups can equally distinguish between 0.001% cinnamon and 0.001% paprika at 11-12 months of age (data not shown).

Anxiety-Like Behaviour and Anhedonia

On the elevated plus maze, 3xTg-AD mice spent less time in the open arms than WT mice (main effect of genotype, permutation $p < 0.001$, $\eta^2_p = 0.310$), but there were no differences between sexes or treatment groups (see Supplementary Figure 8). In the open field, there were no main effects or interactions among factors for the time spent in the centre zone, although 3xTg-AD mice did take longer to approach the centre (permutation $p < 0.001$, $\eta^2_p = 0.121$), visited it less frequently (permutation $p = 0.027$, $\eta^2_p = 0.059$), excreted more fecal boli ($F_{(1, 81)} = 10.346$, $p = 0.002$, $\eta^2_p = 0.110$), spent more time frozen (permutation $p < 0.001$, $\eta^2_p = 0.215$), and froze more often ($F_{(1, 81)} = 25.050$, $p < 0.001$, $\eta^2_p = 0.236$) than WT mice (Supplementary Figure 9).

There were also genotype x sex interactions for freezing time (permutation $p = 0.006$, $\eta^2_p = 0.089$) and frequency ($F_{(1, 81)} = 6.677$, $p = 0.012$, $\eta^2_p = 0.076$), but no other effects or interactions for these measures (Supplementary Figure 9). Follow-up comparisons for the simple main effect of genotype for each sex revealed that 3xTg-AD females, but not males, froze for longer (AD females vs. WT females, permutation $p = 0.002$; AD males vs. WT males, permutation $p = 0.081$) and more often (AD females vs. WT females, $t_{(40)} = 5.867$, $p < 0.001$; AD males vs. WT males, $t_{(45)} = 1.652$, $p = 0.106$) than their WT counterparts. 3xTg-AD mice (especially females) therefore exhibit robust anxiety-like behaviour at 11-12 months of age, which was evident on all elevated plus maze and open field measures except for the time spent in the centre zone of the open field. Moreover, time spent on the open arms of the elevated plus maze is the most sensitive of these measures for detecting the genotype difference according to the observed effect sizes ($\eta^2_p = 0.310$).

A comparison of groups' sucrose preference, a measure of anhedonia, using a 2 x 2 x 2 factorial permutation test, did not reveal any significant main effects or interactions, indicating that neither male nor female 3xTg-AD mice exhibit anhedonia (data not shown).

Object Recognition Memory and Working Memory

An ANOVA of novel object preference scores did not uncover any main effects or interactions. Working memory scores, as indexed by the average alternation rate across trials in the spontaneous alternation test, also did not differ between groups, suggesting that neither object recognition memory nor visuospatial working memory differed between groups at 11-12 months of age (data not shown).

Visuospatial Learning and Memory

Water Maze

There were differences between 3xTg AD mice and WT mice in terms of swimming speed on the cued trials ($F_{(5, 365)} = 8.571$, $p = 0.005$, $\eta^2_g = 0.012$; data not shown), so swim path length to locating the escape platform was used as a measure of performance on the water maze instead of escape time. A repeated measures ANOVA of distance swam on the cued trials did not uncover any effects or interactions (data not shown), demonstrating that the visual acuity of each group was similar. On the hidden platform acquisition trials, there was a main effect of trial (general improvement across trials; $F_{(8, 584)} = 4.050$, $p < 0.001$, $\eta^2_g = 0.048$) and a trial x genotype interaction ($F_{(8, 584)} = 4.050$, $p < 0.001$, $\eta^2_g = 0.048$), but no other significant effects or interactions. Follow-up Holm-corrected pairwise t-tests for the simple main effect of genotype for each trial revealed that 3xTg-AD mice swam farther than WT mice while searching for the hidden platform on the 1st and 7th trials, but WT swam farther than 3xTg-AD mice before escaping on the 10th trial (trial 1, $t_{(1150)} = 3.041$, $p = 0.002$; trial 7, $t_{(1150)} = 1.969$, $p = 0.049$; trial 10, $t_{(1150)} = -2.252$, $p = 0.025$; Supplementary Figure 10). With respect to long term memory for the 1st platform location, on 24-hour delayed probe trials, WT mice spent more time in the target quadrant than 3xTg-AD mice (main effect of genotype, $F_{(1, 79)} = 4.213$, $p = 0.043$, $\eta^2_p = 0.051$; Supplementary Figure 11). The ANOVA also detected a diet x sex interaction ($F_{(1, 79)} = 4.076$, $p = 0.047$, $\eta^2_p = 0.049$; Supplementary Figure 11). Subsequent examination of the simple main effect of diet in males and females separately revealed that MDS-supplemented females spent less time in the target quadrant than vehicle-control females ($t_{(40)} = -2.978$, $p = 0.005$), while there was no treatment effect in males ($t_{(43)} = -0.198$, $p = 0.844$). However, Holm-corrected one tailed *t*-tests comparing the performance of each group to chance (>30s/120s or 25% of the trial) suggested that all groups, including MDS-supplemented females, could still recall the trained platform location at above-chance levels (AFD, $t_{(10)} = 4.216$, $p = 0.002$; AFV, $t_{(8)} = 7.864$, $p <$

0.001; AMD, $t_{(10)} = 5.506$, $p < 0.001$; AMV, $t_{(10)} = 7.653$, $p < 0.001$; WFD, $t_{(9)} = 4.363$, $p = 0.002$; WFV, $t_{(11)} = 12.826$, $p < 0.001$; WMD, $t_{(10)} = 8.154$, $p < 0.001$; WMV, $t_{(11)} = 6.413$, $p < 0.001$).

On the hidden platform reversal trials, as was observed for acquisition, there was a main effect of trial (general improvement across trials; $F_{(3, 211)} = 9.459$, $p < 0.001$, $\eta^2_g = 0.066$) and a genotype x trial interaction ($F_{(3, 211)} = 3.786$, $p = 0.014$, $\eta^2_g = 0.027$; Supplementary Figure 10). An analysis of the simple main effect of genotype for each trial revealed that 3xTg-AD mice swam farther than WT mice when trying to locate the hidden platform on the 1st reversal trial only (trial 1, $t_{(270)} = -2.600$, $p = 0.010$; trial 2, $t_{(270)} = -0.945$, $p = 0.346$; trial 3, $t_{(270)} = 1.015$, $p = 0.311$; trial 4, $t_{(270)} = 0.877$, $p = 0.381$). In contrast to the acquisition and probe trials, there was also a trend towards a sex x diet interaction ($F_{(1, 78)} = 2.844$, $p = 0.093$, $\eta^2_g = 0.015$; Supplementary Figure 10). Holm-corrected one-tailed tests for the simple (beneficial) main effect of the MDS in females and males separately determined that MDS-supplemented females marginally outperformed vehicle-control females ($t_{(165)} = -1.973$, $p = 0.050$ [unadjusted $p = 0.025$]), while this benefit of the MDS was not observed for males ($t_{(178)} = 1.149$, $p = 0.874$). Thus, 3xTg-AD mice exhibit an overall impairment in learning and remembering both platform locations. In addition, MDS-supplemented females exhibited impaired long-term retention for the 1st platform location but were able to learn the 2nd location marginally faster than vehicle-control females.

Barnes maze

Across acquisition trials in the Barnes maze there was the expected reduction (improvement) in locomotor distance before locating the escape box for all groups (repeated-measures ANOVA, main effect of trial, $F_{(4, 308)} = 8.026$, $p < 0.001$, $\eta^2_g = 0.076$). 3xTg-AD males, but not females, were also impaired relative to the WT control group (genotype x sex interaction, $F_{(1, 77)} = 4.134$, $p = 0.045$, $\eta^2_g = 0.011$; simple main effect of genotype among: males, $t_{(77)} = 2.758$, $p = 0.007$; females, $t_{(77)} = -0.197$, $p = 0.844$; data not shown). On the 24-hour delayed acquisition probe trial, the 2 x 2 x 2 ANOVA did not reveal any significant main effects or interactions, and Holm-corrected one-way *t*-tests confirmed that all groups spent more than 25% of the trial in the target quadrant (AFD, $t_{(10)} = 6.181$, $p < 0.001$; AFV, $t_{(8)} = 4.051$, $p = 0.009$; AMD, $t_{(9)} = 3.634$, $p = 0.011$; AMV, $t_{(10)} = 3.133$, $p = 0.015$; WFD, $t_{(9)} = 3.494$, $p = 0.015$; WFV, $t_{(11)} = 5.794$, $p < 0.001$; WMD, $t_{(10)} = 2.155$, $p = 0.028$; WMV, $t_{(11)} = 4.676$, $p = 0.002$; data not shown).

On the reversal trials, 3xTg-AD mice of both sexes were impaired relative to the WT strain (main effect of genotype, $F_{(1, 75)} = 4.118$, $p = 0.046$, $\eta^2_g = 0.024$; data not shown), but all groups exhibited the expected improvement across trials (main effect of trial, $F_{(4, 305)} = 5.021$, $p = 0.001$, $\eta^2_g = 0.036$). Although a factorial permutation test did not reveal any significant main

effects or interactions on the probe trial for the reversal escape box location, Holm-corrected one-way *t*-tests revealed that only vehicle-control 3xTg-AD females failed to spend more than chance level (25% of the trial) time in the target quadrant (AFD, $t_{(10)} = 4.149$, $p = 0.003$; AFV, $t_{(8)} = 1.619$, $p = 0.075$; AMD, $t_{(9)} = 3.634$, $p = 0.011$; AMV, $t_{(10)} = 3.133$, $p = 0.015$; WFD, $t_{(9)} = 3.494$, $p = 0.015$; WFV, $t_{(11)} = 5.794$, $p < 0.001$; WMD, $t_{(10)} = 2.155$, $p = 0.028$; WMV, $t_{(11)} = 4.676$, $p = 0.002$; Figure 6). Taken together, on the Barnes maze 11-12 month old 3xTg-AD males (not females) exhibit impaired learning but no long term memory deficit during training for the 1st escape box location, both sexes of 3xTg-AD mice have greater difficulty learning the 2nd escape box location, and the MDS preserves the long term memory of 3xTg-AD females on the probe trial for the 2nd location.

Spleen, Whole Brain, and Hippocampal Size

Examination of the spleen weights (Figure 7) in a factorial permutation test revealed that the spleen was larger in 3xTg-AD mice than WT mice (main effect of genotype, $p = 0.001$, $\eta^2_p = 0.302$) and larger in males than females (main effect of sex; $p = 0.014$, $\eta^2_p = 0.067$). There was also a sex x genotype interaction ($p = 0.013$, $\eta^2_p = 0.069$) and a trend towards a genotype x diet interaction ($p = 0.084$, $\eta^2_p = 0.037$). Follow-up two-tailed permutation tests determined that the sex effect was only present in the 3xTg-AD strain (males > females; AD, $p = 0.016$; WT, $p > 0.999$), while one-tailed tests evaluating the beneficial simple main effect of diet in each genotype revealed that the MDS attenuated the splenomegaly seen in 3xTg-AD mice ($p = 0.030$) but did not reduce spleen size in WT mice ($p = 0.666$).

With respect to brain size, brain weight to body weight ratios were smaller in 3xTg-AD mice than WT mice (ANOVA, main effect of genotype, $F_{(1, 79)} = 26.030$, $p < 0.001$, $\eta^2_p = 0.248$), and smaller in males than females (main effect of sex, $F_{(1, 79)} = 10.929$, $p = 0.001$, $\eta^2_p = 0.123$). However, there was also a genotype x sex interaction ($F_{(1, 79)} = 15.646$, $p < 0.001$, $\eta^2_p = 0.165$; Supplementary Figure 12), and simple main effects analysis determined that the brains of 3xTg-AD females were smaller than WT females ($t_{(79)} = -6.282$, $p < 0.001$), but there was no strain difference between males ($t_{(79)} = -0.827$, $p = 0.411$). Within the hippocampus, the volume of CA1 (main effect of genotype, permutation $p < 0.001$, $\eta^2_p = 0.457$) and the DG (main effect of genotype, permutation $p < 0.001$, $\eta^2_p = 0.542$) were only smaller in 3xTg-AD mice relative to the WT control strain (Supplementary Figure 13) and did not differ between males and females or between treatment groups (data not shown). Thus, 3xTg-AD males have larger spleens than WT males, 3xTg-AD females have smaller brains than WT females, and both sexes of 3xTg-AD mice have smaller hippocampi than their WT counterparts. The MDS may also attenuate the splenomegaly seen in 3xTg-AD males.

Cortical Aβ

The amount of TBS-soluble Aβ was used as a measure of Aβ burden present in cortical tissue. 3xTg-AD mice had higher levels of TBS-soluble Aβ than WT mice (main effect of genotype; permutation $p = 0.004$, $\eta^2_p = 0.101$; Supplementary Figure 14), but there were no differences between sexes or treatment groups (data not shown).

Hippocampal BDNF, neurogenesis, and c-Fos expression

Examination of BDNF mRNA levels in hippocampal tissue did not detect a difference between genotypes, but did reveal a sex x diet interaction ($F_{(1, 79)} = 4.753$, $p = 0.032$, $\eta^2_p = 0.054$). Follow-up tests for the simple main effect of diet in each sex determined that there was a beneficial effect of the MDS on hippocampal BDNF mRNA in females ($t_{(79)} = 2.265$, $p = 0.026$) but not males ($t_{(79)} = -0.790$, $p = 0.432$; Figure 8). No such interaction was observed for the number of DCX+ cells in the SGZ, where hippocampal neurogenesis was depleted only in 3xTg-AD mice relative to the WT strain (main effect of genotype, permutation $p < 0.001$, $\eta^2_p = 0.655$; Figure 9). When the number of c-Fos+ cells in DG, CA1, and CA3 were examined, however, a different pattern emerged. In the DG, vehicle-control males had more c-Fos+ neurons than MDS-supplemented males, while there was no difference between female treatment groups (sex x diet interaction; permutation $p = 0.019$; simple main effect of diet in males, $p = 0.033$; simple main effect of diet in females, $p = 0.341$). In CA1 and CA3, there were genotype x sex x diet interactions for the quantity of c-Fos+ cells (CA1, permutation $p = 0.026$, $\eta^2_p = 0.045$; CA3, permutation $p = 0.039$, $\eta^2_p = 0.062$), and subsequent permutation tests for the simple main effect of diet in each sex x genotype group revealed that vehicle-control (but not MDS-supplemented) 3xTg-AD males exhibited an abnormal increase in the number of c-Fos immunoreactive cells in both CA1 (AMD vs. AMV, $p = 0.001$; AFD vs. AFV, $p = 0.486$; WMD vs. WMV, $p = 0.568$; WFD vs. WFV, $p > 0.999$) and CA3 (AMD vs. AMV, $p = 0.004$; AFD vs. AFV, $p = 0.256$; WMD vs. WMV, $p = 0.536$; WFD vs. WFV, $p > 0.999$; Figure 10) in response to water maze reversal training. Alternatively, when the simple main effect of genotype for each male treatment group was examined instead, it was also significant for vehicle-control males only in both CA1 (AMV vs. WMV, $p = 0.004$; AMD vs. WMD, $p = 0.926$). and CA3 (AMV vs. WMV, $p = 0.027$; AMD vs. WMD, $p = 0.926$), confirming that vehicle control, but not MDS-supplemented 3xTg-AD males recruited more neurons than WT controls in these two structures. A summary of the results for Experiment 2 is presented in Table 4.

Discussion

Here we report, for the first time, that vehicle-treated 11-12-month-old 3xTg-AD male, but not female, mice exhibit an abnormal pattern of neuronal activation across hippocampal sub-

regions (as assessed by upregulation of the expression of the immediate early gene [IEG] c-Fos) after water maze reversal learning, relative to age-matched WT mice. Specifically, whereas wild type mice predominantly activated the dentate gyrus after reversal learning, 3xTg-AD males predominantly activated the CA3 and CA1 sub-regions. This finding differs from previous reports of a decrease in overall hippocampal c-Fos expression in the hippocampus of 3xTg-AD mice (Chen et al., 2012; Rodriguez-Ortiz, 2014), however, in those studies the level of c-Fos was not measured after behavioural training as it was here, nor was it assessed separately within each hippocampal sub-region. That there were no behavioural differences between groups on the last reversal trial of the water maze, which triggered the activation of these cells, suggests that this abnormal IEG upregulation in CA1 and CA3 by 3xTg-AD males may be a compensatory mechanism that enables them to perform as well as the other groups. Moreover, the additional engagement of CA1 and CA3 was prevented in the MDS-supplemented group, suggesting that the MDS may improve the efficiency of visuospatial encoding by the hippocampus in 11-12-month-old 3xTg-AD males. However, optogenetic labelling of these cells (e.g. Rashid et al., 2016) will need to be done in future studies to confirm their involvement in water maze engram formation.

The MDS also attenuated the splenomegaly which was observed in both sexes of the 3xTg-AD strain. It is worth noting that this autoimmune feature was worse in 3xTg-AD males than females, consistent with previous findings (Kapadia et al., 2018). In 3xTg-AD mice, this splenomegaly has also been associated with alterations in T-splenocyte populations (Kapadia et al., 2018) and an increase in pro-inflammatory cytokines (Yang et al., 2015), suggesting that the beneficial impact of the MDS on spleen weights in the present study may be driven in part by the anti-inflammatory actions of the MDS (Lemon et al., 2003; Lemon et al., 2008). The splenomegaly and other autoimmune manifestations (Marchese et al., 2014; Kapadia et al., 2018) in the 3xTg-AD model may partly be responsible for the anxiety-like behaviour we observed in both sexes of these mice, despite a lack of an effect of the MDS on anxiety in the present study, since both appear very early in their lifespan, prior to when we began treatment (before 2 months of age; Hutton et al., 2018; personal observations by B.S., M.K., and C.P.H.). For example, altered splenocyte function has been associated with anxiety-like behaviour in WT mouse strains (Kinsey et al., 2007), and treatment with the anti-inflammatory antibiotic minocycline prevents the migration of peripheral leukocytes to the brain and subsequent expression of anxiety-like behaviour in mice following chronic stress exposure (Jarrett et al., 2014). In further support of this, pro-inflammatory cytokines or immune challenge (via lipopolysaccharide injections) decrease mRNA levels of BDNF and other neurotrophins (NGF, NT-3) in the rat hippocampus (Guan & Fang, 2006). The MDS also upregulated hippocampal BDNF mRNA levels in both sexes of 3xTg-AD mice at 6-7 months of age (Experiment 1) and females at 11-12 months of age (Experiment 2). The fact that the MDS was able to increase hippocampal BDNF expression only in females and not in males at 11-12 months of age may be due to the greater degree of immunopathology in males than females at this age (Kapadia et al., 2018), which may exceed the anti-inflammatory capacity of the MDS at the dose used here. Alternatively, considering that

BDNF was also upregulated by the MDS in 11-12 month old WT females, there may be a synergistic interaction between the MDS and estrogen, which is an established up-regulator of BDNF levels in the cortex and hippocampus of rats (Singh et al., 1995). In support of this, one of the flavonoids in the MDS (rutin) was recently shown to increase BDNF levels in rat hippocampus, an effect that was partially blocked by an estrogen receptor antagonist (Liu et al., 2018). These possibilities both merit further investigation, particularly since inflammation is implicated in the conversion from mild cognitive impairment to AD in humans (Tarkowski et al., 2003), and the post-menopausal decline in estrogen in women has been proposed as an explanation for the higher prevalence of AD among women than men (Mielke et al., 2014).

Unexpectedly, we did not observe a downregulation of BDNF in the 3xTg-AD brain, in contrast to previous findings in this strain (e.g. Caccammo et al., 2010; Kapadia et al., 2018). This discrepancy may be due to methodological differences such as assaying BDNF levels in cortical or whole brain homogenates (Walker et al., 2013; Castello et al., 2012; Kapadia et al., 2018), examining tissue from mice at different ages (e.g. 6 months of age; Caccamo et al., 2010), and/or examining BDNF protein (Blurton-Jones et al., 2009; Caccamo et al., 2010) rather than mRNA levels as was done in the present study. It is also possible that by 11-12 months of age there is no longer a significant difference in hippocampal BDNF expression between strains, since a decrease in the 3xTg-AD strain was reported at 6 months of age (Caccamo et al., 2010), but not at 24 months of age (Castello et al., 2012). This suggests that 3xTg-AD mice may exhibit acceleration of the normal age-related decline in BDNF signalling (Karege et al., 2002; Hayashi et al., 2001; Lommatzsch et al., 2005; Webster et al., 2006). Whether or not this decline occurs more rapidly in the hippocampus than the cortex of 3xTg-AD mice or human AD patients remains to be determined and warrants further investigation.

Although we have previously shown that the MDS can fully preserve working memory (spontaneous alternation) and spatial learning (water maze reversal) in both sexes of 3xTg-AD mice at 3-4 months of age (Hutton et al., 2018), the data presented here clearly show that some behavioural benefits persist in females, but not males, by 6-7 and 11-12 months of age, perhaps due to the aforementioned (possible) interaction between the MDS, estrogen, and BDNF. For example, the MDS rescued performance of 11-12-month-old 3xTg-AD females on the probe trial for the 2nd escape location on the Barnes maze, in contrast to the vehicle-control group who were at chance, suggesting a long-term benefit of the MDS on spatial memory. Supporting this conclusion, there was a trend towards an improvement on the water maze reversal trials among MDS-supplemented females, but not males. Importantly, the benefits of the MDS are more limited at 11-12 months of age in the 3xTg-AD mouse model, in contrast to the wide-ranging benefits observed in older WT mice (e.g. Aksenov et al., 2013; Lemon et al., 2016). Specifically, at 11-12 months of age, the MDS did not maintain the performance of 3xTg-AD mice at WT levels on the acquisition training portion of the water maze (1st hidden platform location), the reversal training on the Barnes maze (in both sexes) or water maze (males only), and even appeared to have a detrimental effect on the water maze probe trial in females. The fact that this

impairment was not observed on the probe trials for the Barnes maze suggests that there may be an interaction between the MDS and glucocorticoid signalling, based upon the previously mentioned evidence that water maze testing evokes a larger stress response in female than male 3xTg-AD mice (Clinton et al., 2007). This result does warrant caution in interpreting future translational studies of similar polyphenol-based complex supplements in preclinical AD models or humans, particularly when cognitive evaluations are conducted under stressful conditions. For instance, age-related stereotype threat (pertaining to memory decline) can negatively bias the performance of older adults, exaggerating age differences in memory tests (Rahhal et al., 2001) and increasing the risk of misdiagnosis during predementia screening (Mazerolle et al., 2016). We have also found that the MDS is much more effective at improving hippocampal function in chronically stressed WT mice when combined with aerobic exercise (Hutton et al., 2015), suggesting that such a combination would likely yield greater benefits in an AD model as well.

Consistent with our previously published findings in these mice at 3-4 months of age (Hutton et al., 2018), 3xTg-AD mice at 11-12 months of age showed elevated anxiety-like behaviour that was not reversed by the MDS. Importantly, this elevated anxiety behaviour was exhibited by 3xTg-AD mice beginning at 1-2 months of age, prior to the start of treatment (2 months of age). This suggests that the MDS or other similar supplements (e.g. Parachikova et al., 2010) are more likely to be effective as preventative interventions for AD than for anxiety disorders. Lastly, we did observe several biological changes in the 3xTg-AD mice that were not affected by the MDS but are consistent with previous reports, namely, an increase in soluble Aβ in the cortex (Kapadia et al., 2018), as well as severely depleted levels of SGZ neurogenesis (Demars et al., 2010), and reduced overall brain size and hippocampal volume (in APP/PS1 AD model mice; Breyhan et al., 2009). These features are consistent with human AD (e.g. Fox et al., 1996; Michalski et al., 2015; Briley et al., 2016), providing further support for the validity of the 3xTg-AD mouse as an AD model. The fact that some behavioural benefits of the MDS were still observed in the present study without affecting these features is remarkable, supporting other evidence that synaptic changes (Terry et al., 1991) and neurotrophic factors such as BDNF which promote plasticity (Peng et al., 2005) may be more fruitful therapeutic targets in AD research than Aβ or tau (e.g. Cummings et al., 2014).

GENERAL DISCUSSION

Together, these results not only show that long term treatment with the MDS can delay the progression of several symptoms in the 3xTg-AD model (working memory, learning and delayed recall under conditions of high interference), but also provide further evidence that sex differences in AD models cannot be ignored. For example, while others have reported different trajectories for male and female 3xTg-AD mice in terms of Aβ accumulation (Carroll et al., 2010) and some behavioural differences (e.g. Clinton et al., 2007; Stover et al., 2015). it was previously unclear how the behavioural decline across a wide range of measures diverges

between males and females after initial presentation. This was mainly because most other studies on the 3xTg-AD mouse that have examined behaviour beginning at 6 months of age or older (Sternickzuk et al., 2010; Fillai et al., 2012) did not use more than one behavioural test (Billings et al., 2005; Stevens & Brown, 2015), used only one sex (Davis et al., 2013; Marchese et al., 2014), or reported conflicting differences between sexes (Clinton et al., 2007; Stevens & Brown, 2015). In the present study, the use of a comprehensive battery of tests, testing at multiple age points (1-2, 3-4, 6-7, and 11-12 months of age) and both sexes of 3xTg-AD mice has enabled us to clarify the behavioural phenotype of this strain.

Previous studies have suggested that 3xTg-AD mice are not impaired prior to 2 months of age (e.g. Oddo et al., 2003; Billings et al., 2005) and begin to exhibit anxiety-like behaviour at 2-4 months of age (males; Marchese et al., 2014; Davis et al., 2017) or 4-6 months of age (females; Pietropaolo et al., 2014; Garcia-Mesa et al., 2011). Others have consistently shown that learning and memory impairments begin at around 3-6 months of age in both sexes (Attar et al., 2013; Marchese et al., 2014; Davis et al., 2013; Billings et al., 2005; Billings et al., 2007; Stevens & Brown, 2014; Stover et al., 2015; Clinton et al., 2007). However, females are more impaired on the water maze and inhibitory avoidance task than males from 4-12 months of age (Clinton et al., 2007), while males perform worse than females on the 8-arm radial maze from 2-15 months of age (Stevens & Brown, 2014). Object recognition memory and motor function is impaired in both sexes at 4 months of age (Garcia-Mesa et al., 2011) and at 9-12 months of age (Clinton et al., 2007; Marchese et al., 2014). Deficits in working memory (spontaneous alternation) occur starting a bit later at 6-8 months of age (Marchese et al., 2014; Davis et al., 2017; Carroll et al., 2010), with males initially performing worse than females and then vice versa by 12-14 months of age (Carroll et al., 2010). Reductions in exploratory behaviour (Garcia-Mesa et al., 2011), motor co-ordination (Garcia-Mesa et al., 2011; Stover et al., 2015) and olfactory function (Marchese et al., 2014; Roddik et al., 2016) have also been reported starting at around 4-6 months of age.

Our results provide additional insight into the behavioural decline of 3xTg-AD mice, in that anxiety and olfactory alterations (females only) were previously observed to appear first (1-2 months of age), followed by impairments in object recognition, working memory, and reversal (high interference) learning at 3-4 months of age in both sexes (Hutton et al., 2018). Furthermore, in the present report, we show that after 3-4 months of age, males and females diverge, with females performing worse on tests of working memory and long term spatial memory, and males scoring worse on measures of learning at 6-7 months of age. However, by 11-12 months of age the behavioural alterations of both sexes converge again, in that they exhibit increased olfactory sensitivity, impairments in spatial learning under low and high interference conditions, anxiety-like behaviour, but no impairment in object recognition memory. The one exception to this is a greater impairment in 3xTg-AD females than males on the water maze and Barnes maze probe trials. With respect to the novel object test results, we previously reported that the preference of WT, but not 3xTg-AD, mice for the novel object increases from 1-2 to 3-4

months of age (Hutton et al., 2018), suggesting that novel object preference may increase in youth in the WT mice and then decline again with age, rather than declining more rapidly in 3xTg-AD mice. Thus, our data support the work of others (Stevens & Brown, 2014; Stover et al., 2015) indicating that cognitive flexibility measures such as spatial reversal learning and spontaneous alternation are more sensitive at differentiating between these two strains than other memory tests like the novel object. We did also detect some motor impairments in the 3xTg-AD strain (e.g. beam walking test), but these differences were no longer significant after accounting for differences in body weight, indicating that motor alterations may not be a reliable feature of the disease phenotype in this model prior to 12 months of age.

This phenotype mirrors the progression of human AD in some ways and less so in others. For example, with respect to sex differences, it has been estimated that there are 50% more women than men with AD, while the opposite is true for mild cognitive impairment (Mielke et al., 2014). Unfortunately, the trajectory of decline in specific cognitive domains remains incompletely understood, but in general, women appear to be at greater risk of MCI (prodromal for AD; Morris et al., 2001) than men (at ages > 80), while men (irrespective of age) consistently present with the non-amnestic form of MCI (affecting domains like executive function and visuospatial ability; prodromal for non-AD dementias) more often than women (Mielke et al., 2014). In humans, MCI is mainly characterized by impairments in prospective, semantic, and/or episodic memory, with lesser deficits in executive function or visuospatial ability (Morris et al., 2001). Like the 3xTg-AD mouse, MCI patients are also impaired on tests of memory under conditions of high interference between stimuli (Ally et al., 2013).

MCI patients commonly also exhibit several behavioural alterations beyond the expected memory impairments. These symptoms can include (Mega et al., 1996): apathy (72% of AD patients), agitation/aggression (60% of patients), irritability (42 % of patients), dysphoria (38% of patients), aberrant motor function (38% of patients), disinhibition/impulsivity (36 % of patients), delusions (22 % of patients), and hallucinations (10 % of patients). While not present in all cases, symptoms of anxiety or depression (Palmer et al., 2007; Donovan et al., 2015), impairments in olfactory function (Devanand et al., 2000; Djordjevic et al., 2008) and deficits in fine/complex motor co-ordination (e.g. Perdue pegboard test; Kluger et al., 1997) can each distinguish individuals with MCI from unimpaired older adults (or AD patients) and may predict conversion from MCI to AD. Once the disease has progressed to AD, the symptoms which were present in MCI generally become worse, and patients gradually develop additional symptoms such as impairments in gross motor co-ordination (e.g. timed up and go; Kluger et al., 1997; Pettersson et al., 2005), language (Morris et al., 2001), working memory (Morris et al., 2001), copying (mirroring) behaviour (Morris et al., 2001), irritability (Mega et al., 1996), hallucinations/delusions (Mega et al., 1996), aggression, and impulsivity (Bidzan et al., 2012). The relative timing or relevance of such additional behavioural symptoms in the progression of MCI to AD is obviously of great interest from a diagnostic standpoint, yet presently remains incompletely understood and should be a priority of future AD research. Considered together, of

the cognitive symptoms which can be measured in mice, the anxiety-like behaviour, olfactory alterations, learning, and memory impairments exhibited by the 3xTg-AD mouse in the present study further support its role as an excellent model of AD.

ACKNOWLEDGEMENTS

We would like to thank undergraduate students J. Tran, M. Lysenko-Martin, Z. Patel, N. Ramseyer, M. Kryzewska, G. Kaur, N. Elsaadawy, D. Chok, H. Shahid, M. Campbell, L. Ribeiro, L. Babatinca, K. Thomas, B. Terpou, A. Mizzi, M. Radenovic and I. Perera for their substantial contributions to this study, including behavioral testing, scoring of behavioral video recordings, and administration of the dietary supplement. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to S.B. (RGPIN-2014-03580), D.R.B. (RGPIN 238495-10), and J.M.W. (RGPIN 194616-11). It was also supported by a grant from the Canadian Institutes of Health Research to J.M.W. (MOP 11927), grants from the Alzheimer's Society of Canada (grant #17-04) and the Canadian Consortium on Neurodegeneration in Aging Team 2 to M.F., and an NSERC Canada Graduate Scholarship to C.H.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

REFERENCES

Aizenstein, H. J., Nebes, R. D., Saxton, J. A., Price, J. C., Mathis, C. A., Tsopelas, N. D., … Klunk, W. E. (2008). Frequent amyloid deposition without significant cognitive impairment among the elderly. *Archives of Neurology*, *65*(11), 1509–1517.

Akers, K.G., Martinez-Canabal, A., Restivo, L., Yiu, A.P., De Cristofaro, A., Hsiang, H.-L., Wheeler, A. L., Guskjolen, A., Niibori, Y., Shoji, H., Ohira, K., Richards, B.A., Miyakawa, T., Josselyn, S. A., Frankland, P.W. (2014) Hippocampal neurogenesis regulates forgetting during adulthood and infancy. Science, 344:598–602.

Aksenov, V., Long, J., Lokuge, S., Foster, J. A., Liu, J., & Rollo, C. D. (2010). Dietary amelioration of locomotor, neurotransmitter and mitochondrial aging. *Experimental Biology and Medicine*, *235*(1), 66-76.

Aksenov, V., Long, J., Liu, J., Szechtman, H., Khanna, P., Matravadia, S., & Rollo, C. D. (2013). A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. *Age (Dordrecht, Netherlands)*, *35*(1), 23 33.

Ally, B. A., Hussey, E. P., Ko, P. C., & Molitor, R. J. (2013). Pattern separation and pattern completion in Alzheimer's disease: evidence of rapid forgetting in amnestic mild cognitive impairment. *Hippocampus*, *23*(12), 1246-1258.

Anderson, M., & Braak, C. T. (2003). Permutation tests for multi-factorial analysis of variance. *Journal of statistical computation and simulation*, *73*(2), 85-113.

Attar, A., Liu, T., Chan, W. T. C., Hayes, J., Nejad, M., Lei, K., & Bitan, G. (2013). A shortened Barnes maze protocol reveals memory deficits at 4-months of age in the triple-transgenic mouse model of Alzheimer's disease. *PloS one*, *8*(11), e80355.

Bakeman, R. (2005). Recommended effect size statistics for repeated measures designs. *Behavior research methods*, *37*(3), 379-384.

Barnes, C. A. (1979). Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. *Journal of comparative and physiological psychology*, *93*(1), 74.

Benjamini, Y., & Braun, H. (2002). John Tukey's contributions to multiple comparisons. *ETS Research Report Series*, *2002*(2).

Bidzan, L., Bidzan, M., & Pąchalska, M. (2012). Aggressive and impulsive behavior in Alzheimer’s disease and progression of dementia. *Medical science monitor: international medical journal of experimental and clinical research*, *18*(3), CR182.

Billings, L. M., Green, K. N., McGaugh, J. L., & LaFerla, F. M. (2007). Learning decreases Aβ*56 and tau pathology and ameliorates behavioral decline in 3xTg-AD mice. *Journal of Neuroscience*, *27*(4), 751-761.

Billings, L. M., Oddo, S., Green, K. N., McGaugh, J. L., & LaFerla, F. M. (2005). Intraneuronal Aβ causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. *Neuron*, *45*(5), 675-688.

Blackwell, B. N., Bucci, T. J., Hart, R. W., & Turturro, A. (1995). Longevity, body weight, and neoplasia in ad libitum-fed and diet-restricted C57BL6 mice fed NIH-31 open formula diet. *Toxicologic pathology*, *23*(5), 570-582.

Blanchard, J., Wanka, L., Tung, Y. C., del Carmen Cárdenas-Aguayo, M., LaFerla, F. M., Iqbal, K., & Grundke-Iqbal, I. (2010). Pharmacologic reversal of neurogenic and neuroplastic abnormalities and cognitive impairments without affecting Aβ and tau pathologies in 3xTg-AD mice. *Acta neuropathologica*, *120*(5), 605-621.

Blurton-Jones, M., Kitazawa, M., Martinez-Coria, H., Castello, N. A., Müller, F. J., Loring, J. F., ... & LaFerla, F. M. (2009). Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. *Proceedings of the National Academy of Sciences*, pnas-0901402106.

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathologica*, *82*(4), 239–259.

Breyhan, H., Wirths, O., Duan, K., Marcello, A., Rettig, J., & Bayer, T. A. (2009). APP/PS1KI bigenic mice develop early synaptic deficits and hippocampus atrophy. *Acta neuropathologica*, *117*(6), 677-685.

Briley, D., Ghirardi, V., Woltjer, R., Renck, A., Zolochevska, O., Taglialatela, G., & Micci, M. A. (2016). Preserved neurogenesis in non-demented individuals with AD neuropathology. *Scientific Reports*, *6*.

Burgess, N., Maguire, E. A., & O'Keefe, J. (2002). The human hippocampus and spatial and episodic memory. *Neuron*, *35*(4), 625-641.

Caccamo, A., Maldonado, M. A., Bokov, A. F., Majumder, S., & Oddo, S. (2010). CBP gene transfer increases BDNF levels and ameliorates learning and memory deficits in a mouse\ model of Alzheimer's disease. *Proceedings of the National Academy of Sciences*, *107*(52), 22687-22692.

Cahill, S. P., Yu, R. Q., Green, D., Todorova, E. V., & Snyder, J. S. (2017). Early survival and delayed death of developmentally-born dentate gyrus neurons. *Hippocampus*, *27*(11), 1155-1167.

Carroll, J.C., Rosario, E.R., Kreimer, S., Villamagna, A., Gentzschein, E., Stanczyk, F.Z., & Pike, C.J. (2010). Sex differences in β-amyloid accumulation in 3xTg-AD mice: role of neonatal sex steroid hormone exposure. *Brain Res,* 1366:233–245.

Chen, Y., Tian, Z., Liang, Z., Sun, S., Dai, C. L., Lee, M. H., ... & Gong, C. X. (2012). Brain gene expression of a sporadic (icv-STZ Mouse) and a familial mouse model (3xTg-AD mouse) of Alzheimer's disease. *PLoS One*, *7*(12), e51432.

Clinton, L. K., Billings, L. M., Green, K. N., Caccamo, A., Ngo, J., Oddo, S., ... & LaFerla, F. M. (2007). Age-dependent sexual dimorphism in cognition and stress response in the 3xTg-AD mice. *Neurobiology of disease*, *28*(1), 76-82.

Cohen, J. (1969). *Statistical power analysis for the behavioural sciences.* New York: Academic Press.

DaRocha-Souto, B., Coma, M., Perez-Nievas, B. G., Scotton, T. C., Siao, M., Sánchez-Ferrer, P., ... & Serenó, L. (2012). Activation of glycogen synthase kinase-3 beta mediates β amyloid induced neuritic damage in Alzheimer's disease. *Neurobiology of disease*, *45*(1), 425-437.

Davis, K. E., Easton, A., Eacott, M. J., & Gigg, J. (2013). Episodic-like memory for what-where which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *Journal of Alzheimer's Disease*, *33*(3), 681-698.

Demars, M., Hu, Y.-S., Gadadhar, A., & Lazarov, O. (2010). Impaired neurogenesis is an early event in the etiology of familial Alzheimer's disease in transgenic mice. *Journal of Neuroscience Research*, *88*(10), 2103–17.

Déry, N., Goldstein, A., & Becker, S. (2015). A role for adult hippocampal neurogenesis at multiple time scales: A study of recent and remote memory in humans. *Behavioral neuroscience*, *129*(4), 435.

Déry, N., Pilgrim, M., Gibala, M., Gillen, J., Wojtowicz, J. M., MacQueen, G., & Becker, S. (2013). Adult hippocampal neurogenesis reduces memory interference in humans: opposing effects of aerobic exercise and depression. *Frontiers in neuroscience*, *7*, 66.

Devan, B.D., Goad, E.H., & Petri, H.L. (1996). Dissociation of hippocampal and striatal contributions to spatial navigation in the water maze. *Neurobiol Learn Mem,* 66:305–323.

Devanand, D. P., Lee, S., Manly, J., Andrews, H., Schupf, N., Doty, R. L., ... & Mayeux, R. (2015). Olfactory deficits predict cognitive decline and Alzheimer dementia in an urban community. *Neurology*, 10-1212.

Devanand, D. P., Michaels-Marston, K. S., Liu, X., Pelton, G. H., Padilla, M., Marder, K., ... & Mayeux, R. (2000). Olfactory deficits in patients with mild cognitive impairment predict Alzheimer's disease at follow-up. *American Journal of Psychiatry*, *157*(9), 1399-1405.

Diorio, D., Viau, V., & Meaney, M. J. (1993). The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. *Journal of Neuroscience*, *13*(9), 3839-3847.

Djordjevic, J., Jones-Gotman, M., De Sousa, K., & Chertkow, H. (2008). Olfaction in patients with mild cognitive impairment and Alzheimer's disease. *Neurobiology of aging*, *29*(5), 693-706.

Donovan, N. J., Hsu, D. C., Dagley, A. S., Schultz, A. P., Amariglio, R. E., Mormino, E. C., ... & Marshall, G. A. (2015). Depressive symptoms and biomarkers of Alzheimer's disease in cognitively normal older adults. *Journal of Alzheimer's Disease*, *46*(1), 63-73.

Eichstaedt, K. E., Kovatch, K., & Maroof, D. A. (2013). A less conservative method to adjust for familywise error rate in neuropsychological research: the Holm's sequential Bonferroni procedure. *NeuroRehabilitation*, *32*(3), 693-696.

Fahnestock, M. (2011). Brain-derived neurotrophic factor: the link between amyloid-β and memory loss. *Future Neurology*, *6*(5), 627-639.

Fahnestock, M., Marchese, M., Head, E., Pop, V., Michalski, B., Milgram, W. N., & Cotman, C. W. (2012). BDNF increases with behavioral enrichment and an antioxidant diet in the aged dog. *Neurobiology of Aging*, 33(3), 546–54.

Filali, M., Lalonde, R., Theriault, P., Julien, C., Calon, F., & Planel, E. (2012). Cognitive and non-cognitive behaviors in the triple transgenic mouse model of Alzheimer's disease expressing mutated APP, PS1, and Mapt (3xTg-AD). *Behavioural brain research*, *234*(2), 334-342.

Fox, N. C., Warrington, E. K., Freeborough, P. A., Hartikainen, P., Kennedy, A. M., Stevens, J. M., & Rossor, M. N. (1996). Presymptomatic hippocampal atrophy in Alzheimer's disease: a longitudinal MRI study. *Brain*, *119*(6), 2001-2007.

Fox, J., Weisberg, S. (2011) *An {R} companion to applied regression*, 2nd ed., Sage Publications, Thousand Oaks, CA.

Furr, R.M. (2004). Interpreting effect sizes in contrast analysis. *Understanding Statistics* **3**, 1 25.

Fuster, J. M., & Alexander, G. E. (1971). Neuron activity related to short-term memory. *Science*, *173*(3997), 652-654.

Garzon, D. J., & Fahnestock, M. (2007). Oligomeric amyloid decreases basal levels of brain derived neurotrophic factor (BDNF) mRNA via specific downregulation of BDNF transcripts IV and V in differentiated human neuroblastoma cells. *Journal of Neuroscience*, *27*(10), 2628-2635.

Geddes, J. W., Tekirian, T. L., Soultanian, N. S., Ashford, J. W., Davis, D. G., & Markesbery, W. R. (1997). Comparison of neuropathologic criteria for the diagnosis of Alzheimer's disease. *Neurobiology of aging*, *18*(4), S99-S105.

Guzowski, J. F., Setlow, B., Wagner, E. K., & McGaugh, J. L. (2001). Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. *Journal of Neuroscience*, *21*(14), 5089 5098.

Hall, A. M., & Roberson, E. D. (2012). Mouse models of Alzheimer's disease. *Brain research bulletin*, *88*(1), 3-12.

Hamilton, L. K., Aumont, A., Julien, C., Vadnais, A., Calon, F., & Fernandes, K. J. L. (2010). Widespread deficits in adult neurogenesis precede plaque and tangle formation in the 3xTg mouse model of Alzheimer's disease. *European Journal of Neuroscience*, *32*(6), 905–920.

Hardy, J. A., & Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science*, *256*(5054), 184–185.

Harrison, F. E., Hosseini, A. H., & McDonald, M. P. (2009). Endogenous anxiety and stress responses in water maze and Barnes maze spatial memory tasks. *Behavioural brain research*, *198*(1), 247-251.

Hayashi, M., Mistunaga, F., Ohira, K., & Shimizu, K. (2001). Changes in BDNF-immunoreactive structures in the hippocampal formation of the aged macaque monkey. *Brain research*, *918*(1), 191-196.

Heisz, J. J., Clark, I. B., Bonin, K., Paolucci, E. M., Michalski, B., Becker, S., & Fahnestock, M. (2017). The effects of physical exercise and cognitive training on memory and neurotrophic factors. *Journal of cognitive neuroscience*, *29*(11), 1895-1907.

Herrup, K. (2015). The case for rejecting the amyloid cascade hypothesis. *Nat Neurosci*, *18*(6), 794–799.

Holm, S. (1979). A simple sequentially rejective multiple test procedure. *Scandinavian journal of statistics*, 65-70.

Howells, D. W., Porritt, M., Wong, J. Y. F., Batchelor, P. E., Kalnins, R., Hughes, A. J., & Donnan, G. A. (2000). Reduced BDNF mRNA expression in the Parkinson's disease substantia nigra. *Experimental neurology*, *166*(1), 127-135.

Hutton, C.P., Déry, N., Rosa, E., Lemon, J., Rollo, C.D., Boreham, D., Fahnestock, M., deCatanzaro, D., Wojtowicz , J.M., & Becker, S. (2015). Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice. *Neuroscience*, 308, 180-193.

Hutton, C.P., Lemon, J.A., Sakic, B., Rollo, C.D., Boreham, D.R., Fahnestock, M., Wojtowicz, J. M., & Becker, S. (2018). Early intervention with a multi-ingredient dietary supplement improves mood and spatial memory in a triple transgenic mouse model of Alzheimer's disease. *Journal of Alzheimer's Disease*, (Preprint), 1-23.

Jarrett, B., Godbout, J., McKim, D., & Sheridan, J. (2014). 80. Minocycline attenuates stress induced changes in leukocyte activation and anxiety-like behavior. *Brain, Behavior, and Immunity*, *40*, e23-e24.

Jones, L. V., & Tukey, J. W. (2000). A sensible formulation of the significance test. *Psychological methods*, *5*(4), 411.

Kaczmarek, L. (1993). Molecular biology of vertebrate learning: is c-fos a new beginning? *Journal of Neuroscience Research*, *34*(4), 377–381.

Kapadia, M., Mian, M. F., Michalski, B., Azam, A., Ma, D., Salwierz, P., ... & Fahnestock, M. (2018). Sex-dependent differences in spontaneous autoimmunity in adult 3xTg-AD mice. *Journal of Alzheimer's Disease*, (Preprint), 1-15.

Kapadia, M., Mian, M. F., Ma, D., Hutton, C., Azam, A., Narkaj, K., Cao, C., Brown, B., Michalski, B., Morgan, D., Zovkic, I., Fahnestock, F., & Sakic, B. (Submitted). Behavioural and systemic effects of sustained immunosuppression in the 3xTg-AD model of Alzheimer's disease. 1-10.

Karege, F., Schwald, M., & Cisse, M. (2002). Postnatal developmental profile of brain-derived neurotrophic factor in rat brain and platelets. *Neuroscience letters*, *328*(3), 261-264.

Kempermann, G., Kuhn, H. G., & Gage, F. H. (1997). More hippocampal neurons in adult mice living in an enriched environment. *Nature*, *386*(6624), 493.

Kinsey, S. G., Bailey, M. T., Sheridan, J. F., Padgett, D. A., & Avitsur, R. (2007). Repeated social defeat causes increased anxiety-like behavior and alters splenocyte function in C57BL/6 and CD-1 mice. *Brain, behavior, and immunity*, *21*(4), 458-466.

Kluger, A., Gianutsos, J. G., Golomb, J., Ferris, S. H., George, A. E., Franssen, E., & Reisberg, B. (1997). Patterns of motor impairment in normal aging, mild cognitive decline, and

early Alzheimer'Disease. *The Journals of Gerontology Series B: Psychological Sciences and Social Sciences*, *52*(1), P28-P39.

Knoth, R., Singec, I., Ditter, M., Pantazis, G., Capetian, P., Meyer, R. P., … Kempermann, G. (2010). Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PloS One*, *5*(1), e8809.

Kubota, K., & Niki, H. (1971). Prefrontal cortical unit activity and delayed alternation performance in monkeys. *Journal of neurophysiology*, *34*(3), 337-347.

Lee, J., Duan, W., & Mattson, M. P. (2002). Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. *Journal of neurochemistry*, *82*(6), 1367-1375.

Lemon, J. A., Aksenov, V., Samigullina, R., Aksenov, S., Rodgers, W. H., Rollo, C. D., & Boreham, D. R. (2016). A multi-ingredient dietary supplement abolishes large-scale brain cell loss, improves sensory function, and prevents neuronal atrophy in aging mice. *Environmental and molecular mutagenesis*, *57*(5), 382-404.

Lemon, J. A., Boreham, D. R., & Rollo, C. D. (2003). A dietary supplement abolishes age related cognitive decline in transgenic mice expressing elevated free radical processes. *Experimental biology and medicine*, *228*(7), 800-810.

Lemon, J. A., Rollo, C. D., McFarlane, N. M., & Boreham, D. R. (2008). Radiation-induced apoptosis in mouse lymphocytes is modified by a complex dietary supplement: the effect of genotype and gender. *Mutagenesis*, *23*(6), 465-472.

Lenth, R. (2018). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.1.2. https://CRAN.R-project.org/package=emmeans

Linnarsson, S., Willson, C. A., & Ernfors, P. (2000). Cell death in regenerating populations of neurons in BDNF mutant mice. *Molecular Brain Research*, 75(1), 61-69.

Lommatzsch, M., Zingler, D., Schuhbaeck, K., Schloetcke, K., Zingler, C., Schuff-Werner, P., & Virchow, J. C. (2005). The impact of age, weight and gender on BDNF levels in human platelets and plasma. *Neurobiology of aging*, *26*(1), 115-123.

Liu, H., Zhong, L., Zhang, Y., Liu, X., & Li, J. (2018). Rutin attenuates cerebral ischemia/reperfusion injury in ovariectomized rats via estrogen receptor-mediated BDNF-TrkB and NGF-TrkA signaling. *Biochemistry and Cell Biology*, (0),1-35.

Luu, P., Sill, O. C., Gao, L., Becker, S., Wojtowicz, J. M., & Smith, D. M. (2012). The role of adult hippocampal neurogenesis in reducing interference. *Behavioral Neuroscience*, *126*(3), 381.

Marchese, M., Cowan, D., Head, E., Ma, D., Karimi, K., Ashthorpe, V., ... & Sakic, B. (2014). Autoimmune manifestations in the 3xTg-AD model of Alzheimer's disease. *Journal of Alzheimer's Disease*, *39*(1), 191-210.

Mastrangelo, M. A., & Bowers, W. J. (2008). Detailed immunohistochemical characterization of temporal and spatial progression of Alzheimer's disease-related pathologies in male triple-transgenic mice. *BMC Neuroscience*, *9*, 81.

Mazerolle, M., Régner, I., Barber, S. J., Paccalin, M., Miazola, A. C., Huguet, P., & Rigalleau, F. (2016). Negative aging stereotypes impair performance on brief cognitive tests used to screen for predementia. *Journals of Gerontology Series B: Psychological Sciences and Social Sciences*, *72*(6), 932-936.

Mega, M. S., Cummings, J. L., Fiorello, T., & Gornbein, J. (1996). The spectrum of behavioral changes in Alzheimer's disease. *Neurology*, *46*(1), 130-135.

Méndez, M., Méndez-López, M., López, L., Begega, A., Aller, M. Á., Arias, J., & Arias, J. L. (2010). Reversal learning impairment and alterations in the prefrontal cortex and the hippocampus in a model of portosystemic hepatic encephalopathy. *Acta Neurologica Belgica*, *110*(3), 246.

Michalski, B., Corrada, M. M., Kawas, C. H., & Fahnestock, M. (2015). Brain-derived neurotrophic factor and TrkB expression in the "oldest-old," the 90+ Study: correlation with cognitive status and levels of soluble amyloid-beta. *Neurobiology of Aging*, *36*(12), 3130–3139.

Mielke, M. M., Vemuri, P., & Rocca, W. A. (2014). Clinical epidemiology of Alzheimer's disease: assessing sex and gender differences. *Clinical epidemiology*, *6*, 37.

Morris, J. C., Storandt, M., Miller, J. P., McKeel, D. W., Price, J. L., Rubin, E. H., & Berg, L. (2001). Mild cognitive impairment represents early-stage Alzheimer disease. *Archives of neurology*, *58*(3), 397-405.

Nagahara, A. H., Merrill, D. A., Coppola, G., Tsukada, S., Schroeder, B. E., Shaked, G. M., ... & Rockenstein, E. (2009). Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nature medicine*, *15*(3), 331.

Navarro, D. J. (2015). *Learning statistics with R: A tutorial for psychology students and other beginners.* University of Adelaide. Adelaide, Australia

Niibori, Y., Yu, T. S., Epp, J. R., Akers, K. G., Josselyn, S. A., & Frankland, P. W. (2012). Suppression of adult neurogenesis impairs population coding of similar contexts in hippocampal CA3 region. *Nature communications*, *3*, 1253.

Oddo, S., Caccamo, A., Shepherd, J., Murphy, M., Golde, T., Kayed, R., … LaFerla, F. (2003). Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. *Neuron*, *39*(3), 409–421.

O'Keefe, J., & Dostrovsky, J. (1971). The hippocampus as a spatial map: Preliminary evidence from unit activity in the freely-moving rat. *Brain research*.

O'Leary, T. P., & Brown, R. E. (2013). Optimization of apparatus design and behavioral measures for the assessment of visuo-spatial learning and memory of mice on the Barnes maze. *Learning & Memory*, *20*(2), 85-96.

Olson, A. K., Eadie, B. D., Ernst, C., & Christie, B. R. (2006). Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus*, *16*(3), 250-260.

Palmer, K., Berger, A. K., Monastero, R., Winblad, B., Bäckman, L., & Fratiglioni, L. (2007). Predictors of progression from mild cognitive impairment to Alzheimer disease. *Neurology*, *68*(19), 1596-1602.

Parachikova, A., Green, K. N., Hendrix, C., & LaFerla, F. M. (2010). Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. *PloS one*, *5*(11), e14015.

Penfield, W., & Milner, B. (1958). Memory deficit produced by bilateral lesions in the hippocampal zone. *AMA Archives of Neurology & Psychiatry*, *79*(5), 475-497.

Peng, S., Wuu, J., Mufson, E. J., & Fahnestock, M. (2005). Precursor form of brain-derived neurotrophic factor and mature brain-derived neurotrophic factor are decreased in the pre-clinical stages of Alzheimer's disease. *Journal of Neurochemistry*, *93*(6), 1412–1421.

Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *BMJ: British Medical Journal*, *316*(7139), 1236.

Pettersson, A. F., Olsson, E., & Wahlund, L. O. (2005). Motor function in subjects with mild cognitive impairment and early Alzheimer's disease. *Dementia and geriatric cognitive disorders*, *19*(5-6), 299-304.

Rahhal, T. A., Hasher, L., & Colcombe, S. J. (2001). Instructional manipulations and age differences in memory: Now you see them, now you don't. *Psychology and Aging*, *16*(4), 697.

Rashid, A. J., Yan, C., Mercaldo, V., Hsiang, H. L. L., Park, S., Cole, C. J., ... & Deisseroth, K. (2016). Competition between engrams influences fear memory formation and recall. *Science*, *353*(6297), 383-387.

R Core Team (2017). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing. URL: https://www.R-project.org/

Revelle, W. (2017). *psych: Procedures for Personality and Psychological Research*, Northwestern University, Evanston, Illinois, USA, URL: https://CRAN.Rproject.org/package=psych

Richardson, J. T. (2011). Eta squared and partial eta squared as measures of effect size in educational research. *Educational Research Review* **6**, 135-147.

Rodríguez, J. J., Jones, V. C., Tabuchi, M., Allan, S. M., Knight, E. M., LaFerla, F. M., … & Verkhratsky, A. (2008). Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. *PloS One*, *3*(8), e2935.

Rodriguez-Ortiz, C. J., Baglietto-Vargas, D., Martinez-Coria, H., LaFerla, F. M., & Kitazawa, M. (2014). Upregulation of miR-181 decreases c-Fos and SIRT-1 in the hippocampus of 3xTg-AD mice. *Journal of Alzheimer's Disease*, *42*(4), 1229-1238.

Rosa, E., & Fahnestock, M. (2015). CREB expression mediates amyloid β-induced basal BDNF downregulation. *Neurobiology of Aging*, *36*(8), 2406-2413.

Rosa, E., Mahendram, S., Ke, Y.D., Ittner, L.M., Ginsberg, S.D., Fahnestock, M. (2016) Tau downregulates BDNF expression in animal and cellular models of Alzheimer's disease. *Neurobiology of Aging*, *48*, 135-142.

Rosnow, R.L., & Rosenthal, R. (2003). Effect sizes for experimenting psychologists. *Canadian Journal of Experimental Psychology/Revue canadienne de psychologie expérimentale* **57**, 221.

Schenk, F., & Morris, R.G.M. (1985). Dissociation between components of spatial memory in rats after recovery from the effects of retrohippocampal lesions. *Exp Brain Res*, 58:11 28.

Scoville, W. B., & Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of neurology, neurosurgery, and psychiatry*, *20*(1), 11.

Sekeres, M. J., Bonasia, K., St-Laurent, M., Pishdadian, S., Winocur, G., Grady, C., & Moscovitch, M. (2016). Recovering and preventing loss of detailed memory: differential rates of forgetting for detail types in episodic memory. *Learning & Memory*, *23*(2), 72 82.

Selkoe, D. J., & Hardy, J. (2016). The amyloid hypothesis of Alzheimer's disease at 25 years. *EMBO Molecular Medicine*, (e201606210), 1–14.

Shankar, G. M., Li, S., Mehta, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., … Selkoe, D. J. (2008). Amyloid-beta protein dimers isolated directly from Alzheimer's brains impair synaptic plasticity and memory. *Nature Medicine*, *14*(8), 837–42.

Singh, M., Meyer, E. M., & Simpkins, J. W. (1995). The effect of ovariectomy and estradiol replacement on brain-derived neurotrophic factor messenger ribonucleic acid expression in cortical and hippocampal brain regions of female Sprague-Dawley rats. *Endocrinology*, *136*(5), 2320-2324.

Singmann, H., Bolker, B., Westfall, J., & Aust, F. (2016). afex: analysis of factorial experiments. *The R project for statistical computing*. URL: https://CRAN.R-project.org/package=afex

Snyder, J. S., Radik, R., Wojtowicz, J. M., & Cameron, H. A. (2009). Anatomical gradients of adult neurogenesis and activity: young neurons in the ventral dentate gyrus are activated by water maze training. *Hippocampus*, *19*(4), 360-370.

Snyder, J. S., Soumier, A., Brewer, M., Pickel, J., & Cameron, H. A. (2011). Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature*, *476*(7361), 458 461.

Stark, S. M., Stevenson, R., Wu, C., Rutledge, S., & Stark, C. E. (2015). Stability of age-related deficits in the mnemonic similarity task across task variations. *Behavioral neuroscience*, *129*(3), 257.

Stelzmann, R. A., Schnitzlein, H. N., & Murtagh, F. R. (1995). An English translation of Alzheimer's 1907 paper, "uber eine eigenartige erkankung der hirnrinde." *Clinical Anatomy*, *8*(6), 429–431.

Sterniczuk, R., Antle, M. C., LaFerla, F. M., & Dyck, R. H. (2010). Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 2. Behavioral and cognitive changes. *Brain research*, *1348*, 149-155.

Stover, K. R., & Brown, R. E. (2012). Age-related changes in visual acuity, learning and memory in the APPswe/PS1dE9 mouse model of Alzheimer's disease. *Behavioural brain research*, *231*(1), 75-85.

Stover, K. R., Campbell, M. A., Van Winssen, C. M., & Brown, R. E. (2015). Analysis of motor function in 6-month-old male and female 3xTg-AD mice. *Behavioural brain research*, 281, 16-23.

Stevens, L.M., & Brown, R.E. (2014). Reference and working memory deficits in the 3xTg-AD mouse between 2 and 15-months of age: A cross-sectional study. *Behav Brain Res*, 278:496–505.

Stover, K.R., Campbell, M.A., Van Winssen, C.M., & Brown, R.E. (2015). Early detection of cognitive deficits in the 3xTg-AD mouse model of Alzheimer's disease. *Behav Brain Res* 289:1–10.

Tarkowski, E., Andreasen, N., Tarkowski, A., & Blennow, K. (2003). Intrathecal inflammation precedes development of Alzheimer's disease. *Journal of Neurology, Neurosurgery, and Psychiatry*, *74*(9), 1200–5.

Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., … Katzman, R. (1991). Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Annals of Neurology*, *30*(4), 572–580.

Thal, D. R., Rüb, U., Orantes, M., & Braak, H. (2002). Phases of A beta-deposition in the human brain and its relevance for the development of AD. *Neurology*, *58*(12), 1791–1800.

Vitolo, O. V., Sant'Angelo, A., Costanzo, V., Battaglia, F., Arancio, O., & Shelanski, M. (2002). Amyloid β-peptide inhibition of the PKA/CREB pathway and long-term potentiation: reversibility by drugs that enhance cAMP signaling. *Proceedings of the National Academy of Sciences*, *99*(20), 13217-13221.

Walker, M. P., LaFerla, F. M., Oddo, S. S., & Brewer, G. J. (2013). Reversible epigenetic histone modifications and BDNF expression in neurons with aging and from a mouse model of Alzheimer's disease. *Age*, *35*(3), 519-531.

Watanabe, T., & Niki, H. (1985). Hippocampal unit activity and delayed response in the monkey. *Brain research*, *325*(1-2), 241-254.

Webster, M. J., Herman, M. M., Kleinman, J. E., & Weickert, C. S. (2006). BDNF and trkB mRNA expression in the hippocampus and temporal cortex during the human lifespan. *Gene Expression Patterns*, *6*(8), 941-951.

Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A., & Van Eldik, L. J. (2014). Using mice to model Alzheimer's dementia: an overview of the clinical disease and the\ preclinical behavioral changes in 10 mouse models. *Frontiers in genetics*, *5*, 88.

Wesnes, K. A., Annas, P., Basun, H., Edgar, C., & Blennow, K. (2014). Performance on a pattern separation task by Alzheimer's patients shows possible links between disrupted dentate gyrus activity and apolipoprotein E∈ 4 status and cerebrospinal fluid amyloid-β42 levels. *Alzheimer's research & therapy*, *6*(2), 20.

Wheeler, R.E., & Torchiano, M. (2016). *lmPerm*: *Permutation tests for linear models in R*. The R project for statistical computing. URL: https://github.com/mtorchiano/lmPerm

Wickham, H. (2009). *ggplot2: elegant graphics for data analysis*, Springer-Verlag, New York.

Winocur, G., Becker, S., Luu, P., Rosenzweig, S., & Wojtowicz, J. M. (2012). Adult hippocampal neurogenesis and memory interference. *Behavioural brain research*, *227*(2), 464-469.

TABLES

Table 1. Ingredients included in the multiple ingredient dietary supplement.

Ingredient	Daily Dose (mg)	Ingredient	Daily Dose (mg)
Acetyl L-Carnitine	14.4	L-Glutathione	0.36
Acetylsalicylic Acid	2.5	Magnesium	0.72
Ascorbic Acid (Vit. C)	3.6	Melatonin	0.01
Bioflavinoids	4.32	N-Acetyl Cystein	7.2
Cholecalciferol (Vit. D)	0.0000625	Niacin (Vit. B3)	0.72
Chromium Picolinate	0.00144	Potassium	0.36
Cobalamin (Vit. B12)	0.00072	Pyridoxine Hydrochloride (Vit. B6)	0.72
Curcumin	1.8	Quercitin	0.9
D-α-tocopherol (Vit. E)	0.965	Rutin	0.72
Folic Acid	0.01	Selenium	0.00108
Garlic	0.0216	Thiamine (Vit. B1)	0.72
Ginger	7.2	Ubiquinone (CoQ10)	0.44
Gingko Biloba	1.44	Wild Fish Oil	21.6
Ginseng	8.64	α-Lipoic Acid	0.72
Green Tea	7.2	β-Carotene	0.03

Table 2. Mouse behavioural test battery

Day(s)	Test	Measures
-4 to 0	Animal Handling	n/a
1	visual placing response, righting reflex, hind limb placing response, postural reflex, negative geotaxis	basic neurological functioning
	Rotarod	sensorimotor coordination
2	Hanging Basket	muscle strength
3 - 8	Olfactory Acuity and Discrimination	Olfactory Acuity and Discrimination
9	Beam Walking	balance/motor coordination
10 - 11	Open Field	anxiety (agoraphobia/photophobia)
12	Elevated Plus Maze	anxiety (acrophobia/photophobia)
13 - 16	Novel Object Recognition	novelty detection
17 - 20	INBEST Monitoring	spontaneous activity, food and water intake, sucrose preference
21-26	Spontaneous Alternation	visuospatial working memory and navigation strategy
27 - 33	Barnes Maze Reversal Learning	Spatial learning/memory, cognitive flexibility
34 – 42	Morris Water Maze	visual acuity, Spatial learning/memory, cognitive flexibility

Table 3. Effects of the MDS in 3xTg-AD mice at 6-7 months of age.

Behavioral Domain	Test	Differences Between Sexes	Effect of MDS
visual acuity	water maze cued trials	none	null
anxiety-like behavior (photophobia/agoraphobia)	open field	none	null
	elevated plus maze	none	null
recognition memory	novel object	none	null
working memory	spontaneous alternation	lower in females	increased in females
long term memory	water maze probe trial (low interference)	none	null
	Barnes maze acquisition probe (low interference)	none	null
	Barnes maze reversal probe (high interference)	worse performance in females	enabled females to perform above chance
spatial learning and memory	water maze acquisition trials (low interference)	none	null
	Barnes maze acquisition trials (low interference)	none	null
	water maze reversal trials (high interference)	worse performance in males	null
	Barnes maze reversal trials (high interference)	worse performance in males	null
Biomarker	**Measure**	**Differences Between Sexes**	**Effect of MDS**
spleen size	wet weight	larger in males than females	null
brain size	brain weight/body weight ratio	none	null
neurogenesis	DCX+ cells in the SGZ	more cells in females than males	null
	DCX+ cells in the SVZ	none	null
BDNF	hippocampal BDNF mRNA	none	increased in both sexes

note: there were no effects of behavioural testing on any biomarkers.

Table 4. Effects of the MDS in 3xTg-AD vs. WT mice at 11-12 months of age.

Behavioral Domain	Test	Alterations in 3xTg-AD mice	Effect of MDS
motor (co-ordination, strength)	rotarod	none	null
	hanging basket	none	increased in females (both genotypes)
	beam walking	none	null
sensory (olfaction and vision)	olfactory acuity	increased sensitivity in both sexes	null
	olfactory discrimination	none	null
	water maze cued trials	none	null
anxiety-like behavior (photophobia/agoraphobia)	open field	impaired in both sexes, more freezing in females	null
	elevated plus maze	impaired in both sexes	null
depressive-like behavior (anhedonia)	sucrose preference	none	null
recognition memory	novel object	none	null
working memory	spontaneous alternation	none	null
long term memory	water maze probe trial (low interference)	impaired in both sexes	negative effect in females (although they still performed above chance)
	Barnes maze acquisition probe (low interference)	none	null
	Barnes maze reversal probe (high interference)	impaired in females only	prevented deficits (only vehicle control 3xTg-AD females failed to perform above chance)
spatial learning and memory	water maze acquisition trials (low interference)	impaired in both sexes	null
	Barnes maze acquisition trials (low interference)	none	null
	water maze reversal trials (high interference)	impaired in both sexes	trend toward beneficial effect in females (both genotypes)
	Barnes maze reversal trials (high interference)	impaired in both sexes	null
Biomarker	**Measure**	**Alterations in 3xTg-AD mice**	**Effect of MDS**
spleen size	wet weight	enlarged in both sexes (to a greater degree in males)	protective
brain size	brain weight/body weight ratio	smaller in females only	null
hippocampal size	DG volume	smaller in both sexes	null
	CA1 volume	smaller in both sexes	null
neuropathology	cortical TBS-soluble Aβ	elevated in both sexes	null
neurogenesis	DCX+ cells in the SGZ	depleted in both sexes	null
BDNF	hippocampal BDNF mRNA	none	increased in females (both genotypes)
activity-dependent plasticity in the hippocampus	c-Fos+ cells in the DG	increased in males (both genotypes)	normalized (both genotypes)
	c-Fos+ cells in CA1	increased in males	normalized
	c-Fos+ cells in CA3	increased in males	normalized

FIGURES

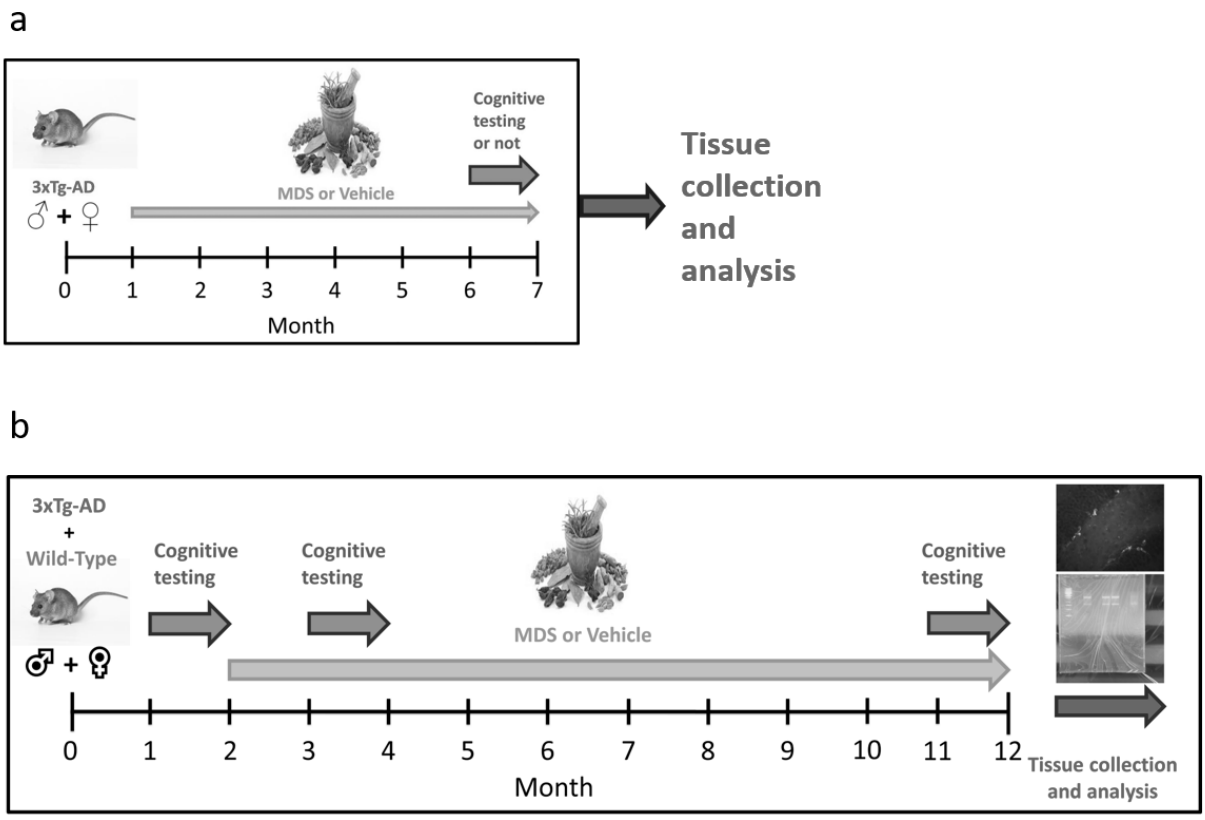


Figure 1. Study design and experimental timeline diagram. In Experiment 1 **(a)**, 3xTg-AD males and females were treated with the MDS or vehicle control from 1-7 months of age, and half of them were tested in the behavioural battery from 6-7 months of age (n = 6 per group). In Experiment 2 **(b)**, both sexes of 3xTg-AD and WT mice were tested in the behavioural battery at 1-2 months of age (baseline), then were randomly assigned to treatment with the MDS or vehicle control until 12 months of age (n = 9-12 per group). Behaviour was re-evaluated in all animals at 3-4 months of age and 11-12 months of age to determine the short- and long-term benefits of MDS supplementation in the 3xTg-AD model. After the behavioural testing was completed (7 months of age in Experiment 1; 12 months of age in Experiment 2), mice were euthanized, and tissues were collected for biomarker assays (see text). Note that the behavioural data for the baseline and 3-4 month testing of animals in Experiment 2 has been published elsewhere (Hutton et al., in press), while the data from Experiment 1 and the 11-12 month testing age in Experiment 2 (including biomarkers) are included in this report.

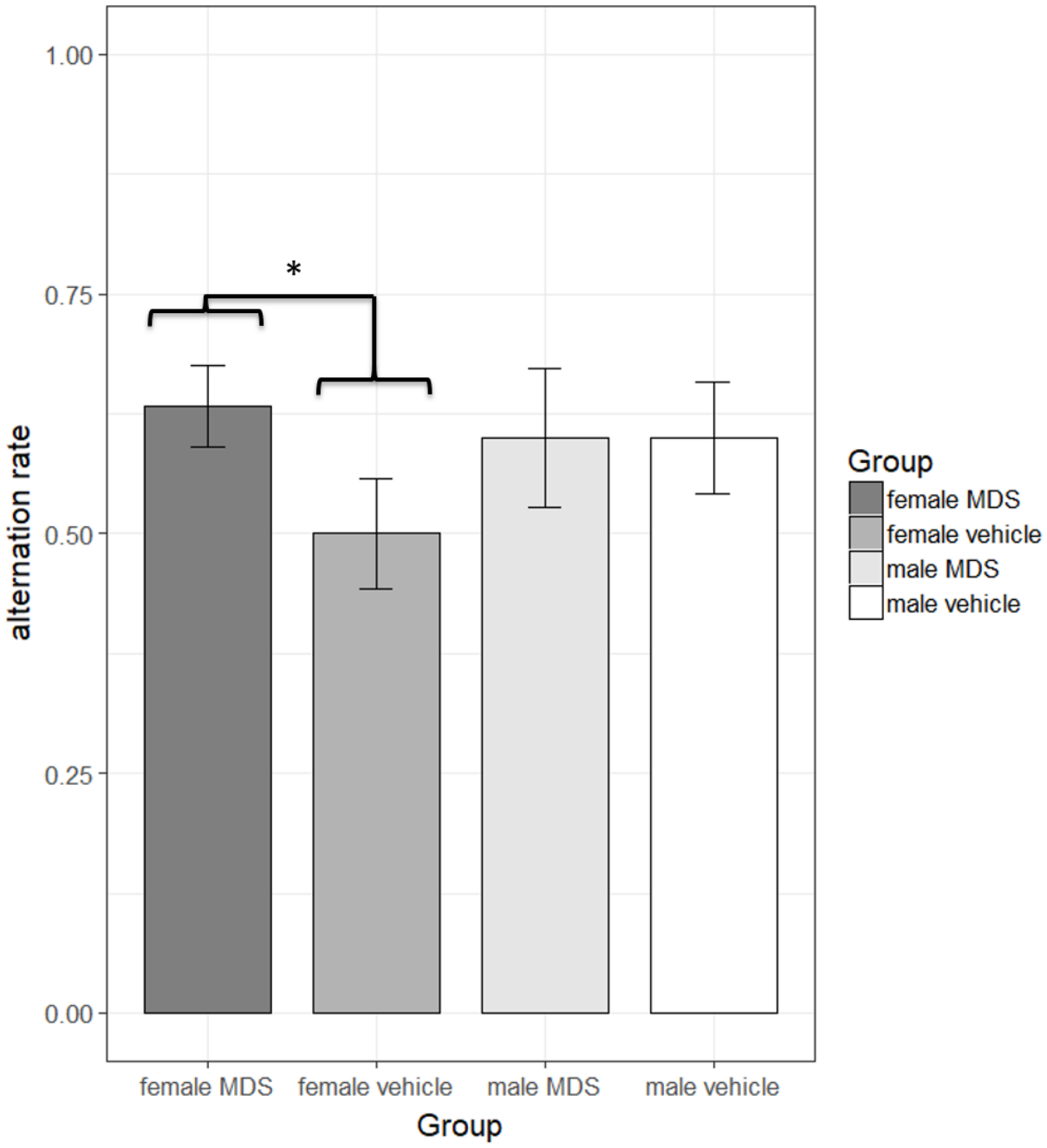


Figure 2. Alternation rate (mean ± SE, n = 6 for all groups) during days 1-5 of spontaneous alternation testing of 6-7- month-old 3xTg-AD mice in Experiment 1. MDS-supplemented 3xTg-AD females alternated more frequently than vehicle-control 3xTg-AD females ($t_{(10)} = 1.865$, *p = 0.046 [unadjusted] or 0.097 [with Holm adjustment]; d = 1.077).

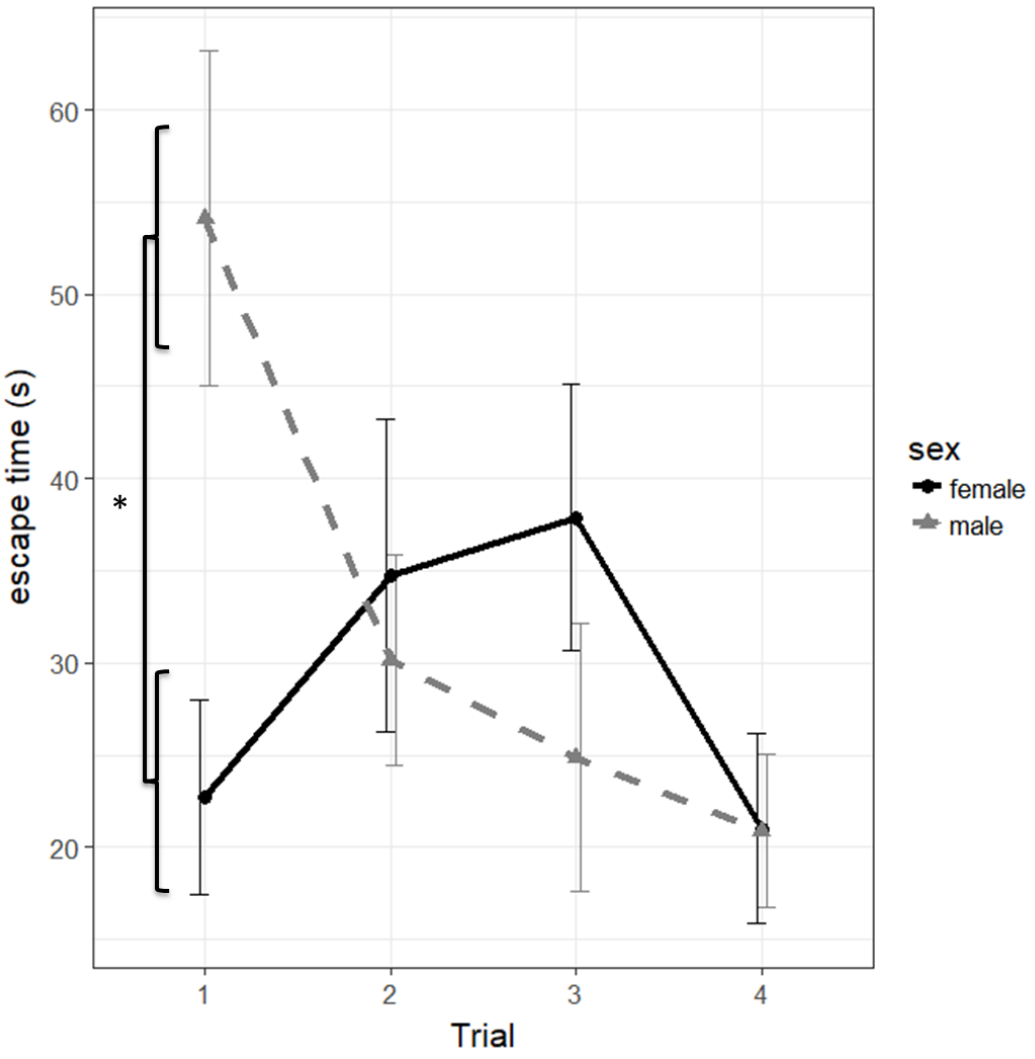


Figure 3. Escape time (s, mean ± SE) of 6-7-month-old 3xTg-AD mice on the hidden platform reversal trials of the water maze (platform in 2nd training location). 3xTg-AD males took longer to escape the maze on the 1st hidden platform reversal trial than females (2 x 2 x 4 ANOVA, sex x trial interaction, $F_{(2, 41)} = 2.957$, *p = 0.048, $\eta^2_g = 0.078$). There were 6 mice in each group.

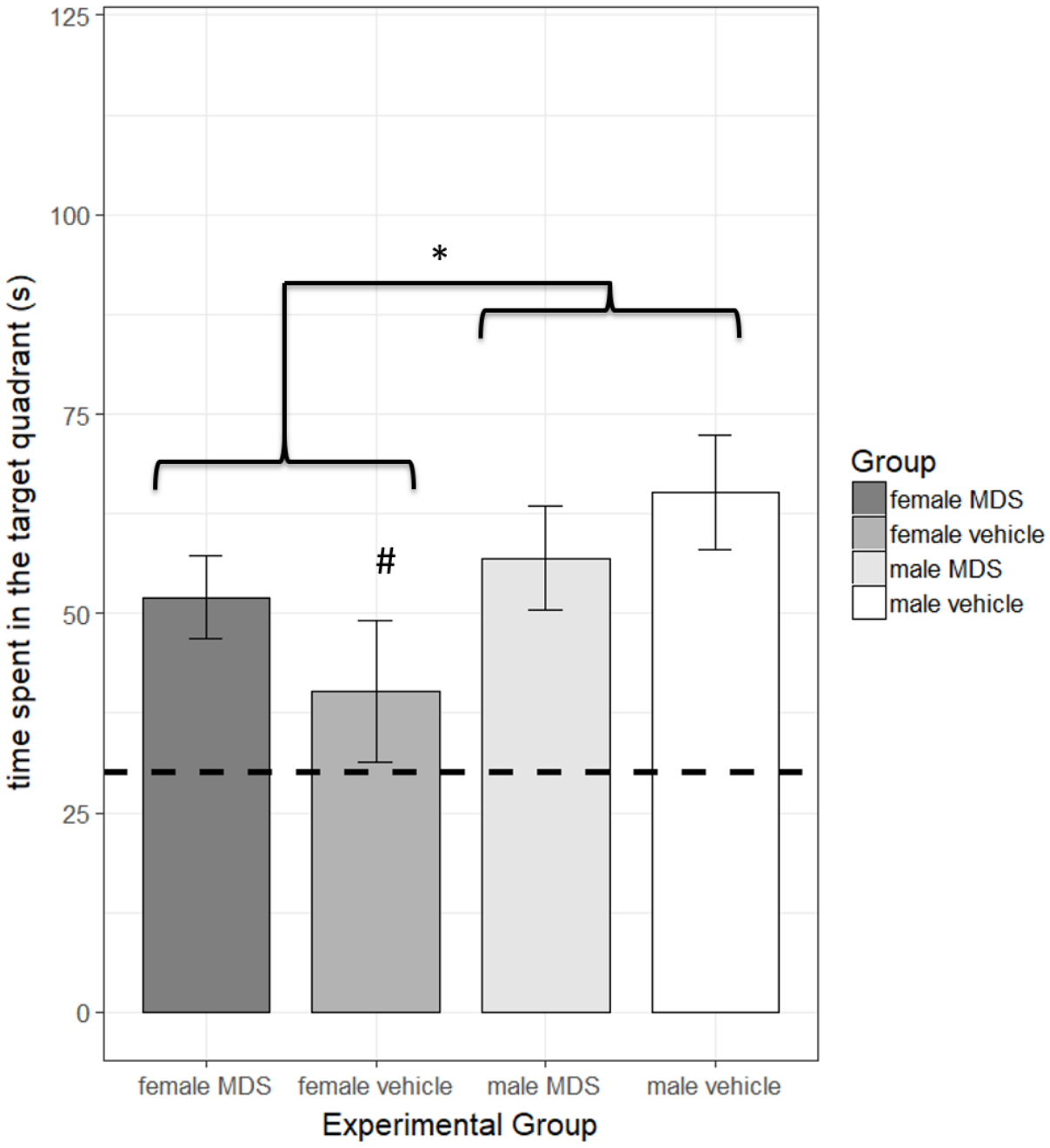


Figure 4. Time spent by 6-7-month-old 3xTg-AD mice in the target quadrant (s, mean ± SE) on the reversal probe trial (24 hours after the final acquisition trial) of the Barnes maze. Females performed worse than males (2 x 2 ANOVA, main effect of sex, $F_{(1, 19)} = 4.454$, *p = 0.048, $\eta^2_p = 0.190$). In addition, vehicle-control females were the only group which did not perform above chance, indicating a failure to recall the 2nd platform location (Holm-corrected *t*-tests: MDS-supplemented females, n = 6, $t_{(5)} = 4.233$, p = 0.016, d = 2.179; vehicle-control females, n = 6, $t_{(5)} = 1.156$, #p = 0.150, d = 1.851; MDS-supplemented males, n = 6, $t_{(5)} = 4.119$, p = 0.016, d = 2.160; vehicle-control males, n = 6, $t_{(5)} = 4.872$, p = 0.015, d = 2.180).

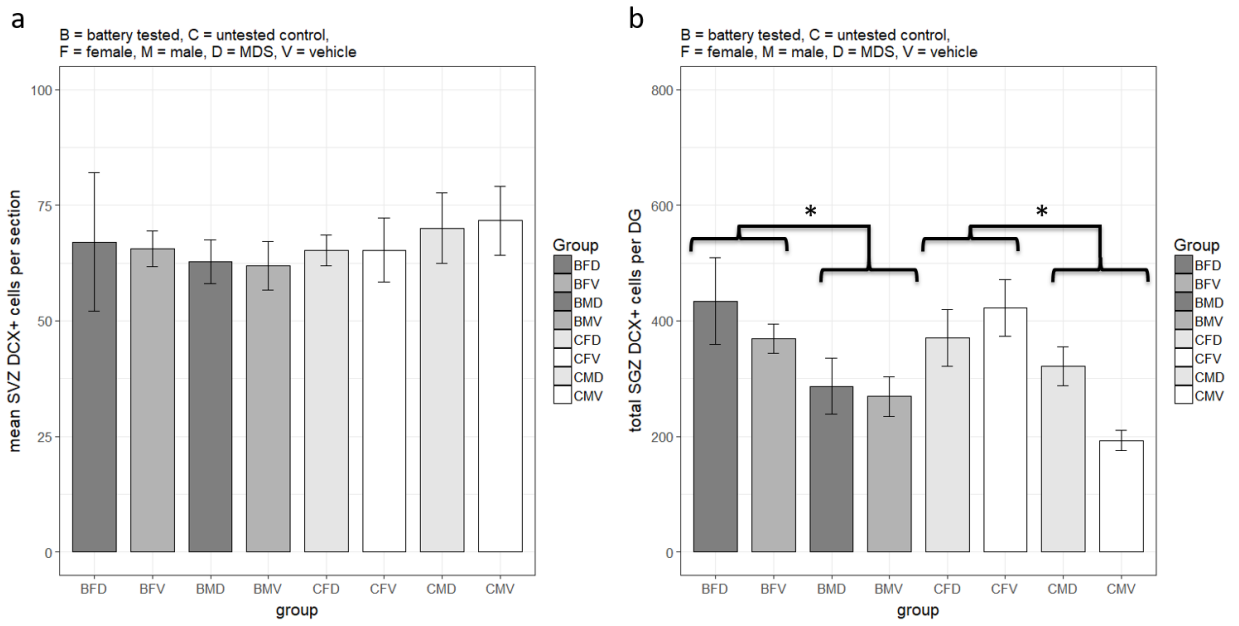


Figure 5. Effects of sex, diet, and behavioural testing on hippocampal neurogenesis in 6-7-month-old 3xTg-AD mice. **(A)** Quantity of doublecortin (DCX)-positive cells in the subventricular zone (SVZ; mean ± SE) per 40-um section for each group (n = 6). There were no differences between groups in the level of SVZ neurogenesis. **(B)** In the subgranular zone (SGZ) of the hippocampus, the estimated total number of DCX cells per dentate gyrus (DG; mean ± SE) was higher in females than males (2 x 2 x 2 ANOVA, main effect of sex; ($F_{(1, 40)}$ = 5.390, *p = 0.025, η^2_p = 0.119). The number of DCX+ neuroblasts was not altered by behavioural testing in either region.

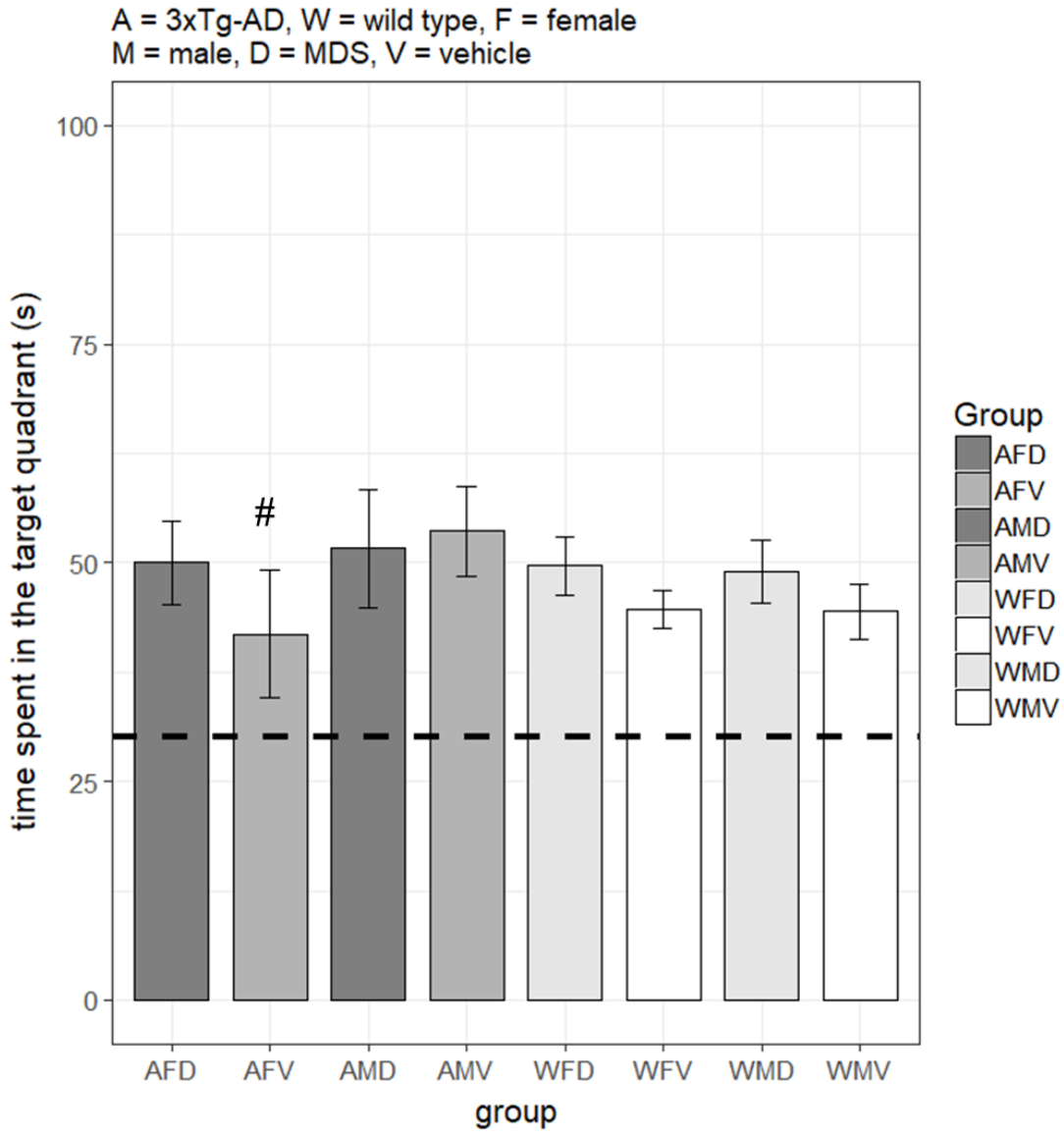


Figure 6. Time spent in the target quadrant (s, mean ± SE) by 11-12-month-old 3xTg-AD and WT mice during the reversal probe trial (2nd hidden platform location) of the Barnes maze. Vehicle-control 3xTg-AD females were the only group which failed to spend more than 25% (30s, dashed black line) of the trial in the target quadrant (Holm-corrected one-way *t*-tests; AFD, n = 11, $t_{(10)} = 4.149$, p = 0.003; AFV, n = 9, $t_{(8)} = 1.619$, #p = 0.075; AMD, n = 10, $t_{(9)} = 3.634$, p = 0.011; AMV, n = 10, $t_{(10)} = 3.133$, p = 0.015; WFD, n = 10, $t_{(9)} = 3.494$, p = 0.015; WFV, n = 11, $t_{(11)} = 5.794$, p < 0.001; WMD, n = 10, $t_{(10)} = 2.155$, p = 0.028; WMV, n = 12, $t_{(11)} = 4.676$, p = 0.002). There was no effect of the MDS in males.

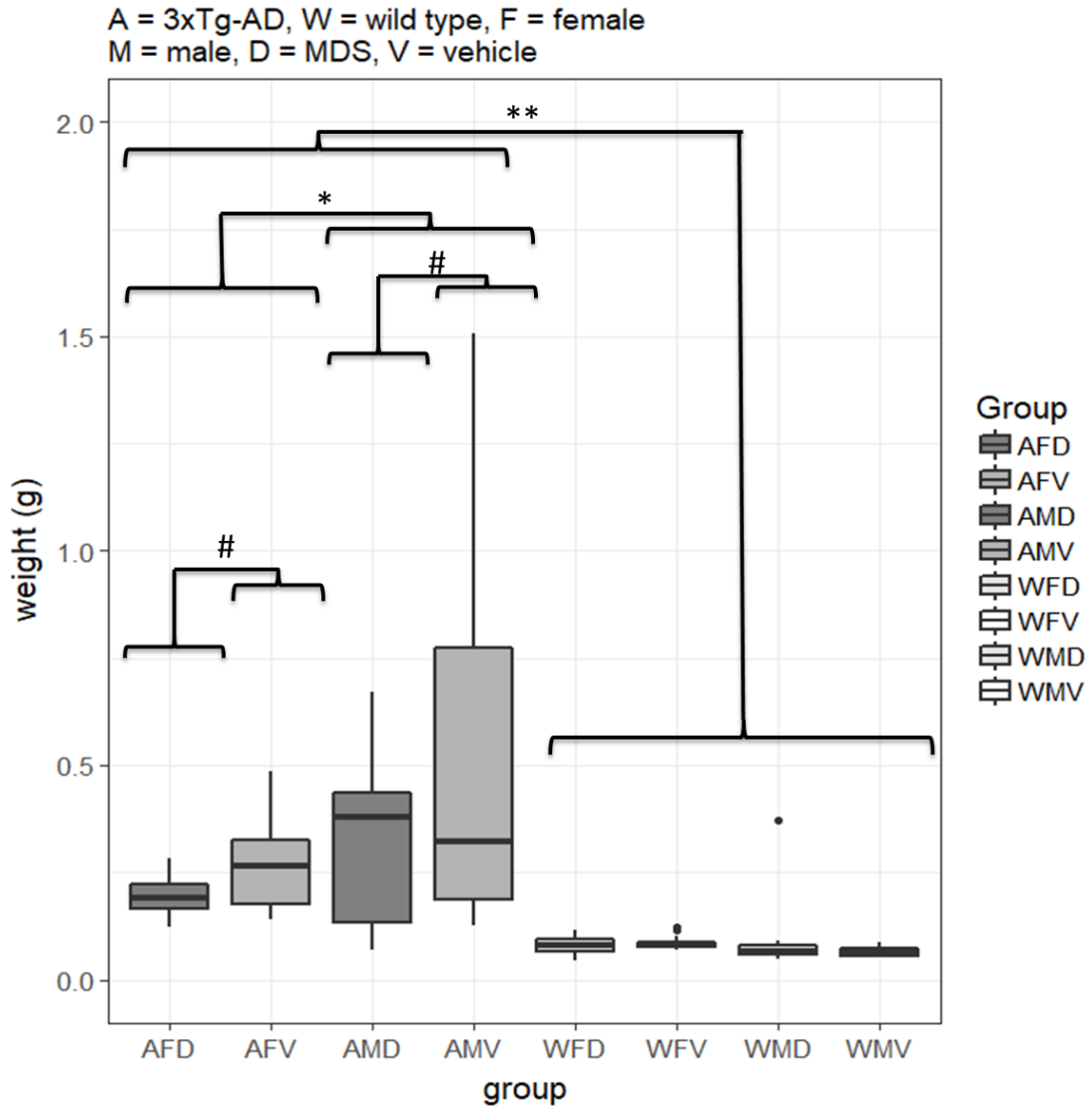


Figure 7. Boxplots showing the spleen sizes of 11-12-month-old 3xTg-AD and WT mice. 3xTg-AD mice had larger spleens than WT mice (factorial permutation test, main effect of genotype, **$p = 0.001$, $\eta^2_p = 0.302$). This splenomegaly was more pronounced in 3xTg-AD males than 3xTg-AD females (sex x genotype interaction; *$p = 0.013$, $\eta^2_p = 0.069$), and was attenuated in both sexes by the MDS (trend towards a genotype x diet interaction: $p = 0.084$, $\eta^2_p = 0.037$; one-tailed tests for simple main effect of diet in each genotype: 3xTg-AD, #$p = 0.030$; WT, $p = 0.666$). Group sample sizes were: AFD, n = 11; AFV, n = 9; AMD, n = 10; AMV, n = 10; WFD, n = 10; WFV, n = 11; WMD, n = 10; WMV, n = 12.

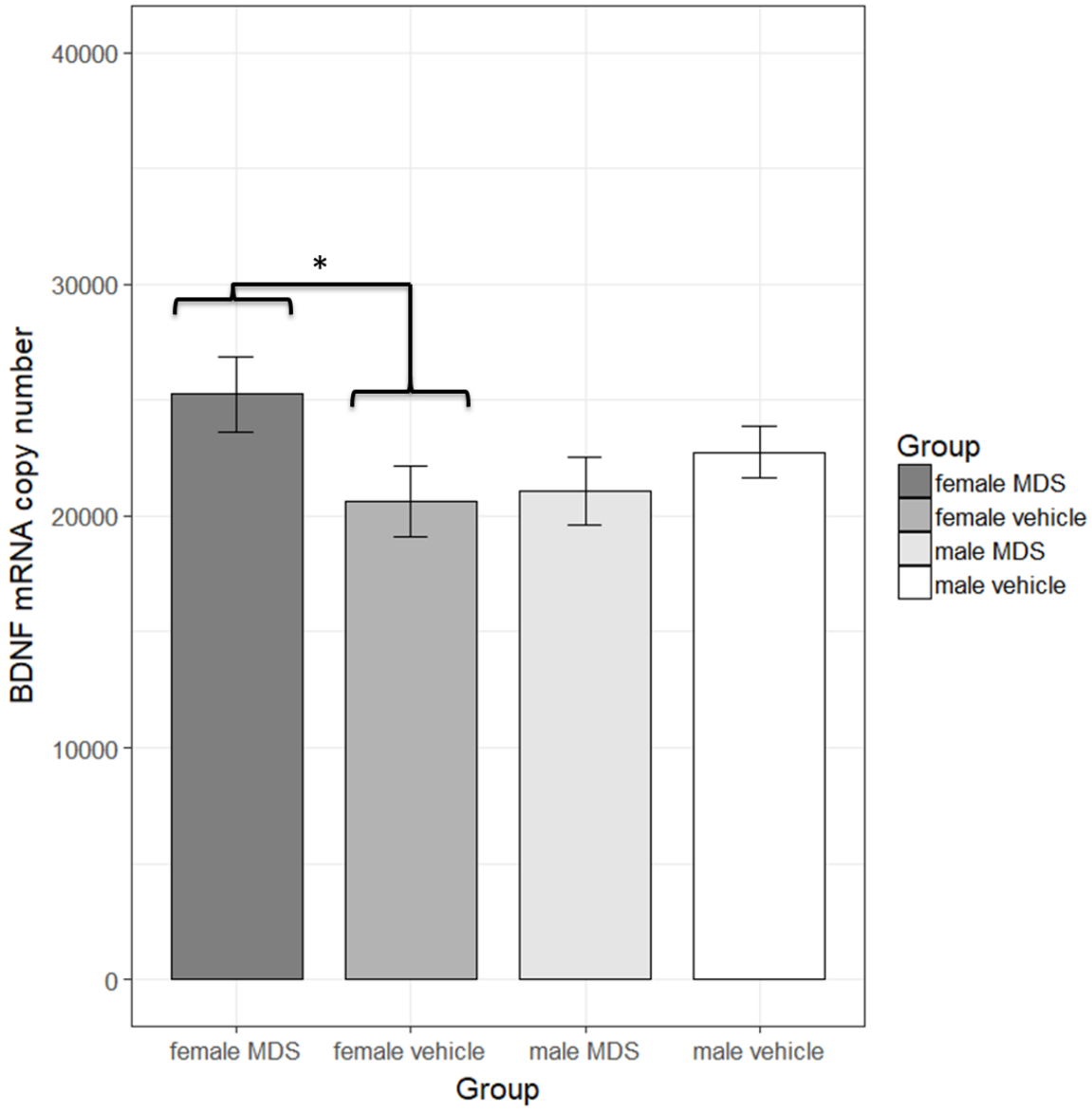


Figure 8. Hippocampal BDNF mRNA copies (mean +/- SE) for male and female MDS and vehicle control treatment groups of 11-12-month-old mice (genotypes combined). BDNF was higher in MDS-supplemented than vehicle-control females, but not males, of both genotypes (ANOVA, sex x diet interaction; $F_{(1, 79)} = 4.753$, *p = 0.032, $\eta^2_p = 0.054$). Group sample sizes were: female MDS, n = 21; female vehicle, n = 19; male MDS, n = 20; male vehicle, n = 22.

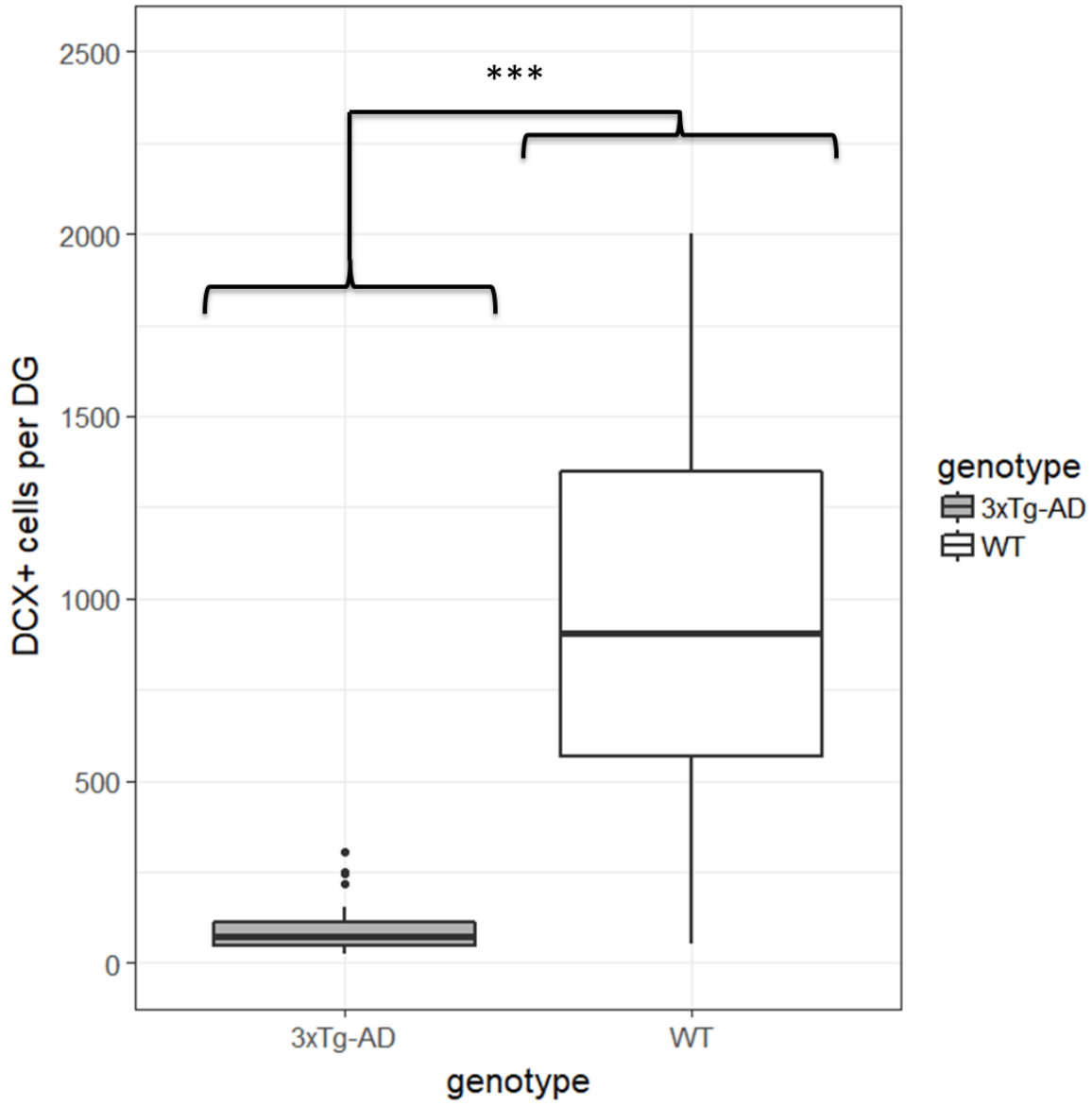


Figure 9. Box-and-whisker plots showing the median number of doublecortin (DCX)+ neuroblasts per dentate gyrus (DG), a measure of hippocampal neurogenesis, for 3xTg-AD and WT mice (treatment groups and sexes combined, n = 43) at 11-12 months of age. Horizontal lines on the tops and bottoms of the boxes represent the 25th and 75th percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range. Adult hippocampal neurogenesis is dramatically depleted in 3xTg-AD mice (n = 40) at this age (factorial permutation test, main effect of genotype, ***$p < 0.001$, $\eta^2_p = 0.655$).

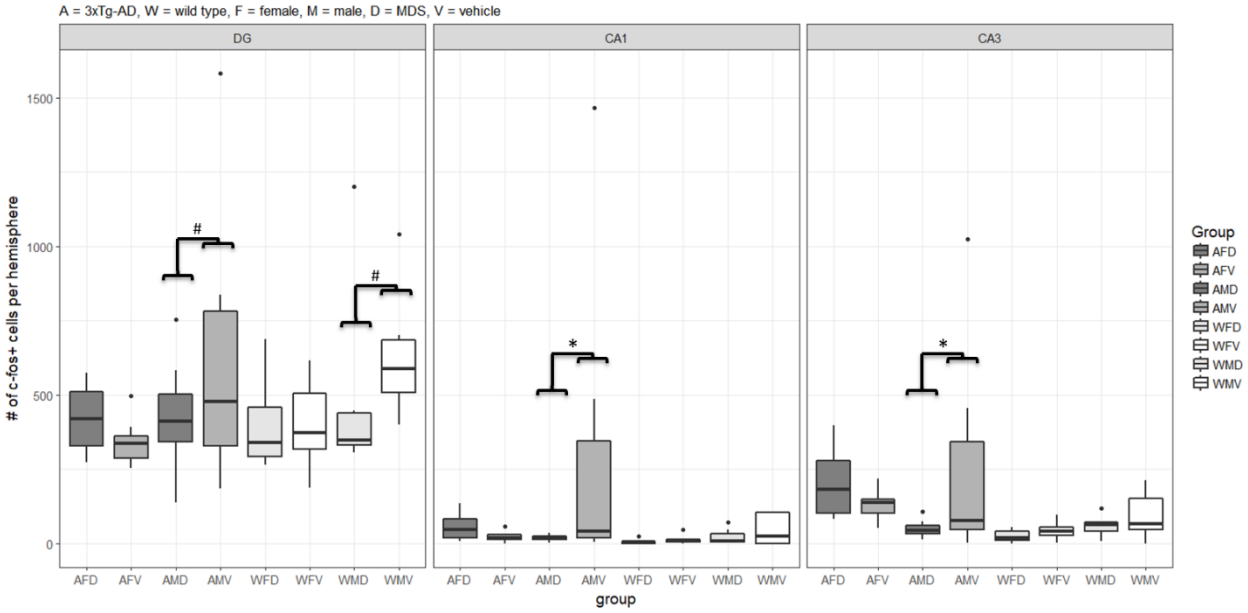


Figure 10. Box-and-whisker plots showing the quantity of c-Fos+ neurons in the dentate gyrus (DG), *cornu ammonis* 1 (CA1) and *cornu ammonis* 3 (CA3) subfields of the hippocampus 90 minutes after the last reversal trial on the Morris water maze. Horizontal lines on the tops and bottoms of the boxes represent the 25th and 75th percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range. Vehicle control males, but not females, of both genotypes exhibited more c-Fos+ cells in the DG than MDS-supplemented males (sex x diet interaction; permutation #p = 0.019). There was also an abnormal increase c-Fos immunoreactivity for 3xTg-AD males (not females) in CA1 and CA3, which was prevented by the MDS (genotype x sex x diet interaction: CA1, permutation *p = 0.026, $\eta^2_p = 0.045$; CA3, permutation *p = 0.039, $\eta^2_p = 0.062$). Group sample sizes were: AFD, n = 11; AFV, n = 9; AMD, n = 10; AMV, n = 10; WFD, n = 10; WFV, n = 11; WMD, n = 10; WMV, n = 12.

5. Discussion and Future Directions

5.1. Empirical Summary and Implications

This work clearly demonstrates that lifestyle-based interventions, particularly those which counteract biological mechanisms of aging such as inflammation (Walford, 1964) and oxidative stress (Harman, 1956), can improve the resilience of mice to hippocampal damage (and the associated behavioural sequelae) due to chronic unpredictable stress or accumulation of Aβ. Moreover, these protective effects may be due to a combination of impacts on the primary targets of the MDS (inflammation, oxidative stress, membrane integrity, mitochondrial function, and insulin resistance; Lemon et al., 2003). The key insights from each chapter, and contributions to the overall thesis, are summarized below.

In **Chapter 2** we demonstrated, for the first time, that the upregulation of hippocampal neurogenesis by aerobic exercise in mice does not occur under conditions of chronic stress. As expected, we also found chronic unpredictable stress to increase the size of the adrenal glands and induce depressive-like behaviour (anhedonia) in the mice. The combination of dietary supplementation and exercise, but neither intervention alone, alleviated this anhedonia, increased hippocampal BDNF expression, neurogenesis levels, and enlarged the hippocampus of chronically stressed mice. These findings strongly suggest that a combination of nutritional intervention with exercise may be much more effective than either intervention alone in counteracting at least one core symptom of MDD in humans (at least in men). We are currently evaluating this prediction using the data from the Canadian Longitudinal Study of Aging (see section 5.3 for details). This work furthers our knowledge into the component of the thesis related to depression, in that it supports the merit of using diet and exercise to buffer against stress-related depression via beneficial changes in the hippocampus.

In **Chapter 3** it was shown that only 2 months of supplementation with the MDS is sufficient to prevent supplemented 3xTg-AD mice from exhibiting the early impairments in spatial learning or working memory that were observed in the vehicle-treated controls. These were robust benefits that were obtained in a shorter treatment period than has been reported using the MDS with other animal models (e.g. Aksenov et al., 2013) or other complex dietary supplements with AD rodent models (e.g. Parachikova et al., 2010). The MDS also increased sucrose preference in both sexes of 3xTg-AD and WT mice at 3-4 months of age, suggesting that it may improve the resilience of younger adults against stress-related depression. Translational work in humans might also see greater benefits with extended periods of treatment, ideally for at least several years (2 months is ~5-8% of the average lifespan for these mouse strains; Kane et al., 2018). As intended, this timeline is more consistent with a lifestyle-based approach to prevent or delay the onset of symptoms than attempting to reverse the damage after it has already been done. Unexpectedly, the 3xTg-AD mice displayed anxiety-like behaviour prior to the start of treatment or the appearance of impairments in learning or memory. That this anxiety was not altered by the MDS is further evidence that similar interventions for protecting against AD are more likely to be successful if administered prophylactically. Furthermore, with respect to the

overall goal of the thesis, these results support the merit of further research into diet-based interventions for the prevention of AD cognitive symptoms in both men and women, at least in the early stages of the disease.

Chapter 4 extended the work of the previous chapter by showing that the MDS continues to improve long term spatial memory in 3xTg-AD females up to 11-12 months of age. To my knowledge, this is also the first report that 3xTg-AD males exhibit an abnormal pattern of activity across the DG, CA1, and CA3 subfields of the hippocampus during the spatial encoding, and that this abnormality can be prevented using the MDS. The MDS also increased hippocampal BDNF levels in both sexes at 6-7 months of age, a benefit that persisted in females only by 11-12 months of age, and which may have been partially responsible for the memory improvement seen in MDS-supplemented 3xTg-AD females. The MDS also attenuated the marked splenomegaly that has been reported for 3xTg-AD mice previously (Marchese et al., 2014; Kapadia et al., 2018), and which is worse in 3xTg-AD males than females. However, in comparison to WT mice, the MDS did not prevent the depletion of hippocampal neurogenesis, hippocampal volume, or elevation in cortical Aβ that was present among 3xTg-AD males and females. These results support the hypothesis that the MDS can produce lasting increases in hippocampal BDNF expression in an AD mouse model, with concomitant benefits on tests of learning and memory. Moreover, these behavioural benefits occurred without altering Aβ, neurogenesis, hippocampal volume, or overall brain size. With respect to the overall goal of the thesis, supplementation with the MDS can yield some lasting benefits in delaying the progression of an AD-like phenotype in a mouse model, however these benefits seem to diminish over time and differ markedly between males and females. This work provides further evidence that sex or gender differences should be examined in future AD research in clinical populations and that dietary supplementation may be more effective at delaying AD in women than men. Additional considerations and some of the limitations of this collective empirical work are discussed below.

5.2. Limitations and Future directions

The mouse work presented in Chapters 2-4 strongly suggests that a combination of dietary supplementation with aerobic exercise may protect against depression (at least in men; Hutton et al., 2015), and long-term supplementation with the MDS may greatly delay the progression of AD (at least in women; Hutton et al., 2018; Hutton et al., submitted). However, it is important to keep in mind that mouse models are only models of human processes or afflictions. For example, one cannot ask a mouse how depressed or apathetic it feels on a scale from 1 to 10, and although the 3xTg-AD mouse used here also harbours human mutations associated with familial AD and tauopathies, only a very small fraction of the dementia patient population (~2%; for a review see Herrup, 2015) carries mutations in APP, PS1 or tau, while the remaining majority of cases are late onset and "sporadic" in nature with an etiology that evades

our understanding to this day. Progress in developing novel therapeutics for AD (or other conditions like depression), therefore critically depends upon advances in our basic understanding of the disease.

A promising alternative to the amyloid cascade hypothesis (Hardy & Higgins, 1992) is already emerging. Compared to the massive research efforts based on amyloid and tau (Cummings et al., 2014), the relative neglect of the vascular contribution to AD is surprising because it has been known for a long time that vascular amyloid deposition, or cerebral amyloid angiopathy (CAA), is present in around 90% of AD cases and roughly half of the population over 90 years of age (Vinters, 1987). This cerebrovascular co-morbidity combined with the fact that most conditions affecting cerebral perfusion (ex. heart disease, atherosclerosis, stroke, smoking) are also risk factors for AD led to the vascular hypothesis of AD (de la Torre, 2000; Zlokovic, 2005). The vascular hypothesis posits that vascular senescence combined with injury or disease (or another vascular risk factor) triggers a vicious cycle of amyloid accumulation, blood-brain-barrier (BBB) breakdown, neuroinflammation, and neurovascular uncoupling driven by impaired perfusion and ultimately leading to neural degeneration.

Perhaps the strongest evidence supporting the vascular hypothesis comes from rodent models of chronic cerebral hypoperfusion (CCH), induced by transient or permanent occlusion of some of the vessels supplying the brain (de la Torre, 2000). CCH in non-transgenic animals can lead to physiological and behavioural changes that are remarkably like those seen in transgenic AD model animals, including the accumulation of Aβ and synaptic atrophy in the hippocampus accompanied by deficits in visuospatial memory (Wang et al., 2010). Long term CCH rodent studies (de la Torre, 2000) varying the degree of hypoperfusion (2- vs 3-vessel occlusion) have even shown that reduced cerebral blood flow can lead to eventual atrophic necrosis in the hippocampus, parietal and frontal cortices, mimicking the pattern seen in human AD (Braak & Braak, 1991). Despite the obvious causality of neurodegeneration in CCH rodent models, the full extent to which chronic hypoperfusion and BBB breakdown contributes to AD pathogenesis in humans remains unknown and should be investigated further.

The potential for dietary interventions in protecting against the development of impaired Aβ clearance in sporadic AD is apparent from evidence that anti-oxidant/anti-inflammatory supplements (e.g. α-Lipoic acid) can protect against the accelerated age-related breakdown of the BBB under conditions of chronic inflammation in mice maintained on a high fat diet (Takechi et al., 2013a, b). It has also been shown in wild-type rodents that NFκB activation and BBB disruption induced by the infection mimetic LPS can be reduced by an ingredient in the MDS (Lemon et al., 2003), the polyphenol epigallocatechin-3-gallate (EGCG) from green tea (Li et al., 2012). Green tea polyphenols may also protect against BBB degradation under ischemic conditions (Liu et al., 2013). Orally administered pre-treatment with green tea extract for 30 days prior to temporary middle cerebral artery occlusion in rats abolished the loss of tight junctions and nearly prevented the increase in BBB permeability to Evans blue dye after ischemia (Liu et al., 2013). Recently, it has also been found that green tea polyphenols also

potently inhibit the Aβ-producing enzyme β-secretase in cultured neurons (Cox et al., 2015) and synergistically interact with aerobic exercise to reduce brain amyloid levels and spatial learning deficits in AD model mice (Walker et al., 2015). Thus, green tea polyphenols, or supplements containing them, may protect against amyloid accumulation by counteracting inflammation in cerebrovascular cells, limiting BBB breakdown, and inhibiting production of Aβ by β-secretase. The cerebrovascular impacts of dietary supplementation in AD animal models are therefore likely to be a fruitful area of future research.

While EGCG may slow down the impaired clearance of Aβ resulting from vascular degeneration, a phenolic compound from olive oil, oleocanthal (not present in the MDS), may be more effective at a later stage in the process. Oleocanthal administration in APP mutant mice, cultured APP-transfected SH-SY5Y neurons, or an *in vitro* BBB monolayer (hCMEC/D3 cells) improved Aβ clearance (by up to ~50%) via multiple mechanisms (Qosa et al., 2015). Specifically, it was found to upregulate efflux transporters P-glycoprotein, LRP-1 and ApoE, reduce IL-1β levels and even slightly increase the expression of the Aβ-degrading enzymes IDE and NEP. Similar effects were also seen in wild type animals following central infusion of Aβ (Abuznait et al., 2013). These benefits may have been due to the activation of the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ) that was also observed (Qosa et al., 2015), which (among other effects) forms heterodimers with the retinoid-X receptor (RXR) to reduce the expression of pro-inflammatory cytokines by acting as an NFκB antagonist (Ricote et al., 1998).

Support for this conclusion is provided by an equally impressive line of work on RXR activation by the omega-3 fatty acid docosahexaenoic acid (DHA; de Urquiza et al., 2000) and a pharmacological agonist, bexarotine. Either DHA or bexarotine enhances ApoE expression, attenuates the release of cytokines and reduces levels of Aβ in both AD model mice (5xFAD) and LPS-stimulated glial cells *in vitro* (Casali et al., 2015). In the AD mice, DHA and bexarotine also attenuate deficits in working memory (spontaneous alternation) in an additive manner. Part of the improved Aβ clearance rate may be due to increased levels of soluble ApoE in peripheral circulation, reported in an earlier study on DHA supplementation in humans (Buckley et al., 2004). Remarkably, activation of RXR by bexarotene was even able to reverse the accumulation of Aβ, tau, reduction of synaptic proteins, and spatial learning impairments (water maze) in ApoE4 transgenic mice (Boehm-Cagan & Michaelson, 2014). These effects were linked to amelioration of lipidation deficiency of ApoE in ApoE4 animals (not present in ApoE2/3 controls; Boehm-Cagan & Michaelson, 2014), which affects the ability of ApoE to bind Aβ (Tokuda et al., 2000). Thus, dietary polyphenols (specifically EGCG and oleocanthal) and DHA are promising candidates that may protect against initial BBB degeneration and amyloid production as well as promote Aβ clearance and may restore ApoE functionality in ApoE4 carriers.

There are also additional limitations in using animal models to study disease. For instance, although the 3xTg-AD mouse model is among the best currently available (Bilkei-

Gorzo, 2014), there is a massive loss of neurons in the CA1 subfield of the hippocampus in human AD patients (West et al., 2014) that is not seen in the brains of 3xTg-AD mice (Manaye et al., 2013). Translational studies (i.e. clinical trials) in humans are therefore needed before we can be certain how helpful the lifestyle based prophylactic approach presented in the thesis will ultimately be. Part of the problem may have to do with differences in how human and animal research is conducted. Indeed, the disconnect between the approaches taken in most animal (e.g. monotherapeutic and using a very limited number of tests) vs. human research may explain, at least partially, why so many pharmaceutical or nutritional interventions initially appear very promising based on the results of cell culture and animal studies but fail to live up to their hype in clinical trials in patients with AD or MCI. An example of such a nutritional supplement is Souvenaid (or Fortasyn Connect), which contains primarily DHA, phostatidylcholine and uridine monophosphate as core ingredients (Cansev & Wurtman, 2007; Scheltens et al., 2010). Work in gerbils showed that such a combination can improve synaptic protein levels (Cansev & Wurtman, 2007), suggesting a potential benefit as a treatment, which was subsequently found to provide some small benefits in terms of memory among early to moderate stage AD patients (Sheltens et al., 2010; Scheltens et al., 2012), yet ultimately failed to prevent the progression of the disease in such patients (Shah et al., 2013). The lesson to be learned from Souvenaid is twofold: first, that promising results for a new treatment based on animal research should be evaluated in the context of a wider variety of tests such as the battery used in our work with the 3xTg-AD mouse (Chapters 3 and 4), which is more similar to the way in which humans are tested. Second, that monotherapeutic approaches to treating complex age-related diseases like AD may be less effective than combining multiple treatments, like nutritional supplements and exercise (Bredesen & John, 2013; Mattson, 2015).

The same logic of capitalizing upon synergistic effects between components of a multi-ingredient supplement applies here. In fact, the most successful pilot longitudinal study in AD patients to date, the metabolic enhancement for neurodegeneration (MEND) program (Bredesen & John, 2013; Bredesen, 2014), has taken such an approach with unprecedented success. Although only 10 patients have been studied as part of the program to date, the majority (9/10) have exhibited lasting improvements in cognitive functioning to the extent in some cases (6/10) that they are able to return to work. Although the MEND program is personalized, it is noteworthy that many of the treatment plans for the subjects include nutraceuticals also present in our MDS (e.g. fish oil and curcumin), as well as exercise. Thus, a multi-ingredient supplement with consistent supporting evidence across multiple model systems, such as the MDS (Lemon et al., 2003; Lemon et al., 2008; Aksenov et al., 2013; Hutton et al., 2015; Hutton et al., 2018), may be well suited to be included in similar programs on a larger scale (i.e. for a standardized preventative approach, rather than personalized treatment approach).

A more scalable, standardized, treatment approach would be well-informed using data from longitudinal observational studies, such as the Canadian Longitudinal Study on Aging (CLSA). The CLSA is among the most comprehensive research platforms on healthy aging in

the world, making use of a wide range of information about the evolving biomedical, psychological, social and economic circumstances of people's lives. Last year, the CLSA completed baseline data collection from over 50,000 community-dwelling women and men aged 45 to 85 years throughout Canada. Of the 51,352 participants, 21,241 provided information through telephone interviews (tracking cohort) and 30,111 through in-home interviews and physical assessments at one of eleven data collection sites across Canada (comprehensive cohort; https://datapreview.clsa-elcv.ca/). Participants in the comprehensive cohort were also asked to provide a blood and urine sample (optional). Repeated waves of CLSA data collection will occur at three-year intervals for at least 20 years, bolstered by a mid-wave maintaining contact questionnaire (MCQ) administered via telephone to maximize retention and collect a small amount of additional data. Dr. Becker, Melanie Lysenko-Martin, and I are currently examining baseline data collected from the comprehensive cohort (n = 30,111) of the CLSA in collaboration with McMaster epidemiologists Parminder Raina, Lauren Griffith, and Anne Gilsing.

Our long-term goal for the project is to understand the interactive effects of antioxidant / anti-inflammatory factors and exercise on healthy ageing and resilience to depression or memory impairment. The specific goals of the study are to examine the baseline data from the CLSA to determine: 1) if exercise levels are positively associated with mood and hippocampal-dependent cognition, 2) if consumption of an anti-oxidant and/or anti-inflammatory diet is beneficial on its own or modifies the relationship between exercise, mood and cognition, 3) if such associations are present for diet and/or exercise do they differ for middle-aged adults and older adults, 4) or for males and females. However, given that participants with cognitive impairment at the time of recruitment were excluded from the CLSA (www.clsa-elcv.ca), these relationships cannot be examined in patients with AD until later waves of data become available.

To achieve these goals, we are examining CLSA questionnaire items and test scores related to age, sex, physical activity, diet, depression and other stress-related disorders and hippocampus-dependent cognition. For example, measures considered to evaluate hippocampal-dependent cognition include the prospective memory test (PMT; Ferbinteanu & Shapiro, 2003; Schacter et al., 2007) and the Rey auditory verbal learning test (RAVLT; Manns et al., 2003; O'Brien et al, 2010), but not the Stroop neurological screening test (STP; Adleman et al., 2002), controlled oral word association test (FAS; Nichols et al., 2006) or choice reaction time test (CRT; Eshel et al., 2007). To quantify exercise levels, we used participant exercise questionnaire responses to construct a physical activity scale variable following the method recommended by Washburn et al. (1993). In addition, given that inflammation and oxidative balance were not explicitly considered in the CLSA questionnaires, we have adapted indices of dietary inflammation (Cavicchia et al., 2009; Shivappa et al., 2014) and oxidative balance (Wright et al., 2004; Kong et al., 2014) from the literature which have already been validated with biomarkers of inflammation (ex. C-reactive protein) and oxidative stress (ex. F2-isoprostanes). These indices are being used as the primary dietary predictor variables during data analysis.

Participant scores on these modified indices were calculated using data from relevant items on the diet and nutritional risk questionnaires. These include both similar items to those used in previous work on the dietary indices and others that are unique to the CLSA and are known from the literature to be associated (negatively or positively) with inflammation and/or oxidative stress. For example, iron or ferritin intake was considered pro-inflammatory or pro-oxidative while β-carotene intake from food items like carrots was considered anti-inflammatory or anti-oxidative (Shivappa et al., 2014; Kong et al., 2014). Since the consumption of individual nutrients such as β-carotene or flavonols (specified in the prior indices) are not specified in the CLSA short diet questionnaire, we instead created scores for each participant based on the z-score of their frequency of food item consumption compared to the rest of the sample, then assigned a positive or negative weight for that item if it is presumed to be protective (e.g. green tea) or harmful (e.g. processed red meat) respectively. The sum of assigned scores for all relevant CLSA questionnaire items was then used to generate a total score, enabling the evaluation of each predictor in linear and logistic regression models for each of the outcome variables (mood and cognition). We also plan to score dietary supplement intake similarly (for items like green tea extract) and factor those items into the total index scores. A full list of the items used (and assigned weights) for both of the derived dietary indices will be disclosed in supplementary tables with the project manuscript following study completion, sometime later this year.

To begin with, we reviewed the literature on food items that were included in the CLSA questionnaires to determine how they should be characterized in terms of their anti-inflammatory/pro-inflammatory or anti-oxidative/pro-oxidative effects, then constructed a single pilot (combined) anti-inflammatory/anti-oxidative dietary index based on a subset of these items. We then evaluated these variables as predictors of mood and cognitive function. As hypothesized, this initial analysis revealed beneficial associations between physical activity and mood (depression questionnaire scores), a prior diagnosis of major depressive disorder, and cognitive functions known to be involved in age-related cognitive decline: delayed recall on the RAVLT, immediate recall on the RAVLT (i.e. working memory) and executive function (stroop test). Furthermore, a stronger relationship between exercise and memory was observed in older adults (over 65 years of age) than middle-aged adults (45-64 years of age). Middle-aged adults were also found to have higher levels of depression than older adults. Across both sexes, exercise accounted for more than double the variance in memory test results between middle-age and old-age adults (i.e. the effect of age). Unexpectedly, the pilot dietary index was only found to be significantly correlated with exercise.

To examine the relationship between nutrition and mental health in more detail, we subsequently created two separate dietary indices based on a more comprehensive review of the literature (an inflammatory diet scale [IDS], and an oxidative diet scale [ODS]). Since the number of white blood cells (a biomarker of inflammation; Willems et al., 2010) and lung function (a biomarker of physical fitness; Cheng et al., 2003) were available in the sample, we also examined correlations between physical activity scale for the elderly (PASE) score vs. lung

function and IDS score vs. WBC count. PASE score was found to be positively correlated with average forced vital capacity, while WBC count was negatively correlated with AIS scores (high AIS score = high anti-inflammatory food consumption), suggesting that both may be considered valid indicators of physical fitness and dietary inflammation, respectively. Unfortunately, biomarkers of oxidative stress are not presently available in the CLSA data, so the ODS scale will need to be validated in the future if appropriate biomarker data becomes available.

When re-analysing the data using these new dietary indices, adherence to an anti-inflammatory, but not anti-oxidative, diet and exercise were both found to be positively associated with mood (i.e. lower CESD-10 scores). Interestingly, adherence to an anti-inflammatory diet was found to be a stronger predictor of mood than physical activity (both significant) in men and women 65 years of age or older, while the reverse was true for men and women between 45-64 years of age. Moreover, this trend was also observed for immediate and delayed recall on the RAVLT and executive function (mental alternation test). In contrast, exercise, but neither dietary index, was associated with event and time-based prospective memory, while neither exercise nor the dietary indices were associated with psychomotor speed (choice reaction time). These results suggest that exercise may have a larger impact on mood, executive function, and associative memory (RAVLT) than the consumption of anti-oxidant or anti-inflammatory foods among Canadians in middle age (between 45-64 years of age), while consumption of an anti-inflammatory diet seemed to benefit these aspects of mental health more than exercise did in seniors (65+ years of age). In addition, physical activity, but not adherence to an anti-inflammatory or anti-oxidant diet, was positively associated with prospective memory.

Ongoing work, led by my collaborators, will determine if these relationships are affected by potential covariates such as income or education level, to ensure that our findings are robust before submitting them for publication. We are also exploring some alternative methods of examining the relationship between nutrition and mental health. Specifically, we are working on determining whether adherence to a common dietary pattern that has some overlap with the IDS (the Mediterranean diet) exhibits a similar pattern of results. These two hypothesis-driven analyses will then be compared to an exploratory machine learning approach, the latter of which may identify a novel dietary pattern (based on foods included in the CLSA questions) for optimizing the mood and cognitive function of Canadians in middle and old age.

MDD contributes to age-related mental and physical decline, and symptoms of depression are twice as prevalent in adults over 65 years of age relative to those under 65 (Fiske et al., 2009). MDD has been associated with multiple age-related diseases (Fiske et al., 2009) including type II diabetes (15% of cases), heart disease (20-25% of cases) and Alzheimer's disease (10-15% of cases), emphasizing the need to identify factors common to MDD, aging and age-related illness which may be counteracted to protect against MDD. Ours and future studies with the CLSA data have the potential to profoundly impact the physical and mental well-being of Canadians.

A final nontrivial consideration is that use of genetically modified animals as model systems of human age-related diseases neglects the influence of the perinatal environment and nutrition in the development of the body's stress response system, the hypothalamic-pituitary-adrenal (HPA) axis. This is particularly relevant for the 3xTg-AD mouse, since dams of the strain also exhibit impairments in nesting and maternal care behaviours, as well as difficulty breeding (personal observations; Torres-Lista & Giménez-Llort, 2013; Sterniczuk et al., 2010).

The physiological responses of the mammalian body to threats or challenges (i.e. stress) are mediated by a neuroendocrine system referred to as the HPA axis (See Lupein et al., 2009 for a review). Briefly, when a threat is detected, the paraventricular nucleus (PVN) of the hypothalamus secretes corticotropin-releasing hormone/factor (CRH) and arginine vasopressin, which trigger the secretion of adrenocorticotropic hormone (ACTH) into circulation by the pituitary gland, which in turn stimulates the release of corticosteroids by the adrenal cortex (Lupein et al., 2009). Corticosteroids then produce a number of changes by acting upon glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs) to prepare the body to deal with the threat (e.g. suppressing the immune system, stimulating glycogenolysis to provide energy to muscles). When the hippocampus, which contains GRs and MRs, has detected that the threat has passed, it sends inhibitory feedback to the PVN to shut off the release of corticosteroids (Lupein et al., 2009). It is worth noting that the PVN responds to both psychological-derived threats and disturbances to metabolic homeostasis, detected by changes in the circulating levels of molecules such as leptin, insulin, glucose, and ghrelin (both in adulthood and early life; Lucassen et al., 2013). Thus, both nutrient imbalances and psychological threats trigger a similar HPA-axis mediated stress response that prepares and motivates the animal to resolve the issue (e.g. an infant crying for milk).

Inadequate maternal care likely also leads to nutritional deficiencies. Moreover, epidemiological evidence in humans suggests that nutritional deficiency (e.g. famine) during the last trimester of gestation is associated with a 50% increased risk of mood/anxiety disorders (Brown et al., 2000), as well as worse performance on tests of selective attention (but not learning or memory) in middle age (de Rooij et al., 2010; de Groot et al., 2011). It is encouraging to note, however, that a meta-analysis of 20 controlled trials examining the effects of breast-feeding vs. formula on cognitive function found breast-feeding to improve cognitive outcomes, especially (larger magnitude effect) in adults who had been born underweight (Anderson et al., 1999). The provision of nutritious milk after birth may therefore attenuate some of the damage done by a nutritional insult/deficiency during gestation. More recent research has shown that protein restriction during gestation or lactation also elicits anxiety- (plus maze, open field) and depressive-like behaviour (tail suspension test) in mice (Belluscio et al., 2014). In humans, low birth weight is associated with elevated levels of glucocorticoid metabolites excreted in the urine of children (Clark et al., 1996). In adults, low birth weight is also associated with increased basal plasma cortisol levels, and an abnormally high adrenocortical responsiveness to ACTH challenge (Phillips et al., 1998).

In addition to negative effects of nutritional deficiency on brain development, there is evidence that both perinatal psychological stress (in the mother before birth or the offspring afterwards) and nutritional stress both induce lasting changes, or programming effects, on the HPA axis. For example, a 50% food restriction intervention in pregnant rats during embryonic days 19-21 was found to increase maternal glucocorticoid levels, placental glucocorticoid transfer (decreased placental 11β-hydroxysteroid dehydrogenase expression), glucocorticoid exposure by the fetus (higher levels detected in cord blood and at birth) and decrease offspring adrenal and body weight (Lesage et al., 2001). In that study, maternal food restriction also reduced GR and MR receptor expression in the hippocampus, CRH expression by the PVN, and plasma ACTH levels, suggesting that the development of the HPA-axis had been perturbed. Notably, these HPA-axis changes were not observed if maternal corticosterone levels were kept at basal levels (adrenalectomy supplemented with corticosterone injections), confirming that elevated maternal corticosteroid levels were responsible for the HPA-axis programming that occurred after food restriction.

Although the mechanisms by which nutritional insults alter the programming of the extended (including the hippocampus) HPA-axis are not directly known, some progress has been made on this front by studying the effects of maternal separation, abuse, or restraint stress perinatally. In rats, maternal separation between postnatal days 2-13 has been shown to increase plasma corticosteroid levels and CRH mRNA expression in the PVN following acute restraint stress, concomitant with hypomethylation of the (CRE) promoter for CRH in the PVN (Chen et al., 2012). A post mortem study of ~30-year-old male suicide victims found that those with a history of child abuse vs. those without a history of abuse (or individuals who did not die of suicide) had decreased expression of GRs, as well as an increase in cytosine methylation of the neuron-specific GR promoter NR3C1 in the hippocampus (McGowan et al., 2009). Thus, epigenetic changes to the PVN and hippocampus may mediate the reprogramming of the HPA-axis by perinatal stress or malnutrition.

5.3. Final Remarks and Conclusions

In Chapter 2, it was shown that in chronically stressed mice, exercise combined with a complex dietary supplement, but neither treatment alone, was sufficient improve numerous measures of hippocampal health under conditions of chronic stress (Hutton et al, 2015). Specifically, a combination of voluntary aerobic exercise and supplementation with anti-oxidant and anti-inflammatory factors reduced markers of hippocampal degeneration (reduced volume, BDNF and neurogenesis) and symptoms of anhedonia (a diagnostic factor in MDD). That exercise alone was insufficient to increase BDNF in chronically stressed mice is consistent with the human literature, in that aerobic exercise seems to increase circulating levels of BDNF in healthy adults (Dinoff et al., 2016) but may not in patients with MDD (Kurebayashi & Otaki, 2018). Others have found that in rodents, supplementation with the omega-3 fatty acid DHA reduced hippocampal levels of oxidized proteins and enhanced exercise-induced increases in BDNF, synaptic plasticity and spatial memory (Wu et al., 2008; Chytrova et al., 2010). Similarly, in elderly humans (70-79yrs), serum markers of inflammation (tumor necrosis factor-α, C-reactive protein) were decreased by both exercise and antioxidant vitamins (Vitamin E, β-carotene; Colbert et al., 2004). In addition, risks of age-related cognitive impairment, dementia and mortality were reduced in men (initially aged 45-59 and then followed for 30 years) who engaged in multiple healthy lifestyle behaviours, including high fruit/vegetable consumption and regular exercise (alcohol consumption, smoking and BMI were also examined), relative to those who engaged in a single healthy behaviour (or none; Elwood et al., 2013). Thus, under conditions of elevated oxidative stress and inflammation, exercise and dietary interventions may act synergistically to protect against MDD and cognitive impairment.

Chapters 3 and 4 supported the idea that dietary inventions may be effective prophylactic measures against AD. This work showed that MDS supplementation from 2-12 months of age had numerous benefits in the 3xTg-AD mouse model. Most notably, it maintained spatial learning and working memory in 3xTg-AD mice at WT levels at 3-4 months of age, upregulated hippocampal BDNF in 3xTg-AD mice (both sexes at 6-7 months of age, females only at 11-12 months of age), attenuated the splenomegaly exhibited by the 3xTg-AD strain at 11-12 months of age, and normalized the altered pattern of hippocampal activity after spatial learning that was detected in 3xTg-AD males. The idea of preventing AD via healthy eating is also garnering increasing recognition in the literature (e.g. Solfrizzi et al., 2007; Mattson, 2015). For instance, adherence to a Mediterranean style diet, emphasizing consumption of fruits, vegetables, nuts, fish, olive oil (also low in dairy, saturated fats and red meat), and including low-moderate alcohol consumption has been associated with delayed cognitive decline and a 45% reduced risk of converting from MCI to AD in both American and French populations over 65 years of age (Féart et al., 2010). The Mediterranean diet has also been associated with the preservation of white matter connectivity, a metric of functional brain aging, along with a very strong anti-aging effect (8-10-year delay) on episodic memory, one of the primary cognitive functions impacted by AD (Pelletier et al., 2015). A study on the effects of the Mediterranean dietary pattern and

exercise in cognitively-normal middle-aged individuals (Matthews et al., 2014) found using PET scans that both were linked to reduced cerebral amyloid levels and improvements in cerebral glucose metabolism, both AD risk factors.

Plant derived polyphenols and unsaturated fatty acids appear to underlie the benefits ascribed to the Mediterranean diet. The omega-3 fatty acid docosahexaenoic acid (DHA; which is present in the MDS) alone can account for a surprising range of neurological benefits ascribed to the Mediterranean diet or fish consumption. A longitudinal (9-year) study on initially healthy older adults found that those in the 75% percentile for plasma phospholipid-associated DHA had a 47% reduced risk of AD (Schaefer et al., 2006). Six months of a low dose (1.16 g/d DHA) in healthy 18-45-year-old adults was found to improve reaction times on measures of episodic memory (females), working memory (males), and attention, effects which were much larger for ApoE4 carriers (Stonehouse et al., 2013). Major challenges in dietary intervention studies are the variability of food nutrients from different sources (or preparation methods) and requirement of high adherence for benefits to be observed. In contrast, the use of supplements facilitates adherence and enables consistent dosing, suggesting that it may be a more reliable approach to preventing AD than simply providing advice on healthy eating.

The more recent vascular hypothesis of AD (de la Torre, 2000), which proposes that cerebrovascular degeneration is the primary cause of late-onset AD, offers a promising alternative to the popular amyloid cascade hypothesis (Hardy & Higgins, 1992). This is particularly apparent based on evidence in rodents (de la Torre, 2000) that cerebral hypoperfusion leads to degeneration of the hippocampus, parietal and frontal cortices, like the pattern seen in human AD (Braak & Braak, 1991). Moreover, that cerebrovascular degeneration may be amenable to remediation using polyphenol-containing supplements (Liu et al., 2013) strongly recommends their use future research on AD prevention.

In addition, perinatal dysregulation of the HPA axis due to inadequate maternal care may explain part of the reason why 3xTg-AD mice, especially females, exhibit a heightened glucocorticoid response to stress, which can impact performance on common tests like the Morris water maze (Clinton et al., 2007). It would therefore be insightful to either place 3xTg-AD pups under the care of WT dams or begin therapeutic interventions at the start of gestation. Whether or not this also occurs in other AD model rodent strains remains to be examined. Perinatal stress could therefore lock the stress response system of AD into a mode of operating that more strongly initiates corticosteroid-stress responses, but more weakly terminates those responses. Consequently, this developmental programming would result in chronic stress exposure, which may damage the hippocampus over time and lead to learning impairments and difficulty regulating mood, ultimately predisposing affected offspring to develop clinically significant learning disabilities, depression, or dementia later in life.

Finally, our ongoing work with the CLSA dataset affords a unique opportunity to examine the complex associations amongst dietary factors and exercise, in both males and

females of various ages, while controlling for numerous covariates such as illness burden. Importantly, by comparing middle-aged (45-65) and older (over 65) adults, this work has the potential to reveal nutritional, dietary and exercise patterns that may reduce the risk of MDD or cognitive impairment in older individuals. In the longer term, this work will lay the foundation for further longitudinal studies with the CLSA dataset that may uncover causal relationships between diet, exercise and mental health in humans.

References

Abuznait, A. H., Qosa, H., Busnena, B. a., El Sayed, K. a., & Kaddoumi, A. (2013). Olive-oil derived oleocanthal enhances ß-amyloid clearance as a potential neuroprotective mechanism against Alzheimer's disease: In vitro and in vivo studies. *ACS Chemical Neuroscience*, *4*, 973–982.

Adleman, N. E., Menon, V., Blasey, C. M., White, C. D., Warsofsky, I. S., Glover, G. H., & Reiss, A. L. (2002). A developmental fMRI study of the Stroop color-word task. *Neuroimage*, *16*(1), 61-75.

Aksenov, V., Long, J., Liu, J., Szechtman, H., Khanna, P., Matravadia, S., & Rollo, C. D. (2013). A complex dietary supplement augments spatial learning, brain mass, and mitochondrial electron transport chain activity in aging mice. *Age (Dordrecht, Netherlands)*, *35*(1), 23 33.

Anderson, J. W., Johnstone, B. M., & Remley, D. T. (1999). Breast-feeding and cognitive development: a meta-analysis. *The American journal of clinical nutrition*, *70*(4), 525-535.

Belluscio, L. M., Berardino, B. G., Ferroni, N. M., Ceruti, J. M., & Cánepa, E. T. (2014). Early protein malnutrition negatively impacts physical growth and neurological reflexes and evokes anxiety and depressive-like behaviors. *Physiology & behavior*, *129*, 237-254.

Bilkei-Gorzo, A. (2014). Genetic mouse models of brain ageing and Alzheimer's disease. *Pharmacology & therapeutics*, *142*(2), 244-257.

Bredesen, D. E. (2014). Reversal of cognitive decline: A novel therapeutic program. *Aging (Albany NY)*, *6*(9), 707.

Bredesen, D. E., & John, V. (2013). Next generation therapeutics for Alzheimer's disease. *EMBO molecular medicine*, *5*(6), 795-798.

Braak, H., & Braak, E. (1991). Neuropathological stageing of Alzheimer-related changes. *Acta Neuropathologica*, *82*(4), 239–259.

Brown, A. S., van Os, J., Driessens, C., Hoek, H. W., & Susser, E. S. (2000). Further evidence of relation between prenatal famine and major affective disorder. *American Journal of Psychiatry*, *157*(2), 190-195.

Buckley, R., Shewring, B., Turner, R., Yaqoob, P., & Minihane, A. M. (2004). Circulating triacylglycerol and apoE levels in response to EPA and docosahexaenoic acid supplementation in adult human subjects. *British Journal of Nutrition*, *92*, 477.

Cansev, M., & Wurtman, R. J. (2007). Chronic administration of docosahexaenoic acid or eicosapentaenoic acid, but not arachidonic acid, alone or in combination with uridine,

increases brain phosphatide and synaptic protein levels in gerbils. *Neuroscience*, *148*(2), 421-431.

Casali, B. T., Corona, A. W., Mariani, M. M., Karlo, J. C., Ghosal, K., & Landreth, G. E. (2015). Omega-3 fatty acids augment the actions of nuclear receptor agonists in a mouse model of Alzheimer's disease. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *35*, 9173–81.

Cavicchia, P. P., Steck, S. E., Hurley, T. G., Hussey, J. R., Ma, Y., Ockene, I. S., & Hébert, J. R. (2009). A new dietary inflammatory index predicts interval changes in serum high sensitivity C-reactive protein. *The Journal of nutrition*, *139*(12), 2365-2372.

Chen, J., Evans, A. N., Liu, Y., Honda, M., Saavedra, J. M., & Aguilera, G. (2012). Maternal deprivation in rats is associated with corticotrophin-releasing hormone (CRH) promoter hypomethylation and enhances CRH transcriptional responses to stress in adulthood. *Journal of neuroendocrinology*, *24*(7), 1055-1064.

Cheng, Y. J., Macera, C. A., Addy, C. L., Sy, F. S., Wieland, D., & Blair, S. N. (2003). Effects of physical activity on exercise tests and respiratory function. *British journal of sports medicine*, *37*(6), 521-528.

Chytrova, G., Ying, Z., & Gomez-Pinilla, F. (2010). Exercise contributes to the effects of DHA dietary supplementation by acting on membrane-related synaptic systems. *Brain research*, *1341*, 32-40.

Clark, P. M., Hindmarsh, P. C., Shiell, A. W., Law, C. M., Honour, J. W., & Barker, D. J.(1996). Size at birth and adrenocortical function in childhood. *Clinical endocrinology*, *45*(6), 721-726.

Clinton, L. K., Billings, L. M., Green, K. N., Caccamo, A., Ngo, J., Oddo, S., ... & LaFerla, F. M. (2007). Age-dependent sexual dimorphism in cognition and stress response in the 3xTg-AD mice. *Neurobiology of disease*, *28*(1), 76-82.

Colbert, L. H., Visser, M., Simonsick, E. M., Tracy, R. P., Newman, A. B., Kritchevsky, S. B., ... & Harris, T. B. (2004). Physical activity, exercise, and inflammatory markers in older adults: findings from the Health, Aging and Body Composition Study. *Journal of the American Geriatrics Society*, *52*(7), 1098-1104.

Cox, C. J., Choudhry, F., Peacey, E., Perkinton, M. S., Richardson, J. C., Howlett, D. R., … Williams, R. J. (2015). Dietary (-)-epicatechin as a potent inhibitor of β-secretase amyloid precursor protein processing. *Neurobiology of Aging*, *36*, 178–187.

Cummings, J. L., Morstorf, T., & Zhong, K. (2014). Alzheimer's disease drug-development pipeline: few candidates, frequent failures. *Alzheimer's research & therapy*, *6*(4), 37.

de Groot, R. H., Stein, A. D., Jolles, J., van Boxtel, M. P., Blauw, G. J., Van de Bor, M., & Lumey, L. H. (2011). Prenatal famine exposure and cognition at age 59 years. *International Journal of Epidemiology*, *40*(2), 327-337.

de la Torre, J. C. (2000). Critically attained threshold of cerebral hypoperfusion: can it cause Alzheimer's disease? *Annals of the New York Academy of Sciences*, *903*, 424–436.

de Rooij, S. R., Wouters, H., Yonker, J. E., Painter, R. C., & Roseboom, T. J. (2010). Prenatal undernutrition and cognitive function in late adulthood. *Proceedings of the National Academy of Sciences*, *107*(39), 16881-16886.

de Urquiza, A. M., Liu, S., Sjoberg, M., Zetterstrom, R. H., Griffiths, W., Sjovall, J., & Perlmann, P. (2000). Docosahexaenoic acid, a ligand for the retinoid X receptor in mouse brain. *Science*, *290*, 2140–2144.

Dinoff, A., Herrmann, N., Swardfager, W., Liu, C. S., Sherman, C., Chan, S., & Lanctôt, K. L. (2016). The effect of exercise training on resting concentrations of peripheral brain derived neurotrophic factor (BDNF): a meta-analysis. *PLoS One*, *11*(9), e0163037.

Eshel, N., Nelson, E. E., Blair, R. J., Pine, D. S., & Ernst, M. (2007). Neural substrates of choice selection in adults and adolescents: development of the ventrolateral prefrontal and anterior cingulate cortices. *Neuropsychologia*,*45*(6), 1270-1279.

Féart, C., Samieri, C., & Barberger-Gateau, P. (2010). Mediterranean diet and cognitive function in older adults. *Current opinion in clinical nutrition and metabolic care*, *13*(1), 14.

Ferbinteanu, J., & Shapiro, M. L. (2003). Prospective and retrospective memory coding in the hippocampus. *Neuron*, *40*(6), 1227-1239.

Fiske, A., Wetherell, J. L., & Gatz, M. (2009). Depression in older adults. *Annual review of clinical psychology*, 5, 363.

Hardy, J. A., & Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science*, *256*(5054), 184.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology*, *11*, 298–230.

Herrup, K. (2015). The case for rejecting the amyloid cascade hypothesis. *Nat Neurosci*, *18*(6), 794–799.

Kane, A. E., Shin, S., Wong, A. A., Fertan, E., Faustova, N. S., Howlett, S. E., & Brown, R. E. (2018). Sex differences in healthspan predict lifespan in the 3xTg-AD mouse model of Alzheimer's disease. *Frontiers in Aging Neuroscience*, *10*, 1-10.

Kapadia, M., Mian, M. F., Michalski, B., Azam, A., Ma, D., Salwierz, P., ... & Fahnestock, M. (2018). Sex-Dependent Differences in Spontaneous Autoimmunity in Adult 3xTg-AD Mice. *Journal of Alzheimer's Disease*, (Preprint), 1-15.

Kong, S. Y. J., Bostick, R. M., Flanders, W. D., McClellan, W. M., Thyagarajan, B., Gross, M. D., ... & Goodman, M. (2014). Oxidative balance score, colorectal adenoma, and markers of oxidative stress and inflammation. *Cancer Epidemiology Biomarkers & Prevention*, *23*(3), 545-554.

Kurebayashi, Y., & Otaki, J. (2018). Does physical exercise increase brain-derived neurotrophic factor in major depressive disorder? A meta-analysis. *Psychiatria Danubina*, *30*(2), 129 135.

Lemon, J. A., Boreham, D. R., & Rollo, C. D. (2003). A dietary supplement abolishes age-related cognitive decline in transgenic mice expressing elevated free radical processes. *Experimental biology and medicine*, *228*(7), 800-810.

Lemon, J. A., Rollo, C. D., McFarlane, N. M., & Boreham, D. R. (2008). Radiation-induced apoptosis in mouse lymphocytes is modified by a complex dietary supplement: the effect of genotype and gender. *Mutagenesis*, *23*(6), 465-472.

Lesage, J., Blondeau, B., Grino, M., Breant, B., & Dupouy, J. P. (2001). Maternal undernutrition during late gestation induces fetal overexposure to glucocorticoids and intrauterine growth retardation, and disturbs the hypothalamo-pituitary adrenal axis in the newborn rat. *Endocrinology*, *142*(5), 1692-1702.

Li, J., Ye, L., Wang, X., Liu, J., Wang, Y., Zhou, Y., & Ho, W. (2012). (-)-Epigallocatechin gallate inhibits endotoxin-induced expression of inflammatory cytokines in human cerebral microvascular endothelial cells. *Journal of Neuroinflammation*, *9*, 161.

Liu, X., Wang, Z., Wang, P., Yu, B., Liu, Y., & Xue, Y. (2013). Green tea polyphenols alleviate early BBB damage during experimental focal cerebral ischemia through regulating tight junctions and PKCalpha signaling. *BMC Complementary and Alternative Medicine*, *13*, 187.

Lucassen, P. J., Naninck, E. F., van Goudoever, J. B., Fitzsimons, C., Joels, M., & Korosi, A. (2013). Perinatal programming of adult hippocampal structure and function; emerging roles of stress, nutrition and epigenetics. *Trends in neurosciences*, *36*(11), 621-631.

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature reviews neuroscience*, *10*(6), 434.

Mah, L., Binns, M. A., Steffens, D. C., & Alzheimer's Disease Neuroimaging Initiative. (2015). Anxiety symptoms in amnestic mild cognitive impairment are associated with medial

temporal atrophy and predict conversion to Alzheimer disease. *The American Journal of Geriatric Psychiatry*, *23*(5), 466-476.

Manaye, K. F., Mouton, P. R., Xu, G., Drew, A., Lei, D. L., Sharma, Y., ... & Turner, S. (2013). Age-related loss of noradrenergic neurons in the brains of triple transgenic mice. *Age*, *35*(1), 139-147.

Manns, J. R., Hopkins, R. O., Reed, J. M., Kitchener, E. G., & Squire, L. R. (2003). Recognition memory and the human hippocampus. *Neuron*, *37*(1), 171-180.

Marchese, M., Cowan, D., Head, E., Ma, D., Karimi, K., Ashthorpe, V., ... & Sakic, B. (2014). Autoimmune manifestations in the 3xTg-AD model of Alzheimer's disease. *Journal of Alzheimer's Disease*, *39*(1), 191-210.

Matthews, D. C., Davies, M., Murray, J., Williams, S., Tsui, W. H., Li, Y., ... & de Leon, M. J. (2014). Physical activity, Mediterranean diet and biomarkers-assessed risk of Alzheimer's: a multi-modality brain imaging study. *Advances in molecular imaging*, *4*(4), 43.

Mattson, M. P. (2015). Late-onset dementia: a mosaic of prototypical pathologies modifiable by diet and lifestyle. *npj Aging and Mechanisms of Disease*, *1*, 15003.

McGowan, P. O., Sasaki, A., D'alessio, A. C., Dymov, S., Labonté, B., Szyf, M., ... & Meaney, M. J. (2009). Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. *Nature neuroscience*, *12*(3), 342.

Nichols, E. A., Kao, Y. C., Verfaellie, M., & Gabrieli, J. D. (2006). Working memory and long-term memory for faces: Evidence from fMRI and global amnesia for involvement of the medial temporal lobes. *Hippocampus*, *16*(7), 604.

O'brien, J. L., O'keefe, K. M., LaViolette, P. S., DeLuca, A. N., Blacker, D., Dickerson, B. C., & Sperling, R. A. (2010). Longitudinal fMRI in elderly reveals loss of hippocampal activation with clinical decline. *Neurology*, *74*(24), 1969-1976.

Parachikova, A., Green, K. N., Hendrix, C., & LaFerla, F. M. (2010). Formulation of a medical food cocktail for Alzheimer's disease: beneficial effects on cognition and neuropathology in a mouse model of the disease. *PloS one*, *5*(11), e14015.

Pelletier, A., Barul, C., Féart, C., Helmer, C., Bernard, C., Periot, O., ... & Catheline, G. (2015). Mediterranean diet and preserved brain structural connectivity in older subjects. *Alzheimer's & Dementia*, *11*(9), 1023-1031.

Phillips, D. I. W., Barker, D. J. P., Fall, C. H. D., Seckl, J. R., Whorwood, C. B., Wood, P. J., & Walker, B. R. (1998). Elevated plasma cortisol concentrations: a link between low birth

weight and the insulin resistance syndrome?. *The Journal of Clinical Endocrinology & Metabolism*, *83*(3), 757-760.

Prince, M., Bryce, R., Albanese, E., Wimo, A., Ribeiro, W., & Ferri, C. P. (2013). The global prevalence of dementia: a systematic review and metaanalysis. *Alzheimer's & Dementia*, *9*(1), 63-75.

Qosa, H., Batarseh, Y. S., Mohyeldin, M. M., El Sayed, K. A., Keller, J. N., & Kaddoumi, A. (2015). Oleocanthal Enhances Amyloid-β Clearance from the Brains of TgSwDI Mice and in Vitro across a Human Blood-Brain Barrier Model. *ACS Chemical Neuroscience*, *6*, 1849–1859.

Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., & Glass, C. K. (1998). The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature*, *391*, 79–82.

Schacter, D. L., Addis, D. R., & Buckner, R. L. (2007). Remembering the past to imagine the] future: the prospective brain. *Nature Reviews Neuroscience*, *8*(9), 657-661.

Schaefer, E. J., Bongard, V., Beiser, A. S., Lamon-Fava, S., Robins, S. J., Au, R., ... & Wolf, P. A. (2006). Plasma phosphatidylcholine docosahexaenoic acid content and risk of dementia and Alzheimer disease: the Framingham Heart Study. *Archives of neurology*, *63*(11), 1545-1550.

Shah, R. C., Kamphuis, P. J., Leurgans, S., Swinkels, S. H., Sadowsky, C. H., Bongers, A., ... & Bennett, D. A. (2013). The S-Connect study: results from a randomized, controlled trial of Souvenaid in mild-to-moderate Alzheimer's disease. *Alzheimer's research & therapy*, *5*(6), 59.

Shivappa, N., Steck, S. E., Hurley, T. G., Hussey, J. R., Ma, Y., Ockene, I. S., ... & Hébert, J. R. (2014). A population-based dietary inflammatory index predicts levels of C-reactive protein in the Seasonal Variation of Blood Cholesterol Study (SEASONS). *Public health nutrition*, *17*(08), 1825-1833.

Solfrizzi, V., D'Introno, A., Colacicco, A. M., Capurso, C., Del Parigi, A., Caselli, R. J., ... & Panza, F. (2007). Incident occurrence of depressive symptoms among patients with mild cognitive impairment–the Italian longitudinal study on aging. *Dementia and geriatric cognitive disorders*, *24*(1), 55-64.

Sterniczuk, R., Antle, M. C., LaFerla, F. M., & Dyck, R. H. (2010). Characterization of the 3xTg-AD mouse model of Alzheimer's disease: part 2. Behavioral and cognitive changes. *Brain research*, *1348*, 149-155.

Stonehouse, W., Conlon, C. A., Podd, J., Hill, S. R., Minihane, A. M., Haskell, C., & Kennedy, D. (2013). DHA supplementation improved both memory and reaction time in healthy young adults: a randomized controlled trial. *The American journal of clinical nutrition*, *97*(5), 1134-1143.

Takechi, R., Pallebage-Gamarallage, M. M., Lam, V., Giles, C., & Mamo, J. C. (2013)a. Aging related changes in blood-brain barrier integrity and the effect of dietary fat. *Neurodegenerative Diseases*, *12*, 125–135.

Takechi, R., Pallebage-Gamarallage, M. M., Lam, V., Giles, C., & Mamo, J. C. (2013)b. Nutraceutical agents with anti-inflammatory properties prevent dietary saturated-fat induced disturbances in blood-brain barrier function in wild-type mice. *Journal of Neuroinflammation*, *10*, 73.

Tokuda, T., Calero, M., Matsubara, E., Vidal, R., Kumar, A., Permanne, B., … Ghiso, J. (2000). Lipidation of apolipoprotein E influences its isoform-specific interaction with Alzheimer's amyloid β peptides. *Biochemical Journal*, *348*, 359–365.

Torres-Lista, V., & Giménez-Llort, L. (2013). Impairment of nesting behaviour in 3xTg-AD mice. *Behavioural brain research*, *247*, 153-157.

Vinters, H. V. (1987). Cerebral amyloid angiopathy. A critical review. *Stroke; a Journal of Cerebral Circulation*, *18*, 311–24.

Walford, R. L. (1964). The immunologic theory of aging. *The Gerontologist*,*4*(4), 195-197.

Walker, J. M., Klakotskaia, D., Ajit, D., Weisman, G. a, Wood, W. G., Sun, G. Y., … Schachtman, T. R. (2014). Beneficial Effects of Dietary EGCG and Voluntary Exercise on Behavior in an Alzheimer's Disease Mouse Model. *Journal of Alzheimer's Disease : JAD*, 1–12.

Wang, X., Xing, A., Xu, C., Cai, Q., Liu, H., & Li, L. (2010). Cerebrovascular hypoperfusion induces spatial memory impairment, synaptic changes, and amyloid-β oligomerization in rats. *Journal of Alzheimer's Disease*, *21*, 813–822.

Washburn, R. A., Smith, K. W., Jette, A. M., & Janney, C. A. (1993). The Physical Activity Scale for the Elderly (PASE): development and evaluation. *Journal of clinical epidemiology*, *46*(2), 153-162.

West, M. J., Coleman, P. D., Flood, D. G., & Troncoso, J. C. (1994). Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. *The Lancet*, *344*(8925), 769-772.

Willems, J. M., Trompet, S., Blauw, G. J., Westendorp, R. G., & de Craen, A. J. (2010). White blood cell count and C-reactive protein are independent predictors of mortality in the oldest old. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences*, *65*(7), 764-768.

Wright, M. E., Mayne, S. T., Stolzenberg-Solomon, R. Z., Li, Z., Pietinen, P., Taylor, P. R., ... & Albanes, D. (2004). Development of a comprehensive dietary antioxidant index and application to lung cancer risk in a cohort of male smokers. *American journal of epidemiology*, *160*(1), 68-76.

Wu, A., Ying, Z., & Gomez-Pinilla, F. (2008). Docosahexaenoic acid dietary supplementation enhances the effects of exercise on synaptic plasticity and cognition. *Neuroscience*, *155*(3), 751-759.

Zlokovic, B. V. (2005). Neurovascular mechanisms of Alzheimer's neurodegeneration. *Trends in Neurosciences*, *28*, 202–208.

Appendix A: Supplementary Materials for Chapter 3

Supplementary Experimental Procedures

Motor Coordination and Muscle Strength

The beam walking test also measures motor coordination but was included in the battery, as it may be a more sensitive measure than the rotarod [1] and may therefore reveal motor deficits in the 3xTg-AD mouse prior to those seen in the hanging basket or rotarod tests.

The rotarod. Animals were placed on the rotating drum (diameter = 3.2 cm, height above padded trough = 16.5 cm), which then accelerated from 4 up to 40 rpm over 5 minutes. The apparatus was cleaned with a 1.5% acetic acid aqueous solution between trials.

The Hanging Basket Test. Each mouse was tested on 2 trials with approximately a 3 h break between trials.

Beam Walking Test. The platforms (and attached beam) were each supported 66 cm above a table by a stainless steel ring stand and attached buret clamp. A 1000 W halogen lamp facing the long axis of the beam was positioned ~ 15 cm from the small rectangular platform and a triangular black Plexiglas shelter (sides: 13.5 cm^2 x 19 cm, height = 14 cm) with a small doorway (4 cm x 6 cm) was placed on the large circular platform. During the shaping and test trial, after climbing onto the large platform with the shelter, the mouse was permitted to explore it for 45s before the next trial was started. The beam was cleaned using 1.5% acetic acid between trials.

Olfactory Acuity and Discrimination

MCI patients exhibit impairments in olfactory sensitivity, while deficits in discrimination ability seem to manifest later, in AD [2]. Thus, deficits in olfactory acuity may appear prior to those in discrimination ability in the 3xTg-AD mouse as well.

Acuity testing. Video recordings of all trials were scored using Observer XT software (Noldus, Toronto, ON) for sniffing time when the mouse was observed to be sniffing within 1 cm of the filter paper. Cages were cleaned before and after each trial using 1.5% acetic acid in aqueous solution.

Open Field

The automated scoring of freezing time and frequency by Ethovision was performed using detection setting parameters that have been described previously and validated for such purposes [3]. The open field was cleaned between mice using a 1.5% aqueous solution of acetic acid.

Integrated Behavioral Station (INBEST)

INBEST boxes were cleaned with Quatricide® between animals. Due to technical issues with the lickometers and food dispenser of the INBEST apparatus in the present study, food and liquid consumption were measured by weighing water bottles and food before and after testing.

Novel Object Recognition Test

An assortment of glass, plastic, polished rock and painted metal statues were used as objects, all approximately 0.5-1 kg in weight and 200-500 cm^3 in size, which were chosen because they were sufficiently heavy and irregularly shaped that the mice could not easily move or climb on them. A different set of objects was used for baseline and 3-4 month testing.

Each trial was initiated by placing the mouse in a corner (that did not contain an object nearby) of the testing box. The second of the two trials was recorded using a video camera, and the recordings were scored for interaction time with each object using Ethovision XT (Noldus, Toronto, ON). Mice were considered to be interacting with an object when their nose was within 1 cm of the object. The interaction time data for each object was then used to calculate preference scores reported in Figure 3.

Morris Water Maze

Each trial was initiated by releasing the mouse at one of 4 starting locations (N, E, S, or W; each used once per day). A trial ended when the mouse either climbed onto the platform or spent 2 min searching for it. It was then left (or placed) on the platform for 45 s to observe the visual cues, dried off, and returned to its home cage.

References

[1] Stanley JL, Lincoln RJ, Brown TA, McDonald LM, Dawson GR, Reynolds DS (2005) The mouse beam walking assay offers improved sensitivity over the mouse rotarod in

determining motor coordination deficits induced by benzodiazepines. *J. Psychopharmacol.* **19**, 221–227.

[2] Djordjevic J, Jones-Gotman M, De Sousa K, Chertkow H (2008) Olfaction in patients with mild cognitive impairment and Alzheimer's disease. *Neurobiol. Aging* **29**, 693–706.

[3] Pham J, Cabrera SM, Sanchis-segura C, Wood MA (2009) Automated scoring of fear related behavior using EthoVision software. *J. Neurosci. Methods* **178**, 323–326.

Supplementary Tables

Supplementary Table 1. Statistical results for the rotarod test. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	mean time (s) before falling		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.903, ***p < 0.001	W = 0.814, ***p < 0.001	W = 0.977, *p = 0.017
Levene's test (homogeneity of variance)	F(7, 89) = 0.656, p = 0.709	F(7, 89) = 0.491, p = 0.839	F(7, 89) = 0.551, p = 0.794
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test
main effect of genotype	p = 0.083, η2p = 0.060	p = 0.142, η2p = 0.024	p = 0.943, η2p < 0.001
main effect of sex	p = 0.945, η2p = 0.004	p = 0.251, η2p = 0.015	p = 0.692, η2p = 0.002
main effect of diet	p = 0.684, η2p = 0.003	p = 0.398, η2p = 0.008	p = 0.829, η2p < 0.001
genotype x sex interaction	p = 0.254, η2p = 0.026	p = 0.877, η2p < 0.001	p = 0.167, η2p = 0.021
genotype x diet interaction	p = 0.083, η2p < 0.001	p = 0.883, η2p < 0.001	p = 0.658, η2p = 0.002
sex x diet interaction	p = 0.558, η2p = 0.007	p = 0.574, η2p = 0.004	p = 0.336, η2p = 0.010
genotype x sex x diet interaction	p = 0.552, η2p < 0.001	p = 0.744, η2p = 0.001	p = 0.377, η2p = 0.009

Supplementary Table 2. Statistical results for the beam walking test. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	time (s) to cross the beam			number of foot slips		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.786, ***p < 0.001	W = 0.308, ***p < 0.001	W = 0.384, ***p < 0.001	W = 0.786, ***p < 0.001	W = 0.864, ***p < 0.001	W = 0.987, p = 0.439
Levene's test (homogeneity of variance)	F(7, 89) = 0.383, p = 0.9101	F(7, 89) = 1.489, p = 0.181	F(7, 89) = 1.815, p = 0.094	F(7, 89) = 0.383, p = 0.910	F(7, 89) = 1.039, p = 0.410	F(7, 89) = 1.178, p = 0.323
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial ANOVA
main effect of genotype	p = 0.368, η2p = 0.010	p = 0.172, η2p = 0.018	p = 0.434, η2p = 0.009	p = 0.096, η2p = 0.032	p = 0.306, η2p = 0.012	F(1, 89) = 0.325, p = 0.570, η2p = 0.004
main effect of sex	p = 0.555, η2p = 0.004	p = 0.957, η2p = 0.001	p = 0.952, η2p < 0.001	p = 0.153, η2p = 0.024	p = 0.169, η2p = 0.021	F(1, 89) = 0.016, p = 0.901, η2p < 0.001
main effect of diet	p = 0.537, η2p = 0.005	p = 0.143, η2p = 0.019	p = 0.279, η2p = 0.015	p = 0.362, η2p = 0.010	p = 0.924, η2p < 0.001	F(1, 89) = 1.178, p = 0.281, η2p = 0.013
genotype x sex interaction	p = 0.189, η2p = 0.020	p = 0.839, η2p = 0.004	p = 0.783, η2p = 0.001	p = 0.113, η2p = 0.030	p = 0.060, η2p = 0.039	F(1, 89) = 0.008, p = 0.929, η2p < 0.001
genotype x diet interaction	p = 0.254, η2p = 0.015	p = 0.138, η2p = 0.020	p = 0.501, η2p = 0.006	p = 0.805, η2p < 0.001	p = 0.413, η2p = 0.008	F(1, 89) = 0.181, p = 0.672, η2p = 0.002
sex x diet interaction	p = 0.185, η2p = 0.020	p = 0.949, η2p = 0.006	p = 0.893, η2p < 0.001	p = 0.207, η2p = 0.019	p = 0.571, η2p = 0.004	F(1, 89) = 0.279, p = 0.599, η2p = 0.003
genotype x sex x diet interaction	p = 0.726, η2p = 0.001	p = 0.949, η2p = 0.006	p = 0.610, η2p = 0.004	p = 0.709, η2p = 0.002	p = 0.797, η2p < 0.001	F(1, 89) = 0.019, p = 0.890, η2p < 0.001

Supplementary Table 3. Statistical results for the hanging basket test. Significant results ($p < 0.05$) are highlighted in grey. (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	mean time (s) before falling		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.904, ***p < 0.001	W = 0.946, ***p < 0.001	W = 0.978, p = 0.106
Levene's test (homogeneity of variance)	F(7, 89) = 1.350, p = 0.237	F(7, 89) = 1.941, p = 0.072	F(7, 89) = 1.934, p = 0.07344
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial ANOVA
main effect of genotype	p = 0.506, η2p = 0.005	p = 0.536, η2p = 0.004	F(1, 89) = 0.134, p = 0.715, η2p = 0.002
main effect of sex	**p = 0.002, η2p = 0.104	p = 0.215, η2p = 0.017	F(1, 89) = 0.701, p = 0.405, η2p = 0.008
main effect of diet	p = 0.960, η2p < 0.001	p = 0.016, η2p = 0.032	F(1, 89) = 0.968, p = 0.328, η2p = 0.011
genotype x sex interaction	p = 0.086, η2p = 0.005	p = 0.282, η2p = 0.046	F(1, 89) = 0.968, p = 0.328, η2p = 0.011
genotype x diet interaction	p = 0.539, η2p = 0.004	p = 0.992, η2p < 0.001	F(1, 89) = 0.136, p = 0.713, η2p = 0.002
sex x diet interaction	p = 0.238, η2p = 0.016	p = 0.522, η2p = 0.005	F(1, 89) = 0.263, p = 0.610, η2p = 0.003
genotype x sex x diet interaction	p = 0.509, η2p = 0.005	p = 0.390, η2p = 0.008	F(1, 89) = 0.017, p = 0.897, η2p < 0.001

Supplementary Table 4. Statistical results for the olfactory acuity test. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measures: r2c = r contrast squared, η2g = generalized eta-squared

	measure	sniffing time (s)	
	data set	1-2 months of age	3-4 months of age
	omnibus statistical test used to compare groups	2 x 2 x 2 x 5 mixed effects ANOVA	2 x 2 x 2 x 5 mixed effects ANOVA
	main effect of genotype	F(1, 89) = 1.714, p = 0.194, η2g = 0.009	F(1, 89) = 0.066, p = 0.798, η2g < 0.001
	main effect of sex	F(1, 89) = 2.711, p = 0.103, η2g = 0.014	F(1, 89) = 4.051, *p = 0.047, η2g = 0.021
	main effect of diet	F(1, 89) = 0.426, p = 0.516, η2g = 0.002	F(1, 89) = 1.386, p = 0.242, η2g = 0.007
	genotype x sex interaction	F(1, 89) = 3.061, p = 0.084, η2g = 0.023	F(1, 89) = 0.289, p = 0.592, η2g = 0.002
	genotype x diet interaction	F(1, 89) = 0.007, p = 0.933, η2g < 0.001	F(1, 89) = 0.425, p = 0.516, η2g = 0.002
	sex x diet interaction	F(1, 89) = 0.054, p = 0.817, η2g < 0.001	F(1, 89) = 5.740, *p = 0.019, η2g = 0.029
	genotype x sex x diet interaction	F(1, 89) = 11.780, p = 0.186, η2g = 0.009	F(1, 89) = 0.857, p = 0.357, η2g = 0.004
	main effect of concentration	F(4, 356) = 19.950, ***p < 0.001, η2g = 0.109	F(4, 356) = 25.009, ***p < 0.001 , η2g = 0.130
	genotype x concentration interaction	F(4, 356) = 6.422, p = 0.758, η2g = 0.003	F(4, 356) = 0.287, p = 0.851, η2g = 0.002
	sex x concentration interaction	F(4, 356) = 2.303, p = 0.070, η2g = 0.014	F(4, 356) = 2.605, *p = 0.047, η2g = 0.015
	diet x concentration interaction	F(4, 356) = 1.272, p = 0.283, η2g = 0.008	F(4, 356) = 0.917, p = 0.439, η2g = 0.005
	genotype x sex x concentration interaction	F(4, 356) = 2.102, p = 0.093, η2g = 0.013	F(4, 356) = 0.368, p = 0.793, η2g = 0.002
	genotype x diet x concentration interaction	F(4, 356) = 0.456, p = 0.774, η2g = 0.003	F(4, 356) = 0.477, p = 0.714, η2g = 0.003
	sex x diet x concentration interaction	F(4, 356) = 0.250, p = 0.880, η2g = 0.002	F(4, 356) = 3.159, *p = 0.022, η2g = 0.019
	genotype x sex x diet x concentration interaction	F(4, 356) = 1.058, p = 0.371, η2g = 0.006	F(4, 356) = 2.434, p = 0.597, η2g = 0.014
	posthoc test	Tukey HSD corrected first order (linear) polynomial contrasts to evaluate a linear change in sniffing time across concentrations in a one-way ANOVA for each genotype x sex group separately (1-2 months of age only)	
	3xTg-AD males	t (1, 374) = 6.955, ***p < 0.001, r2c = 0.115	-
	3xTg-AD females	t(1, 374) = 1.567, p = 0.118, r2c = 0.007	-
	WT males	t(1, 374) = 4.537, ***p < 0.001, r2c = 0.052	-
	WT females	t(1, 374) = 3.201, **p = 0.002, r2c = 0.027	-
odorant concentration	posthoc test	Tukey HSD corrected comparisons for sex x diet groups for each concentration (3-4 months of age only)	
0% peanut butter (ctrl)	MDS females vs. MDS males	-	t(1, 308) = -1.584, p = 0.389, r2c = 0.008
	MDS females vs. vehicle females	-	t(1, 308) = -1.850, p = 0.252, r2c = 0.011
	MDS females vs. vehicle males	-	t(1, 308) = -1.036, p = 0.729, r2c = 0.003
	MDS males vs. vehicle females	-	t(1, 308) = -0.346, p =0.986 , r2c < 0.001
	MDS males vs. vehicle males	-	t(1, 308) = 0.556, p = 0.942, r2c = 0.001
	vehicle females vs. vehicle males	-	t(1, 308) = 0.886, p = 0.812, r2c = 0.003
0.001% peanut butter	MDS females vs. MDS males	-	t(1, 308) = -2.899, *p = 0.021, r2c = 0.027
	MDS females vs. vehicle females	-	t(1, 308) = -0.682, p = 0.904, r2c = 0.002
	MDS females vs. vehicle males	-	t(1, 308) = -1.999, p = 0.191, r2c = 0.013
	MDS males vs. vehicle females	-	t(1, 308) = 2.081, p = 0.162, r2c = 0.014
	MDS males vs. vehicle males	-	t(1, 308) = 0.932, p = 0.787, r2c = 0.003
	vehicle females vs. vehicle males	-	t(1, 308) = -1.214, p = 0.618, r2c = 0.005
0.01% peanut butter	MDS females vs. MDS males	-	t(1, 308) = -1.550, p = 0.409, r2c = 0.008
	MDS females vs. vehicle females	-	t(1, 308) = -0.504, p > 0.999 , r2c < 0.001
	MDS females vs. vehicle males	-	t(1, 308) = -2.400, p = 0.079, r2c = 0.018
	MDS males vs. vehicle females	-	t(1, 308) = 1.424, p = 0.485, r2c = 0.007
	MDS males vs. vehicle males	-	t(1, 308) = -0.837, p = 0.835, r2c = 0.002
	vehicle females vs. vehicle males	-	t(1, 308) = -2.232, p = 0.117, r2c = 0.016
0.1% peanut butter	MDS females vs. MDS males	-	t(1, 308) = -1.960, p = 0.206, r2c = 0.012
	MDS females vs. vehicle females	-	t(1, 308) = -2.243, p = 0.114, r2c = 0.016
	MDS females vs. vehicle males	-	t(1, 308) = -0.452, p = 0.969, r2c = 0.001
	MDS males vs. vehicle females	-	t(1, 308) = -0.381, p = 0.981, r2c < 0.001
	MDS males vs. vehicle males	-	t(1, 308) = 1.532, p = 0.419, r2c = 0.008
	vehicle females vs. vehicle males	-	t(1, 308) = 1.839, p = 0.257, r2c = 0.011
1% peanut butter	MDS females vs. MDS males	-	t(1, 308) = -2.875, *p = 0.022, r2c = 0.026
	MDS females vs. vehicle females	-	t(1, 308) = -3.546, **p = 0.003, r2c = 0.039
	MDS females vs. vehicle males	-	t(1, 308) = -2.056, p = 0.170, r2c = 0.014
	MDS males vs. vehicle females	-	t(1, 308) = -0.815, p = 0.848, r2c = 0.002
	MDS males vs. vehicle males	-	t(1, 308) = 0.850, p = 0.830, r2c = 0.002
	vehicle females vs. vehicle males	-	t(1, 308) = 1.630, p = 0.363, r2c = 0.009

Supplementary Table 5. Statistical results for the olfactory discrimination test. Significant results ($p < 0.05$) are highlighted in grey (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Effect size measure: $\eta 2p$ = partial eta-squared.

measure	change in sniffing time from trial 4 (last 0.001% cinnamon trial) to trial 5 (1st/only 0.001% paprika trial)	
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**
Shapiro-Wilk test (normality)	W = 0.157, ***p < 0.001	W = 0.977, p-value = 0.096
Levene's test (homogeneity of variance)	F(7, 89) = 0.722, p = 0.579	F(7, 89) = 1.177, p = 0.324
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial ANOVA
main effect of genotype	p > 0.999, η2p < 0.001	F(1, 89) = 0.052, p = 0.471, η2p = 0.006
main effect of sex	p > 0.999, η2p < 0.001	F(1, 89) = 0.657, p = 0.420, η2p = 0.007
main effect of diet	p = 0.383, η2p = 0.019	F(1, 89) = 0.123, p = 0.727, η2p = 0.001
genotype x sex interaction	p > 0.999, η2p < 0.001	F(1, 89) = 0.002, p = 0.968, η2p < 0.001
genotype x diet interaction	p = 0.854, η2p = 0.009	F(1, 89) = 0.011, p = 0.919, η2p < 0.001
sex x diet interaction	p = 0.384, η2p = 0.019	F(1, 89) = 1.048, p = 0.309, η2p = 0.012
genotype x sex x diet interaction	p = 0.852, η2p = 0.009	F(1, 89) = 0.617, p = 0.434, η2p = 0.007

Supplementary Table 6. Statistical results for the elevated plus maze. Significant results ($p < 0.05$) are highlighted in grey (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Effect size measure: $\eta 2p$ = partial eta-squared.

measure	time spent in the open arms (s)		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.989, p = 0.678	W = 0.989, p = 0.581	W = 0.988, p = 0.619
Levene's test (homogeneity of variance)	F(7, 89) = 2.745, *p = 0.013	F(7, 89) = 1.239, p = 0.290	F(7, 89) = 1.565, p = 0.157
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA
main effect of genotype	***p < 0.001, η2p = 0.293	F(1, 89) = 67.585, ***p < 0.001, η2p = 0.440	F(1, 89) = 0.823, p = 0.367, η2p = 0.009
main effect of sex	p = 0.319, η2p = 0.011	F(1, 89) = 0.12, p = 0.730, η2p = 0.001	F(1, 89) = 0.009, p = 0.922, η2p < 0.001
main effect of diet	p = 0.218, η2p = 0.017	F(1, 89) = 0.399, p = 0.529, η2p = 0.005	F(1, 89) = 0.675, p = 0.413, η2p = 0.008
genotype x sex interaction	p = 0.879, η2p < 0.001	F(1, 89) = 0.917, p = 0.341, η2p = 0.011	F(1, 89) = 2.720, p = 0.103, η2p = 0.031
genotype x diet interaction	p = 0.938, η2p < 0.001	F(1, 89) = 0.012, p = 0.913, η2p < 0.001	F(1, 89) = 0.975, p = 0.326, η2p = 0.011
sex x diet interaction	p = 0.293, η2p = 0.013	F(1, 89) = 0.005, p = 0.945, η2p < 0.001	F(1, 89) = 0.078, p = 0.780, η2p < 0.001
genotype x sex x diet interaction	p = 0.837, η2p < 0.001	F(1, 89) = 3.098, p = 0.082, η2p = 0.035	F(1, 89) = 0.078, p = 0.780, η2p = 0.027

Supplementary Table 7. Statistical results for the open field test. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	time spent in the center (s)			latency to approach the center (s)		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.926, p < 0.001	W = 0.923, p < 0.001	W = 0.963, p = 0.007	W = 0.668, p < 0.001	W = 0.796, p < 0.001	W = 0.722, p < 0.001
Levene's test (homogeneity of variance)	F(7, 89) = 2.787, p = 0.045	F(7, 89) = 0.391, p = 0.906	F(7, 89) = 1.927, p = 0.074	F(7, 89) = 1.125, p = 0.355	F(7, 89) = 1.551, p = 0.161	F(7, 89) = 1.309, p = 0.255
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test
main effect of genotype	p = 0.062, η2p = 0.039	p = 0.148, η2p = 0.025	p = 0.271, η2p = 0.014	p = 0.718, η2p = 0.002	p = 0.131, η2p = 0.026	p = 0.765, η2p = 0.001
main effect of sex	p = 0.067, η2p = 0.037	*p = 0.015, η2p = 0.064	p = 0.886, η2p < 0.001	p = 0.193, η2p = 0.020	*p = 0.022, η2p = 0.054	p = 0.698, η2p = 0.002
main effect of diet	p = 0.857, η2p < 0.001	p = 0.745, η2p = 0.001	p = 0.932, η2p < 0.001	p = 0.147, η2p = 0.024	p = 0.851, η2p < 0.001	p = 0.542, η2p = 0.005
genotype x sex interaction	*p = 0.013, η2p = 0.067	p = 0.099, η2p = 0.031	p = 0.241, η2p = 0.016	*p = 0.039, η2p = 0.048	p = 0.154, η2p = 0.023	p = 0.240, η2p = 0.016
genotype x diet interaction	p = 0.151, η2p = 0.023	p = 0.496, η2p = 0.005	p = 0.368, η2p = 0.009	p = 0.623, η2p = 0.003	p = 0.952, η2p < 0.001	p = 0.767, η2p = 0.001
sex x diet interaction	p = 0.795, η2p < 0.001	p = 0.738, η2p = 0.001	p = 0.368, η2p = 0.009	p = 0.218, η2p = 0.018	p = 0.963, η2p < 0.001	p = 0.531, η2p = 0.005
genotype x sex x diet interaction	p = 0.608, η2p = 0.003	p = 0.877, η2p < 0.001	p = 0.946, η2p < 0.001	p = 0.231, η2p = 0.017	p = 0.644, η2p = 0.003	p = 0.714, η2p = 0.002
simple main effect of genotype in males	p = 0.823, η2p < 0.001	-	-	*p = 0.013, η2p = 0.090	-	-
simple main effect of genotype in females	***p < 0.001, η2p = 0.214	-	-	p = 0.367, η2p = 0.029	-	-
measure	**number of fecal boli**			**time spent immobile/frozen (s)**		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.986, p = 0.414	W = 0.979, p = 0.129	W = 0.989, p = 0.581	W = 0.772, ***p < 0.001	W = 0.861, ***p < 0.001	W = 0.818, ***p < 0.001
Levene's test (homogeneity of variance)	F(7, 89) = 0.539, p = 0.803	F(7, 89) = 0.615, p = 0.742	F(7, 89) = 1.239, p = 0.290	F(7, 89) = 0.288, p = 0.834	F(7, 89) = 1.457, p = 0.193	F(7, 89) = 0.834, p = 0.563
omnibus statistical test used to compare groups	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test
main effect of genotype	F(1, 89) = 5.437, *p = 0.022, η2p = 0.055	F(1, 89) = 0.320, p = 0.573, η2p = 0.004	F(1, 89) = 0.518, p = 0.474, η2p = 0.006	**p = 0.004, η2p = 0.135	p = 0.209, η2p = 0.012	p = 0.834, η2p = 0.001
main effect of sex	F(1, 89) = 4.644, *p = 0.034, η2p = 0.048	**F(1, 89) = 11.301, p = 0.001, η2p = 0.113	F(1, 89) = 0.309, p = 0.580, η2p = 0.003	F(1, 89) = 5.556, *p = 0.021, η2p = 0.058	p = 0.061, η2p = 0.038	p = 0.216, η2p = 0.027
main effect of diet	F(1, 89) = 0.002, p = 0.962, η2p < 0.001	F(1, 89) = 0.009, p = 0.926, η2p < 0.001	F(1, 89) = 0.962, p = 0.329, η2p = 0.011	p = 0.287, η2p = 0.020	p = 0.071, η2p = 0.036	p = 0.057, η2p = 0.062
genotype x sex interaction	F(1, 89) = 0.111, p = 0.740, η2p = 0.001	F(1, 89) = 1.564, p = 0.214, η2p = 0.017	F(1, 89) = 0.174, p = 0.678, η2p = 0.002	p = 0.901, η2p < 0.001	p = 0.680, η2p = 0.002	p = 0.243, η2p = 0.024
genotype x diet interaction	F(1, 89) = 0.556, p = 0.458, η2p = 0.006	F(1, 89) = 1.065, p = 0.305, η2p = 0.012	F(1, 89) = 0.426, p = 0.516, η2p = 0.005	p = 0.287, η2p = 0.020	p = 0.892, η2p < 0.001	p = 0.517, η2p = 0.007
sex x diet interaction	F(1, 89) = 0.001, p = 0.992, η2p < 0.001	F(1, 89) = 0.489, p = 0.486, η2p = 0.005	F(1, 89) = 0.999, p = 0.320, η2p = 0.011	p = 0.914, η2p < 0.001	p = 0.821, η2p < 0.001	p = 0.500, η2p = 0.008
genotype x sex x diet interaction	F(1, 89) = 0.083, p = 0.774, η2p = 0.001	F(1, 89) = 1.075, p = 0.303, η2p = 0.012	F(1, 89) = 0.435, p = 0.511, η2p = 0.005	p = 0.414, η2p = 0.012	p = 0.924, η2p < 0.001	p = 0.754, η2p = 0.002

Supplementary Table 8. Statistical results for INBEST testing. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	sucrose preference = (volume of 0.01% sucrose water consumed) / (total volume of liquid consumed)			total ambulatory distance over 10 h (cm)		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.979, p = 0.121	W = 0.965, *p = 0.012	W = 0.989, p = 0.657	W = 0.876, ***p < 0.001	W = 0.923, ***p < 0.001	W = 0.983, p = 0.243
Levene's test (homogeneity of variance)	F(7, 89) = 1.093, p = 0.375	F(7, 89) = 1.456, p = 0.193	F(7, 89) = 0.539, p = 0.802	F(7, 89) = 0.122, p = 0.947	F(7, 89) = 0.668, p =0.698	F(7, 89) = 0.863, p = 0.539
omnibus statistical test used to compare groups	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial ANOVA
main effect of genotype	F(1, 89) = 0.667, p = 0.416, η2p = 0.007	p = 0.089, η2p = 0.032	F(1, 89) = 0.395, p = 0.531, η2p = 0.005	p = 0.058, η2p = 0.028	p = 0.075, η2p = 0.035	F(1, 89) = 0.709, p = 0.402, η2p = 0.008
main effect of sex	F(1, 89) = 5.556, *p = 0.021, η2p = 0.058	p = 0.278, η2p = 0.013	F(1, 89) = 0.971, p = 0.327, η2p = 0.011	p = 0.619, η2p < 0.001	p =0.880 , η2p < 0.001	F(1, 89) = 0.196, p = 0.659, η2p = 0.002
main effect of diet	F(1, 89) = 0.401, p = 0.528, η2p = 0.005	p = 0.104, η2p = 0.030	F(1, 89) = 6.370, *p = 0.013, η2p = 0.069	p = 0.487, η2p = 0.002	p = 0.447, η2p = 0.006	F(1, 89) = 1.191, p = 0.278, η2p = 0.013
genotype x sex interaction	F(1, 89) = 0.969, p = 0.327, η2p = 0.011	p = 0.319, η2p = 0.011	F(1, 89) = 0.001, p = 0.974, η2p < 0.001	p = 0.997, η2p < 0.001	p = 0.756, η2p < 0.001	F(1, 89) = 0.334, p = 0.564, η2p = 0.004
genotype x diet interaction	F(1, 89) = 0.033, p = 0.857, η2p < 0.001	p = 0.874, η2p < 0.001	F(1, 89) = 0.003, p = 0.974, η2p < 0.001	p = 0.061, η2p = 0.013	p = 0.951, η2p < 0.001	F(1, 89) = 0.023, p = 0.881, η2p < 0.001
sex x diet interaction	F(1, 89) = 0.017, p = 0.898, η2p < 0.001	p = 0.570, η2p = 0.004	F(1, 89) = 0.342, p = 0.560, η2p = 0.004	p = 0.751, η2p < 0.001	p = 0.629, η2p = 0.003	F(1, 89) = 0.097, p = 0.757, η2p = 0.001
genotype x sex x diet interaction	F(1, 89) = 0.069, p = 0.794, η2p < 0.001	p = 0.715, η2p = 0.002	F(1, 89) = 0.001, p = 0.994, η2p < 0.001	p = 0.708, η2p < 0.001	p = 0.629, η2p = 0.003	F(1, 89) = 1.935, p = 0.168, η2p = 0.021
measure	**mean ambulatory speed over 10 h**					
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**			
Shapiro-Wilk test (normality)	W = 0.961, **p = 0.006	W = 0.949, ***p < 0.001	W = 0.974, *p = 0.048			
Levene's test (homogeneity of variance)	F(7, 89) = 1.399, p = 0.248	F(7, 89) = 0.570, p = 0.778	F(7, 89) = 1.164, p = 0.332			
omnibus statistical test used to compare groups	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test	2 x 2 x 2 factorial permutation test			
main effect of genotype	**p = 0.004, η2p = 0.088	p = 0.631, η2p = 0.003	*p = 0.011, η2p = 0.069			
main effect of sex	p = 0.160, η2p = 0.022	p = 0.936, η2p < 0.001	p = 0.340, η2p = 0.010			
main effect of diet	p = 0.968, η2p < 0.001	p = 0.699, η2p = 0.002	p = 0.759, η2p = 0.001			
genotype x sex interaction	p = 0.918, η2p < 0.001	p = 0.508, η2p = 0.005	p = 0.616, η2p = 0.003			
genotype x diet interaction	p = 0.077, η2p = 0.035	p = 0.615, η2p = 0.003	p = 0.083, η2p = 0.033			
sex x diet interaction	p = 0.946, η2p < 0.001	p = 0.685, η2p = 0.002	p = 0.762, η2p = 0.001			
genotype x sex x diet interaction	p = 0.750, η2p = 0.001	p = 0.751, η2p = 0.001	p = 0.964, η2p < 0.001			

Supplementary Table 9. Statistical results for the novel object test. Significant results ($p < 0.05$) are highlighted in grey. (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	(time spent interacting with the novel object) / (total time spent interacting with objects)		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.980, p = 0.155	W = 0.983, p = 0.238	W = 0.984, p = 0.266
Levene's test (homogeneity of variance)	F(7, 89) = 0.733, p = 0.644	F(7, 89) = 0.532, p = 0.809	F(7, 89) = 1.227, p = 0.296
omnibus statistical test used to compare groups	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA
main effect of genotype	F(1, 89) = 0.951, p = 0.332, η2p = 0.011	F(1, 89) = 18.107, ***p < 0.001 , η2p = 0.169	F(1, 89) = 54.527, ***p < 0.001 , η2p = 0.336
main effect of sex	F(1, 89) = 0.394, p = 0.532, η2p = 0.004	F(1, 89) = 0.011, p = 0.916, η2p < 0.001	F(1, 89) = 1.416, p = 0.237, η2p = 0.016
main effect of diet	F(1, 89) < 0.001, p = 0.998, η2p < 0.001	F(1, 89) = 1.523, p = 0.220, η2p = 0.017	F(1, 89) = 0.635, p = 0.428, η2p = 0.007
genotype x sex interaction	F(1, 89) = 2.605, p = 0.110, η2p = 0.028	F(1, 89) = 1.499, p = 0.224, η2p = 0.016	F(1, 89) = 0.659, p = 0.419, η2p = 0.007
genotype x diet interaction	F(1, 89) < 0.001, p = 0.995, η2p < 0.001	F(1, 89) = 0.027, p = 0.871, η2p < 0.001	F(1, 89) = 0.619, p = 0.434, η2p = 0.006
sex x diet interaction	F(1, 89) = 0.017, p = 0.897, η2p < 0.001	F(1, 89) = 2.009, p = 0.160, η2p = 0.028	F(1, 89) = 1.178, p = 0.281, η2p = 0.013
genotype x sex x diet interaction	F(1, 89) = 0.188, p = 0.666, η2p = 0.002	F(1, 89) = 1.396, p = 0.241, η2p = 0.019	F(1, 89) = 0.538, p = 0.465, η2p = 0.005

Supplementary Table 10. Statistical results for the spontaneous alternation test. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2p = partial eta-squared.

measure	alternation rate		
data set	**1-2 months of age (raw data)**	**3-4 months of age (raw data)**	**change from 1-2 to 3-4 months of age (difference score)**
Shapiro-Wilk test (normality)	W = 0.982, p = 0.217	W = 0.988, p = 0.507	W = 0.998, p = 0.972
Levene's test (homogeneity of variance)	F(7, 89) = 0.616, p = 0.741	F(7, 89) = 0.616, p = 0.741	F(7, 89) = 0.473, p = 0.812
omnibus statistical test used to compare groups	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA	2 x 2 x 2 factorial ANOVA
main effect of genotype	F(1, 89) = 0.319, p = 0.574, η2p = 0.004	F(1, 89) = 2.674, p = 0.106, η2p = 0.029	F(1, 89) = 0.243, p = 0.122, η2p = 0.027
main effect of sex	F(1, 89) = 0.550, p = 0.460, η2p = 0.006	F(1, 89) = 2.174, p = 0.144, η2p = 0.024	F(1, 89) = 0.811, p = 0.370, η2p = 0.009
main effect of diet	F(1, 89) = 0.397, p = 0.530, η2p = 0.004	F(1, 89) = 0.003, p = 0.954, η2p < 0.001	F(1, 89) = 0.032, p = 0.857, η2p < 0.001
genotype x sex interaction	F(1, 89) = 1.163, p = 0.284, η2p = 0.013	F(1, 89) = 6.555, *p = 0.012, η2p = 0.069	F(1, 89) = 5.231, *p = 0.025, η2p = 0.056
genotype x diet interaction	F(1, 89) = 0.296, p = 0.588, η2p = 0.003	F(1, 89) = 2.479, p = 0.119, η2p = 0.027	F(1, 89) = 5.359, *p = 0.023, η2p = 0.057
sex x diet interaction	F(1, 89) = 0.184, p = 0.669, η2p = 0.002	F(1, 89) = 0.568, p = 0.453, η2p = 0.006	F(1, 89) = 0.134, p = 0.715, η2p = 0.002
genotype x sex x diet interaction	F(1, 89) = 0.796, p = 0.375, η2p = 0.009	F(1, 89) = 0.601, p = 0.440, η2p = 0.007	F(1, 89) = 0.003, p = 0.959, η2p < 0.001
simple main effect of genotype in males	-	F(1, 49) = 0.146, p = 0.749, η2p = 0.002	F(1, 49) = 0.226, p = 0.636, η2p = 0.005
simple main effect of genotype in females	-	F(1, 44) = 14.151, ***p < 0.001, η2p = 0.243	F(1, 44) = 7.039, *p = 0.011, η2p = 0.138
simple main effect of genotype in MDS-supplemented mice	-	-	F(1, 47) = 0.314, p = 0.578, η2p = 0.007
simple main effect of genotype in vehicle-control mice	-	-	F(1, 46) = 5.64, *p = 0.022, η2p = 0.112

Supplementary Table 11. Statistical test results for the water maze cued acquisition and cued reversal trials. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measure: η2g = generalized eta-squared.

behavioral test	water maze cued acquisition			
measure	mean swimming speed (cm/s)		swim path length (cm)	
data set	1-2 months of age	3-4 months of age	1-2 months of age	3-4 months of age
omnibus statistical test used to compare groups	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA
main effect of genotype	F(1, 89) = 18.590, ***p < 0.001, η2g = 0.081	F(1, 89) = 33.494, ***p < 0.001 , η2g = 0.087	F(1, 89) = 0.321, p = 0.573, η2g = 0.001	F(1, 89) = 3.496, p = 0.073, η2g = 0.011
main effect of sex	F(1, 89) = 2.154, p = 0.146, η2g = 0.010	F(1, 89) = 0.728, p = 0.396, η2g = 0.002	F(1, 89) = 1.670, p = 0.200, η2g = 0.005	F(1, 89) = 0.130, p = 0.719, η2g < 0.001
main effect of diet	F(1, 89) = 0.589, p = 0.445, η2g = 0.003	F(1, 89) = 0.600, p = 0.589, η2g = 0.005	F(1, 89) = 0.548, p = 0.461, η2g = 0.002	F(1, 89) = 3.131, p = 0.080, η2g = 0.010
genotype x sex interaction	F(1, 89) = 2.313, p = 0.132, η2g = 0.011	F(1, 89) = 0.311, p = 0.579, η2g < 0.001	F(1, 89) = 0.003, p = 0.955, η2g < 0.001	F(1, 89) = 0.474, p = 0.493, η2g = 0.002
genotype x diet interaction	F(1, 89) = 0.841, p = 0.362, η2g = 0.004	F(1, 89) = 0.145, p = 0.705, η2g < 0.001	F(1, 89) = 0.287, p = 0.593, η2g = 0.001	F(1, 89) = 0.262, p = 0.636, η2g = 0.001
sex x diet interaction	F(1, 89) = 1.772, p = 0.187, η2g = 0.008	F(1, 89) = 3.797, p = 0.055, η2g = 0.012	F(1, 89) = 0.432, p = 0.513, η2g = 0.001	F(1, 89) = 1.030, p = 0.313, η2g = 0.003
genotype x sex x diet interaction	F(1, 89) = 2.301, p = 0.133, η2g = 0.011	F(3, 250) = 2.742, p = 0.101, η2g = 0.008	F(1, 89) = 2.145, p = 0.147, η2g = 0.006	F(1, 89) = 2.148, p = 0.146, η2g = 0.007
main effect of trial	F(3, 250) = 17.038, ***p < 0.001 , η2g =0.101	F(3, 250) = 2.873, *p = 0.046, η2g = 0.024	F(3, 250) = 42.611, ***p < 0.001, η2g = 0.268	F(3, 250) = 4.307,**p = 0.009, η2g = 0.034
genotype x trial interaction	F(3, 250) = 0.258, p = 0.845, η2g = 0.002	F(3, 250) = 1.692, p = 0.118, η2g = 0.014	F(3, 250) = 1.396, p = 0.248, η2g = 0.012	F(3, 250) = 0.538, p = 0.626, η2g = 0.004
sex x trial interaction	F(3, 250) = 1.940, p = 0.127, η2g = 0.013	F(3, 250) = 0.602, p = 0.586, η2g = 0.005	F(3, 250) = 2.296, p = 0.123, η2g = 0.019	F(3, 250) = 0.357, p = 0.749, η2g = 0.003
diet x trial interaction	F(3, 250) = 0.234, p = 0.862, η2g = 0.002	F(3, 250) = 1.153, p = 0.325, η2g = 0.009	F(3, 250) = 0.096, p = 0.832, η2g = 0.001	F(3, 250) = 1.465, p = 0.230, η2g = 0.012
genotype x sex x trial interaction	F(3, 250) = 0.274, p = 0.833, η2g = 0.002	F(3, 250) = 1.366, p = 0.256, η2g = 0.012	F(3, 250) = 0.049, p = 0.892, η2g < 0.001	F(3, 250) = 0.695, p = 0.532, η2g = 0.006
genotype x diet x trial interaction	F(3, 250) = 1.835, p = 0.145, η2g = 0.012	F(3, 250) = 2.399, p = 0.079, η2g = 0.020	F(3, 250) = 1.504, p = 0.227, η2g = 0.013	F(3, 250) = 2.727, p = 0.054, η2g = 0.022
sex x diet x concentration interaction	F(3, 250) = 0.765, p = 0.508, η2g = 0.005	F(3, 250) = 2.826, p = 0.056, η2g = 0.021	F(3, 250) = 2.455, p = 0.109, η2g = 0.021	F(3, 250) = 3.746, p = 0.017, η2g = 0.030
genotype x sex x diet x concentration interaction	F(3, 250) = 0.852, p = 0.461, η2g = 0.006	F(3, 250) = 1.210, p = 0.305, η2g = 0.010	F(3, 250) = 0.538, p = 0.518, η2g = 0.005	F(3, 250) = 1.395, p = 0.248, η2g = 0.011
behavioral test	water maze cued reversal			
measure	mean swimming speed (cm/s)		swim path length (cm)	
data set	1-2 months of age	3-4 months of age	1-2 months of age	3-4 months of age
omnibus statistical test used to compare groups	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA
main effect of genotype	F(1, 89) = 28.532, ***p < 0.001 , η2g = 0.084	F(1, 89) = 12.084, **p = 0.001, η2g = 0.023	F(1, 89) = 1.393, p = 0.241, η2g = 0.004	F(1, 89) = 2.999, p = 0.087, η2g = 0.008
main effect of sex	F(1, 89) = 0.218, p = 0.642, η2g = 0.001	F(1, 89) = 1.343, p = 0.250, η2g = 0.003	F(1, 89) = 0.262, p = 0.610, η2g = 0.001	F(1, 89) = 2.135, p = 0.148, η2g = 0.006
main effect of diet	F(1, 89) = 1.092, p = 0.299, η2g = 0.003	F(1, 89) = 1.208, p = 0.275, η2g = 0.002	F(1, 89) = 1.252, p = 0.266, η2g = 0.004	F(1, 89) = 0.654, p = 0.421, η2g = 0.002
genotype x sex interaction	F(1, 89) = 0.208, p = 0.650, η2g = 0.001	F(1, 89) = 0.622, p = 0.432, η2g = 0.001	F(1, 89) = 0.457, p = 0.501, η2g = 0.001	F(1, 89) = 0.227, p = 0.635, η2g = 0.001
genotype x diet interaction	F(1, 89) = 1.763, p = 0.188, η2g = 0.006	F(1, 89) < 0.001 , p = 0.985, η2g < 0.001	F(1, 89) = 2.093, p = 0.256, η2g = 0.006	F(1, 89) = 0.324, p = 0.571, η2g =0.001
sex x diet interaction	F(1, 89) = 2.096, p = 0.151, η2g = 0.007	F(1, 89) = 0.083, p = 0.774, η2g < 0.001	F(1, 89) = 0.091, p = 0.764, η2g < 0.001	F(1, 89) = 0.001, p = 0.974, η2g < 0.001
genotype x sex x diet interaction	F(1, 89) = 0.120, p = 0.730, η2g < 0.001	F(1, 89) = 0.912, p = 0.342, η2g = 0.002	F(1, 89) = 0.216, p = 0.643, η2g = 0.001	F(1, 89) = 2.576, p = 0.112, η2g = 0.007
main effect of trial	F(3, 250) = 19.332, ***p < 0.001 , η2g = 0.136	F(3, 250) = 4.004, **p = 0.008, η2g = 0.044	F(3, 250) = 21.225, ***p < 0.001 , η2g = 0.152	F(3, 250) = 3.834, *p = 0.014, η2g = 0.032
genotype x trial interaction	F(3, 250) = 2.543, p = 0.065, η2g = 0.020	F(3, 250) = 0.806, p = 0.446, η2g = 0.007	F(3, 250) = 0.802, p = 0.376, η2g = 0.007	F(3, 250) = 1.359, p = 0.258, η2g = 0.011
sex x trial interaction	F(3, 250) = 1.760, p = 0.163, η2g = 0.014	F(3, 250) = 0.185, p = 0.828, η2g = 0.002	F(3, 250) = 0.127, p = 0.728, η2g = 0.001	F(3, 250) = 1.153, p = 0.326, η2g = 0.010
diet x trial interaction	F(3, 250) = 0.200, p = 0.872, η2g = 0.002	F(3, 250) = 0.815, p = 0.442, η2g = 0.008	F(3, 250) = 0.713, p = 0.404, η2g = 0.006	F(3, 250) = 1.171, p = 0.319, η2g = 0.010
genotype x sex x trial interaction	F(3, 250) = 5.185, **p = 0.003, η2g = 0.041	F(3, 250) = 1.541, p = 0.217, η2g = 0.014	F(3, 250) = 0.248, p = 0.625, η2g = 0.002	F(3, 250) = 0.990, p = 0.391, η2g = 0.008
genotype x diet x trial interaction	F(3, 250) = 0.891, p = 0.435, η2g = 0.007	F(3, 250) = 0.184, p = 0.828, η2g = 0.002	F(3, 250) = 3.620, p = 0.059, η2g = 0.030	F(3, 250) = 0.662, p = 0.558, η2g = 0.006
sex x diet x concentration interaction	F(3, 250) = 0.856, p = 0.451, η2g = 0.007	F(3, 250) = 0.611, p = 0.541, η2g = 0.006	F(3, 250) = 0.061, p = 0.811, η2g = 0.001	F(3, 250) = 1.281, p = 0.282, η2g = 0.011
genotype x sex x diet x concentration interaction	F(3, 250) = 0.229, p = 0.850, η2g = 0.002	F(3, 250) = 0.146, p = 0.861, η2g = 0.001	F(3, 250) = 0.039, p = 0.849, η2g < 0.001	F(3, 250) = 0.433, p = 0.705, η2g = 0.004

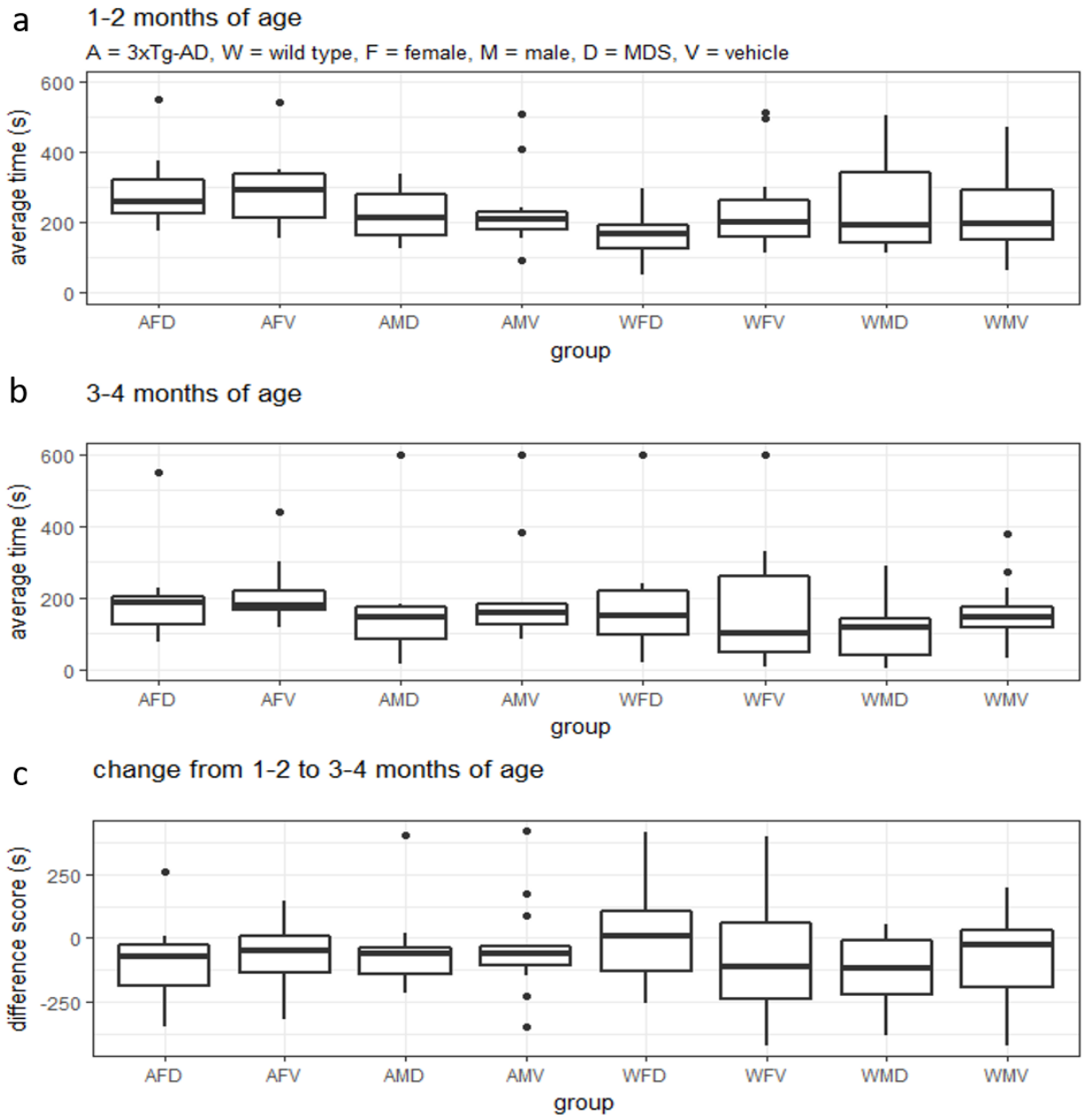
Supplementary Table 12. Statistical test results for the water maze acquisition and reversal trials. Significant results ($p < 0.05$) are highlighted in grey (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Effect size measures: r2c = r contrast squared, η2g = generalized eta-squared.

behavioral test	water maze acquisition		water maze reversal	
measure	swim path length (cm)		swim path length (cm)	
data set	1-2 months of age	3-4 months of age	1-2 months of age	3-4 months of age
omnibus statistical test used to compare groups	2 x 2 x 2 x 16 mixed effects ANOVA	2 x 2 x 2 x 16 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA	2 x 2 x 2 x 4 mixed effects ANOVA
main effect of genotype	F(1, 89) = 0.732, p = 0.395, η2g = 0.001	F(1, 89) = 1.563, p = 0.214, η2g = 0.002	F(1, 89) = 0.021, p = 0.886, η2g < 0.001	F(1, 89) = 1.189, p = 0.278, η2g = 0.006
main effect of sex	F(1, 89) = 0.037, p = 0.848, η2g < 0.001	F(1, 89) = 0.424, p = 0.516, η2g = 0.001	F(1, 89) = 0.512, p = 0.476, η2g = 0.002	F(1, 89) = 0.249, p = 0.619, η2g = 0.001
main effect of diet	F(1, 89) = 0.119, p = 0.731, η2g < 0.001	F(1, 89) = 0.027, p = 0.869, η2g < 0.001	F(1, 89) = 0.005, p = 0.947, η2g < 0.001	F(1, 89) = 0.230, p = 0.633, η2g = 0.001
genotype x sex interaction	F(1, 89) = 1.960, p = 0.165, η2g = 0.004	F(1, 89) = 0.005, p = 0.943, η2g < 0.001	F(1, 89) = 0.205, p = 0.652, η2g = 0.001	F(1, 89) = 0.050, p = 0.823, η2g < 0.001
genotype x diet interaction	F(1, 89) = 0.008, p = 0.927, η2g < 0.001	F(1, 89) = 0.207, p = 0.650, η2g < 0.001	F(1, 89) = 0.746, p = 0.390, η2g = 0.002	F(1, 89) = 0.294, p = 0.589, η2g = 0.001
sex x diet interaction	F(1, 89) = 0.752, p = 0.388, η2g = 0.001	F(1, 89) = 0.137, p = 0.712, η2g < 0.001	F(1, 89) = 0.574, p = 0.451, η2g = 0.002	F(1, 89) = 0.320, p = 0.573, η2g = 0.002
genotype x sex x diet interaction	F(1, 89) = 0.030, p = 0.863, η2g < 0.001	F(1, 89) = 0.011, p = 0.915, η2g < 0.001	F(1, 89) = 0.125, p = 0.724, η2g < 0.001	F(1, 89) = 2.377, p = 0.127, η2g = 0.012
main effect of trial	F(15, 1335) = 7.154, ***p < 0.001 , η2g = 0.063	F(10, 855) = 3.399, ***p < 0.001 , η2g = 0.032	F(3, 255) = 4.009, *p = 0.011, η2g = 0.033	F(3, 255) = 3.280, *p = 0.023 , η2g = 0.020
genotype x trial interaction	F(15, 1335) = 0.600, p = 0.763, η2g = 0.006	F(10, 855) = 1.673, p = 0.086, η2g = 0.016	F(3, 255) = 1.096, p = 0.348, η2g = 0.009	F(3, 255) = 0.627, p = 0.591, η2g = 0.004
sex x trial interaction	F(15, 1335) = 1.050, p = 0.395, η2g = 0.010	F(10, 855) = 1.766, p = 0.066, η2g = 0.017	F(3, 255) = 0.798, p = 0.484, η2g = 0.007	F(3, 255) = 0.066, p = 0.975, η2g < 0.001
diet x trial interaction	F(15, 1335) = 0.781, p = 0.608, η2g = 0.007	F(10, 855) = 1.013, p = 0.429, η2g = 0.010	F(3, 255) = 1.300, p = 0.276, η2g = 0.011	F(3, 255) = 1.993, p = 0.118, η2g = 0.012
genotype x sex x trial interaction	F(15, 1335) = 2.004, p = 0.054, η2g = 0.018	F(10, 855) = 1.057, p = 0.393, η2g = 0.010	F(3, 255) = 1.343, p = 0.262, η2g = 0.011	F(3, 255) = 1.864, p = 0.139, η2g = 0.012
genotype x diet x trial interaction	F(15, 1335) = 1.862, p = 0.070, η2g = 0.017	F(10, 855) = 0.589, p = 0.818, η2g = 0.006	F(3, 255) = 1.462, p = 0.229, η2g = 0.012	F(3, 255) = 2.129, p = 0.100, η2g = 0.013
sex x diet x trial interaction	F(15, 1335) = 0.686, p = 0.690, η2g = 0.006	F(10, 855) = 1.108, p = 0.353, η2g = 0.011	F(3, 255) = 0.759, p = 0.504, η2g = 0.006	F(3, 255) = 0.468, p = 0.696, η2g = 0.003
genotype x sex x diet x trial interaction	F(15, 1335) = 0.899, p = 0.510, η2g = 0.008	F(10, 855) = 0.921, p = 0.511, η2g = 0.009	F(3, 255) = 0.294, p = 0.807, η2g = 0.002	F(3, 255) = 0.824, p = 0.477, η2g = 0.005
first order (linear) polynomial contrast for linear change across trials	t (1, 1335) = -5.652, ***p < 0.001, r2c = 0.023	t (1, 1335) = -5.670, ***p < 0.001, r2c = 0.024	t (1, 265) = -2.967, ***p < 0.001, r2c = 0.032	F (1, 368) = 1.175, p = 0.311, r2c = 0.003
a priori contrast for the hypothesis that [(3xTg-AD x MDS) > (3xTg-AD x vehicle = WT x MDS = WT x vehicle)]	-	-	-	F (1, 368) = 3.301, p = 0.070, r2c = 0.009
Interaction between the linear change across trials and the hypothesis contrast [(3xTg-AD x MDS) > (3xTg-AD x vehicle = WT x MDS = WT x vehicle)]	-	-	-	F (1, 368) = 6.628, *p = 0.011, r2c = 0.018

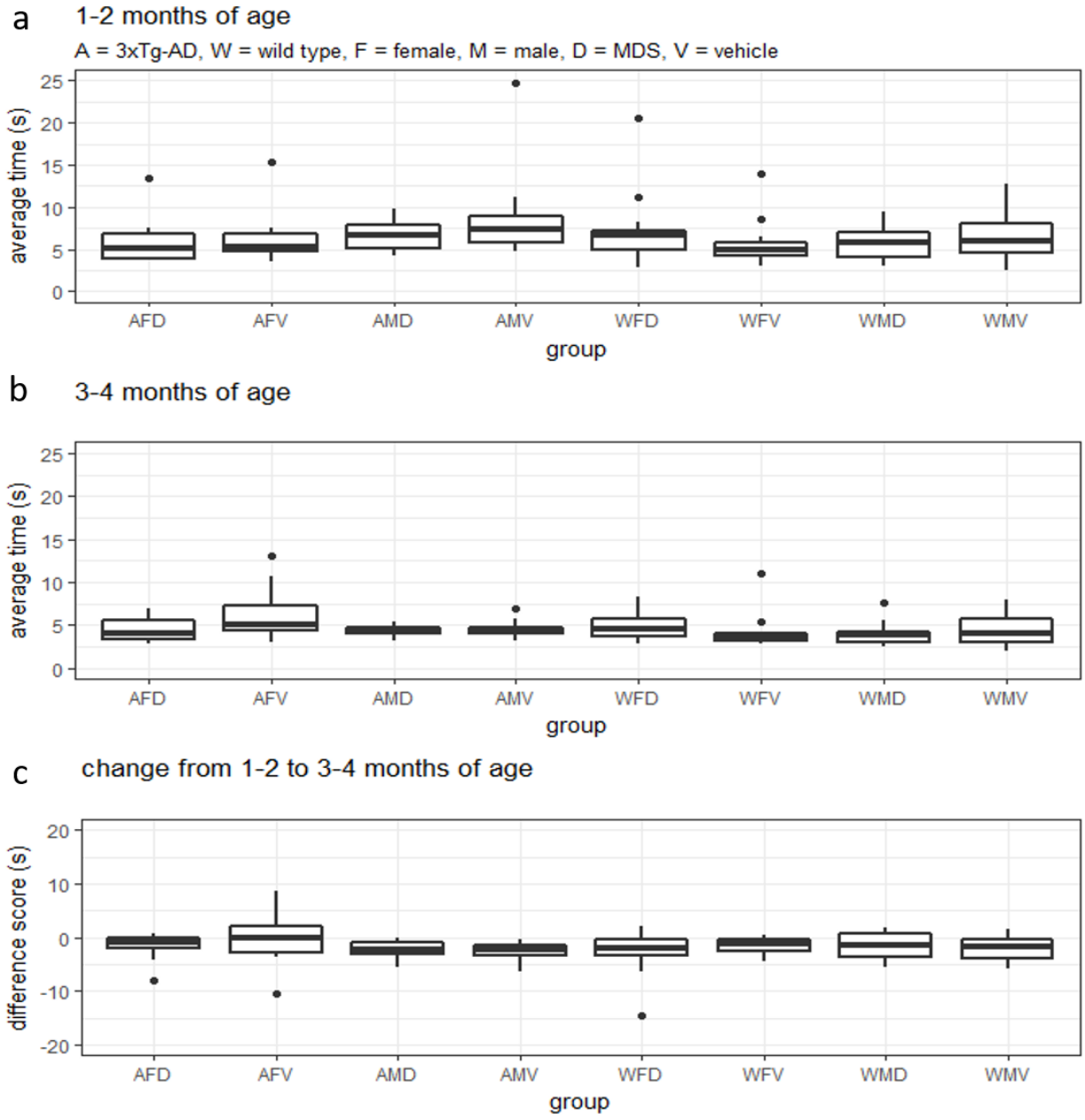
Supplementary Table 13. Statistical test results for the water maze probe trials. Significant results ($p < 0.05$) are highlighted in grey (*p < 0.05, **p < 0.01, ***p < 0.001). Effect size measures: r2c = r contrast squared, η2g = generalized eta-squared, d = cohen's d

	measure	time spent in the target quadrant (s)	
	data set	1-2 months of age	3-4 months of age
	omnibus statistical test used to compare groups	2 x 2 x 2 x 16 mixed effects ANOVA	2 x 2 x 2 x 16 mixed effects ANOVA
	main effect of genotype	F(1, 89) = 1.895, p = 0.756, η2g = 0.001	F(1, 89) = 0.097, p = 0.756, η2g = 0.001
	main effect of sex	F(1, 89) = 6.500, *p = 0.013, η2g = 0.042	F(1, 89) = 0.026, p = 0.872, η2g < 0.001
	main effect of diet	F(1, 89) = 1.192, p = 0.278, η2g = 0.008	F(1, 89) = 0.172, p = 0.679, η2g = 0.001
	genotype x sex interaction	F(1, 89) = 0.030, p = 0.863, η2g < 0.001	F(1, 89) = 0.718, p = 0.423, η2g = 0.005
	genotype x diet interaction	F(1, 89) = 0.241, p = 0.624, η2g = 0.002	F(1, 89) = 1.173, p = 0.282, η2g = 0.008
	sex x diet interaction	F(1, 89) =0.334 , p = 0.565, η2g = 0.002	F(1, 89) = 0.893, p = 0.347, η2g = 0.006
	genotype x sex x diet interaction	F(1, 89) = 0.006, p = 0.939, η2g < 0.001	F(1, 89) = 0.060, p = 0.807, η2g < 0.001
	main effect of trial	F(3, 267) = 11.427, ***p < 0.001, η2g = 0.049	F(3, 267) = 18.036, ***p < 0.001 , η2g = 0.072
	genotype x trial interaction	F(3, 267) = 1.837, p = 0.148, η2g = 0.008	F(3, 267) = 2.271, p = 0.087, η2g = 0.010
	sex x trial interaction	F(3, 267) = 1.141, p = 0.330, η2g = 0.005	F(3, 267) = 0.356, p = 0.766, η2g = 0.002
	diet x trial interaction	F(3, 267) = 1.958, p = 0.129, η2g = 0.009	F(3, 267) = 0.581, p = 0.612, η2g = 0.002
	genotype x sex x trial interaction	F(3, 267) = 1.134, p = 0.333, η2g = 0.005	F(3, 267) = 0.359, *p = 0.020, η2g = 0.015
	genotype x diet x trial interaction	F(3, 267) = 0.550, p = 0.626, η2g = 0.002	F(3, 267) = 1.237, p = 0.297, η2g = 0.005
	sex x diet x trial interaction	F(3, 267) = 1.429, p = 0.238, η2g = 0.006	F(3, 267) = 1.620, p = 0.189, η2g = 0.007
	genotype x sex x diet x trial interaction	F(3, 267) = 1.476, p = 0.225, η2g = 0.007	F(3, 267) = 0.301, p = 0.807, η2g = 0.001
	first order (linear) polynomial contrast to evaluate a linear change in swim path lengh (cm) across trials (all groups) in a one-way ANOVA	t(1, 288) = -4.940, ***p < 0.001, r2c = 0.078	t(1, 288) = -7.116, ***p < 0.001, r2c = 0.159
	posthoc t-test comparing 3xTg-AD females to change performance (30s spent in target quadrant) on trial 1	-	t(19) = 2.832, *p = 0.011, d = 0.633
trial	posthoc test	Tukey HSD corrected comparisons for genotype x sex groups for each trial (3-4 months of age only)	
1	3xTg-AD females vs. 3xTg-AD males	-	t(1, 207) = -1.610, p = 0.471, r2c = 0.009
	3xTg-AD females vs. WT females	-	t(1, 207) = -3.506, **p = 0.003, r2c = 0.056
	3xTg-AD females vs. WT males	-	t(1, 207) = -1.318, p = 0.553, r2c = 0.008
	3xTg-AD males vs. WT females	-	t(1, 207) = -0.967, p = 0.768, r2c = 0.005
	3xTg-AD males vs. WT males	-	t(1, 207) = 1.363, p = 0.524, r2c = 0.009
	WT females vs.WT males	-	t(1, 207) = 2.318, p = 0.097, r2c = 0.025
2	3xTg-AD females vs. 3xTg-AD males	-	t(1, 207) = -1.422, p = 0.487, r2c = 0.010
	3xTg-AD females vs. WT females	-	t(1, 207) = -2.119, p = 0.151, r2c = 0.021
	3xTg-AD females vs. WT males	-	t(1, 207) = 0.212, p = 0.997, r2c < 0.001
	3xTg-AD males vs. WT females	-	t(1, 207) = -0.751, p = 0.876, r2c = 0.003
	3xTg-AD males vs. WT males	-	t(1, 207) = 1.742, p = 0.305, r2c = 0.014
	WT females vs.WT males	-	t(1, 207) = 2.483, p = 0.066, r2c = 0.029
3	3xTg-AD females vs. 3xTg-AD males	-	t(1, 207) = -1.085, p = 0.699, r2c = 0.006
	3xTg-AD females vs. WT females	-	t(1, 207) = -1.235, p = 0.605, r2c = 0.007
	3xTg-AD females vs. WT males	-	t(1, 207) = 0.184, p = 0.998, r2c < 0.001
	3xTg-AD males vs. WT females	-	t(1, 207) = -0.163, p = 0.998, r2c < 0.001
	3xTg-AD males vs. WT males	-	t(1, 207) = 1.353, p = 0.531, r2c = 0.009
	WT females vs.WT males	-	t(1, 207) = 1.512, p = 0.432, r2c = 0.011
4	3xTg-AD females vs. 3xTg-AD males	-	t(1, 207) = -0.319, p = 0.989, r2c < 0.001
	3xTg-AD females vs. WT females	-	t(1, 207) = 0.410, p = 0.977, r2c = 0.001
	3xTg-AD females vs. WT males	-	t(1, 207) = 0.601, p = 0.932, r2c = 0.002
	3xTg-AD males vs. WT females	-	t(1, 207) = 0.783, p = 0.862, r2c = 0.003
	3xTg-AD males vs. WT males	-	t(1, 207) = 0.984, p = 0.759, r2c = 0.005
	WT females vs.WT males	-	t(1, 207) = 0.207, p = 0.997, r2c < 0.001

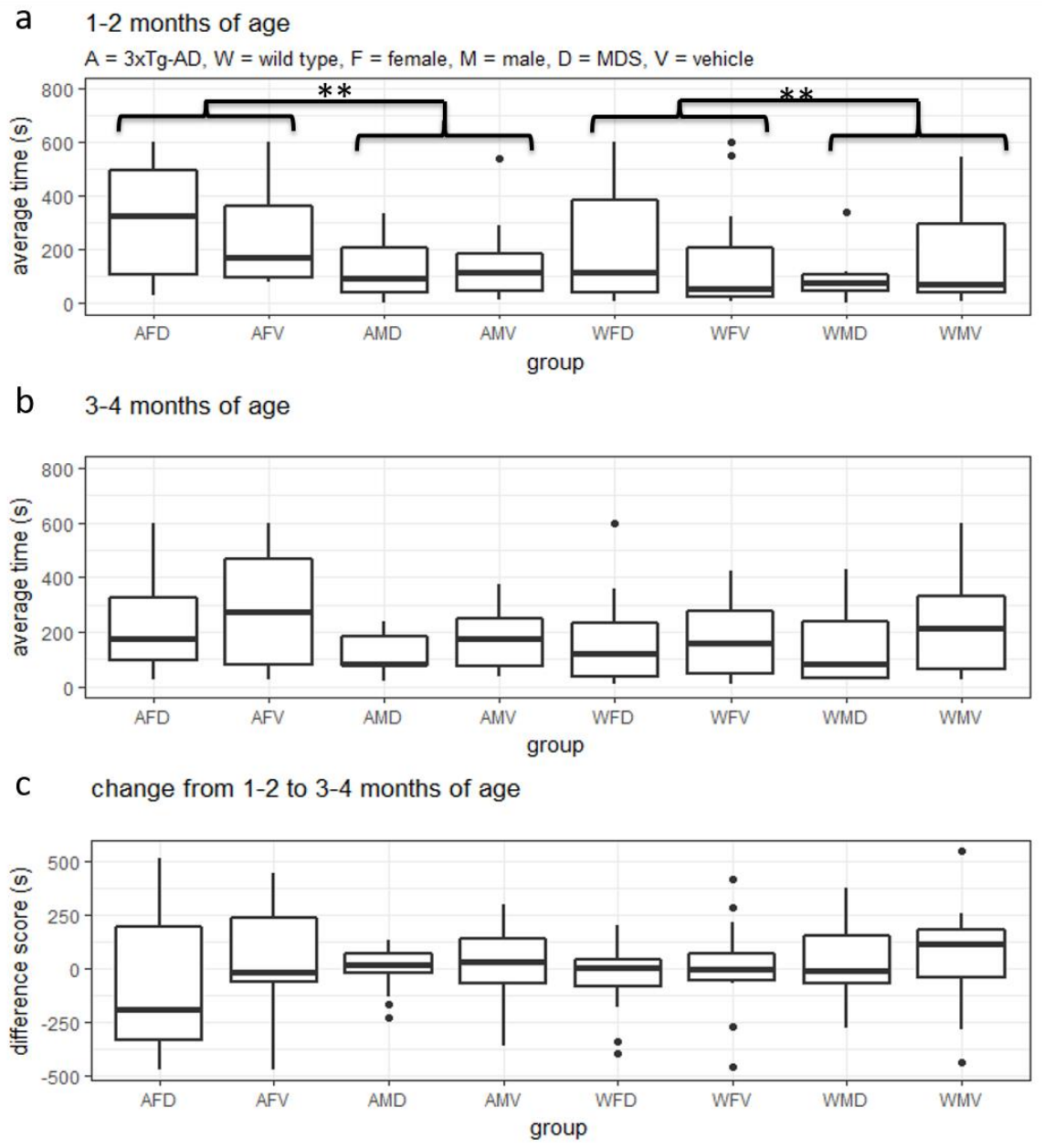
Supplementary Figures



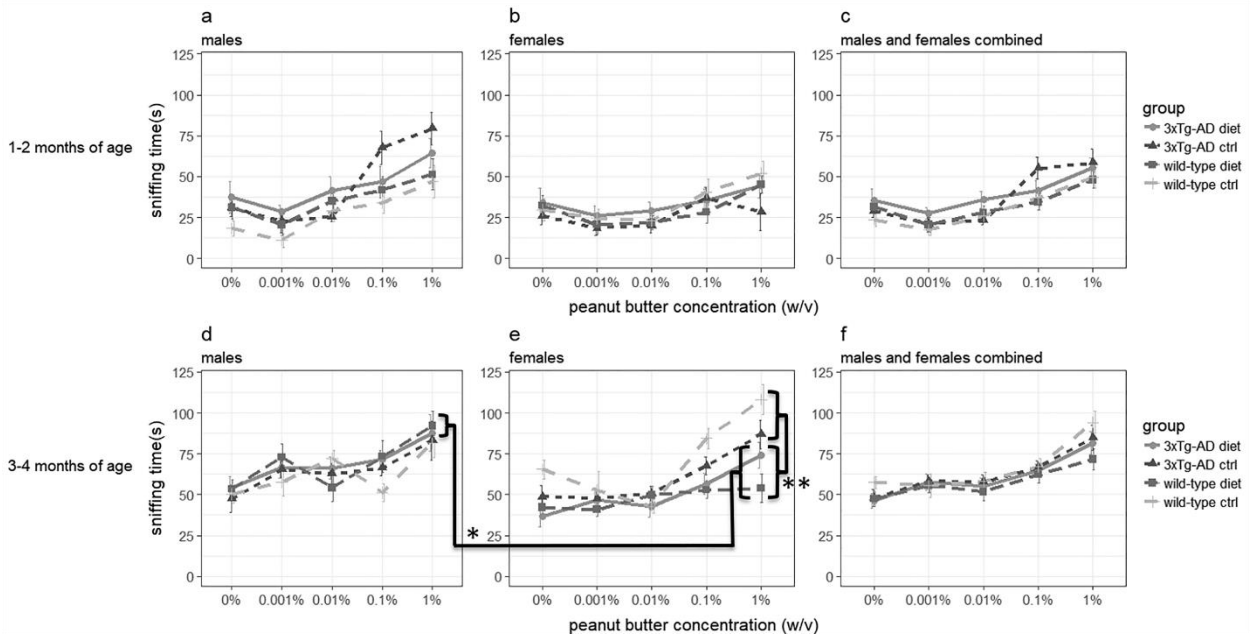
Supplementary Figure 1. Box plots of the average time (s) to fall from the rota-rod at 1-2 months of age (a), 3-4 months of age (b), and the difference scores (c) for each group between 1-2 and 3-4 months of age. There were no significant differences between groups when body weight was accounted for. (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



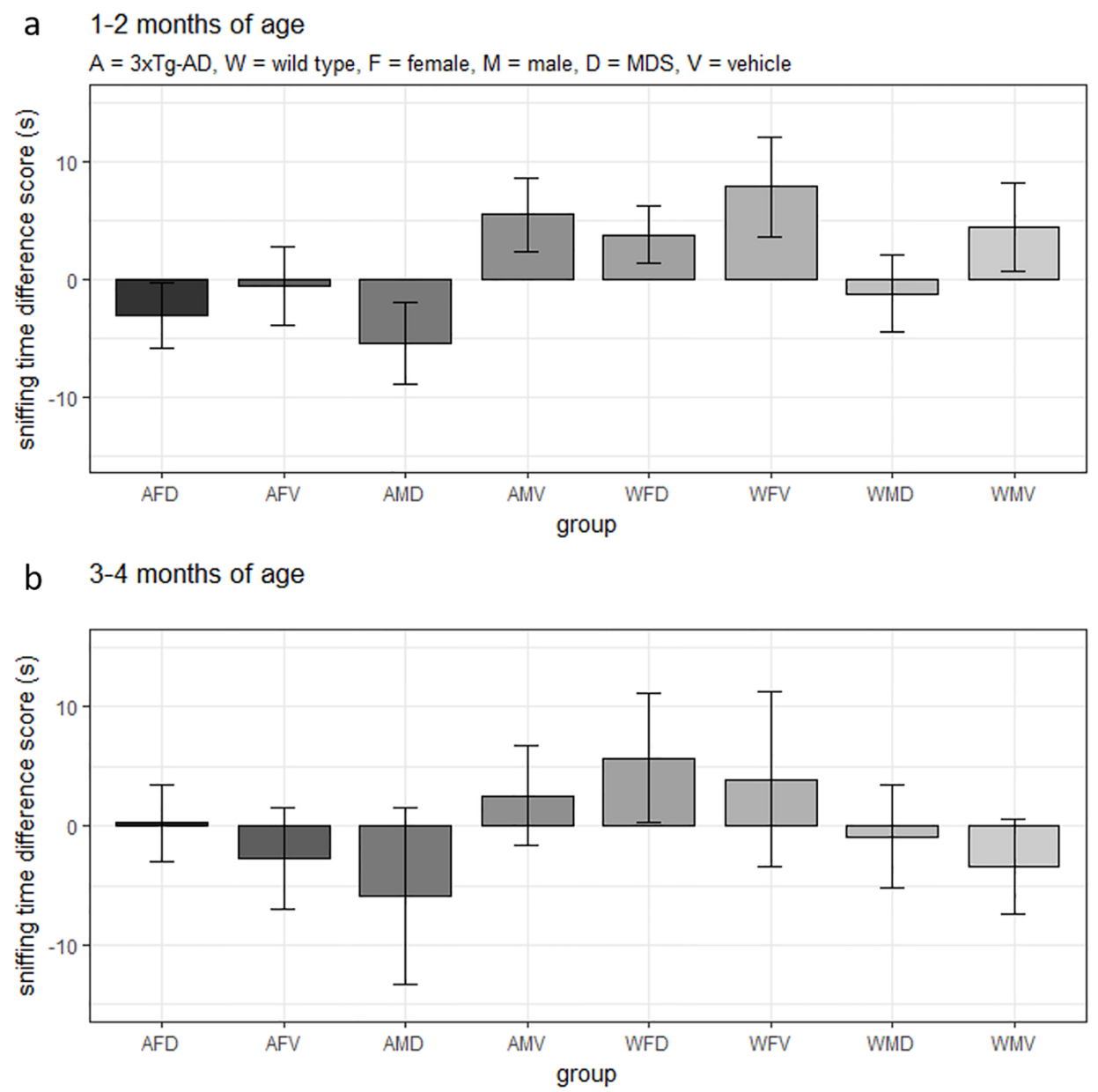
Supplementary Figure 2. Box plots of the time (s) to cross during the beam walking test at 1-2 months of age (a), 3-4 months of age (b), and the difference scores (c) for each group between 1-2 and 3-4 months of age. There were no significant differences between groups. (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



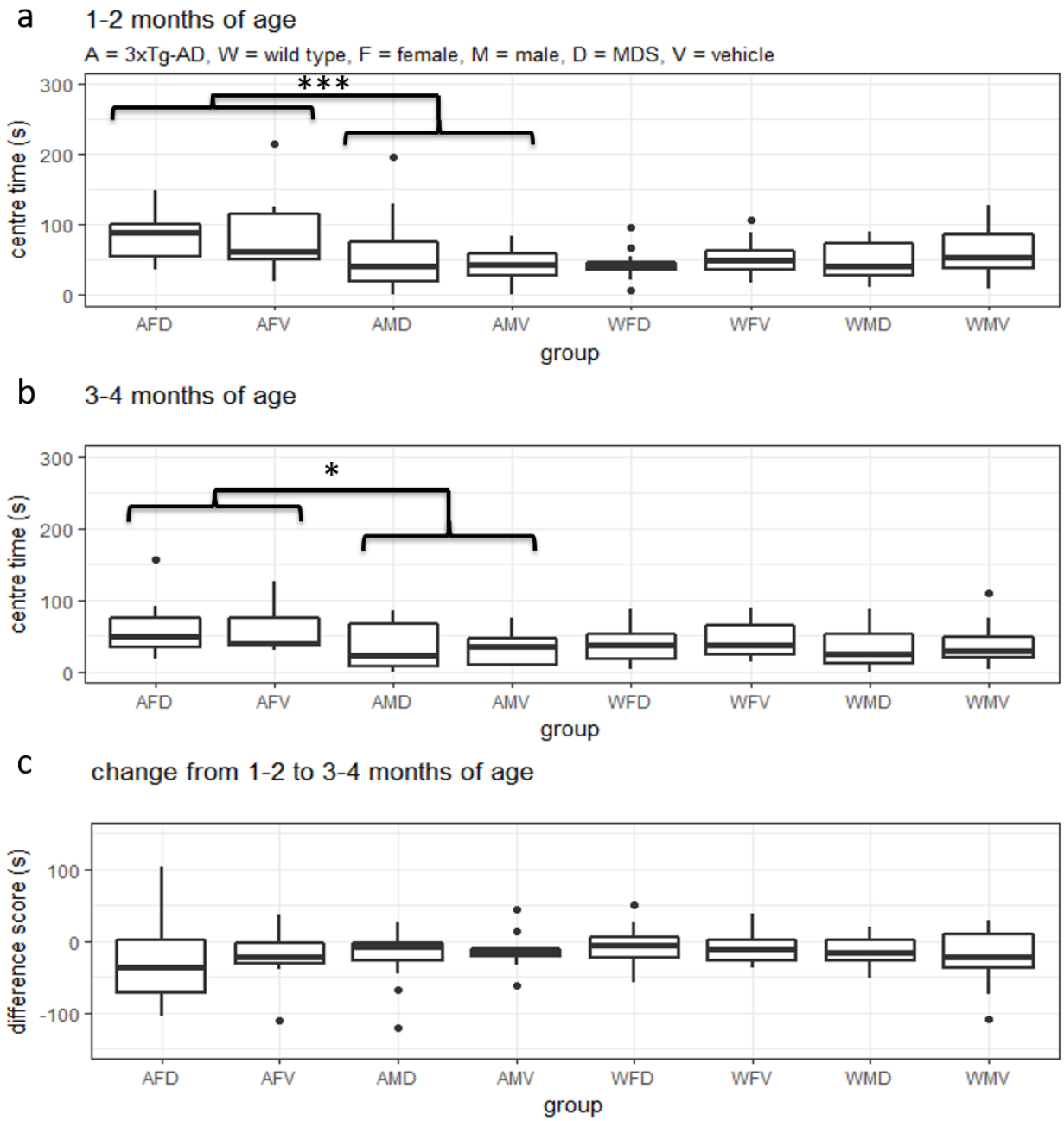
Supplementary Figure 3. Box plots of the average fall time (s) during the hanging basket test at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. Females held on longer than males at baseline only (2 x 2 x 2 factorial permutation test, **p = 0.002). (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



Supplementary Figure 4. Time (s, mean ± SE) spent sniffing the peanut butter soaked filter paper at different concentrations (w/v) during olfactory acuity testing at 1-2 **(a-c)** and 3-4 **(d-f)** months of age. **a & b.** Only 3xTg-AD females did not show a linear increase in sniffing time with increasing concentration (repeated measures one-way ANOVA with a linear contrast across concentrations for each group; p-values < 0.05 for all groups except 3xTg-AD females). **d and e**. At 3-4 months of age, MDS-supplemented females spent less time sniffing the peanut butter than MDS-supplemented males at the 2nd lowest and highest concentrations (Tukey-HSD *post-hoc* comparisons between sex x diet groups for each concentration 0.001% PB; $t_{(1, 308)} = -2.899$, *p = 0.021; 1% PB, $t_{(1, 308)} = -2.875$, *p = 0.022). **e.** Sniffing time was also lower for MDS-supplemented females than vehicle-supplemented females at the highest concentration (1% PB, $t_{(1, 308)} = -3.546$, **p = 0.003). Additionally, only MDS-supplemented wild type females did not exhibit a linear increase in sniffing time with increasing concentration (**e**, repeated measures one-way ANOVA with a linear contrast across concentrations for each genotype x sex x diet group; p-values < 0.05 for all groups except MDS-supplemented WT females).

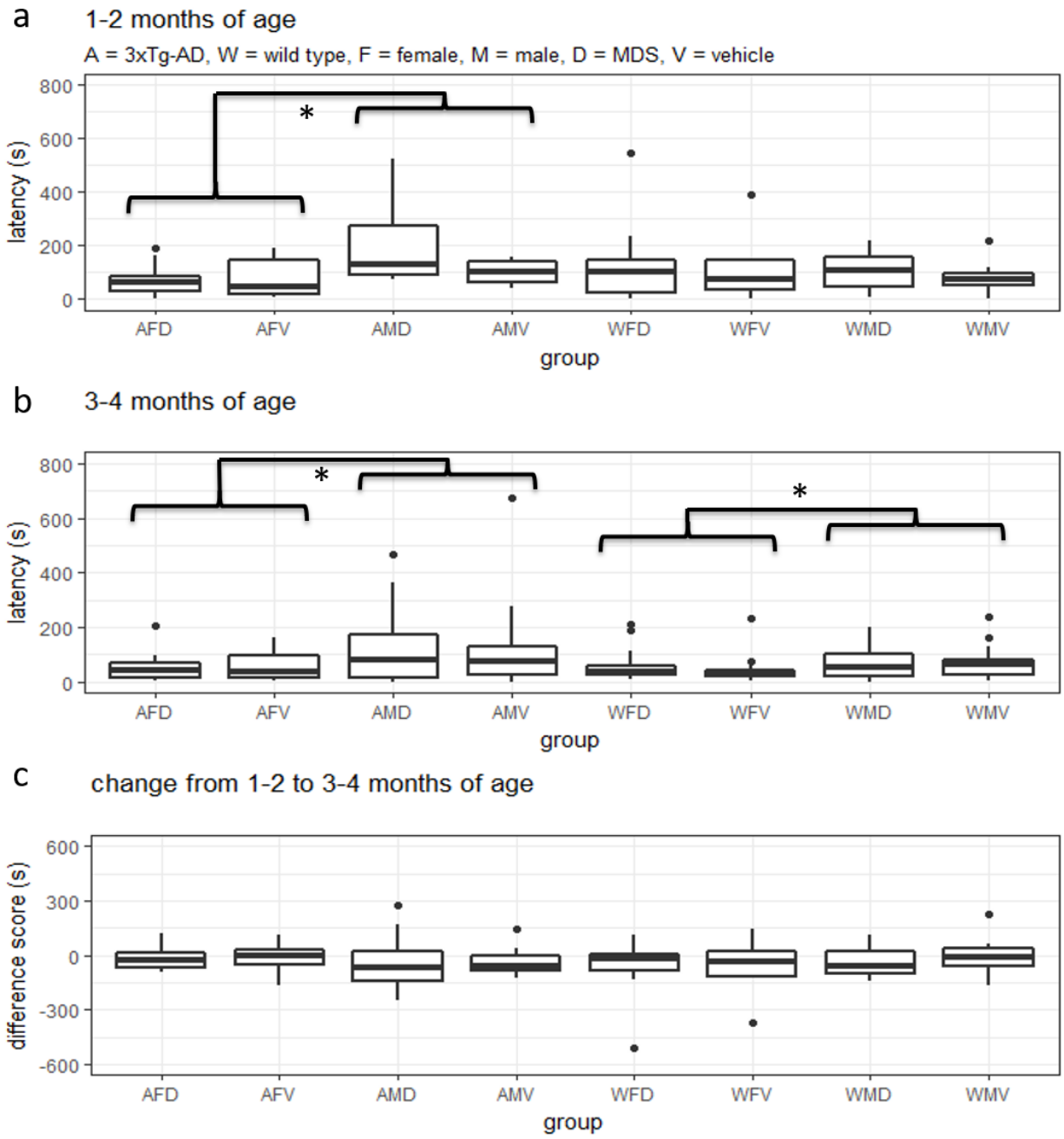


Supplementary Figure 5. Change in sniffing time (s, mean ± SE) between the 4th trial with 0.001% cinnamon (w/v) as the odorant and the trial with 0.001% paprika (w/v) as the odorant during olfactory discrimination testing at 1-2 months of age **(a)** and 3-4 months of age **(b)**. There were no significant differences between groups. (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



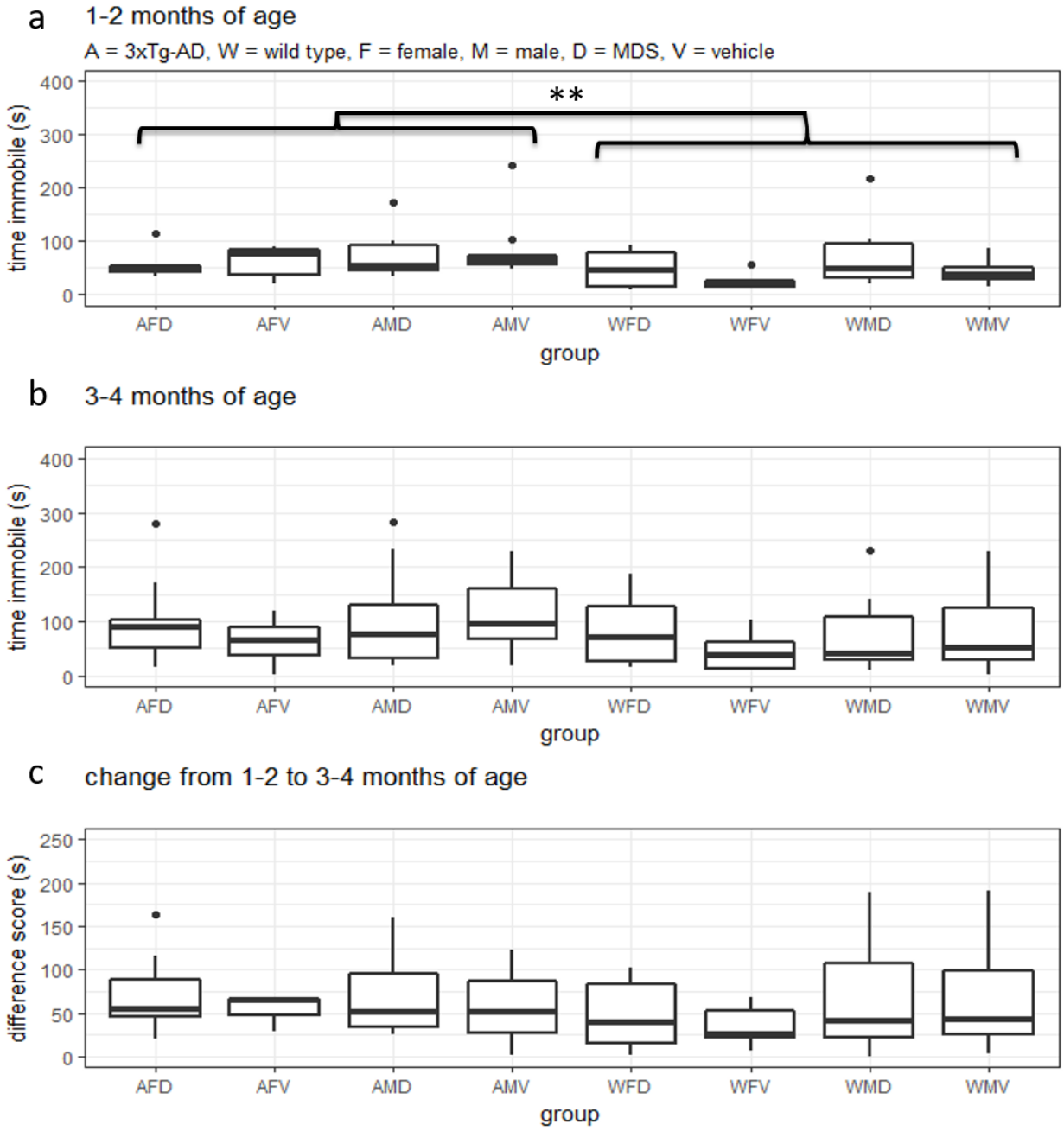
Supplementary Figure 6. Box plots of the time (s) spent in center of the open field at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. **A)** At baseline, 3xTg-AD female mice spent more time than WT female mice in the center (2 x 2 x 2 factorial permutation test, genotype x sex interaction, $p = 0.013$; simple main effect of genotype for females only, permutation ***$p < 0.001$). **B)** At 3-4 months of age, females spent more time in the centre than males (2 x 2 x 2 factorial permutation test, main effect of sex, *$p = 0.014$; simple main effect of genotype for females only, permutation *$p < 0.022$). **C)** There was a trend toward a relative decrease in the open arm time of 3xTg-AD females between 1-2 and 3-4 months of age (2 x 2 x 2 factorial permutation test, genotype x sex interaction, $p = 0.083$). (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD

females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



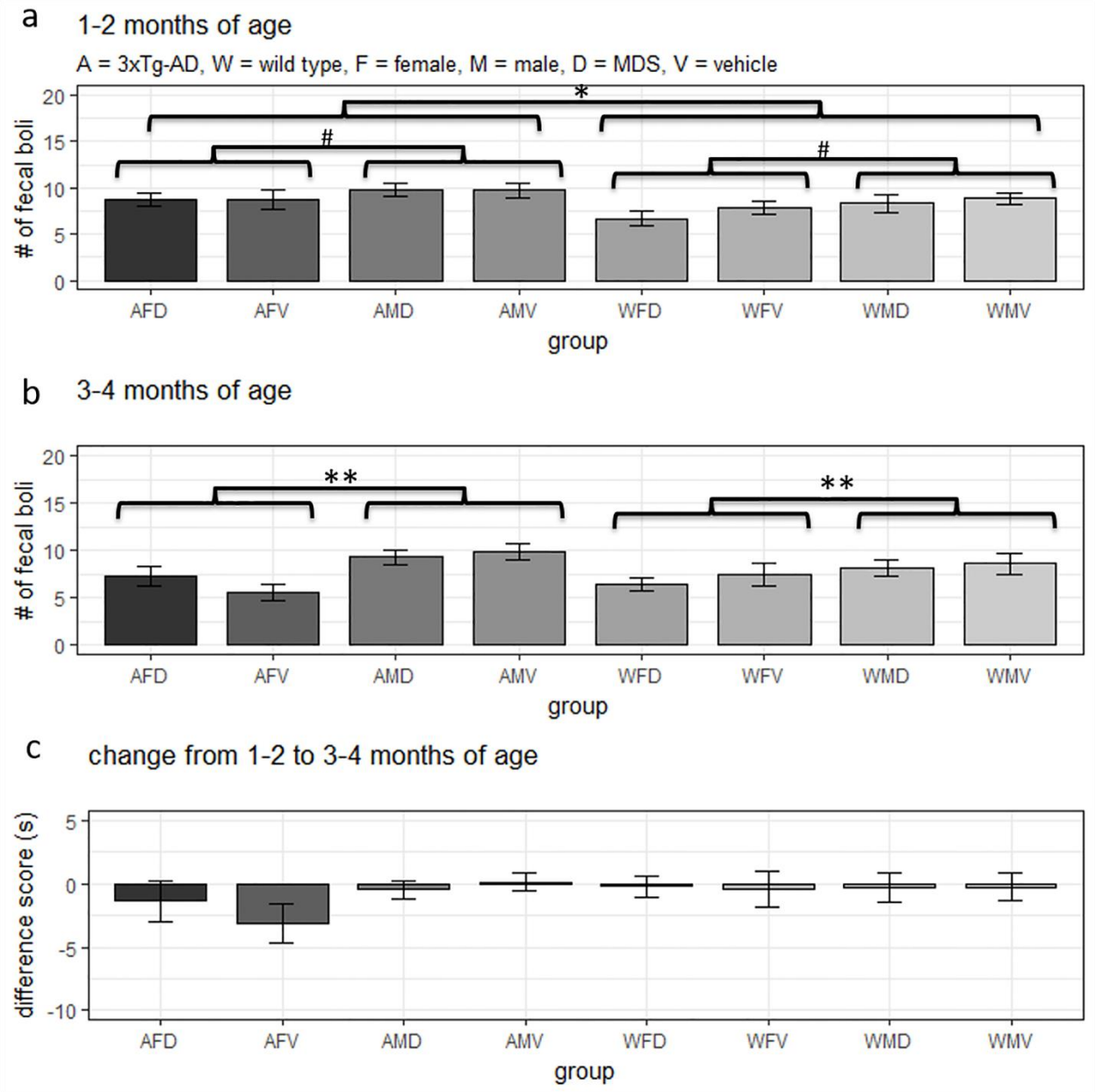
Supplementary Figure 7. Box plots of the latency (s) to approach the center of the open field at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. **A)** At baseline, 3xTg-AD males took longer to approach the center than 3xTg-AD females (2 x 2 x 2 factorial permutation test, p = 0.039). **B)** At 3-4 months of age, males of both genotypes took longer to approach the center than females (2 x 2 x 2 factorial permutation test, main effect of sex, p = 0.022). **C)** There were no significant effects or

interactions between groups for the difference scores. (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])

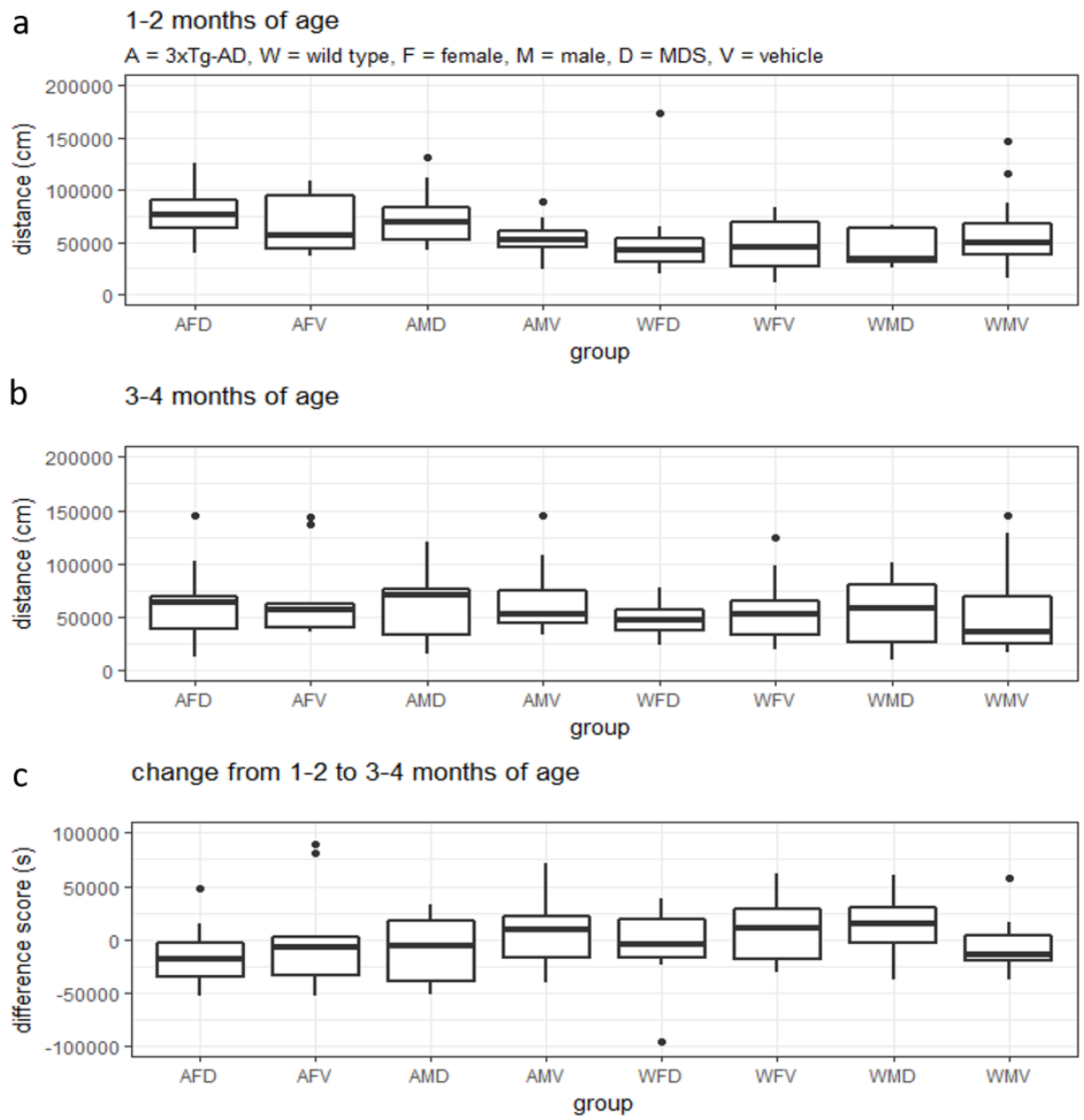


Supplementary Figure 8. Box plots of the time spent immobile/frozen (s) during open field testing at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. **A)** At baseline, 3xTg-AD mice froze more than WT mice (2 x 2 x 2 factorial permutation test, **p = 0.004). There were no significant differences between groups at 3-4 months of age **(b)** or using the difference scores between 1-2 months of

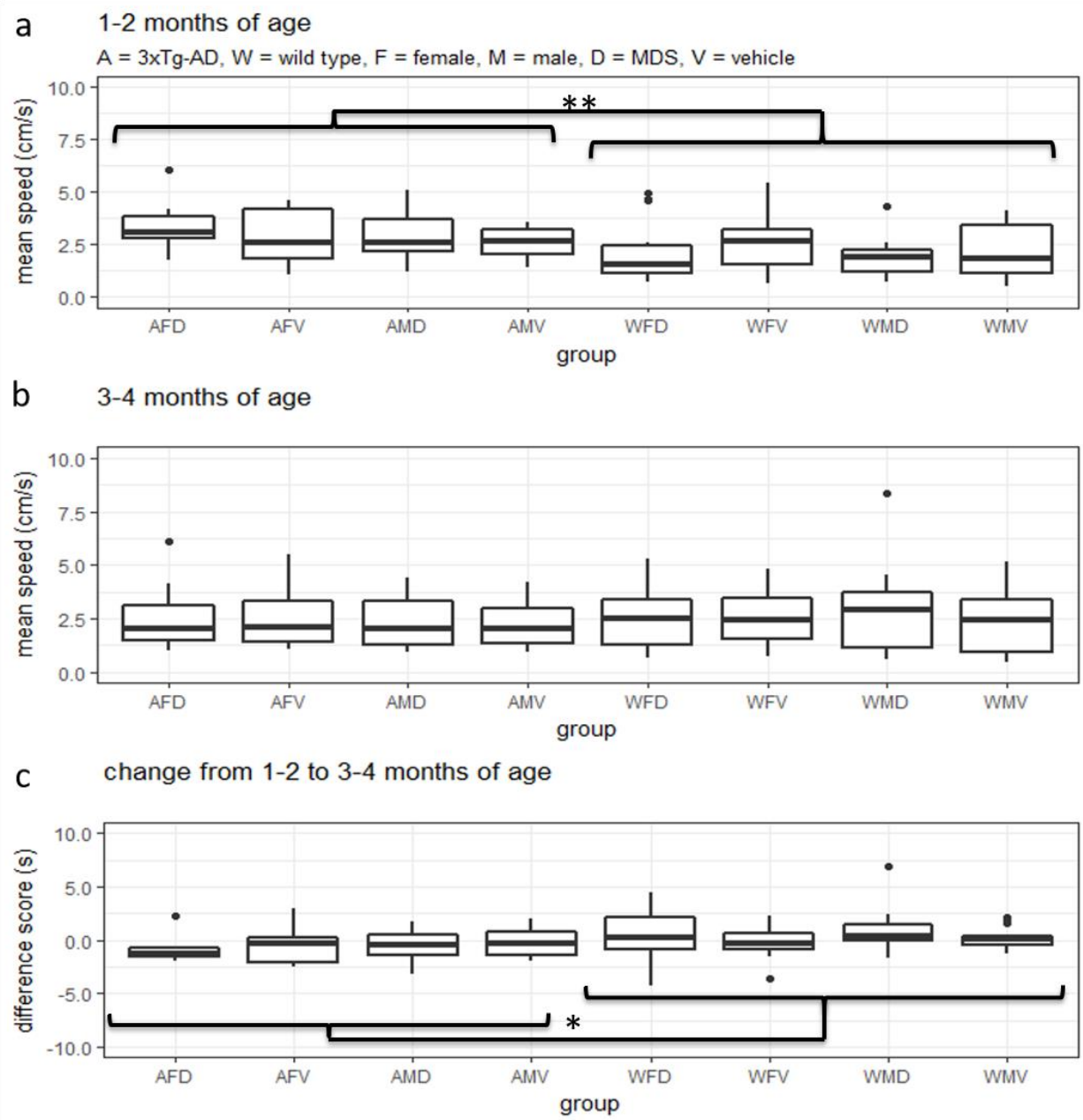
age and 3-4 months of age **(c)**. (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



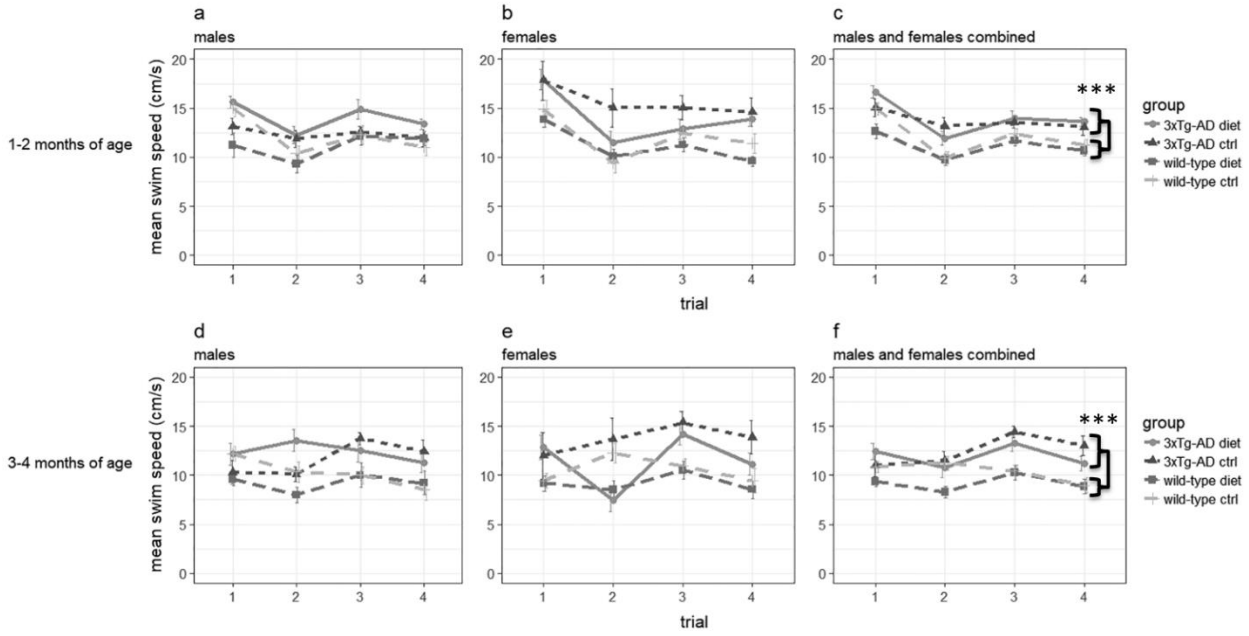
Supplementary Figure 9. Number of fecal boli secreted (mean +/- SE) during open field testing at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. **A)** At baseline, 3xTg-AD mice produced more fecal boli than WT mice and males produced more than females (2 x 2 x 2 ANOVA; main effect of genotype, *p = 0.026; main effect of sex, $^{\#}$p = 0.041). **B)** The difference between genotypes was no longer significant at 3-4 months of age (2 x 2 x 2 ANOVA, main effect of sex only, **p = 0.001). (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



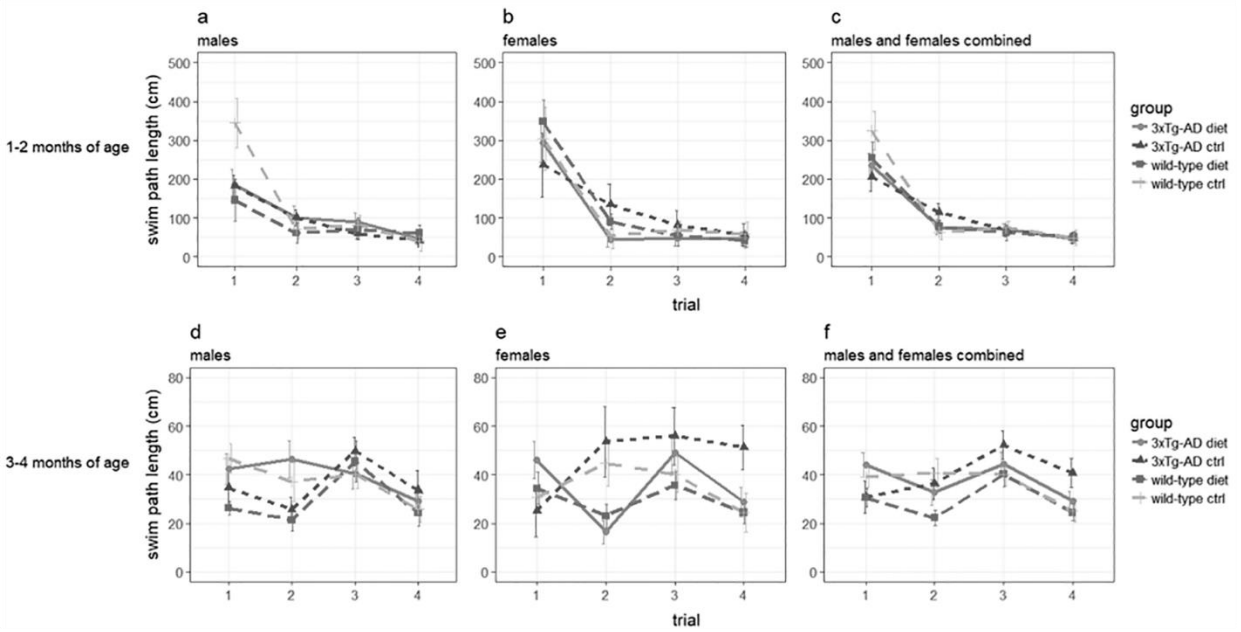
Supplementary Figure 10. Box plots of the total ambulatory distance (cm) of mice during INBEST testing at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. There were no differences between groups. (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



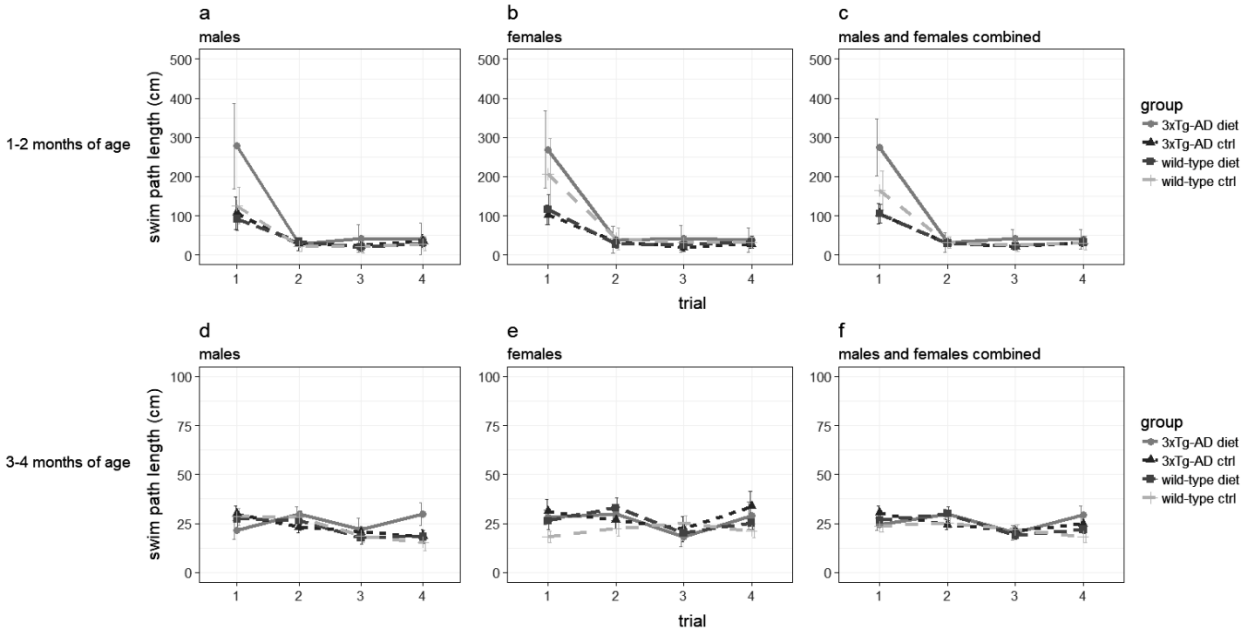
Supplementary Figure 11. Box plots of the mean ambulatory speed (cm/s) of mice during INBEST testing at 1-2 months of age **(a)**, 3-4 months of age **(b)**, and the difference scores **(c)** for each group between 1-2 and 3-4 months of age. 3xTg-AD mice are more active than WT mice at baseline **(a)** (2 x 2 x 2 factorial permutation test, main effect of genotype, **p = 0.004), but not by 3-4 months of age **(b)**, a developmental change also captured using difference scores (**c**; 2 x 2 x 2 factorial permutation test, main effect of genotype, *p = 0.011). (AFD = MDS 3xTg-AD females [n = 10]; AFV = vehicle 3xTg-AD females [n = 10]; AMD = MDS 3xTg-AD males [n = 13]; AMV = vehicle 3xTg-AD males [n = 13]; WFD = MDS WT females [n = 13]; WFV = vehicle WT females [n = 13]; WMD = MDS WT males [n = 12]; WMV = vehicle WT males [n = 13])



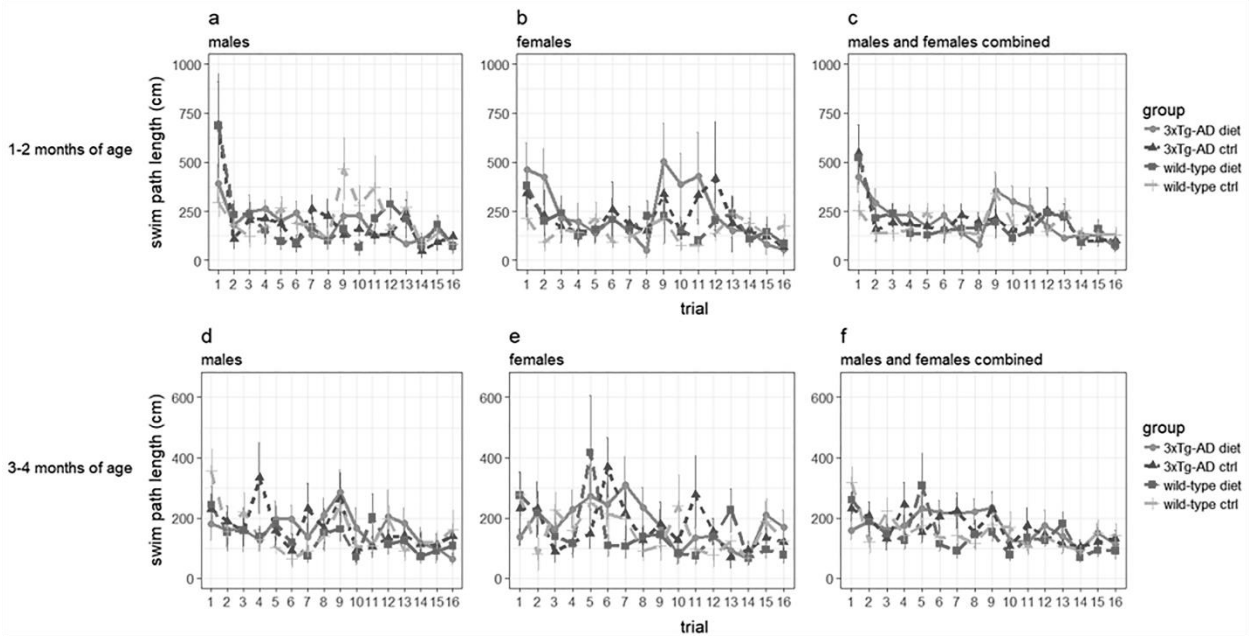
Supplementary Figure 12. Mean swimming speed (cm/s, mean ± SE) on the cued acquisition trials of the water maze at 1-2 **(a-c)** and 3-4 months of age **(d-f)**. At both testing ages, 3xTg-AD mice swam faster than WT mice (2 x 2 x 2 x 4 ANOVA, genotype main effect, ***$p < 0.001$).



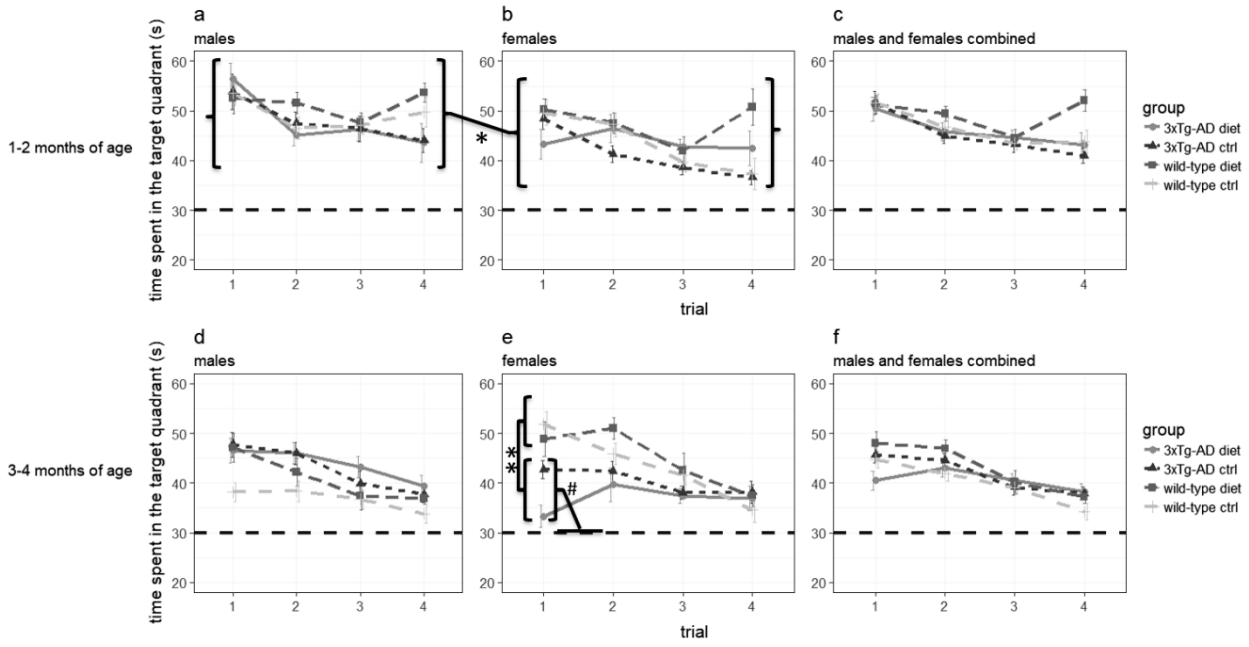
Supplementary Figure 13. Swim path length (cm, mean ± SE) on the cued acquisition trials of the water maze at 1-2 **(a-c)** and 3-4 months of age **(d-f)**. There were no significant differences between groups at either testing age.



Supplementary Figure 14. Swim path length (cm, mean ± SE) on the cued reversal trials of the water maze at 1-2 **(a-c)** and 3-4 months of age **(d-f)**. There were no differences between groups at either testing age.



Supplementary Figure 15. Swim path length (cm, mean ± SE) on the acquisition trials (1st hidden platform location) of the water maze at 1-2 **(a-c)** and 3-4 months of age **(d-f)**. There were no differences between groups at 1-2 months of age **(a-c)**. At both testing ages, all mice improved equally across trials (2 x 2 x 2 x 16 mixed effects ANOVA, main effect of trial, $p < 0.001$ at baseline and 3-4 months of age).

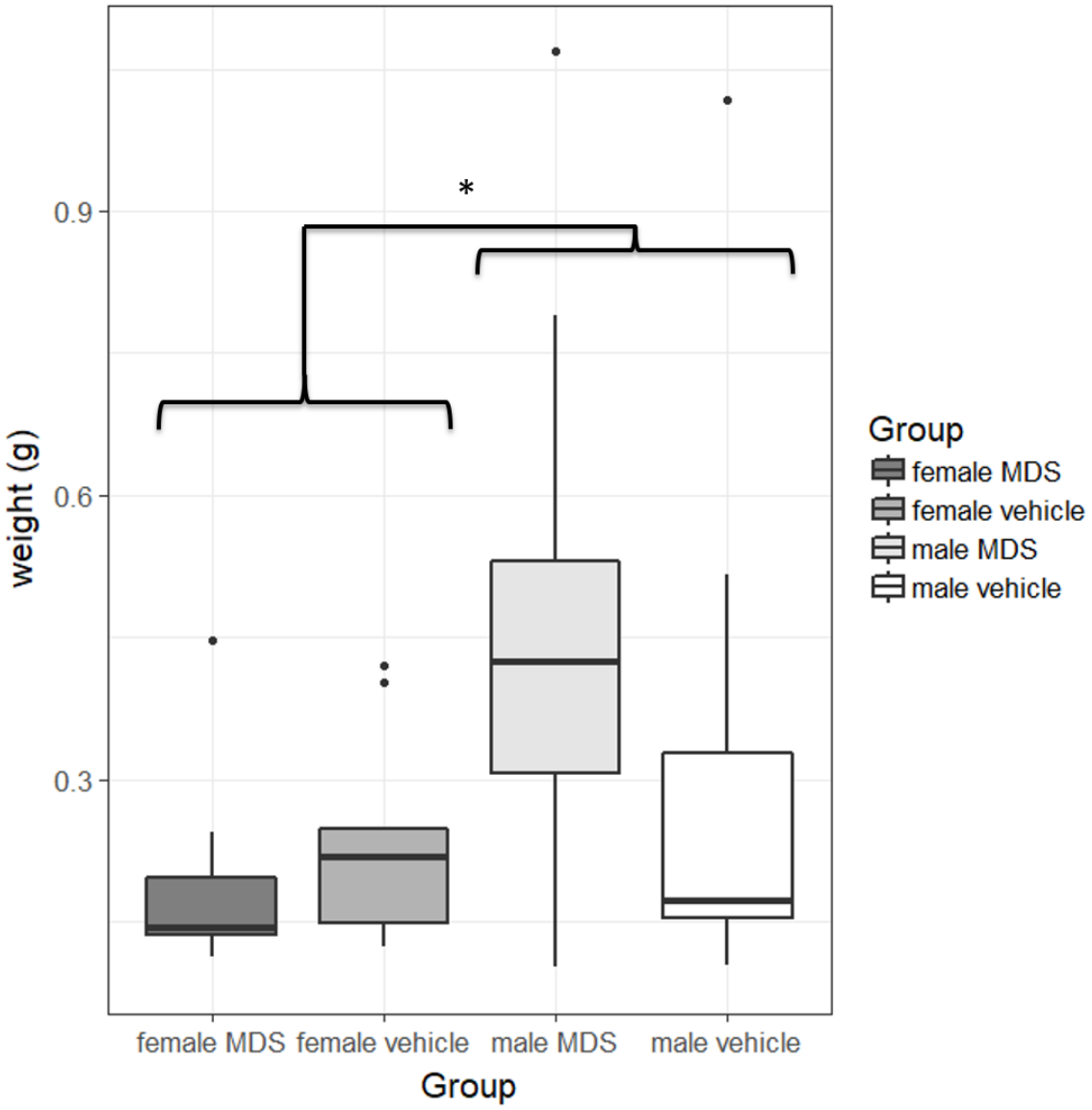


Supplementary Figure 16. Time spent in the target quadrant (s, mean ± SE) on the probe trials of the water maze at 1-2 **(a-c)** and 3-4 months of age **(d-f)**. At 1-2 months age, males spent more time in the target quadrant than females (2 x 2 x 2 x 4 ANOVA, main effect of sex, **a<->b**,*p =

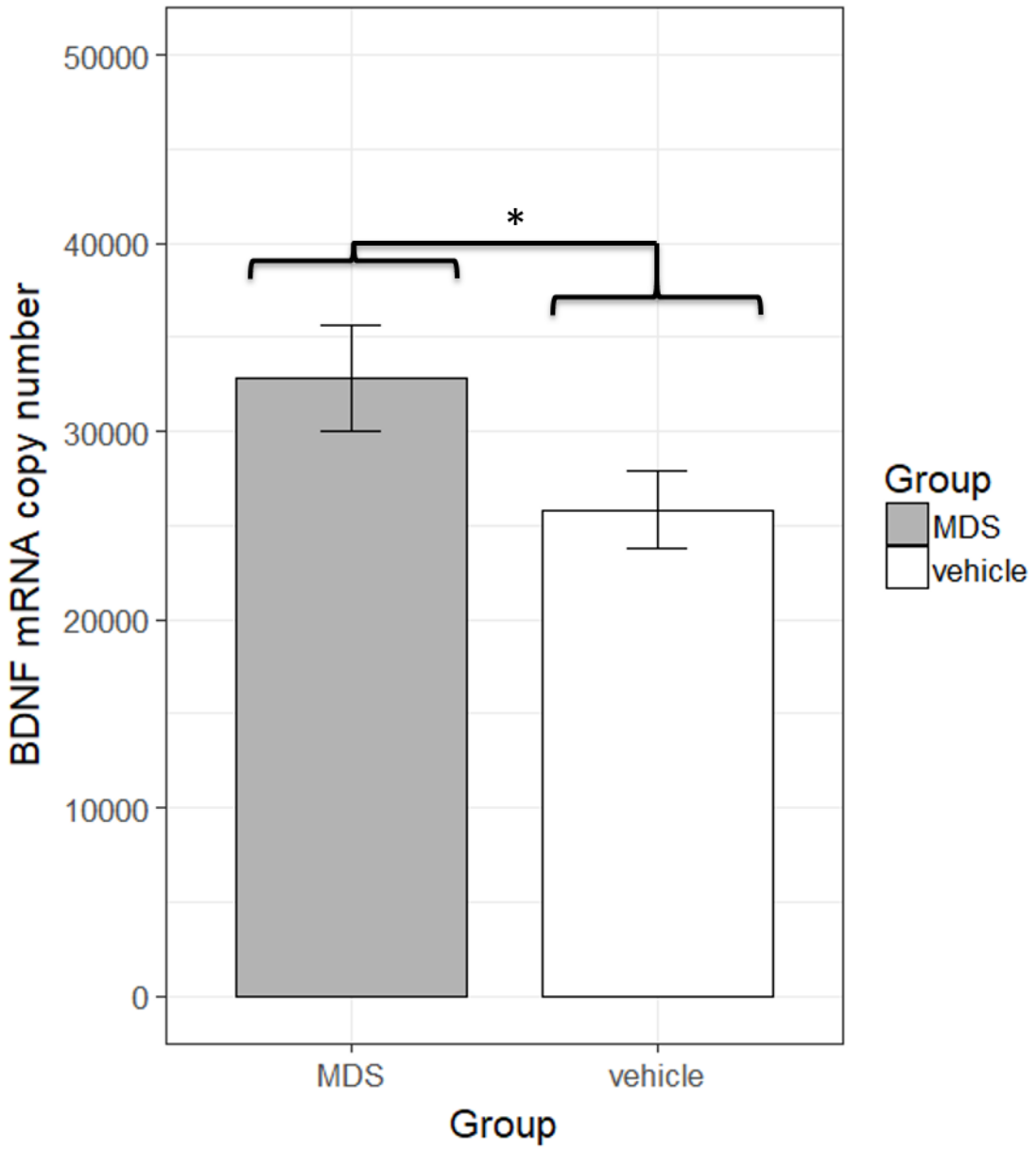
0.013). At 3-4 months of age, 3xTg-AD females spent less time in the target quadrant on the 1st probe trial than WT females (**e**, 2x2x2x4 ANOVA, genotype x sex x trial interaction, $p = 0.02$; Tukey HSD comparisons of genotype x sex groups for each trial, trial 1 [3xTg-AD females vs. WT females], $^{**}p = 0.003$). However, 3-4 month old 3xTg-AD females **(e)** did still spent more than 25% of the trial (30s) in the target quadrant (t-test, $^{\#}p = 0.011$), indicating that they could still recall the platform location.

Appendix B: Supplementary Materials for Chapter 4

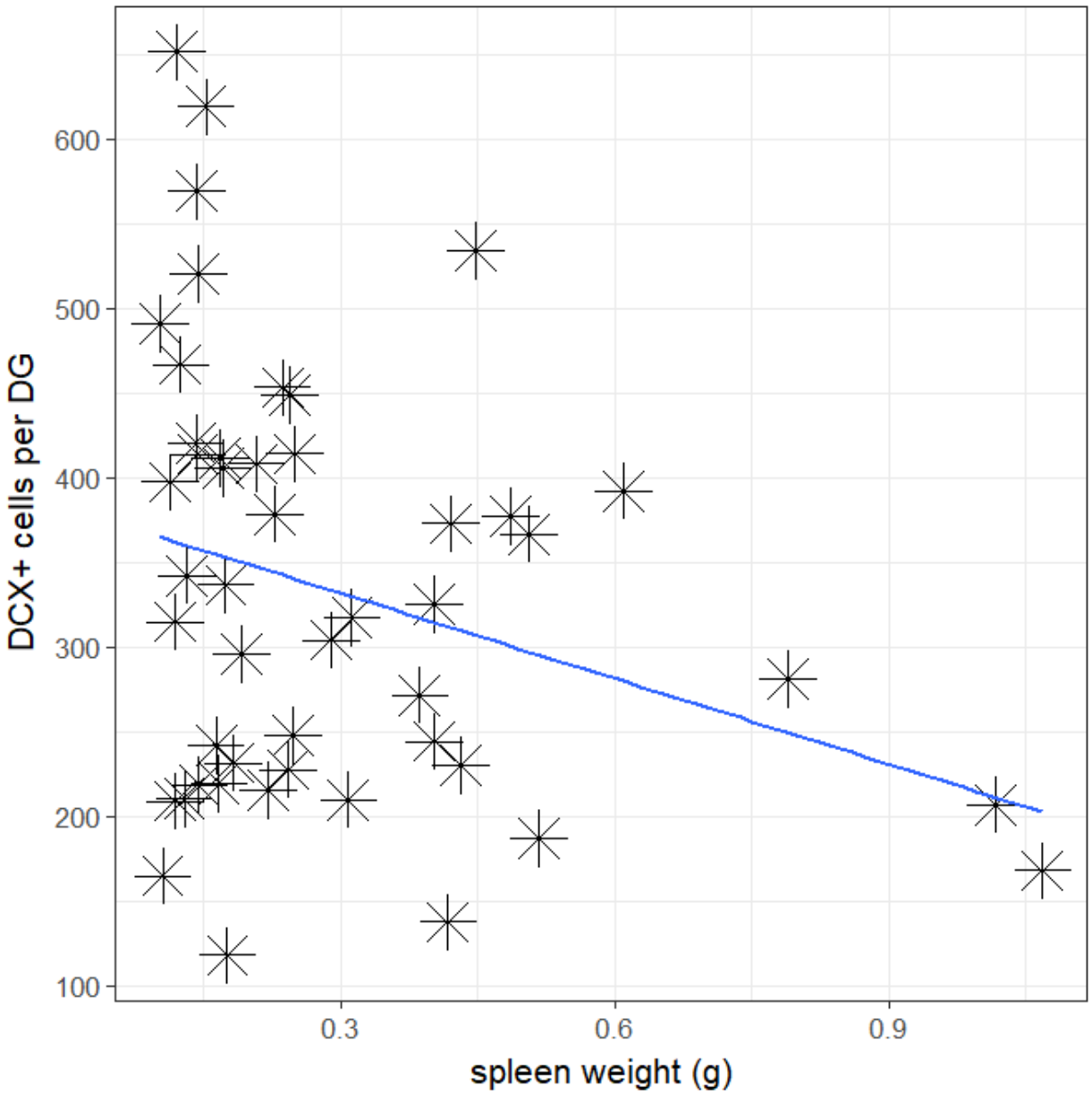
Supplementary Figures



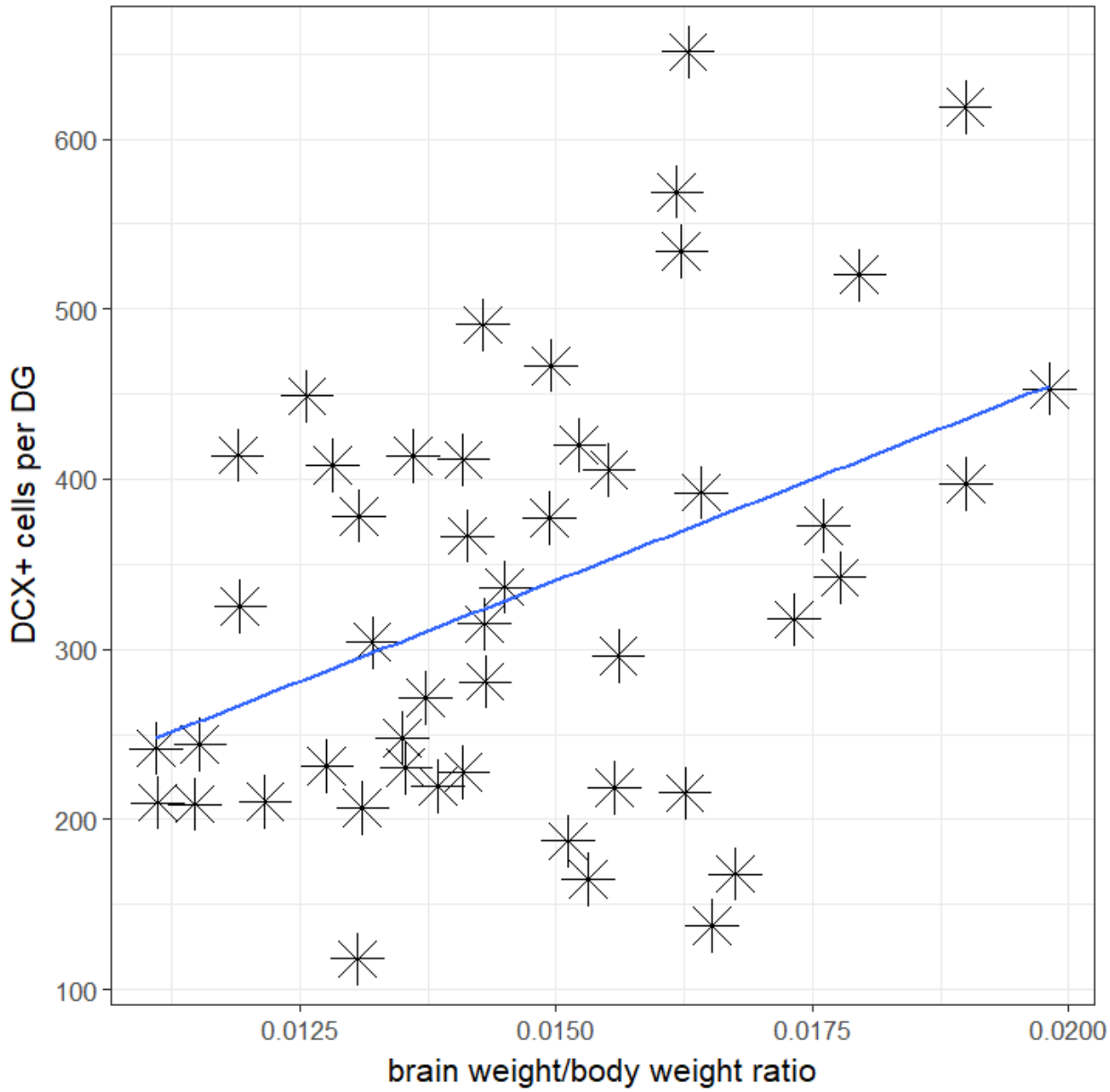
Supplementary Figure 1. Box-and-whisker plots showing the effects of sex and diet on spleen size (g) in 6-7-month-old 3xTg-AD mice (behavioural testing and untested controls combined, n = 12). Males had larger spleens than females (factorial permutation test, main effect of sex, *p = 0.003, $\eta^2_p = 0.178$). Horizontal lines on the tops and bottoms of the boxes represent the 25th and 75th percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range.



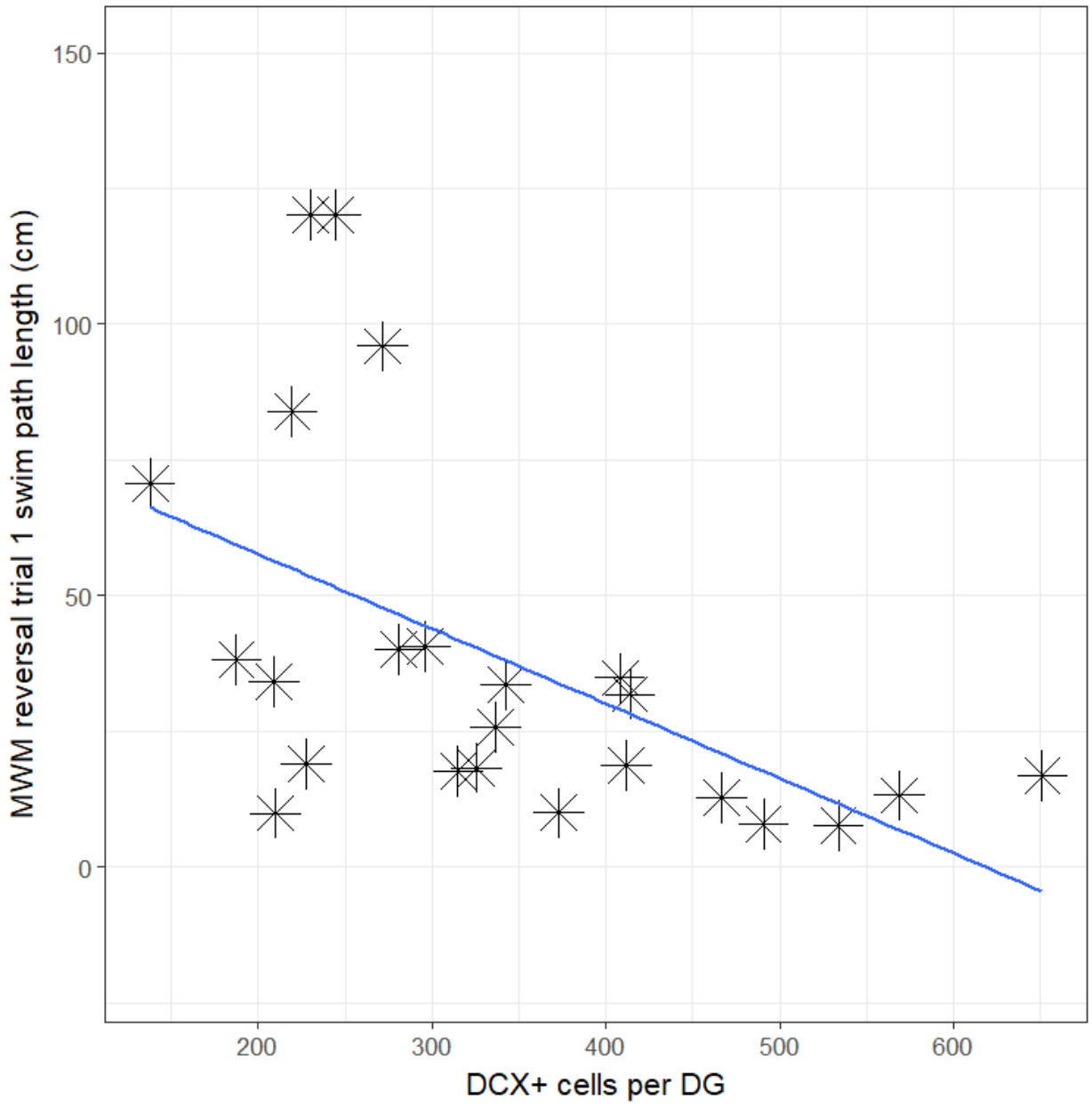
Supplementary Figure 2. Hippocampal brain-derived neurotrophic factor (BDNF) mRNA copies (mean +/- SE) for MDS and vehicle control treatment groups of 6-7-month-old 3xTg-AD mice (both sexes and behavioural testing groups combined; n = 24). BDNF was significantly higher in MDS-treated mice vs. vehicle controls (ANOVA, main effect of diet; $F_{(1, 41)} = 4.081$, *p = 0.049, $\eta^2_p = 0.091$).



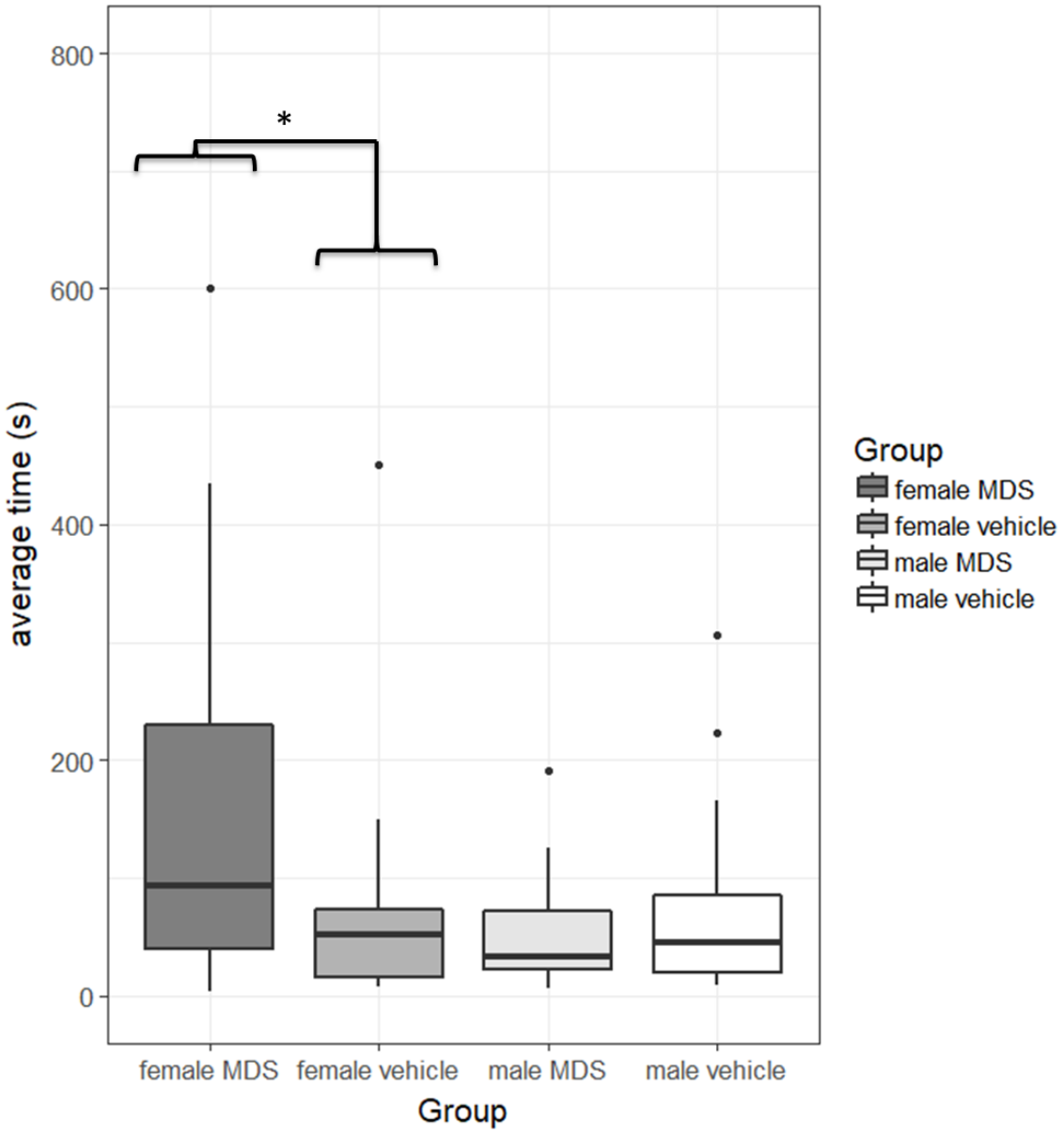
Supplementary Figure 3. Scatterplot showing the relationship between hippocampal neurogenesis and spleen weight fitted with a linear regression line. The number of doublecortin (DCX)-positive cells per dentate gyrus (DG) was negatively correlated with spleen size ($r_{(48)}$ = -0.289, p = 0.046), indicating that 6-7-month-old 3xTg-AD mice with larger spleens had lower levels of hippocampal neurogenesis.



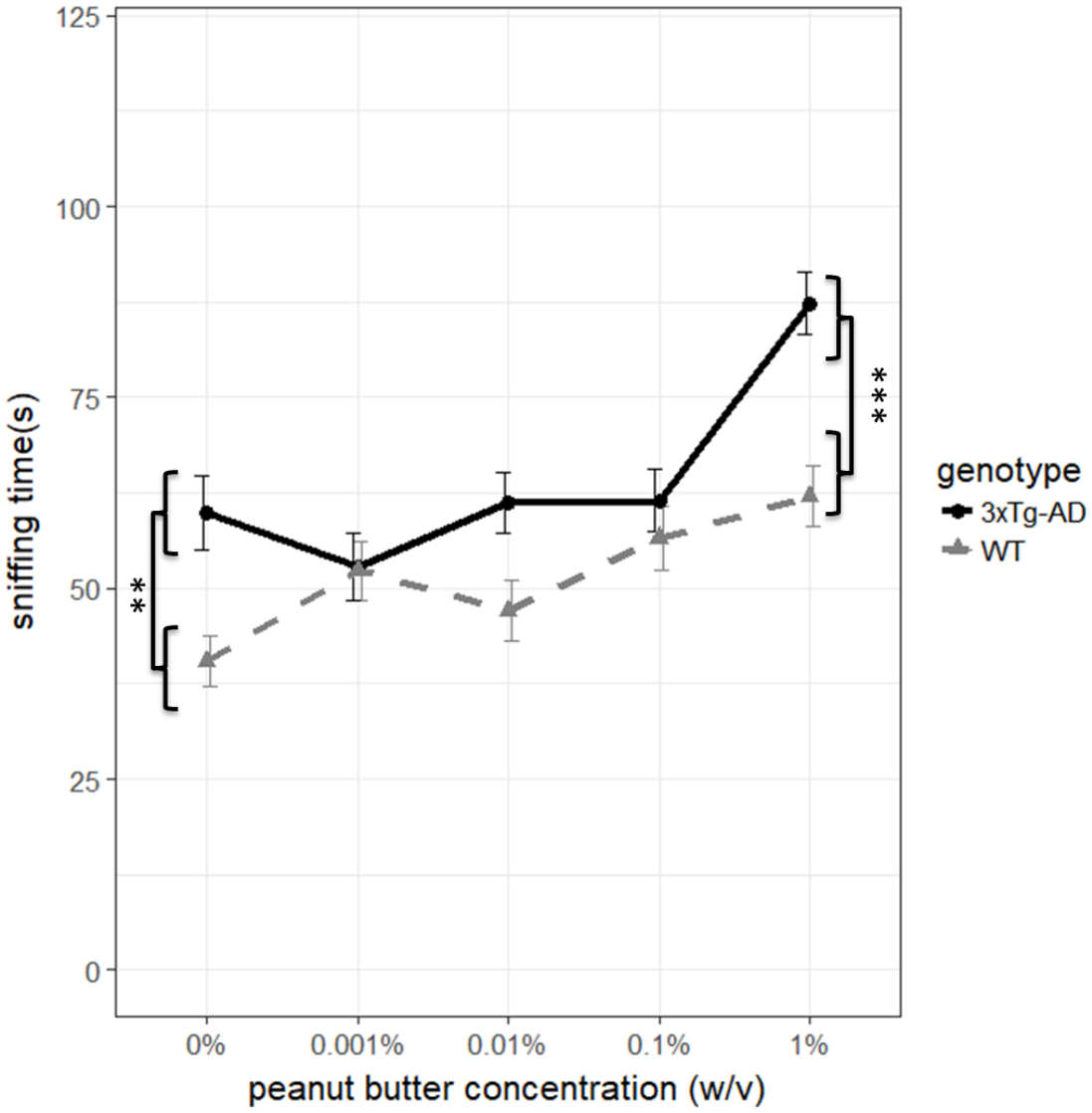
Supplementary Figure 4. Scatterplot showing the relationship between hippocampal neurogenesis (doublecortin [DCX]-positive cells per dentate gyrus [DG]) and brain size (brain weight(g)/body weight(g)) fitted with a linear regression line. The number of DCX+ cells per DG was positively correlated with brain size ($r_{(48)}$ = .403, p = 0.005), indicating that 6-7-month-old 3xTg-AD mice with larger brains also had higher levels of hippocampal neurogenesis.



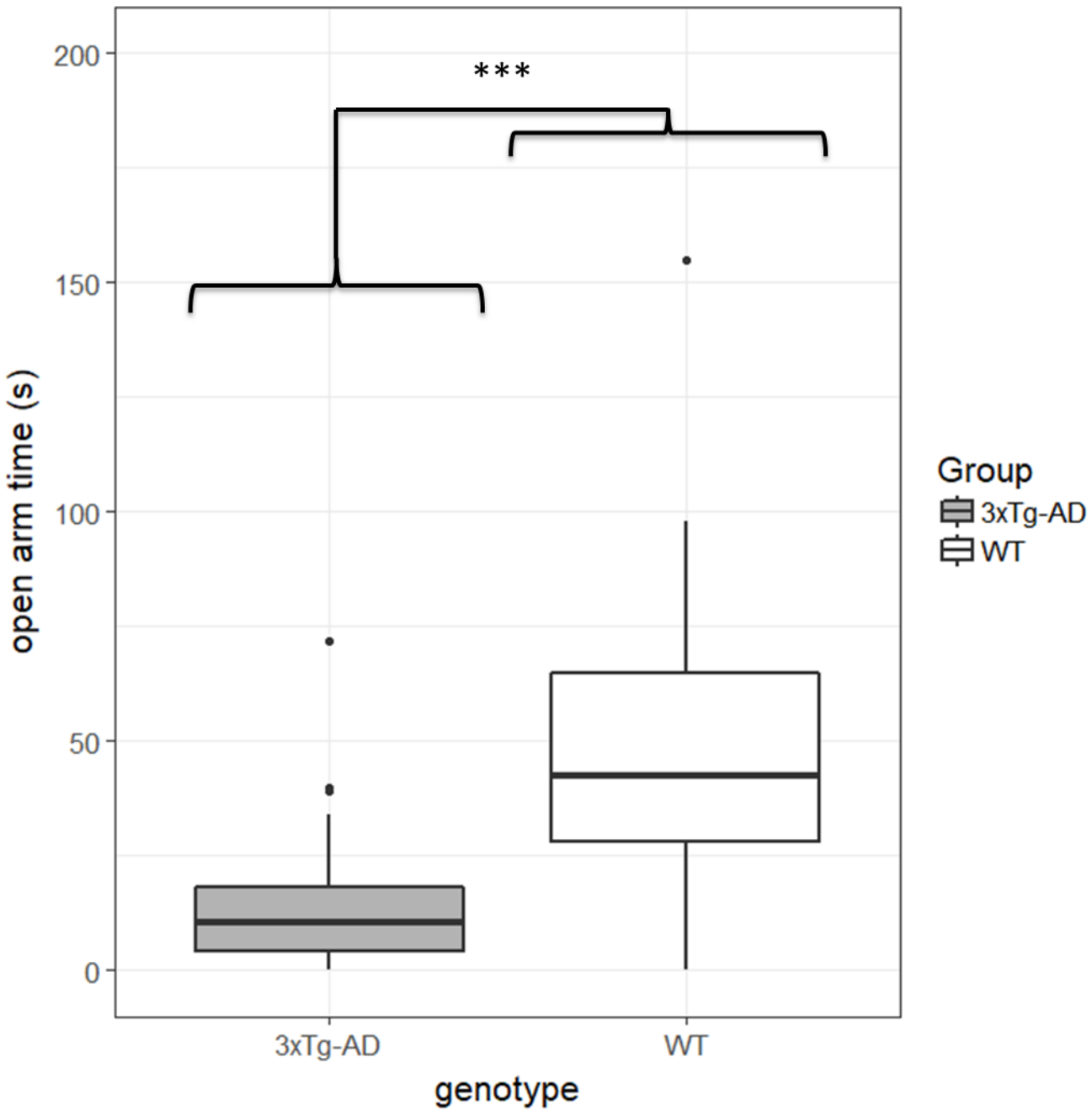
Supplementary Figure 5. Scatterplot showing the relationship between hippocampal neurogenesis and performance (swim path length [cm]) of the mice on the first hidden platform reversal trial of the Morris water maze fitted with a linear regression line. The number of DCX+ cells per DG was negatively correlated with swim path length ($r_{(24)}$ = -.530, p = 0.008), indicating that 6-7 month old 3xTg-AD mice with higher levels of neurogenesis swam more directly towards the hidden platform location.



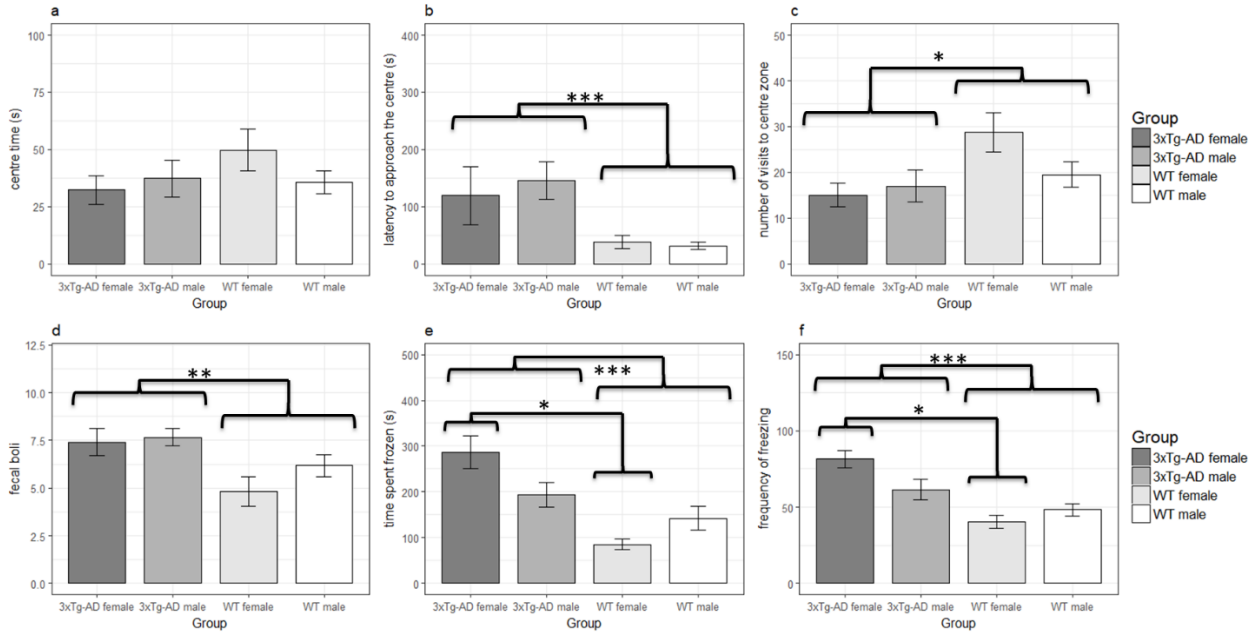
Supplementary Figure 6. Box-and-whisker plots of the average time (s) before falling on the hanging basket test for 3xTg-AD and WT mice at 11-12 months of age (genotypes combined). MDS-supplemented females (n = 21) held on for longer than vehicle-control females (n = 20) or males of either treatment group (male MDS, n = 20; male vehicle, n =22; 2 x 2 x 2 factorial permutation test; sex x diet interaction, *p = 0.021; $\eta^2_p = 0.178$). Horizontal lines on the tops and bottoms of the boxes represent the 25th and 75th percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range.



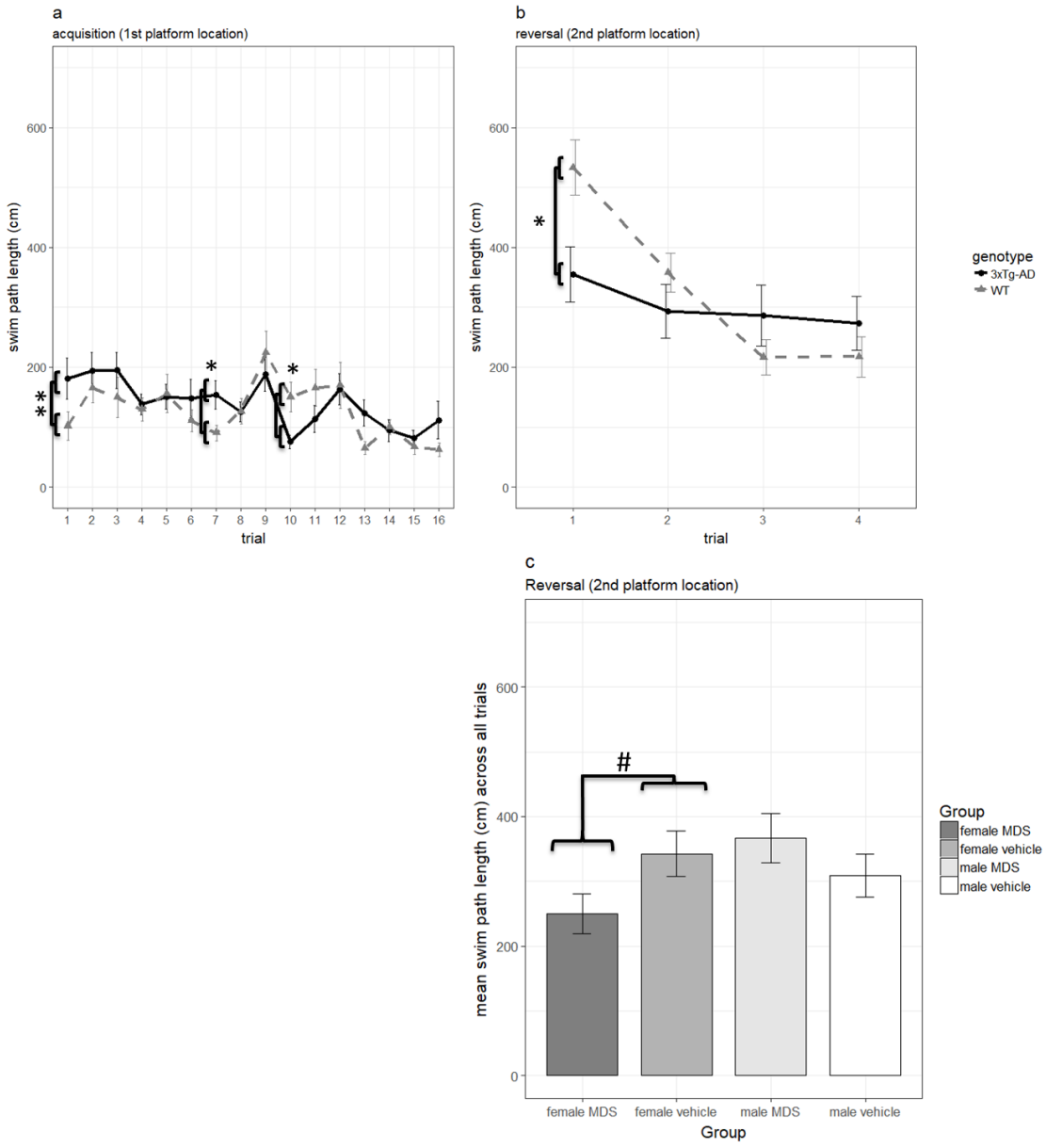
Supplementary Figure 7. Time (s, mean ± SE) spent sniffing the peanut butter (PB) soaked filter paper at different concentrations (w/v) during olfactory acuity testing at 11-12 months of age (sex and diet groups combined). 3xTg-AD mice spent more time sniffing the filter paper at the lowest (0% PB/control) and highest (1% PB) concentrations (repeated-measures ANOVA, genotype x concentration interaction ($F_{(4, 307)} = 3.093$, $p = 0.018$, $\eta^2_g = 0.023$; simple main effect of genotype at each concentration: 0% PB/control, $t_{(324)} = 2.626$, **$p = 0.009$, $d = 0.146$; 0.001% PB, $t_{(324)} = -0.117$, $p = 0.907$, $d = 0.006$; 0.01% PB, $t_{(324)} = 1.825$, $p = 0.069$, $d = 0.101$; 0.1% PB, $t_{(324)} = 0.490$, $p = 0.623$, $d = 0.027$; 1% PB, $t_{(324)} = 3.635$, ***$p < 0.001$, $d = 0.201$).



Supplementary Figure 8. Box-and-whisker plots of the time spent by 11-12- month-old 3xTg-AD (n = 40) and WT mice (n = 43; sex and diet groups combined) in the open arms of the elevated plus maze. 3xTg-AD mice spent less time than WT mice in the open arms (2 x 2 x 2 factorial permutation test; main effect of genotype, ***$p < 0.001$, $\eta 2p = 0.310$). Horizontal lines on the tops and bottoms of the boxes represent the 25th and 75th percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range.

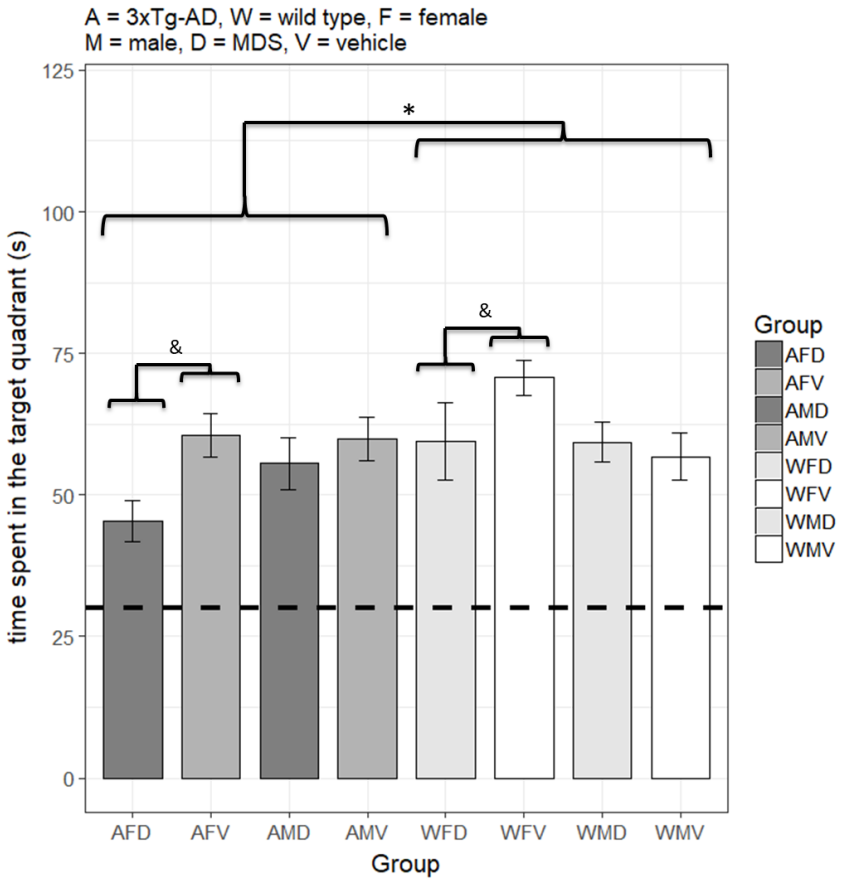


Supplementary Figure 9. Bar graphs showing the performance (mean +/- SE) of male and female 3xTg-AD and WT mice (diet groups combined) on each measure of the open field test. **A)** There were no differences between groups in the time spent (s) in the centre zone. **B)** 3xTg-AD mice took longer (s) to approach the centre (permutation ***$p < 0.001$, $\eta^2_p = 0.121$), **(c)** visited it less frequently (permutation *$p = 0.027$, $\eta^2_p = 0.059$), and **(d)** excreted more fecal boli ($F_{(1, 81)} = 10.346$, **$p = 0.002$, $\eta^2_p = 0.110$) than WT mice. 3xTg-AD females, but not males, also spent more time frozen/immobile **(e)** spent more time frozen (genotype x sex interaction, permutation $p = 0.006$, $\eta^2_p = 0.089$), and **(f)** froze more often (genotype x sex interaction, $F_{(1, 81)} = 6.677$, $p = 0.012$, $\eta^2_p = 0.076$) than WT mice of the same sex.

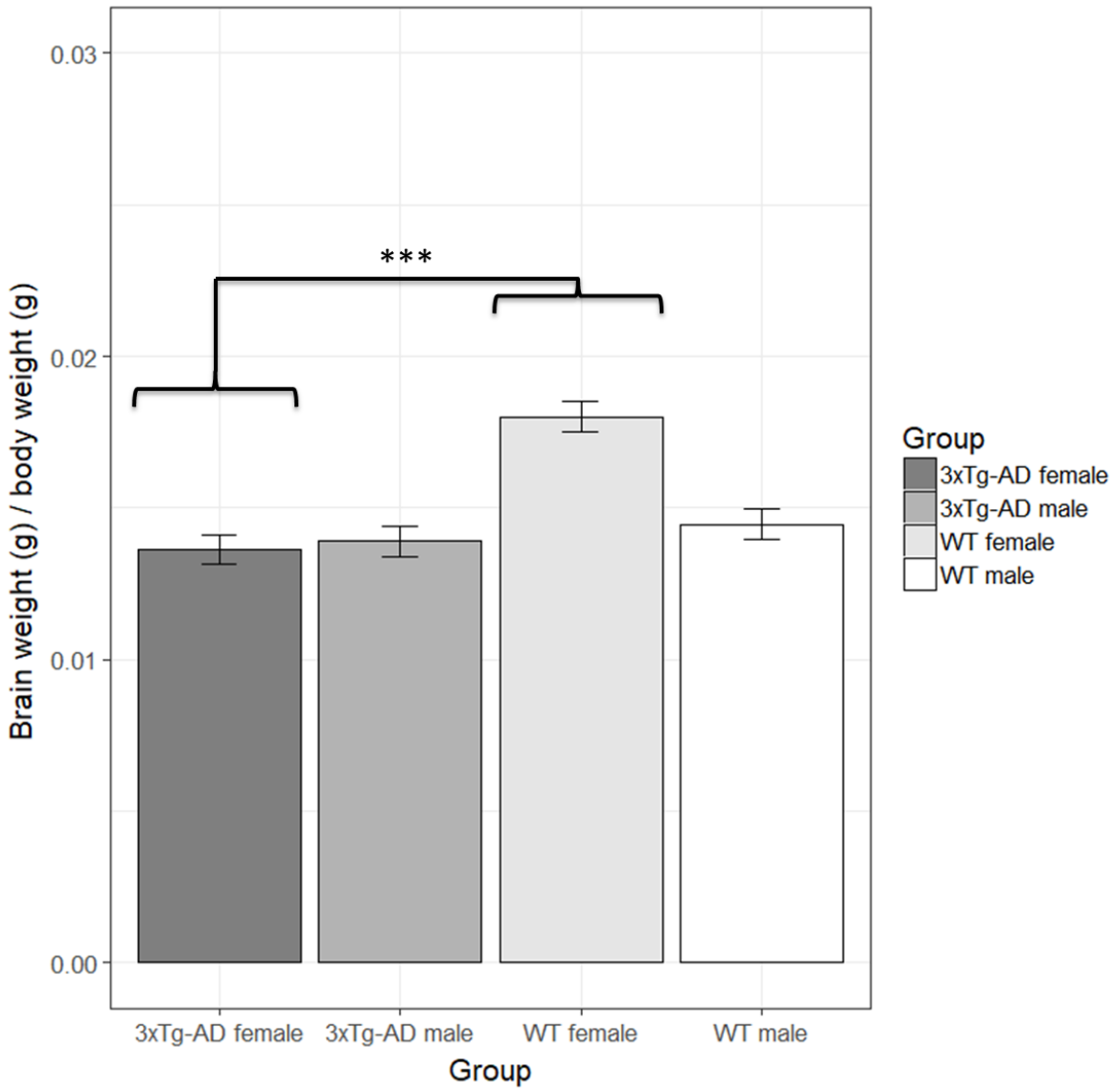


Supplementary Figure 10. Swim path length (cm, mean +/- SE) of 3xTg-AD and WT mice on the acquisition and reversal phases of the Morris water maze. **A)** During the acquisition phase, 3xTg-AD mice swam farther than WT mice (both sexes and diet groups combined) while searching for the hidden platform on the 1st and 7th trials, but WT swam farther than 3xTg-AD mice before escaping on the 10th trial (repeated measures ANOVA, trial x genotype interaction, $F_{(8, 584)} = 4.050$, $p < 0.001$, $\eta^2_g = 0.048$; trial 1, $t_{(1150)} = 3.041$, **p = 0.002; trial 7, $t_{(1150)} = 1.969$, *p = 0.049; trial 10, $t_{(1150)} = -2.252$, *p = 0.025). **B)** On the reversal phase (2nd platform location), 3xTg-AD mice again performed worse than WT mice (both sexes and diet groups combined) on the 1st trial only (genotype x trial interaction ($F_{(3, 211)} = 3.786$, *p = 0.014, $\eta^2_g = 0.027$). **C)** Across

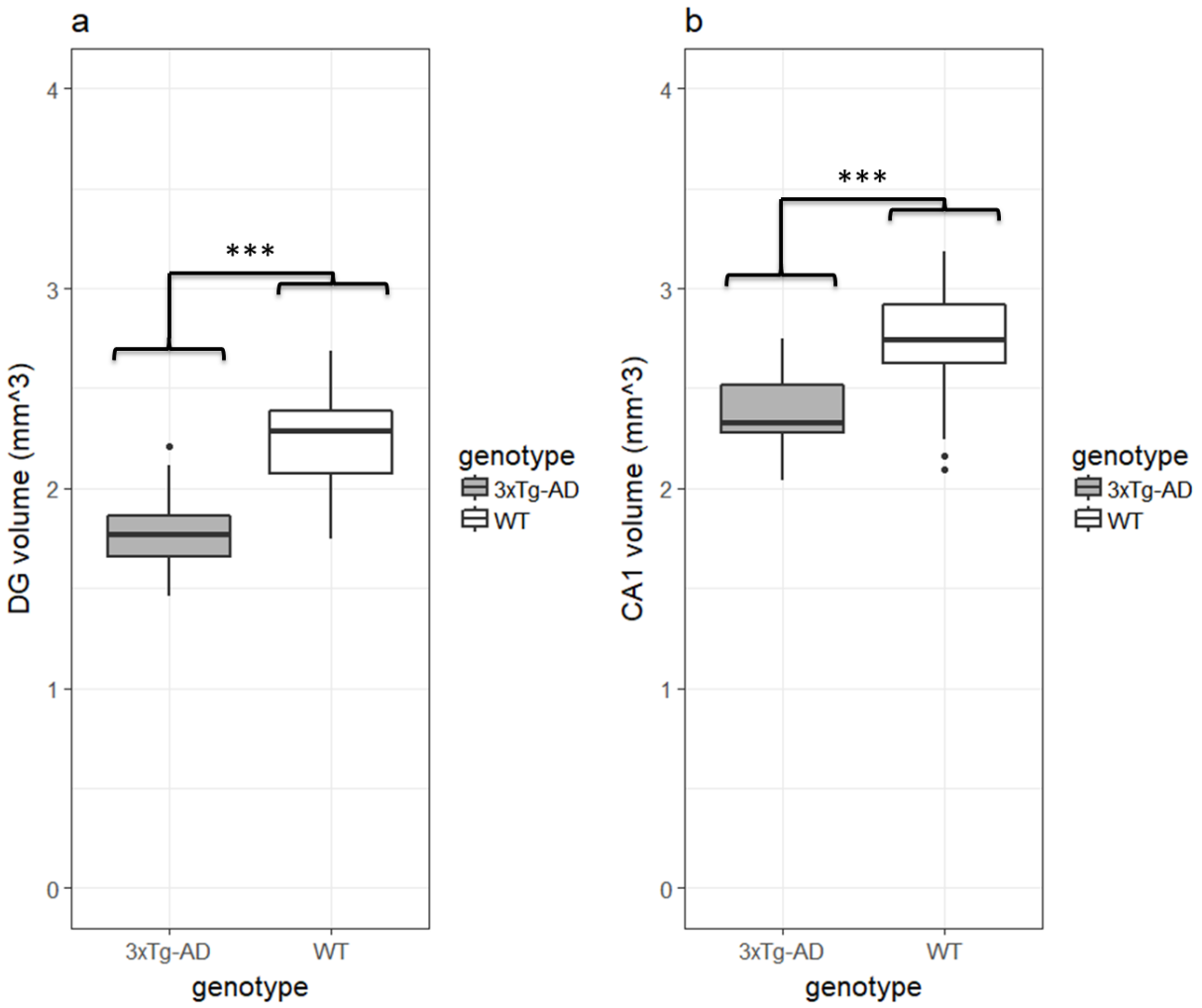
all reversal trials, there was also a trend towards shorter swimming distances in MDS-supplemented females vs. vehicle-control females (shown with genotypes combined; ANOVA, sex x diet interaction; $F_{(1, 78)} = 2.844$, $p = 0.093$, $\eta^2_g = 0.015$; female MDS vs. female vehicle $t_{(165)} = -1.973$, $^{\#}p = 0.050$ [unadjusted $p = 0.025$]; male MDS vs. male vehicle, $t_{(178)} = 1.149$, $p = 0.874$).



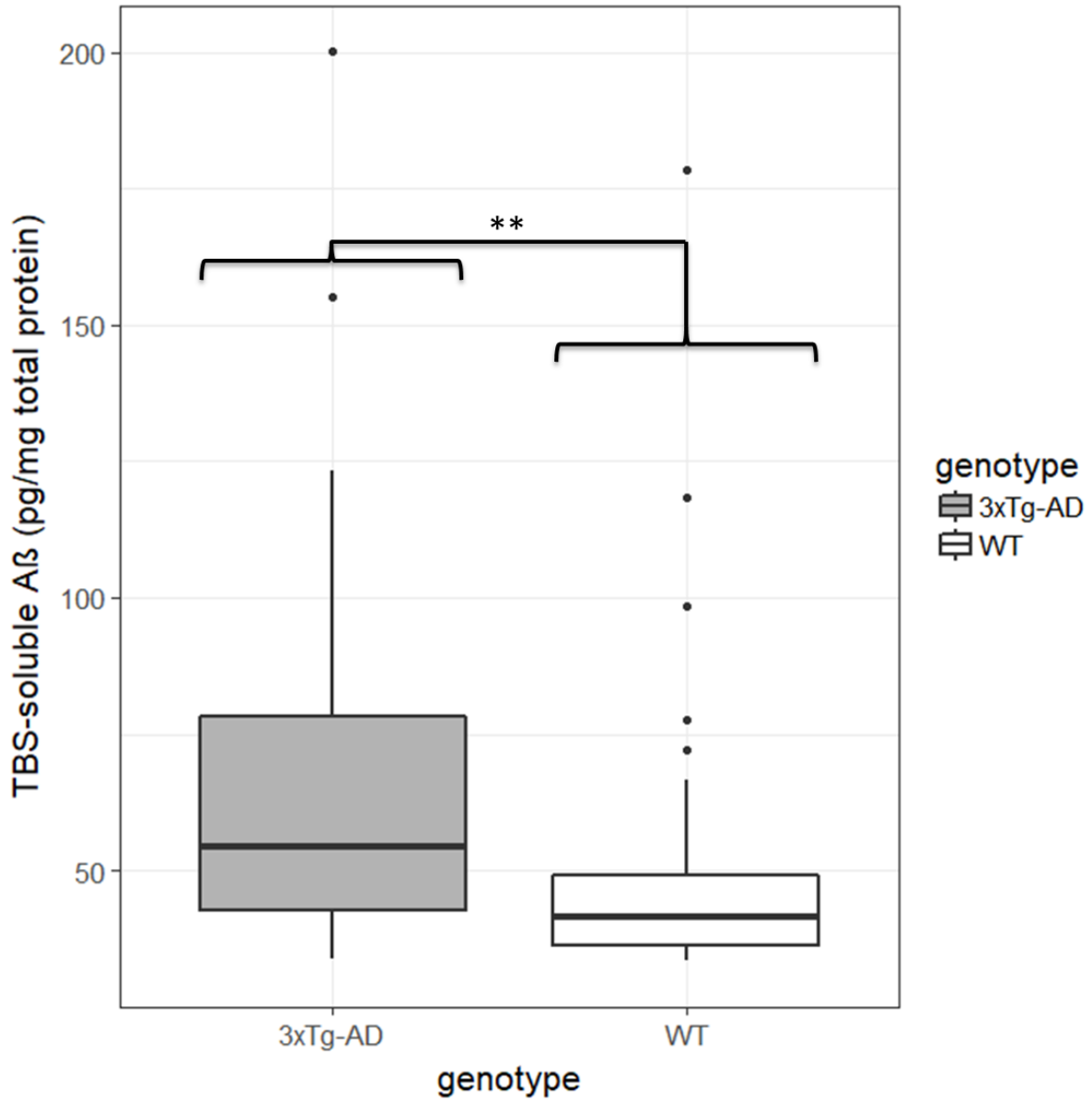
Supplementary Figure 11. Time (s, mean +/- SE) spent by 11-12-month-old mice in the target quadrant of the Morris water maze on the probe trial for the 1st trained platform location. WT mice spent more time in the target quadrant than 3xTg-AD mice (main effect of genotype, $F_{(1, 79)} = 4.213$, $^{*}p = 0.043$, $\eta^2_p = 0.051$). MDS-supplemented females also spent less time in the target quadrant than vehicle-control females (diet x sex interaction; $F_{(1, 79)} = 4.076$, $^{\&}p = 0.047$, $\eta^2_p = 0.049$). However, Holm-corrected one tailed *t*-tests comparing the performance of each group to chance (>30s/120s or 25% of the trial; dashed black line) suggested that all groups, including MDS-supplemented females, could still recall the trained platform location (AFD, $t_{(10)} = 4.216$, $p = 0.002$; AFV, $t_{(8)} = 7.864$, $p < 0.001$; AMD, $t_{(10)} = 5.506$, $p < 0.001$; AMV, $t_{(10)} = 7.653$, $p < 0.001$; WFD, $t_{(9)} = 4.363$, $p = 0.002$; WFV, $t_{(11)} = 12.826$, $p < 0.001$; WMD, $t_{(10)} = 8.154$, $p < 0.001$; WMV, $t_{(11)} = 6.413$, $p < 0.001$).



Supplementary Figure 12. Brain weight/body weight ratio for 11-12-month-old male and female 3xTg-AD and WT mice (diet groups combined). The brains of 3xTg-AD females were smaller than WT females, but there was no difference between 3xTg-AD males and WT males (ANOVA; genotype x sex interaction; $F_{(1, 79)} = 15.646$, ***$p < 0.001$, $\eta^2_p = 0.165$).



Supplementary Figure 13. Box-and-whisker plots of the volume (mm^3) of the DG **(a)** and CA1 **(b)** per hemisphere for 11-12-month-old 3xTg-AD and WT mice (both sexes and diet groups combined). Both hippocampal subfields were smaller in 3xTg-AD mice than WT mice (factorial permutation tests; DG, main effect of genotype, ***$p < 0.001$, $\eta^2_p = 0.542$; CA1, main effect of genotype, ***$p < 0.001$, $\eta^2_p = 0.457$). Horizontal lines on the tops and bottoms of the boxes represent the 25^{th} and 75^{th} percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range.



Supplementary Figure 14. Box-and-whisker plots of the TBS-soluble (oligomeric) Aβ present in the cortical tissue homogenates (pg Aβ/mg total protein) of 11-12-month-old 3xTg-AD and WT mice (both sexes and diet groups combined). Cortical TBS-soluble Aβ levels were higher in 3xTg-AD mice than WT mice (factorial permutation test, main effect of genotype; **p = 0.004, η2p = 0.101). Horizontal lines on the tops and bottoms of the boxes represent the 25th and 75th percentiles, vertical whiskers represent ± 1.5 x the interquartile range, and dots represent potential outliers which are beyond this range.