Use of Dietary Supplementation of Unsaturated Fatty Acids to Delay Onset of Learning and Memory Deficits in TgCRND8 Mice

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Abstract

Alzheimer's disease (AD) is a complex neurodegenerative disorder, involving metabolic dysfunction, pathogenic aggregation of amyloid beta, and deteriorating cognitive function. Patients exhibit deficiency in omega-3,-6,-9 unsaturated fatty acids (UFAs) in plasma and brain membrane phospholipids, suggesting aberrant fatty acid metabolism influences pathology. Cognitive benefits of omega UFAs in AD remain unknown. Here, I examined effects of a four-month dietary supplementation with UFAs for capacity to alter learning and memory behaviour in an AD mouse model. Cognitive impairment in a fifth generation backcross (N5) C57BL/6Crl X C3H/HeJ TgCRND8 (Tg) mice was compared to control (NonTg) littermates, with respect to both males and females, at six months of age using the Morris Water Maze (MWM). Impairment differed between sexes; female Tg mice were severely impaired, whereas male Tg mice displayed delayed learning. A reduced visual acuity in Tg and NonTg mice, shown by adapted SLAG reflex test, did not impair spatial navigation in cued MWM. A four-month omega-6/-9 UFA oral treatment (75 mg/kg/day) improved learning and memory of Tg mice as compared to vehicle and untreated controls. Omega-3 UFAs, or vehicle alone, did not alter learning and memory of Tg and NonTg mice. Thus, dietary supplementation, particularly when enriched in omega-6/9 UFAs, can affect neural function, and delay conversion from a pre-symptomatic to symptomatic state in the TgCRND8 mouse model.

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List of Abbreviations

Aβ	amyloid beta
AICD	amyloid intracellular domain
AD	Alzheimer's disease
ALA	α-linolenic acid
Alb	albumin
ANOVA	analysis of variance
APP	amyloid precursor protein
BACE1	beta-site amyloid precursor protein cleaving enzyme
cGMP	cyclic guanosine monophosphate
cGMP-PDE	cyclic guanosine monophosphate phosphodiesterase
CNS	central nervous system
CRND	centre of research in neurodegenerative diseases
CSF	cerebrospinal fluid
DHA	docosahexaenoic acid
EOFAD	early onset familial Alzheimer's disease
EPA	eicosapentaenoic acid
F	female
FA	fatty acid
FABP	fatty acid binding protein
FATP	fatty acid transport protein
GC	gas chromatography
hAPP	human amyloid precursor protein
IC_{50}	half-maximal inhibitory concentration
IDE	insulin degrading enzyme
IGF	insulin-like growth factor
LA	linoleic acid
LOAD	late onset Alzheimer's disease
M	male
MCI	mild cognitive impairment
MUFA	monounsaturated fatty acid
MWM	Morris Water Maze
N5	fifth generation backcross
NFT	neurofibrilary tangles
NMDA	N-methyl-D-aspartate
NonTg	non-transgenic
OA	oleic acid
PC	phosphatidylcholine
PDGF	platelet-derived growth factor beta chain
PDE	phosphodiesterase
$Pde6b^{rd1/rd1}$	phosphodiesterase 6 beta gene rd1/rd1 mutation
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PrP	prion protein
PS1	presenilin 1

PS2	presenilin 2
PUFA	polyunsaturated fatty acid
sAPP α	soluble amyloid precursor protein alpha
sAPP β	soluble amyloid precursor protein beta
SFA	saturated fatty acid
SLAG	slow angled-descent forepaw grasping
SP	signal peptide
τ	tau
Tg	N5 C57BL/6Crl X C3H/HeJ TgCRND8
Thy-1	thymocyte differentiation antigen 1
TTR	transthyretin
Tyr^{C}	tyrosinase gene locus
UFA	unsaturated fatty acid
VWB	visual water box
ZT	zeitgeiber time
ω	omega

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Chapter 1 – General Introduction

1.1 AD and the amyloid cascade hypothesis

In 1906, Alois Alzheimer defined the clinicopathological syndrome of AD in the reported case of Auguste Deter, an elderly woman presenting several of the cardinal manifestations observed in patients today (1). Dementia is a general term for the deterioration of memory and cognitive abilities, and AD is the most common form of dementia (2). AD is a progressive, neurodegenerative disorder characterized by a decline in cognitive function and alterations in mood and behaviour (1). Symptoms present as a worsening ability to recall information and memories (2,3). Individuals may display symptomatic predementia; clinically termed mild cognitive impairment (MCI) due to AD, exhibiting symptoms of cognitive deficits yet functional cognition (4). Core clinical criteria for diagnosis of possible AD dementia meets described criteria for all-cause dementia and additionally has characteristics of gradual onset, with history of worsening cognition and decline in two or more cognitive domains (i.e., amnestic presentation, language presentation, visuospatial presentation or executive dysfunction) (5). Also, diagnosis of probable AD dementia is not applied when there is evidence for other concurrent cognitive impairment or other dementia types (stroke, Lewy bodies), although it is now recognized that few “pure” AD cases exist without progressive vascular involvement (5). Generally, individuals decline in cognitive and functional abilities, requiring aid with basic activities of daily living (2). Advanced AD patients are bed-bound, have lost ability to

communicate, and fail to recognize loved ones (2). AD may ultimately lead to death by consequence of pneumonia (2).

AD has no cure, and few therapeutic options currently exist (6). Current treatments with cholinesterase inhibitors (Donepezil, Galantamine, Rivastigmine, Tacrine) or N-methyl-D-aspartate (NMDA) receptor antagonists (Memantine) offer symptomatic relief at best, but there is no disease-modifying treatment for AD (3,7). AD and AD-type dementias with vascular involvement influence approximately 36 million people worldwide (8). In 2008, AD affected 480,600 people of the Canadian population, and this number is predicted to increase to 1.1 million by 2038 (9). Individuals with dementia, their families and friends, are afflicted with a tremendous burden of personal, emotional, and financial levels (8). The Alzheimer Society of Canada predicts an economic burden of $872 billion and a ten-fold increased demand for long-term care over the next 30 years (9). This dementia is significantly affecting every worldwide healthcare system (8), and the impact on individual families is insufficiently appreciated.

AD is often divided into two forms: (1) Mendelian inheritance familial cases of early-onset (<60 years, EOFAD), and (2) sporadic cases of late onset with no familial aggregation (>60 years, late-onset AD (LOAD)) (10,11). The majority of reported cases are LOAD with an unknown cause, whereas EOFAD accounts for 1–10% of cases and is linked to several autosomal dominant mutations of either the amyloid precursor protein (12) (12), presenilin 1 (PS1), or presenilin 2 (PS2) (1,11). Identification of other underlying genetic determinants of risk is an area of intense research (1). EOFAD and LOAD forms are phenotypically similar. Both

forms present common pathologies post-mortem and similar clinical manifestations pre-mortem although with different time courses of onset (1).

Two central neuropathologies define AD: (1) intraneuronal accumulation of neurofibrillary tangles composed of hyperphosphorylated tau, and (2) aberrant processing of the APP forming toxic amyloid β (Aβ) fragments (10,13). The amyloid cascade hypothesis postulates an imbalance between the production and clearance of Aβ as the root cause of AD (14-16). As the *APP* gene is located on chromosome 21, Down syndrome patients develop neuropathological characteristics of AD early by midlife and this example supports the amyloid cascade hypothesis (6,17,18). Aβ isolated from amyloid plaques originate from transmembrane APP (19). APP is a type I transmembrane protein with a large extracellular amino-terminal domain and a small intracellular cytoplasmic domain (20). APP can be processed along two pathways: non-amyloidogenic and amyloidogenic (Figure 1.1) (21). First, APP can be cleaved by either α-secretase or β-secretase, leading to the removal of the amino-terminal fragment and the release of soluble APP (sAPPα and sAPPβ) (21). In non-amyloidogenic processing, α-secretase removes an APP amino terminal fragment to produce sAPPα and a carboxyl-terminal fragment C83 peptide remains membrane-bound. γ-secretase can then cleave C83 to yield P3 and an amyloid intracellular domain (AICD) (21). In amyloidogenic processing, APP is cleaved by β-secretase yielding the sAPPβ fragment and a membrane-bound C99 fragment. γ-secretase then cleaves C99 creating AICD and Aβ peptides (21). In brief, Aβ is produced by the sequential cleavage of APP by β-secretase and γ-secretase, and its

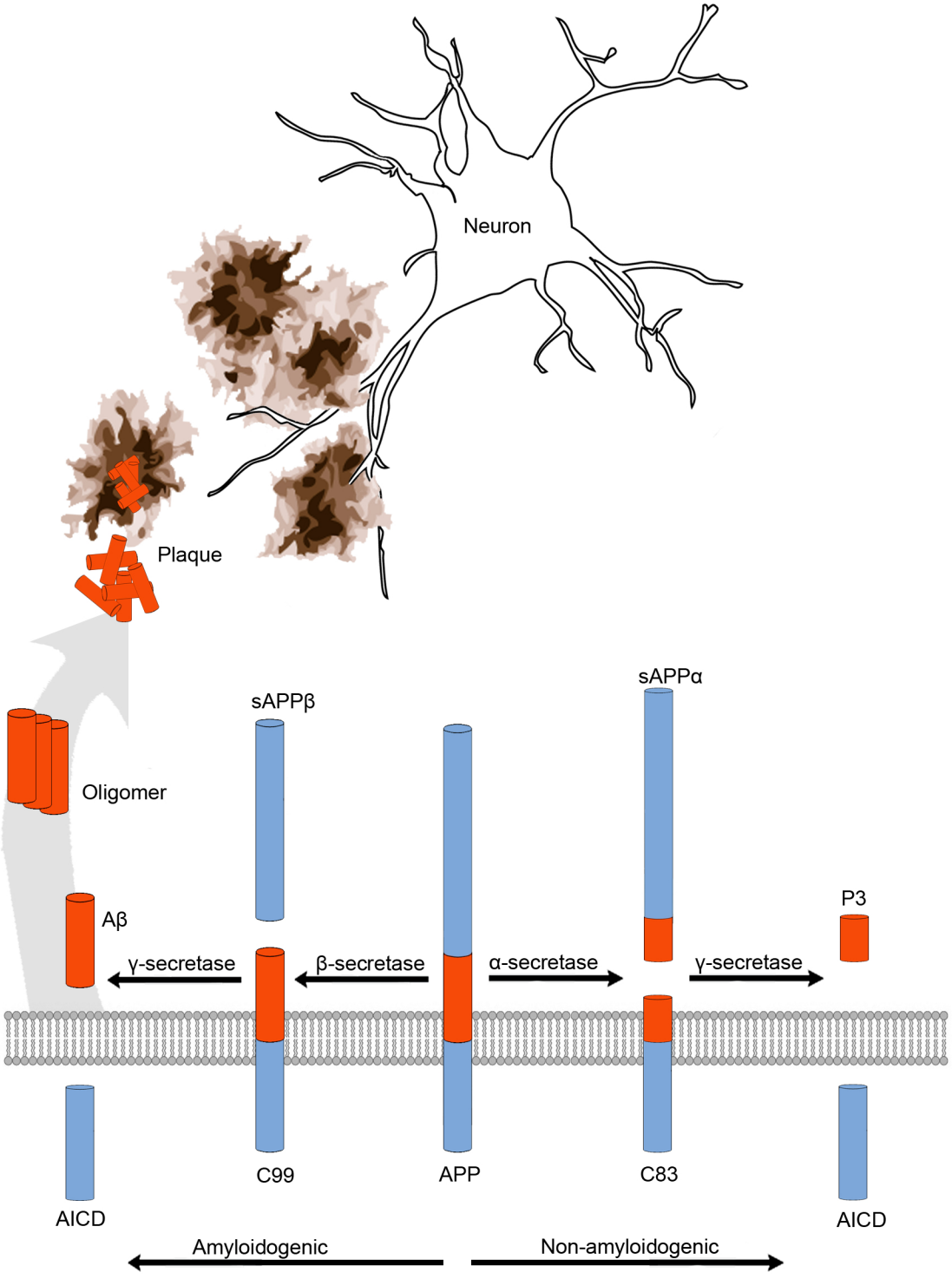

Neuron
Plaque
Oligomer
Aβ
sAPPβ
sAPPα
P3
γ-secretase
β-secretase
α-secretase
γ-secretase
C99
APP
C83
AICD
AICD
Amyloidogenic
Non-amyloidogenic

Figure 1.1.

Figure 1.1 Amyloid cascade hypothesis

The amyloid hypothesis postulates that AD pathogenesis is driven by an imbalance between production and clearance of Aβ. Aβ originates from the transmembrane APP. APP can be processed along two main pathways non-amyloidogenic (22) and amyloidogenic (left). In non-amyloidogenic processing: APP is cleaved by α-secretase which removes the amino-terminal fragment sAPPα and a C83 peptide remains membrane-bound, next γ-secretase cleaves C83 to yield P3 and an AICD. In amyloidogenic processing: APP is cleaved by β-secretase which yields sAPPβ fragment and a membrane-bound C99 peptide, next γ-secretase cleaves C99 creating AICD and Aβ peptides. Aβ can spontaneously self-aggregate into oligomers (2-6 peptides), accumulate to form fibrils, and ultimately arrange into β-pleated sheets forming the insoluble fibers of neurotoxic amyloid plaques.

formation can be precluded in non-amyloidogenic processing. Aβ ranges from 36 to 43 amino acid long as the C99 peptide can be cleaved by the γ-secretase at several positions, with $A\beta_{40}$ and $A\beta_{42}$ being highly neurotoxic (3,16). $A\beta_{42}$ is the primary pathogenic form. Aβ monomers exist as alpha helices but spontaneous conversion to a β-pleated sheet configuration promotes self-aggregation into oligomers (2-6 peptides) that impair synaptic function and signal neuronal loss (15,16,23,24). Ultimately, oligomers assemble into protofibrils, fibrils, and finally, these fibrils aggregate into the insoluble extracellular amyloid plaques that define disease pathology post-mortem (16,21). Additional pathogenic consequences in AD are vascular amyloid deposits, neuroinflammation, loss of synapses and neurons, microvascular damage, hypomyelination, and cholinergic dysfunction (10,25).

While the amyloid deposition cycle is certainly a primary determinant of disease, reports of Aβ accumulation detected in cognitively unimpaired elderly and transgenic mouse models with little learning and memory impairment (26,27), suggest other factors may be required for AD conversion. It may be that amyloid deposition is integrated into a sequence of pathogenic events, thus a primary, yet only one of multiple determinants, required for conversion from a presymptomatic to symptomatic state (28). Although research has provided immense insight of AD, mechanisms behind phenoconversion from preclinical to clinical presentation remain unclear. Environmental and biological risk factors of AD onset include age, family history, trauma, depression, diabetes, diet, sex and education (2,10). Age is one of the largest risk factors for AD (28,29). Women are

at greater risk than males (29). Aβ plaques and neurofibrillary tangles observed in post-mortem could be considered as macroscopic tombstones of an advanced stage of the disease, whereas insights to etiology and early pathogenesis may involve metabolic dysfunction occurring prior to these lesions (1,28). Here, I ask whether alterations in lipid composition, conferred by dietary supplementation, could delay onset of learning and memory impairment in a mouse model of AD.

1.2 Mouse models of AD

AD is arguably one of the most complex diseases of the human nervous system (28). There are no naturally occurring rodent forms of AD thus humanized mouse models have been generated to offer pathogenic insight (30). As amyloid accumulation can be considered a contributor to AD pathogenesis, imperfect, but highly useful, animal models have been developed to study early features of the disease and attempt in identifying therapeutic interventions (1). Overexpression of human *APP* (hAPP) in transgenic mouse models of AD results in amyloid plaques similar to those in the human condition, but in a substantially condensed timeframe (6,30,31). Generally, hAPP transgenic lines develop robust amyloid pathology, some model synaptotoxicity, and are associated with learning and memory deficits assessed behaviourally (32).

Three isoforms of hAPP (695, 751, 770) have been used to generate transgenic lines (31). The major APP isoform in the human central nervous system (CNS) is APP695, with isoforms expressed at a ratio of 20:10:1 (APP695:APP751:APP770) in cerebral cortex (33). Overexpression of the *hAPP*

gene did not induce plaque deposition or relevant neuropathological dysfunction (34,35); transgenic insertion of *hAPP* with EOFAD-associated mutations do (31,36). The mutations in hAPP used are named according to geographic location from which affected families resided: Swedish (K670N & M671L) (37), London (V717I) (38), Indiana (V717F) (39), Dutch (E693Q) (40), and Arctic (E693G) (41). The Swedish mutation conformationally changes APP to favor β-secretase cleavage. The London and Indiana mutations favour γ-secretase cleavage, increasing production of neurotoxic $A\beta_{42}$ levels relative to $A\beta_{40}$ (32). Dutch and Arctic mutations increase Aβ forms that enhance fibrillogenesis or peptide resistance to proteolysis (32).

Moreover, various promoters have been used in the construction of transgenics to drive APP over-expression preferentially or exclusively in CNS including the platelet-derived growth factor beta chain (PDGF), thymocyte differentiation antigen 1 (Thy-1), and hamster prion protein (PrP) promoters (31,32). The PDGF promoter drives expression primarily in brain, widespread across regions, and selectively in neurons (32,42). The Thy-1 promoter drives higher levels of expression selectively in neurons, but expression is not active until between postnatal day six to ten (32,43). The PrP promoter induces the strongest expression among the above-described promoters (up to 15-fold above endogenous levels), but expression is not restricted to neurons as PrP is ubiquitously expressed in neurons, glia and extraneural tissues (32,44,45).

Background strain can also impact the phenotype of AD models. Mouse strains vary in their sensorimotor abilities, and some are prone to hearing and

vision impairments, all of which can affect performance on behavioural tests used to study higher order cognitive functions (32). For example, FVB/N mice undergo severe perinatal retinal degeneration that affects motor and cognitive performances compared to fully sighted C57BL/6 mice (46). Strains also differ in learning and memory abilities (47,48). Some strains are more susceptible to amyloidogenic pathology effects, for instance the TgCRND8 line used in this study, whereby the mutant *APP* gene is driven by the PrP promoter, reports a more severe cognitive deficit in the MWM relative to NonTg controls, and greater plaque deposition on a 129SvEvTac/C57 than on C3H/C57 hybrid background (49). Close examination of these data, however, indicate that Tg performance is comparable and the severity of the deficit is due to the robust performance of NonTg 129SvEvTac/C57 littermates compared to the poor performance of NonTg C3H/C57 mice (49). Thus, analysis manifests as a greater cognitive impairment in the 129SvEvTac/C57. Here, I seek to address why C3H/C57 MWM performance is less robust than other lines. As reported (45,50), a valid animal model for AD should exhibit: (i) progressive AD-related neuropathology, (ii) cognitive deficits, (iii) phenotypic dysfunction should be due to hAPP mutations and absent in controls, (iv) should be verified in several laboratories, and/or in independent Tg lines harbouring the same construct. The TgCRND8 line for instance is a particularly aggressive AD mouse model that meets these criteria, albeit with strain differences described above. TgCRND8 mice originate from the Centre for Research in Neurodegenerative Diseases (CRND) of the University of Toronto generated by Dr David Westaway (30). These transgenic

mice express *hAPP695* holding Swedish (KM670/671NL) and Indiana (V717F) mutations driven by the PrP promoter (30). On an C3H/C57 background, this model develops AD-like pathology (extracellular Aβ deposits) evident by three months of age (30), and behavioural impairment can be assessed between four and six months of age (49,51,52).

It is important to note that different transgenic lines develop varying phenotypes of AD dysfunction, and no existing mouse model mirrors all clinical and pathological features of AD (32). AD mouse models are invaluable reductionist tools wherein pathologies related to aberrant APP processing associated with EOFAD can be investigated under conditions of controlled experimental manipulation (32). Mouse models in AD research offer advantages: they have abundant progeny with short gestation, and allow long-term controlled studies or invasive manipulations which cannot be feasible in humans (31). Commonalities between human AD and transgenic AD conditions are: Aβ overproduction and aggregation, synaptic dysfunction, inflammation, and cognitive impairment (31). Mouse models are well suited for identification of prevention strategies and preclinical validation of innumerable therapeutic agents and have formed the basis of clinical trials, albeit with little success to date (27,32,53). However, animal models still provide immense *in vivo* mechanistic insight to AD pathology and our current understanding is, in part, owing to the experiments that could not have been performed in humans.

1.3 Behavioural measures of learning and memory

Principal outcome measures in mouse models are either neuropathological (e.g. plaque deposition) or functional (e.g. learning and memory deficits) (32). AD mouse model reports focused on pathological outcome measures are typically robust but monogenic or, at best, bigenic in nature (32). Functional outcome measures of behavioural studies, although more variable, reflect the symptomatic deficits of AD and may be more relevant and predictive in efficacy of therapeutic agents designed to reduce or prevent cognitive decline (as compared to failed trials of Aβ degradation) (32).

How do preclinical mouse behavioural tests relate to clinical neuropsychological assessments in human AD? Behavioural paradigms aim to measure memory reflecting cognitive domains vulnerable to damage in AD (54,55). Neuropathological changes in AD affecting episodic memory localize to the hippocampus, entorhinal cortex, and medial temporal lobe (45,56). Episodic memory deficits are followed by semantic memory deficits, both developing before emergence of impairments in other cognitive domains such as attention, visuospatial memory, or executive function (55,57). Humans are not uniformly affected in these domains, deficits in some domains may occur earlier than others throughout progression of the disease, but generally a progressive decline in memory functioning occurs as a person progresses from preclinical, to amnestic MCI, to AD (5,55,58). Ideally, murine behavioural tasks would assess identical cognitive domains as those examined in neuropsychological assessments. Reference memory, working memory and executive function have

been extensively modelled, while other cognitive domains (attention and episodic memory) less so, or cannot be modelled in rodent models (i.e., clinical verbal acuity and recall tasks) (55). Reference memory most closely reflects human semantic memory, i.e., learned knowledge for a constant aspect of a task throughout the behavioural task (55). Working memory refers to the mental processing of holding transitory information over a short-term, manipulating and adapting this store based on contextual changes to guide behaviour (55). Recognition memory, classified as long-term declarative memory, refers to ability to recognize previously encountered objects, individuals or events (55). Various existing behavioural methods assess AD-like cognitive deficits in mice. Common murine behavioural tasks of reference memory, working memory and associative learning and memory include the MWM (59), Radial Arm Maze (60), Radial Arm Water Maze (61), Barnes Maze (62), T-Maze/Y-Maze (63,64), passive/active avoidance (65,66), as well as cued and contextual fear conditioning (67). Behavioural tasks such as novel object recognition (68) and the What-Where-Which Task (69) evaluate recognition memory and episodic-like memory. Attention, executive function and cognitive flexibility can further be investigated by Multiple-Choice Serial Reaction Time Task (70), attentional set-shifting tasks (71), and reversal learning (72).

Spatial based working and reference memory tasks are heavily employed in mouse models of AD as paradigms of clinical visuospatial memory tasks. The most widely used protocols involve maze type tasks (55). The MWM has become a gold standard paradigm to demonstrate spatial learning and memory

impairment in mouse models of AD (55,73,74). The MWM was developed by Morris in 1984 (59) as a behavioural task in rodents to assess hippocampal-dependent spatial learning and long-term memory (54). The MWM is a task of learning and memory wherein mice learn how to use spatial cues to navigate from entry points around the perimeter of a swimming arena to locate a submerged hidden escape platform (75). The MWM protocol can be modified further to examine reference and working memory. The capacity of a mouse to retain learned information can be assessed by a probe test (wherein after a learning period the escape platform is removed and the amount of time a mouse searches the prior area is assessed), or the flexibility to relearn new strategies can be assessed by relocating the escape platform to a new location after a learning period (reversal) (76). Advantages of MWM task include: (1) accurate and reproducible study of reference and working memory, (2) sensitivity to assess damage to the hippocampus, (3) simplicity in execution, (4) ease of learning (reported to require two-fold less the number of trials to acquire the task than radial maze), and (5) the use of water creates both a motivating stimulus (eliminating need for food/water deprivation) and reduces aromatic cues (54,73,76). On the other hand, the MWM task relies on basic functional abilities (capacity to see and swim) such that motoric deficits, elevated anxiety levels, or impaired visual acuity could confound the phenotype of a cognitive impairment (75). Additional factors to influence MWM performance are apparatus (e.g. number and distance of cues), training procedure, background strain, sex, age, health, and stress (77). For example, C57BL/6 strain mice are reported to exhibit

a robust performance in the MWM task, whereas the FVB/N strain displays impaired performance as a result of visual deficits (46), while clearly capable of learning in vision-independent tasks (e.g. contextual fear conditioning) (78). Altogether, functional outcome measures of murine cognitive behaviour provide insight into learning and memory impairments related to AD symptomology and can prove predictive therapeutic agent efficacy.

1.4 Benefits of omega-3,-6,-9 UFA

Intriguingly, Alois Alzheimer described ‘adipose inclusions’ and ‘lipoid granules’ in the brain as another pathological characteristic of AD, suggesting aberrant fatty acid (FA) metabolism may be a critical, but modifiable, factor in transition from presymptomatic to symptomatic AD (13,79,80). A FA is a carboxylic acid with long unbranched aliphatic chain (81). FAs can be divided into two categories: saturated (SFA) and UFA based on the number of carbon bond unsaturations (81). SFAs do not contain double bonds, whereas UFAs contain at least one double bond and thus can be termed monounsaturated (one double bond present) (MUFA) or polyunsaturated (many double bonds present along the carbon chain) (PUFA) (81). UFAs can further be defined as omega-3, omega-6 and omega-9, based on the location of the first double bond from the omega (methyl) end (81). In 1930, linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) were identified as essential FAs from diet (82). Omega UFAs are critical in development and maintenance of normal CNS structure and function, modulating cell structure, gene expression, enzyme activity, receptors and

second messengers through their incorporation into membrane phospholipids, sphingolipids, and cholesterols (81,83,84).

Epidemiological studies have reported decreased coronary heart disease and cognitive benefit in Mediterranean diet as compared to Western style habits (84-86). Mediterranean diet habits are characterized by high consumption of fruit, vegetables, legumes, fish, and the use of olive oil as main source of fats (84,86). Mediterranean diet's beneficial active components arise from both soybean and olive oils which contain high UFAs LA and oleic acid (18:1n-9, OA), and fish oils containing docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) (87,88). Adherence to a Mediterranean diet has shown to affect risk of AD and progression of MCI and AD (84,86,89). Clinical trials report that higher adherence to Mediterranean diet is associated with: reduced risk of AD (90,91), reduced risk of mortality in AD patients (92), reduced risk of MCI (93,94), and reduced rate of cognitive decline (95,96). Moreover, compared to healthy controls, MCI and AD patients are reported to have lower plasma levels of LA (97) and OA (98), with CNS membrane phospholipids substantially depleted of OA sn-1 and sn-2 carbon chains (99-101). This suggests a deficiency in omega-3, omega-6, and/or omega-9 UFAs, which could play an important role in the development of AD.

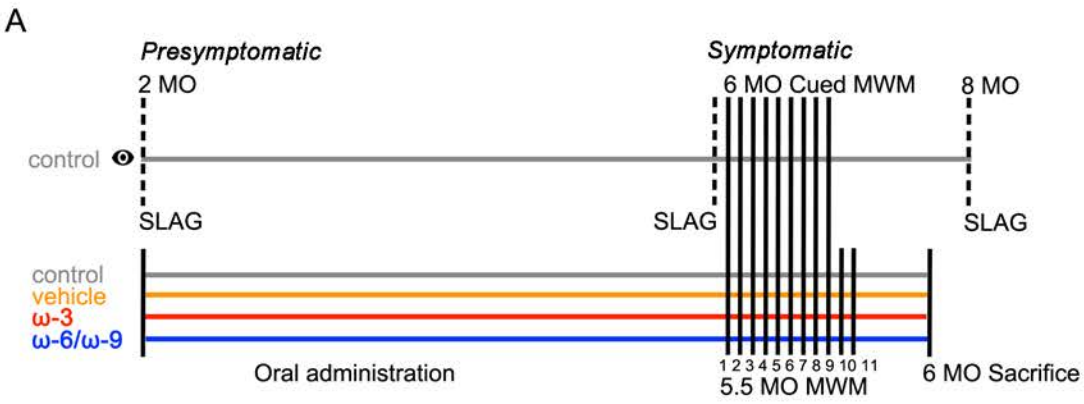
Omega-3 UFAs are particularly crucial in CNS development (83). Omega-3 UFAs have also been reported beneficial in a multitude of conditions including cardiovascular disease (88,102), cancer (103,104), attention deficit hyperactivity disorder (105), depression and schizophrenia (106,107). Although omega-3

UFAs can be synthesized endogenously from ALA, the rate of conversion is low and the main source is diet, such as fish oil (83). Deficiencies in omega-3 UFAs, particularly DHA, have been reported as a risk factor for AD (108-110). AD patients have reduced brain and serum DHA levels as compared to healthy controls (111-113), and decreased DHA proved correlative with severity of dementia (111). Thus, a deficiency in omega UFAs, constituents of the Mediterranean diet, could play an important role in the development of AD. If deficient omega UFA levels are associated with AD pathology, remodelling of neuronal membranes by dietary intervention may hold exciting therapeutic promise.

1.5 Rationale, Hypothesis and Objectives

CNS lipids may be critical determinants of AD conversion: (i) lipids regulate proteolytic activity and trafficking of membrane-bound proteins, thus perhaps a crucial role in amyloidogenesis, (ii) structural cellular membrane lipid metabolism is perturbed as a result of Aβ cytotoxicity, (iii) lipids themselves may modulate effects of Aβ and thwart metabolic disruptions engendering transition from presymptomatic to a symptomatic state (13,79). Identifying and targeting cognitive deficits occurring early in AD is critical for maximum impact of treatment on cognitive function and quality of life, thus monitoring effect of lipid supplements designed to alter membrane composition represents an area of intense interest in AD research (55).

At present, drug therapies for AD are primarily focused on symptomatic treatment. There is no preventative or disease-modifying treatments for AD. AD pathology includes dietary deficiencies, and perhaps symptoms may be delayed by enhanced nutritional strategies to modify brain lipid composition (114). Benefits of dietary intervention in neurodegenerative conditions require further investigation. In this thesis, I have examined whether treatment with different combinations of UFAs affect behavioural indices of learning and memory in a mouse model of AD. I hypothesized that four-month enhanced nutritional therapy of omega UFAs would improve learning and memory performance in TgCRND8 mice. To test this hypothesis, the first objective of this study was to establish the degree of cognitive decline in the Tg mouse model and NonTg littermates, with respect to both males and females, at six months of age using the hippocampal-dependent spatial task of MWM and further ascertain whether performance in MWM was indicative of learning and memory impairment or affected by visual deficits potentially present in the genetic background of our TgCRND8 model. Next, cognitive benefit of a dietary intervention was assessed (Schematic of timeline of experimental procedures, Figure 1.2). The effect of a four month dietary supplementation with vehicle (25% sweetened condensed milk), omega-3 UFAs or omega-6/-9 UFAs treatment on learning and memory was assessed in Tg and NonTg mice at six months of age using the MWM task. Confounds during MWM such as motoric function and anxiety were also evaluated compared to their NonTg controls. Four specific aims were addressed:


A
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2 MO
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8 MO
control
SLAG
SLAG
SLAG
control
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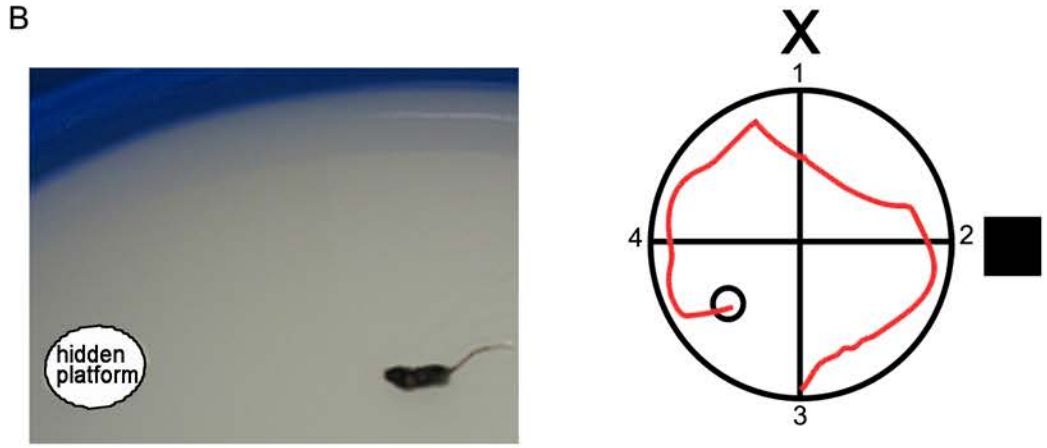
Oral administration
1 2 3 4 5 6 7 8 9 10 11
5.5 MO MWM
6 MO Sacrifice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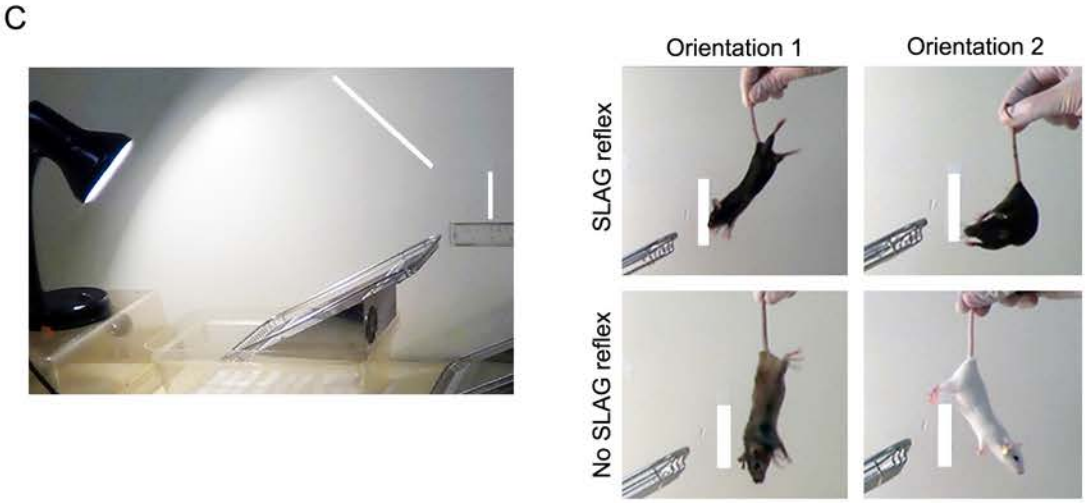
C
Orientation 1
Orientation 2
SLAG reflex
No SLAG reflex

Figure 1.2.

Figure 1.2 Treatment and behavioural testing paradigm

Schematic of the timeline of experimental procedures assessing learning and memory performance, effect of omega UFAs supplementation, and inherent visual acuity (A). At two months of age, presymptomatic TgCRND8 and NonTg mice of both sex received treatments: control, sweetened condensed milk vehicle, omega-3 UFAs, omega-6/-9 UFAs (lower panel of A). All mice had *ad libitum* access to control diet, treatments were orally administered daily by syringe as an enhanced diet (75 mg/kg/day). After four months of treatment, behavioural symptoms of learning and memory were assessed by the hippocampal-dependent navigational task of Morris Water Maze (MWM). MWM testing consisted of four trials per day for eight consecutive days, a ninth test day was conducted as probe trial, and additional tenth and eleventh test days assessed reversal learning. Animals received daily treatment until sacrificed at six months of age. Visual acuity of control (C57/BL6, FVB/N, BALB/c and CD1), TgCRND8 and NonTg mice was established with respect to both sexes (upper panel of A). TgCRND8 and NonTg mice were tested at 2-3, 5-6, 8-10 months of age by adapted slow angled-descent forepaw grasping (SLAG) reflex test. TgCRND8 and NonTg mice were tested at 5 months of age in cued MWM. The cued MWM paradigm was identical to below described, except extra-maze cues were removed and escape platform made visible by a flag. The MWM is a task of learning and memory for mice that relies on spatial cues to navigate from entry points around the perimeter of a swimming pool to locate a submerged hidden escape platform (B). The pool was filled with water rendered opaque by white nontoxic paint to conceal a hidden escape platform positioned one centimeter below the surface. The pool (overhead view) can be bisected into four imaginary quadrants representative of the position of two extra-maze cues (black solid square and black “X”) and the four entry points per test trial (right panel of B). A representative swim path in the pool marks a mouse’s navigation to the platform from the third entry location. Adapted SLAG reflex test assessed visual placing response of mice to establish visual function (C). A mouse was suspended by the tail and slowly lowered following a marked 20 cm descent toward a cage wire lid fixed with the edge at an upward angle, and at 4 cm adjacent the wire lid the SLAG reflex was anticipated. SLAG reflex presence entailed; directed sustained extension or multiple reaches of the forepaws toward the wire lid, additional head elevation or extension of hind limbs, and twisting of the body to reach the wire lid. Each mouse received six trials, three per orientation (orientation 1 ventral descent, orientation 2 dorsal descent).

1. Establish the degree of learning and memory impairments in male and female Tg mice at six months of age using the MWM task.
2. Determine whether impairments were the result of cognitive decline or visual acuity.
3. Assess effect of a four-month omega-3 UFA treatment on learning and memory outcomes in Tg and NonTg male and female mice at six months of age.
4. Assess effect of a four-month omega-6/-9 UFA treatment effect on learning and memory outcomes in Tg and NonTg male and female mice at six months of age.

Chapter 2 – Materials and Methods

2.1 Animals

F1 C57BL/6 X C3H/HeJ TgCRND8 mice generated by Dr David Westaway and colleagues (30) were kindly provided by Dr Paul Fraser (University of Toronto). Mice were backcrossed for five generations (N5) to a C57BL/6Crl lineage in the Bennett laboratory using C57BL/6Crl mice obtained from Charles Rivers Laboratories (Senneville, Canada). Heterozygous Tg and NonTg littermates were maintained by filial breeding of NonTg females with Tg males. Here, a total of 94 females, Tg (n=52) and NonTg (n=42), and a total of 91 males, Tg (n=48) and NonTg (n=43) were analyzed. For genotyping, genomic DNA was isolated from biopsied tail and PCR amplification was 3 min at 94 ° C followed by 35 cycles of 20 s at 94 ° C, 20 s at 68 ° C, and 90 s at 72 ° C terminating after the last cycle with 7 min at 73 ° C. Primers were as follows: 5′ -GGC CGC GGA GAA ATG AAG AAA CGC CAA GCG CCG TGA CT-3′ (forward) and 5′ - TGT CCA AGA TGC AGC AGA ACG GCT ACG AAA A-3′ (36). Tg mice produce a 1 kB amplicon. To verify template integrity, all animals were genotyped for the platelet-activating factor receptor. PCR amplification was 10 min at 95 ° C followed by 30 cycles of 20 s at 94 ° C, 20 s at 65 ° C, and 50 s at 70 ° C. Primers were as follows 5′ -TAT GGC TGA CCT GCT CTT CCT GAT-3′ (forward) and 5′ - TAT TGG GCA CTA GGT TGG TGG AGT-3′ (36). Tg and NonTg mice produced a 289-kB amplicon. Mice began treatments at nine

weeks of age and behavioural assessments were performed at six months of age.

The following inbred control strains were chosen to be assessed in SLAG reflex test with *a priori* knowledge of genetically determined visual function: C57BL/6, FVB/NCrl, C3H/HeNCrl, BALB/c and CD1. C57BL/6 between two and eight months of age were from our own breeding colony of C57BL/6Crl controls and tested. FVB/NCrl and C3H/HeNCrl retired breeders between 6 and 12 months of age were obtained from Charles Rivers Laboratories. BALB/c mice between one and three months of age were generously provided by Dr Mona Nemer (University of Ottawa). CD1 mice were generously provided by Dr Marie-Andrée Akimenko (University of Ottawa) at three months of age. Controls were tested with respect to both males (M) and females (F) as follows: C57BL/6-M (n=8), C57BL/6-F (n=6), FVB/NCrl-M (n=5), FVB/NCrl-F (n=5), C3H/HeNCrl-M (n=5), C3H/HeNCrl-F (n=5), BALB/c-M (n=6), CD1-M (n=2), CD1-F (n=3). All mice were maintained on a 12-h light and 12-h dark cycle and all behavioural testing was performed during the light cycle. All animal manipulations were performed in strict accordance with the ethical guidelines for experimentation established by the Canadian Council for Animal Care and with the approval of the University of Ottawa Animal Care Committee for the ethical treatment of experimental animals.

2.2 MWM learning and memory paradigm

Learning and memory was assessed in the MWM. The apparatus consisted of a plastic blue pool (Med Associates Inc., Water maze pool for mouse, CA# ENV-514M-B) measuring 127 cm in diameter and 42 cm deep with floor insert (internal measurements). The pool was filled with water rendered opaque with white water-soluble nontoxic paint. A hidden escape platform (10 cm diameter) was positioned in the center of the back-right quadrant of the pool, 1 cm below the surface of the water. Water temperature was maintained at 21°C. Four quadrants were defined in *post hoc* digital analysis by two principal axes bisecting the pool perpendicular to one another. The end of each axes demarcated four cardinal entry points where the mouse was placed into the pool: front, back, right and left. Two visual cues were placed on walls of the test room (2.98 m × 3.97 m × 2.62 m): (1) a black solid square located above left entry point and (2) a black "X" located above front entry point. Mouse cages were changed (fresh food, bedding and water) one day prior to testing and remained untouched until completion of MWM paradigm. On each test day, mice were habituated to the test room for 1 h. Constant white noise (white noise generator – San Diego Instruments, Serial # SDI 000141) at 70 dB was provided during acclimatization and testing to ensure no auditory disturbances. The room was lit with overhead white light of 100 lux, regularly checked by Light meter (ExTech, light meter 401025). Testing was conducted over eight consecutive days with each mouse completing four trials per day at zeitgeiber time (ZT) nine (tested 9 h after lights on, based on a schedule of 12 h lights on/12 h lights off). Each trial

lasted until the animal found the platform or for a maximum of 60 s. A correct trial involved a mouse locating the platform within this time period and remaining on the platform for 5 s. Mice that failed to find the platform within this time period were guided to the platform by the experimenter, placed on the platform for 10 s and then removed from the pool. Each trial was randomized to one of the four possible cardinal entry points. Mice were run in cohorts of 6–12. Each mouse received an inter-trial interval of 20 min to recover and avoid risk of hypothermia (74). A ninth day of testing was conducted as probe trial. The platform was removed and the amount of time each animal spent exploring the correct target zone wherein the escape platform had been located was recorded over a 60 s test period. Probe was followed by an additional two testing days (testing day ten and eleven) wherein the platform was relocated from its original position 180° to the opposing quadrant (front-left). Two sets of four trials per day were conducted during this reversal testing. Reversal learning revealed whether or not animals could extinguish their learned expectation of the platform's position to acquire a different direct path of a new goal position (75). Performance was recorded with a video camera (Bosch, LTC0355/20; Pentax 3.5-8 mm Ins, TS2V314BED) mounted directly above the pool and data acquired and analyzed with Ethovision v8 (Noldus Information Technology, Ethovision XT v8.0.516) software. In a separate set of experiments, mice were tested in the cued water maze wherein extra maze cues were removed and the escape platform was made readily visible by a flag (52). Cued MWM testing was conducted over eight days and a probe trial on day nine.

2.3 MWM data analysis

Multiform assessments of MWM performance were used to ascertain learning and memory, motoric function, anxiety levels, and visual function of mice. The parameters of cognitive function analysis in MWM experiments involved: (A) escape latency, (B) number of platform-site cross-overs during probe trial, (C) reversal escape latency, (D) path efficacy, and (E) search strategy. Search strategy was assessed by two methods: (1) total incidence of strategies used over the eight test days, and (2) incidence of strategies used per test day. Additionally, if subjects were impaired in motoric abilities or exhibited elevated anxiety levels during MWM testing, it may have hindered performance and was not reflective of higher order cognitive function of learning and memory (75). Motoric abilities were investigated by analysis of distance moved (F) in conjunction with velocity (G) of swimming during the MWM task, and anxiety levels were measured by percent time spent in thigmotaxis (H). Measures of distance moved, velocity, and thigmotaxis were calculated as an average over the first eight test days. Performance on each test day was established as an average of all four trials. This is with exception to probe testing, in which case mice searched for one trial. Details are as follows:

(A) Escape latency – represented the time elapsed for a mouse to locate the hidden platform. This value, in seconds, should theoretically, progressively, decrease as a mouse learned to accurately locate the platform. In each trial, a mouse must have located the platform within 60 s. If a mouse failed to locate the platform it was assigned a 60 s maximum score. Escape latency is an often

reported measure of performance in MWM (74,115). This measure was also used to assess cued MWM performance.

(B) Number of platform-site cross-overs during probe trial – represented the frequency of crossing the platform location area during a probe trial. In a probe trial the platform was removed, and animals indicating memory of the platform location displayed a search bias to the former location (74,75). Frequency of crossing over the platform area (78.53 cm^2) was assessed.

(C) *Reversal escape latency* – represented ability of mice to use spatial cues when the challenges of the task have been modified; such as relocation of the platform to a novel quadrant (opposite front-left quadrant). This testing measured the ability of mice to extinguish initial learning of the platform's position and ability to acquire a direct path to the new escape platform position (75).

(D) Path efficacy – represented the learning and memory of a mouse by its capacity to attain the platform by the most direct means. The total distance moved of the mouse was compared to the direct path distance to the platform. Thus, path efficacy = (direct path distance)/(total distance moved). If the mouse did not reach the platform it was assigned a score of 0.

(E) Search strategy – trajectory a mouse adopted to locate the platform via spatial cues. This analysis helped identify the strategy and nature of memory impairment of the animal (74). Mice may switch strategies during testing (116). As described by Janus in 2004 and, Brody and Holtzman in 2006, there were four categories of search strategy: (1) spatial, (2) systematic non-spatial, (3) repetitive looping, (4) floating. Spatial strategies included: *spatial direct* (swam

directly to the platform, *spatial indirect* (swam to platform with at most one loop), and *focal correct* (swam directly and searched in the quadrant containing the platform). Systematic non-spatial strategies included: *scanning* (searched the interior of the pool without a spatial bias), *random* (searched the entire pool without any bias), and *focal incorrect* (searched a small portion of the pool that did not contain the platform). Repetitive looping strategies included: *chaining* (swim was circular at an approximately fixed distance greater than 15 cm from the pool wall), *peripheral looping* (persistently swam around the pool edge), and *circling* (swam in tight circles) (52,116). Floating behaviour (remained motionless with less than 6 cm/s velocity and an escape latency greater than 50 s) was classified here as an additional category. Mice that exhibited an average velocity less than 6 cm/s and an average escape latency greater than 50 s on a test day for greater than five of the eight learning days were considered floaters, unmotivated to perform the learning task, and excluded from subsequent analysis.

(F) Distance moved – represented a measure of total swim path length. Analysis of distance moved paired with velocity, reflected the motoric ability of mice during MWM (74).

(G) Velocity – represented mouse swim speed during testing time. If a mouse exhibited a low velocity in conjunction with a low distance moved over a trial this was indicative of a motoric impairment, and should be considered when evaluating conditions of learning and memory.

(H) Thigmotaxis – represented percent time that a mouse spent swimming within the 15 cm periphery of the pool wall (115) throughout eight test days. High levels of anxiety during MWM testing in rodents were manifested behaviourally as thigmotaxis, otherwise termed 'wall hugging' (115). An animal displaying excessive thigmotaxis was indicative of inability to focus on the task appropriately, because the animal must first have learned there is no escape located around the perimeter of the tank (75). It is widely accepted that the MWM spatial task is acutely stressful owing to the nature of the task (wet, brightly lit, open-field task) thus enhanced anxiety must be evaluated (77,115,117). Thigmotaxis is a reliable non-invasive indicator of anxiety during MWM testing (115,118,119).

2.4 Adapted slow angled descent forepaw grasping (SLAG) reflex test to assess visual acuity of mice

The SLAG reflex test was used as an innate behavioural task useful to identify functional vision in mice (120). Mice were assessed two days before cued MWM testing according to a modified protocol of Gil-Pagés *et al.*, (120). Briefly, testing apparatus included a videocamera (Sony High Definition Camcorder, HDR-CX210/R), a low-heat (20 W, 12 V) desk lamp (Illuminada Gooseneck Desk Lamp 17341-000) at 600-700 lux, and clean wire-bar stainless steel cage lids for routine housing (one per mouse). Each steel wire lid was set with the edge at an upward 45° angle in a clear plastic cage lid base. The background was marked with a diagonal piece of tape representing a 20 cm path

and a second vertical piece of tape delineated a 4 cm distance from the wire. SLAG reflex test paradigm involved six total trials, three trials per orientation. In orientation 1 (Figure 1.2C), the mouse was suspended by its tail, such that the mouse's ventral aspect was toward the same side as the edge of raised wire lid. In this orientation, the wire lid was within the mouse's immediate field of vision. In orientation 2 (Figure 1.2C), the mouse was suspended by the tail such that the mouse's dorsal aspect was toward the edge of the raised wire lid. In this orientation, the wire lid was excluded from the mouse's field of vision until passing adjacent to the wire lid. The mouse was lowered, in each trial, along the 20 cm diagonal path until it reached the 4 cm distance adjacent to the wire lid. There was a pause interval of 5 s to determine whether the subject would grasp the adjacent lid. Upon completing a trial, the mouse was placed to rest on the wire lid for 15 s and returned to its home cage. All cohorts were randomized in orientation and genotypes. Cohorts were tested four-six mice at a time with each mouse receiving a 5 min inter-trial rest.

Following testing, all trial videos were individually scored for SLAG reflex presence (score=1) or absence (score=0). A SLAG reflex entailed one of the following behaviours: (1) directed sustained extension of the forepaws toward wire lid, (2) multiple reaches of the forepaws toward wire lid, (3) forepaw extension with head elevation or hind limb extension in attempt to reach wire lid, or (4) twisting of the body to reach wire lid. Absence of SLAG reflex was defined by: (1) no extension of forepaws toward the wire lid, (2) twisting of body to reach

the hind limb or tail and not the wire lid, (3), or only extension of forepaws toward wire lid at a distance less than 4 cm thus guided by whisker proprioception.

The originally described SLAG reflex test only distinguished impaired/unimpaired conditions (120). Scoring criteria was further adapted to distinguish conditions of no visual impairment, mild visual impairment, and moderate to severe visual impairment following analysis of mice with no visual impairment (C57BL/6), mild to moderate visual impairment (BALB/c and CD1), and moderate to severe visual impairment (blind) (FVB/NCrl, and C3H/HeNCrl). Data were categorized based on SLAG reflex performance of the three trials in each orientation. In orientation 1, mice were subdivided into two categories of (1) no to mild impairment (≥ score of 2), or (2) impairment (< score of 2). In orientation 2, those mice that were categorized as no to mild impairment in orientation 1 were further classified based on their orientation 2 scores, these are: (1) no impairment (≥ score of 2), or (2) mild impairment (< score of 2). Those mice that were categorized as impaired in orientation 1 were also further subdivided into two categories based on their orientation 2 scores; these are: (1) moderate impairment (≥ score of 2), or (2) severe impairment (≤ score of 1). *Post hoc* video analysis of mouse behaviour was used to determine presence or absence of SLAG reflex during trails. Four investigators were trained in visual placing response reflex, viewed the same collection of videos, and scored the mice. Phenotypes of strains were achieved by inter-rater scoring based on majority consensus among investigators.

2.5 Dietary interventions: UFA supplementation via oral administration

NonTg and presymptomatic Tg mice began receiving dietary intervention at nine weeks of age (51). Control groups were fed only the standard diet. All mice had *ad libitum* access to standard diet (Harlan Laboratories – Teklad Global 18% Protein Rodent Diet), which contained no animal protein or fish meal. Tg and NonTg mice received: (1) only control diet, (2) supplementation with vehicle (25% diluted President's Choice Sweetened Condensed Milk), (3) supplementation of omega-3 (ω-3) FAs emulsified in vehicle, (4) supplementation of omega-6/omega-9 (ω-6/ω-9) FAs emulsified in vehicle. Omega-3 FA supplementation was from fish oil (Ocean Nutrition 40/20 EE 1000 mg Fish Oil Capsules, Lot # 21623 and #26533) and capsules contained 1.2 g EPA, 0.6 g DHA, with 0.1 g other omega-3 FAs. Omega-6 and omega-9 FAs were a 50:50 of corn:soybean oil mix with less than 0.12 g omega-3 FAs (Ocean Nutrition 1000 mg Corn/Soybean Capsules, Lot # 21624 and # 26532). Previous studies regarding FA composition determined by capillary gas chromatography (GC) revealed corn oil rich in FAs; LA (47.189%) and OA (36.994%) (121), whereas composition of soybean oil by capillary GC showed high FAs; LA (52.18%) and OA (23.27%) (122,123). Due to oxidative properties of omega FAs, all supplements were stored at – 20 °C in aliquots sufficient for a 7-14 day administration.

Mice were trained to feed from a syringe and randomly assigned to vehicle, omega-3, or omega-6/-9 supplement groups for a treatment period of four months. Syringe feeding allowed for exact non-invasive daily dosage to be

administered based on individual mouse weight. Administration occurred at ZT 6; behavioural testing commenced between ZT 7-10 depending on test. The supplements and dosage used in the animal study doubled that of a human study being performed in collaboration with Dr Krista Lanctôt at Sunnybrook Health Science Centre of the University of Toronto. Mice received a dosage of 75 mg/kg/day (treatment groups) or 5 ml/kg/day (vehicle groups). Daily dosage considered weight, and the higher metabolic rates of mice. The following assumptions were made in volume calculation: (1) the human intake of three capsules daily (1000 mg each), (2) average body weight of a human patient as 80 kg, (3) doubled enhanced metabolism of mice. Daily mouse weight was measured and age-dependently ranged from 15-40 g.

Intervention studies required a minimum n=10 per group: ω-3-NonTg-F, ω-3-NonTg-M, ω-3-Tg-F, ω-3-Tg-M, ω-6/ω-9-NonTg-F, ω-6/ω-9-NonTg-M, ω-6/ω-9-Tg-F, ω-6/ω-9-Tg-M, vehicle-NonTg-F, vehicle-NonTg-M, vehicle-Tg-F, vehicle-Tg-M, control-NonTg-F, control-NonTg-M, control-Tg-F, control-Tg-M. Final subject numbers are reported in the text. MWM behavioural testing was initiated at 5.5 months of age and continued for 2 weeks with mice sacrificed between 6-6.3 months of age by lethal injection with sodium pentobarbital and decapitation. Brain tissue was dissected and collected for additional lipidomic assessments performed by other members of the Bennett laboratory. It should be noted that animals continued to receive daily supplementation throughout MWM testing until sacrifice.

2.6 Statistical analysis

Analyses were performed using Prism 6.0a (GraphPad). Statistical analysis of escape latency, reversal escape latency and path efficacy were each analyzed by repeated measures two-way analysis of variance (124). Data of search strategy were analyzed by two-way ANOVA with a *post hoc* of Holm Sidak's. Data of average distance moved, average velocity of mice, average percent thigmotaxis and number of platform-site cross-overs during probe trial, were each analyzed by a Student's *t* test. Statistical significance was shown below a minimum of $p<0.05$. All data are represented as means ±SEM.

Chapter 3 – Sex differences in MWM performance in the TgCRND8 mouse model of AD

3.1 Objective of this study

Our laboratory has backcrossed hybrid C57BL/6 X C3H/HeJ TgCRND8 mice obtained from Dr Paul Fraser (uToronto) for five generations to a C57BL/6NCrl genetic background. The primary objective of this study was to establish whether N5 C57BL/6NCrl X C3H/HeJ females and males (referred to as Tg mice) exhibit learning and memory, anxiety, or motoric impairments by six months of age in comparison to wild-type littermates (referred to as NonTg mice).

3.2 Statement of author contributions

Thank you to Dr Diane Lagace and Mirela Hasu for access to the University of Ottawa Behavioural Core Facility. All experiments were performed by the author of this thesis with the following exceptions: Matthew Granger tested control-Tg-M, control-Tg-F, control-NonTg-M and control-NonTg-F. Dr Carolina Cieniak and Fida Ahmed, with Bettina Franko, handled, administered vehicle daily by syringe feeding, and tested vehicle-Tg-M and vehicle-NonTg-M. Dr Hongbin Xu and Dr Carolina Cieniak assisted me in study design and analysis.

3.3 Introduction

The TgCRND8 mouse model of AD expresses a *hAPP* transgene with Swedish and Indiana familial AD mutations (KM670/671NL+V717F) driven by the PrP promoter (30). This early onset mouse model of AD results in pathogenic

increases in extracellular Aβ deposition and cognitive impairment in MWM by eleven weeks of age (30). We have backcrossed this line for five generations (N5) to a C57BL/6NCrl lineage and we have published that female mice exhibit significant Aβ plaque deposition and learning and memory impairment at six but not two months of age (51). Phenoconversion of male mice has not been assessed.

The MWM is frequently used as a navigation task to assess hippocampal-dependent spatial learning and memory (59,74), however many studies indicate performance is dependent upon endogenic variables such as strain (47,48,125,126), sex (127), age (128), nutrition (22,129), and stress (115,130), and exogenic influences such as room apparatus, test method, and test paradigm (75,77,131). For example, the C3H/HeN strain exhibits poor MWM performance due to a severe visual impairment (See Chapter 4); while C57BL/6 mice exhibit robust performance in MWM (48,125). Differences in MWM performance are also reported between sexes of rodents, such that male animals show advantage in spatial learning (77,132-134). Male C57BL/6 mice also show significantly better working and reference memory than female mice in a water-escape motivated radial arm maze task (127). Furthermore, some studies report postnatal handling of animals may affect stress and outcomes of MWM (135,136), while others report no effect (137,138).

TgCRND8 mice on an F1 C57BL/6 X C3H/HeJ hybrid, exhibit the least postnatal lethality compared to the same mice placed on a 129SvEv/Tac or FVB/N lineage (30). The C3H/HeN strain however, has shown poor MWM

performance due to a phosphodiesterase 6 beta gene rd1/rd1 mutation ($Pde6b^{rd1/rd1}$) mutation resulting in severe retinal degeneration (see Chapter 4) (48,139). To address this issue, we backcrossed F1 mice to a fully sighted C57BL/6Crl lineage. While we had intended to generate a congenic (>N10) TgCRND8 C57BL/6 lineage, we found that perinatal lethality increased significantly by an N6 generation thus we halted backcrossing and maintained our mice at incipient congenic (N5) generation. I report here characterization of cognitive and sensorimotor behaviour phenotypes in both male and female Tg mice, as well as address whether daily handling and oral administration of vehicle (25% sweetened condensed milk) affected sex-specific phenotypic cognitive conversion. These latter studies were performed in anticipation of our planned intervention studies (Chapter 5 and 6).

3.4 Results

NonTg and TgCRND8 mice receiving daily vehicle treatment displayed no different learning and memory performance than untreated mice in MWM

Tg mice and NonTg mice of both sexes were orally administered vehicle treatment (25% sweetened condensed milk) by hand daily via a syringe for four months and effect on cognitive behaviour was evaluated at six months of age in the MWM task. Indices of learning and memory in the MWM were determined by parameters of escape latency, path efficacy, number of platform area cross-overs in probe trial, and escape latency in reversal learning testing (Table A1). Female NonTg mice receiving vehicle supplementation did not perform differently than

control NonTg mice in escape latency ($F_{(1,20)}$=0.01499, Figure A1). No difference was observed in number of platform area cross-overs between vehicle treated and control female NonTg mice ($t_{(20)}$=1.100, Figure A1B). Reversal learning escape latency values were not different between vehicle treated and untreated female NonTg mice ($F_{(1,12)}$=0.4943, Figure A1C). The ability to attain the platform by the most direct means, termed path efficacy, was no different between vehicle treated and control female NonTg mice ($F_{(1,20)}$=8.051e^{-005}, Figure A1D).

Male NonTg mice receiving vehicle treatment did not perform differently than control mice in escape latency to the hidden platform ($F_{(1,20)}$=0.01665, Figure A2A). No difference was observed in number of platform area cross-overs between vehicle treated and control male NonTg mice ($t_{(20)}$=0.3783, Figure A2B). Reversal learning test was conducted on control male NonTg mice, but not vehicle male NonTg mice (Figure A2C). Path efficacy was no different between vehicle treated and control male NonTg mice ($F_{(1,20)}$=0.1560, Figure A2D).

Female Tg mice receiving vehicle supplementation did not perform differently than control Tg mice in escape latency measures ($F_{(1,28)}$=3.404, Figure A3A). In MWM probe trial, vehicle treated and untreated female Tg mice performed similarly in number of platform area cross-overs ($t_{(28)}$=1.595, Figure A3B). In MWM reversal learning test days, vehicle treated and control female Tg mice performed comparably in escape latency values ($F_{(1,16)}$=2.124, Figure A3C). Capability to attain the platform most direct swim path was not significantly different between vehicle treated and untreated female Tg mice ($F_{(1,28)}$=2.253, Figure A3D).

Male Tg mice receiving vehicle supplementation performed comparable to untreated controls in escape latency values ($F_{(1,21)}$=0.0903, Figure A4A). In MWM probe trial, no difference was observed in number of platform area cross-overs between vehicle treated and untreated male Tg mice ($t_{(21)}$=0.8703, Figure A4B). In MWM reversal learning test days, escape latency values were not different between vehicle and control groups of male Tg mice ($F_{(1,15)}$=0.1305, Figure A4C). Vehicle treated male Tg mice performed no different than untreated controls in path efficacy ($F_{(1,21)}$=0.4089, Figure A4D). In sum, no significant difference was observed in the cognitive performance of vehicle treated mice and untreated control mice in the MWM task.

Handling and vehicle supplementation did not alter motoric or anxiety behaviours among NonTg and TgCRND8 mice

Effect of non-cognitive confounds, such as motoric deficit or elevated anxiety during MWM was assessed (Figure A5). Motoric abilities were assessed by average distance moved and velocity swam (Table A1). No significant main effect of diet was observed when average distance moved of female vehicle treated and control Tg and NonTg groups were compared by a two-way ANOVA ($F_{(1,48)}$=0.7749, Figure A5A). No difference was observed in average velocity of vehicle-Tg-F, control-Tg-F, vehicle-NonTg-F, control-NonTg-F groups when compared by two-way ANOVA ($F_{(1,48)}$=0.3227, Figure A5B). A significant main effect of genotype was observed in motoric abilities of these groups, as shown by distanced moved ($F_{(1,48)}$=27.07, $p<0.0001$) and velocity ($F_{(1,48)}$=5.157, $p=0.0277$).

Anxiety levels were investigated by thigmotaxis behaviour. When vehicle-Tg-F, control-Tg-F, vehicle-NonTg-F and control-NonTg-F groups were assessed by a two-way ANOVA, a main effect of diet was observed ($F_{(1,48)}$=5.884, p=0.0191, Figure A5C). This main effect is a comparison in which both Tg and NonTg groups are collapsed and only control is compared to vehicle. To determine an effect of vehicle with respect to individual Tg and NonTg genotypes, a *post hoc* Holm-Sidak's test was performed and showed no significance between vehicle-Tg-F and control-Tg-F (p=0.1490) or vehicle-NonTg-F and control-NonTg-F (p=0.1490). Additionally, percentage values of thigmotaxis of all animals were below 40%.

Male Tg and NonTg mice were subsequently assessed in non-cognitive MWM parameters of distance moved, velocity and thigmotaxis. No significant main effect of treatment was observed when average distance moved of vehicle-Tg-M, control-Tg-M, vehicle-NonTg-M, control-NonTg-M groups were compared by two-way ANOVA ($F_{(1,41)}$=1.487, Figure A5D). No difference was observed in average velocity between vehicle treated and control Tg mice, or vehicle treated and control NonTg mice ($F_{(1,41)}$=1.540, Figure A5E). A significant main effect of genotype was observed in motoric abilities of these groups, as shown by distanced moved ($F_{(1,41)}$=48.08, p<0.0001) and velocity ($F_{(1,41)}$=18.80, p<0.0001) measures. No significant main effect of treatment was observed in percent thigmotactic behaviour of vehicle treated and control Tg mice, or vehicle treated and control NonTg mice ($F_{(1,41)}$=1.324, Figure A5F). Taken together, a four month

vehicle treatment had no influence on motoric abilities or anxiety levels of Tg or NonTg mice with respect to either sex.

Female Tg mice at six months of age display learning and memory impairments in MWM compared to NonTg littermates

Behaviour of female Tg mice was assessed in MWM navigation task and learning and memory indices were analyzed in comparison to NonTg littermates by four parameters of MWM performance: (1) escape latency, (2) path efficacy, (3) number of crossings over the platform location during the probe trial, and (4) performance when the escape platform was reallocated by 180° (reversal). Firstly, I determined the effect of handling on performance of NonTg and Tg female mice in the MWM. Handling and daily administration of 25% sweetened condensed milk (5 ml/kg/day) did not significantly affect performance. Based on these results, I analyzed both control and vehicle treated animals together when comparing Tg to NonTg mice.

In the MWM, a high escape latency value without progressive improvements over successive test days (Figure 3.1A) is indicative of poor learning and memory. NonTg females displayed a progressive decline in escape latency most pronounced over the first four test days. A main effect of genotype was observed with Tg females clearly impaired as evidenced by significantly higher escape latency values compared to NonTg littermates ($F_{(1,50)}$=15.24, $p<0.001$). A *post hoc* Holm-Sidak's test further showed test days two (p=0.009), three (p=0.0019), six (p=0.0070) and eight (p=0.0027) as significantly different

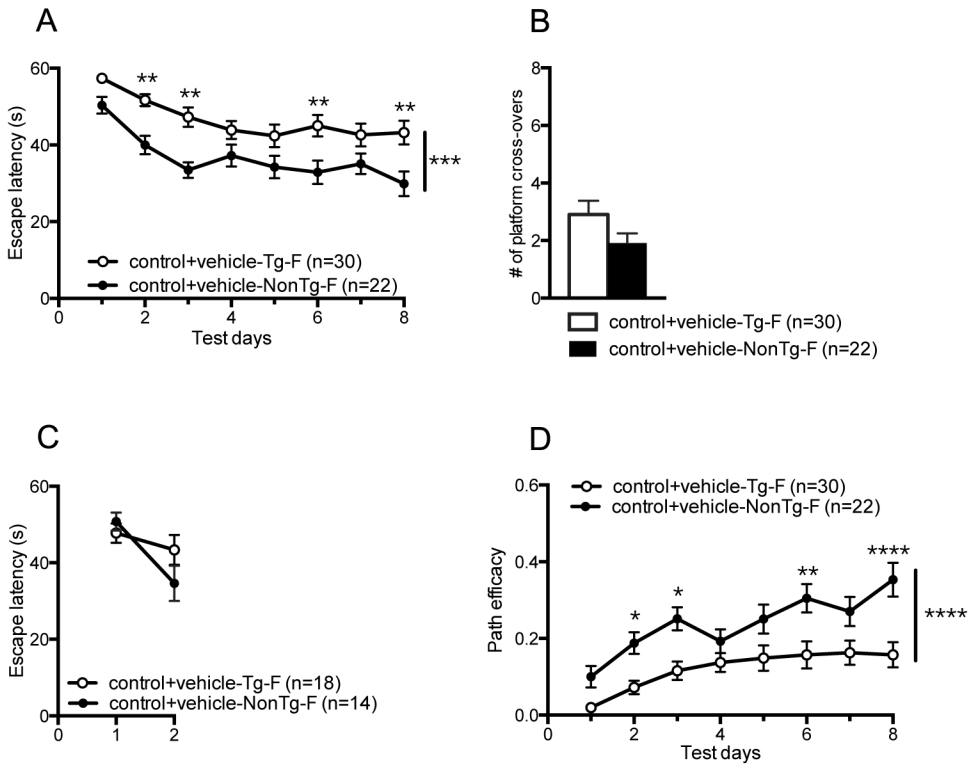
A
Escape latency (s)
60
40
20
0
0
2
4
6
8
Test days
**
**
**
**
***
control+vehicle-Tg-F (n=30)
control+vehicle-NonTg-F (n=22)
B
# of platform cross-overs
8
6
4
2
0
control+vehicle-Tg-F (n=30)
control+vehicle-NonTg-F (n=22)
C
Escape latency (s)
60
40
20
0
0
1
2
control+vehicle-Tg-F (n=18)
control+vehicle-NonTg-F (n=14)
D
Path efficacy
0.6
0.4
0.2
0.0
0
2
4
6
8
Test days
control+vehicle-Tg-F (n=30)
control+vehicle-NonTg-F (n=22)
*
*
**
****
****

Figure 3.1.

Figure 3.1 MWM analysis of control female Tg compared to NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) was done by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Data shown are mean ± SEM.

between female Tg and NonTg mice. During the probe trial on day nine, where the escape platform was removed and the amount of time mice spent searching (i.e., crossing over) the former location of the escape platform, NonTg mice exhibited more platform cross-overs than Tg mice although this measure did not reach statistical significance when analyzed by an unpaired two-way Student's *t* test ($t_{(50)}$=1.553, Figure 3.1B). No difference in the ability of NonTg or Tg mice to adapt to new task requirements was observed when the platform was relocated (reversal) on test day ten and eleven (Figure 3.1C). A significant main effect of the repeated measures test days was observed ($F_{(1,30)}$=14.63, p=0.0006), indicating both genotypes progressively decreased in escape latency value. However reversal learning escape latency values were not significantly different between Tg and NonTg mice ($F_{(1,30)}$=0.4836).

A second measure of cognitive performance is the ability to attain the platform by the most direct means, termed path efficacy (Figure 3.1D). When analyzed with a two-way repeated measures ANOVA, a main effect of genotype was observed ($F_{(1,50)}$=18.36, p<0.0001). NonTg mice swam more directly to the platform compared to Tg mice (Figure 3.1D). A *post hoc* Holm-Sidak's test further supported path efficacy difference between female Tg and NonTg mice on test days two (p=0.0403), three (p=0.0118), six (p=0.0053) and eight (p<0.0001).

Male Tg mice at six months of age do not perform as well as NonTg mice when introduced into the MWM but are capable of progressive learning and memory over successive test days

Learning and memory indices in the MWM with respect to male transgenic mice were assessed analogous to those of female mice. As with females, there were no significant differences in MWM performance of control and vehicle treated mice in either genotype, thus vehicle and controls were examined together in subsequent analyses (Table A1). Unlike females, male Tg mice displayed progressively decreased escape latency values over successive test days, indicative of robust learning and memory (Figure 3.2A). When overall performance of Tg mice was compared to NonTg littermates in escape latency with two-way repeated measures ANOVA, a main effect between genotypes was observed ($F_{(1,43)}$=6.946,p=0.0116, Figure 3.2A). This difference was restricted to initial performance between genotypes, a *post hoc* Holm-Sidak's test showed statistical difference between male Tg and NonTg mice on test days one (p=0.0164), two (p=0.0188) and four (p=0.0438). This difference could be attributed to the fact that male NonTgs were able to escape the maze very rapidly upon initial exposure (i.e., Tgs took longer to reach the same escape latencies immediately obtained by NonTgs) (Figure 3.2A). Performance during probe ($t_{(44)}$=0.4872) and reversal ($F_{(1,19)}$=0.6273) testing were comparable between genotypes (Figure 3.2B,C). A significant main effect of the repeated measures test days was observed ($F_{(1,30)}$=14.63, p=0.0006), indicating both genotypes progressively decreased in escape latency value over the two days of

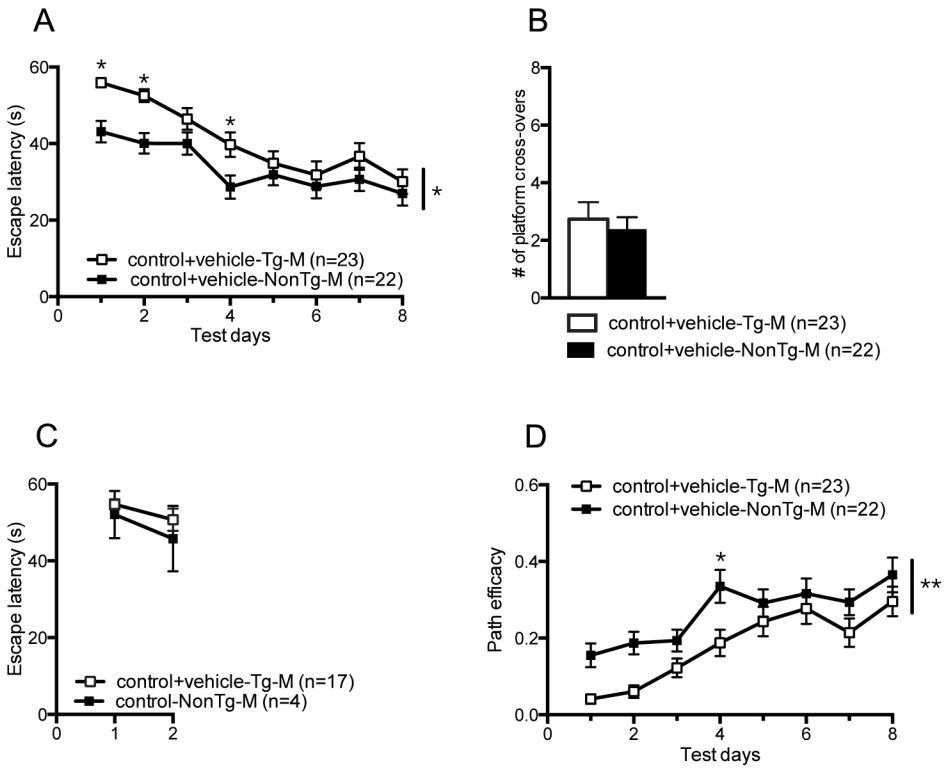
A
Escape latency (s)
Test days
control+vehicle-Tg-M (n=23)
control+vehicle-NonTg-M (n=22)
B
# of platform cross-overs
control+vehicle-Tg-M (n=23)
control+vehicle-NonTg-M (n=22)
C
Escape latency (s)
control+vehicle-Tg-M (n=17)
control-NonTg-M (n=4)
D
Path efficacy
Test days
control+vehicle-Tg-M (n=23)
control+vehicle-NonTg-M (n=22)

Figure 3.2.

Figure 3.2 MWM analysis of control male Tg compared to NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) was done by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was done by Student's *t* test. *$p<0.05$; **$p<0.01$. Data shown are mean ± SEM.

reversal testing. However reversal learning escape latency values were not significantly different between Tg and NonTg mice. These differences between genotypes in initial performance but not rate of adopting a more direct path to the escape platform was reflected in measures of path efficacy. A two-way repeated measures ANOVA analysis of path efficacy performance revealed a main effect between genotypes ($F_{(1,43)}$=9.211,p=0.0041, Figure 3.2D) and further *post hoc* Holm-Sidak's test showed significant difference on test day four (p=0.0184).

Sex differences in learning and memory performance are not due to motoric deficits or elevated anxiety levels

Non-cognitive indices were additionally measured during MWM to verify confounds of motoric deficits or high anxiety with respect to both male and female Tg mice compared to NonTg controls at six months of age (Table A1). Motoric abilities were assessed by distance moved and velocity swam during MWM task. When analyzed by a two-way ANOVA, no significant main effect of sex was observed in the average distance moved of control+vehicle-Tg-F, control+vehicle-Tg-M, control+vehicle-NonTg-F and control+vehicle-NonTg-M groups ($F_{(1,69)}$=3.592, p=0.0623, Figure 3.3A). No effect of sex was observed in average velocity between female and male Tg mice, or female and male NonTg mice ($F_{(1,63)}$=0.2258, p=0.6363, Figure 3.3B). A significant main effect of genotype was observed in motoric abilities of these groups as Tg mice displayed significantly increased distance moved ($F_{(1,93)}$=74.78, p<0.0001) and greater velocity ($F_{(1,93)}$=74.78, p<0.0001). A further *post hoc* Holm-Sidak's test indicated

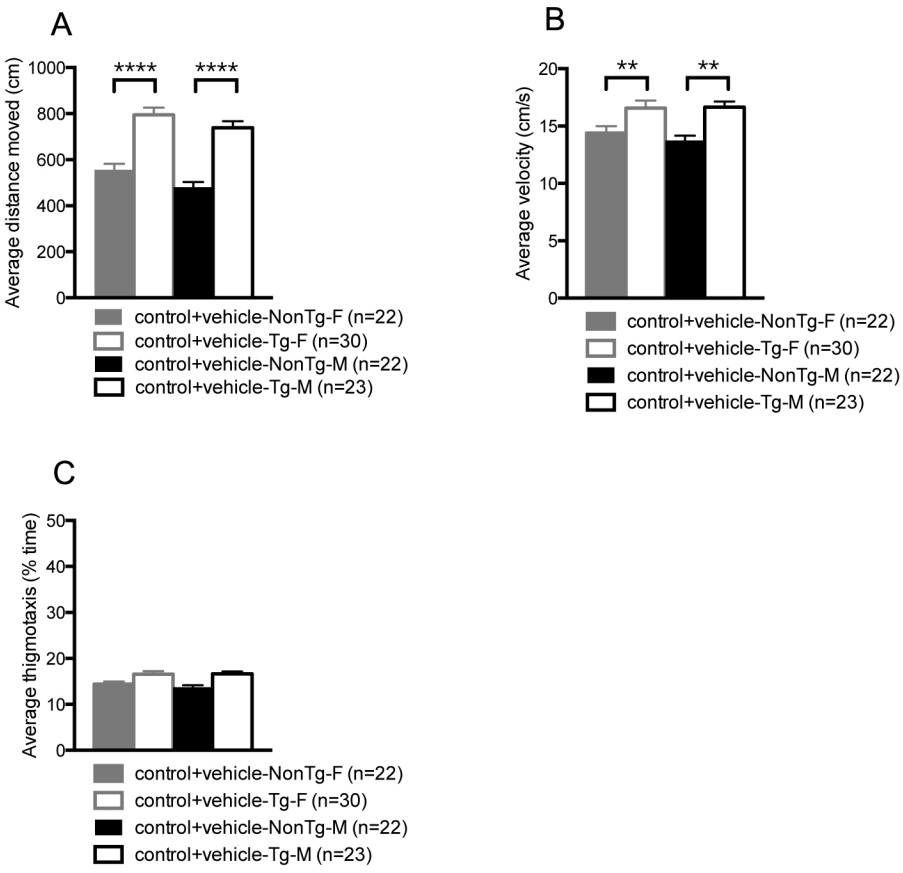
A
Average distance moved (cm)
1000
800
600
400
200
0
****
****
control+vehicle-NonTg-F (n=22)
control+vehicle-Tg-F (n=30)
control+vehicle-NonTg-M (n=22)
control+vehicle-Tg-M (n=23)
B
Average velocity (cm/s)
20
15
10
5
0
**
**
control+vehicle-NonTg-F (n=22)
control+vehicle-Tg-F (n=30)
control+vehicle-NonTg-M (n=22)
control+vehicle-Tg-M (n=23)
C
Average thigmotaxis (% time)
50
40
30
20
10
0
control+vehicle-NonTg-F (n=22)
control+vehicle-Tg-F (n=30)
control+vehicle-NonTg-M (n=22)
control+vehicle-Tg-M (n=23)

Figure 3.3.

Figure 3.3 Analysis of non-cognitive indices in MWM of control male and female Tg and NonTg mice.

During MWM testing the motoric function was assessed by average distance moved (140) (A) and average velocity (cm/s) (B), and anxiety levels during the task were determined by average time spent in thigmotaxis (%) (C) with respect to Tg and NonTg mice of both sexes. Statistical analysis of average distance moved, average velocity and average percent thigmotaxis were each done by two-way ANOVA. **p<0.01; ****p<0.0001. Data shown are mean ± SEM.

significance of genotype to occur in distance moved of females ($p<0.0001$) and males (0.0001), and velocity of females ($p=0.0081$) and males ($p=0.0020$). Anxiety levels were investigated by quantifying thigmotaxis behaviour defined as amount of time spent swimming in periphery of MWM pool. No effect of sex was observed in percent thigmotaxis between female and male Tg mice, or female and male NonTg mice ($F_{(1,66)}=1.045$, $p=0.3105$, Figure 3.3C). No difference was observed in thigmotaxis behaviour of Tg compared to NonTg mice of either sex ($F_{(1,66)}=0.08425$, $p=0.7725$). Taken together, Tg mice of both sexes displayed no motoric deficit or elevated anxiety levels, variables that would differentially influence MWM performance.

Sex differences in MWM performance of six month old Tg mice

Next, escape latency and path efficacy were contrasted and compared for male and female Tg mice. A novel sex difference was observed between the cognitive performances of male and female Tg mice in the MWM at six months of age (Figure 3.4A). When escape latency was analyzed with two-way repeated measures ANOVA, a main effect between sexes was observed. Female Tg mice displayed significantly greater overall escape latency values compared to male Tg mice ($F_{(1,51)}=4.611$, $p=0.0365$, Figure 3.4A). A *post hoc* Holm-Sidak's test further showed test days six ($p=0.0049$) and eight ($p=0.0049$) as significantly different between female and male Tg mice. Performance during probe testing were comparable between female and male Tg mice ($t_{(51)}=0.8318$, Figure 3.4B). In reversal learning of MWM (Figure 3.4C), a main effect of sex was observed

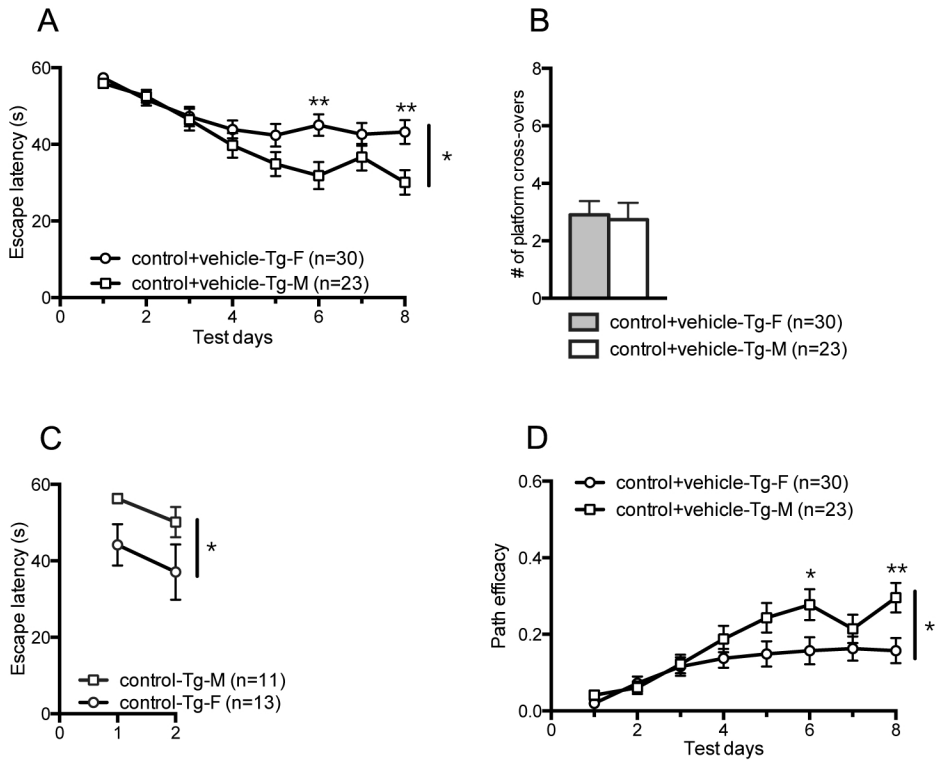
A
Escape latency (s)
60
40
20
0
0
2
4
6
8
Test days
**
**
*
control+vehicle-Tg-F (n=30)
control+vehicle-Tg-M (n=23)
B
# of platform cross-overs
8
6
4
2
0
control+vehicle-Tg-F (n=30)
control+vehicle-Tg-M (n=23)
C
Escape latency (s)
60
40
20
0
0
1
2
*
control-Tg-M (n=11)
control-Tg-F (n=13)
D
Path efficacy
0.6
0.4
0.2
0.0
0
2
4
6
8
Test days
control+vehicle-Tg-F (n=30)
control+vehicle-Tg-M (n=23)
*
**
*

Figure 3.4.

Figure 3.4 MWM analysis of control male compared to female Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) was done by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was done by Student's *t* test. *$p<0.05$; **$p<0.01$. Data shown are mean ± SEM.

($F_{(1,33)}$=4.627, p=0.0389), indicating over all reversal test days collapsed, male Tgs displayed more difficulty to acquire reversal task than female Tgs. However, a *post hoc* Holm-Sidak's test indicated no significant difference between male and female Tg mice on individual test days ten (p=0.1691) and eleven (p=0.1412). Additionally, no main effect of the repeated measures test days was observed ($F_{(1,33)}$=3.358, p=0.0759), indicating both male and female Tgs did not progressively decrease escape latency values thus did not display significant difference in learning rate.

Differences between sexes were also reflected in path efficacy (Figure 3.4D). Analysis by two-way repeated measures ANOVA detected a main effect between sexes ($F_{(1,51)}$=4.596, p=0.0368, Figure 3.4D) with *post hoc* Holm-Sidak's analysis indicating that male Tg mice swam more directly to the platform than females on days six (p=0.0292) and eight (p=0.0080). Taken together, these behavioural indices of cognition in the MWM indicate that male Tg mice at six months of age performed very differently than age-matched female Tg mice.

3.5 Discussion

In this study, I assessed whether Tg mice exhibit behavioural impairments in learning and memory by six months of age. I found that handling and daily feeding with 25% sweetened condensed milk did not alter performance of Tg and NonTg males and females. This determination was important for analysis of the concurrent treatment studies (Chapter 5 and 6) as it allowed me to increase my control numbers by combining vehicle and untreated cohorts and thus increase statistical power (see Chapter 5 and 6). Further, I show that female Tg mice

display significant learning and memory impairments compared to NonTg mice at six months of age, whereas impairment of male Tg mice is mild, significantly different from females, and restricted to a difference in performance upon exposure to the MWM task as compared to a marked impairment of learning and memory over the course of the entire test period. These differences were independent of sex or genotype differences in motoric impairments or anxiety behaviours in the MWM. This study represents the first comparison of sex differences in the TgCRND8 mouse model of AD.

Escape latency is a well-documented measure of performance in MWM (74,115), and robust performance is indicated by a decline in escape latency over progressive test days. Here, female Tg mice displayed high escape latency, indicating failure to learn to improve in the task or acquire the task. These results match previous work of our laboratory indicating impairment in female TgCRND8 mice at six months of age (51). These data are also consistent with reported F1 TgCRND8 impairment wherein mice of both sexes were tested and data pooled (30,49,52). Intriguingly, performance of male Tg mice differed from that of females. At six months of age, male Tg mice displayed robust learning curves statistically different from females although impaired with respect to initial performance upon exposure to the MWM compared to age-matched NonTg males. Previous reports have shown learning and acquisition of the MWM task to occur by the first six days of training (141), and that inhibition of the NMDA receptors of hippocampal CA3 pyramidal cells affect the memory acquisition of novel hidden platform locations in MWM (142,143). Inability to initially acquire the

task, displayed in early trials of the MWM test of male Tg mice may be a phenotype comparable to AD neuropathology. Male Tg mice do not process the spatial cues as rapidly as NonTg mice; they exhibit significantly impaired navigational capacity on early test days (one, two and four). However male Tg mice exhibit a different impairment in the MWM compared to female Tg mice; male Tgs are significantly less impaired during later test days (six and eight). Female Tgs are severely impaired, whereas male Tgs are mildly impaired, in spatial navigation.

Cognitive impairment in Tg mice was additionally assessed by ability to attain the platform by most direct path length. Path efficacy is a novel approach comparing the total distance swam per trial to a measured direct path distance, with respect to each entry point. This is a more stringent measure than path length measure alone, as the latter may be an inaccurate reflection of learning given that cardinal start positions are not equidistant from the platform (75). This parameter is also independent of time and swimming speed (75). TgCRND8 mice displayed agitated behaviour by increased locomotion and swim speed, which is consistent with reports (144-146), but not due to altered stress. Mice daily handled and vehicle treated displayed no difference in thigmotaxis behaviour, were non-invasively manipulated as opposed to gavage (147), and we predict had unaltered corticosterone levels. Increased swim could nonetheless increase coincidental chance of obtaining the platform without reflecting spatial learning. Here, I show that not only are female mice impaired with respect to escape latency but they also swim less directly to the platform than NonTg mice

consistent of learning and memory impairment. Conversely, males, initially swim indirectly to the platform, but with time adopt the same direct swim path as NonTg males.

Previous work (47,48,125,126), has indicated that different genetic backgrounds display varied MWM performance. Our NonTg mice are a mixed C3H/C57 hybrid background and are backcrossed for five generations to a C57BL/6 lineage. Their performance is consistent with the work of Glazner *et al.* in 2010 and Janus in 2004 using C3H/C57F1 TgCRND8 mice (49,52). It must be noted, however, that this performance of a spatial memory task is not as robust as what is seen on a pure C57BL/6 background (48,49). Glazner and colleagues additionally have shown that important differences in MWM performance exist between two TgCRND8 background strains; 129SvEvTac/C57F1 and C3H/C57F1 (49). Greater deficits were found in 129SvEvTac/C57F1 strain in several parameters of MWM relative to control (49). Search behaviour also differed, as C3H/C57F1 transgenics predominantly used non-spatial systematic search strategies, whereas 129SvEvTac/C57F1 transgenics displayed no preference (49). Thus, the failure of all of our animals to improve their escape latencies to below an average of 30 seconds likely reflects strain differences or other comorbid impairments such as visual deficits (I address the latter in Chapter 4).

The sex difference reported here are the first established for the TgCRND8 mouse model of AD, and concurrent with previous studies that have indicated AD is manifested at higher prevalence among women (148-150).

Certainly, MWM performance is influenced by sex of subjects; male animals exhibit better spatial learning than females (77,132-134). Male C57BL/6 mice compared to females, have also exhibited better spatial working and reference memory performances (127). Also, female APP/PS1 transgenic mice exhibited greater deficits in MWM learning task than males (151). It may be that these sex-based differences in spatial learning are consequence of aberrant APP processing. Female mice exhibited more aggressive plaque pathology (29,152-156). It has also been reported that female transgenic mice compared to male mice exhibit increased β-secretase and γ-secretase activity, increased microglial activation, as well as decreased activity of Aβ-degrading enzymes neprilysin and insulin degrading enzyme (IDE) (29,151,153). Female F1 TgCRND8 mice were also reported to have greater $A\beta_{40}$ and $A\beta_{42}$ levels than males (157). Our laboratory has previously reported significant plaque load in female Tg mice at this time point (51); male mice have yet to be assessed. Thus, it will be important in future studies to compare the $A\beta_{42}/A\beta_{40}$ ratio and plaque burden in male and female Tg mice at six months of age.

Taken together, the data presented in this chapter contribute to the learning and memory phenotype of the TgCRND8 mouse model of AD. This study assessed multiple behavioural indices of cognition in the MWM with respect to an incipient congenic C57BL/6 background. Most interestingly, a novel sex difference was observed; male Tg mice differed from female mice by displaying unimpaired performance at six months of age that could not be explained by differences in motoric performance or anxiety behaviours and

perhaps manifest as result of differences of sex in amyloidogenic processing. This hypothesis will be pursued in future investigations.

Chapter 4 – Visual acuity of N5 C57BL/6NCrl X C3H/HeJ NonTg and Tg mice

4.1 Objective of this study

Because our N5 TgCRND8 mouse model is on a mixed genetic background of sighted and visually impaired animals, I sought to address whether the performance in the MWM and sex differences demonstrated in Chapter 3 were due to differences in learning and memory and not the result of visual impairment. Two tests were implemented to identify visual function: (1) adapted SLAG test and (2) the cued MWM task.

4.2 Statement of author contributions

Thank you to Dr Diane Lagace and Mirela Hasu for access to the University of Ottawa Behavioural Core Facility. Dr Alexandra Pettit, Dr Carolina Cieniak and Matthew Granger assisted me in study design, analysis, and visual assessment as independent raters of the SLAG reflex.

4.3 Introduction

Significant strain differences in visual ability have previously been reported to result in varied MWM performance (48,125,158). Varied visual acuity has been shown in inbred C3H/HeN, FVB/N, CD1 and BALB/c strains as compared to C57BL/6 mice. The frequently used C57BL/6 strain displays robust performance in MWM (158,159). In fundus and retina examinations, C57BL/6 mice display no defects (160,161). Furthermore, C57BL/6 mice do not display visual impairment in optokinetic response test (161,162) and normal vision is reported in both the

visual water box test (VWB) (125,161,163) and cued MWM tests (48,125,158). Relevant to this thesis, the C3H/HeN strain has severe visual impairment due to retinal degeneration (139,160,164). The C3H/HeN strain is homozygous for retinal degeneration mutation Pde6b$^{rd1/rd1}$, resulting in early visual impairment due to a rapid loss of rod photoreceptor cells and cones (139). *Pde6b* encodes the beta subunit of the cyclic guanosine monophosphate phosphodiesterase (cGMP-PDE). Mutations result in progressive degeneration of retinal rods and early severe visual impairment (160,165,166). Rod cGMP-PDE is an important enzyme in phototransduction, serving as regulator between rhodopsin-activated transducing and cGMP-gated cationic channels present in photoreceptor plasma membrane (79). PDE activation results in hydrolysis of cGMP, closure of cationic channels and plasma membrane hyperpolarization, thus modifying cell signalling (79). Rod photoreceptors in Pde6b$^{rd1/rd1}$ mutants attain a fraction of normal length and progressive degeneration of whole photoreceptors (164). Head-tracking reactions in optokinetic response is impaired in C3H/HeN by ten weeks of age (139,167,168). C3H/HeN mice also displayed visual impairment in the VWB and cued MWM tests (48,125,163,169). Intriguingly, homozygous mice with Pde6b$^{rd1/rd1}$ retinal degeneration are nevertheless able to synchronize circadian rhythms to light-dark conditions, and spend more time in dark than illuminated areas (170).

Like C3H/HeN mice, FVB/N mice are commonly used for generation of transgenic animals because of their vigorous breeding performance and size of oocyte pronuclei that facilitates microinjection (165,171). FVB/N are functionally

blind by weaning due to two unrelated genetic defects (165). Firstly, FVB/N mice are an albino strain, caused by a dysfunctional tyrosinase locus (*Tyr*^{C}) (165). This locus is known to encode tyrosinase, the rate-limiting enzyme in the production of melanin pigment (172,173). Albino mice exhibit impaired visual projections at the optic chiasm, decreased rod photoreceptors and spatiotemporal defects (172). Additionally, albino mice have inadequate ipsilateral inputs leading to impaired visual acuity and depth perception (174). Secondly, FVB/N mice, also carry the Pde6b$^{rd1/rd1}$ mutation. In behavioural measures of vision, FVB/N strain displayed severe impairment in VWB test (125,163) and cued MWM (169,175). FVB/N mice performed poorly in MWM (158,169,175), and proved unsuitable in vision-based testing of learning and memory. However, in hippocampus-dependent tasks that rely less heavily on visual input (such as fear conditioning or odour habituation), FVB/N mice displayed learning and memory comparable to C57BL/6 strain (78).

Intermediate visual acuity is observed in CD1 and Balb/c strains. The CD1 strain is sensitive to light intensities due to albinism resulting from a dysfunctional *Tyr*^{C} and exhibit visual impairment in optokinetic response test (176). Photophobia in testing conditions is observed in light/dark box test; CD1 mice spend significantly more time in dark box than light box, suggesting, despite visual impairment, an ability to perceive brightness (176). Finally, BALB/c mice are another strain with oculocutaneous albinism as a result of dysfunctional *Tyr*^{C} (177). BALB/c mice also display significantly lowered optokinetic response values than the pigmented C57BL/6 strain (161,177), and intermediate visual

accuracy compared to normal visual acuity of C57BL/6 mice and severely impaired acuity of FVB/N and C3H/HeN strains in tests of VWB (125,161,163) and cued MWM (48,126).

Here, visual acuity of N5 TgCRND8 and NonTg mice was assessed in both males and females at six months of age. Two tests were implemented to unambiguously identify functional vision; the SLAG test and the cued MWM test. As SLAG reflex test is a novel reported test of murine spatial vision (120), the test was further adapted to distinguish a scope of visual conditions (no visual impairment, mild visual impairment, and moderate to severe visual impairment). To validate the SLAG test, C57BL/6, FVB/N, C3H/HeN, CD1 and BALB/c mice were tested as genetically predetermined control strains before comparison to Tg and NonTg mice at two-three, five-six, and eight-ten months of age. I report that Tg and NonTg mice have reduced visual acuity, this impairment does not affect their performance at six months of age in spatial navigation of cued MWM test. Performances in SLAG reflex test and cued MWM were irrespective of sex or age.

4.4 Results

Adapted SLAG reflex test identifies varied degrees of visual acuity in C57BL/6, CD1, BALB/c, C3H/HeN and FVB/N strains

Functional vision in male and female mice in C57BL/6, CD1, BALB/c, C3H/HeN and FVB/N strains was assessed using an adapted SLAG reflex test (Figure 4.1A). These strains were chosen as representative controls with *a priori*

knowledge of visual acuities to validate modified scoring criteria. The SLAG reflex test quantifies an animal's use of the visual placing response. Adaptations applied to the analysis of SLAG reflex test (described in Chapter 2) permitted distinction of no visual impairment, mild visual impairment and moderate to severe visual impairment as compared to the binary blind or sighted criteria described in the original paper (120). Here, successfully displaying a visual placing response repeatedly within six trials and within each orientation was indicative of robust vision, whereas mice that failed to display this reflex were categorized based on their degree of impairment. Phenotypes of control strains were achieved by inter-rater scoring based on majority consensus among four investigators. In adapted SLAG reflex test, as expected, all C57BL/6 mice displayed no visual impairment (Figure 4.1A). The CD1 strain predominantly displayed mild visual impairment. Additionally, the BALB/c, C3H/HeN and FVB/N strains predominantly displayed moderate-severe visual impairment (Figure 4.1A). Performance in SLAG reflex test was irrespective of sex; males and females exhibited comparable vision-dependent reflex behaviours (Figure 4.1A).

Tg and NonTg mice exhibit reduced visual acuity in adapted SLAG test

Having validated our scoring criteria, visual acuity of male and female Tg and NonTg mice was individually assessed in adapted SLAG reflex test at two-three, five-six, and eight-ten months of age (Figure 4.1B). NonTg mice predominantly displayed mild visual impairment, throughout ages of two-three, five-six and eight-ten months of age (Figure 4.1B). NonTgs exhibited a high

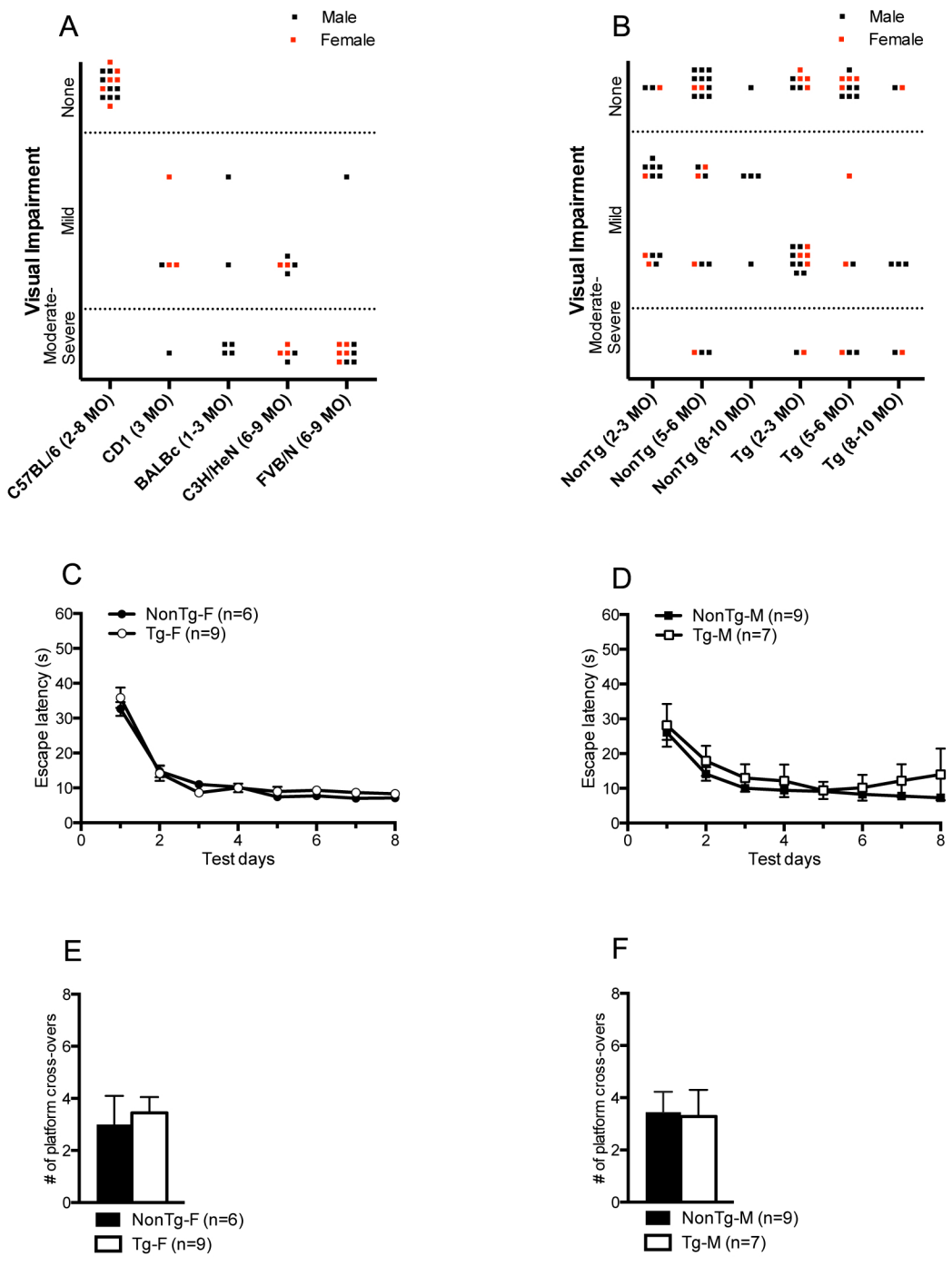
A
Male
Female
None
Mild
Moderate-
Severe
Visual Impairment
C57BL/6 (2-8 MO)
CD1 (3 MO)
BALBc (1-3 MO)
C3H/HeN (6-9 MO)
FVB/N (6-9 MO)
B
Male
Female
None
Mild
Moderate-
Severe
Visual Impairment
NonTg (2-3 MO)
NonTg (5-6 MO)
NonTg (8-10 MO)
Tg (2-3 MO)
Tg (5-6 MO)
Tg (8-10 MO)
C
NonTg-F (n=6)
Tg-F (n=9)
Escape latency (s)
Test days
D
NonTg-M (n=9)
Tg-M (n=7)
Escape latency (s)
Test days
E
# of platform cross-overs
NonTg-F (n=6)
Tg-F (n=9)
F
# of platform cross-overs
NonTg-M (n=9)
Tg-M (n=7)

Figure 4.1.

Figure 4.1 Visual acuity of Tg and NonTg mice as compared to sighted and visually impaired strains.

Adapted SLAG reflex test of control groups (A): C57BL/6 (2-8 months of age), CD1 (3 months of age), BALB/c (1-3 months of age), C3H/HeN (6-9 months of age), FVB/N (6-9 months of age). Adapted SLAG reflex test of Tg (2-3 months of age, 5-6 months of age, 8-10 months of age) and NonTg littermates (2-3 months of age, 5-6 months of age, 8-10 months of age) (B). In adapted SLAG reflex test, six trials were conducted, three per each orientation. Each point represents a mouse (red as female, black as male). Cued MWM was conducted over eight consecutive test days, and represented as average escape latency values of four trials per day. A ninth testing day was conducted as probe trial. Female Tg and NonTg mice were compared in cued MWM (C) and probe trial (E). Male Tg and NonTg mice were compared in cued MWM (D) and probe trial (F). Statistical analysis of (C) and (D) were done by repeated measures two-way ANOVA. Statistical analysis of (E) and (F) were analyzed by Student's *t* test. MWM data shown are mean ± SEM.

none-mild and lower moderate-severe (19:3) abilities, comparable to CD1 mice (4:1). A similar phenotype was observed in Tg littermates indicating that overexpression of the mutant *APP* transgene did not exacerbate visual impairment (Figure 4.1B).

Visual impairment in Tg and NonTg mice does not impair performance in the cued MWM

To test whether this impairment altered capacity to perceive spatial cues in the MWM, Tg and NonTg mice were assessed in cued MWM task at six months of age. Testing environment remained identical to the conventional MWM paradigm described in Chapters 2 and 3, except extra maze cues were removed and the hidden platform was rendered visible by a flag, allowing a direct sightline to the platform location in visually intact mice. Visual acuity performance in cued MWM was analyzed by escape latency values on eight successive test days. Males and females were analysed separately; no sex differences were observed in performance (Figure 4.1C,D). When assessed using a two-way repeated measures ANOVA, no difference was observed between female Tg and NonTg mice ($F_{(1,13)}$=0.4919, Figure 4.1C). Escape latency performance was not significantly different between male Tg and NonTg mice ($F_{(1,14)}$=0.6014, Figure 4.1D). Both Tg and NonTg mice navigated readily to the visible platform, and significantly improved their performance over progressive test days (significant effect of test days with respect to females $F_{(7,91)}$=93.30, $p<0.0001$ and males $F_{(7,98)}$=29.88, $p<0.0001$), reaching the test threshold of a 10 s escape latency

score. Taken together, this suggests both male and female Tg and NonTg mice possess sufficient eyesight to repetitively locate and navigate to the spatial cue on the platform. After cued MWM testing, an additional probe trial day was executed which involved no spatial cues and no platform. Comparison between female Tg and NonTg mice displayed no difference in number of platform area cross-overs ($t_{(13)}$=0.3854, Figure 4.1E). Male Tg mice displayed no difference in number of platform area cross-overs than NonTg mice ($t_{(14)}$=0.1259, Figure 4.1F). Taken together, probe trial indicated spatial accuracy was no different between Tg and NonTg mice with respect to either sex.

4.5 Discussion

The experiments presented in this chapter aimed to establish the visual acuity of the Tg and NonTg mice with respect to both males and females at six months of age. Murine visual function was assessed by two tests; adapted SLAG reflex test and cued MWM task. I show here that while Tg and NonTg mice are visually impaired, this impairment is not sufficient to affect visually-cued performance in the MWM.

The SLAG reflex test is a novel test which evokes innate behaviour of the visual placing response (120). As described in (178); the visual system is an integrated sensory-motor system. Visuo-motor reflexes enable image stability on the retina, and as these reflexes are automatic, they provide measure visual function while precluding reinforcement training (178). This reflex has been described in SmithKline Beecham Harwell Imperial college Royal London

hospital phenotype assessment protocol (179) and Institutional Animal Care and Use Committee neurological exam (180). As a mouse is suspended by the tail above an object (in this case a cage wire lid) and slowly lowered near it, the mouse may display behaviours of reflexively reaching, extending its forepaws, elevating its head, and twisting its body towards toward the object dependent on functional vision (see Chapter 2 for SLAG reflex characterization details). This reflex-based, non-invasive assay immediately and clearly identifies sight, without training or conditioning (120). As originally described, the SLAG paradigm (120), involved two trials per mouse in two different orientations of descent (see Chapter 2) and only distinguished between sighted and blind conditions. To avoid vision scoring ambiguity or errors, I adapted the SLAG reflex test protocol as follows: six total trials were conducted (three per orientation), descent was marked by a 20 cm path at a 45° angle from the wire lid base, mice were held for a 5 s interval at a 4 cm distance adjacent to the wire lid, mice received a 15 s rest on the wire lid per trial and a 5 min inter-trial rest. Mice that failed to display SLAG reflex were categorized based on performance in both orientations (see Chapter 2 for scoring method). Using genetic controls, vision assesssments were comparable with previous investigations in optokinetic response (161,162), cued MWM performance (48,125,158), VWB performance (125,161,163), and fundus and retina examinations (160,161). The C57BL/6 strain displayed no visual impairment in SLAG reflex test; C3H/HeN and FVB/N strains were severely impaired comparable to reported impaired performances in VWB test (125,163) and cued MWM task (48,169,175). The CD1 and BALB/c strains hold the

melanin synthesis disorder of oculocutaneous albinism, leading to lack of melanin pigment and visual system abnormalities (177). As previously reported (181), reduced melanin in retinal pigment epithelium of albino mice resulted in a 30% decrease of number of rod photoreceptors, underdevelopment of the retina and distortion of chiasmatic projection to the brain. Both CD1 and BALB/c mice carry missense mutation in *Tyr*^{c}, causing a cysteine to serine change at amino acid 103 of the protein, and dysfunction of this key enzyme of melanin synthesis (177,182). In adapted SLAG reflex test, I found that CD1 mice predominantly displayed a mild visual impairment whereas BALB/c mice exhibited a moderate-severe visual impairment. This result is consistent with a previous report of optokinetic response test performances (178). Optokinetic response thresholds of both BALB/c and CD1 mice were reported lower than that of pigmented C57/BL6 strain (176,178), but CD1 mice had substantially better threshold in optokinetic test than BALB/c mice (178).

With this validated test in hand, Tg and NonTg mice were assessed over time. Previous work of Francis and colleagues demonstrated functional visual ability by optokinetic response test in nine week old F1 TgCRND8 and NonTg mice and no significant impairment (183), however, visual acuity in Tg and NonTg mice at six months of age has never previously been investigated. I found a range of visual impairments and it may be that mice displaying moderate to severe impairment phenotypes, with respect to either genotype, may reflect penetrance of the C3H/HeN background (3.12% of the loci in the genome of

each N5 animal (184)). This hypothesis can be tested by examining each animal for presence of a heterozygote Pde6b$^{rd1/wt}$ mutation by PCR.

Functional vision and ability to respond to visual cues are essential in the MWM task; otherwise performance does not reflect learning and memory. As a second measure to unambiguously identify visual function of male and female Tg and NonTg mice at six months of age, cued MWM task was implemented. In the present study, despite varying degrees of visual acuity, all of the Tg and NonTg mice tested in both SLAG and cued MWM at six months of age were able to effectively locate and navigate to the spatial cue on the platform, indicating sufficient functional vision to perform in visually cued learning and memory tasks. This result is corroborated by previous work of Janus, in which six-seven month old F1 TgCRND8 and NonTg mice were investigated in a similar cued MWM paradigm (52). Similar to those F1 TgCRND8 and NonTg mice, N5 mice displayed an initial difference during the first day of training, perhaps due to subtle differences in reactivity, thigmotaxis behaviour, or an exaggerated systematic strategy response to a novel environment as reported (52,185), notwithstanding any deficiencies were abated by next testing trials. F1 TgCRND8 and NonTg mice exhibited comparable escape latency performance in the cued MWM test, both genotypes reached 5-10 s escape latency threshold performance in the four days tested (52).

Taken together, the data presented in this chapter is the first to establish visual acuity of this Tg mouse model with respect to both male and female mice at six months of age indicating that despite some degree of visual impairment Tg

and NonTg mice possess sufficient vision to navigate the MWM and thus performance likely reflects learning and memory rather than result of visual impairment.

Chapter 5 – Assessing effect of four-month omega-3 UFA supplementation on learning and memory performance in Tg and NonTg mice at six months of age

5.1 Objective of this study

The purpose of this study was to investigate cognitive benefit of a dietary intervention in an AD mouse model. The experiments presented in this chapter assess the effect of a four-month omega-3 UFA supplementation on learning and memory performance in male and female Tg and NonTg mice at six months of age. Additionally, the effect of omega-3 UFA treatment was evaluated with respect to non-cognitive behaviours such as motoric abilities and anxiety levels, which may confound MWM performance.

5.2 Statement of author contributions

Thank you to Dr Diane Lagace and Mirela Hasu for access to the University of Ottawa Behavioural Core Facility. Matthew Granger tested the following groups; control-Tg-M, control-Tg-F, control-NonTg-M and control-NonTg-F. Dr Carolina Cieniak and Fida Ahmed tested the following groups; vehicle-Tg-M and vehicle-NonTg-M. Dr Hongbin Xu and Dr Carolina Cieniak assisted me in study design and analysis.

5.3 Introduction

Omega-3 UFAs are essential for neuronal function. Epidemiological and animal studies suggest that omega-3 UFAs, particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), may be neuroprotective and reduce cognitive decline (186-188). However, studies investigating dietary omega-3 UFA intake in relation to AD and aging have reported conflicting results (88,189). Randomized controlled trials of EPA and DHA supplementation to healthy old individuals displayed no benefit (190,191). Studies involving omega-3 UFA supplementation to subjects with early dementia (i.e age-related cognitive decline or MCI) report small cognitive benefit (187,192), or improved mood and mental health (193). However, interpretation of clinical trials must be cautious. Positive effects of nutritional interventions may be group-specific (194). Some cognitive benefits are reported occurred in patients with previous history of heart disease or stroke (195-197). Comprehensive therapeutic approaches and rigorous validations are required (194).

Although AD subjects show omega-3 UFAs deficit, individuals with AD receiving omega-3 UFAs supplements display no significant cognitive improvement. Deficiencies in omega-3 UFAs have been reported in plasma (109,111), and decreased DHA has been reported in post-mortem brains (98,112,198) of AD patients. This suggests a deficient diet or, alternatively, a failure to incorporate omega-3 fatty acids into membrane lipids effectively could affect AD progression or severity (188,199). However clinical trials have reported no success; AD patients supplemented omega-3 UFAs showed no significant

improvement in cognitive function nor delay in rate of decline (200-202). Many omega-3 UFAs clinical investigations focus primarily on cognitive effects of DHA, rather than a diet of EPA and DHA oil-rich fish thus do not directly address whether diet can be used to modulate AD progression (203).

Studies of nutrition's connection to dementia may be inherently compromised by a lack of full dietary information (194). Currently, reports are lacking with respect to long-term dietary regimens, and effectivity in mild, moderate, or severe stage of AD has not been assessed (203). Timing of nutritional intervention, analogous to pharmacological intervention, is critical for maximal efficacy (194). Animal models of AD offer controlled long-term assessment to address these questions (203). However, investigations of omega-3 supplementation on cognitive benefit in animal models, as with clinical trials, are confounded by varied reported dosage, mode of administration, treatment duration, treatment composition as single species or mixed lipids in natural substances, sex, age, background and number of subjects tested (194,204). Additionally, dietary intervention studies in mouse models often neglect assessment of individual sexes. As women have been reported to hold greater AD risk (148), this cannot be ommited.

To establish whether chronic dietary supplementation of male and female Tg and NonTg mice affects learning and memory performance in the MWM, mice were treated with 75 mg/kg/day omega-3 UFAs for four months before testing. This treatment regimen was designed to match an ongoing human pilot study treating human controls with the same supplements. Here, I show that omega-3

UFA supplementation did not benefit learning and memory performance of Tg mice or NonTg mice of either sex.

5.4 Results

Four-month omega-3 UFA supplementation did not significantly affect behavioural indices of learning and memory in NonTg mice

Omega-3 UFA treatment was administered for four months to male and female NonTg mice and behavioural indices of learning and memory were assessed at six months of age in the MWM. Learning and memory indices in MWM navigation task were analyzed (Table A1), by the following parameters of performance: (1) escape latency, (2) path efficacy, (3) search strategy, (4) number of crossings over the platform location during the probe trial, and (5) performance when the escape platform was reallocated by 180° (reversal).

Female NonTg mice receiving omega-3 UFA treatment for four months displayed no difference in escape latency values compared to untreated NonTg mice ($F_{(1,29)}$=1.694, Figure 5.1A). A significant main effect of the repeated measures test days was observed for females ($F_{(7,203)}$=11,37, $p<0.0001$) of both treatment groups, suggestive of learning by progressively decreased escape latency values. In MWM probe trial, the escape platform was removed and the amount of time mice spent crossing the former location was measured. Omega-3 treated NonTg mice performed comparable to untreated NonTg mice ($t_{(30)}$=0.1443, Figure 5.1B). No difference was observed between omega-3 treated NonTg and untreated NonTg females in ability to adapt to new task

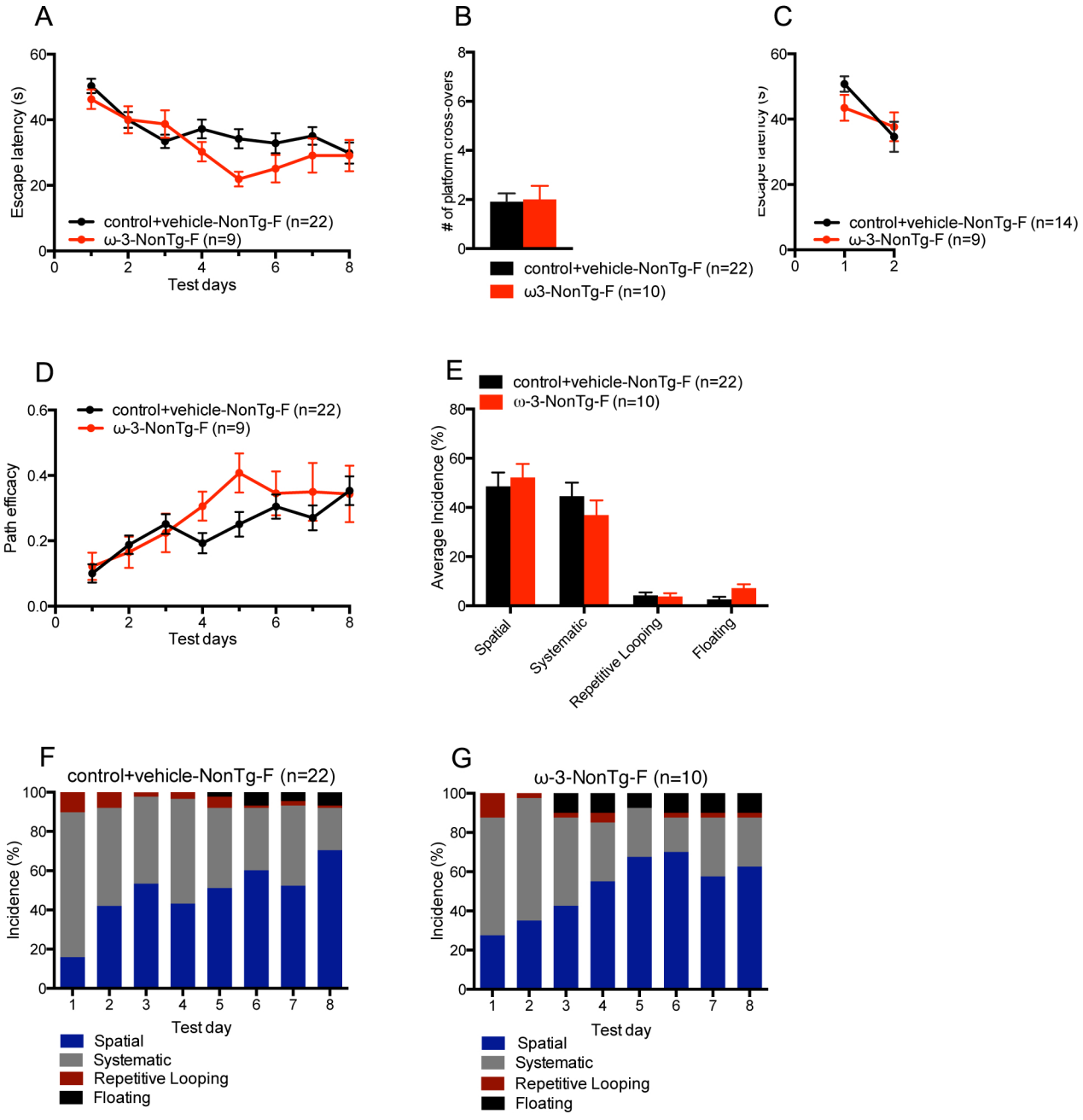
A
Escape latency (s)
Test days
control+vehicle-NonTg-F (n=22)
ω-3-NonTg-F (n=9)
B
# of platform cross-overs
control+vehicle-NonTg-F (n=22)
ω3-NonTg-F (n=10)
C
control+vehicle-NonTg-F (n=14)
ω-3-NonTg-F (n=9)
D
Path efficacy
Test days
control+vehicle-NonTg-F (n=22)
ω-3-NonTg-F (n=9)
E
Average Incidence (%)
control+vehicle-NonTg-F (n=22)
ω-3-NonTg-F (n=10)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-NonTg-F (n=22)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-3-NonTg-F (n=10)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 5.1.

Figure 5.1 MWM analysis of control compared to omega-3 treated female NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

requirements upon platform relocation of reversal test days ten and eleven ($F_{(1,21)}$=0.1942, Figure 5.1C). Omega-3 treated and untreated female NonTg mice also exhibited no difference in their ability to attain the platform by the most direct means, path efficacy ($F_{(1,29)}$=1.434, Figure 5.1D). An additional measure of cognitive performance was examining search behaviour a mouse employs to locate the escape platform via spatial cues, termed search strategy (Figure 5.1E). Types of search strategy used during MWM task were no different between omega-3 treated and untreated female NonTg mice ($F_{(1,56)}$=7.398e-007).

Likewise, omega-3 treated male NonTg mice displayed no difference in MWM performance compared to untreated NonTg mice in parameters of escape latency ($F_{(1,29)}$=0.6705, Figure 5.2A). A significant main effect of the repeated measures test days was observed ($F_{(7,203)}$=12.16, $p<0.0001$) indicating both progressively learned. No difference was observed in number of platform area cross-overs between omega-3 treated and untreated male NonTg mice ($t_{(30)}$= 0.8503, Figure 5.2B). Reversal learning escape latency values were not different between omega-3 treated and untreated male NonTg mice ($F_{(1,11)}$=0.03606, Figure 5.2C). Omega-3 treated male NonTg mice performed no differently than untreated controls in path efficacy ($F_{(1,29)}$= 0.2778) and search strategy ($F_{(1,56)}$=7.487e-015) measures (Figure A5.2D,E). Taken together, four-month supplementation with omega-3 UFAs did not induce improvement in learning and memory performance in the MWM of NonTg mice at six months of age.

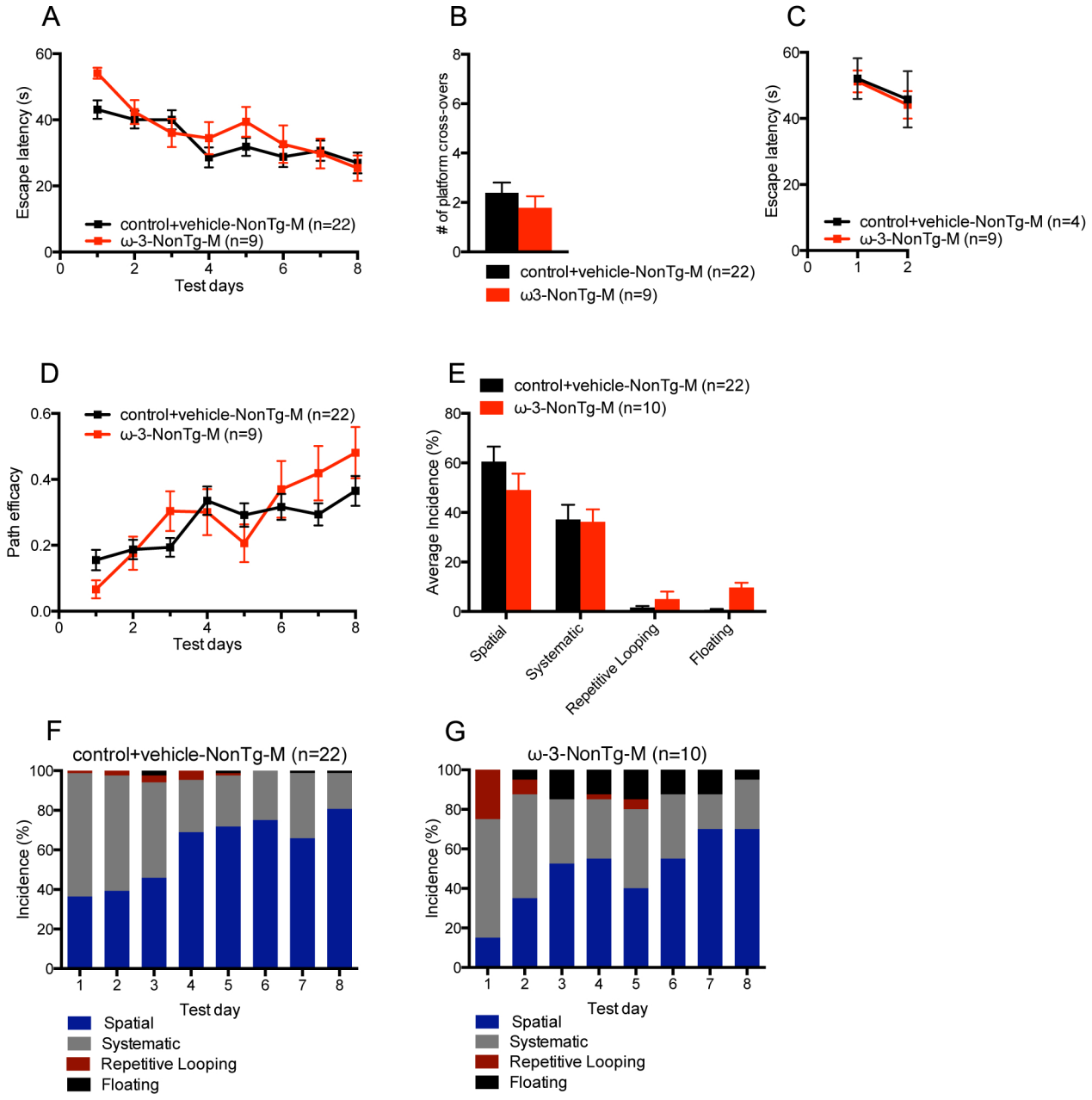
A
Escape latency (s)
Test days
control+vehicle-NonTg-M (n=22)
ω-3-NonTg-M (n=9)
B
# of platform cross-overs
control+vehicle-NonTg-M (n=22)
ω3-NonTg-M (n=9)
C
Escape latency (s)
control+vehicle-NonTg-M (n=4)
ω-3-NonTg-M (n=9)
D
Path efficacy
Test days
control+vehicle-NonTg-M (n=22)
ω-3-NonTg-M (n=9)
E
control+vehicle-NonTg-M (n=22)
ω-3-NonTg-M (n=10)
Average Incidence (%)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-NonTg-M (n=22)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-3-NonTg-M (n=10)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 5.2.

Figure 5.2 MWM analysis of control compared to omega-3 treated male NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

Four-month omega-3 UFA supplementation did not significantly affect behavioural indices of learning and memory in TgCRND8 mice at six months of age in the MWM

Male and female Tg mice were supplemented with omega-3 UFAs and behavioural indices of learning and memory were evaluated at six months of age in the MWM. As aforementioned, cognitive performance in the MWM was analyzed by measures of escape latency, path efficacy, search strategy, probe trial platform cross-overs, and reversal learning escape latency (Table A1). Female Tg mice receiving omega-3 supplementation did not perform differently than untreated Tg mice in escape latency values ($F_{(1,34)}$=1.729, Figure 5.3A). In MWM probe trial, no difference was observed in number of platform area cross-overs between omega-3 treated and untreated female Tg mice ($t_{(40)}$=0.7822, Figure 5.3B). In reversal learning task, performance was comparable between omega-3 treated and untreated female Tg mice ($F_{(1,28)}$=0.7367, Figure 5.3C). Omega-3 treated and untreated female NonTg mice exhibited no difference in path efficacy ($F_{(1,40)}$=1.973, Figure 5.3D). We further expanded our assessments to consider search strategy employed; Figure 5.3E represents assessment of overall search strategies, while Figure 5.3F and G indicate incidence per strategy per test day for control-Tg-F and omega-3-Tg-F groups respectively. Search strategies used were comparable between control and omega-3 treated female Tg mice ($F_{(1,56)}$=8.705e-007, Figure 5.3E).

Male Tg mice receiving omega-3 UFA supplementation performed comparable to control Tgs in escape latency values ($F_{(1,32)}$=4.075, Figure 5.4A),

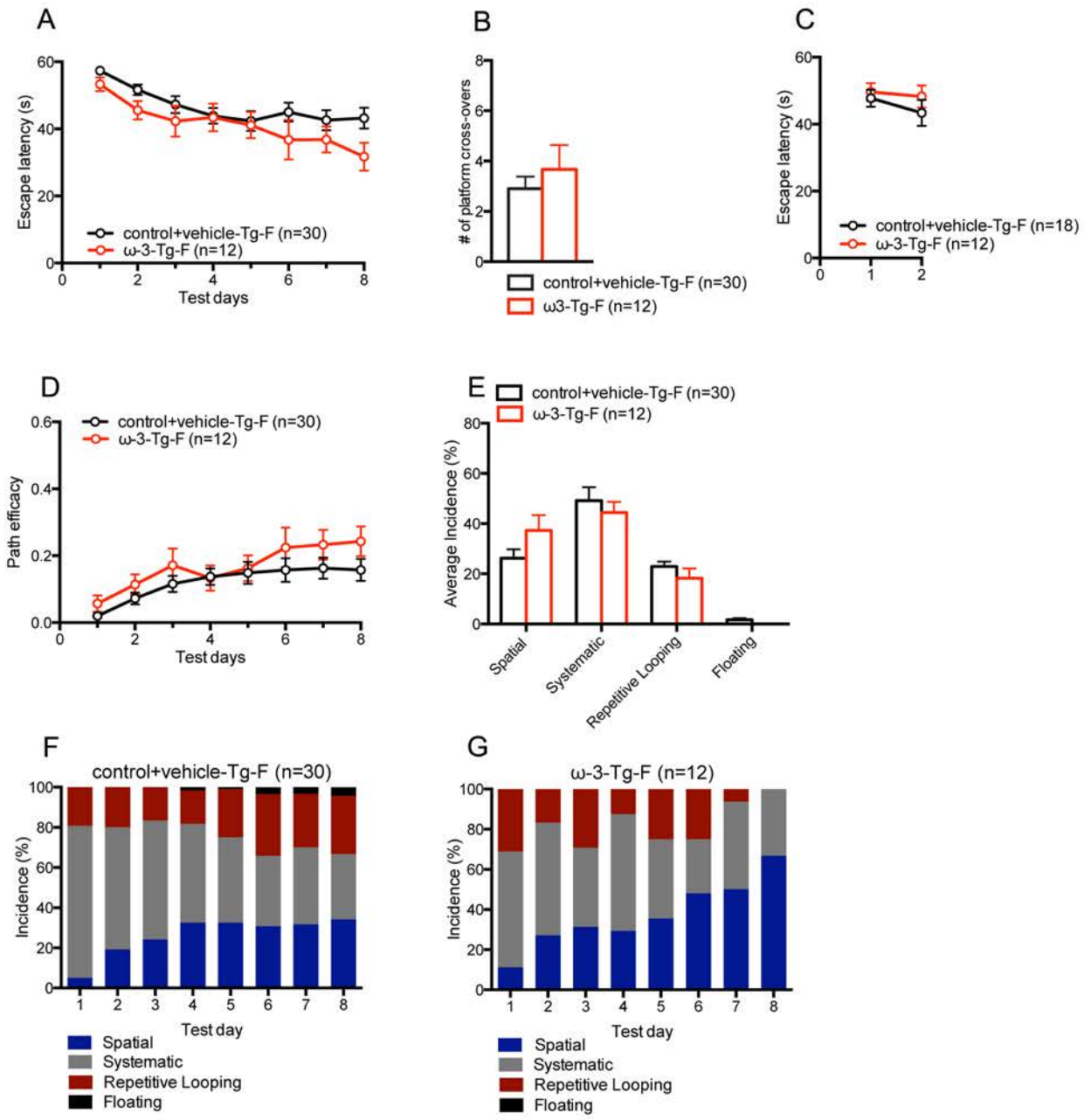
A
Escape latency (s)
Test days
control+vehicle-Tg-F (n=30)
ω-3-Tg-F (n=12)
B
# of platform cross-overs
control+vehicle-Tg-F (n=30)
ω3-Tg-F (n=12)
C
Escape latency (s)
control+vehicle-Tg-F (n=18)
ω-3-Tg-F (n=12)
D
Path efficacy
Test days
control+vehicle-Tg-F (n=30)
ω-3-Tg-F (n=12)
E
control+vehicle-Tg-F (n=30)
ω-3-Tg-F (n=12)
Average Incidence (%)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-Tg-F (n=30)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-3-Tg-F (n=12)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 5.3.

Figure 5.3 MWM analysis of control compared to omega-3 treated female Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

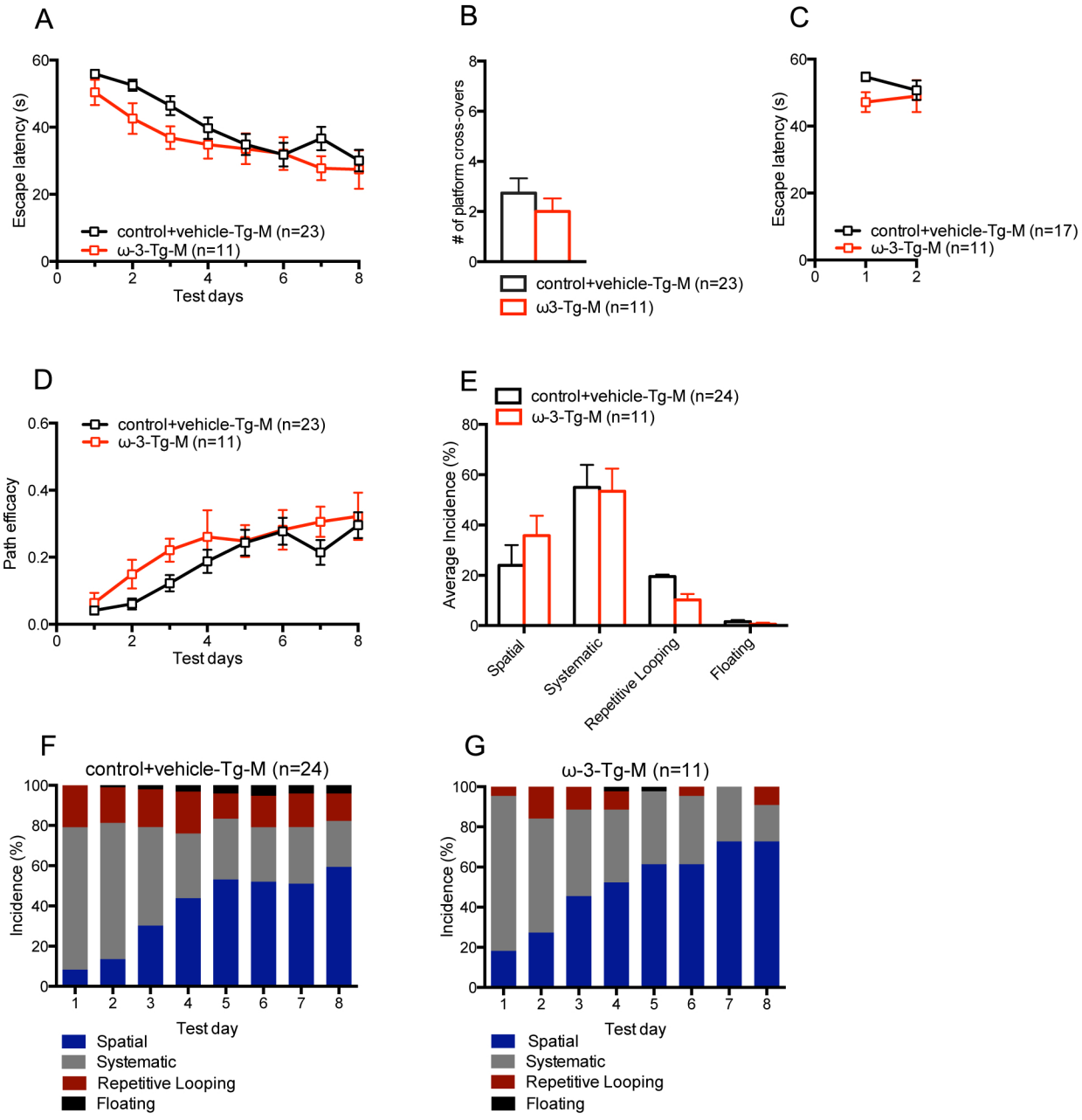
A
Escape latency (s)
Test days
control+vehicle-Tg-M (n=23)
ω-3-Tg-M (n=11)
B
# of platform cross-overs
control+vehicle-Tg-M (n=23)
ω3-Tg-M (n=11)
C
Escape latency (s)
control+vehicle-Tg-M (n=17)
ω-3-Tg-M (n=11)
D
Path efficacy
Test days
control+vehicle-Tg-M (n=23)
ω-3-Tg-M (n=11)
E
control+vehicle-Tg-M (n=24)
ω-3-Tg-M (n=11)
Average Incidence (%)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-Tg-M (n=24)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-3-Tg-M (n=11)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 5.4.

Figure 5.4 MWM analysis of control compared to omega-3 treated male Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

perhaps due to the robust progressive learning male Tg mice displayed in successive test days, as described in Chapter 3. No difference was observed between omega-3 treated and untreated male Tg mice in probe trial platform cross-overs ($t_{(32)}$=0.8016, Figure 5.4B), or reversal learning escape latency values ($F_{(1,26)}$=1.765, Figure 5.4C). Omega-3 UFA treated male Tg mice performed no different than untreated controls in parameters of path efficacy ($F_{(1,32)}$=1.761, Figure 5.4D), or search strategy ($F_{(1,56)}$=5.634e-014, Figure 5.4E). Assessment of search strategy per test days (Figure 5.4F,G) suggested robust learning may be a result of increased use spatial strategy. Collectively, these data indicate that an enriched diet, chronically supplemented with omega-3 UFAs did not significantly affect the phenoconversion of Tg mice.

Supplementation with omega-3 UFA does not alter motoric performance or anxiety levels among TgCRND8 and NonTg mice

Effect of four-month omega-3 UFA supplementation on non-cognitive behaviours such as motoric ability and anxiety levels were assessed during MWM (Table A1). Motor function was determined by MWM parameters of average distance moved and velocity swam. Firstly, effect of treatment was analyzed between female omega-3 treated and untreated Tgs, and omega-3 treated and untreated NonTgs by a two-way ANOVA. No significant main effect of treatment was observed among these groups with respect to average distance moved ($F_{(1,69)}$=0.1399, Figure 5.5A), or average velocity swam ($F_{(1,69)}$=2.752, Figure 5.5B). As described in Chapter 3, a significant main effect of genotype

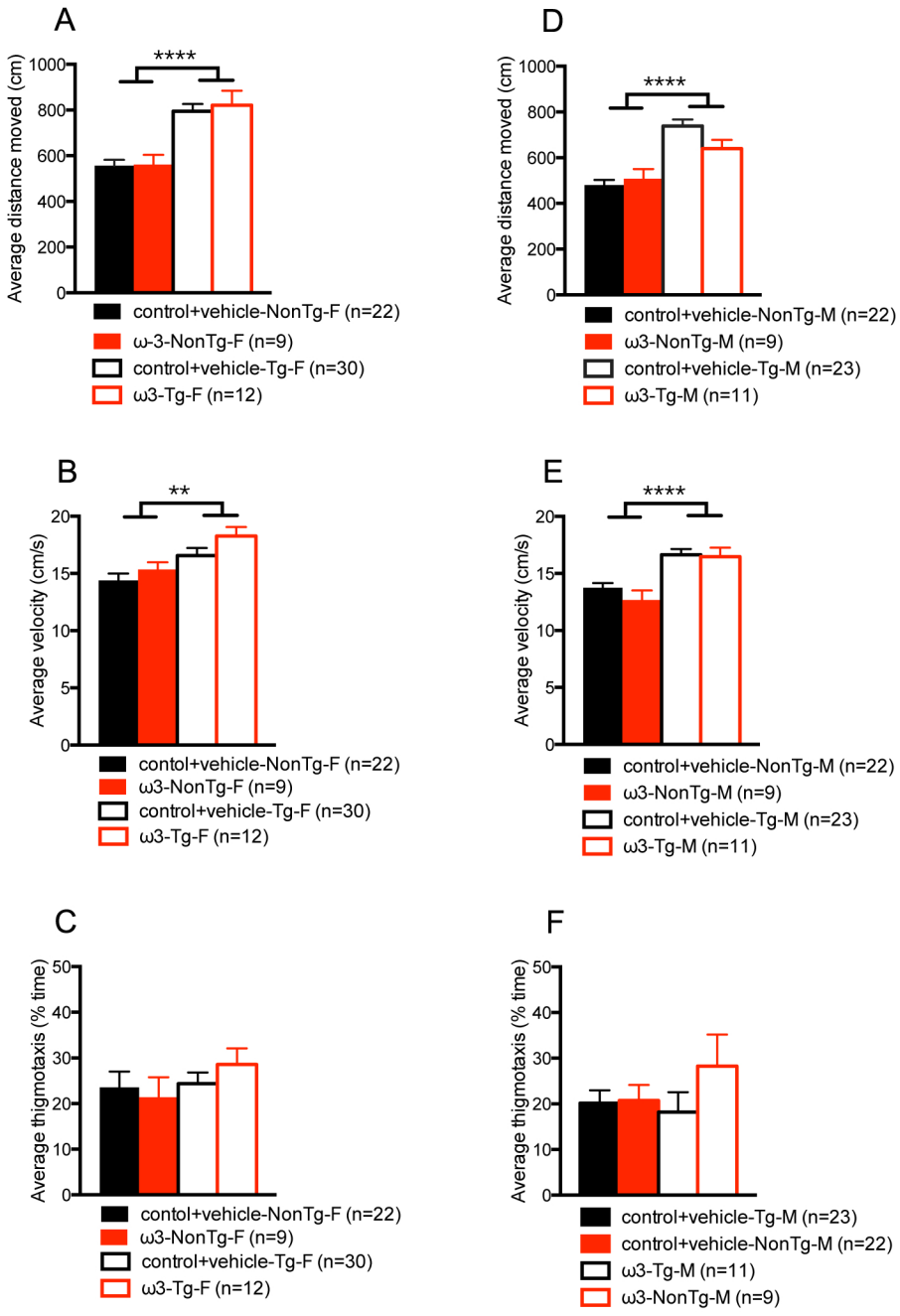
A
Average distance moved (cm)
1000
800
600
400
200
0
****
control+vehicle-NonTg-F (n=22)
ω-3-NonTg-F (n=9)
control+vehicle-Tg-F (n=30)
ω3-Tg-F (n=12)
D
Average distance moved (cm)
1000
800
600
400
200
0
****
control+vehicle-NonTg-M (n=22)
ω3-NonTg-M (n=9)
control+vehicle-Tg-M (n=23)
ω3-Tg-M (n=11)
B
Average velocity (cm/s)
20
15
10
5
0
**
contol+vehicle-NonTg-F (n=22)
ω3-NonTg-F (n=9)
control+vehicle-Tg-F (n=30)
ω3-Tg-F (n=12)
E
Average velocity (cm/s)
20
15
10
5
0
****
control+vehicle-NonTg-M (n=22)
ω3-NonTg-M (n=9)
control+vehicle-Tg-M (n=23)
ω3-Tg-M (n=11)
C
Average thigmotaxis (% time)
50
40
30
20
10
0
contol+vehicle-NonTg-F (n=22)
ω3-NonTg-F (n=9)
control+vehicle-Tg-F (n=30)
ω3-Tg-F (n=12)
F
Average thigmotaxis (% time)
50
40
30
20
10
0
control+vehicle-Tg-M (n=23)
control+vehicle-NonTg-M (n=22)
ω3-Tg-M (n=11)
ω3-NonTg-M (n=9)

Figure 5.5.

Figure 5.5 Analysis of effect of omega-3 treatment on non-cognitive indices in MWM for male and female Tg and NonTg mice.

During MWM testing the motoric function was assessed by average distance moved (140) (A, D) and average velocity (cm/s) (B,E), and anxiety levels during the task was determined by average time spent in thigmotaxis (%) (C,F).Female Tg and NonTg mice (159) and males (D-F) were analyzed separately. Statistical analysis of average distance moved, average velocity and average percent thigmotaxis were each analyzed by two-way ANOVA. **p<0.01; ****p<0.0001. Data shown are ±SEM.

was observed in motoric abilities; transgenic mice irrespective of diet, exhibited significantly increased distance moved ($F_{(1,69)}$=34.40, p<0.0001) and swim velocity ($F_{(1,69)}$=10.04, p=0.0023). Anxiety during MWM was assessed by thigmotactic behaviour; measuring amount of time spent swimming in periphery of MWM pool. No significant effect of treatment was observed in thigmotaxis behaviour of female omega-3 treated and control Tg mice, or omega-3 treated and control NonTg mice ($F_{(1,69)}$=0.08100, Figure 5.5C).

Secondly, male Tg and NonTg mice were assessed in non-cognitive behavioural indices of MWM. Effect of treatment was analyzed by two-way ANOVA between male omega-3 treated and untreated Tgs, and omega-3 treated and untreated NonTgs. No significant main effect of treatment was observed among these groups with respect to average distance moved ($F_{(1,61)}$=1.111, Figure 5.5D), or average velocity swam ($F_{(1,69)}$=0.9750, Figure 5.5E). A significant main effect of genotype was observed in motoric abilities; transgenic mice displayed significantly increased average distance moved ($F_{(1,61)}$=33.44, p<0.0001) and swim velocity ($F_{(1,61)}$=28.79, p<0.0001). No significant effect of treatment was observed in thigmotaxis behaviour between male omega-3 treated and control Tg mice, or omega-3 treated and control NonTg mice ($F_{(1,61)}$=0.4355, Figure 5.5F). Taken together, four-month omega-3 UFA treatment did not alter motoric ability or anxiety levels in the MWM of Tg and NonTg mice, with respect to either sex.

5.5 Discussion

The experiments presented in this chapter aimed to determine effect of a long-term enriched treatment of omega-3 UFAs on learning and memory performance in the MWM in male and female Tg and NonTg mice. Spatial learning and memory was evaluated by escape latency, path efficacy and search strategy measures of MWM navigation performance. Our data revealed, that a four-month omega-3 UFA supplementation did not alter learning and memory, anxiety, or motoric performance of either male or female Tg or NonTg mice. Collectively, these data indicate that an enriched diet of omega-3 UFAs does not prevent or reduce cognitive decline in this AD model.

Although AD patients have been reported deficient in omega-3 UFAs (98,112,198), and some clinical studies report cognitive benefit of omega-3 treatment in mild dementia (187,192), none demonstrate significant cognitive improvements in AD (200-202,205). Our data are consistent with this lack of effect despite chronic administration. As in this study, Arendash and colleagues reported a 5.5 month omega-3 UFA supplementation did not improve cognitive performance of $APP_{K670N,M671L}$+PS1 transgenic mice in six cognitive-based tasks (Y-maze, Morris water maze, circular platform, platform recognition, and radial arm water maze), and did not prove any cognitive benefit to normal NonTg mice (206). High omega-3 UFA diet did not induce changes in soluble or insoluble hippocampal Aβ in transgenic mice (206). Additionally, omega-3 UFA did not affect sensorimortor-based and anxiety-based performance (measured by open field activity, balance beam, string agility and elevated plus maze) (206). Our

results contrast the findings of a recent meta-analysis focusing on treatment periods >10% of total life span that suggest long-term omega-3 UFA treatment may reduce Aβ levels in experimental models of AD (203). Subgroup analyses, however, showed that treatment effect on cognitive function was greater in rats than in mice, although a sex difference was detected with males exhibited greater reductions in Aβ levels compared to females (203). Interestingly, decreases in Aβ levels associated with supplementation failed to improve spatial learning in the MWM consistent with this study (207). Calon and colleagues reported in 2004 and 2005, that dietary reduction of omega-3 UFAs in Tg2576 mice led to synaptic protein loss and behavioural deficits but that these effects were ameliorated by treatment with omega-3 UFAs (208,209). However these effects are result of prior depletion. As described (194), an inherent difficulty of clinical trials is varied nutritional status among participants prior to start of the test. For example, individuals selected with insufficient folate nutrition were randomized to receive folic acid or placebo supplement for three years, and treatment revealed slower cognitive decline, but no benefit occurred in individuals that already consumed recommended daily allowances (194,210,211). Taken together, the findings presented in this chapter clearly indicate that chronic dietary supplementation of omega-3 UFAs has no effect on learning and memory in the TgCRND8 mouse model or NonTg littermates of either sex.

This study also examined impairment in Tg mice by search behaviour during MWM. As described (52,116), analysis of search strategies extends characterization of cognitive abilities of mice as they attempt to locate the escape

platform via spatial cues. Mice often display a switch in strategies; each subsequent day should theoretically show a concomitant increase in use of effective search strategies (spatial) and decrease in use of less effective search strategies (systematic and repetitive looping) (52,116). Similar to work shown by Janus, control NonTg mice increased use of spatial strategy in MWM, indicating greater learning potential and focused search in the correct quadrant (52). Similar to reported F1 Tg impairment (49,52), Tg impairment displayed of females was characterized by significantly decreased use of effective spatial strategies and predominant use of less effective systematic and repetitive looping strategies. Male Tgs displayed surprisingly comparable search behaviour to NonTg control mice in the last four test days, this increased use of spatial search suggested established memory for the hidden platform. We conclude that female Tg mice exhibit severe learning and memory deficits due to a failure to predominantly adopt spatial strategies. Inability to adopt spatial strategy may be owing to high repetitive looping phenotype throughout test days and an inability to transition from systematic to spatial strategies, thus underlying the observed cognitive deficits. Tg females, as compared to Tg males, are more severely impaired because of failure to develop effective spatial strategy. Collectively, two outcomes were identified in this chapter: (1) AD-like pathology disrupts spatial strategies of TgCRND8 mice with greater extent in females, (2) cognitive performances were unaltered by chronic omega-3 UFA supplementation.

Chapter 6 – Assessing effect of four-month omega-6/-9 UFA supplementation on learning and memory performance in TgCRND8 and NonTg mice at six months of age

6.1 Objective of this study

The experiments presented in this chapter assess the effects of a four month omega-6/omega-9 UFA supplementation on learning and memory performance in male and female Tg and NonTg mice at six months of age. Additionally, effect of omega-6/-9 UFA treatment on non-cognitive behaviours during MWM was examined as not to confound cognitive indices.

6.2 Statement of author contributions

Thank you to Dr Diane Lagace and Mirela Hasu for access to the University of Ottawa Behavioural Core Facility. Dr Hongbin Xu and Dr Carolina Cieniak assisted me in study design and analysis.

6.3 Introduction

AD patients' plasma and brain display deficiencies in omega-6/-9 UFAs perhaps due to altered metabolism or nutrition (97). For instance, compared with healthy controls, MCI and AD patients plasma display lower levels of LA (18:2n-6) (97,98) with progressive decline of omega-6 UFA correlated with severity of disease (97). LA is an essential FA that cannot be synthesized by humans thus suggesting an impoverished diet or impaired uptake (97). These findings also

suggest that LA deficiency starts early in AD perhaps with important roles in pathology (97). Additionally, compared to healthy controls, MCI and AD patients' plasma fatty acid profiles were also lower in the MUFA, OA (18:1n-9) (98). Incorporation into membrane phospholipids, such as phosphatidylethanolamine, phosphatidylinositol and phosphatidylcholine is also impaired (99,100). AD patients hippocampus exhibit significantly decreased PE-derived and PI-derived incorporated OA (99). Guan and colleagues also reported decreased OA PE plasmalogens (100). If these dietary deficiencies are linked to AD, perhaps symptoms may be prevented or delayed by nutritional interventions that naturally remodel brain lipid content and confer resistance to pathogenesis (114). At present, AD therapies are focused symptomatic amelioration rather than a targeted disease-modifying or preventative treatment approach.

In the present chapter, male and female Tg and NonTg mice were orally administered enhanced diet of omega-6/-9 UFAs for four months, and submitted to varied MWM tasks at six months of age to establish effect on learning and memory competency. As with our omega-3 study, animals were not depleted of omega-6/-9 fatty acid prior to supplementation. Here, I show that performance in the MWM of female, but not male, Tg mice receiving omega-6/-9 UFA supplement was improved compared to untreated mice. These results provide preliminary evidence to indicate that dietary interventions to increase omega-6/-9 UFAs may be an important novel tool to delay onset or slow progression of AD in females.

6.4 Results

Four-month omega-6/-9 UFA supplementation did not significantly affect behavioural indices of learning and memory in NonTg mice

Omega-6/-9 UFA treatment was supplemented for four months to female and male NonTg mice, and effect on learning and memory was assessed at six months of age in the MWM. Cognitive performance in the MWM was analyzed by parameters of escape latency, path efficacy, search strategy, probe trial platform cross-overs, and reversal learning escape latency (Table A1). Treatment and testing of female and male mice occurred in concert, each sex was analyzed separately. Female NonTg mice receiving omega-6/-9 supplementation did not perform differently than untreated NonTg mice in escape latency ($F_{(1,30)}$=0.01027, Figure 6.1A). A significant main effect of the repeated measures test days was observed ($F_{(7,210)}$=7.471, $p<0.0001$) indicating, irrespective of treatment, both groups exhibited robust learning. During MWM probe trial, both groups spent comparable time crossing the platform area ($t_{(30)}$=0.1499, Figure 6.1B). In reversal learning task, omega-6/-9 treated and untreated female NonTg mice performed similarly ($F_{(1,22)}$=0.1548, Figure 6.1C) and both groups exhibiting progressively decreased escape latency values over successive test days ($F_{(1,22)}$=10.52, $p=0.0037$). Omega-6/-9 treated compared to control, also displayed no difference in ability to attain the platform by the most direct means ($F_{(1,30)}$=0.01847, Figure 6.1D). Types of search strategy used during MWM task to effectively locate the escape platform was no different between omega-6/-9 treated and control female NonTg mice ($F_{(1,56)}$=8.254e-007, Figure 6.1E).

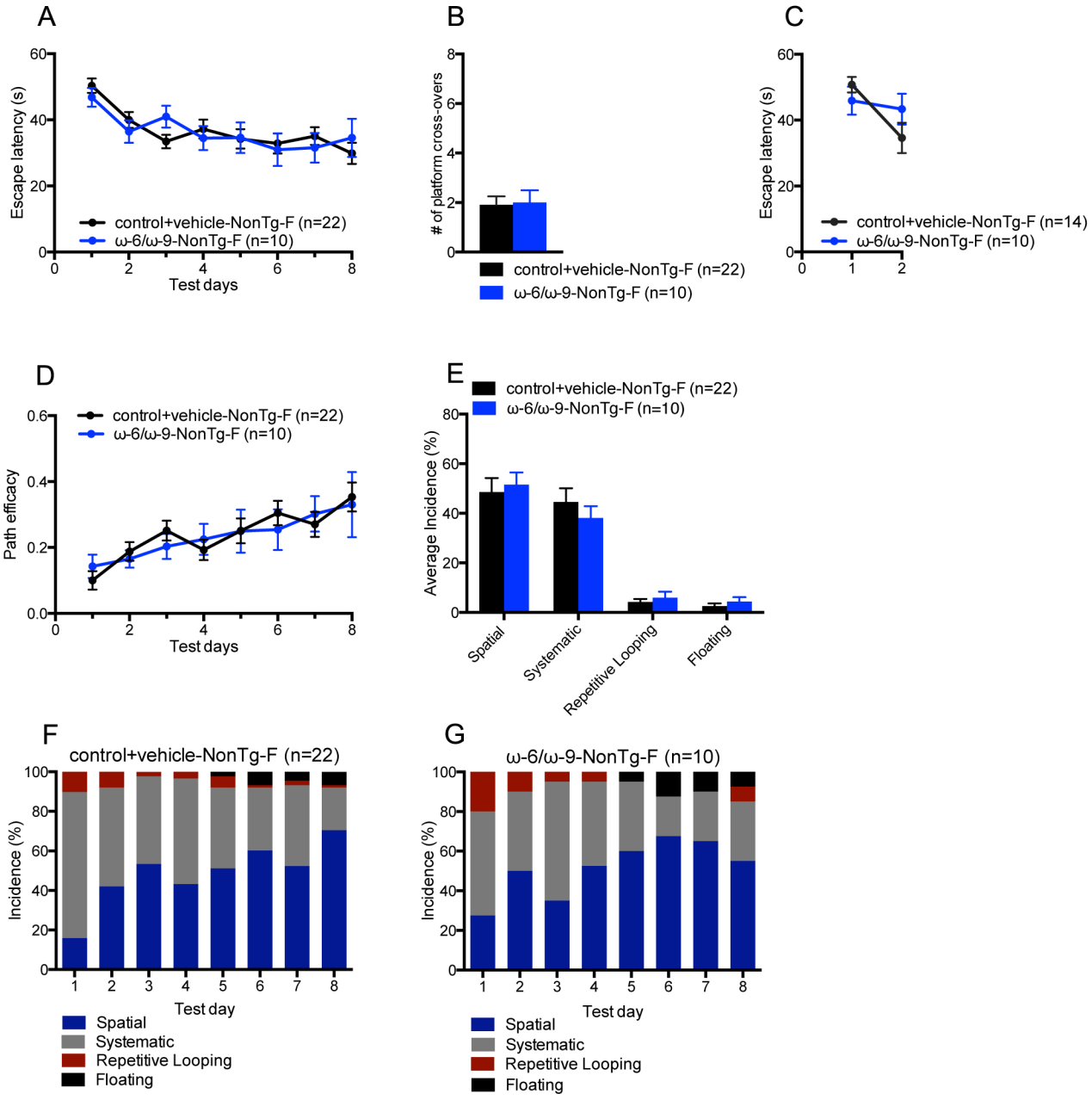
A
Escape latency (s)
Test days
control+vehicle-NonTg-F (n=22)
ω-6/ω-9-NonTg-F (n=10)
B
# of platform cross-overs
control+vehicle-NonTg-F (n=22)
ω-6/ω-9-NonTg-F (n=10)
C
Escape latency (s)
control+vehicle-NonTg-F (n=14)
ω-6/ω-9-NonTg-F (n=10)
D
Path efficacy
Test days
control+vehicle-NonTg-F (n=22)
ω-6/ω-9-NonTg-F (n=10)
E
Average Incidence (%)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-NonTg-F (n=22)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-6/ω-9-NonTg-F (n=10)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 6.1.

Figure 6.1 MWM analysis of control compared to omega-6/-9 treated female NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

Akin to cognitive performance displayed by female NonTg mice, male NonTg mice treated with omega-6/-9 UFAs did not display significant different cognitive function compared control (Figure 6.2). No significant effect of treatment was observed between omega-6/-9 treated male NonTg mice and control male NonTg mice in escape latency values ($F_{(1,31)}$=1.022, Figure 6.2A). Irrespective of treatment, both groups displayed learning by significant progressive decreased escape latency values over subsequent test days ($F_{(7,217)}$=12.08, p<0.0001). No difference was observed between omega-6/-9 treated and untreated male NonTg mice in probe trial platform cross-overs ($t_{(32)}$=0.2913, Figure 6.2B), or reversal learning escape latency values ($F_{(1,13)}$=0.5377, Figure 6.2C). Irrespective of treatment, both groups displayed significant effect of repeated measures test days ($F_{(1,13)}$=5.464, p=0.0361). Four-month omega-6/-9 UFA treatment given to male NonTg mice did not alter spatial navigation capacities compared to control untreated NonTg mice, as demonstrated by parameters of path efficacy ($F_{(1,31)}$=0.1189, Figure 6.2D) and search strategy ($F_{(1,56)}$=6.335e-014, Figure 6.2E). These behavioural indices of cognition in the MWM indicated that when both male and female NonTg mice were treated with omega-6/-9 UFA for four months, performance was unaltered compared to untreated NonTg mice. Considering results described in Chapter 5, this collectively demonstrates that a four month supplementation of omega-3,-6,-9 UFAs had no influence on learning and memory performance of NonTg mice in the MWM.

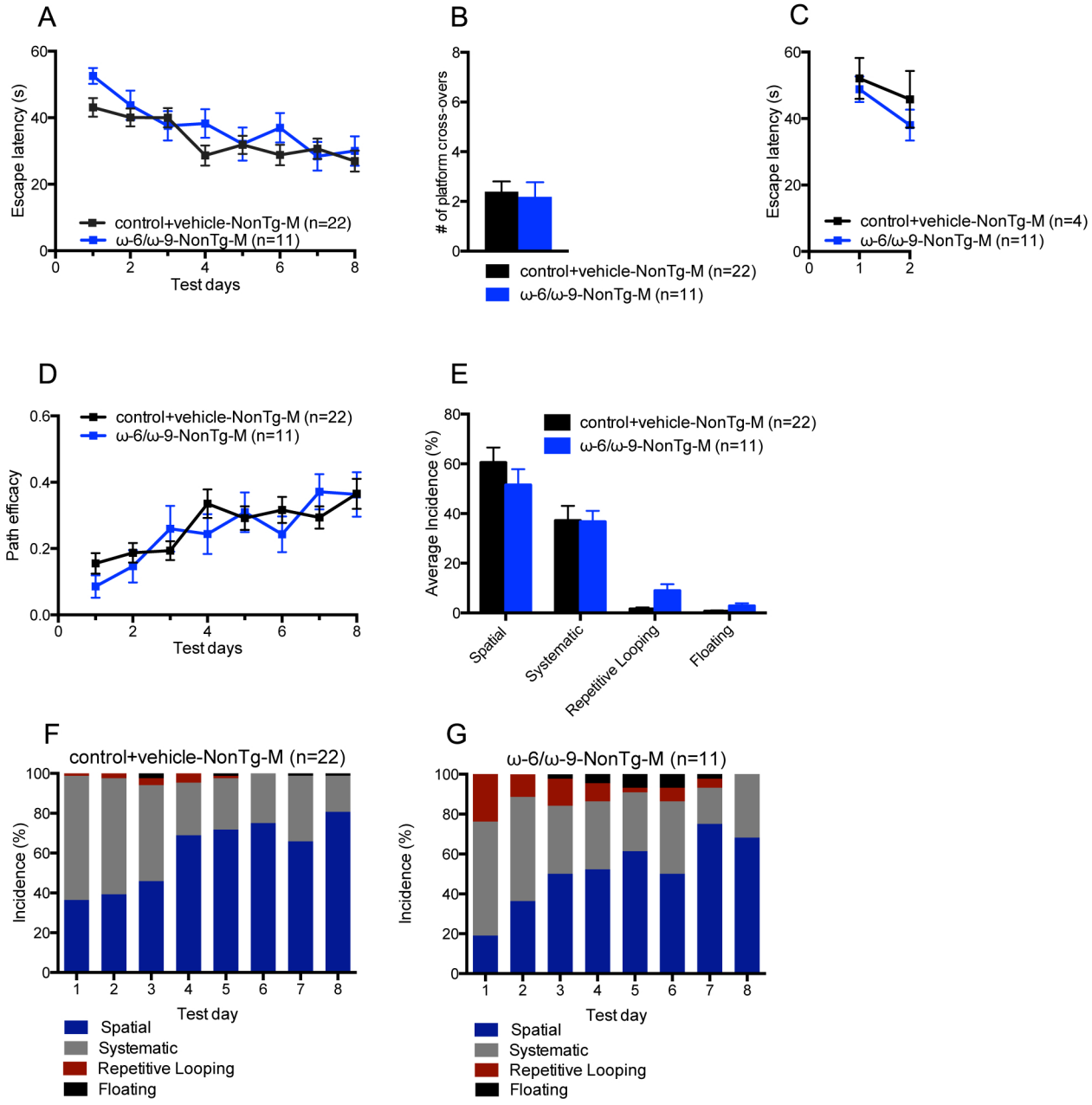
A
Escape latency (s)
Test days
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
B
# of platform cross-overs
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
C
Escape latency (s)
control+vehicle-NonTg-M (n=4)
ω-6/ω-9-NonTg-M (n=11)
D
Path efficacy
Test days
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
E
Average Incidence (%)
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-NonTg-M (n=22)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-6/ω-9-NonTg-M (n=11)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 6.2.

Figure 6.2 MWM analysis of control compared to omega-6/-9 treated male NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

Female TgCRND8 mice receiving omega-6/-9 UFA supplementation display improved performance in MWM

Female Tg mice were given omega-6/-9 UFA treatment for four months and behavioural indices of learning and memory were evaluated at six months of age in the MWM. These indices of cognition during MWM were escape latency, path efficacy, search strategy, probe trial platform cross-overs, and reversal learning escape latency (Table A1). Remarkably, omega-6/-9 treated female Tg mice displayed significantly decreased escape latency performance compared to untreated Tg mice (Figure 6.3A). When analyzed with two-way repeated measures ANOVA, a main effect of diet was observed ($F_{(1,38)}$=4.346,p=0.0439), indicating omega-6/-9 improved escape latency performance, particularly during test days four-eight, and causing treated Tg mice to exhibit less cognitive impairment than untreated Tg littermates. A *post hoc* Holm-Sidak's test demonstrated a significant difference between treated compared to untreated Tg mice on test day eight (p=0.0083). This effect was further mirrored in else described search strategy analysis.

No difference was observed between omega-6/-9 treated and untreated female Tg mice in probe trial platform cross-overs ($t_{(38)}$=0.6582, Figure 6.3B), or reversal learning escape latency values ($F_{(2,39)}$=0.2220, Figure 6.3C). Omega-6/-9 treated compared to untreated female Tg mice exhibited more effective capacity to acquire the platform by direct path, although this measure did not reach statistical significance when analyzed by a two-way repeated measures ANOVA ($F_{(1,38)}$=2.852, p=0.0994, Figure 6.3D). Although the measure of path

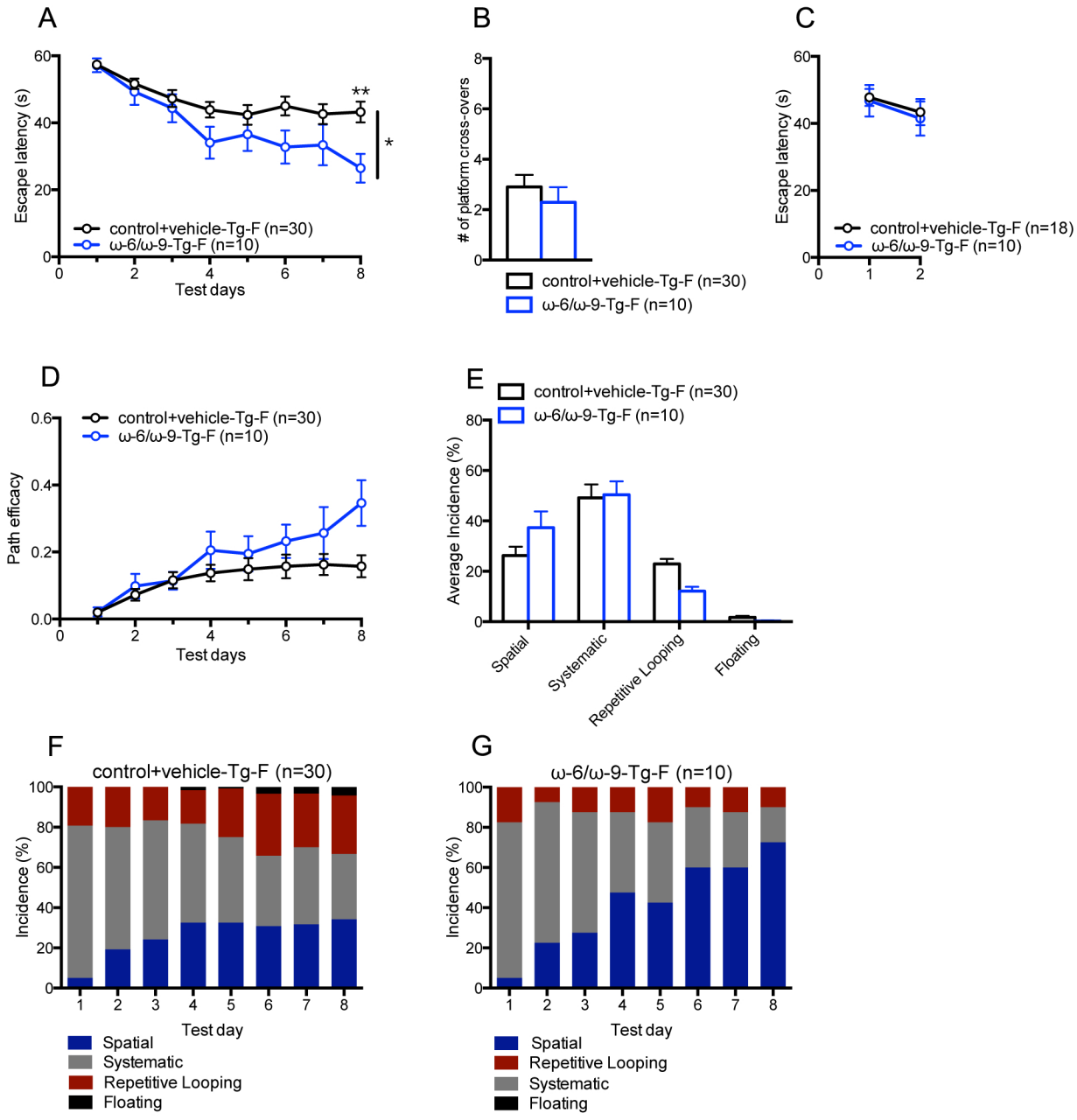
A
Escape latency (s)
Test days
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
**
*
B
# of platform cross-overs
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
C
Escape latency (s)
control+vehicle-Tg-F (n=18)
ω-6/ω-9-Tg-F (n=10)
D
Path efficacy
Test days
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
E
Average Incidence (%)
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-Tg-F (n=30)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-6/ω-9-Tg-F (n=10)
Incidence (%)
Test day
Spatial
Repetitive Looping
Systematic
Floating

Figure 6.3.

Figure 6.3 MWM analysis of control compared to omega-6/-9 treated female Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM. *$p<0.05$; **$p<0.01$.

efficacy as cognitive performance precludes factors of time or swim velocity during a trial, it may be too stringent, and near implausible for a mouse to exactly follow a straight line constant swim path to the escape platform (perfect score of 1). Reported search behaviours mice adopt during MWM task to locate the platform can be characterized to determine effective search, and offer insight of impaired or improved phenotypes displayed by escape latency. Search strategies employed by omega-6/-9 treated and untreated Tg female mice were comparable ($F_{(1,56)}$=3.381e-006, Figure 6.3E), when expressed as types of strategies used over averaged eight test days. Upon examination of percent incidence of each strategy per test day (Figure 6.3F,G), omega-6/-9 UFA treated mice exhibited more effective search strategy in the last four days of the test period as compared to control. Groups compared by a two-way ANOVA with *post hoc* of Holm-Sidak's for test days four-eight revealed that treated Tg mice adopted significantly greater spatial strategy ($p<0.0001$) and significantly less repetitive looping strategy ($p<0.0001$) than untreated. This phenotypic divergence occurring on test day four between omega-6/-9 UFA treated and untreated control Tg females, as shown in escape latency scores, may be consequent to a shift in search strategies. Control Tg females remained unchanged throughout test days, predominantly relying on systematic or repetitive looping strategies of search, whereas omega-6/-9 treated mice substantially incrementally increase use of more effective spatial strategy. Taken together, these cognitive parameters of performance suggested that female Tg mice, when treated with omega-6/-9 for four months, displayed no learning and memory impairment in the

MWM. A diet enriched in omega-6/-9 UFA could delay the transition from a presymptomatic to a symptomatic phenotype. These data indicate that dietary intervention holds neuroprotective potential in an aggressive pathogenic mouse model and that omega-6/-9 UFAs could be a novel adjuvant therapy in AD.

Male TgCRND8 mice receiving omega-6/-9 UFA supplementation do not display significant improvement in learning and memory

Male Tg mice were given omega-6/-9 UFA treatment for four months and cognitive behaviour was evaluated at six months of age in the MWM by parameters of escape latency, path efficacy, search strategy, probe trial platform cross-overs, and reversal learning escape latency (Table A1). As described in Chapter 3, control male Tg mice at six months of age displayed mild impairment compared to NonTg, but less severe impairment than female Tg littermates, thus further amelioration in cognitive performance may be minimal. Male Tg mice treated with omega-6/-9 supplement did not perform differently than untreated Tg mice in escape latency parameter ($F_{(1,34)}$=0.3856, Figure 6.4A), suggesting omega-6/-9 treatment did not alter cognitive function. Irrespective of treatment, both groups displayed progressively decreased escape latency values over successive test days ($F_{(7,238)}$=20.26, $p<0.0001$), and attained learning behaviour to the extent of NonTgs. In MWM probe trial, no difference was observed in number of platform area cross-overs between omega-6/-9 treated and untreated male Tg mice ($t_{(34)}$=0.3932, Figure 6.4B). When reversal learning test

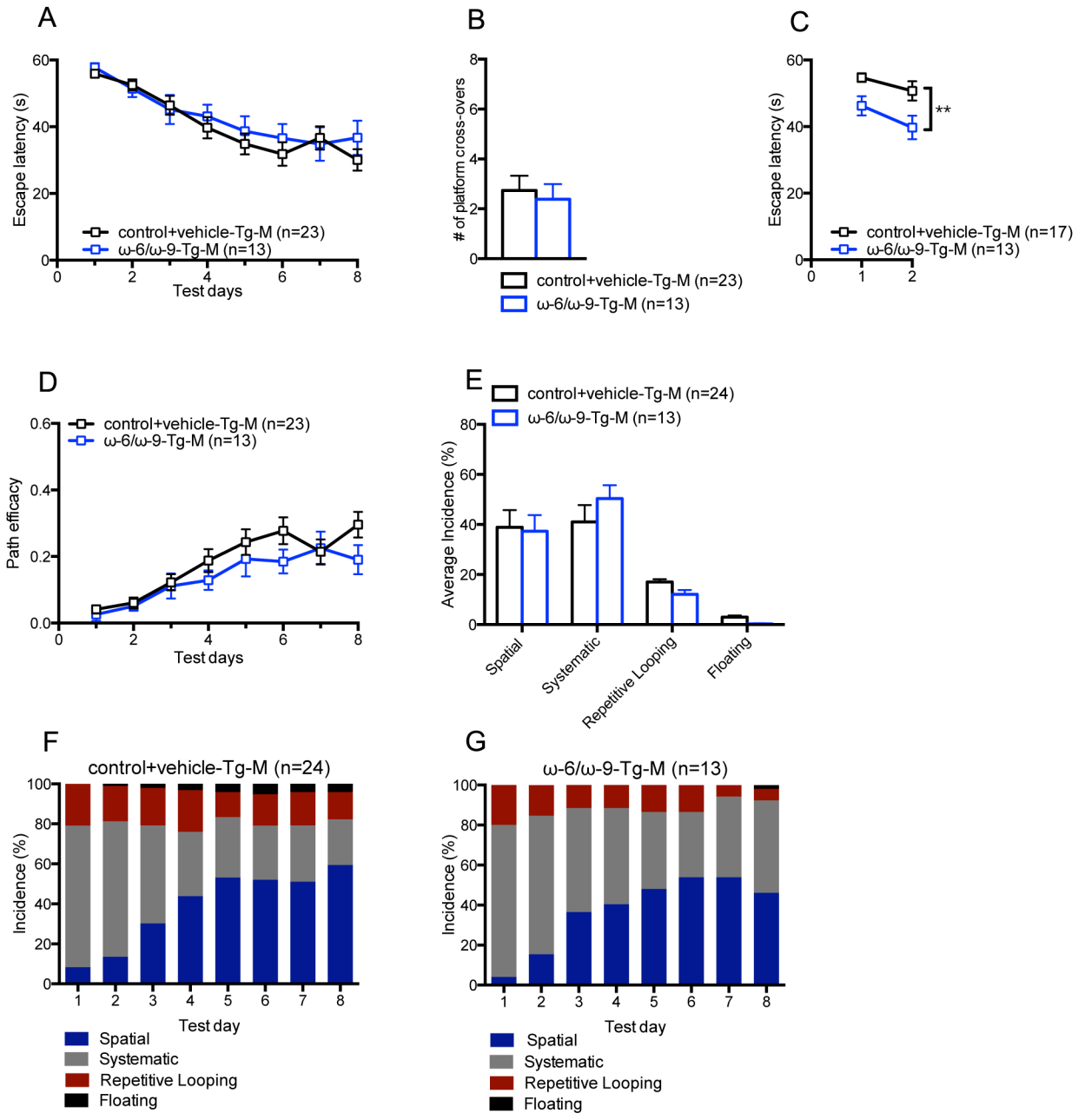
A
Escape latency (s)
Test days
control+vehicle-Tg-M (n=23)
ω-6/ω-9-Tg-M (n=13)
B
# of platform cross-overs
control+vehicle-Tg-M (n=23)
ω-6/ω-9-Tg-M (n=13)
C
Escape latency (s)
**
control+vehicle-Tg-M (n=17)
ω-6/ω-9-Tg-M (n=13)
D
Path efficacy
Test days
control+vehicle-Tg-M (n=23)
ω-6/ω-9-Tg-M (n=13)
E
control+vehicle-Tg-M (n=24)
ω-6/ω-9-Tg-M (n=13)
Average Incidence (%)
Spatial
Systematic
Repetitive Looping
Floating
F
control+vehicle-Tg-M (n=24)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating
G
ω-6/ω-9-Tg-M (n=13)
Incidence (%)
Test day
Spatial
Systematic
Repetitive Looping
Floating

Figure 6.4.

Figure 6.4 MWM analysis of control compared to omega-6/-9 treated male Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), path efficacy (D), search strategy (197). Percent incidence of search strategies per test days was shown with respect to control (F) and treated (G) groups. Statistical analysis of (A) and (C-E) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM. **$p<0.01$.

performance was analyzed by two-way repeated measures ANOVA, a significant main effect of the diet was observed ($F_{(1,28)}$=11.69, p=0.0019, Figure 6.4C) and further *post hoc* Holm-Sidak's test revealed significance on test day ten (p=0.300) and eleven (p=0.0108), suggesting omega-6/-9 treated Tg males were more able than control Tg males to adapt to new task requirements upon platform relocation.

The above described result of omega-6/-9 UFA treatment on male Tg mice as compared to control Tg mice with respect to escape latency performance, was mimicked in spatial navigation capacities, no significant difference was observed between groups in path efficacy ($F_{(1,34)}$=1.743, Figure 6.4D) or search strategy ($F_{(1,56)}$=5.986e-007, Figure 6.4E) parameters. Taken together; male Tg mice, unlike females, displayed little cognitive impairment at six months of age and supplement of omega-6/-9 UFA for four months did not further ameliorate overall performance.

Supplementation with omega-6/-9 UFA does not alter motoric performance or anxiety levels among TgCRND8 and NonTg mice

Effect of four-month omega-6/-9 UFA supplementation on non-cognitive behaviours such as motoric ability and anxiety levels were assessed during MWM (Table A1). Firstly, effect of treatment was analyzed between female omega-6/-9 treated and untreated Tgs, and omega-6/-9 treated and untreated NonTgs by a two-way ANOVA. Omega-6/-9 treatment did not affect motoric function, as no significant main effect of treatment was observed among these

groups with respect to average distance moved ($F_{(1,68)}$=0.01532, Figure 6.5A), or average velocity swam ($F_{(1,68)}$=2.121, Figure 6.5B). A significant main effect of genotype was observed in motoric abilities; transgenic mice irrespective of diet, exhibited significantly increased distance moved ($F_{(1,68)}$=25.97, p<0.0001) and swim velocity ($F_{(1,68)}$=10.03, p=0.0023). Anxiety during the MWM was assessed by thigmotactic behaviour, a measure of amount of time spent swimming in pool periphery. No significant effect of treatment was observed in thigmotaxis behaviour of female omega-6/-9 treated and control Tg mice, or omega-6/-9 treated and control NonTg mice ($F_{(1,68)}$=0.0004316, p=0.9835, Figure 6.5C).

Secondly, effect of treatment on non-cognitive behavioural indices during MWM was assessed between male omega-6/-9 treated and untreated Tgs, and omega-6/-9 treated and untreated NonTgs by two-way ANOVA. No significant main effect of treatment was observed among these groups with respect to average distance moved ($F_{(1,65)}$=1.948, Figure 6.5D), or average velocity swam ($F_{(1,65)}$=0.02519, Figure 6.5E). A significant main effect of genotype was observed in motoric function; transgenic mice displayed significantly increased distance moved ($F_{(1,65)}$=55.49, p<0.0001), and swim velocity ($F_{(1,65)}$=31.01, p<0.0001) than nontransgenic mice. No significant effect of treatment was observed in thigmotaxis behaviour between male omega-6/9 treated and control Tg mice, or omega-6/-9 treated and control NonTg mice ($F_{(1,65)}$=3.203, Figure 6.5F). Taken together, four-month omega-6/-9 UFA treated Tg and NonTg mice did not alter motoric function or anxiety levels in the MWM task, as compared to untreated mice with respect to either sex.

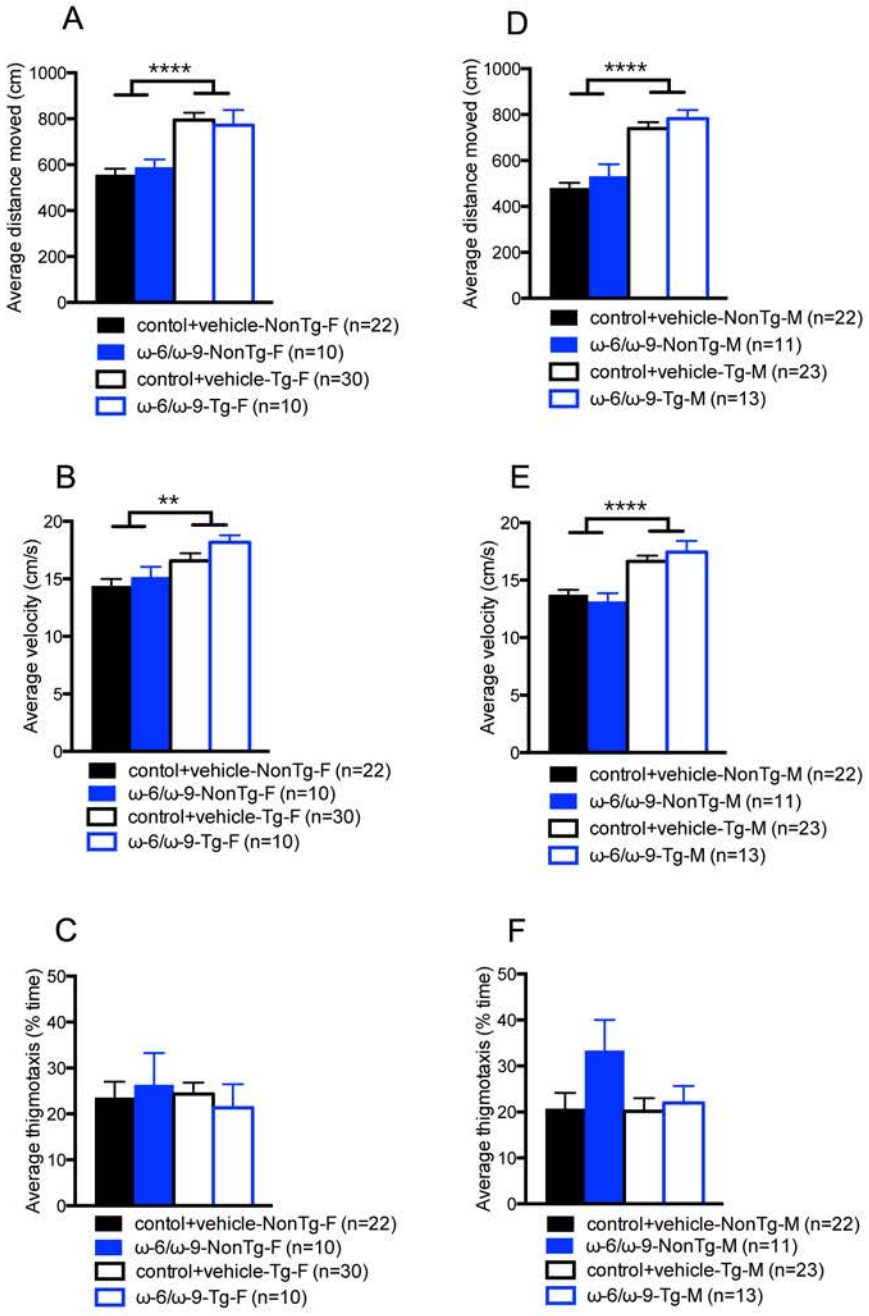
A
Average distance moved (cm)
1000
800
600
400
200
0
****
contol+vehicle-NonTg-F (n=22)
ω-6/ω-9-NonTg-F (n=10)
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
D
Average distance moved (cm)
1000
800
600
400
200
0
****
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
control+vehicle-Tg-M (n=23)
ω-6/ω-9-Tg-M (n=13)
B
Average velocity (cm/s)
20
15
10
5
0
**
contol+vehicle-NonTg-F (n=22)
ω-6/ω-9-NonTg-F (n=10)
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
E
Average velocity (cm/s)
20
15
10
5
0
****
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
control+vehicle-Tg-M (n=23)
ω-6/ω-9-Tg-M (n=13)
C
Average thigmotaxis (% time)
50
40
30
20
10
0
contol+vehicle-NonTg-F (n=22)
ω-6/ω-9-NonTg-F (n=10)
control+vehicle-Tg-F (n=30)
ω-6/ω-9-Tg-F (n=10)
F
Average thigmotaxis (% time)
50
40
30
20
10
0
control+vehicle-NonTg-M (n=22)
ω-6/ω-9-NonTg-M (n=11)
control+vehicle-Tg-M (n=23)
ω-6/ω-9-Tg-M (n=13)

Figure 6.5.

Figure 6.5 Analysis of effect of omega-6/-9 treatment on non-cognitive indices in MWM for male and female Tg and NonTg mice.

During MWM testing the motoric function was assessed by average distance moved (140) (A, D) and average velocity (cm/s) (B,E), and anxiety levels during the task was determined by average time spent in thigmotaxis (%) (C,F).Female Tg and NonTg mice (159) and males (D-F) were analyzed separately. Statistical analysis of average distance moved, average velocity and average percent thigmotaxis were each analyzed by two-way ANOVA. **p<0.01; ****p<0.0001. Data shown are ±SEM.

6.5 Discussion

I show here, for the first time, that female Tg, but not male Tg, mice receiving omega-6/-9 UFA supplement for four months prior to testing display improved learning and memory performance in the MWM compared to vehicle- and unsupplemented animals. Enhanced performance was evident by a decrease in escape latency, progressive improvements in path efficacy over the test period, a significantly increased use of spatial search strategies and decreased repetitive looping strategies, particularly in the last four days of the task. A minor effect in reversal learning was detected in males in that supplementation was associated with improvements in performance on the first day of platform reversal, however, improvements proceeded at the same rate in both omega-6/-9 UFA treated and control Tg animals. It may be that omega-6/-9 had little effect on males simply because Tg males display little to no learning and memory impairment in the MWM at six months of age relative to females (Chapter 3). Overall, the primary effect of omega 6/9 supplementation was in female Tg mice.

As I did not examine later time points, I cannot conclude whether a diet supplemented with omega-6/omega-9 UFAs prevents phenoconversion in female Tg mice or simply delays the transition from a presymptomatic to a symptomatic state. The omega-6/omega-9 UFA supplements used in this study are enriched in both LA (18:2n-6) and OA (18:1n-9), and previous studies (212-216) have suggested these UFAs to be neuroprotective in AD. The cognitive enhancing effects observed here may be related to changes in APP processing. *In vitro,*

supplementing the media with LA and OA (alone or combined) of APP695 and SP-C99 transfected COS-7 cells reduced secreted Aβ levels (212-214). Our results are also consistent with reports that TgCRDN8 mice fed a high-protein, low-fat, cholesterol-free diet enriched with OA show a reduced $A\beta_{42}/A\beta_{40}$ ratio, thus lower levels of the more pathogenic $A\beta_{42}$ peptide (214). Additionally, mice maintained on this diet exhibited decreased β-secretase activity, increased sAPPα, increased expression of Aβ clearance proteins and reduced amyloid plaque burden in the brain (214). Sex-specific effects or cognitive performance however, was not investigated. LA and OA have also been shown to specifically affect the equilibrium of Aβ processing by decreasing amyloidogenic process and increasing amyloid clearance (12,214,217,218) (described in depth in Chapter 7). If increased plaque deposition is related to greater impairment in the MWM (49), then we expect that females, but not male, omega-6/-9 UFA treated mice will show a reduced plaque burden compared to untreated controls. It is also tempting to speculate that female Tg mice treated with omega-6/-9 UFAs may have improved cognitive behaviour in response to environmental contingencies, otherwise termed an improved behavioural flexibility (219,220). Behavioural flexibility is analogous to human cognitive flexibility, representative of mental ability to contemplate multiple concepts simultaneously (219). Future work would involve investigating behavioural flexibility of omega-6/-9 UFA treated Tg mice by additional reversal learning test days to observe rate of learning or a repeated learning paradigm involving a set of reversal or shift phases serially (75).

AD therapies are currently primarily focused on treating symptoms rather than preventing cognitive decline. As AD patients show early deficits in omega-6/-9 UFAs (97-100), it will be crucial to investigate neuroprotective potential of dietary interventions that target this deficiency. A Mediterranean diet is rich in omega-6/-9 LA and OA (84). Only a few clinical studies have investigated of association between cognitive benefit and Mediterranean diet, most studies only focus on effects of omega-3 UFAs (86). Clinical trials report that higher adherence to Mediterranean diet is associated with; reduced risk of AD (90,91), reduced risk of mortality in AD patients (92), reduced risk of MCI (93,94), and reduced rate of cognitive decline (95,96). Adherence to a Mediterranean diet (84,86,89), and therein omega-6/-9 UFAs neuroprotective effects, may alter cognitive deficits observed in AD pathology. Dietary UFAs and their lipid metabolites display a plethora of important roles such as maintaining membrane structural integrity and fluidity, modifying receptors, ion channels and membrane-bound enzymes (221), which may themselves, or act synergistically, as benefit in cognitive decline. This study is the first to provide preclinical evidence of omega-6/-9 UFAs beneficial effect in cognitive function. It may be that a diet enriched in omega-6/-9 can modify the brain lipid content and thus be capable of delaying transition from a presymptomatic to a symptomatic phenotype, as suggested here in an aggressive pathogenic mouse model of AD. Thus, omega-6/-9 UFAs should be investigated in more detail for adjuvant therapeutic potential.

Chapter 7 – General Discussion

7.1 Summary

In this thesis, I tested whether long-term enhanced nutritional supplementation with omega-3, or omega-6 and omega-9 UFAs would prevent (or at least delay) phenoconversion apparent in behavioural learning and memory deficits in an AD mouse model. Specifically, I established whether a four-month vehicle, omega-3 UFA, or omega-6/-9 UFA oral treatment (75 mg/kg/day) altered performance of male and female N5 C57BL/6Crl X C3H/HeJ TgCRND8 and NonTg mice in the MWM learning and memory paradigm.

Previous animal model and clinical trial studies have shown inconsistencies with respect to the cognitive benefit of omega UFAs and comprehensive experimental analyses are required. Contention in these reports are due to discrepancies in appropriate dosage, mode of administration, treatment duration, treatment composition as single species or mixed lipids in natural substances, sex, age, background and number of subjects tested. Clinical reports are often lacking regarding long-term nutritional intervention (203). Human studies are generally constrained by inability to experimentally manipulate variables and conclusions are descriptive or correlational, whereas mouse models enable inference of causal relationships under controlled conditions (32). AD mouse models are invaluable reductionist tools; they provide *in vivo* mechanistic insight to pathology, facilitate controlled long-term assessment and identification of novel therapeutic agents (32). Although transgenic lines of AD are models of genetically determined EOFAD thus

representing fewer cases than LOAD, both AD forms are phenotypically similar (comparable in histological assessment and clinical manifestations), suggesting mechanisms represented in these genetically determined models will be relevant to pathogenesis in non-familial forms (1).

I show here that, in this hybrid genetic background, female Tg mice display significant learning and memory impairments compared to NonTg mice at six months of age, whereas impairment of male Tg mice is mild, significantly different from females, and manifests as a delayed learning in MWM. These data may reflect a sex difference in amyloidogenic processing as female mice have been reported to have a greater plaque burden than males (29,152-156). To test this hypothesis, it will be important to compare the $A\beta_{42}/A\beta_{40}$ ratio and plaque burden in male and female Tg mice at six months of age in future studies.

Additionally, I demonstrate that handling and daily treatment of vehicle (25% sweetened condensed milk) did not significantly alter performance of Tg and NonTg mice. I have also conclusively shown that non-cognitive confounds of motoric function and anxiety levels during MWM did not affect learning and memory performances and was further unaffected by omega-3,-6,-9 UFAs. Moreover, four-month omega-3 UFA treatment did not significantly affect learning and memory performance compared to untreated controls. This result indicated that an enriched diet of omega-3 UFAs did not delay or improve cognitive decline in an AD transgenic model, consistent with previous animal (206) and clinical studies (200,205,222). Intriguingly, female Tg mice receiving four-month omega-6/-9 UFA treatment displayed improved MWM performance and exhibited a more

effective spatial searching strategy in MWM compared to untreated Tg mice. Thus, simple dietary supplementation (above and beyond core changes in diet) is sufficient to affect neural function.

Not surprisingly, behavioural learning and memory tests dependent on visual cues rely on functional vision. Visual impairment would hinder performance and inaccurately characterize cognitive function. Visual acuity of male and female Tg and NonTg mice was assessed by cued MWM and adapted SLAG reflex test. Adapted SLAG reflex test proved sensitive to distinguish degrees of impaired conditions, demonstrating that our N5 model and NonTg littermates exhibit a reduced visual acuity. This mild impairment, however, did not impair spatial navigation in the cued MWM test, concurrent with previous F1 Tg reports (52,183). Performances in SLAG reflex test and cued MWM were irrespective of sex, thus sex-differences in visual competency do not underlie sex-differences in MWM performance of either control or omega UFA supplemented mice.

Although research has provided immense insight into AD, mechanisms behind phenoconversion from a preclinical to a symptomatic AD pathology remain unknown. It may be that amyloid deposition should be considered integrated into a sequence of triggered events, rather than causal itself, and correlated with cellular ‘change of state’ wherein converging metabolic disruptions result in disease conversion (28). At present, drug therapies for AD are primarily focused on symptomatic treatment; there are no preventative or disease-modifying treatments for AD. Because AD pathology includes dietary deficiencies manifested in systemic changes in lipid content (114), optimization of

nutrition related to AD decline should ideally be initiated as a presymptomatic strategy to lower risk of AD. In AD, nutritional intervention may improve quality of life, and a combinatorial approach with pharmacological intervention may provide maximum benefit in cognitive decline (194).

7.2 Mechanism of omega-6/-9 UFA role in amyloidogenesis

Cognitive benefits emphasized in the Mediterranean diet, have reported omega-3, omega-6, and omega-9 UFAs as key bioactive compounds with potential to reduce risk of AD and/or modify disease progression (84,86,88). Here, I show that omega-6/-9 supplementation is more effective in ameliorating behavioural indices of learning and memory than omega-3 supplementation. Previous reports indicate AD patients' plasma (109,111), and post-mortem brains (98,112,198) are deficient in omega-3 UFAs, particularly DHA (22:6n-3). MCI and AD patients also exhibited substantially decreased omega-6/-9 UFAs, LA (18:2n-6) and OA (18:1n-9), in plasma (97,98), and CNS membrane phospholipids (99,100). Thus suggesting UFAs could be a contributing role in etiology and pathology of AD. The biochemical basis whereby a Mediterranean regime may prevent or improve AD pathology remains unclear. Multiple *in vitro* and *in vivo* preclinical assessments have elucidated mechanisms influencing amyloidogenic processing. For example, *in vitro* supplementation of LA and OA (alone and combined) to APP695 and SP-C99 transfected COS-7 cells lead to reduced total Aβ levels (212-214). TgCRND8 mice fed high-protein, low-fat, cholesterol-free diet enriched with OA exhibited decreased plaque number in neocortical and

hippocampal regions, significantly decreased pathogenic $A\beta_{42}$ peptide, decreased β-secretase activity, and increased neuroprotective sAPPα (214). Whereas, $APP_{K670N,M671L}$+PS1 mice fed omega-3 UFAs did not alter Aβ levels or cognitive performance (206). I propose here that cognitive benefits are mediated by omega-6/-9 and not omega-3. These effects are result of not only decreased amyloid production but also increased amyloid clearance. In Figure 7.1, I present current mechanistic knowledge of omega-6/-9 UFA effect on AD pathogenesis, specifically on the equilibrium between production and clearance of Aβ processing (12,214,217,218).

The major β-secretase in neurons, responsible for amyloid aggregation, is β-site APP cleaving enzyme (BACE1) (223,224). BACE1 is a key rate-limiting enzyme in amyloidogenesis and potential target for drug therapy, but unlike γ-secretase, absence does not create adverse effects or lethality (225,226). OA and LA are natural BACE1 inhibitors (217). OA dose-dependently inhibits BACE1 activity with a half-maximal inhibitory concentration (IC_{50}) value of 61.31 μM, while LA attenuates BACE1 activity with an IC_{50} of 235 μM (217). The inhibition constant values of OA and LA are 34.3 and 186 μM respectively, and does not influence other proteases (217). *In silico* docking simulations support the direct interaction of OA and LA with BACE1 (217). OA and LA prefer different, allosteric binding sites of BACE1, rather than the active catalytic center (Asp32 and Asp228) (217). The OA binding sites are formed by residues: Ser10, Ala157, Gln303, Arg307, Pro308, Cys319, Tyr320, and Val336 (217). The hydrogen atom of the carboxylic acid moiety of OA forms two hydrogen bonds with the hydroxyl

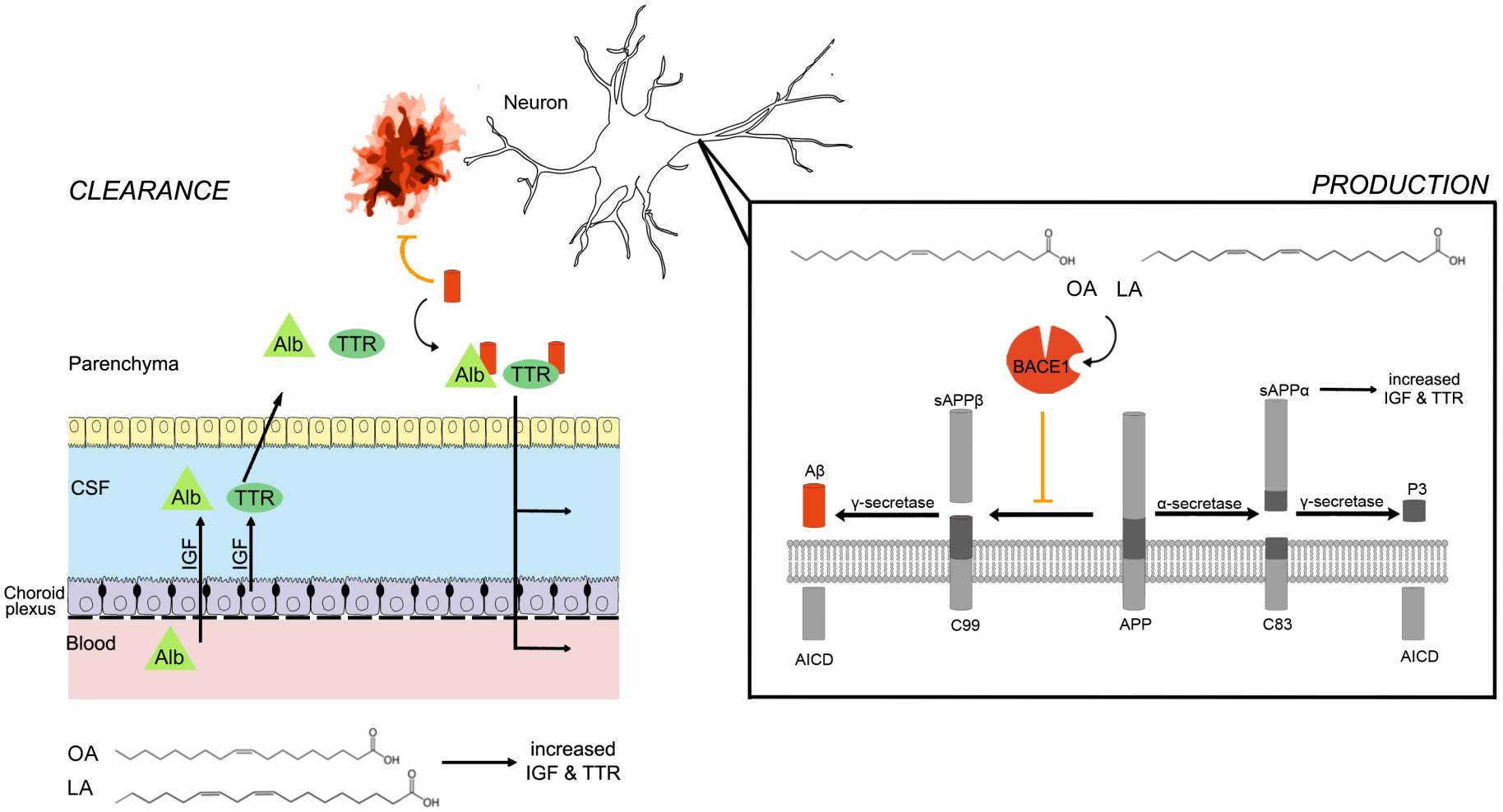
CLEARANCE
Neuron
PRODUCTION
Alb
TTR
Parenchyma
CSF
IGF
Choroid plexus
Blood
OA
LA
increased IGF & TTR
OA LA
BACE1
sAPPβ
sAPPα
increased IGF & TTR
Aβ
γ-secretase
α-secretase
γ-secretase
P3
C99
APP
C83
AICD
AICD

Figure 7.1.

Figure 7.1 Model of omega-6/-9 UFA mechanistic role in amyloidogenesis

Omega-6/9 UFAs OA and LA are neuroprotective by decreasing amyloid production (22) and increasing amyloid clearance (left). OA and LA both decrease amyloidogenesis by inhibiting rate-limiting enzyme BACE1 by directly interacting with allosteric sites. OA and LA increase TTR and IGF and thereby increase amyloid clearance. OA increased neuroprotective sAPPα, which can also induce expression of IGF and TTR. Aβ clearance by Aβ binding proteins, TTR and albumin (Alb), occurs by the following cascade: (1) serum IGF induces secretion of TTR from choroid plexus into CSF, as well as induces passage of albumin from the blood into CSF, (2) TTR and Alb accumulate in brain parenchyma where they bind to Aβ and transport it into the CSF, (3) Aβ-bound to TTR or albumin efflux in the CSF into the bloodstream via either active or passive transport. Carrier protein bound Aβ is cleared by via active transport of the choroid plexus and endothelial cell barrier of capillary vessels, or passive transport of CSF efflux into venous circulation. OA and LA affect AD pathogenesis by decreasing production of Aβ and clearing Aβ, thus preventing further fibrillization and maintaining equilibrium.

of Cys319 and with the amine of Tyr320 (217). The LA binding sites are formed by residues: Tyr14, Ala157, Val170, Thr232, Gln304, Ala323, Ala335, Glu339, His360, and Val361(217). LA forms a hydrogen bond with the carbonyl of Gln304 (217). Thus, OA and LA noncompetitively attenuate BACE1 activity by stably interacting with allosteric sites. Although inhibitory activity of OA and LA is not as strong as synthetic BACE1 inhibitors such as P10-P40StatVal, OM99-1, and OM99-2, these synthetic inhibitors are impaired by a limited blood-brain barrier penetration due to large molecular weight (227,228). OA and LA hold potential as safe, stable, natural source BACE1 inhibitors. Passage of OA and LA from blood to the brain may occur by either diffusion, fatty acid transport proteins (FATP-1 and FATP-4), fatty acid binding protein (FABP5) and fatty acid translocase/CD36, in brain microvessel endothelial cells (229,230). As OA and LA exert significant noncompetitive inhibition of BACE1 activity, these UFAs are not only energy sources but natural inhibitors in prevention of treatment of AD (217).

Enriched OA diet *in vivo* lead to significant increases in amyloid clearance proteins transthyretin (88) and insulin-like growth factor (IGF-II) (214). Additionally, OA and LA have been shown to enhance the growth promoting effects of IGF (12). In smooth muscle cells, membrane incorporated OA and LA enhanced growth-promoting effects of IGF-I by phospholipase D hydrolysis and subsequent generation of diacylglycerol (12). I speculate that OA and LA may increase IGF or subsequent neuroprotective pathway in CNS. Trophic factors IGF-I and IGF-II are neuroprotective against Aβ toxicity (231,232). IGFs ability to clear brain Aβ occurs by enhancing transport of Aβ carrier proteins such as

albumin and TTR (218,232). Albumin, present most abundantly in plasma, binds to oligomeric and polymeric Aβ, preventing further fibrillization (233,234). TTR, also known as prealbumin, is a carrier protein able to bind to thyroid hormones and Aβ (235,236). TTR monomer (and dimer) complexes with soluble $A\beta_{1-42}$ peptides (236,237), oligomers and fibrils (238) thus inhibiting aggregation and deposition. TTR mutations underlie familial amyloidotic polyneuropathy, a senile amyloidosis characterized by amyloid deposition in peripheral nervous system and heart (239).

Aβ clearance by Aβ binding proteins occurs by the following cascade. Serum IGF induces; (1) secretion of TTR from the choroid plexus into the cerebrospinal fluid (CSF), (2) passage of albumin from the bloodstream into the CSF (218). These carrier proteins accumulate in brain parenchyma where they bind to Aβ and transport it into the CSF (218). Efflux of Aβ-bound carrier protein in the CSF into the bloodstream occurs via either: (1) active transport of the choroid plexus and endothelial cell barrier of capillary vessels, or (2) passive transport of CSF efflux into venous circulation (218). In sum, IGF induces clearance carrier proteins albumin and TTR to efflux carrier-bound Aβ from brain parenchyma to the bloodstream through the CSF (218). Moreover, the reported (214) effect of OA increasing IGF-II as well as increasing neuroprotective sAPPα may be related. sAPPα has been shown neuroprotective against Aβ toxicity *in vitro* (240,241) and *in vivo* (242) and can induce expression of neuroprotective proteins IGF-II and TTR (242). Taken together, the omega-6/-9 UFAs OA and LA, found abundantly in a Mediterranean diet, are neuroprotective by decreasing

amyloidogenic process and increasing amyloid clearance, thereby preventing phenoconversion or altering cognitive deficit.

7.3 Significance of research

AD is arguably one of the most complex diseases of the human nervous system (28). Beyond the devastating symptoms of each patient, a heavy burden is placed upon loved ones and society as prevalence continues to rise. There is currently a lack of understanding of AD etiology, and a greater lack in therapeutic options available. This imperatively calls for development of preventative or disease-modifying treatments and regenerative approaches. Our laboratory and that of Herrup's has suggested that phenoconversion to AD is initiated by progressive amyloid deposition, aggravated by chronic neuroinflammation, that triggers a cellular 'change in state' wherein cell metabolism, specifically metabolism of structural membrane lipids, is primed towards neurodegeneration in place of homeostatic function (i.e., an inability to metabolize omega-6/-9 UFAs resulting in their depletion in structural membrane lipids in the CNS) (13,28). AD development is a multifactorial result that may begin years prior to emergence of symptoms (2). Naturally altering the CNS glycerophospholipidome in neuronal and glial membranes by dietary interventions may promote AD resistance. Monounsaturated and polyunsaturated fatty acids may have essential and neuroprotective benefits thus hold exciting therapeutic promise (81,114). In this thesis, I have shown that dietary intervention of omega-6/-9 UFAs should be further explored as a treatment strategy to modify the brain lipid content and thus

delay transition from a presymptomatic to a symptomatic state. Data presented in this thesis is the first to provide preclinical evidence of a beneficial effect of long-term omega-6/-9 UFAs treatment on cognitive function tests. This research holds importance as there is currently no preventative or disease-modifying treatments for AD and drug therapies are primarily focused on symptomatic treatment. An individual diagnosed with AD has by definition undergone considerable cognitive decline; thus optimization of nutrition should be initiated as a presymptomatic strategy to lower risk or delay development of AD (194). In AD, nutritional intervention may improve quality of life, and a combinatorial approach may provide maximum benefit (194). For example, the additive effects of lipid mixtures (natural to human diet rather than single species), such as OA and LA combined appeared to have a stronger inhibitory effect on Aβ production (212). Comprehensive therapeutic approaches should also encompass: nutritional intervention alongside pharmacological treatments, as well as mental, physical, and social stimulation (194).

Appendix I – Supplemental Analysis

Table A1. Statistical analysis of MWM performance.

MWM performance parameters with respect to treatment, genotype, and sex were compared and statistical significance analyzed. Statistical analysis of escape latency, path efficacy, search strategy and reversal escape latency were each analyzed by repeated measures two-way ANOVA. Statistical analysis of average distance moved, average velocity and average percent thigmotaxis were each analyzed by two-way ANOVA. Main effect of genotype or treatment are shown, with respect to groups compared, refer to text for further *post hoc* assessments. Statistical analysis of number of platform-site cross-overs during probe trial were each analyzed by Student's *t* test. Statistical significance were shown above a minimum of $p<0.05$. Groups compared listed in order of appearance in chapters. Abbreviations listed: v, vehicle, c, control, cc, cued control, ω3, omega-3 UFAs, ω-6/ω-9, omega-6/-9 UFAs.

Groups compared	Escape latency	Path efficacy	Search strategy	Platform cross-overs	Reversal	Distance moved	Velocity	Thigmotaxis
v-NonTg-F c-NonTg-F	p=0.9038	p=0.9929	p>0.9999	p=0.2846	p=0.4954	p=0.5698	p=0.8527	p=0.1490
v-NonTg-M c-NonTg-M	p=0.8986	p=0.6970	p>0.9999	p=0.7092	/	p=0.5698	p=0.5769	p=0.5116
v-Tg-F c-Tg-F	p=0.0756	p=0.1445	p>0.9999	p=0.1219	p=0.1643	p=0.3461	p=0.8527	p=0.1490
v-Tg-M c-Tg-M	p=0.7668	p=0.5294	p=0.9995	p=0.3940	p=0.7230	p=0.9978	p=0.5769	p=0.5685
v+c-Tg-F v+c-NonTg-F	p=0.0003	p<0.0001	p<0.0001	p=0.1268	p=0.4921	p<0.0001	p=0.0081	p=0.9745
v+c-Tg-M v+c-NonTg-M	p=0.0116	p=0.0041	p>0.9999	p=0.6285	p=0.4381	p<0.0001	p=0.0020	p>0.9999
v+c-Tg-F v+c-Tg-M	p=0.0365	p=0.0368	p<0.0001	p=8318	p=0.0389	p=0.2892	p=0.7848	p=0.7164
cc-Tg-F cc-NonTg-F	p=0.4955	/	/	p=0.7061	/	/	/	/
cc-Tg-M cc-NonTg-M	p=0.4509	/	/	p=0.9016	/	/	/	/
ω-3-NonTg-F c+v-NonTg-F	p=0.2033	p=0.2408	p<0.0001	p=0.8862	p=0.6640	p=0.9332	p=0.4381	p=0.7036
ω-3-NonTg-M c+v-NonTg-M	p=0.4196	p=0.6022	p<0.0001	p=0.4019	p=0.8528	p=0.5763	p=0.4287	p=0.4061
ω-3-Tg-F c+v-Tg-F	p=0.1973	p=0.1678	p<0.0001	p=0.4387	p=0.3980	p=0.8677	p=0.2017	p=0.6192
ω-3-Tg-M c+v-Tg-M	p=0.0520	p=0.1939	p<0.0001	p=0.4287	p=0.1955	p=0.686	p=0.8551	p=0.7353
ω-6/ω-9-NonTg-F c+v-NonTg-F	p=0.9199	p=0.8928	p=0.993	p=0.8818	p=0.6978	p=0.8269	p=0.5173	p=0.8476
ω-6/ω-9-NonTg-M c+v-NonTg-M	p=0.3200	p=0.7326	p>0.9999	p=0.7727	p=0.4764	p=0.5131	p=0.6006	p=0.0694
ω-6/ω-9-Tg-F c+v-Tg-F	p=0.0439	p=0.0994	p=0.9985	p=0.5144	p=0.7704	p=0.8269	p=0.2917	p=0.8476
ω-6/ω-9-Tg-M c+v-Tg-M	p=0.5387	p=0.1956	p=0.9994	p=0.6966	p=0.0019	p=0.5131	p=0.6006	p=0.7454

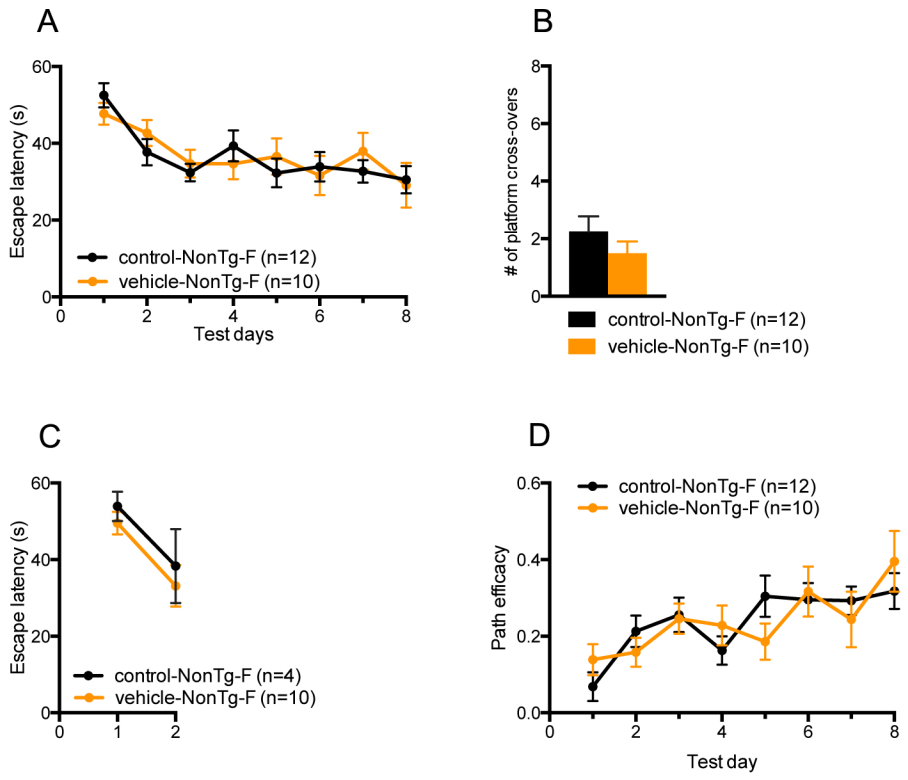
A
Escape latency (s)
60
40
20
0
0
2
4
6
8
Test days
control-NonTg-F (n=12)
vehicle-NonTg-F (n=10)
B
# of platform cross-overs
8
6
4
2
0
control-NonTg-F (n=12)
vehicle-NonTg-F (n=10)
C
Escape latency (s)
60
40
20
0
0
1
2
control-NonTg-F (n=4)
vehicle-NonTg-F (n=10)
D
Path efficacy
0.6
0.4
0.2
0.0
0
2
4
6
8
Test day
control-NonTg-F (n=12)
vehicle-NonTg-F (n=10)

Figure A1.

Figure A1 MWM analysis of control compared to vehicle in female NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

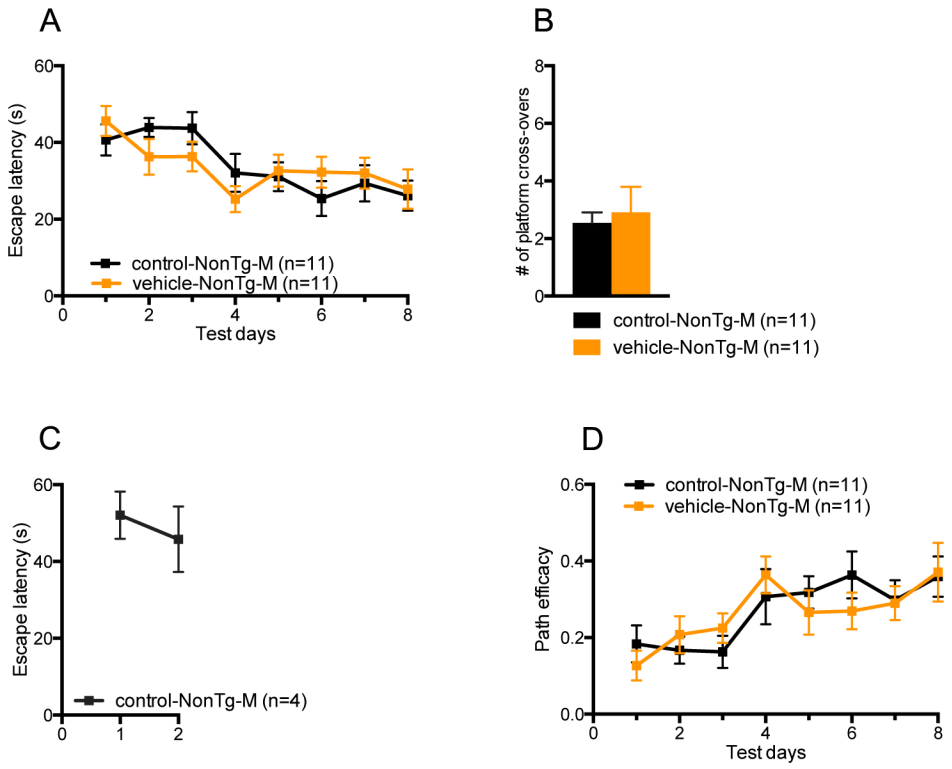
A
Escape latency (s)
60
40
20
0
0
2
4
6
8
Test days
control-NonTg-M (n=11)
vehicle-NonTg-M (n=11)
B
# of platform cross-overs
8
6
4
2
0
control-NonTg-M (n=11)
vehicle-NonTg-M (n=11)
C
Escape latency (s)
60
40
20
0
0
1
2
control-NonTg-M (n=4)
D
Path efficacy
0.6
0.4
0.2
0.0
0
2
4
6
8
Test days
control-NonTg-M (n=11)
vehicle-NonTg-M (n=11)

Figure A2.

Figure A2 MWM analysis of control compared to vehicle in male NonTg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

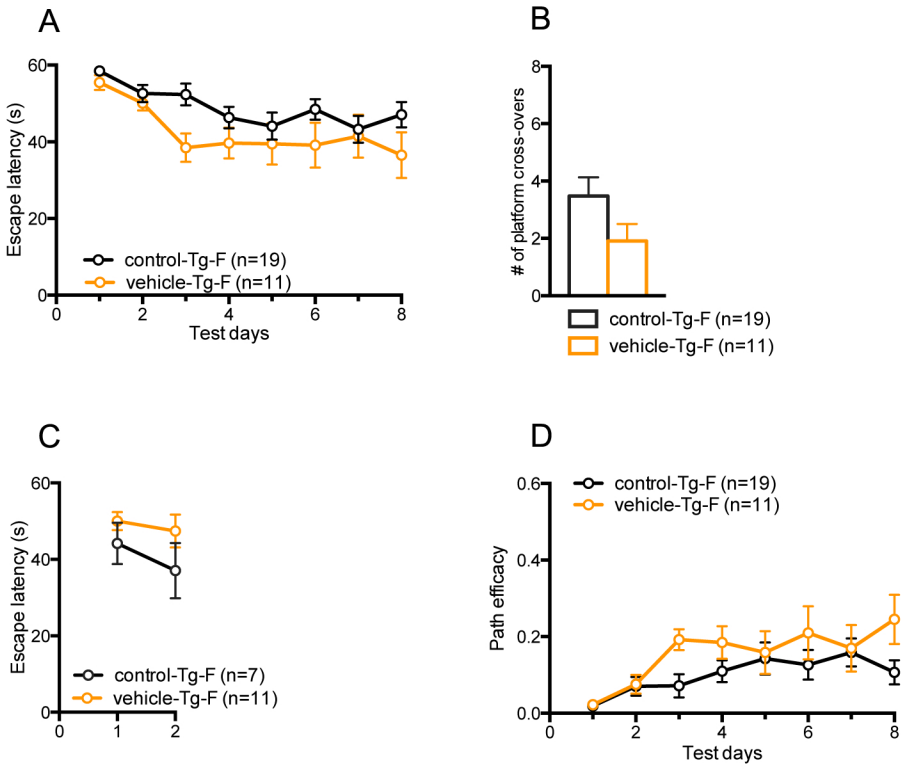
A
Escape latency (s)
60
40
20
0
0
2
4
6
8
Test days
control-Tg-F (n=19)
vehicle-Tg-F (n=11)
B
# of platform cross-overs
8
6
4
2
0
control-Tg-F (n=19)
vehicle-Tg-F (n=11)
C
Escape latency (s)
60
40
20
0
0
1
2
control-Tg-F (n=7)
vehicle-Tg-F (n=11)
D
Path efficacy
0.6
0.4
0.2
0.0
0
2
4
6
8
Test days
control-Tg-F (n=19)
vehicle-Tg-F (n=11)

Figure A3.

Figure A3 MWM analysis of control compared to vehicle female Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

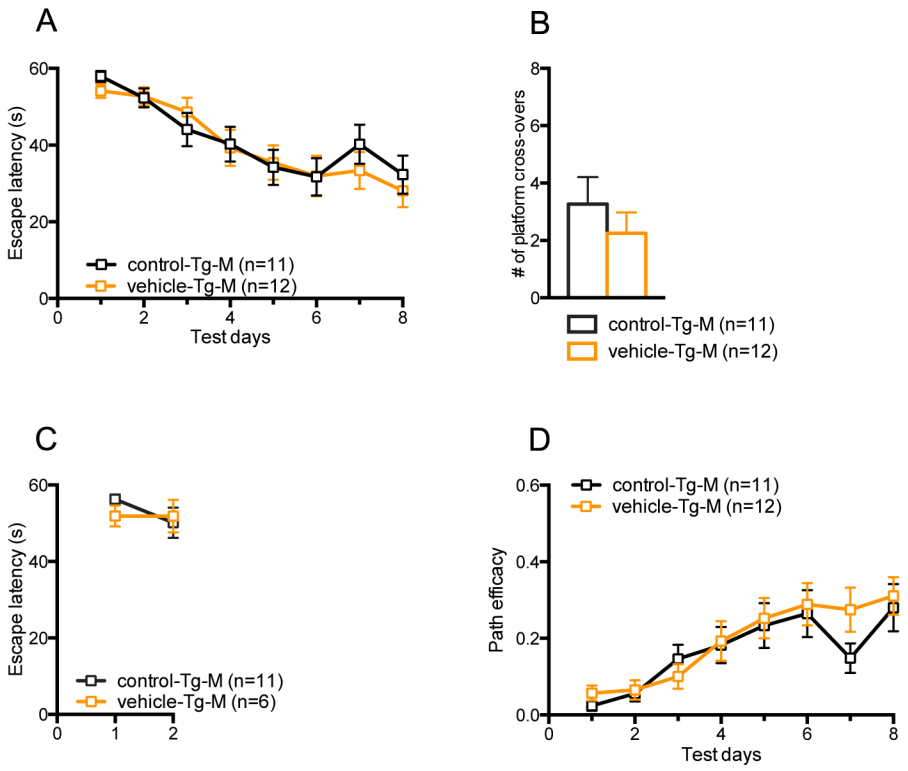
A
Escape latency (s)
60
40
20
0
0
2
4
6
8
Test days
control-Tg-M (n=11)
vehicle-Tg-M (n=12)
B
# of platform cross-overs
8
6
4
2
0
control-Tg-M (n=11)
vehicle-Tg-M (n=12)
C
Escape latency (s)
60
40
20
0
0
1
2
control-Tg-M (n=11)
vehicle-Tg-M (n=6)
D
Path efficacy
0.6
0.4
0.2
0.0
0
2
4
6
8
Test days
control-Tg-M (n=11)
vehicle-Tg-M (n=12)

Figure A4.

Figure A4 MWM analysis of control compared to vehicle male Tg mice.

MWM testing was conducted over eight consecutive days. A ninth test day was conducted as probe trial. Test days ten and eleven involved reversal learning testing. Four trials per day were conducted; average values are shown, with the exception of probe trial. Analysis parameters involved; average escape latency (s) (A), number of platform-site cross-overs during probe trial (B), reversal escape latency (s) (C), and path efficacy (D). Statistical analysis of (A), (C) and (D) were analyzed by repeated measures two-way ANOVA, with a *post hoc* of Holm-Sidak's multiple comparisons test. Statistical analysis of (B) was analyzed by Student's *t* test. Data shown are ±SEM.

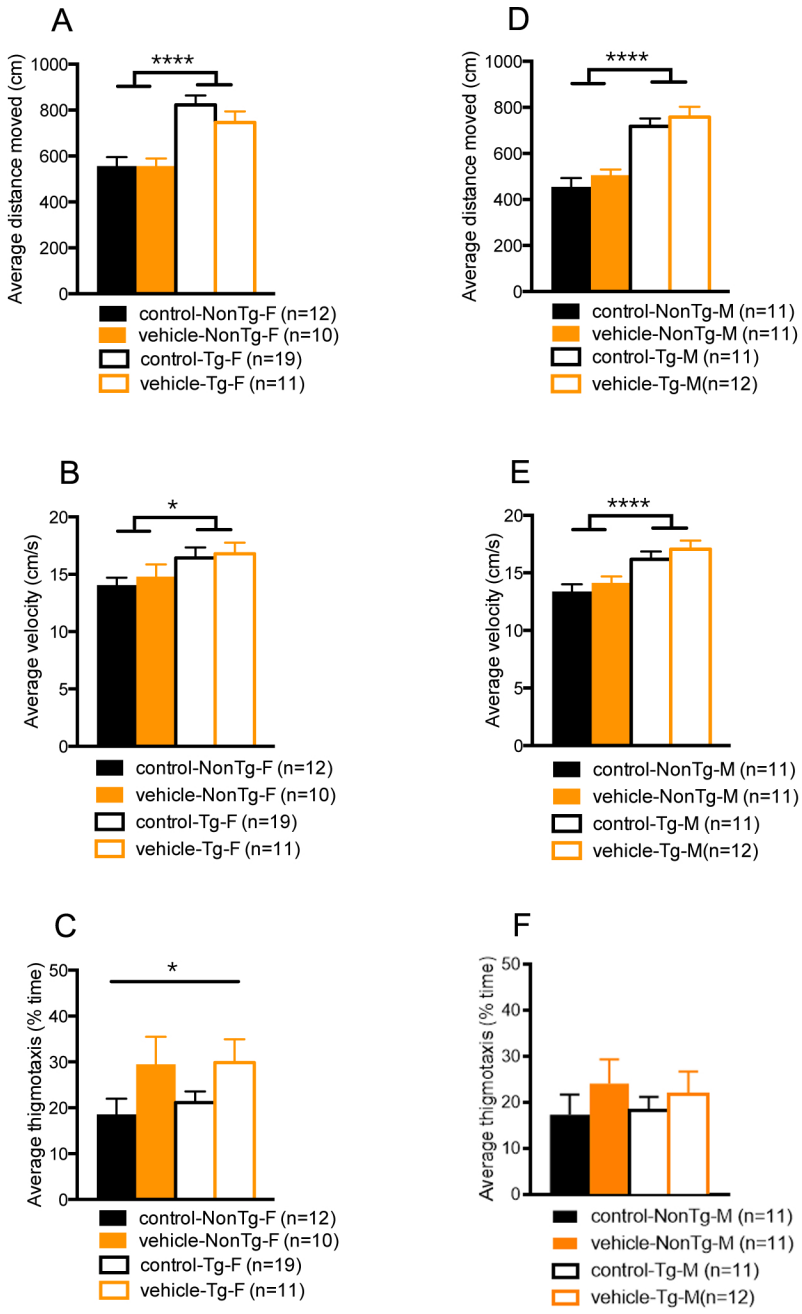
A
Average distance moved (cm)
1000
800
600
400
200
0
****
control-NonTg-F (n=12)
vehicle-NonTg-F (n=10)
control-Tg-F (n=19)
vehicle-Tg-F (n=11)
D
Average distance moved (cm)
1000
800
600
400
200
0
****
control-NonTg-M (n=11)
vehicle-NonTg-M (n=11)
control-Tg-M (n=11)
vehicle-Tg-M(n=12)
B
Average velocity (cm/s)
20
15
10
5
0
*
control-NonTg-F (n=12)
vehicle-NonTg-F (n=10)
control-Tg-F (n=19)
vehicle-Tg-F (n=11)
E
Average velocity (cm/s)
20
15
10
5
0
****
control-NonTg-M (n=11)
vehicle-NonTg-M (n=11)
control-Tg-M (n=11)
vehicle-Tg-M(n=12)
C
Average thigmotaxis (% time)
50
40
30
20
10
0
*
control-NonTg-F (n=12)
vehicle-NonTg-F (n=10)
control-Tg-F (n=19)
vehicle-Tg-F (n=11)
F
Average thigmotaxis (% time)
50
40
30
20
10
0
control-NonTg-M (n=11)
vehicle-NonTg-M (n=11)
control-Tg-M (n=11)
vehicle-Tg-M(n=12)

Figure A5.

Figure A5 Analysis of effect of vehicle treatment on non-cognitive indices in MWM for male and female Tg and NonTg mice.

During MWM testing the motoric function was assessed by average distance moved (140) (A, D) and average velocity (cm/s) (B,E), and anxiety levels during the task was determined by average time spent in thigmotaxis (%) (C,F).Female Tg and NonTg mice (159) and males (D-F) were analyzed separately. Statistical analysis of average distance moved, average velocity and average percent thigmotaxis were each analyzed by two-way ANOVA. *$p<0.05$;****$p<0.0001$. Data shown are ±SEM.

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