ASSESSING MEMORY IN AN ALDEHYDE DEHYDROGENASE 2 KNOCKOUT MODEL OF ALZHEIMER'S DISEASE

by

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Abstract

The study of Alzheimer's Disease (AD) has been hindered by the absence of animal models of late-onset/age-related AD (also termed sporadic AD) (95% of AD cases) since current transgenic mouse models exhibit pathological changes dependent on overexpression of mutant human genes linked to early-onset, familial AD (5% of cases). Oxidative stress is considered to be a causative factor in age-related AD, and we have found that aldehyde dehydrogenase 2 (Aldh2) null mice exhibit not only oxidative stress, but also display many AD-like pathologies. The current study used behavioral analysis to assess whether $Aldh2^{-/-}$ mice also exhibit memory and cognition deficits. Male and female wild type and $Aldh2^{-/-}$ mice were tested monthly beginning at three months of age, using the open field novel object recognition test (a measure of recognition memory), as well as spontaneous alternations in the Y-maze (a measure of spatial working memory). In both tasks, significant decreases in performance occurred in $Aldh2^{-/-}$ mice by 3.5-4 months of age, and this progressively declined over the next three months compared to wild type mice. Sex-related differences in memory impairment were not observed. These results, together with the findings that AD-like pathologies are also present, suggest that $Aldh2^{-/-}$ mice represent a new, oxidative stress-based model of age-related cognitive impairment and AD. This model may prove useful both for assessing AD therapeutics and for gaining better insight into the pathogenesis of AD.

Co-Authorship

The studies presented herein were performed by Ahmed Mohamed Elharram with the following co-authorships and technical assistance:

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List of Abbreviations

3xTg-AD: triple transgenic mouse model
$A\beta_{42}$: amyloid beta peptide 1-42
ACh: acetylcholine
AD: Alzheimer's disease
ALDH2: aldehyde dehydrogenase 2
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA: analysis of variance
APoE: apolipoprotein E
APP: amyloid precursor protein
BACE: beta-site APP-cleaving enzyme
CAM: calmodulin
CNS: central nervous system
CDK5: cyclin-dependent kinase-5
CREB: cyclic adenosine monophosphate response element binding protein
DAG: diacylglycerol
ERK: extracellular signal-regulated kinase
FAD: familial Alzheimer's disease
HNE: 4-hydroxynonenal
IP3: inositol 1,4,5-trisphosphate
LTP: long term potentiation
LTM: long term memory
LRP-1: LDL receptor- related protein 1
MAP: microtubule associated protein
MAPK: mitogen-activated protein kinase
MWM: Morris water maze
NFTs: neurofibrillary tangles
NMDA: N-methyl-D-aspartate receptor
NOR: novel object recognition task
PSEN1: presenilin 1
PSEN2: presenilin 2
PSD95: post-synaptic density protein 95
PSEN: presenilin gene
RAM: radial arm maze
RNS: reactive nitrogen species
ROS: reactive oxygen species
sAPPβ: soluble N-terminal amyloid precursor protein fragment
STM: short term memory
Tg2576: Transgenic 2576 animal model

Introduction

1.1 Statement of the Research Problem

Alzheimer's disease (AD) is a very complex disease with a number of different molecular pathologies and behavioral impairments. Abnormal amyloid precursor protein (APP) processing leading to the formation of amyloid beta (Aβ) plaques, the accumulation of neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein, and neurodegeneration and neuronal cell loss are the key pathologies of AD and are the manifestation of alterations of a number of different molecular cascades. The amyloid cascade hypothesis has driven much of current AD research efforts, and has led to the development of a number of different animal models of AD that have greatly helped further our understanding of the disease and how these pathologies can impact the brain. However, recent results from clinical trials have demonstrated a lack of efficacy of therapeutics that target the amyloid cascade and aim to reduce Aβ plaque formation in AD brains. These results are also mirrored in currently used animal models of AD. The majority of animal models currently used to study AD are mouse models that exhibit pathological changes dependent on the overexpression of mutant human genes linked to early-onset, familial AD which only accounts for a small proportion of all AD cases. These transgenic animal models produce significantly higher levels of Aβ deposits in the brain that often do not correlate with neuronal or synaptic loss. Furthermore, these animals exhibit cognitive deficits prior to the formation of amyloid plaques. These data question the validity of aspects of the amyloid cascade hypothesis and its role in the

progression of AD, and emphasize the need to develop novel animal models that are based on alternative factors involved in the progression of AD.

A considerable amount of evidence suggests that oxidative stress is an initiating factor in sporadic AD. In particular, a lipid peroxidation marker of oxidative stress, 4-hydroxy-2-nonenal (HNE), has been shown to play a role in driving AD pathogenesis, and has been found at significantly higher levels (and at a very early stage) in AD brains. HNE has been shown to covalently modify tau protein, accelerate the aggregation of Aβ via conjugate additions at multiple locations along the Aβ peptide, as well as alter glucose and glutamate transport systems. One of the primary metabolizers of HNE is aldehyde dehydrogenase 2 (ALDH2), and its levels are significantly higher in AD brains, suggesting that increased ALDH2 expression may serve as a protective response to the increased lipid peroxidation that can occur during the progression of AD. These results emphasize the critical role HNE and oxidative stress can have on the progression of AD, and the significance of ALDH2 in metabolizing HNE as well as other products of oxidative stress.

Aldh2$^{-/-}$ mice have been previously studied in our laboratory in studies of organic nitrate tolerance. However, after reviewing the link between ALDH2 and its metabolism of HNE, as well as the critical role HNE and oxidative stress may have in initiating AD, we propose that Aldh2$^{-/-}$ mice could represent a new, oxidative stress-based model of age-related cognitive impairment with AD-like pathologies. In order to further characterize these mice as a model of cognitive impairment and AD, appropriate behavioral analyses are required to determine whether these molecular changes translate into behavioral and memory impairments. Thus, the main goal of the proposed research was to determine

whether memory and cognition were impaired in Aldh2$^{-/-}$ mice, and to determine if there was an age-dependent decline in memory in these mice. The findings of this research, coupled with existing molecular data may serve to further establish Aldh2$^{-/-}$ mice as a novel oxidative stress-based model of age-related cognitive impairment and AD. This model may prove useful both for assessing AD therapeutics and for gaining better insight into the pathogenesis of AD.

1.2 Epidemiology

Alzheimer's disease (AD) is a global epidemic that will affect more than 150 million people within the next 5 years. Reports from the UN Aging Program project that the number of older people (those aged 65 or older) in the world is expected to increase from 420 million in 2000 to approximately 1 billion by 2030 (with developing nations among the most significantly impacted), with an increase in the proportion of older people increasing from 7% to 12% [1]. Because AD is strongly associated with increasing age, it is expected to pose huge issues to public health care across the world as populations continue to age.

AD is the most common form of dementia, a term which encompasses a group of symptoms including memory loss, cognitive dysfunction, and impairment in attention, language, and problem solving, as well as many other areas of cognitive function [2]. Worldwide prevalence of dementia is estimated to be approximately 3.9% in those aged 60 and over, with regional prevalence estimated to be 1.6% in Africa, 4.0% in China, 4.6% in Latin America, 5.4% in Western Europe, and 6.4% in North America [1]. Several meta-analyses have yielded similar data regarding age-specific prevalence of AD across

the world [1]. The incidence rate of AD increases exponentially with age until approximately age 85, although it is unknown whether this continues to increase or plateaus at a particular age. Currently, AD affects more than half a million Canadians, with data showing that approximately 1 in 11 Canadians over the age of 65 is affected by AD, and the likelihood of being afflicted with AD increasing to 1 in 3 after the age of 80 [3].

At the individual level, AD shortens life expectancy, decreases overall quality of life, and is a primary cause of physical disability and institutionalization amongst the elderly [1]. Studies have shown that nearly half of the elderly who develop some form of functional dependence on others (commonly occurring in those suffering from stroke, musculoskeletal disorders, and other cardiovascular diseases) suffer from a two-to-five-fold increase in the risk of death, and overall, the median survival time for people with newly diagnosed AD ranges from 3 to 6 years [4].

Alzheimer's disease has also had a substantial effect on the worldwide economy and global health care systems, which will continue to grow as incidence increases. Long-term institutional care is the main cost in many developed countries for patients with dementia and AD, and it is estimated that about 43% of AD patients require significant care, including that in nursing homes. In the United States, nearly 10 million Americans provided unpaid care for loved ones suffering from AD or dementia [1]. The worldwide societal costs of dementia were estimated to be more than US$300 billion, including one third for informal care, and in the United States, annual costs for patients with AD were estimated at nearly US$148 billion [1]. It is evident that AD will have a significant economic impact on families worldwide because of the requirement for constant care and

therapy, and that this will continue to become a major strain on the world economy in the years ahead.

1.3 Sporadic vs. Genetic AD

Genetic and sporadic AD are two distinct forms of AD, each with varying risk factors and pathologies. Sporadic AD accounts for the vast majority (nearly 95%) of all AD cases, and typically strikes much later in life, usually after the age of 65. While genetics can play an important role, the primary risk factor for sporadic AD is age, and the majority of cases are based on an age-related accumulation of malfunctions, although other factors including hypertension, diabetes, and cardiovascular disease can also increase the likelihood of developing AD [5]. The most established genetic risk factor for the development of sporadic AD is the inheritance of the *ε4* allele of the *apolipoprotein E (APOE)* gene, and individuals with one *ε4* allele are at a 2-3 fold increased risk of developing AD (rising to nearly 12 fold with the inheritance of two *ε4* alleles) [6]. The APOE protein is important for the transport of lipoproteins, vitamins, and cholesterol into the lymph system and the blood, and has a key role in the development of cardiovascular diseases, although its direct role in the development of AD is very poorly understood [7]. It is believed that other alleles of *APOE* enhance the proteolytic breakage of amyloid-$\beta_{1\text{-}42}$ ($A\beta_{42}$) peptide, whereas the *ε4* allele actually promotes aggregation and formation of amyloid plaques, possibly due to its inability to clear Aβ from the brain, although the mechanism by which this occurs is unknown [7]. Over 50% of people with AD carry at least one *APOE ε4* allele and it is likely that there are other mutations that also play a role in the development of sporadic AD, although none have been definitively determined [6].

Other factors that may influence the development of sporadic AD include estrogen levels, head injury, diet, and physical activity [8].

Genetic AD accounts for approximately 5-10% of all cases, and can be highly aggressive at an earlier age than sporadic AD cases (usually striking between the ages of 50 and 60) [9]. A true understanding of the genetics behind AD began with the discovery that individuals with Down's syndrome inevitably develop the pathological features typical of AD early in adulthood, suggesting a link between AD and a critical gene located on chromosome 21 [6]. Subsequent research led to the discovery of $A\beta_{42}$ and eventually the amyloid precursor protein (APP) gene located on chromosome 21. Further research into the APP gene led to the discovery of the genetic form of AD, termed familial Alzheimer's Disease (FAD), in which patients have pathological features similar to sporadic AD (abundant amyloid plaques and NFTs) but have a much shorter survival time, and exhibit other symptoms including seizures and myoclonus [9]. FAD typically involves genetic mutations in three main genes, the most common of which can be found on the gene encoding APP. There are currently 32 mutations in APP that have been categorized into three main classes: The first type of mutation, commonly known as the Swedish mutation, is located next to the β-cleavage site of APP, where it causes a 10-fold increase in the rate of β-cleavage triggering a significant increase in amyloid plaque formation [10]. The second group of mutations is near the γ-site of APP and changes the specificity of γ-secretase cleavage, causing an increase in the production of $A\beta_{42}$ [10]. Finally, point mutations within the actual $A\beta_{42}$ sequence also appear to increase the aggregation of $A\beta_{42}$[10]. Other mutations that cause an increase in APP expression (such as promoter mutations, or gene duplication) can also lead to FAD [6]. Further research

into the genetics of AD has uncovered two other distinct genes linked to early-onset FAD: presenilin-1 (*PS1*) on chromosome 14, and presenilin-2 (*PS2*) on chromosome 1 [6]. Individuals with mutations of these genes often have additional symptoms including seizures, ataxia, myoclonus, and other psychiatric abnormalities [9]. The presenilins are catalytic proteins in the γ-secretase enzyme complex, and mutations in *PS1* can alter its processing enzymatic activity leading to an increase in the amount of $A\beta_{42}$ (despite appearing to actually decrease total Aβ levels, although the mechanism by which this happens is not fully understood).

1.4 Pathogenesis of AD: Plaques, Tangles, and Neurodegeneration

Despite the fact that AD has been described for over a century, the main etiology of the disease remains uncertain. Three characteristics are commonly seen in AD cases: the accumulation of β-amyloid (Aβ) plaques (numerous dense toxic deposits), the formation of neurofibrillary tangles (NFTs) containing hyperphosphorylated tau protein, and which interfere with normal cellular processes in the brain, and neuronal cell loss and neurodegeneration [3]. Together, these pathologies are associated with the impaired memory, thinking and behaviour typically seen in AD patients. Prior to investigations linking oxidative stress to AD, it was believed that the main pathogenesis of AD involved the accumulation of Aβ plaques consisting primarily of the $A\beta_{42}$ peptide that could then lead to further pathological events including the formation of NFTs, and neuronal degeneration (Amyloid Cascade Hypothesis) [6]. Aβ plaques are made up of small peptide fragments that are created from the proteolytic cleavage of transmembrane APP. APP is a single transmembrane domain protein with several alternate transcripts and,

despite significant research, its functions are not well understood [11]. Two different pathways are involved in the processing of APP one of which results in the formation of $A\beta_{42}$ (summarized in Figure 1)**.** In the amyloidogenic pathway, N-terminal cleavage of APP at the β-site by an aspartyl proteinase known as BACE (beta-site APP-cleaving enzyme) generates soluble APPα and C99 fragments [6]. This C99 fragment is a substrate for γ-secretase which catalyzes C-terminal cleavage of C99, resulting in the formation of $A\beta_{42}$ [12]. The $A\beta_{42}$ peptides contain hydrophobic amino acids which allow them to form aggregates in aqueous solutions, beginning as small assemblies of dimers and trimers, and eventually leading to the formation of oligomers and large insoluble fibrils that are seen in amyloid plaques [6]. Conversely, in the non-amyloidogenic pathway, α-secretase cleaves APP at the α-position within the $A\beta_{42}$ domain generating the APPα and C83 fragments and so precluding the formation of the $A\beta_{42}$ peptide [12]. It has been hypothesized that $A\beta_{42}$ is not always associated with neurotoxicity (especially at low concentrations), and that it may actually have a neuroprotective role. $A\beta_{42}$ is found in a number of different cell types (and also plasma and cerebrospinal fluid) and studies have shown that a peptide consisting of the first 28 amino acids of Aβ can enhance the survival of neurons in the hippocampus, can induce the survival of developing neurons during periods of trophic-factor deprivation, and can protect mature neurons against cell death [13]. $A\beta_{42}$ has also been linked to an essential role in synaptic function, learning, memory, and plasticity and studies have shown that treatment of hippocampal slices with lower concentrations of $A\beta_{42}$ increased long-term potentiation (LTP), and stimulated NMDA receptor-mediated

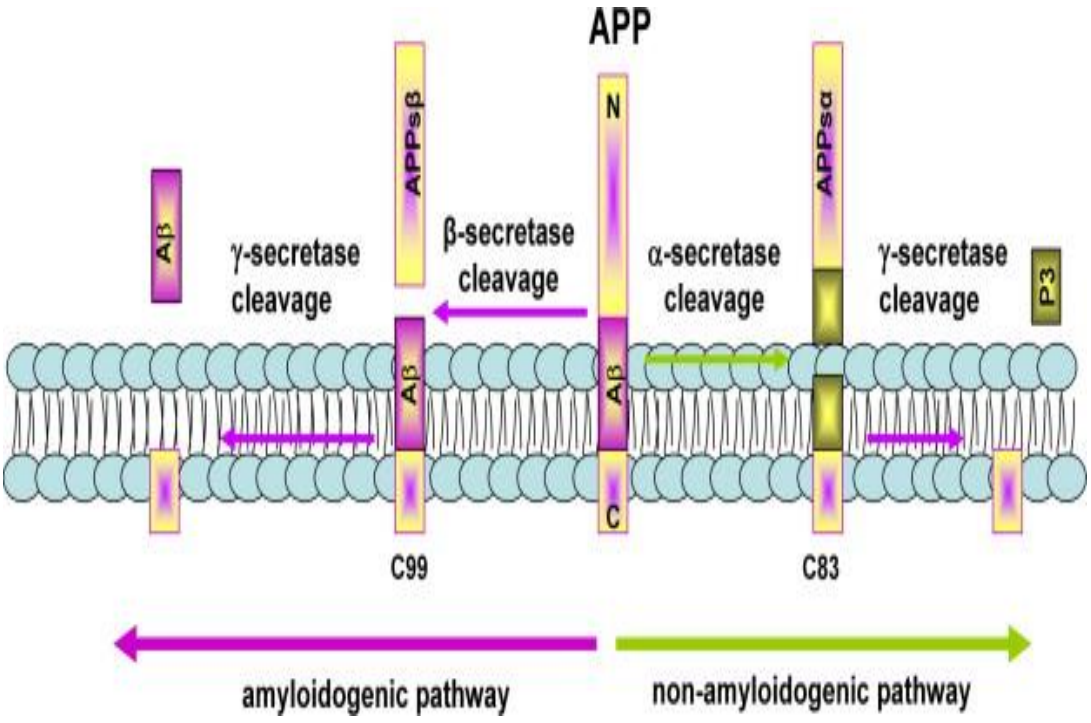

Figure 1.Amyloidogenic and Non-Amyloidogenic Processing of APP
APP can be cleaved by β-secretase and γ-secretase to generate $A\beta_{42}$ in the amyloidogenic pathway. APP can also be hydrolyzed by α-secretase and γ-secretase in the non-amyloidogenic pathway, precluding the formation of $A\beta_{42}$ [88].

synaptic currents [14]. In short, whereas the accumulation of amyloid plaques in the brain due to the abnormal processing of APP has long been thought of as the driving factor behind the pathogenesis of AD, varying neuroprotective and synaptic roles have shown that Aβ can have a much more diverse impact on neuronal cells.

NFTs or tauopathies are an accumulation of tangles that can increase as the severity of AD increases. Early stages of AD are characterized by small abnormal fibrillary inclusions within affected nerve cells which aggregate into large bundles that fill the entire neuronal cytoplasm, eventually forming NFTs [15]. Immunocytochemical analysis has shown that although NFTs can contain phosphorylated neurofilaments and ubiquitin, the major component is the hyperphosphorylated and aggregated tau protein, an important microtubule-associated protein (MAP) in neurons that promotes the assembly and stability of microtubules and vesicle transport [3]. Under normal conditions, tau can be phosphorylated at numerous sites that function to regulate the interaction between tau protein and its targeted microtubules [16]. However, during the progression of AD, tau can become hyperphosphorylated, leading to its dissociation from microtubules into a soluble cellular pool and eventual aggregation into paired helical filaments that ultimately form NFTs [16]. Despite the importance of tau in the progression of AD, evidence suggests that the formation and accumulation of Aβ plaques precede tau hyperphosphorylation and aggregation, indicating that the formation of NFTs may be a consequence of Aβ formation [16]. In a study performed by Götz *et al* [17], synthetic $A\beta_{42}$ fibrils were injected into the somatosensory cortex and the hippocampus of 6 month-old tau transgenic mice and it was only after a significant accumulation of amyloid plaques (approximately 18 days later) that NFTs began to aggregate. However, the

mechanistic basis for the aggregation of tau proteins remains unknown and the majority of research on AD has focused on Aβ plaque formation.

Neurodegeneration is possibly the most important pathological hallmark of AD, with patients suffering a significant loss in neuronal cells as they age. It has long been hypothesized that the deposition of insoluble and toxic $A\beta_{42}$ can lead to neurodegeneration, characterized initially by synaptic injury followed by a loss of cortical and hippocampal neurons [18]. This is followed by astrogliosis, microglial cell proliferation, and the development of NFTs [19]. However, the role of $A\beta_{42}$ in promoting neurodegeneration is widely debated. Some studies have shown that $A\beta_{42}$ can accumulate in the neuronal endoplasmic reticulum as well as extracellularly, and that neurodegeneration and synaptic loss may occur as a result of the abnormal accumulation of $A\beta_{42}$ oligomers which may interfere with synaptic function by altering synaptic proteins such as post-synaptic density-95 (PSD95), or glutamate receptors [19]. Other possibilities currently under investigation include the idea that Aβ may cause the formation of pore-like structures with channel activity, mitochondrial dysfunction, lysosomal failure, or other alterations in cell signalling cascades that are involved in synaptic plasticity or neuronal cell death [19]. Alterations in other signalling proteins, including cyclin-dependent kinase-5 (CDK5), and members of the mitogen activated protein kinase (MAPK) family such as extracellular signal-regulated kinase (ERK) may also lead to the abnormal phosphorylation and aggregation of tau proteins, again promoting neurodegeneration. Hyperactivation of CDK5 (the predominant CDK found in the brain, and a key protein involved in synaptic plasticity and neuronal development) and its activators p35 and p25 have been observed in the brains of AD patients [20]. CDK5 is

a protein kinase that phosphorylates a variety of different synaptic proteins, including PSD95, synapsin, and cadherin, as well as other transcription factors. The primary activator of CDK5 is p35; however, under high calcium conditions, p35 can be cleaved to p25 which can hyperactivate CDK5 and lead to abnormal phosphorylation of other substrates, including tau which may play a key role in the pathogenesis of AD [21].

Cell death via the activation of caspases has also been implicated in neurodegeneration in AD. Apoptotic cell death (or programmed cell death) can occur in an area that is not affected by injury, and is the predominant form of cell death in neurodegenerative diseases such as AD [22]. The major proteases involved in apoptosis are the caspases which, when activated, initiate cell death by destroying key components of cellular infrastructure, and activating factors that damage cells [22]. At least 7 different caspases (caspases 1, 2, 3, 6, 8, 9, and 12) are thought to play a role in regulating neuronal cell death in response to changes in Aβ in the brain of AD patients [23]. Caspases can be divided into two major groups, interleukin 1β converting enzyme-like (which are involved in the proteolytic processing of cytokines and have an indirect effect on apoptosis via modulation of inflammatory responses), and cell death protein-3-like (which are directly involved in apoptosis and can be further subdivided into initiator and downstream effector caspases) [23]. There are two main pathways by which caspase-dependent cell death can occur: the extrinsic pathway, in which a cell death ligand can bind and trigger caspase-8 activation, and the intrinsic pathway whereby the mitochondria release cytochrome c into the cytosol to trigger cell death [22]. It has been hypothesized that caspases may play a role in AD pathogenesis via the caspase-3-mediated cleavage of APP which may facilitate the formation of $A\beta_{42}$, and through caspase activation and cleavage of tau which may

facilitate the formation of NFTs [24]. Although caspases have been extensively studied, their complete role in the progression of AD is not well understood and remains a key target of investigation.

In addition to loss of synapses, neurodegenerative processes in AD have been shown to be accompanied by alterations in neurogenesis, indicating a possible two pronged approach contributing to neurodegeneration in AD [19]. Neurogenesis in a healthy nervous system occurs throughout adult life, and is a complex process characterized by several important steps including proliferation, migration, differentiation, and maturation through growth and synaptogenesis, and can be regulated by a number of distinct molecular mechanisms including key markers of cell division [19]. Although it is not well understood, it is possible that the pathologies of AD may trigger a change in one, or a combination of these underlying processes of neurogenesis that may slow the development of neurons. Coupled with an increase in neuronal cell loss, this may be a key contributing factor towards the progressive neurodegeneration seen in AD.

In summary, the accumulation of amyloid plaques, NFTs, and neurodegeneration and cell death are the three main pathological hallmarks of AD. Despite the extensive amount of research that has been done to determine the mechanisms behind their pathologies, much remains unknown about how they contribute to AD pathogenesis, although they are all thought to be intrinsically linked.

1.5 Memory and Behavioral Impairments

As mentioned previously, there are a number of different behavioral changes associated with AD, including memory loss, and cognitive dysfunction. Researchers have found that early stages of AD can be characterized by a decline in executive function, processing speed, verbal capacity, visual-spatial abilities, and attention [2]. These changes appear to arise from brain lesions originating in the temporal lobe, which eventually lead to impairments in a number of different types of memory. Long-term and short-term memory (LTM and STM respectively) are the two major memory systems that have been distinguished in humans and depending on the stage of AD, patients have been shown to experience some sort of long-term or short-term memory impairment.

Short-term memory refers to the capacity of the brain to hold a small amount of information in an active and readily available state for a short period of time. STM has a fairly limited capacity (between 5 and 9 items can be typically stored), and a limited duration [25]. STM is also involved in the selection, initiation, and termination of information-processing functions such as encoding, storing, and retrieving data [25]. Although they share some characteristics, spatial working memory, one of the most commonly studied forms of memory in AD, is now understood to be a distinct and evolved form of STM [26].While STM can simply refer to only the temporary storage of information, working memory is a more evolved concept that refers to the processes used to temporarily store, organize, and manipulate information [26]. Spatial memory refers to the part of memory responsible for recording information about the environment and its spatial orientation, and is responsible for the successful learning and navigation in complex environments [27]. Working memory refers to a limited capacity system that

allows one to temporarily store and process information, and is often linked with spatial memory in order to understand and process information about the surrounding environment [27]. Spatial disorientation is a common symptom of AD that can have a significant impact on patients and their caregivers, and it is an early indicator of impaired spatial and working memory. Neuroimaging techniques have shown that parietal and temporal cortices (areas of the brain involved with short-term memory) as well as medial temporal structures, including the hippocampus, are damaged early in AD indicating a link between neurodegeneration and memory impairment [28].

As AD progresses LTM can also become significantly impaired. LTM refers to the memory in which associations amongst items are stored, and can be sub-divided into two main categories, declarative (or explicit) and non-declarative (or implicit) memory [29]. Declarative memory refers to the conscious recollection of facts and events, and it is thought to be under the control of the hippocampus and other related temporal lobe connections [30]. Non-declarative or implicit memory refers to the unconscious acquisition of information and can be further subdivided into other forms such as non-associative learning, and motor, perceptual or cognitive skill acquisition, all of which are independent of the medial temporal lobe, and diencephalon [30]. One of the most commonly tested forms of declarative memory is recognition memory, a form of memory that refers to ability to recognize previously encountered events, objects, or people [29]. The dual-processing theory of recognition memory states that recognition depends on two types of memory processing: familiarity (based on the ability to determine a general similarity between test items and studied items), and recollection (based on the retrieval of item-specific information of studied items, including things like physical

characteristics, and contextual information) [31]. Many neuroimaging studies have shown that both familiarity and recollection are correlated with activities in various areas of the brain; familiarity is related to activity in the parahippocampus and anterior medial temporal lobe, whereas recollection is correlated with activity in the hippocampus, prefrontal lobe, and parietal lobe [31]. During the process of normal aging, the frontal lobe degenerates at a relatively early stage, thereby causing a decline in recollection memory over time while keeping familiarity processing relatively intact [32]. However, in AD, pathological changes in both the frontal lobe and hippocampus result in impaired recollection and familiarity processing [32]. As a result, AD patients show a significant decline in correct recognition and an increase in false recognition.

As mentioned above, the brain areas associated with learning and memory systems include the cerebral cortex, the hippocampus, and the parahippocampal region. The cortical areas provide perceptual and motor information to the hippocampus; an area of the brain composed of several cell layers and organized in a manner that allows information to be processed through a series of defined circuits [33]. Through the use of cellular models, it has been hypothesized that the transfer of information within the hippocampus occurs through glutamatergic receptors and via long-term potentiation (LTP), a key process associated with plasticity and the formation of long-term memories whereby a long-lasting enhancement in signal transmission between two neurons can form as a result of high-frequency electrical stimulation[33]. Early experiments by Morris and colleagues demonstrated that glutamatergic NMDA receptors were important for spatial learning and synaptic plasticity via LTP when they showed that NMDA receptor antagonists directly infused into the hippocampus resulted in impaired spatial working

memory in rats as well as impaired LTP [34]. Additional experiments with homozygous mice lacking NMDA receptors, specifically in the CA1 region of the hippocampus, demonstrated impairment of a variety of LTP-induced spatial working, and non-spatial memory tasks [35].

Although the mechanism underlying LTP and its relationship with memory are not fully understood, it is thought that a sufficient electrical stimulation of the presynaptic neuron in the hippocampus causes the release of glutamate from the axon terminal, which can then bind to AMPA receptors on the postsynaptic neuron, depolarizing the membrane sufficiently to relieve the magnesium ion block of the NMDA receptor channel. This renders the channel responsive to glutamate, allowing the entry of calcium and sodium ions through the channel and subsequent activation of several cell signalling cascades [36]. Specifically, calcium ions can bind to calmodulin, leading to calcium/calmodulin-dependant activation of several protein kinases, which can further affect AMPA receptors, increasing their permeability to sodium ions, and increasing the availability of AMPA receptors in the membrane, thereby increasing the response strength to a stimulus, and strengthening the synapse [36]. Also, these protein kinases can translocate to the nucleus, where they can activate cAMP response element-binding protein (CREB), a transcription factor that has been shown to be important in spatial memory, and long-term potentiation [37]. This strengthening of the synapse via glutamate-induced LTP and through the activation of CREB (summarized in Figure 2) is thought to be the major cellular mechanism that underlies learning and memory. Of relevance, $A\beta_{42}$ has been shown to impair glutamate release, induce cell death pathways, and disturb the mitochondrial membrane, leading to cellular dysfunction and a reduction in LTP [36].

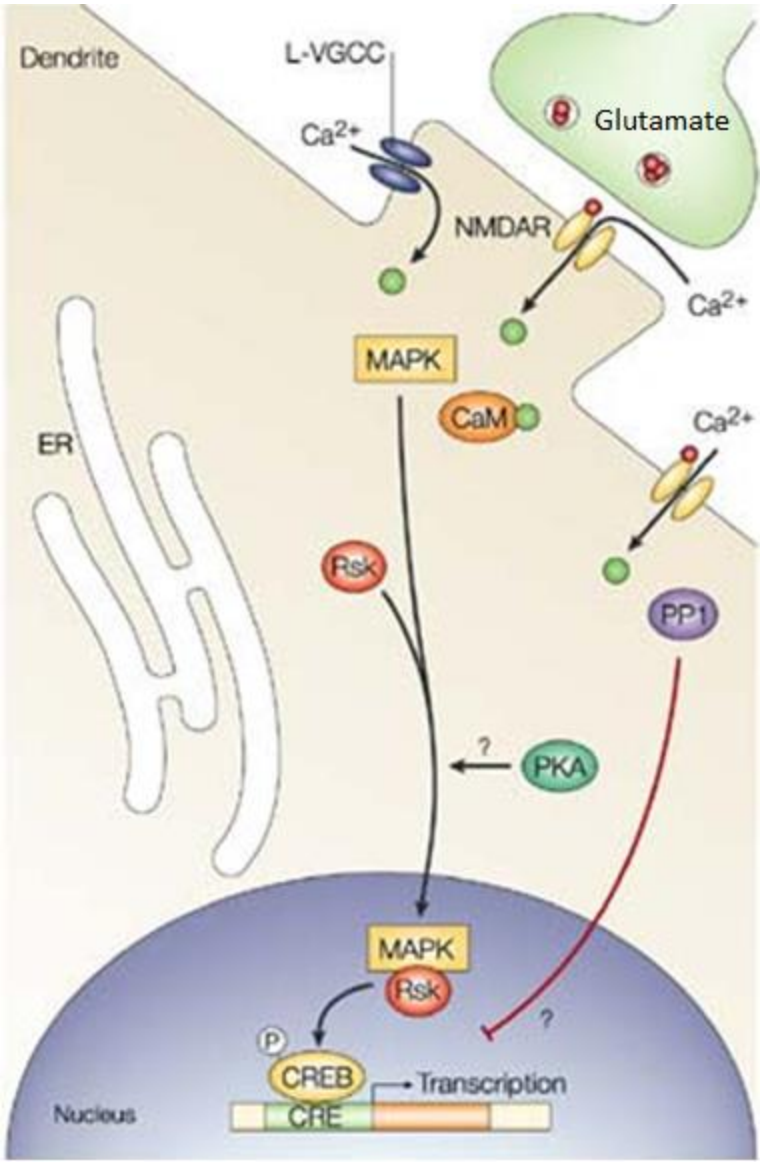

Figure 2. Glutamate and Calcium Induced Activation of CREB
In response to glutamate, calcium flows into the cell via NMDA receptors (as well as through voltage-gated ion channels). The calcium ions can then bind to calmodulin (CaM), leading to calcium/calmodulin-dependant activation of protein kinases (such as PKA) which can eventually translocate to the nucleus where it can phosphorylate and activate CREB, leading to downstream changes in LTP and memory (modified from [37])

There are a number of ways in which memory can be tested in animal models of AD. For example, the Morris Water Maze (MWM) was extensively used to study the effects of NMDA-induced LTP on spatial learning and memory in rodent models. The test is conducted in a large circular pool of water with a platform that has been hidden beneath the water in one specific area of the pool. The animal uses spatial cues surrounding the pool to navigate and find the hidden platform and, as it learns the location of the platform over repeated trials, its swim time decreases, and a higher proportion of time is spent swimming in the area near the platform [38]. Although the MWM has been extensively used because of its relative ease and, the speed in which the animals can learn the task or demonstrate memory impairments, it has a number of limitations. These include the significant amount of stress that is placed on the animals during the test, and that the task can have much less specificity when testing different types of memory, as well as different areas of the brain. Also, a number of variables can influence the results obtained from a MWM test including the dimensions of the pool, water temperature, and training schedules [38].

The radial arm maze (RAM) and Y-maze have also been commonly used to study spatial and non-spatial memory in animals, as well as working and reference memory. The RAM consists of a central area from which eight arms of identical size radiate outward. Spatial cues are placed surrounding the maze (or along the walls of the maze), and food rewards are placed in some of the arms. Animals must remember the location of the food using the spatial cues surrounding the maze. Over repeated trials, the animals learn to avoid re-entry into arms where food has been retrieved, and in general, behavior is measured as the number of arms the animal enters before repeated entry into an arm, as

well as the total time required to retrieve all food (animals with impaired memory take longer to retrieve all food, and repeatedly enter a previously visited arm) [39]. The Y-maze is a simpler version of the RAM, designed with three arms branching out from a central area in the shape of a Y. In general, spontaneous alternation is used as a measure of spatial working memory whereby the animals are placed in the center of the maze and allowed to explore for a set period of time, and the number of arms entered and the sequence of entries are recorded in order to determine an alternation rate. An alternation occurs when an animal enters an arm that is different from two previously visited arms, and a higher alternation rate generally equates to sustained memory and cognition, whereas a low alternation rate indicates memory impairment [40] The advantage of both the Y-maze and the RAM is that they are very simple and cost-effective ways to test spatial working memory in animals, with very few trials required for the animals to learn the maze or display memory impairment (especially with the Y-maze). Also in contrast to the MWM, both the RAM and the Y-maze avoid the significant stress placed on the animals as a result of swimming in a pool of water. The main disadvantage for the RAM is that the animals need to be food deprived for a period of time prior to testing to motivate them to explore the maze adequately.

Object recognition is a fast and effective way to assess recognition memory in animals and is based on the innate preference of an animal to explore a novel object rather than a familiar object [41]. Initially described by Ennanceur and Delacour [41] the animal is placed into an arena or open field with two identical objects and is allowed to explore them for a set period of time. After a period of exploration, the animal is removed and a delay is imposed which can vary between a few minutes to several hours. After the delay,

the animal is placed back into the arena where one of the objects is replaced with a completely novel object (usually of similar size, but differing in shape and colour), and the amount of time spent sniffing, or examining the novel and familiar object is recorded. Animals with sustained recognition memory should spend the majority of their time with a novel object rather than the familiar object. The novel object recognition task (NOR) is a simple method that can be completed in a short amount of time and that does not require external motivation rewards or punishment (although it does require a training period) and therefore limits stress placed on the animals. The main limitation to this test, however, is that the level of exploration by the animals can be low or inconsistent, although there are experimental manipulations that can be performed in order to control for inactivity [42].

The study of memory systems in the brain is extremely complex and depends on a number of different factors and mechanisms that are not completely understood. Although there are a number of different cognitive and behavioral impairments associated with AD, the impairment of memory throughout the progression of the disease is one of the most important factors for both patients and caregivers, significantly impacting quality of life. A number of paradigms have been developed to test the behavior in animal models of AD, and these have contributed to a further understanding of the impact of AD pathologies on memory and cognition. Further research into the types of memory that can be affected by AD, and the regions of the brain involved will help support the development of new techniques to study memory in animal models and improve our understanding of the way in which AD affects normal cognitive function.

1.6 Current Models of AD

Much of what is understood about the pathogenesis of AD was derived from a variety of animal models that have been developed to study different aspects of AD. Also, because of the uncertainty and challenge behind clinical trials and their ability to safely test the efficacy of novel agents in human subjects, the development of animal models that adequately mirror the pathologies seen in human AD is crucial to the development of novel therapeutic agents. Since the early development of transgenic mouse models that displayed minimal characteristics of AD neuropathology, a number of different animal models have been developed that can display more aggressive and complex phenotypes. Species including dogs, cats, bears, as well as many primates spontaneously develop plaque pathology (and some exhibit tauopathies), but have limited use in research due to availability, and economic or ethical reasons [43]. Rodents have been the animal of choice for research of AD because of their economic viability, and availability, despite the fact that ageing rodents do not spontaneously develop AD-like pathological hallmarks [43]. The majority of these are transgenic mouse models that rely on the overexpression of genes that contain mutations associated with FAD, resulting in a significant increase in the accumulation of Aβ in the brain, but showing minimal neurodegeneration or hyperphosphorylated tau. The PDAPP mouse model was the earliest transgenic model, developed in the mid-1990s, and was followed in subsequent years by the development of the Tg2576 and APP23 mouse models [44]. The PDAPP mouse model expresses human APP with the Indiana familial AD mutation, whereas Tg2576 and APP23 mice model express human APP with the Swedish mutation. Data derived from all of these models

support the amyloid cascade hypothesis whereby they display progressive Aβ deposition, cerebral amyloid angiopathy, astrocytosis (abnormal increase in the number of astrocytes due to destruction of nearby neurons), hippocampal atrophy, synaptic and neurotransmitter alterations, and cognitive and behavioral deficits [44]. These animal models have helped describe the central role APP and Aβ can have in AD pathology, and have helped initiate the development of disease-modifying drugs that target the amyloid cascade (described later), despite the fact that they do not show an increase in NFT formation [43].

The discovery of mutations of the PSEN genes associated with FAD led to the development of PSEN1 and PSEN2 transgenic mouse models of AD, which show an increase in $A\beta_{42}$ levels, but do not show plaque pathology, and display very few cognitive or behavioral abnormalities, as well as a lack of NFT formation [45]. However, these models have proven useful in the development of double transgenic APP/PSEN mice which have shown an accelerated accumulation of Aβ pathology as well as neuronal loss, inflammation, and behavioral alterations, which speaks to the importance of PSEN in FAD [45]. In order to overcome the lack of NFTs in these models, mutated human tau mice were developed and the combination of tau and APP mutations showed enhanced amyloid deposition followed by tau hyperphosphorylation, NFT formation, and neuronal cell loss [43].

One of the main limitations of these models was the lack of co-localization of plaques and NFTs in brain regions that are relevant to AD (like the hippocampus or cortex) in these mice. This was addressed with the development of the triple transgenic 3xTg AD mouse model whereby mutant APP and tau constructs were injected into single-

cell embryos from mutant PSEN1 mice, preventing the segregation of APP and tau genes in further generations [45]. These mice develop amyloid plaques prior to NFTs (in accordance with the Aβ cascade hypothesis) and have a temporal and spatial localization of plaques and NFTs equivalent to AD (as well as inflammation, synaptic dysfunction and behavioral impairments) [45].

In order to study tau-related neurodegeneration, single tau-knockout transgenic models were developed, although these mice did not show any overt pathologies [46]. After the discovery that mutations of tau gene were linked to chromosome 17 and were important in dementia and Parkinson's disease, transgenic models using these mutations were developed and displayed a significant increase in tau aggregation and neurodegeneration [46]. The development of these mice greatly supported researchers in their pursuit to uncover the importance of NFTs in AD.

1.7 Pharmacological Treatment of AD

The development of these models has also contributed greatly to drug development efforts by providing a variety of different molecular drug targets. Although there is no current cure for AD, there are a limited number of therapeutic agents available that provide limited benefits to AD patients. The main goal of current therapies in those with mild to moderate AD is to maintain or improve baseline performance while in more progressed cases (where behavioural and cognitive impairments have significantly impaired the health of the patient), the goal of treatment is to slow the rate of decline [43]. The only approved pharmacological approach to treating AD (prior to the approval of memantine), is based on the idea of enhancing cholinergic neurotransmission through

the use of acetylcholinesterase inhibitors. The idea behind this line of treatment was the "cholinergic hypothesis" of AD which theorized that the degeneration of cholinergic neurons in the basal forebrain and the loss of cholinergic neurotransmission in the cerebral cortex were the main factors behind the significant decline in cognitive function seen in AD [47]. The acetylcholinesterase inhibitors donepezil, rivastigmine, and galantamine have all been approved by the Food and Drug Administration (FDA) and are commonly prescribed by physicians [48]. They work by inhibiting the breakdown of acetylcholine by acetylcholinesterase, thereby resulting in greater activation of postsynaptic acetylcholine (ACh) receptors which helps to re-establish cholinergic neurotransmission effects on post-synaptic neurons [47].

The only other approved AD therapeutic is the *N*-methyl-D-aspartate (NMDA) channel blocker memantine. It has been approved for the treatment of moderate-to-severe AD, although its effects are only modest, and there is very little evidence of effect in mild AD. Glutamate is the primary excitatory neurotransmitter in the CNS, with important roles in neurotransmission and synaptic plasticity. The NMDA receptor has a complex structure with binding sites for glutamate and other ligands [49]. In AD, an increase in extracellular glutamate can lead to excessive activation of NMDA receptors leading to an increased calcium influx, initiating a cascade of events that can result in neuronal cell death (glutamate excitotoxicity) [48]. The NMDA receptor channel blocker memantine has been approved by the FDA for the treatment of moderate to severe AD, and has been shown to have a modest effect on memory, attention, and reasoning, and on the ability to perform simple tasks [50]. Memantine is a non-competitive NMDA receptor antagonist that is thought to confer neuroprotection through inhibition of glutamate-mediated

toxicity, although in most cases, memantine does not provide a significant clinical benefit [50].

A variety of other novel approaches have been developed to treat AD but none have successfully completed clinical trials. Because of the critical roles of β-secretase and γ-secretase in the formation of $A\beta_{42}$, their pharmacological inhibition has been proposed as a possible method of treatment to inhibit plaque formation in AD [51]. Several inhibitors have been recently developed and have been tested in animal models of AD, where they have been shown to reduce amyloid plaque accumulation. However, these compounds have failed at clinical trials because of their severe adverse effects and lack of efficacy in reducing human amyloid plaque accumulation [51].

Vaccination, both active and passive, has also been used as a strategy for treating AD. Active immunization using intraperitoneal vaccination of the PDAPP transgenic mouse model of AD with $A\beta_{1-42}$ mixed with Freund's adjuvant (a solution of antigen that can be used as a stimulator of the immune system) has been used as a method of preventing the buildup of amyloid plaques in the brain [48]. Investigators reported an almost complete prevention of Aβ deposition in 6-week-old mice and a general slowing of the progression of AD pathology (a reduction in both neuritic dystrophy and astrogliosis) in older mice [48]. Despite the promising results in animal models, clinical trials failed at phase II when patients developed signs and symptoms of meningoencephalitis, and follow up studies determined that cognitive decline was identical to patients under placebo treatment despite the complete removal of Aβ [52].

Bapineuzumab and solanezumab have been recently developed for the treatment of AD. These agents are humanized monoclonal antibodies, that bind to the central region

of $A\beta_{42}$ with the expectation that they reduce the accumulation of plaques in the brains of AD patients [53]. There was a strong hope that these drugs represented the first effective passive vaccine for AD treatment. Unfortunately, bapineuzumab failed to improve cognitive or functional performance compared with placebo patients (patients with familial AD were excluded from clinical trials), and the results from solanezumab clinical trials were only mildly encouraging (although side effects associated with bapineuzumab were not seen with solanezumab), calling into question the efficacy of drugs targeting amyloid plaques in AD patients [53].

The amyloid cascade hypothesis has been the most generally accepted hypothesis of AD etiology, despite conflicting theories behind the role of $A\beta_{42}$ in the brain. De la Torre (2004) summarised a number of different issues that have been raised with respect to the amyloid cascade hypothesis [18]: (1) $A\beta_{42}$ deposition in the brain does not adequately correlate with dementia severity, (2) patients without dementia have plaques, much in the same way as patients with dementia, (3) despite previous reports amyloid deposition is not the earliest neuropathological event in AD, and is preceded by oxidative stress, (4) many elderly people who are cognitively healthy have plaques but no AD, (5) amyloid deposition does not correlate well with neuronal or synaptic loss, (6) much like in human AD, in transgenic animal models of AD that produce a significantly higher level of Aβ deposits in the brain, there is no correlation between $A\beta_{42}$ levels and neuronal or synaptic loss, (7) and finally these transgenic animals also show cognitive loss before Aβ is found in the brain. Although the amyloid cascade hypothesis and the development of animal models based on the overproduction of Aβ have allowed researchers to better understand AD, it is clear that there are a number of issues with the validity of this

hypothesis and it is likely that alternative molecular mechanisms come into play prior to the appearance of amyloid plaques that may drive the progression of AD.

1.8 Oxidative Stress in AD

Over the last ten years, research has suggested that oxidative stress plays a significant role in the initiation and progression of AD. High brain levels of polyunsaturated fatty acids and redox metals, coupled with high oxygen utilization and modest antioxidant defence, creates an environment especially vulnerable to oxidative damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) [54]. Under normal physiological conditions, the toxic effects of ROS are controlled by a variety of antioxidant systems, including metabolism of toxic aldehydes by aldehyde dehydrogenase. However, under pathological conditions (including the pathogenesis of AD), the accumulation of ROS exceeds the capacity of antioxidant systems, leading to cellular damage and death, especially in neurons particularly vulnerable to oxidative stress [3]. AD brains exhibit significantly higher levels of ROS and RNS, leading to oxidative damage through lipid peroxidation, reactive aldehyde formation, and nucleic acid oxidation. Oxidative damage is often observed prior to the appearance of other AD pathologies, suggesting that oxidative damage could potentially be an initiating event in AD pathogenesis[54]. Redox metals also play a key catalytic role in the production of free radicals, and many metals including iron, aluminum, copper, and zinc, are associated with oxidative stress and may have a role in the progression of AD [55]. Specifically, iron is known to be involved in the formation of the hydroxyl radical, which has well known deleterious effects. The concentration of iron is significantly elevated in AD brains,

particularly in the hippocampus and cerebral cortex, as well as in NFTs and amyloid plaques [55]. Copper is also found in NFTs and amyloid plaques, and its homeostasis is altered in patients with AD, resulting in the formation of ROS through peroxide and hydroxyl radicals [55]. Other sources of oxidative damage and ROS in the pathology of AD include the mitochondria, which are damaged in AD. Mitochondrial enzymes including tricarboxylic acid cycle enzymes, the pyruvate dehydrogenase complex, and cytochrome c oxidase have been shown to have functional abnormalities in AD, and this can result in altered mitochondrial function and energy metabolism leading to an increase in ROS via the production of superoxide and hydrogen peroxide [56].

Transgenic models of AD in which APP is overexpressed also demonstrate a significant increase in oxidative damage prior to the formation of Aβ plaques, consistent with the idea that oxidative damage may be the initiating factor in AD [57]. Tg2576 APP mice, one of the most commonly used animal models of AD, show lipid peroxidation and increases in oxidative stress markers earlier than Aβ plaque deposition, and increases in RNS and ROS correlate with increases in Aβ aggregation [57]. Similar increases in lipid and protein oxidation also occur in the APP/PSEN double-knockout mouse model [58]. Finally, in the 3xTg-AD transgenic mouse model, decreased levels of antioxidant enzymes, and increased levels of lipid peroxidation markers are seen before the appearance of amyloid plaques and NFTs [59]. The early appearance of oxidative stress products in human AD brains and animal models of AD suggest that oxidative damage may be a driving force in AD progression. This has led to the formation of the oxidative stress hypothesis of AD **(**summarized in Figure 3). According to this hypothesis, oxidative stress can increase the formation of Aβ plaques by increasing the expression of APP, or

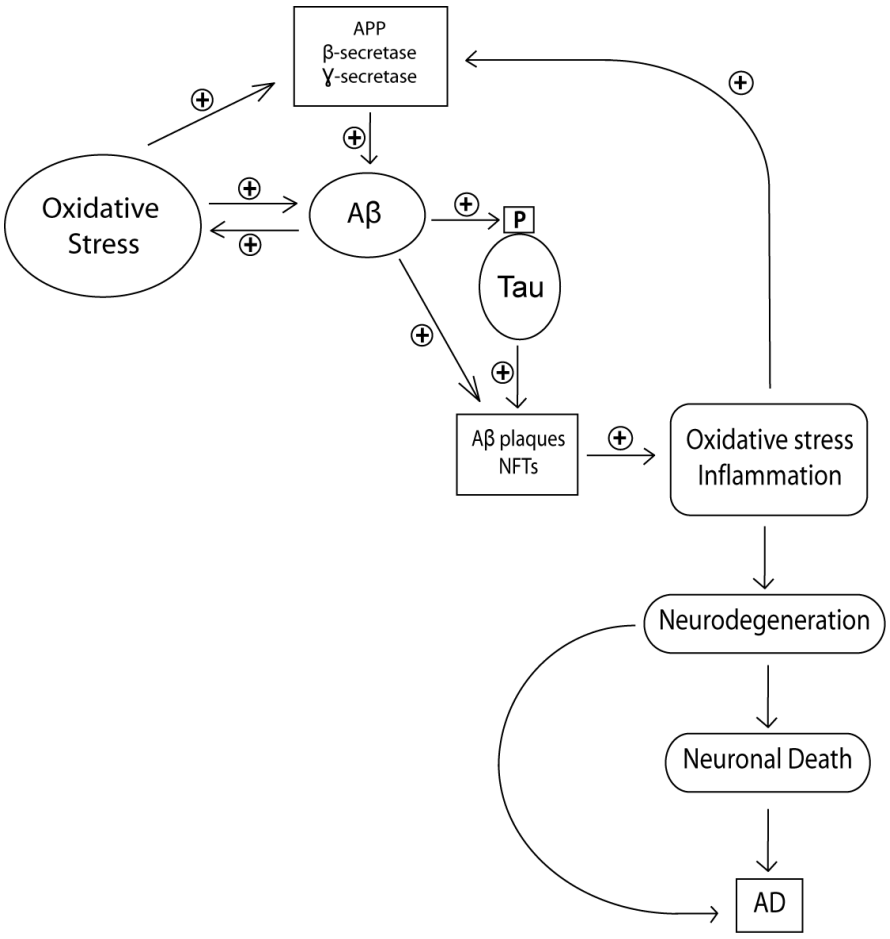

Figure 3 Schematic of the Oxidative Stress Hypothesis of AD.
Oxidative stress can increase the formation of Aβ by directly increasing the expression of APP, or via an increase in the expression or activity of the APP processing enzymes β-secretase and γ-secretase. Aβ can also produce oxidative stress thereby inducing further lipid peroxidation and contributing to a positive feedback on its own formation. This increase in Aβ can lead to the accumulation of amyloid plaques, and hyperphosphorylated tau proteins, resulting in the formations of NFTs. Both NFTs and amyloid plaques contribute to further oxidative stress and inflammation, leading to neurodegeneration and cell death (modified from [54])

via an increase in the expression or activity of the APP processing enzymse, β-secretase and γ-secretase. $A\beta_{42}$ can also produce oxidative stress thereby inducing further lipid peroxidation and contributing to a positive feedback on its own formation. This increase in $A\beta_{42}$ can lead to the accumulation of amyloid plaques, and hyperphosphorylated tau proteins, resulting in the formations of NFTs. Both NFTs and amyloid plaques contribute to further oxidative stress and inflammation, leading to neurodegeneration and cell death.

Increases in oxidative stress in the brain have been shown to have an important role in the molecular pathologies of AD, and also play a significant role in memory and cognition defecits. The cerebral cortex and hippocampus, both of which are key parts of the brain controlling cognitive and motor functions, are particularly sensitive to oxidative stress and damage by free radicals [60]. There are a number of hypothesized ways in which oxidative stress could impact memory and cognition through alterations in synaptic signalling and plasticity. For example, it is thought that oxidative stress induces an abnormal accumulation of synaptic vesicles in the synapse, including the neurotransmitters, and it is believed that oxidative stress may cause a decline in the depolarization of the neuronal membrane, resulting in a deterioration of many neurotransmission systems that are important in the signalling cascades involved in learning and memory [60]. Other products of oxidative stress are thought to affect neuronal growth, axonal transport, and synaptic function by forming covalent bonds and adducts with proteins involved with these processes and by disrupting calcium signalling, which plays many important roles in the formation and function of neuronal circuits and the structural modification of synapses [61]. Oxidative stress may also induce alterations in synaptic signalling that may trigger the death of neurons, possibly via the induction of

apoptosis-related events such as mitochondrial membrane depolarization and caspases activation in synaptic terminals and dendrites, or through neurotrophic factor withdrawal that may trigger the activation of apoptotic cascades in synaptic terminals [61].

As mentioned above, there are many sources of ROS, including redox metals such as iron and copper (which can lead to the production of hydroxyl radical), and non-functioning mitochondrial enzymes [62]**.** One of the most important products of oxidative stress in AD patients is the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) which is found at significantly higher levels in the brain of AD patients and in transgenic animal models of AD in comparison to other products of oxidative stress [63]. Studies have shown high levels of HNE-protein adduct in the hippocampus and amygdala of AD patients, and have shown that HNE specifically accumulates in areas of the brain susceptible to degeneration in AD [64]. HNE is a neurotoxic molecule that can interfere with the synthesis of RNA, DNA, and proteins and can form Michael adducts with protein nucleophiles that can interfere with, and impair cellular metabolism and signalling [65]. HNE can covalently bind to cysteine, lysine, and histidine residues and can modify proteins resulting in protein aggregation, crosslinking, altered phosphorylation, and inactivation of enzyme activity [61]. This has been proposed as one possible mechanism by which Aβ can aggregate; it is believed that HNE can form 1, 4-conjugate additions at multiple locations on the $A\beta_{42}$ peptide, which can allow it to crosslink with other Aβ peptides, thereby accelerating the formation of Aβ plaques [64]**.** HNE is also thought to increase Aβ production by increasing the activity of BACE and γ-secretase [64]. Increased HNE adducts of nicastrin, a component of the γ-secretase complex, have been observed in AD brains and this correlates with an increase in Aβ plaque burden.

Additionally, HNE has also been shown to increase BACE expression through stress-activated protein kinases pathways [66]. HNE adducts of LDL receptor- related protein 1 (LRP-1), a key protein involved in the transport of Aβ across the blood-brain barrier, have been shown to be increased in AD brains, and alteration of this protein can attenuate the clearance of Aβ from the brain, which is thought to play a key role in the accumulation and formation of plaques [67]. HNE can also covalently modify tau as well as other high molecular weight neurofilament proteins, and this can affect neuronal growth, axonal transport and synaptic function [61]. Glucose and glutamate-transporter proteins and GTP-binding proteins have also been shown to be altered by direct covalent binding of HNE, and the molecular function of HNE-adducted ion-motive ATPases and glucose and glutamate transport systems has been shown to be drastically altered in synaptosome preparations, suggesting that HNE and other products of oxidative stress can modify synaptic ion homeostasis, energy metabolism, and glutamatergic transmission [61]. Finally, other proteins that have been shown to be bound to HNE in AD brains include glutamine synthetase (enzyme that plays crucial role in metabolism of nitrogen), manganese superoxide dismutase, and peroxiredoxin (antioxidant enzymes that control peroxide levels) [68]. Together, these studies suggest a significant link between HNE, oxidative stress, and AD pathogenesis.

1.9 Aldh2 and Oxidative Stress

There are three main pathways by which HNE is metabolized in the brain. These include adduction with glutathione (through the enzyme glutathione transferase), reduction by aldo-keto reductase and, most importantly, oxidation by aldehyde

dehydrogenase 2 (ALDH2). Evidence suggests that the primary route of metabolism of HNE in the brain is through ALDH2 [69]. ALDH2 catalyzes the oxidation of HNE to the non-electrophilic and non-reactive metabolite 4-hydroxynon-2-enoic acid (HNA), thus reducing the toxicity of HNE and its impact on neurons in the brain [70]. ALDH2 can be found in many different brain regions, including the hippocampus, frontal and temporal cortex, and cerebellum, and ALDH2 expression and activity are increased in AD brains, and is the only metabolizing enzyme of HNE to do so. These data suggest that higher concentrations of the ALDH2 are needed to cope with increases in free HNE, and that increased expression of ALDH2 may serve as a protective response to lipid peroxidation that can occur during the progression of AD, in order to limit oxidative damage [71]. Population-based studies have examined the association of AD risk in individuals possessing the ALDH2 Glu504Lys polymorphism which is present in approximately 30-50% of the East Asian population [72]. In individuals with this polymorphism, ALDH2 activity is reduced by approximately 90-95%. Although inconsistent findings have been reported, these studies have shown that, whereas there was no increased risk of AD associated with the variant ALDH2, subgroup analysis did indicate that there was a significant association in males [72]. Animal studies have demonstrated that transgenic mice with a dominant-negative form of ALDH2 displayed an increase in HNE formation leading to neuronal degeneration and impaired cognitive function that was age-dependent [69]. These data suggest that ALDH2 is crucial for the detoxification of toxic aldehydes such as HNE, and that there is a link between the accumulation of HNE in the brain and the pathogenesis of AD.

1.10 Rationale, Hypothesis and Objectives

Aldh2$^{-/-}$ mice have been previously used in our laboratory in studies of organic nitrate tolerance. However, after reviewing the link between ALDH2 and its metabolism of HNE, as well as the critical role of HNE and oxidative stress in initiating AD, we propose that Aldh2$^{-/-}$ mice represent a new, oxidative stress-based model of age-related cognitive impairment and AD. Our preliminary data (Figures 4-9, [89]) have shown that Aldh2$^{-/-}$ mice exhibit significant increases in HNE adducts as early as 3 months of age, as well as at 9 months. We have also shown significant increases in intraneuronal $A\beta_{42}$ at 3, 9, and 12 months and in phosphorylated tau protein at 9 months. In addition, we have found significant brain atrophy, increased activated caspases 3 and 6, and decreased PSD-95 and phosphorylated CREB, all of which are key components of AD that are not often seen in current transgenic animal models of AD. Whereas these molecular data show the potential of the Aldh2$^{-/-}$ mice, in order to further characterize these mice as a model of cognitive impairment and AD, appropriate behavioral analyses are required to determine whether these molecular changes translate into behavioral and memory impairments in these mice. Thus, the main goal of the proposed research was to determine whether memory and cognition were impaired in Aldh2$^{-/-}$ mice, and to determine if there was an age-dependent decline in memory in these mice. The findings of this research, coupled with existing molecular data, may serve to further establish Aldh2$^{-/-}$ mice as a novel oxidative stress-based model of age-related cognitive impairment and AD. This model may prove useful both for assessing AD therapeutics and for gaining better insight into the pathogenesis of AD.

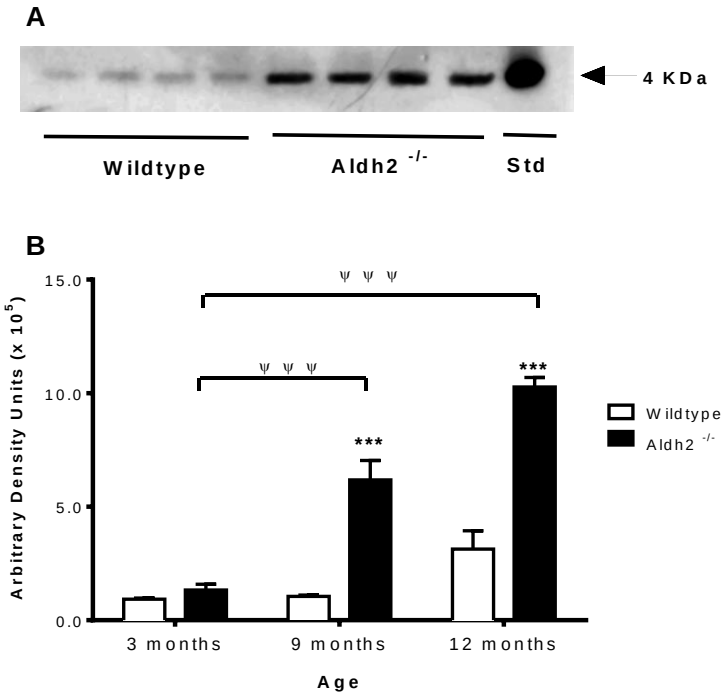

Figure 4.Age-dependent Changes in $A\beta_{42}$

Immunoblot showing age-dependent increase in $A\beta_{42}$ in the hippocampus of $Aldh2^{-/-}$ mice at 3, 9, and 12 months of age (each blot represents a different animal at 9 months of age). Data are presented as the mean ± S.D. (n=4) and were analyzed by a Student's *t*-test for unpaired data, or by a two-way ANOVA. * were used to indicate significant differences from wild type (***$p<0.001$). Ψ were used to indicate significant differences in $Aldh2^{-/-}$ mice from 3 months (ψψψ $p<0.001$) [89].

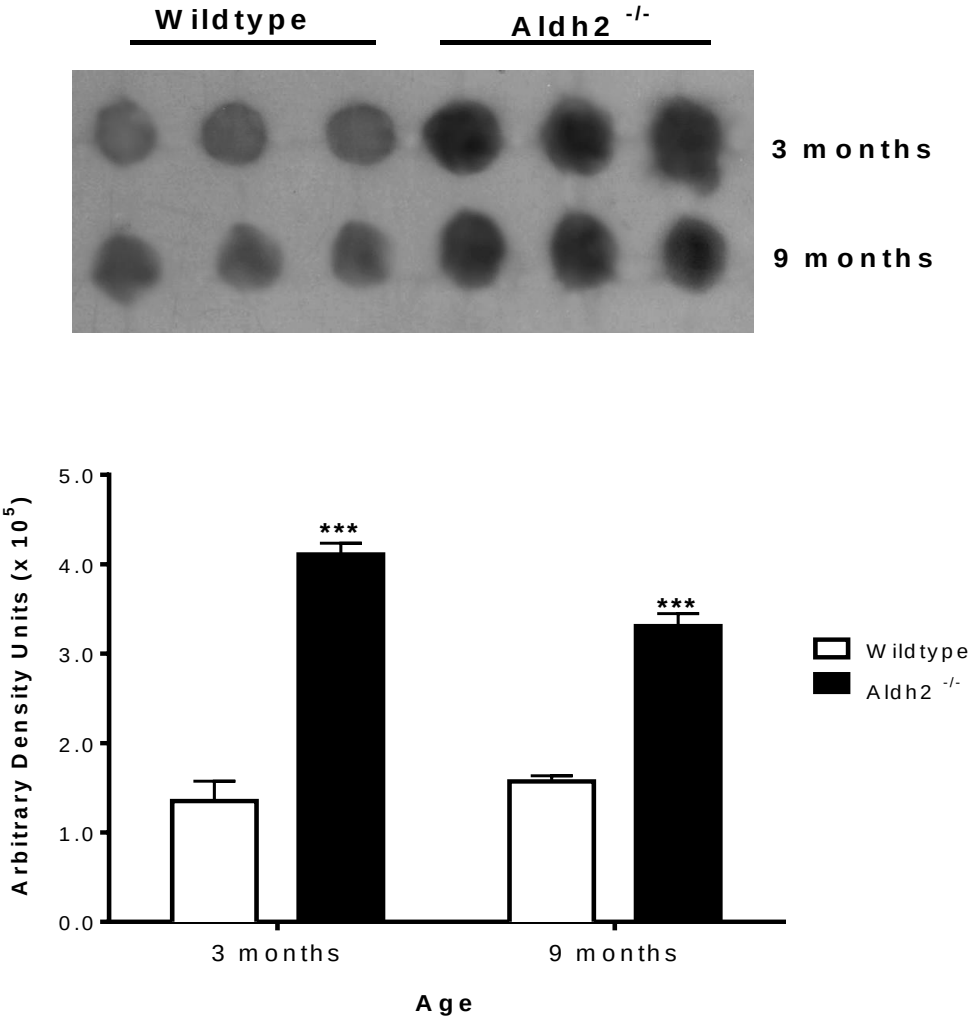

Figure 5. HNE Adduct Formation
Immunoblot showing increased HNE adduct formation in the hippocampus of Aldh2$^{-/-}$ mice at 3 and 9 months of age. Data are presented as the mean ± S.D. (n=3-4) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant differences from wild type (***p<0.001) [89].

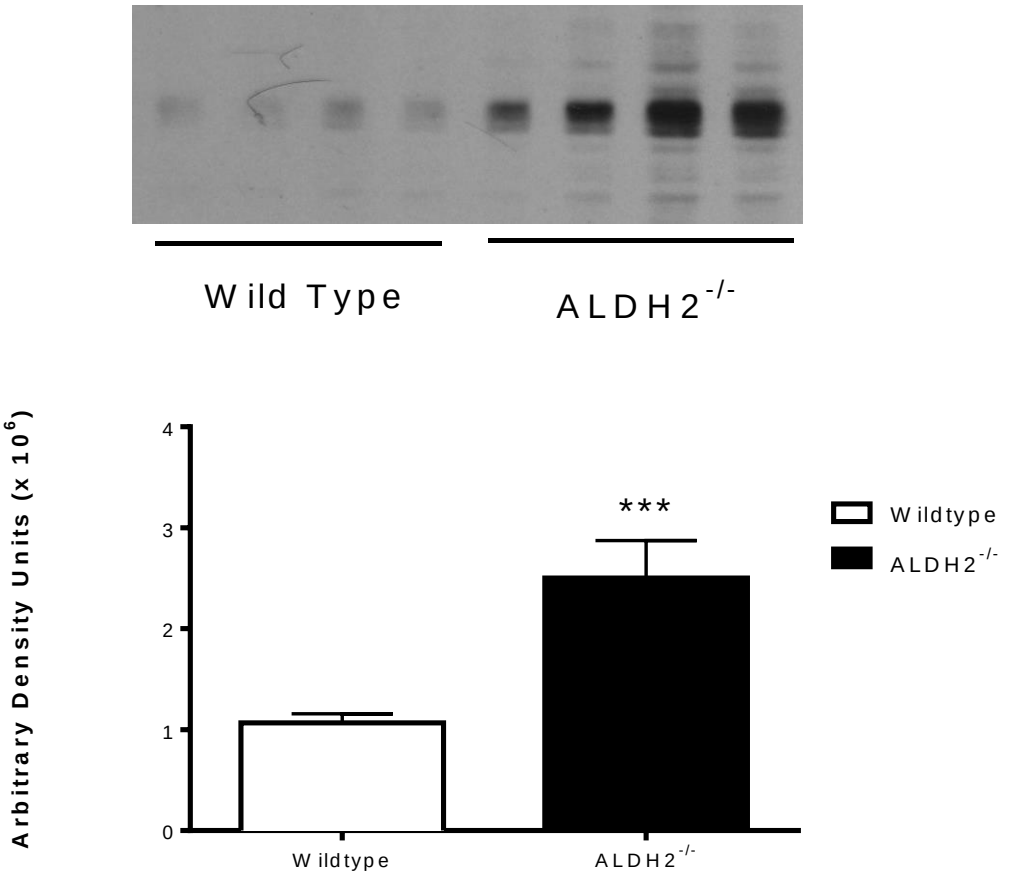

Figure 6. **Hyperphosphorylated tau formation**
Immunoblot showing increased hyperphosphorylated tau formation in the hippocampus of Aldh2$^{-/-}$ mice at 9 months of age. Data are presented as the mean ± S.D. (n=4) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant differences from wild type (***p<0.001) [89].

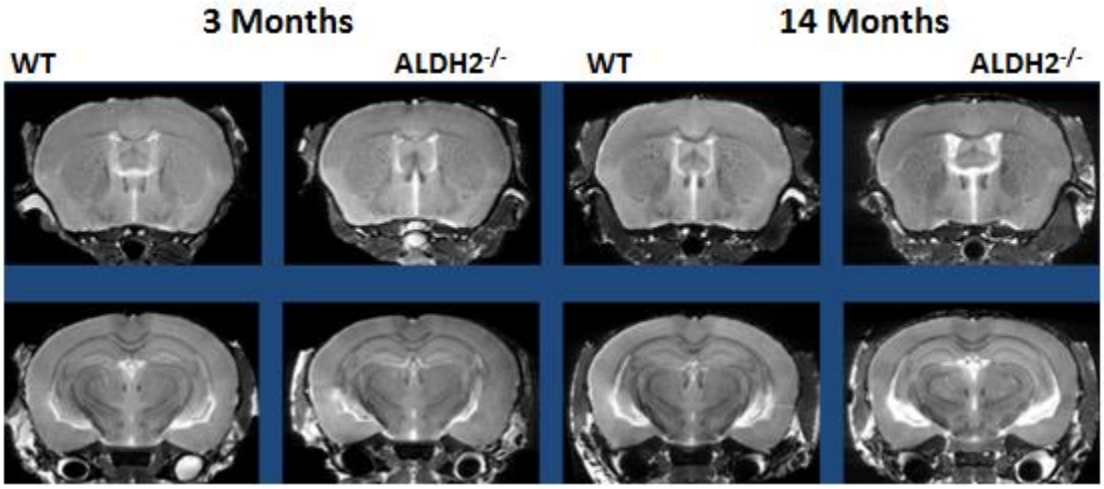

Figure 7. Imaging of Aldh2$^{-/-}$ and Wild Type Brains
MRI imaging of wild type and Aldh2$^{-/-}$ mice at 3 and 14 months of age. At 14 months, enlarged ventricles and a shrinking cortex in Aldh2 $^{-/-}$ mice are evident, implying neurodegeneration and brain atrophy [89].

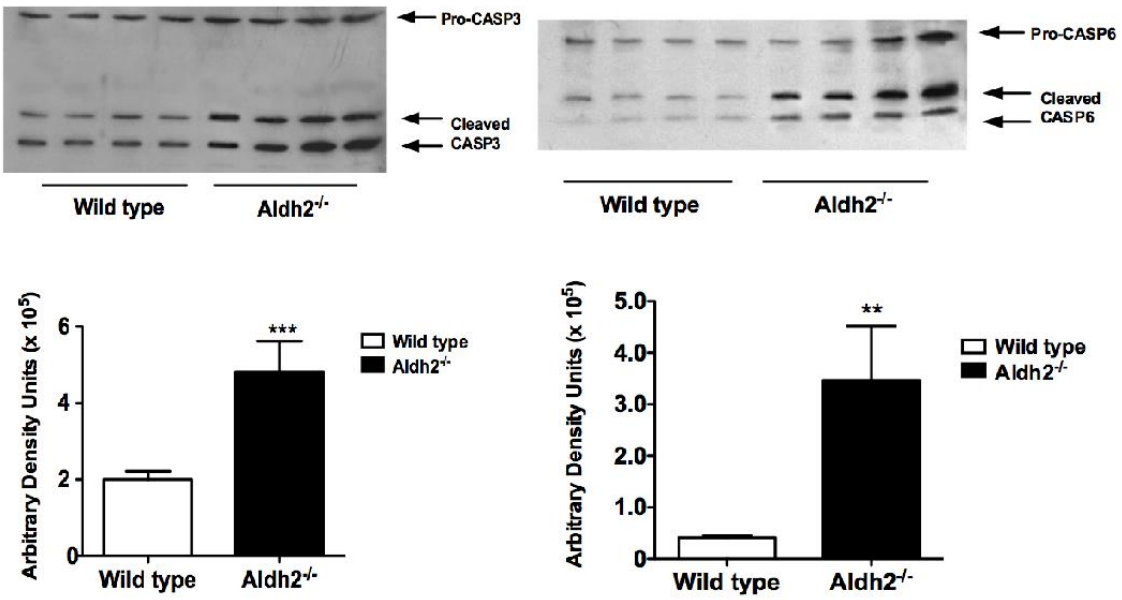

Figure 8. Increased Activated Caspases 3 and 6
Immunoblots showing increased activated caspases 3 and 6 in the hippocampus of Aldh2$^{-/-}$ mice at 9 months of age. Data are presented as the mean ± S.D (n=4) and were analyzed by a Student's t-test for unpaired data. * were used to indicate significant differences from wild type ($^{***}p<0.001$; $^{**}p<0.01$) [89].

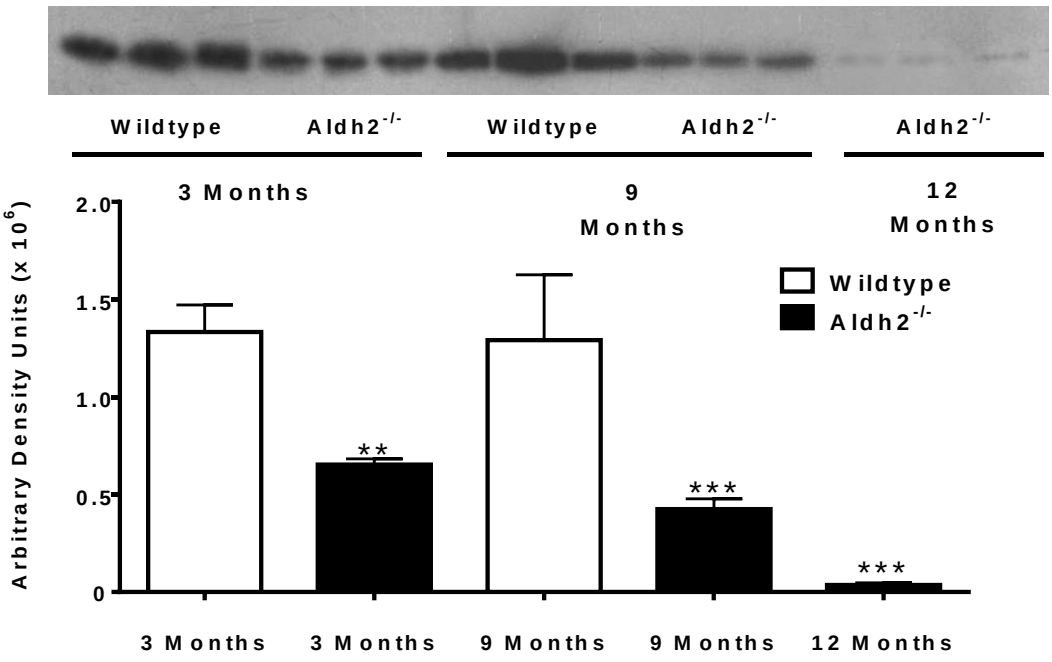

Figure 9. Age-Dependent Changes in Phosphorylated CREB
Immunoblot showing age-dependent decreases in phosphorylated CREB in the hippocampus of Aldh2$^{-/-}$ mice. Data are presented as the mean ± S.D. (n=3) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant differences from wild type (***p<0.001) [89].

1.11 Statement of Hypotheses and Objectives

Two main hypotheses were tested in this thesis work:

Hypothesis 1. Memory and cognition are impaired in Aldh2$^{-/-}$ mice.

Hypothesis 2. There is a progressive age-related decline in memory and cognition in Aldh2$^{-/-}$ mice.

The overall objectives of this thesis work were:

1. To examine spatial working, and recognition memory in male and female wild type and Aldh2$^{-/-}$ mice using the Y-maze and novel object recognition tasks.
2. To assess age-related changes in spatial working and recognition memory monthly, beginning at three months of age in male and female wild type and Aldh2$^{-/-}$ mice.
3. To determine if there are sex-differences in memory impairment in wild type and Aldh2$^{-/-}$ mice.

Materials and Methods

2.1 Generation of Aldh2$^{-/-}$ mice

All procedures for animal experimentation were undertaken in accordance with the principles and guidelines of the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee. Animals were maintained under a 12 h light/dark cycle (lights off at 1:00pm), with free access to food and water, and were individually housed. All behavior testing was performed during the dark phase of the light cycle. The Aldh2$^{-/-}$ mice have a C57BL/6 background (backcrossed with Aldh2$^{-/-}$ mice for more than 10 generations). They were generated by gene targeting knockout [73] and were provided by Dr. T. Kawamoto (University of Occupational and Environmental Health, Kitakyushu, Japan).Wild type male C57BL/6 mice (20 -30 g) were obtained from Jackson Laboratory, (Bar Harbor ME). Initial experiments with 12 month old animals utilized these Aldh2$^{-/-}$ and wild type mice. However, in subsequent experiments we generated wild type mice in-house, obtained by mating Aldh2 $^{-/-}$ mice with C57BL/6 wild type mice, and subsequent mating of the F1 heterozygotes and genotyping of the F2 progeny by PCR analysis of genomic DNA extracted from tail tips. At 3 weeks of age the mice were weaned, ear-punched for identification, and a 1.2cm snip of the end of their tails was taken for genotyping. The tail snip was placed in a DNAase-free Eppendorf tube and, using a Qiagen DNeasy Blood and Tissue Kit, DNA extraction was performed. The tail snip was cut up into small pieces and 20 µl of proteinase K were added. After mixing with a Vortex® mixer and incubation for a minimum of 2 hours, 200µl of 100% ethanol and 200µl of buffer were added and the DNA recovered after passing the samples through

the spin columns provided in the kit. DNA was quantitated using UV spectroscopy and 100ng were used for PCR amplification. Primer sequences were used according to [73]:

Forward: CCGTACTGACTGTCCCATGCAGTGCT
R1M: GGTGGATGTGGAAGTTGTGCGAGGC
R1WT: TCCGCCAATCGGTACAACAGCCG

Where R1M and R1WT are the reverse primers specific for Aldh2 $^{-/-}$ and wild type mice, respectively. PCR was performed using a Qiagen Multiplex PCR Kit and a Thermocycler using the following PCR conditions:

Cycle 1= 95°C for 15 min
Cycle 2= 94°C for 30 sec (denaturation)
Cycle 3= 58°C for 30 sec (annealing)
Cycle 4= 72°C for 30 sec (extension)
Cycle 5= 34 times repeat cycles 2-4
Cycle 6= 72°C for 10 min (final extension)
Cycle 7= 4°C indefinitely

PCR products were separated by DNA gel electrophoresis; Aldh2 $^{-/-}$ mice generated a 280 basepair fragment, whereas wild type mice generated a 208 basepair fragment.

2.2 Novel Object Recognition Task

The novel object recognition task is based on the natural and innate preference of rodents to explore novel objects, and was initially described by Ennaceur and Delacour (1988). All behavioural testing was performed in a dimly lit training room within the animal care facility. The experimenter was blinded with respect to the genotype of animals undergoing behavioural testing. Animals were initially introduced and habituated to the training room in their home cages for one hour prior to all testing. The protocol for the novel object recognition experiment was adopted from several sources and consisted of three consecutive days of testing per trial. The first day of testing consisted of a

habituation period to the training arena (plastic opaque bedding-free container approximately 44×24×20cm). Mice were introduced to the empty training arena individually and were allowed to roam free for 10 minutes. No data were recorded during this component of the experiment. Phase two (training period) was performed 24 hours later. Two identical objects (constructed from Lego® blocks, approximately 5-8cm in height) were added to the training arena approximately 10cm apart from each other. Mice were again introduced individually and allowed to explore the objects for a 10 minute session. On the third day (24 hours after the training period), the arena was set-up so that it contained one of the objects from the second phase, as well as a completely novel object (again, constructed of Lego® blocks, similar in height but differing in shape and colour). Again, the mice were introduced individually, and were allowed to explore the objects until they accumulated a total of 30 seconds of exploration time (exploration was recorded when the nose of the mouse was within approximately 1cm of the object; climbing on the object was not considered exploration). This was done rather than using a set exposure time in the test environment in order to account for any variability in movement and exploration that may occur between the two groups of mice [74] [75]. Also, this method can be particularly advantageous, as the novelty of the objects gradually declines as the test continues. By stopping after an accumulation of 30 seconds of exploration time, the new object remains novel, maximizing the animal's motivation to explore. The location of the novel object was counterbalanced among mice to control for any side biases that may occur. The training arena and objects used in the experiment were cleaned with 70% aqueous ethanol solution between each mouse trial to eliminate odour cues, and were subsequently washed off with water. All behaviour was recorded using a digital camera

mounted on a tripod over the arena. Two measures of behaviour were analyzed and determined based on the video-recorded behaviour: The time the animal spent with its head oriented directly towards and within 1cm of the object, and the frequency of visits to the object. The discrimination index (defined as the difference in time exploring the novel and familiar objects, divided by the total time spent exploring both objects) and the exploratory ratio (defined as the time the animal spent with the novel object divided by the total exploration time) were also determined based on the recorded times. Mice were also initially recorded during training with two identical objects to determine whether the mice had any side biases. Animals that did not show any exploratory behaviour were not included in the data analysis. The novel object recognition task was also modified towards the end of the study to examine a shorter delay (one hour) between the training and testing phases.

2.3 Y-Maze Task

The Y-maze is a simple and commonly used behavioural test for spatial working memory and is based on the mouse's natural exploratory instincts. Spatial working memory in mice is measured by scoring the number of arm alternations that the mouse makes when it travels to all three different arms of the maze without entering the same arm twice in a row. Animals were initially introduced and habituated to the training room in their home cages for one hour prior to all testing. The maze was specially constructed from black odorless wood and was designed as a radial arm maze (8 arms, each approximately 40x15x10cm with a center of approximately 15-20cm in diameter) with detachable arms. Each of the arms can be removed in order to form the shape required for testing (in this case, 5 arms were removed in order to form a Y-maze). The arms are

covered with a clear plexiglass to prevent mice from climbing out of the maze. Each arm also contains a plastic door that can be dropped in order to prevent access into the arm. The maze is directly surrounded by distinct spatial cues so that the mice can distinguish between arms. The protocol for the Y-maze was adopted from several sources and examines spontaneous alternation as a means of measuring spatial working memory in mice. Each of the three arms of the maze remained open, and mice were placed in the center of the maze. The animals were allowed to explore the 3 arms of the maze freely for a 10 minute session. The sequence and total number of arms entered were recorded (an entry is only considered if the hind paws of the mouse have completely entered the arm) and the spontaneous alternation rate was calculated (the total trials containing entries into each of the three arms without repeated entry into a previously visited arm, divided by the total amount of arm entries multiplied by 100(%)). A high alternation rate is indicative of better performance in this spatial working memory task. The maze was completely cleaned with 70% aqueous ethanol solution between each mouse to eliminate odour cues, and all behaviour was recorded using a digital camera mounted on a tripod over the maze for analysis.

A preliminary study began using in-house Aldh2$^{-/-}$ mice and commercial wild type animals, and subsequent studies used Aldh2$^{-/-}$ and wild type siblings. 9 male wild type and 12 male Aldh2$^{-/-}$ mice, as well as 6 female wild type and 6 female Aldh2$^{-/-}$ mice were used in this preliminary study. These mice were approximately 12 months old, and initial behavioral testing using the Y-maze and novel object recognition test was performed in order to determine whether memory was impaired. After testing on 12 month old mice was completed, a new cohort of male and female mice was bred (9 male wild type, 9

female wild type, 8 male Aldh2$^{-/-}$ mice, 9 female Aldh2$^{-/-}$ mice), and beginning at 3 months of age, mice were tested using the Y-maze and novel object recognition task once per month in order to examine age-related changes in spatial working and recognition memory impairment. Male and female Aldh2 heterozygote mice were also test at 6.5-7 months of age using the Y-maze and novel object recognition task.

2.4 Data Analysis

The ratio of time spent with the novel object in relation to the familiar object (exploratory ratio), the discrimination index, and the frequency of visits to the novel object and familiar object were all determined from the novel object recognition test. The spontaneous alternation rate was determined from the Y-maze task. All data are expressed as the mean ± SD and were analyzed by two-way analysis of variance and/or a Student's *t* test for unpaired data, as indicated. A *P*-value of 0.05 or less was considered statistically significant. All data were initially tested using a three-way analysis of variance in order to determine if there were sex differences in any of the memory tasks.

Results

3.1 Performance in the Novel Object Recognition Task at 12 months of Age

Twelve month-old male and female mice were subjected to the novel object recognition task in order to determine if there were memory differences between wild type and Aldh2$^{-/-}$ animals. A two-way ANOVA test was first performed to examine sex differences in memory. No significant differences were found between male and female animals in discrimination index, the time spent with the novel object in relation to the familiar object, and in frequency of visits ($P>0.05$ for all data). As a result, data from male and female mice were combined (thereby increasing sample size and reducing variability). A significant decline in discrimination index was found in Aldh2$^{-/-}$ mice compared to wild type mice (Student's *t*-test for unpaired data, $P<0.0001$) (Figure 10). The ratio of time spent with the novel object in relation to the familiar object was also calculated from the novel object recognition task. Similarly, a significant decline in the time spent with the novel object in relation to the familiar object was also found in Aldh2$^{-/-}$ mice in comparison to wild type mice (Student's *t*-test for unpaired data, $P<0.0001$) (Figure 11). The frequency of visits to the novel and familiar objects was the final parameter calculated from the novel object recognition task. Wild type mice were found to frequent the novel object significantly more than the familiar object (Student's *t*-test for paired data, $P<0.0001$), whereas the Aldh2$^{-/-}$ mice did not frequent the novel object significantly more than the familiar object, but rather, visited both objects with the same frequency ($P>0.05$) (Figure 12). These results suggest significant memory impairment in Aldh2$^{-/-}$ mice at 12 months of age.

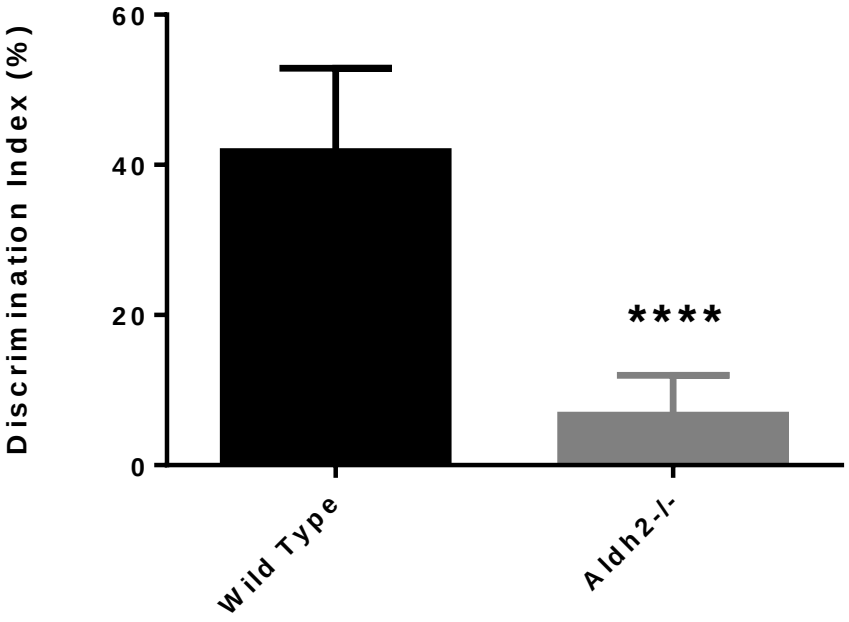

Figure 10. Discrimination Index in the Novel Object Recognition Task at 12 Months of Age

12-month-old male and female mice were subjected to the novel object recognition task consisting of three phases (habituation to an empty cage, training with two identical objects, and testing with one identical and one novel object). Data are presented as the mean ± S.D. (wild type n=15, $Aldh2^{-/-}$ n=20) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant difference from wild type (****$p<0.0001$).

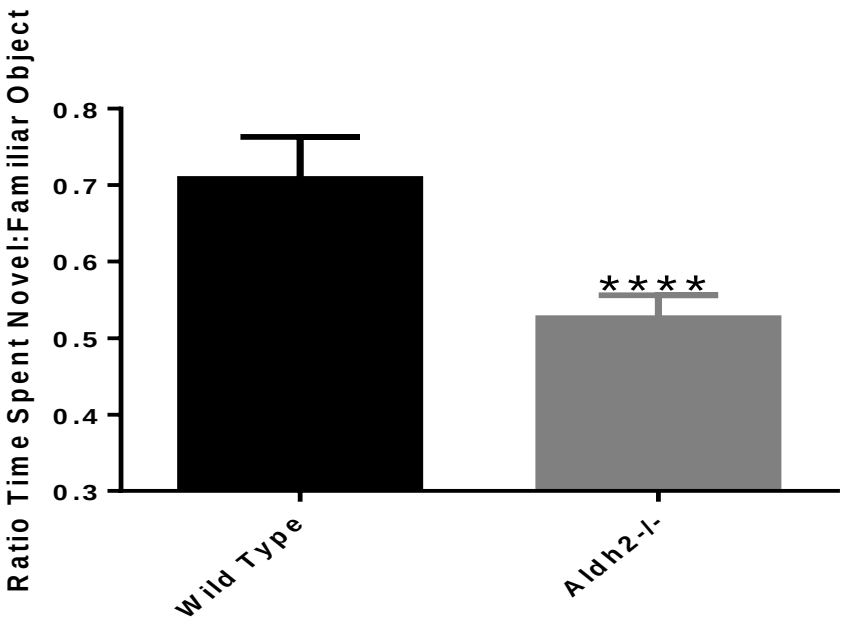

Figure 11. Time Spent with Novel Object in Relation to the Familiar Object in the Novel Object Recognition Task at 12 Months of Age

12-month-old male and female mice were subjected to the novel object recognition where the amount of time spent with each object was determined and the ratio of time spent with the novel object in relation to the familiar object was calculated. The data are presented as the mean ± S.D. (wild type n=15, $Aldh2^{-/-}$ n=20) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant difference from wild type (****p<0.0001).

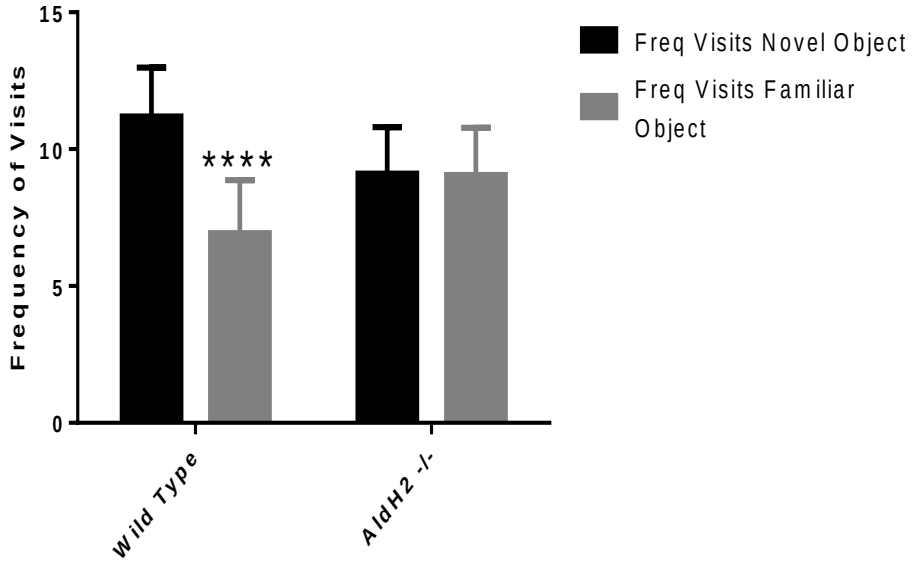

Figure 12. **Frequency of Visits in the Novel Object Recognition Task at 12 Months of Age**

12-month-old male and female mice were subjected to the novel object recognition task where the frequency of visits to the novel and familiar objects was calculated. The data are presented as the mean ± S.D. (wild type n=15, $Aldh2^{-/-}$ n=20) and were analyzed by a Student's *t*-test for paired data. * were used to indicate significant difference from frequency of visits to the novel object (****p<0.0001).

3.2 Performance in the Y-maze at 12 months of Age

Impairments in memory of 12-month-old mice were also examined using spontaneous alternation performance in the Y-maze task. Again, male and female data were combined due to a lack of a significant difference in performance between male and female wild type and Aldh2$^{-/-}$ mice. A significant decrease in the spontaneous alternation rate (approximately 20%) was found in Aldh2$^{-/-}$ mice in comparison to wild type mice (Student's *t*-test for unpaired data, $P<0.0001$) (Figure 13). These results from the Y-maze task suggest significant spatial working memory impairment in Aldh2$^{-/-}$ mice at 12 months of age.

3.3 Assessment of Age-Related Changes in Memory from the Novel Object Recognition Task

A new cohort of mice was bred in order to determine if there were age-related changes in memory in Aldh2$^{-/-}$ mice in relation to wild type littermates. Mice were assessed once per month (beginning at an age of approximately 3.5-4 months) using the Y-maze and novel object recognition task. Again, males and females were tested separately; however a three-way ANOVA test (examining sex differences of wild type and Aldh2$^{-/-}$ mice over time) was performed on all data generated from the Y-maze and novel object recognition tests. No significant differences were found when comparing male and female wild type and Aldh2$^{-/-}$ mice ($P>0.05$) (example of lack of differences shown in Figure 14). As such, male and female data were combined to increase sample size and reduce variability.

The discrimination index was determined from the novel object recognition task

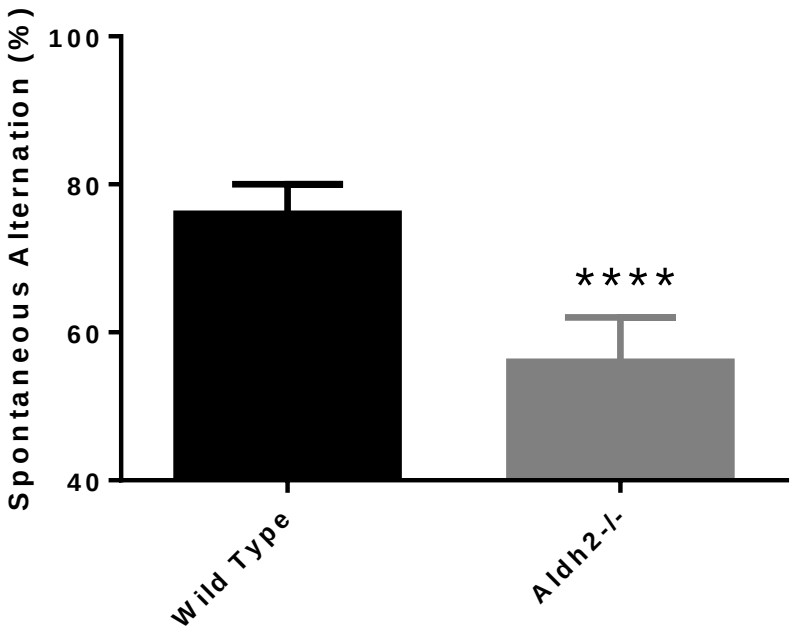

Figure 13. Spontaneous Alternation Rate in the Y-maze Task in 12-Month-Old Mice

12-month-old male and female mice were subjected to the Y –maze task and the spontaneous alternation rate was calculated. Data are presented as the mean ± S.D. (wild type n=15, Aldh2$^{-/-}$ n=20) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant difference from wild type (****p<0.0001).

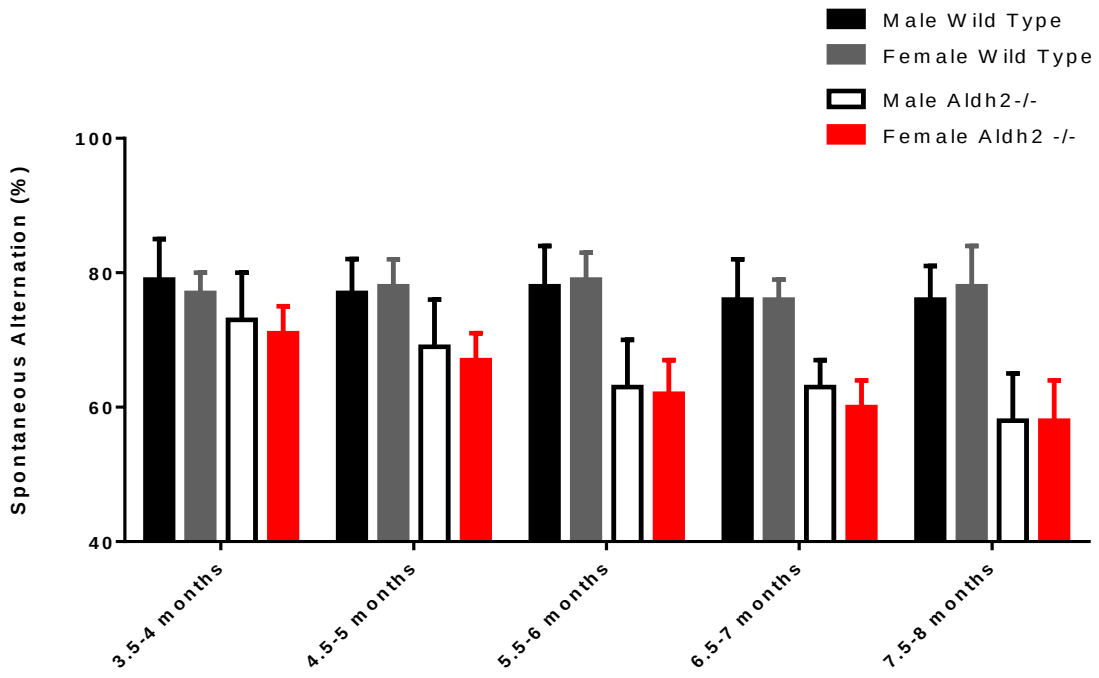

Figure 14. Male and Female Spontaneous Alternation Rate from the Y-Maze Task
Age-dependent decline in male and female spontaneous alternation rate in the Y-maze task illustrating a lack of significant difference between males and females. Data are presented as the mean ± S.D. (wild type males n=9, Aldh2$^{-/-}$ n=9, females wild type n=9, Aldh2$^{-/-}$ n=8). Three-way ANOVA test (examining sex differences of wild type and Aldh2$^{-/-}$ mice over time) was performed on all data generated from the Y-maze and novel object recognition test. No significant differences were found when comparing male and female wild type and Aldh2$^{-/-}$ mice ($P>0.05$)

and was found to be significantly decreased in Aldh2$^{-/-}$ mice in comparison to wild type as early as 3.5-4 months of age, and a significant decline in discrimination index was also evident at every other measured time point (4.5-5 months, 5.5-6 months, 6.5-7 months, and 7.5-8 months of age) in Aldh2$^{-/-}$ mice in comparison to wild type mice (two-way ANOVA, Sidak multiple comparisons test, $P<0.01$ at 3.5-4 months, $P<0.0001$ at all other time points) (Figure 15). The discrimination index was also found to progressively decline over the time course. Aldh2$^{-/-}$ mice performed significantly worse beginning at 5.5-6 months of age than they did at 3.5-4 months of age (two-way ANOVA, Tukey's multiple comparisons test, $P<0.0001$). These significant declines were also seen at 6.5-7 months and 7.5-8 months of age ($P<0.0001$ at both time points). The decline in discrimination index also appeared to plateau at 5.5-6 months, as no significant difference was found when comparing the discrimination index at 5.5-6 months in Aldh2$^{-/-}$ mice with that at 6.5-7 months and 7.5-8 months. Interestingly, wild type mice improved over the time course of testing, with the discrimination index being significantly higher after 5.5-6 months of age in comparison to 3.5-4 months ($P<0.01$).

Similar results were observed when examining the ratio of time spent with the novel object, where it was found to be significantly impaired in Aldh2$^{-/-}$ mice as early as 3.5-4 months of age, and at every other measured time point (two-way ANOVA, Sidak multiple comparisons test, $P<0.01$ at 3.5-4 months, $P<0.0001$ at all other time points) (Figure 16). The time spent with the novel object was also found to progressively decline over the time course of analysis. Aldh2$^{-/-}$ mice performed significantly worse beginning at 5.5-6 months of age than they did at 3.5-4 months of age (two-way ANOVA, Tukey's multiple

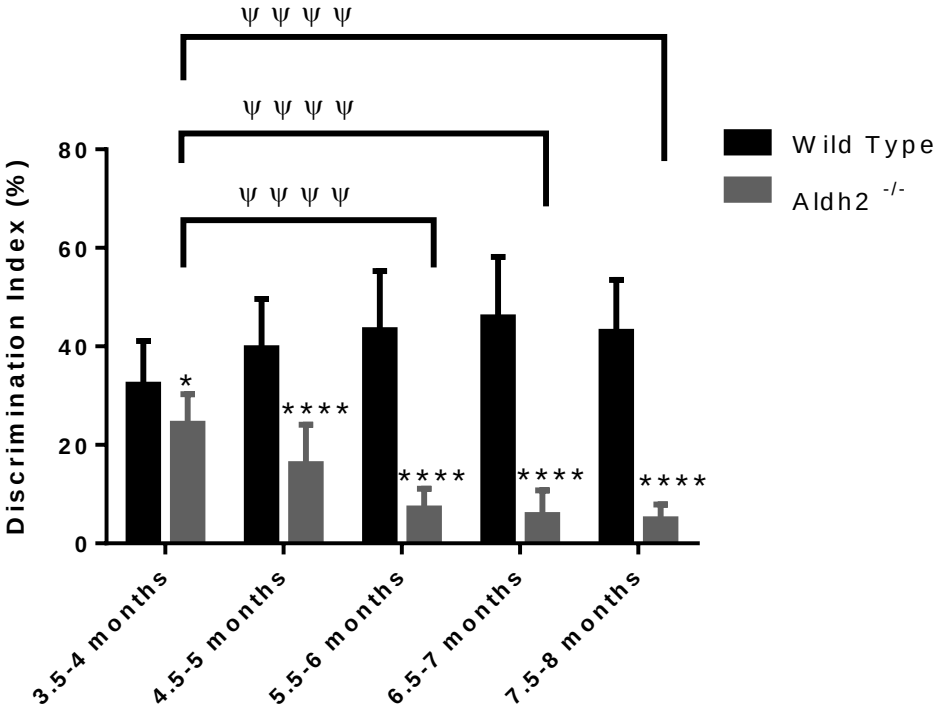

Figure 15. Age-Dependent Decline in Discrimination Index in the Novel Object Recognition Task

Male and female mice were subjected to the novel object recognition task once per month beginning at three months of age. The amount of time spent with each object was determined and the discrimination index was calculated. Data are presented as the mean ± S.D. (wild type n=18, Aldh2$^{-/-}$ n=17) and were analyzed by two-way ANOVA. * were used to indicate significant differences from wild type (*$p<0.05$, ****$p<0.0001$).ψ were used to indicate significant differences in Aldh2$^{-/-}$ mice from 3.5-4 months (ψψψψ $p<0.0001$).

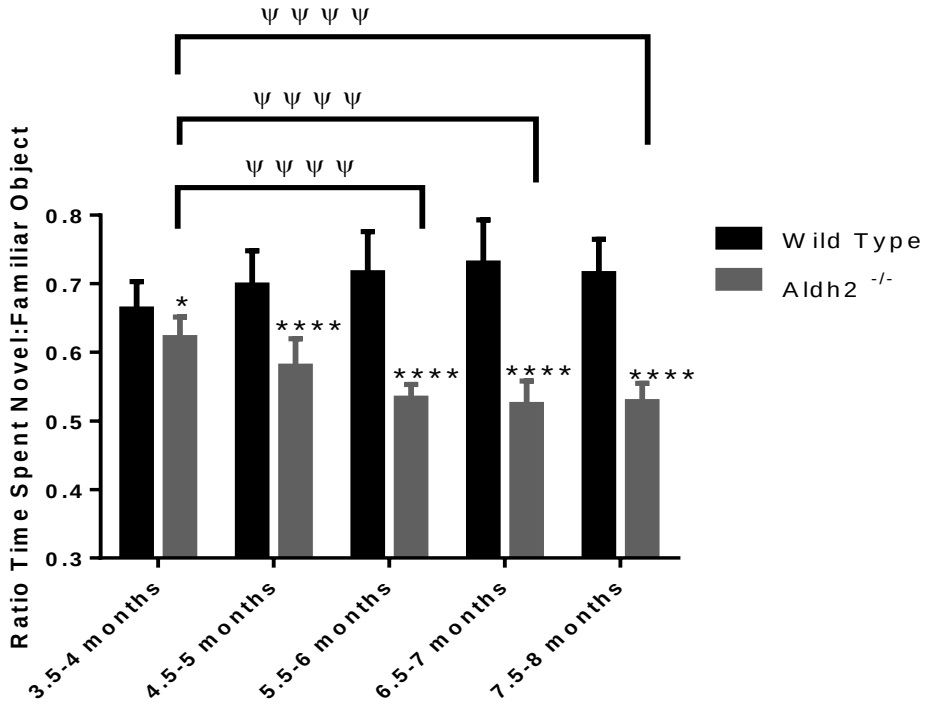

Figure 16. Age-Dependent Decline in Time Spent with Novel Object in the Novel Object Recognition Task

Male and female mice were subjected to the novel object recognition task once per month beginning at three months of age. The amount of time spent with each object was determined and the ratio of time spent with the novel object in relation to the familiar object was calculated. Data are presented as the mean ± S.D. (wild type n=18, Aldh2$^{-/-}$ n=17) and were analyzed by a two-way ANOVA. * were used to indicate significant differences from wild type (*p<0.05, ****p<0.0001).ψ were used to indicate significant differences in Aldh2$^{-/-}$ mice from 3.5-4 months (ψψψψ p<0.0001).

comparisons test, P<0.0001). These significant declines were also seen at 6.5-7 months and 7.5-8 months of age (P<0.0001 at both time points). The decline in the time spent with the novel object also appeared to plateau at 5.5-6 months, as no significant difference was found when comparing the ratio at 5.5-6 months in $Aldh2^{-/-}$ mice with 6.5-7 months and 7.5-8 months. Again, wild type mice appeared to improve over the time as the time spent with the novel object was found to be significantly higher after 5.5-6 months of age compared to 3.5-4 months of age(P<0.01).

The frequency of visits to the novel and familiar objects was also determined from the novel object recognition task at each time point. At 3.5-4 months and at 4.5-5 months of age, the wild type and $Aldh2^{-/-}$ mice frequented the novel object significantly more often than the familiar object (Student's t-test for paired data, P<0.0001 for wild type and $Aldh2^{-/-}$ mice). Beginning at 5.5-6 months of age, the $Aldh2^{-/-}$ mice did not frequent the novel object significantly more often than the familiar object whereas the wild type mice continued to frequent the novel object significantly more often (Student's *t*-test for paired data, P<0.0001 for wild type, P>0.05 for $Aldh2^{-/-}$ mice). This pattern continued for the remaining time points (Figure 17).

3.4 Assessment of Age-Related Changes in Memory in the Y-maze

The new cohort of animals was also tested monthly using the Y-maze task beginning at 3-3.5 months. Again, male and female data were combined, and a spontaneous alternation rate was calculated. Results observed over the time course in the Y-maze task were similar to those seen in the object recognition task. The spontaneous alternation rate was found to be significantly impaired in $Aldh2^{-/-}$ mice in comparison to wild type mice as early as 3.5-4 months of age, and a significant decline was also evident

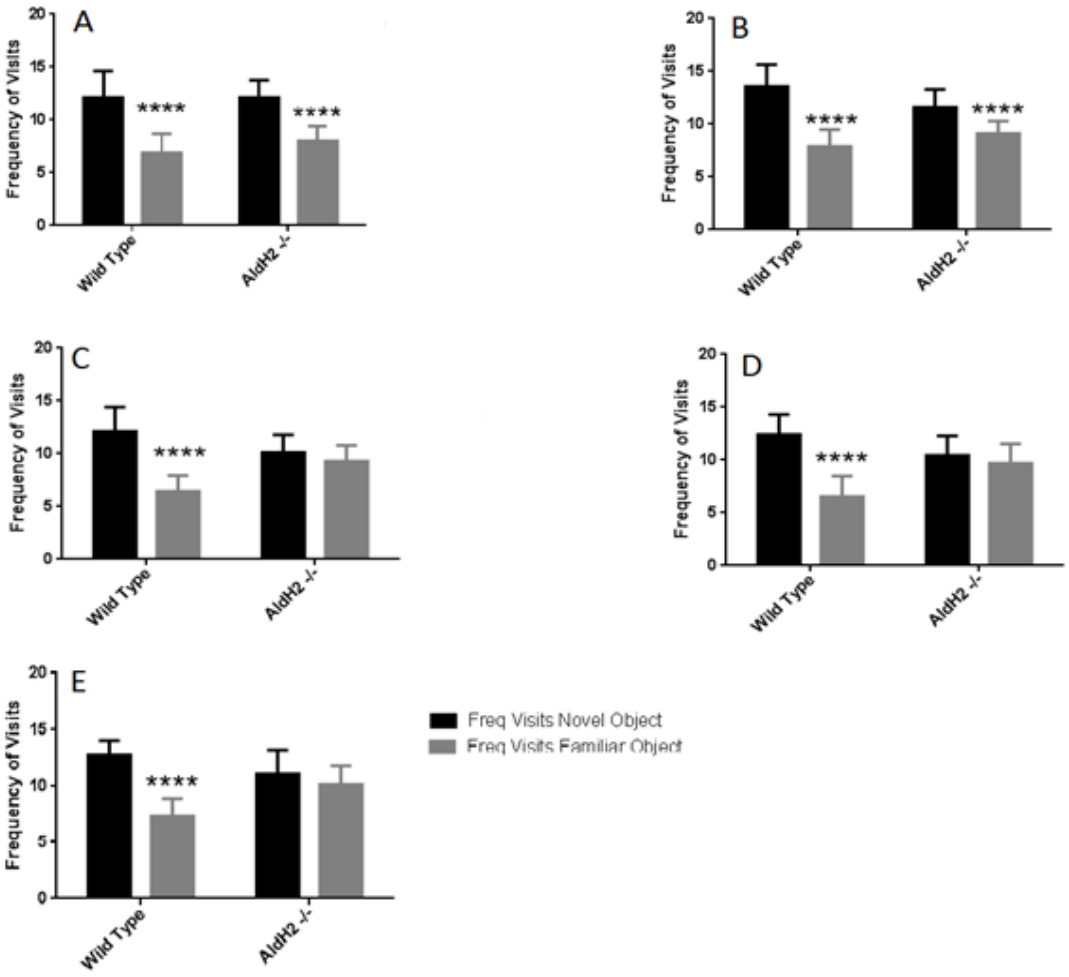

Figure 17. **Frequency of Visits to Novel and Familiar Objects over Time in the Novel Object Recognition Task**

Male and Female mice were subjected to the novel object recognition task where the frequency of visits to the novel and familiar objects was calculated at 5 different time points, 3.5-4 months (A), 4.5-5 months (B), 5.5-6 months (C), 6.5-7 months (D), and 7.5-8 months (E). Data are presented as the mean ± S.D. (wild type n=18, $Aldh2^{-/-}$ n=17) and were analyzed by a Student's *t*-test for paired data. * were used to indicate significant differences from frequency of visits to the novel object (****p<0.0001).

at every other measured time point in Aldh2$^{-/-}$ mice in comparison to wild type mice (two-way ANOVA, Sidak multiple comparisons test, $P<0.01$ at 3.5-4 months, $P<0.0001$ at all other time points) (Figure 18). The spontaneous alternation rate was also found to progressively decline over the time course. Aldh2$^{-/-}$ mice performed significantly worse beginning at 5.5-6 months of age than they did at 3.5-4 months of age (two-way ANOVA, Tukey's multiple comparisons test, $P<0.0001$). These significant declines were also seen at 6.5-7 months and 7.5-8 months of age ($P<0.0001$ at both time points). The decline in spontaneous alternation rate also appeared to plateau at 5.5-6 months, as no significant difference was found when comparing at 5.5-6 months in Aldh2$^{-/-}$ mice with 6.5-7 months and 7.5-8 months. The wild type mice sustained a significantly higher spontaneous alternation rate over the time course but did not appear to significantly improve over time ($P>0.05$ after 5.5-6 months of age). All the data collected from the Y-maze and novel object recognition test over the time course suggest that Aldh2$^{-/-}$ mice display memory impairment early in their life which progressively worsens over time.

3.5 Assessment of Memory Impairments in Aldh2 Heterozygote Mice

Ten male and 10 female Aldh2 heterozygote mice were tested at 6.5-7 months of age using the Y-maze and novel object recognition task. Again, no significant differences were found between males and females in any of the data generated, so male and female data were combined. The discrimination index, ratio of time spent with the

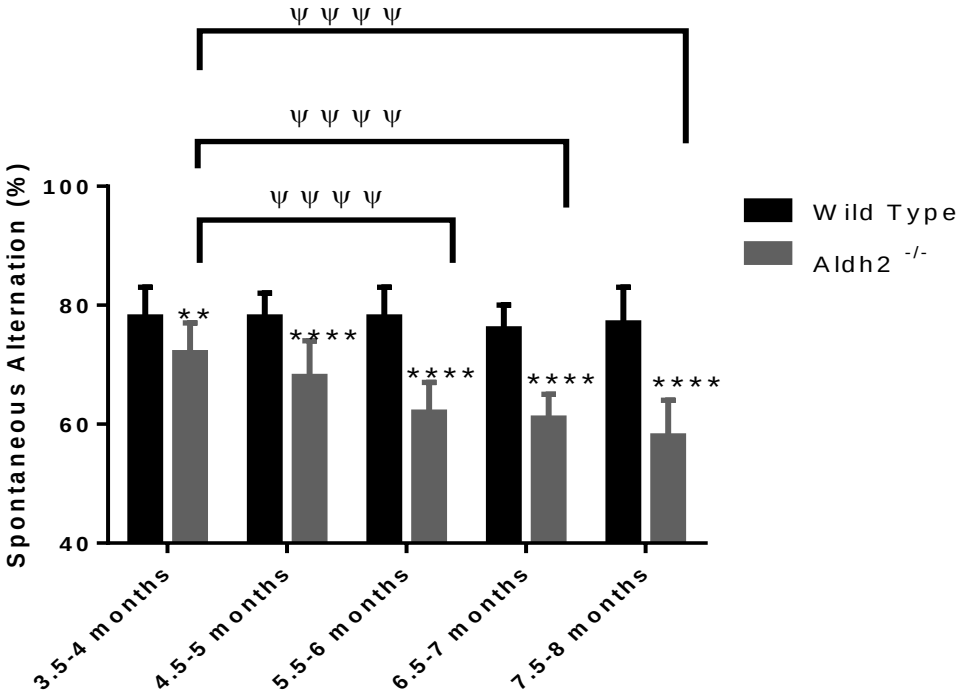

Figure 18. Age-Dependent Decline in Spontaneous Alternation Rate in the Y-maze Task Male and female mice were subjected to the Y-maze task once per month beginning at three months of age and the spontaneous alternation rate was determined. Data are presented as the mean ± S.D. (wild type n=18, Aldh2$^{-/-}$ n=17) and were analyzed by a two-way ANOVA. * were used to indicate significant differences from wild type (**, P<0.01, ****p<0.0001).ψ were used to indicate significant differences in Aldh2$^{-/-}$ mice from 3.5-4 month old Aldh2$^{-/-}$ mice (ψψψψ p<0.0001).

novel object in relation to the familiar object, frequency of visits, and spontaneous alternation rate were all calculated and compared to data obtained from wild type and Aldh2$^{-/-}$ mice at a similar time point (6.5-7 months of age). The discrimination index was determined from the novel object recognition task and was found to be significantly impaired in the heterozygote mice (as well as in Aldh2$^{-/-}$ mice) in comparison to wild type at 6.5-7 months of age (one-way ANOVA, Tukey's multiple comparisons test, $P<0.0001$) (Figure 19). No significant difference was found when comparing the performance of heterozygote mice with Aldh2$^{-/-}$ mice (one-way ANOVA, Tukey's multiple comparisons test, $P>0.05$).

Similar results were observed in the ratio of time spent with the novel object in comparison to the familiar object. Heterozygotes spent significantly less time with the novel object (as did the Aldh2$^{-/-}$ mice) at 6.5-7 months of age in comparison to wild type mice (one-way ANOVA, Tukey's multiple comparisons test, $P<0.0001$), but no significant difference was found when comparing Aldh2$^{-/-}$ mice with heterozygotes (one-way ANOVA, Tukey's multiple comparisons test, $P>0.05$) (Figure 20). Interestingly, heterozygotes did frequent the novel object significantly more often than the familiar object (as did the wild type mice) (Student's *t*-test for paired data, $p<0.05$), whereas the Aldh2$^{-/-}$ mice frequented both objects nearly equally (no significant difference found) (Figure 21).

The spontaneous alternation rate from the Y-maze was also found to be significantly decreased in heterozygotes in comparison to wild type mice at 6.5-7 months of age (one-way ANOVA, Tukey's multiple comparisons test, $P<0.0001$) (Figure 22). Interestingly however, despite this impairment, heterozygotes performed significantly better than

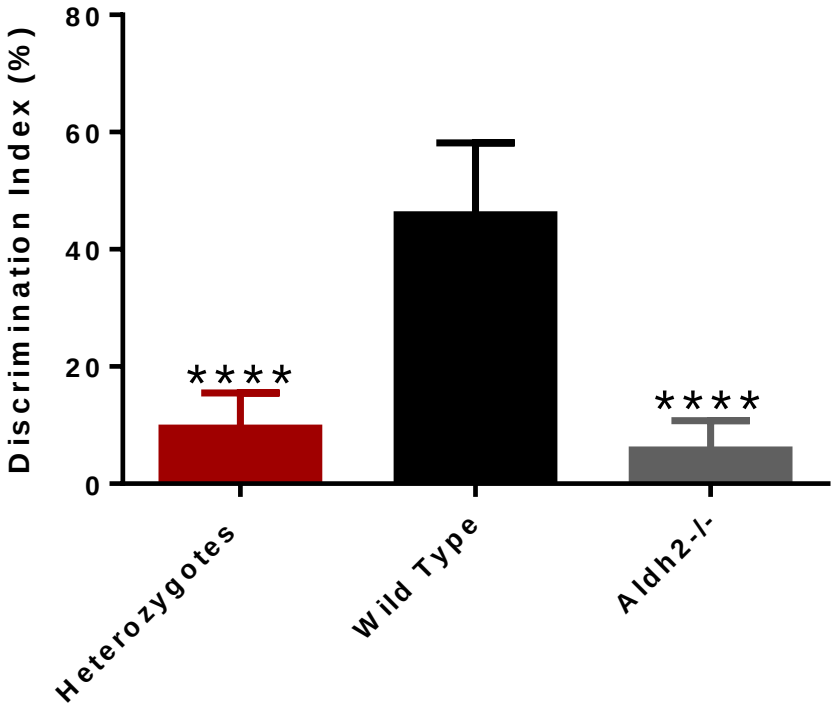

Figure 19. Discrimination Index in the Novel Object Recognition Task at 6.5-7 Months of Age in Heterozygote Mice

Male and female mice subjected to the Novel object recognition task at 6.5-7 months of age. The amount of time spent with each object was determined and the discrimination index was calculated. Data are presented as the mean ± S.D. (wild type n=18, $Aldh2^{-/-}$ n=17, Heterozygotes n=19) and were analyzed by a one-way ANOVA. * were used to indicate significant differences from wild type (****p<0.0001).

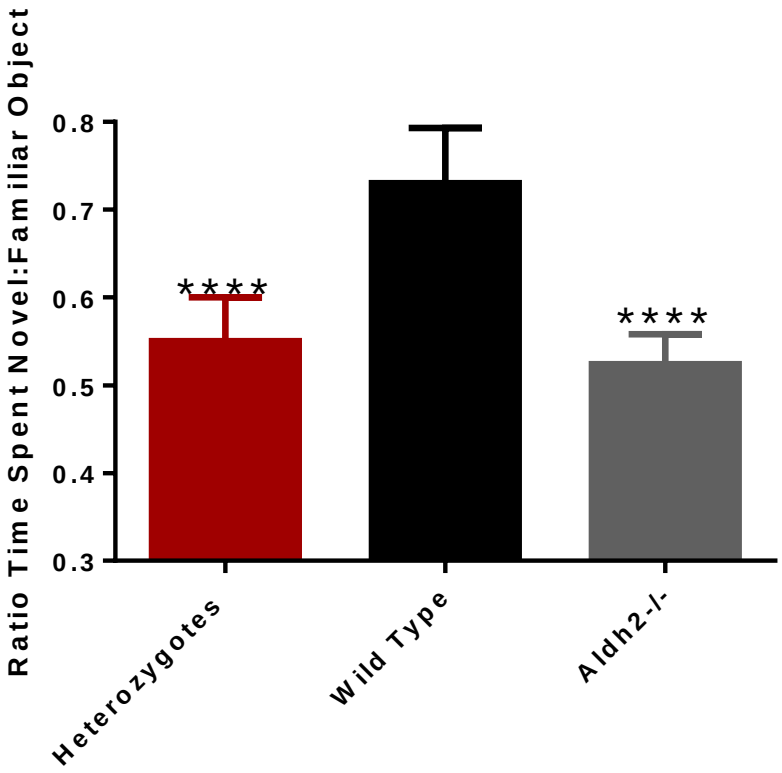

Figure 20. **Time Spent with Novel Object in Relation to the Familiar Object in the Novel Object Recognition Task at 6.5-7 Months of Age in Heterozygote Mice**

The amount of time spent with each object was determined and the ratio of time spent with the novel object in relation to the familiar object was calculated. Data are presented as the mean ± S.D. (wild type n=18, Aldh2$^{-/-}$ n=17, Heterozygotes n=19) and were analyzed by a one-way ANOVA. * were used to indicate significant differences from wild type (****p<0.0001).

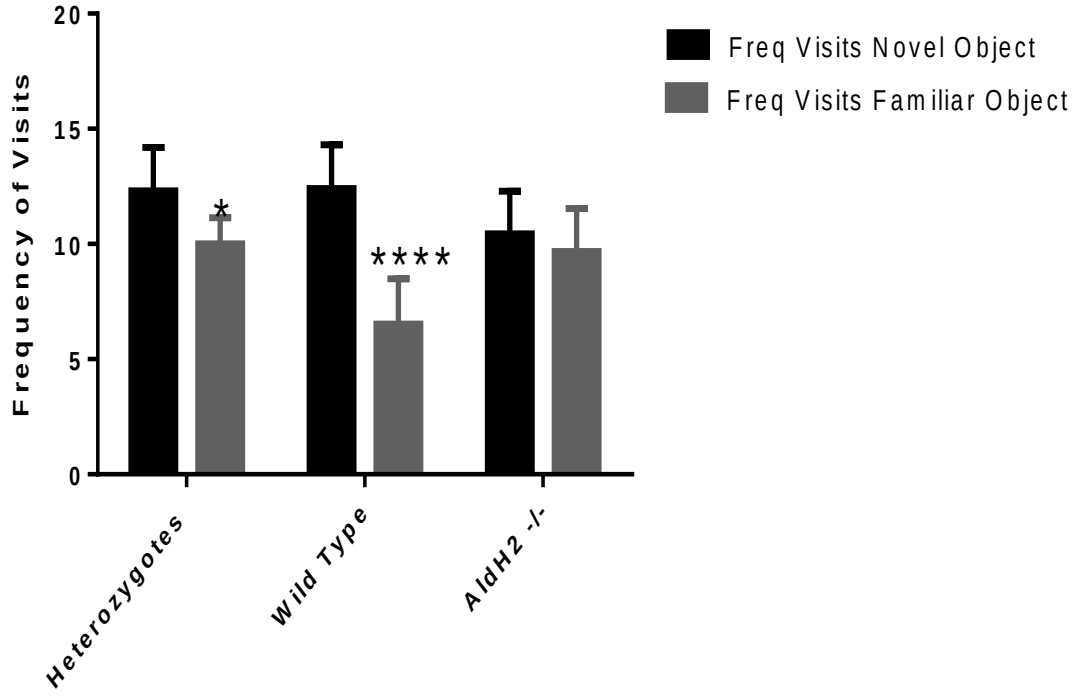

Figure 21. Frequency of Visits in the Novel Object Recognition Task at 6.5-7 Months of Age in Heterozygote Mice

The frequency of visits to each object was determined. Data are presented as the mean ± S.D. (wild type n=18, Aldh2$^{-/-}$ n=17, Heterozygotes n=19) and were analyzed by a Students t-test for paired data. * were used to indicate significant difference from frequency of visits to the novel object (****p<0.0001).

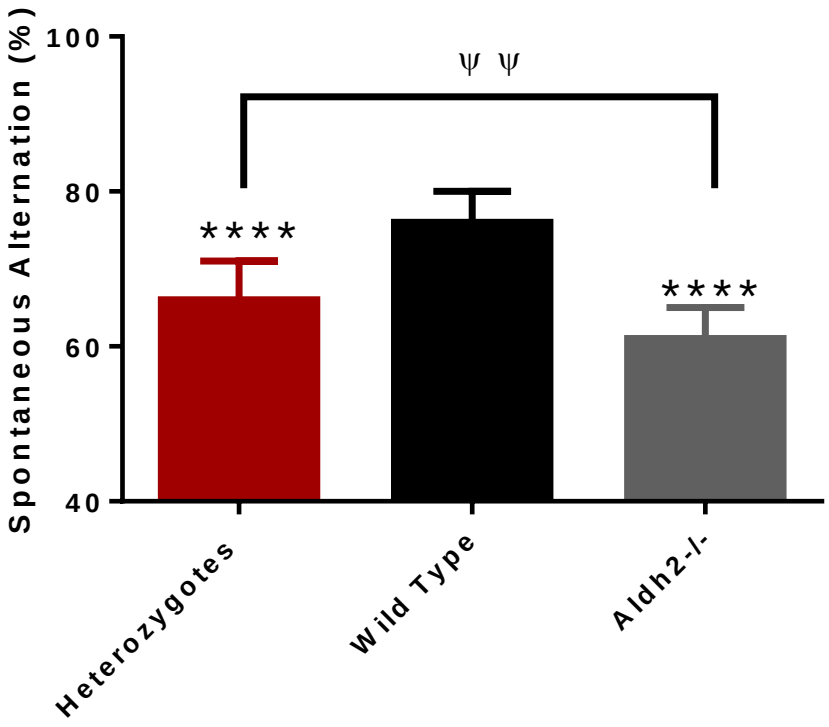

Figure 22. **Spontaneous Alternation Rate in the Y-maze Task at 6.5-7 Months of Age in Heterozygote Mice**

Male and female mice subjected to the Y-maze at 6.5-7 months of age, and the spontaneous alternation rate was calculated. Data are presented as the mean ± S.D. (wild type n=18, Aldh2$^{-/-}$ n=17, Heterozygotes n=19) and were analyzed by a one-way ANOVA. * were used to indicate significant differences from wild type (****p<0.0001), ψ were used to indicate significant difference between heterozygotes and Aldh2$^{-/-}$ mice (ψψ p<0.01).

Aldh2$^{-/-}$ mice at this time point (one-way ANOVA, Tukey's multiple comparisons test, $P<0.01$). The data suggest that heterozygote mice also experience memory impairment; however, they do not appear to be as impaired as Aldh2$^{-/-}$ mice.

3.6 Novel Object Recognition Task with Shorter Delay between Training and Testing

The novel object recognition task was also repeated with a shorter delay between training and testing, using 8-month-old male and female wild type and Aldh2$^{-/-}$ mice. A one hour delay was used between training and testing rather than a 24 hour delay. All data were collected according to the protocols used previously. Similar to a 24 hour delay, the discrimination index and ratio of time spent with the novel object in relation to the familiar object were found to be significantly decreased in Aldh2$^{-/-}$ compared to wild type mice (Student's *t*-test for unpaired data, $P<0.0001$ for both the discrimination index and ratio) (Figures 23 and 24 respectively). Wild type mice also frequented the novel object significantly more often than the familiar object whereas Aldh2$^{-/-}$ mice did not (Student's *t*-test for paired data, $P<0.0001$ for wild type, $P>0.05$ for Aldh2$^{-/-}$ mice) (Figure 25). Interestingly, no significant difference was observed in any of the parameters when comparing performance in the novel object recognition task with a 1 hour delay to that using a 24 hour delay (two-way ANOVA, Sidak multiple comparisons test, $P>0.05$) (Figure 26). These results suggest that a shorter delay between training and testing does not improve memory, thereby emphasizing the drastic impairments in memory and behaviour in Aldh2$^{-/-}$ mice.

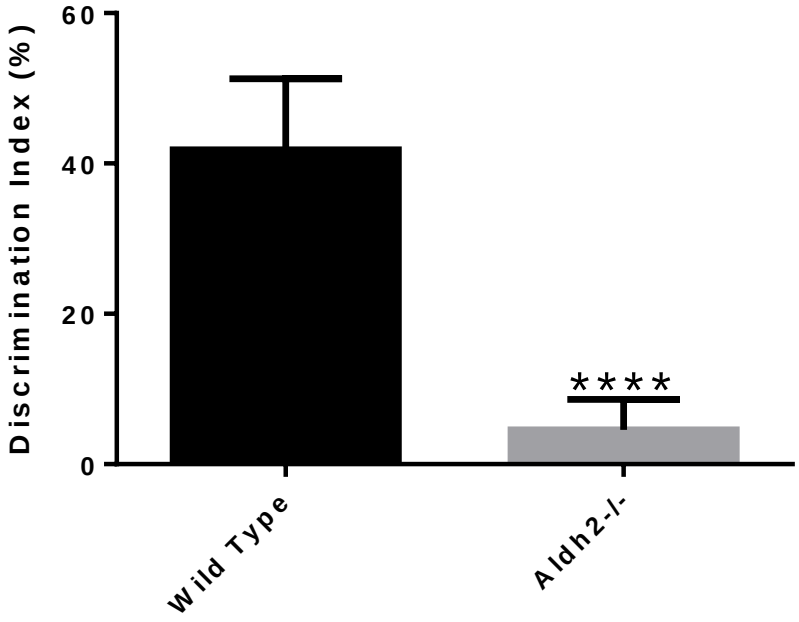

Figure 23. **Discrimination Index in the Novel Object Recognition Task using a One Hour Delay**

Male and female mice were subjected to the novel object recognition task with a short (1 hour) delay rather than a 24 hour delay between training and testing. The discrimination index was calculated as described previously. Data are presented as the mean ± S.D. (wild type n=18, $Aldh2^{-/-}$ n=16) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant difference from wild type (****$p<0.0001$)

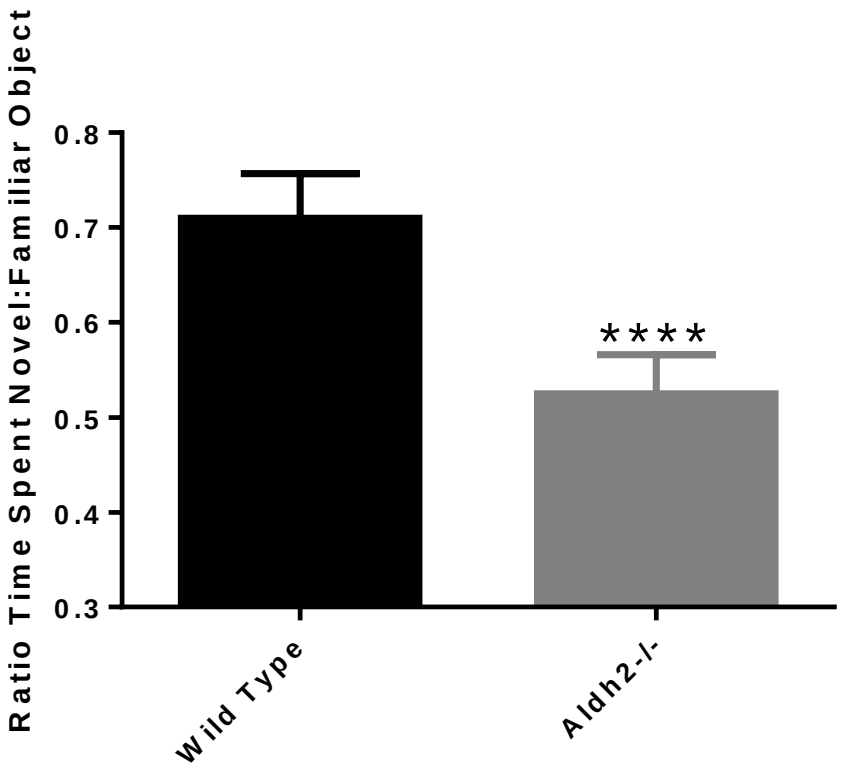

Figure 24. Time Spent with Novel Object in the Novel Object Recognition Task using a One Hour Delay

Male and female mice were subjected to the novel object recognition task with a short (1 hour) delay rather than a 24 hour delay between training and testing. The ratio of time spent with the novel object in relation to the familiar object was calculated as described previously. Data are presented as the mean ± S.D. (wild type n=18, $Aldh2^{-/-}$ n=16) and were analyzed by a Student's *t*-test for unpaired data. * were used to indicate significant difference from wild type (****p<0.0001)

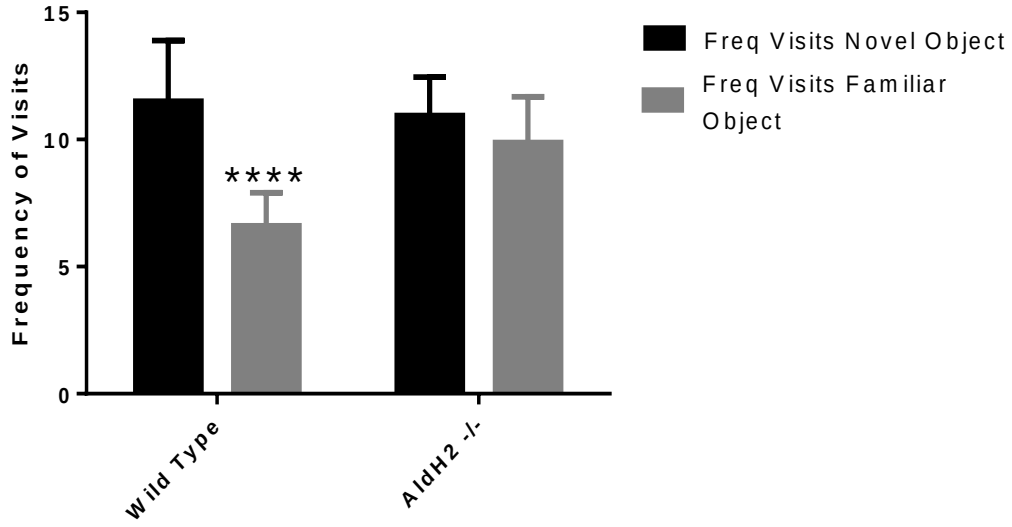

Figure 25. Frequency of Visits in the Novel Object Recognition Task using a One Hour Delay

Male and female mice were subjected to the novel object recognition task with a short (1 hour) delay rather than a 24 hour delay between training and testing. The frequency of visits to each object was calculated as described previously. Data are presented as the mean ± S.D. (wild type n=18, $Aldh2^{-/-}$ n=16) and were analyzed by a Student's *t*-test for paired data. * were used to indicate significant difference in wild type (****$p<0.0001$)

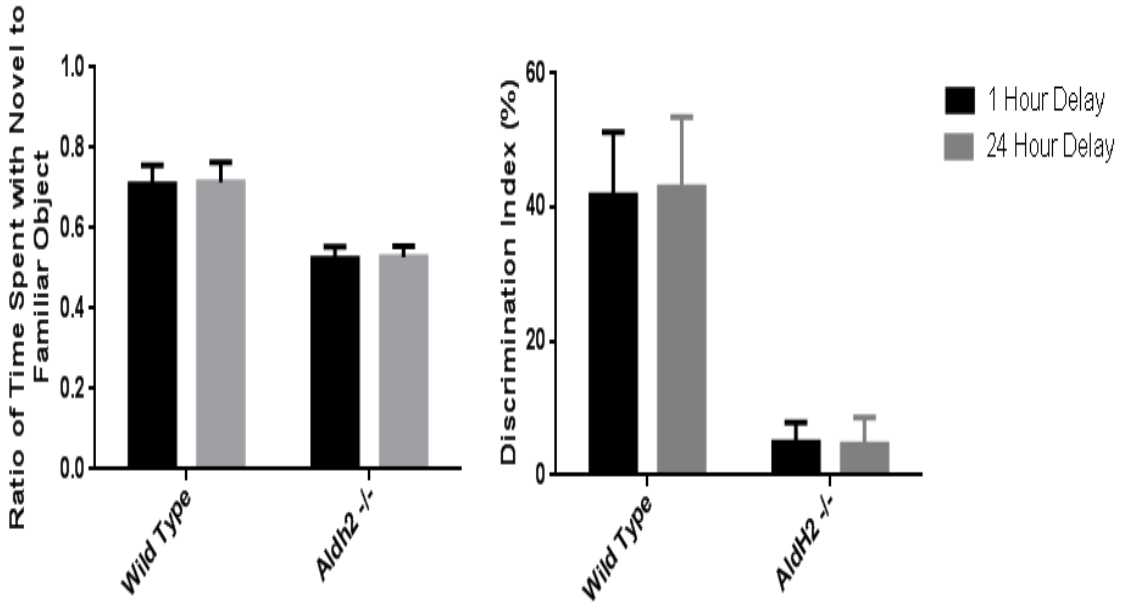

Figure 26. Comparison of Performance in the Novel Object Recognition Task with a 1 Hour Delay to that of a 24 Hour Delay

Male and female mice were subjected to the novel object recognition task with a short (1 hour) delay between training and testing and were compared to that using a 24 hour delay (at 8 months of age). The ratio of time spent with the novel object in relation to the familiar object, and the discrimination index were compared. Data are presented as the mean ± S.D. (wild type n=18, $Aldh2^{-/-}$ n=16) and were analyzed by a two-way ANOVA. No significant difference was found comparing wild type mice with one hour delay to wild type mice with 24 hour delay, or comparing $Aldh2^{-/-}$ mice with one hour delay to $Aldh2^{-/-}$ mice with 24 hour delay ($P>0.05$).

Discussion

Previous work by our laboratory has shown that Aldh2$^{-/-}$ mice exhibit significant increases in HNE adduct formation as early as 3 months of age, as well as at 9 months [89]. There are significant increases in both intraneuronal $A\beta_{42}$ and in phosphorylated tau protein at 9 months, and, we have also found significant brain atrophy, increased activated caspases 3 and 6, and decreased PSD-95 and phosphorylated CREB, all of which are key components of AD that are not often seen in current transgenic animal models of AD [89]. In order to further characterize these mice as a model of cognitive impairment with AD-like pathologies, a Y-maze and novel object recognition task were performed to determine whether these molecular changes correlate with behavioral and memory impairments in these mice. In the present study, Aldh2$^{-/-}$ mice exhibited significant memory impairments as early as 3.5-4 months of age. Performance in the Y-maze and novel object recognition test were significantly impaired for Aldh2$^{-/-}$ mice, even with a shorter delay between training and testing in the object recognition test (Figures 23-26). It was also shown that these mice exhibited an age-related decline in memory that plateaus at approximately 5.5-6 months of age, and that there were no sex-differences in cognitive performance. Wild type mice significantly improved their performance in the novel object recognition task (as determined from the time spent with the novel object in relation to the familiar object) as they aged, however Aldh2$^{-/-}$ mice exhibited a progressive decline. Finally, Aldh2 heterozygote mice also exhibited memory impairments, although these were not as pronounced as those in Aldh2$^{-/-}$ mice. Together, these data suggest significant memory and behavioral impairments in Aldh2$^{-/-}$ that decline progressively, beginning as

early as 3.5 months, and reaching a plateau at approximately 6 months of age, corresponding with similar molecular data collected by our laboratory.

The Y-maze and novel object recognition task are two commonly used animal behavioral tests for spatial working memory and recognition memory respectively. The Y-maze employs the use of a spontaneous alternation rate as a measure of spatial working memory, which is an estimate of an animal's willingness to explore novel stimuli and avoid similar stimuli by using spatial cues surrounding the maze. In light of the general apathy patients suffering from AD typically experience, one might predict that alternation rates would be reduced in murine models of AD [76]. The spontaneous alternation rate is also a measure of short-term memory, and a decline in spontaneous alternation rates may result from behavioral disinhibition and a loss of short-term memory, both of which are also distinct symptoms of AD [76]. Patients suffering from AD are likely to engage in a number of behaviors because of a lack of initiative to try new behaviours, a general lack of curiosity, a fear of trying new things, and/or a tendency to choose impulsive actions that have often been repeated in the past [76]. Similarly, $Aldh2^{-/-}$ mice may impulsively choose the first available arm without considering their previous choice, and may display a lack of curiosity, and/or a fear of novel stimuli thereby lowering spontaneous alternation rates as a result of this behavioral disinhibition, emphasizing a significant loss of short-term spatial working memory.

The novel object recognition task uses familiarization, delay, and test phases, to test an animal's spontaneous preference for novel objects, and provides a valuable measure of declarative or long-term recognition memory. It does not require spatial learning or the application of positive or negative reinforcement, has been extensively

used in monkeys, rodents, and in humans, and exploits an animal's innate preference for novelty rather than requiring explicit learning over time, thus representing a key advantage over other memory tests [77]. Also, the administration of the test is fairly similar regardless of the test subject (specifically rodents, monkeys, or humans) and the behavioural findings are fairly consistent across species [77]. The present study found that performance in the object recognition task (specifically, the time spent with the novel object in relation to the familiar object, the discrimination index, and the frequency of visits to the novel object) was significantly impaired in $Aldh2^{-/-}$ mice in comparison to wild type mice (with either a 24 hour or a 1 hour delay between training and testing). The novel object recognition task has been widely used in the literature to measure exploratory activity and the ability to recognize novel objects in a familiar environment. Semantic memory in humans is known to be used to retrieve and remember information for naming and categorizing objects, and individuals suffering from AD have been shown to have difficulties recognizing objects because of deficits in semantic memory [78]. Similarly in rodents, memory acquisition occurs when an animal perceives an object's physical properties and applies semantic attributes to it, and rodent models of AD have shown impairments in recognition memory because of their inability to distinguish between, and recognize and explore novel objects over familiar objects [78]. This test can also provide information about the exploratory behaviour of rodents which is related to attention, anxiety, and preference for novelty, all of which are important components of sustained cognition, and have been shown to be impaired in AD [78]. With regards to the present study, the significant decline in performance in the novel object recognition task by $Aldh2^{-/-}$ mice, represented by a fall in all measures of exploratory behaviour, mimics these

semantic memory impairments and impaired cognitive function seen not only in other rodent models of AD, but in human AD as well, emphasizing the idea that Aldh2$^{-/-}$ mice suffer from a significant decline in semantic recognition memory.

The significant age-dependent decline in the performance of Aldh2$^{-/-}$ mice in both the novel object recognition task and the Y-maze may have resulted from dysfunctions in brain regions critical for sustained spatial working and recognition memory, specifically the hippocampus. Several studies have reported that hippocampal lesions in rodents result in significant spontaneous alternation deficits and recognition memory impairment as a result of an accumulation of $A\beta_{42}$ or products of oxidative stress, or an interruption in brain circuitries (often times as a result of $A\beta_{42}$ or oxidative stress accumulation) [76]. The present study found that declines in spontaneous alternation, as well as all measures recorded during the novel object recognition test were evident as early as 3.5-4 months of age in Aldh2$^{-/-}$ mice, despite a lack of significant increase in $A\beta_{42}$ at this early age (see Figure 4). Whereas there may be a lack of significant accumulation of Aβ at this time point, other products of oxidative stress, particularly HNE, are significantly increased (see Figure 5), indicating that an overall decline in spatial working and recognition memory may result from general increases in oxidative stress in the brain, which are known to impair synaptic growth and plasticity (as described in the Introduction). Also, many studies have described a lack of correlation between significant accumulations of $A\beta_{42}$ in the brain and memory impairment in AD patients [79]. This mirrors what was observed in the present study since memory impairments were evident well before any significant increases in $A\beta_{42}$.

The results from this study, as well as the results from clinical trials of a number of therapeutic agents question the primary role of $A\beta_{42}$ in initiating the pathogenesis of AD, and emphasize the impact that other factors, particularly oxidative stress, can have on memory. As described in the Introduction, it has been shown that ALDH2 is important for the catabolism of oxidative stress products such as HNE, which have been found at significantly elevated levels in AD patients, and our laboratory has shown that $Aldh2^{-/-}$ mice develop significant increases in oxidative stress as early as 3 months of age. Whereas many details of the mechanisms by which oxidative stress impacts memory and cognition remain unknown, neurological research continues to uncover new mechanisms by which synaptic plasticity can be altered. For example, the cholinergic system is thought to be important for learning and memory, and acetylcholine has been shown to facilitate neuronal firing in neurons through muscarinic receptor stimulation, which leads to several downstream changes in synaptic plasticity signalling cascades. HNE has been shown to decrease the release of acetylcholine, resulting in a disruption in learning and memory mechanisms that rely on cholinergic signalling, leading to decreases in synaptic transmission and plasticity, impairing learning and memory [80]. As mentioned above, our laboratory has shown that $Aldh2^{-/-}$ mice exhibit significantly higher HNE levels at 3 months of age, and the results from this study have shown significantly impaired spatial working and recognition memory in both male and female mice at 3 months of age as well. The lack of ALDH2 enzyme activity in $Aldh2^{-/-}$ mice likely explains the significant increases in HNE that we have observed. In turn, this could be the primary driving force behind the memory impairments that are seen early in the life cycle of $Aldh2^{-/-}$ mice, possibly via impairments in the cholinergic system as mentioned above.

There are a number of other factors important for sustaining memory and cognition that can be impacted by oxidative stress, and which may explain the early impairment in spatial working and recognition memory observed in Aldh2$^{-/-}$ mice. In addition to muscarinic receptor stimulation increasing calcium entry into the cell via the inositol triphosphate (IP3) and diacylglycerol (DAG) pathway (whereby IP_3 can diffuse to the sarcoplasmic reticulum to trigger the opening of calcium channels and increase the release of calcium into the cytoplasm), the activation of neuronal NMDA receptors by glutamate can also increase sodium and calcium entry into a neuronal cell. The increase in calcium leads to the downstream activation of several cell signalling cascades. Specifically, calcium ions can bind to calmodulin and activate several protein kinases, which can translocate into the nucleus, where they can activate CREB, an important transcription factor that has shown to be important in spatial learning and memory [37]. Synaptic strengthening via the activation of CREB (described in the Introduction) is thought to be a major cellular mechanism underlying learning and memory, and oxidative stress has been shown to impair glutamate release, induce cell death pathways, and disturb the mitochondrial membrane, leading to cellular dysfunction which can impair learning and memory in AD brains [36]. Transgenic animal models of AD have shown that oxidative stress can result in a decrease in CREB activation in hippocampal neurons which may contribute to the exacerbation of AD progression including behavioral and memory impairment [81]. Previous work in our laboratory has shown that Aldh2$^{-/-}$ animals exhibit significant decreases in phosphorylated CREB as early as 3 months of age, which progressively worsens over time (Figure 9). Similarly, the results of the present study have shown impairments in the Y-maze and novel object recognition test at

this early time point which also progressively worsens, indicating progressive spatial working and recognition memory impairment. It is possible that the elevated level of oxidative stress in these animals has impaired both muscarinic receptor and NMDA receptor signalling, thereby reducing phosphorylated CREB levels, and causing the decline in spatial working and recognition memory exhibited by Aldh2$^{-/-}$ mice.

Oxidative stress may also cause a decline in the depolarization of the neuronal membrane surface, resulting in a deterioration of neurotransmission systems. It may also affect neuronal growth, axonal transport, and synaptic function by forming adducts with proteins involved in these processes and by the disruption of calcium signalling, which plays many important roles in the formation and function of neuronal circuits [61]. Oxidative stress may also induce changes in synaptic signalling that may trigger the death of neurons, possibly via apoptosis-related events such as caspase activation in synaptic terminals and dendrites, or through neurotrophic factor withdrawal that may trigger the activation of apoptotic cascades in synaptic terminals [61].The induction of these apoptosis-related events can lead to neuronal cell loss, and consequently, general memory and cognitive impairments in patients suffering from AD. Previously performed molecular analyses have shown significant increases in activated caspases 3 and 6 in the hippocampus of Aldh2$^{-/-}$ mice at 9 months of age. The present study has shown a significant and progressive decline in memory and cognition over time that plateaus at approximately 6 months of age (memory impairments were also seen at 12 months of age in a different cohort of mice). Presumably, these memory impairments will continue to be seen at 9 months of age, and the overall decline in memory may have resulted via

oxidative stress-induced alterations in synaptic signalling and elevated caspase activation, disrupting signalling cascades and inducing neuronal cell loss.

All behavior testing was performed on both male and female mice; however, significant sex differences in spatial working and recognition memory were not observed in the present study, both in wild type and Aldh2$^{-/-}$ mice. Species and sex differences have been demonstrated in many cognitive behavioral tests; however, current literature suggests contradicting reports of sex differences in spatial ability and memory in rodents and rodent models of AD [82]. Some transgenic animal models of AD, including the commonly used APPxPS1 model, have shown spatial memory impairments in behavioral paradigms (such as the Barnes maze and the Morris water maze) that differ with sex, while others do not report any sex-differences (and actually combine males and females into one test cohort) [83]. Several explanations have been proposed to account for behavioral sex-differences. For example, there have been reports that males and females differ in their motivation to learn certain tasks (untrained females, for example, are much less motivated to forage for food when deprived of food in comparison to males) and in their displays of anxiety [82]. Other explanations include differences in physiological development of the hippocampus or the effects of certain hormones. Some reports claim that the volume of the hippocampus differs between sexes, and that the total number of receptor sites in the hippocampus (such as the number of benzodiazepine, steroid, or serotonin receptors) differs between males and females [82]. Finally, hormones such as estrogen have been shown to affect hippocampal physiology, although their role in learning and memory remains unknown. Estrogen fluctuations have been shown to be important for performance of females in spatial memory, and high levels of estrogen

(such as that seen in pregnant rodents) have been associated with significantly poorer performance in behavioral tasks such as the Y-maze and radial arm maze, despite the neuroprotective role estrogen has been shown to have in the brain [82]. Several studies in mice support higher risk for AD in females mediated by $A\beta_{42}$ or APP because of sex hormones (although it is unknown how $A\beta_{42}$ or APP may interact with female hormones), and there is some evidence suggesting varied effects of sex hormones on tau proteins (animal models have shown that female mice develop more tangles than males, and that males possess some kind of greater protection against tangle formation); however, sex differences in tau pathology have not been described in humans [84]. Although hormone levels were not measured, the inconclusiveness of sex differences in memory and cognition and their effects on AD pathologies that have been reported in the literature is mirrored in the present study, as no differences between males and females in performance in the Y-maze and novel object recognition task were seen.

Studies on AD patients have produced similar inconclusive results with respect to age-related sex-differences. Increasing age is one of the main risk factors for AD, and since women typically have a higher life expectancy than men, this alone would result in increased incidence of women with AD [85]. Studies have shown that, whereas men have a greater total prefrontal brain volume than women, sex differences in both gray and white matter are not seen in AD patients [85]. Epidemiological studies have reported equal rates of AD in American men and women, whereas European studies have shown higher rates in women [86]. Estrogens, and other gonadal steroids which act on target sites in the brain, have the potential to combat the neurodegenerative pathologies of AD, and the beneficial effects of estrogens on the brain have been proposed as a potential explanation

as to why AD is rarely seen in women prior to menopause and why hormone replacement therapy is associated with a reduced incidence in AD. However, other epidemiological analyses have yet to conclusively prove that estrogen hormones can provide any protective role against AD [86]. In short, the evidence supporting sex differences in AD in humans and in rodent models of AD is inconclusive. Although hormones such as estrogen have been proposed as key factors underlying sex differences, their effects on AD are not completely understood. The present study has shown that although there is a trend towards female Aldh2$^{-/-}$ mice performing slightly worse than male Aldh2$^{-/-}$ mice, no statistical difference was found in either the Y-maze or the novel object recognition task, again emphasizing the inconsistencies in sex-differences in animal models of AD.

Interestingly, the present study showed that Aldh2$^{-/-}$ mice exhibited memory impairments at a relatively early age of 3.5 months. Currently used animal models of AD (such as the commonly used Tg2576 animal model, or the APP+PS1 animal model) exhibit behavioral and memory impairments much later in life. Behavioral tests by Arendash *et al.* [87] using APP and APP + PS1 animal models demonstrated that these animal models exhibited normal memory and cognition at 5 to 7 months of age in a wide range of tasks including the Y-maze, Morris water maze, and radial arm water maze. Memory impairments were only seen after 15 to 17 months of age in these transgenic animals, much later than the impairments seen in Aldh2$^{-/-}$ mice. Similarly, both the Tg2576 and 3xTg-AD transgenic animal models did not exhibit memory impairments until 12 months of age when assessed for spontaneous alternation rates in the Y-maze [76]. Again, these memory impairments appear much later in life than in Aldh2$^{-/-}$ mice and, coupled with the early appearance of significantly increased HNE adducts and

decreased phosphorylated CREB at 3 months of age, they represent a significant advantage that Aldh2$^{-/-}$ mice have over transgenic AD models, because cognitive impairments occur at a more experimentally useful time.

The results from this study also showed that Aldh2 heterozygote mice exhibited significant memory impairments in comparison to wild type mice although these were not as severe as those of Aldh2$^{-/-}$ mice (impairments were found at 6.5-7 months of age). The spontaneous alternation rate was significantly higher in heterozygote mice relative to Aldh2$^{-/-}$ mice, despite the fact that both were significantly different to wild type (Figure 21). The heterozygote mice also frequented the novel object significantly more than the familiar object (at 6.5-7 months of age) whereas Aldh2$^{-/-}$ mice of the same age frequented both objects nearly equally (Figure 20). There were no differences between heterozygotes and Aldh2$^{-/-}$ mice in the discrimination index and in the amount of time spent with the novel object (both genotypes were significantly lower than wild type). ALDH2 activity should be present in brains from heterozygotes, even with a single functioning allele, and as such, the findings of significant memory impairments were somewhat unexpected. Heterozygous ALDH2 should result in an activity level of the enzyme half that of wild type, potentially resulting in increased levels of HNE in the brain. With a single functioning allele, the activity of ALDH2 should be higher than a complete knockout, possibly explaining why performance in the behavioral paradigms was at a level between what was seen in Aldh2$^{-/-}$ mice and in wild type mice at this time point. A similar decline in activity has been seen in humans. Individuals heterozygous for the ALDH2 Glu504Lys polymorphism, present in approximately 30-50% of the East Asian population, have ALDH2 activity levels that are only about 6% of normal enzymatic activity [72]. As

mentioned previously, although these studies have shown that, whereas there was no increased risk of AD associated with the variant ALDH2, subgroup analysis did indicate that there was a significant association in males (although inconsistent results have been reported). Regardless, memory was still significantly impaired in all recorded data in the present study, and a molecular characterization of heterozygous ALDH2 mice should be performed in order to determine whether the activity of the enzyme is affected, and whether levels of HNE or changes in other molecular markers that were observed in $Aldh2^{-/-}$ mice are altered as well.

There are a few limitations to the chosen methodologies in this study. One of the main limitations to the Y-maze is that a decline in spontaneous alternation in $Aldh2^{-/-}$ mice may have been caused by a lack of curiosity or impulsiveness rather than actual spatial working memory impairment. One of the ways to correct for this would be to measure choice latencies by measuring the time it takes for the mice to choose an arm, and one would expect higher latencies in apathetic mice, and lower latencies in impulsive mice. Surprisingly few studies have actually performed this analysis and, whereas a negative result (no difference in latencies despite impairment in spontaneous alternation) may indicate that another factor may have been involved (such as a loss in short-term memory), it remains possible that other factors, including a lack of motivation to explore, are still involved.

An additional limitation involves the delay between training and testing in the novel object recognition task. As mentioned previously, a 24 hour delay was used for the duration of the study, and a one-hour delay was only performed once the mice were approximately 8 months old. Delay-dependant impairment is particularly powerful

evidence of true memory impairment because it helps to exclude other interpretations of behavior based on motivation or attention deficits. It has also been frequently reported in rodents with hippocampal or cortex damage [77]. The study could have been expanded upon had a one hour (or even shorter) delay been used at all of the time points, especially at a young age. It is possible that a shorter delay between training and testing at an early time point (i.e. 3 months of age) may have shown sustained memory and cognition in Aldh2$^{-/-}$ mice even though impairments are evident after a 24 hour delay, emphasizing a decline in long-term memory in this potential AD model.

Finally, human recognition memory can be a function of specific recollection of encountering an item, or a generic feeling of familiarity in the absence of specific recollection [77]. This kind of generic feeling is difficult to measure in animals, and the standard animal tests of recognition memory do not incorporate this distinction, although progress with respect to this distinction is being made in the development of animal tests [77].

4.1 Future directions

Oxidative stress is an important factor in the initiation and progression of AD, and products of oxidative stress, specifically HNE, are present throughout the lifetime of Aldh2$^{-/-}$ mice. The data of the present study indicated significant memory impairment as early as 3.5 months of age that progressively worsens until a plateau at approximately 6 months of age. Although spatial working memory and recognition memory were examined using the Y-maze and novel object recognition tasks respectively, other behavioral tasks could be performed to further explore other types of memory, both short-

term and long-term, and their impairment in Aldh2$^{-/-}$ mice. The Morris water maze or Barnes maze are two widely used paradigms that have the potential to provide more insight into the behaviour of Aldh2$^{-/-}$ mice. Also, behavioral tests examining anxiety or fear (such as the Elevated plus maze, or the step through passive avoidance test) have the potential to provide insight into the learning abilities of Aldh2$^{-/-}$ mice. A month-to-month behavioral characterization of wild type and Aldh2$^{-/-}$ mice, beginning at 3 months of age, using these tests could further show learning and memory impairments in Aldh2$^{-/-}$ mice, and could further show that these behavioral impairments were a result of a decline in memory, and not because of a change in anxiety or other factors. Also, as mentioned above, a molecular characterization of heterozygous Aldh2 animals could provide insight into whether ALDH2 enzymatic activity is reduced or whether other pathological hallmarks of AD are observed, which could help explain the memory impairments that were seen. Finally, previous work by our laboratory has examined a novel organic nitrate, GT1061, which has shown promising effects on the progression of AD in other animal models. In order to further establish Aldh2$^{-/-}$ mice as a potential model for cognitive impairment with AD-like pathology, GT1061, as well as other currently prescribed therapeutics for AD could be tested in Aldh2$^{-/-}$ mice in order to determine whether behavioral deficits can be prevented (by treating animals prior to 3 months of age), or reversed (by treating animals after memory impairments are evident) by drug treatment.

4.2 Conclusions

AD is one of the most significant afflictions in the world, affecting millions of people and costing the economy billions of dollars every year. It is the most common

form of dementia and is characterized by the accumulation of Aβ plaques, NFTs, neurodegeneration, and impaired memory, thinking and behaviour. The study of AD has been hindered by the absence of animal models of late-onset/age-related AD (which accounts for nearly 95% of AD cases) since the majority of transgenic mouse models exhibit pathological changes that are dependent on the overexpression of mutant human genes linked to early-onset, familial AD (which only accounts for approximately 5% of cases). Oxidative stress has been under investigation for a number of years and is considered to be a causative factor in age-related AD. We have found that Aldh2 null mice exhibit not only oxidative stress, but also display many AD-like pathologies, including brain atrophy, increased phosphorylated tau protein, activated caspases, age-related changes in Aβ, PSD-95, and phosphorylated CREB. The current study used the Y-maze and novel object recognition tasks to assess whether $Aldh2^{-/-}$ mice also exhibit memory and cognitive deficits. In both tasks, significant decreases in performance occurred in $Aldh2^{-/-}$ mice as early as 3.5 months of age and this progressively worsened over the next several months. Sex-related differences in memory impairment were not observed, and heterozygote animals also exhibited memory impairments. These results, together with the findings that AD-like pathologies are also present, suggest that $Aldh2^{-/-}$ mice represent a new, oxidative stress-based model of age-related cognitive impairment with AD-like pathologies. This model may prove useful both for assessing AD therapeutics and for gaining better insight into the pathogenesis of AD.

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