NEUROPSYCHIATRIC-LIKE BEHAVIOURAL FEATURES OF THE $ALDH2^{-/-}$ MOUSE MODEL OF ALZHEIMER’S DISEASE

by

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Abstract

Alzheimer’s disease (AD) is a complex, multifaceted neurodegenerative disorder characterized by progressive memory impairment and frequently presents with neuropsychiatric comorbidities, notably anxiety and depression. We have established Aldh2\(^{-/-}\) mice as an oxidative stress-based model of age-related memory loss and cognitive impairment. However, very little is known about non-cognitive behavioural deficits, or if a sexual dimorphism exist in this model, as it does in humans. We performed a behavioural characterization of anxiety-related behaviours in response to aversive stimuli and depression-like behaviours related to despair and anhedonia. The performance of three cohorts at different time points (3-4, 7-8, and 11-12 months) was examined in a battery of behavioural tests consisting of an assessment of mobility and exploration (open field test), anxiety-related behaviour (light/dark box, elevated plus maze), and depression-related behaviour (forced swim test, tail suspension test, sucrose preference test). Compared to wild type mice, male and female Aldh2\(^{-/-}\) mice exhibited increased anxiety-like behaviours that were first observed at 7 months of age. This anxious behaviour was prevented in Aldh2\(^{-/-}\) mice treated with a deuterated polyunsaturated fatty acid (D-PUFA) diet for 10 weeks. Male Aldh2\(^{-/-}\) mice exhibited a diminished preference for sucrose compared to wild type male mice.

Dysfunction in the hypothalamic-pituitary (HPA) axis presents along with AD, anxiety, and depression. It was postulated that HPA dysfunction may underlie anxious and depressed behaviours in this model. This was evaluated using a mild chronic unpredictable stress (CUS) paradigm. The sucrose preference test and serum corticosterone analysis revealed no differences in response to chronic stress or acute stress between Aldh2\(^{-/-}\) and wild type mice. These data reveal previously unreported behavioural abnormalities in the Aldh2\(^{-/-}\) model of AD-like cognitive impairment.
Co-Authorship

The research presented in this thesis was conducted by Nicole Czegledy under the supervision of Dr. Brian Bennett. Data collection and statistical analyses were performed by Nicole Czegledy. Diane Anderson performed all breeding and genotyping of the mouse colony.
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List of Abbreviations

3xTG: Three times transgenic mouse model of Alzheimer’s disease
aMCI: amnestic mild cognitive impairment
Aβ: Amyloid beta peptide
ACE: angiotensin converting enzyme
ACTH: adrenocorticotropic hormone
AD: Alzheimer’s disease
ADAM: a disintegrin and metalloproteinase domain-containing protein
ADHD: attention deficit hyperactivity disorder
ALDH2: aldehyde dehydrogenase 2
ANOVA: analysis of variance
APoE: apolipoprotein E
APP: amyloid precursor protein
ARA: arachidonic acid
BACE1: beta-site amyloid precursor protein cleaving enzyme 1
CUS: chronic unpredictable stress
CREB: cyclic adenosine monophosphate response element binding protein
CRH: corticotrophin releasing hormone
DG: dentate gyrus
DHN: docosahexaenoic acid
DNA: deoxyribonucleic acid
ELISA: enzyme-linked immunosorbent assay
EOAD: early-onset Alzheimer’s disease
EPA: eicosapentaenoic acid
EPM: elevated plus maze
D-PUFA: deuterated-polyunsaturated fatty acid
FST: forced swim test
GABA: gamma-Aminobutyric acid
GC: glucocorticoid
GR: glucocorticoid receptor
H-PUFA: hydrogenated-polyunsaturated fatty acid
HNE: 4-hydroxy-2-nonenal
HPA: hypothalamic-pituitary adrenal
LDB: light/dark box
LOAD: late-onset Alzheimer’s disease
LPO: lipid peroxidation
LTP: long-term potentiation
MCI: mild cognitive impairment
MR: mineralocorticoid receptor
MWM: Morris water maze
NFT: neurofibrillary tangles
OFT: open field test
OS: oxidative stress
NMDAR: N-methyl-D-aspartate receptor
NOR: novel object recognition
p-Tau: hyperphosphorylated tau
PCR: polymerase chain reaction
PSD-95: post-synaptic density protein 95
PSEN: presenilin gene
PSEN1: presenilin 1
PSEN2: presenilin 2
PUFA: polyunsaturated fatty acid
PVN: paraventricular nucleus
ROS: reactive oxygen species
sAPPα: soluble amyloid precursor protein alpha
sAPPβ: soluble amyloid precursor protein beta
SHIRPA: SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment
SPT: sucrose preference test
TST: tail suspension test
VP: vasopressin
Chapter 1

Introduction

1.1 Statement of the Research Problem

Alzheimer’s disease (AD) is a neurodegenerative disorder that is complex in its pathogenesis and debilitating in its pathology. It is characterised by progressive cognitive decline associated with amyloid-β (Aβ) peptide accumulation, neurofibrillary tangles (NFT) containing hyperphosphorylated tau protein, and generalized cortical and hippocampal atrophy (1), and disrupted cholinergic and excitatory neurotransmission (2). Progressive cognitive decline is a core symptom and includes memory impairment and loss of executive function. The brain regions that are most significantly affected in AD include the hippocampus, amygdala, entorhinal cortex, and frontal lobe. Many disease processes co-occur within the AD brain and it remains unclear what the initiating event is. There is considerable heterogeneity in both the multitude of behavioural impairments observed and the age of onset in AD patients. Despite decades of research, the underlying mechanisms responsible for the pathogenesis of AD remain poorly understood. Consequently, there are currently no effective disease-modifying therapies that offer relief to patients or their caregivers.

Currently, transgenic animal models used to understand AD largely do not reflect the latent and progressive onset of impairments that are observed in most human patients. Though these models frequently display significant cognitive impairment, many models fail to demonstrate the non-cognitive comorbidities commonly experienced by AD patients. These include psychiatric symptoms, sleep disturbances, heart diseases, and pain (3). The study of AD is hindered by rodent models that do not demonstrate the entirety of the impairments and disease processes present in AD, suggesting there is a need for novel modelling approaches.

The Aldh2−/− mouse exhibits increased 4-hydroxy-2-nonenal (HNE) protein adducts and deficits in working and spatial memory that begin at age 4 months and show age-related progression (4). These mice
also exhibit numerous pathological, biochemical, and structural changes in the brain parenchyma and cerebral vasculature. We believe the Aldh2−/− mouse model represents an oxidative stress-induced, age-related, AD-like model of cognitive impairment. This model displays a constellation of pathological changes related to AD, however it was not previously known if this model also exhibits non-cognitive behavioural abnormalities commonly comorbid with AD.

1.2 Incidence of AD

Alzheimer’s disease is the most prevalent form of dementia, affecting over 24 million individuals globally (5). The prevalence of AD is expected to rise as the global population ages (6,7) due to its higher incidence during advancing age and its relatively long duration of illness. It is currently estimated that 6.4% of the population in North America over the age of 60 are living with dementia (8). The age-specific incidence rate of AD increases each year after age 60 (1% at age 60, 6% at age 70, 8% at age 85) (6). It remains difficult to accurately depict the incidence and patterns in the progression of AD because the time of clinical diagnosis may not be representative of the true onset of clinical symptoms.

1.3 Clinical Diagnosis of AD

Clinically, AD is diagnosed after excluding other causes of memory impairment. A key feature in the diagnosis of AD is the evolution of the disease. AD is a progressive disorder that can be categorized into the following stages: pre-clinical, amnestic mild cognitive impairment (aMCI), early-stage AD, and late-stage AD. Currently, clinical diagnoses are based on reported symptoms and cognitive tests, rendering the distinctions between the aMCI, early-, and late-stage AD largely subjective. A definitive diagnosis of AD can only be made post-mortem.

AD patients often present with aMCI that appears to be a transition between normal aging and dementia. The diagnostic criteria described by the National Institute on Aging (9) include: (i) concern about a change in cognition from the patient or an informant, (ii) evidence of lower performance in one or more cognitive domains relative to the patient’s age and educational background, (iii) a maintained ability
to live independently, though some impairments in completing tasks of daily living may be present. The cognitive domains impaired may include: memory, executive function, attention, language, and visuospatial skills. It is currently estimated that 10-15% of patients with aMCI will progress to dementia per year (9). Progression from aMCI to early-stage AD is characterized by (i) a decline in cognitive function from a previous higher level, (ii) a decline in one or more areas of cognition in addition to memory, (iii) a clinical dementia rating score of 0.5-1, (iv) impaired activities of daily living, and (v) a clinical evaluation that excludes other explanations of the symptoms present. The disease will then further progress to late-stage AD, characterized by severe dementia with profound global cognitive deficits (10).

1.4 Genetics of AD

Alzheimer’s disease is a genetically dichotomous disease that presents as one of two distinct forms: early onset AD (EOAD) which exhibits typical Mendelian inheritance, and late onset AD (LOAD), or sporadic AD (≥60 years of age) which has no consistent genetic basis. Known mutations in the genes: amyloid precursor protein (APP), tau, and presenilin 1 (PSEN1) (11,12) are implicated in the small minority (<5%) of patients who fall under the subtype of EOAD. There are currently 16 known fully penetrant mutations in APP, 140 in PSEN1, and 10 presenilin 2 (PSEN2) that lead to EOAD (13). The remaining AD patients present with LOAD, the more common sub-type (>95%). The risk for LOAD development is influenced by genetic variations and environmental exposures.

A polymorphism in the apolipoprotein E (ApoE) allele confers additional risk in the development of LOAD. ApoE is a cholesterol carrier that is involved in the transport of cholesterol into neurons(14). Single amino acid differences result in three isoforms with different abilities to bind to their receptors, lipids, and Aβ (15). Risk of LOAD is significantly increased in individuals with one or two copies of the ε4 allele, whereas the ε2 allele has protective effects against AD (5). ApoE ε4 interacts with Aβ and is associated with senile plaques (15), suggesting its increase in risk of developing AD may be exerted by increased Aβ production (16). However, the ε4 allele is present in 15% of the general population and its presence is neither necessary nor sufficient to guarantee the development of LOAD in old age.
Several lifestyle and comorbid factors have been proposed to explain the origins of LOAD pathology. Significant relationships have been observed between LOAD and the following: diabetes, hypertension, stroke, depression, thyroid condition, cancer (any type), and head trauma (17). Several population-based studies have considered these factors, however, a clear consensus has not emerged (8). Population-based studies suggest that environmental factors are a significant factor in determining the rate of progression after AD diagnosis, with factors such as male sex, low education, comorbidities (e.g. diabetes, heart disease, hypertension), and physical disability contributing to shorter survival after diagnosis (8).

A meta-analysis on AlzGene (18), an online database of AD candidate genes, reveals over 40 loci that show nominal significance in risk of LOAD development from genome-wide association studies. Notable examples from this study include genes related to disorders believed to be comorbid with AD. For example, angiotensin converting enzyme 1 (ACE) is involved in controlling blood pressure. Hypertension during midlife is a risk factor for developing LOAD (19), therefore genetic alterations in ACE consequently alter an individual’s risk of developing LOAD. Another example is interleukin 8 (IL8), a cytokine that plays a role in mediating inflammatory responses. Chronic inflammatory illnesses are also believed to be a risk factor in developing LOAD (20). It appears that comorbid conditions may have predictive validity of life-time AD risk and could serve as a point of intervention before AD pathology arises.

1.5 Pathogenesis of AD

The Alzheimer’s brain exhibits a myriad of co-occurring disease processes, rendering it difficult to discern which may be the initiating event. Key features of this neurodegenerative disease include Aβ plaques and NFTs, disrupted neurotransmission, brain atrophy, neuroinflammation, and oxidative stress.

The presence of Aβ and NFTs has become a defining feature of this disease and has been extensively researched in animal models. Plaques form extracellularly when Aβ aggregates, whereas NFTs are formed as a consequence of hyperphosphorylation of the intracellular tau protein. The
pathogenic Aβ peptide arises through abnormal amyloid precursor protein (APP) processing (Figure 1). APP is converted to the amyloid β (Aβ) peptides through sequential proteolytic cleavage by α-, β- and γ-secretases. Presenilin-1 (PSEN1) and presenilin-2 (PSEN2) are proteins of the γ-secretase complex that regulate its activity. Mutations in PSEN1, PSEN2, and APP lead to early-onset AD in an autosomal-dominant manner. These mutations result in the over-expression of Aβ. This formed the basis of the rationale for the Amyloid Cascade Hypothesis of AD, which posits Aβ that is present under normal physiological conditions becomes overproduced and accumulates to initiate the pathological events resulting in AD. This is supported by the observations that soluble Aβ can disturb synaptic function and induce cognitive deficits (21–23). Several variants of the Aβ peptide exist due to differences in the sites of cleavage by gamma-secretase, all differing at the carboxy terminus. Two variants, Aβ-40 and Aβ-42, are the primary forms in AD. Aβ-38 does not appear to be implicated in AD pathology (24). Aβ-42 is more prone to fibril formation and appears to be the more toxic form of the peptide since EOAD brains show an increase in the ratio of Aβ42:Aβ40 (24). However, the amount of Aβ does not correlate well with the clinical impairment observed in AD patients (25) and many clinical attempts to treat AD patients by targeting Aβ with monoclonal antibodies have been unsuccessful in developing a disease-modifying therapy (26). The degree of impairment observed in AD does not appear to correlate with the amount of p-tau accumulation (27) which suggests that NFTs are insufficient to explain the pathogenesis of AD.

An additional pathogenic factor in the AD brain is a state of neuroinflammation. Aggregated proteins trigger an innate immune response, leading to the release of inflammatory mediators that contribute to neurodegeneration. It is likely that neuroinflammation plays a role early in disease progression, as activated microglia and inflammatory markers were elevated in the neocortex of early stage AD patients in a post-mortem study (28). Further, a genetic analysis revealed that increased proinflammatory cytokine production capacity was correlated with a parental history of LOAD (28). Oxidative stress (OS) is an additional cause of disease in the AD brain and will be discussed below. It is clear that many factors are involved in propagating and exacerbating AD pathology. Novel treatments
should be reflective of the complex amalgamation of disease processes that contribute to the observed pathology of AD.
Amyloid beta (Aβ) is generated by the amyloidogenic pathway. Amyloid precursor protein (APP) can be cleaved by β-site amyloid precursor protein cleaving enzyme 1 (BACE1) to generate soluble APPβ (sAPPβ). The γ-secretase complex, which consists of Presenilin 1 or 2, nicastrin anterior pharym-defective-1 (APH-1), and presenilin enhancer 2 (PEN2) cleave sAPPβ to generate Aβ. Disintegrin and metalloproteinase domain-containing proteins (ADAM) contribute to the production of soluble APPα (sAPPα) via the non-amyloidogenic pathway. Modified from reference (29).
1.6 Oxidative Stress in AD

Reactive oxygen species (ROS) are highly reactive species with at least one unpaired electron in the outer shell. ROS are a normal by-product of oxygen metabolism in mitochondrial respiration, enzymatic reactions such as phagocytosis and prostaglandin synthesis (30), and non-enzymatic reactions such as those initiated by ionizing radiation. Examples of ROS are hydrogen peroxide and free radicals such as superoxide anion and hydroxyl radical. At low concentrations, ROS can act as second messengers in cell signalling, especially during inflammation, ischemia, and stress. The immune system utilises ROS adaptively in order to destroy invading pathogens (30). Because ROS are common in cells, numerous antioxidant systems exist to prevent overproduction. Oxidative stress (OS) is a pathological state that arises when ROS are elevated beyond the metabolic capabilities of the antioxidant enzymes present, and results in protein and DNA damage (Figure 2). Excess ROS exert deleterious effects by interacting with proteins, lipids, and DNA, resulting in DNA strand breaks, protein modifications, and disruptions in normal cellular signalling. This contributes to chronic inflammation and ultimately leads to cellular death, suggesting OS plays a role in exacerbating neurodegeneration. The brain is particularly vulnerable to OS due to its high rate of oxygen consumption (neurons require high levels of ATP in order to meet their relatively high metabolic demands) and relatively modest complement of antioxidant defenses.

Oxidative stress is a common feature of neurodegenerative and neuropsychiatric disorders, including AD, Parkinson’s disease, depression, and attention deficit hyperactivity disorder (ADHD). Oxidative stress markers precede the onset of cognitive decline and the appearance of Aβ plaques and NTFs in AD patients (31). Because biomarkers of OS precede the onset of cognitive decline (10,31), OS may be an early event contributing to the progression of AD.
Oxidative stress (OS) from a variety of genetic and environmental factors increases the formation of amyloid beta (Aβ) by increasing the expression and activity of amyloidogenic amyloid precursor protein (APP) processing enzymes such as β-secretase and γ-secretase. Aβ contributes to the generation of OS and lipid peroxidation by reciprocal positive feedback. Elevated levels of Aβ create an environment which favours the hyperphosphorylation of tau protein, resulting in the production of neurofibrillary tangles (NFTs). The aggregation of extracellular Aβ forms amyloid plaques, which in turn contribute to the generation of OS. OS and neuroinflammation contribute to neurodegeneration which leads to neuronal death. Modified from reference (32).
Lipid peroxidation (LPO) is the process by which lipids undergo oxidative degradation to form highly reactive electrophilic aldehydes, most often secondary to states of OS. Lipid peroxidation occurs selectively in membrane lipids and induces cellular damage by increasing membrane rigidity, altering the activity of membrane-bound enzymes, and altering permeability (33,34). As a result, LPO can interfere with neurotransmission and neuronal function. LPO results in the selective production of free radicals in the lipid components of cellular membranes. Two broad categories of by-products are derived through endoperoxide or hydroperoxide intermediates. Endoperoxide intermediates give rise to F₂-isoprostanes derived from arachidonic acid (ARA), F₃ isoprostanes derived from eicosapentaenoic acid (EPA), and F₄ neuroprostanes derived from docosahexanenoic acid (DHA). These metabolites are chemically stable and therefore serve as reliable biomarkers of LPO. Hydroperoxide intermediates decompose to produce reactive aldehydes including: malondialdehyde, acrolein, 4-hydroxy-2-nonenal (HNE), 4- hydroxy-2-hexenal (HHE), and several others. HNE is an alkenal α,β-unsaturated aldehyde formed by the peroxidation of ω-6 polyunsaturated fatty acids (PUFA) (35). HNE can attach to proteins by Michael addition to thiol (-SH) or amino (-NH₂) groups of Cys, His, and Lys residues (35). Though it is unclear if OS is an initiating factor or consequence of AD pathology, it has been demonstrated that HNE covalently modifies Aβ, triggering aggregation via cross-linking of Aβ peptides (36) and HNE is elevated in ventricular fluid of AD patients (37).

Lipid peroxidation (LPO) (Figure 3) occurs following initiation by the abstraction of bisallylic hydrogen atoms from an unsaturated carbon resulting in resonance-stabilized free radicals, which can react with molecular oxygen to produce lipid peroxyls. These newly formed species can, in turn, abstract a hydrogen atom from an adjacent lipid to form a lipid hydroperoxide and a lipid radical, thus propagating the reaction. Termination occurs when two peroxyl radicals react with each other (homologous recombination), or by reaction with chain-terminating antioxidants, such as Vitamin E.
A polyunsaturated fatty acid (PUFA) containing double bonds separated by a methylene group results in a highly reactive hydrogen on the methylene group that is readily abstracted. Abstraction of a hydrogen from a PUFA generates a fatty acid radical that reacts with oxygen to produce a peroxyl fatty acid capable of abstracting a hydrogen from a second PUFA, propagating the generation of fatty acid radicals. Modified from reference (38).

Figure 3. Chain propagation reaction of PUFAs
1.7 Selective Vulnerability of the Hippocampus to Oxidative Stress

The selective pathology observed in AD stems from the increased vulnerability of certain neuronal populations. A division of the hippocampus is one of the structures most affected by AD. The hippocampus is a structure containing densely packed neurons that is subdivided into regions CA1-CA4. Both CA1 and CA3 are composed of pyramidal cells, but respond differently to OS. When exposed to various OS-inducing agents including superoxide anions, ferrous sulphate, and hydrogen peroxide, CA1 neurons exhibit far greater cell death than CA3 neurons (39,40). Transcriptomic analysis shows that CA1 neurons express significantly higher levels of both antioxidant genes and genes related to the production of ROS than CA3 (39). The observed difference in gene expression related to OS may be partially explained by differences in the signalling roles of ROS in the different regions. For example, CA1 neurons require superoxide for synaptic plasticity through long-term potentiation (LTP), as demonstrated by disrupted LTP in CA1, but not CA3, after the introduction of superoxide scavengers (41). This suggests that CA1 neurons exhibit a differential signalling profile that is intrinsically more vulnerable to OS. Mitochondria are the site of energy production and also the primary source of ROS production (42). Transcriptional analysis shows that mitochondria isolated from CA1 neurons produce more ROS than those from CA3 neurons (39). Because ROS are produced in the mitochondria, mitochondrial DNA and proteins are at high risk of accumulating oxidative-induced damage. Accumulating mitochondrial damage may lead to energy shortages that prevent neurons from producing sufficient antioxidant enzymes which in turn exacerbates the production of ROS. Together, this suggests that OS is an important feature that contributes to neurodegeneration in the CA1 region of the hippocampus.

1.8 Neuropsychiatric Disorders Comorbid with AD

Though memory loss has long been considered the defining clinical feature of MCI and AD, clinical populations present with a number of notable neuropsychiatric comorbidities. There is a compelling body of literature supporting the association between the presence of neuropsychiatric
symptoms and the subsequent development of AD, however, it is unclear whether these features are in part responsible for contributing to or are merely a consequence of the pathology of AD. In MCI, 35-75% of patients display neuropsychiatric symptoms including apathy, anxiety, depression, irritability, and agitation (43). The presence of comorbid neuropsychiatric symptoms increases the risk of conversion from MCI to AD in patients with depression (44), apathy (45,46), anxiety (47), and agitation (48).

Considerable variation exists regarding neuropsychiatric symptoms and AD. Depression appears to be more common during prodromal AD but not predictive of rate of functional decline, whereas symptoms of personality change (e.g., agitation, irritability, passivity) and psychosis were associated with a more rapid functional decline (48). The remainder of this review focuses on anxiety and depression symptoms and their role in progressive stages of AD.

Depression is a psychiatric disorder characterized by low mood. The DSM V (49) requires either depressed mood and/or loss of interest or pleasure to be present, and at least five of the following symptoms to be present in a two week period: weight loss or gain, insomnia or hypersomnia, psychomotor agitation or retardation, fatigue, feelings of worthlessness, diminished ability to concentrate, and thoughts of suicide. Anxiety is an umbrella term for several psychiatric disorders characterized by inappropriate or unhelpful worries or nervousness. This includes disorders such as phobias, post-traumatic stress disorder, panic disorder, and generalized anxiety disorder. The aversive emotional state triggered by anxiety may manifest itself as avoidance of a perceived source of danger and activation of the sympathetic nervous system.

It is unclear if neuropsychiatric comorbidities represent an additional feature of the prodromal phase of the disease, or if they are an independent risk factor. Anxiety and depression disorders frequently precede AD (50,51). Depression symptoms have been shown to be predictive of AD in a large-scale cohort where the onset of depression preceded the onset of AD by 25 years of more (52). A similar twin study revealed a history of depression to be associated with AD. This association was present up to 10 years before the onset of memory impairment, however it was strongest when the depressive episodes
occurred less than 10 years before onset of AD symptoms (53). However, it has also been suggested that depressive symptoms are a clinical feature of prodromal AD (54). A meta-analysis of MCI patients revealed that anxiety was the most common symptom (52%) and that symptom frequency increased with increasing clinical severity. A trend toward a significant association between anxiety and conversion to AD was seen (p<0.052) (50). However, in contrast to the positive associations shown, several studies have claimed contrary results (55,56). Thus, the relationship among anxiety, depression, and AD remains unclear and is easily confounded by prior treatment, heterogeneity in these disorders, and many environmental factors. The study of these comorbidities is further complicated by difficulties in obtaining diagnoses and in differing methodology used in studies.

The co-occurrence of anxiety, depression, and AD suggests common pathogenic origins. There is evidence supporting the theories that this may be the result of a common neurodegenerative pathway or sharing similar genetic risk factors. Individuals who sought psychiatric treatment for depression showed positive associations for familial risk of dementia among first degree relatives (57). Oxidative stress could offer a potential pathogenic explanation, as it is a common feature of AD, anxiety, and depression. Markers of OS in the amygdala, hippocampus, and cortex are elevated in anxious and depressed patients (58,59). Understanding the co-occurrence of psychiatric disorders and AD could provide new insights into diagnosis and treatment of the disease. It may be important to treat mood disorders in order to reduce the psychiatric burden of AD (60) and vastly improve the quality of life of patients and their caregivers.

1.9 HPA Dysfunction in AD

The hypothalamic-pituitary-adrenal (HPA) axis is a major neuroendocrine system responsible for regulating the stress response through mediators such as elevated glucocorticoid and epinephrine secretion. An increase in the amount of circulating glucocorticoids has diverse consequences that may be adaptive to an organism under stress in order to meet the metabolic demands of an immediate threat. This includes actions such as increasing available glucose through gluconeogenesis, glycogenolysis, and lipolysis (60,61).
In humans, the principle glucocorticoid is cortisol, whereas the principle glucocorticoid of rodents is corticosterone. In both primates and rodents, neurons of the paraventricular nucleus of the hypothalamus release corticotrophin-releasing hormone (CRH) into the hypothalamic-hypophyseal portal circulation, which acts on the anterior pituitary to stimulate the secretion of adrenocorticotropic hormone (ACTH) that, in turn, controls the release of corticosteroids from the adrenal cortex (Figure 4). Glucocorticoid release follows a discrete pulsatile pattern with peaks during the beginning of the wake cycle. This release is highly plastic and can be influenced by environmental factors and disease states, that may act through modulatory neural inputs and negative feedback loops to rapidly terminate the stress response when it is no longer necessary. Binding of glucocorticoids to receptors in the hippocampus can inhibit ACTH or CRH synthesis and release. Corticosteroid receptors are distributed throughout the brain, though it has been shown in rodents and humans that they are particularly abundant in the hippocampus (63,64). Glucocorticoids exert their effect via two receptors, mineralocorticoid receptors (MR) (high affinity) and glucocorticoid receptors (GR) (lower affinity). MRs are predominantly expressed in the hippocampus. GRs are expressed throughout the brain, but are most dense in the hippocampus (65). At normal physiological levels of circulating glucocorticoids, MRs are occupied and GRs are mostly unoccupied. This leaves GRs primarily responsible for mediating the effects of elevated stress, and therefore adverse effects of chronic stress are likely mediated by GRs, not MRs (66).
The hypothalamic-pituitary-adrenal axis (HPA) controls the stress response. In response to perceived environmental stress, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH) and vasopressin (VP), which stimulate the production and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland, which stimulates the adrenal cortex to release glucocorticoids (GC). Glucocorticoids in circulation inhibit further glucocorticoid release by negative feedback at the anterior pituitary and PVN through glucocorticoid receptors (GR). GC interact with GR in the hippocampus, which inhibits the stress response via GABAergic projections to the PVN. Low levels of GC act on mineralocorticoid receptors (MR) to maintain basal HPA activity. Modified from reference (67).

Figure 4. HPA axis signaling mediated by the hippocampus
The HPA axis can be modulated by multiple feedback mechanisms to rapidly terminate the stress response when it is no longer necessary. In addition to its role in learning and memory, the hippocampus also contributes to the regulation of the stress response by regulating the release of hypothalamic CRH. Circulating glucocorticoids bind to receptors of the anterior pituitary, hypothalamus, and other brain regions including the hippocampus to inhibit ACTH or CRH synthesis and release. Both ACTH and CRH are controlled by negative feedback mechanisms to inhibit their own release. However, chronic activation of the HPA axis becomes maladaptive and can contribute to disease states by causing impaired growth and tissue repair, insulin sensitivity, hypertension, hyperlipidemia, and neuronal death (62). It is well-established that hypercortisolemia causes cognitive impairments, particularly in memory function (60,68).

Glucocorticoids increase mitochondrial respiration and oxidative phosphorylation, which contributes to neuronal oxidative stress. It has been shown that cortical neurons incubated with corticosterone show concentration and time-dependent increases in mitochondrial oxidation, membrane potential, and calcium-holding capacity (69). It has been demonstrated in vivo that the administration of exogenous corticosterone results in increased markers of OS, including LPO, and increased apoptosis in the hippocampus (70). It has also been shown that stress promotes gliogenesis over neurogenesis in hippocampal neural stem cell progenitors (71). Elevated glucocorticoids are also capable of impairing hippocampal LTP (72,73). In animal models, hypercortisolemia induced by chronic unpredictable stress (CUS), which is exposure to variable stressors over a long time period, or by injection of exogenous GCs, exacerbates AD pathology. Collectively, these observations suggest glucocorticoids may play a role in exacerbating the pathology of AD. This is supported by the observation that the hippocampus contains the greatest density of corticosteroid receptors in the brain (74).

In both mood disorders and AD, the hypercortisolemia observed suggests that the feedback mechanisms responsible for decreasing ACTH and CRH in response to elevated glucocorticoids are not acting appropriately, resulting in an insensitivity to feedback inhibition. Lifetime stress and mood disorders are both considered to be risk factors for the development of LOAD (75,76). This effect could
be mediated by the HPA axis, given epidemiological evidence showing elevated glucocorticoid receptors in the hippocampi of depressed elderly adults (77).

1.10 Modelling AD in Rodents

Research in AD has largely focused on examining pathogenic mechanisms and novel therapeutic interventions in animal models. Current mouse models are based primarily on insertions of the mutant human genes known to cause early-onset AD. Because increased production of Aβ peptides is able to cause cognitive deficits that resemble AD in rodents (78), numerous therapeutic interventions have been developed that target the clearance and reduction of Aβ. Despite decades of research, the initiating pathogenic mechanisms underlying AD remain poorly understood and there are currently no disease-modifying therapeutic interventions. It is important to note that, currently, there are no animal models that exhibit all features of the human disease. Though our understanding of the underlying genetic mutations leading to early-onset AD has improved greatly over the past decades, the recent clinical failures suggest additional genetic and environmental factors contribute to the lifetime risk and progression of AD. Two notable examples include OS and HPA axis dysfunction. Additionally, current models frequently fail to exhibit neuropsychiatric comorbidities observed in human patients, such as anxiety, depression, and an altered stress response. This suggests there is a need for a novel approach to AD research that considers additional mechanisms underlying late-onset AD and age-related progression of disease in order to uncover potential therapeutic interventions.

Previous studies examining non-cognitive symptoms of AD-like pathology in mouse models have primarily focused on behavioural differences induced by the expression of aberrant human genes implicated in early-onset AD. The most commonly used mouse models of AD express the aberrant human APP isoforms responsible for causing EOAD in some individuals. These models fail to represent significant neuronal loss (79). PSEN1 mutations alone do not cause mice to develop cognitive impairments (80). The tau pathology can be modeled similarly using human tau with mutations causing frontotemporal dementia, a form of dementia distinct from AD (81). In order to increase the scope of
impairments and create a more relevant animal model, several mutations are combined. APP, PS1, and tau transgenes are present in the 3xTg line, which is commonly used in AD studies. These mice display both Aβ and NFT pathologies (79). A major limitation of this approach is that the initiating factor of the pathology has little relevance to humans. These mutations are each individually rare, comprising only ~5% of the AD population. It would be exceedingly rare for an individual to have several of these mutations.

In these transgenic rodent models of AD, Aβ immunotherapy successfully prevents disease progression (82). Despite this preclinical success, immunotherapy targeting Aβ has largely been unsuccessful, even when circulating Aβ 40 and 42 are successfully reduced (26). It remains unclear why these treatments fail when translated to human populations. It is possible that it is due to an inability to begin treatment sufficiently early, as AD cannot be diagnosed until clinical impairments are manifested. It is also possible that several additional disease processes contribute to the pathogenesis of AD, rendering a singular immunotherapy target inefficient in mitigating the many dysfunctional processes.

1.11 Chronic Stress Exacerbates AD Pathology in Rodents

Stress is a response to environmental or situational demands that threaten the well-being of an individual. It is an unavoidable part of life that disrupts the typical homeostatic balance of our body systems. In both rodents and humans, stress has demonstrated an ability to cause cognitive impairments in learning and memory, and contribute to depression and anxiety (74,83,84). Because the hippocampus is vulnerable to glucocorticoids, and is especially vulnerable in AD, it has been proposed that chronic stress contributes to the risk of developing LOAD. In rodents, chronic stress accelerates the onset and severity of cognitive impairments, which correlates with the amount of extracellular Aβ and tau phosphorylation (85).
1.12 The *Aldh2* Model

Aldehyde dehydrogenase 2 (ALDH2) is a mitochondrial antioxidant enzyme expressed in the cerebral cortex, hippocampus, basal ganglia, and midbrain (42). It is important for the detoxification of endogenous LPO-derived aldehydes, including HNE, a reactive species known to accumulate in AD brains and associate with amyloid plaques (86,87). It is of particular interest in understanding the pathogenic mechanisms behind late-onset AD because of several population-based studies linking ALDH2 mutations as a risk factor for developing late-onset AD in East Asian populations (86,87). Longitudinal studies of individuals possessing the GLu504Lys loss of function mutation of ALDH2 indicate no clear increased risk of AD, however subgroup analysis revealed a significant association between the variant ALDH2 and AD risk in males (88). Additionally, ALDH2 has been localized to reactive glia and Aβ plaques in both the cerebral cortex and hippocampus (42). In addition to the activity of ALDH2, detoxification of HNE occurs by conjugation with glutathione by glutathione transferases and reduction by aldo-keto reductases. Of these three pathways, only ALDH2 activity is increased in the AD brain (42). Glutathione transferase activity in the AD brain is decreased in several regions, including the amygdala and hippocampus and GST protein was shown to be decreased in all brain regions compared to healthy controls (89).

The *Aldh2* mouse model displays AD-like pathological changes and cognitive impairments originating from OS due to the genetic deletion of ALDH2. These mice exhibit the elevated HNE levels observed in AD, and display progressive biochemical, histopathological, and cognitive changes that resemble LOAD in humans. These pathological changes include increased Aβ, p-tau, activated caspases, synaptic loss, deficient CREB signalling, cognitive impairments, and vascular pathologies (4). HNE adduct formation can be observed at age 3 months. Monomeric Aβ and p-tau were significantly elevated at age 6 months and progressively increased until age 12 months. Similarly, impairments in spatial and working memory were observed at age 3.5 months and progressively worsened until reaching a plateau at age 7 months. Because of sequence differences between the N-termini of human and mouse Aβ, mouse
Aβ does not readily aggregate and form amyloid plaques. Transgenic mouse models that express human Aβ exhibit plaque formation, however Aldh2−/− mice only exhibit increases in monomeric and oligomeric Aβ and not Aβ plaques. The lack of plaque formation is arguably a limitation of the model, but it does allow for the examination of the role of Aβ pathology in cognitive loss per se, which is difficult to accomplish with current transgenic mouse models.

1.13 Chronic Unpredictable Stress

Chronic unpredictable stress (CUS) protocols have been widely used to elicit behavioural and immunological stress responses in rodent models. Chronic stress is implicated in the pathophysiology of several psychiatric disorders, including anxiety, depression, and dementia (90). These protocols consist of exposure to randomized, intermittent stressors in order to prevent habituation. The specifics of the stress exposure such as the type of stressor, intensity, and duration can all affect the response observed. Importantly, the perception of, and the ability of an individual to deal with the stressors is dependent on neuroendocrine, neurochemical, and genetic factors (91). It follows that individuals who are experiencing neurological dysfunction may be more susceptible to stressors.

CUS has been widely used to model psychiatric disorders and to exacerbate the pathology observed in AD models. Exposure to chronic stress increases markers of neuroinflammation in the CNS, increases gene expression of inflammatory factors, and increases anxiety- and depression-like behaviours (92). Chronic stress can induce alterations in gene transcription that lead to an increased vulnerability to infectious diseases and auto-immune attacks (75). In AD models, CUS has been shown to accelerate cognitive decline, increase Aβ deposits, and increase the degree of tau phosphorylation (85). In transgenic models of AD, chronic isolation stress caused an increase in GR expression in the hippocampus (93). Together, these data suggest that chronic stress may contribute to the onset and progression of AD.
1.14 D-PUFAs

In disorders resulting from LPO of PUFAs, a potential strategy to combat disease progression could come from restricting the substrate for this process. A potential strategy to reduce LPO is to decrease the abstraction of bis-allylic hydrogen atoms from the PUFAs, the rate-limiting step of the reaction. This is possible by substituting hydrogen (H-PUFA) with deuterium (D-PUFA) at bis-allylic sites of the PUFAs to create an isotope effect resulting in decreased PUFA peroxidation. This strategy has shown decreased LPO in a cell model of Friedrich ataxia (94). Friedrich ataxia is a hereditary ataxia characterized in part by OS, which leads to subsequent neurodegeneration of spinal nerves and spinocerebellar tracts (95).

1.15 Rationale, Hypothesis, Objectives

Despite considerable evidence showing the associations between anxiety, depression, HPA deficits, and dementia, the interactions between these disorders are not well understood in the AD brain. The Aldh2/- mouse is a model of LPO-induced oxidative damage leading to memory impairments and pathological features of AD. It is not currently known if this model displays additional behavioural disturbances present in human AD. The role of OS in a defective HPA axis response within AD-like models is also not known. Here we perform a behavioural characterization of anxiety- and depression-like behaviours in the Aldh2/- mouse model. Three animal groups at of different ages (3-4, 7, and 11 months) were subjected to a battery of behavioural tests including an assessment of mobility and exploration (open field test), anxiety-related behaviour (light/dark box, elevated plus maze), and depression-related behaviour (forced swim test, tail suspension test). Following this, another cohort was subjected to a four-week CUS protocol at ages 3, 7, and 11 months. Depression-related behaviour was measured with the sucrose preference test. A corticosterone analysis in response to acute restraint stress was performed at age 11 months. Additionally, we evaluated whether a diet enriched in D-PUFAs could be protective against the development of anxiety-like behaviours.
Available data show that the Aldh2<sup>−/−</sup> mouse exhibits memory impairments and molecular markers of neurodegeneration induced by OS. Markers of OS in the amygdala, hippocampus, and cortex are elevated in anxious and depressed patients (58,59). Anxiety and depression disorders frequently precede AD (50,51), and may be implicated in predisposing individuals to AD-like cognitive impairment. It was not previously known if a diet enriched in D-PUFAs can prevent the development of non-cognitive behavioural impairments in the Aldh2<sup>−/−</sup> mouse. The stress response is also disturbed in patients with AD (96). The present experiment investigates the potential links between OS, anxiety and depression; environmental stress; and the ability of dietary D-PUFAs to prevent OS-induced behavioural abnormalities.

1.15.1 Statement of Hypothesis and Objectives

The following five hypotheses were tested in this thesis work:

Hypothesis 1. Age-related increases in anxiety- and depression-like behavioural abnormalities occur in the Aldh2<sup>−/−</sup> mouse.

Hypothesis 2. Anxiety-like behaviours can be prevented in the Aldh2<sup>−/−</sup> mouse by the reduction in LPO induced by 10 weeks of a diet enriched in D-PUFAs.

Hypothesis 3. The Aldh2<sup>−/−</sup> mouse is more susceptible to developing anhedonia following three 4-week sessions of mild CUS.

Hypothesis 4. The Aldh2<sup>−/−</sup> mouse develops HPA axis dysfunction in response to acute and chronic stressors following three 4-week sessions of mild CUS.

Hypothesis 5: Male Aldh2<sup>−/−</sup> mice display more behavioural abnormalities than female Aldh2<sup>−/−</sup> mice.

The objectives of this thesis work were:

1. To determine whether anxiety-like behaviours exist in male and female Aldh2<sup>−/−</sup> mice using the light/dark box (LDB) and elevated plus maze (EPM)
2. To determine whether depression-like behaviours in exist in male and female Aldh2−/− mice using the forced swim test (FST), tail suspension test (TST), and sucrose preference test (SPT)

3. To assess the progression of anxiety- and depression-like behaviours throughout aging in male and female Aldh2−/− mice using time points 3, 7, and 11 months of age

4. To assess differences in the ability of male and female Aldh2−/− mice to cope with mild CUS as measured by the corticosterone response to acute stress and at baseline

5. To determine the ability of D-PUFA to prevent the development of in anxiety- and depression-like behaviours in male and female Aldh2−/− mice

6. To determine if sex differences exist between male and female Aldh2−/− mice in anxiety- and depression-like behaviours or stress response before and after mild CUS
Chapter 2

Materials and Methods

2.1 Generation of Aldh2\(^{-/-}\) Mice

Aldh2\(^{-/-}\) mice were generated by a gene targeting knockout as described previously (97) and were provided by Dr. T. Kawamoto (University of Occupational and Environmental Health, Kitakyushu, Japan). Wild type male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, Maine, United States) and served as the background of the strain. Wild type and Aldh2\(^{-/-}\) mice were backcrossed for more than 10 generations. Age-matched male and female mice obtained were littermates from heterozygous matings and genotyped by PCR analyses of DNA obtained from ear punches using the primers as described previously (98).

All protocols for animal use and care were approved by Queen’s University Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines. The animals were maintained on a reversed 12 hour light/dark cycle with free access to food and water. Non-stressed animals were housed in cages with 1-3 same sex littermates (Aldh2\(^{+/+}\), Aldh2\(^{+/}\), Aldh2\(^{-/-}\)), though some males were separated and housed individually due to aggressive behaviours (wild type n=1-4, Aldh2\(^{-/-}\) n=2-4 per cohort). All animals subjected to CUS were housed individually.

2.2 Behavioural Testing Procedures

Males and females from four cohorts with an approximate 1:1 ratio of male to female animals (20-65g) for each Aldh2\(^{-/-}\) and wild type were subjected to a battery of 5 behavioural tests at 3 time points (ages 3-4, 7, and 11 months). Two cohorts were combined to increase sample size in the 11 month group. Separate cohorts were used for different time points and were subjected to the testing sequence only once. After being subjected to a paradigm, each mouse was left for a minimum of 48 hours before beginning the next paradigm.
All behavioural testing was conducted between 11:00 and 16:00, corresponding with the dark (active) period of the reversed light cycle. The animals were maintained in the dark until testing was conducted. Testing took place in a testing room adjacent to the housing room that was maintained at 23-25°C with minimal background noise. The open field test (OFT) was conducted in a separate room where the equipment was housed, that also had minimal background noise and was maintained at 23-25°C. The animals were allowed to habituate to this room while remaining in the dark for a minimum of 1 hour prior to testing. Where applicable, tests were videotaped and scored by the author, who was blind to the genotypes of the animals. Testing arenas were cleaned with 70% ethanol prior to each individual trial. Testing was conducted in order of increasing degree of stress associated with the test, as justified previously (99). Testing was performed in the following order: 1) elevated plus maze, 2) light-dark box, 3) open field test, 4) tail suspension test, 5) forced swim test. A separate cohort was used for the sucrose preference test.

2.2.1 Open Field Test

The open field test (OFT) was used to assess spontaneous locomotor activity during free exploration and was performed as described previously (100). The OFT was conducted using a square Plexiglass® open field apparatus (45cm x 45cm x 25cm). Each mouse was placed individually in the centre and allowed to explore freely for 10 minutes. The test was performed under ambient light conditions and minimal background noise. Spontaneous locomotion was assessed by a grid of infrared beams using a software data acquisition system (TSE Actimot, Scientific Products and Equipment, Concord, ON). The total time spent moving, total distance travelled, and time spent rearing were recorded and analyzed.

2.2.2 Light/Dark Box

The light/dark box (LDB) test was used to assess aversion to bright illumination as a measure of anxiety-like behaviour. The opaque plastic arena (50cm x 25cm x 18cm) was arranged as described (101).
It was divided unequally such that two-thirds of the space was considered the “light” side and one-third was the “dark” side. The compartments were divided by an opaque barrier with a hole (6cm x 6cm) along the bottom to allow free movement between sides. The dark side was enclosed by opaque plastic sheets to block light and the light side was illuminated by a 60W bulb suspended 20 cm above the arena. Animals were placed in the dark side and allowed to explore freely for 6 minutes. An entry was considered when the centre of mass of the animal crossed the division between sides. The latency to enter the light side and total time spent in the light and dark sides over the 6 minute testing period were recorded for analysis.

2.2.3 Elevated Plus Maze

The elevated plus maze (EPM) was used to assess aversion to an elevated open space and was performed as described (102). The maze was elevated 80 cm from the floor and consisted of 4 arms (2 open, 2 closed), measuring 60 cm x 8 cm, with walls 12 cm in height on the closed arms. Testing was conducted under ambient lighting. Each mouse was placed individually in the centre of the maze and allowed to explore freely for 6 minutes. An entry was considered when the centre of mass of the animal crossed the entrance to the arm. The time spent in the open and closed arms was recorded along with number of individual entries into each arm for analysis.

2.2.4 Forced Swim Test

The forced swim test (FST) was used to assess mobility versus immobility in response to an aversive, inescapable situation. The FST was performed in accordance with the procedures described previously (103). Mice were placed into the centre of a 2 L polypropylene cylinder filled with 1600 mL of water maintained at room temperature (23-25°C). The water was sufficiently deep that the mouse’s tail could not touch the bottom of the container. Testing was conducted in a dark room illuminated by red light. Mobility was defined as the concerted movement of 2 limbs resulting in propulsion or any change in the orientation of the body. This definition excludes passive limb movements performed to maintain
floating. The latency to becoming immobile and time spent mobile and immobile were recorded for analysis.

2.2.5 Tail Suspension Test

The tail suspension test (TST) was also used to assess mobility versus immobility in response to an aversive, inescapable situation. The TST was performed in accordance with protocols described previously (103). The tails of the animals were wrapped in 8 double layers of gauze and suspended by a clamp approximately 2 cm from the end of the tail, such that the head was suspended approximately 8 cm from the base of the testing box. Testing took place individually in a white chamber (35cm x 25cm x 20cm) for a duration of 6 minutes. Testing was conducted in a dark room illuminated by red light. Immobility was defined as passive hanging or holding a static position, while mobility included concerted movement of 2 limbs, body shaking, and changes in position. The latency to becoming immobile and time spent mobile and immobile were recorded for analysis.

2.2.6 Sucrose Preference Test

The sucrose preference test (SPT) was performed four weeks after beginning the CUS protocol. The mice were given 48 hours to adapt to the experimental set up (housed singly in a divided cage with 2 bottles of water). For 24 hours, mice were given free choice between two bottles, one with water and the other with 2% sucrose solution. Bottle position was switched every 12 hours, halfway through the light cycle to prevent a side bias. The mice had *ad libitum* access to food and water at all times to minimize the influence of metabolic factors.

Sucrose intake was estimated in mg of sucrose consumed per gram of body weight. The preference for sucrose was calculated in terms of the percentage of sucrose solution consumed over the total amount of liquid consumed during the 24 hour time period. The liquid consumed from each bottle was measured by weight of fluid consumed and licking behaviour over the 24 hour period. Licking behaviour was measured by connecting a metal sipper tube to the input of a standard analog/digital
converter and connecting the animal to the ground via an aluminum cage floor (Figure 5). A single lick produces 100-800mV dc voltage step when the animal’s tongue completes an electrical circuit. This voltage step lasts only for the duration of the contact between the tongue and the spout. This methodology was described previously (104). A “licking event” in this study was recorded when a rate of 10 licks per second was registered. These events were collected for a period of one minute and then the total value during the one minute period was recorded. The percent preference was determined by the number of events interacting with the sucrose bottle divided by the total number of events with both the water and sucrose bottles to give a value relative to each animal’s drinking behaviour.

2.3 Chronic Unpredictable Stress Paradigm

Wild type and knockout mice were subjected to a 28 day chronic unpredictable stress (CUS) paradigm. To our knowledge, this protocol has not been used; and was adapted from protocols conducted previously (105,106). The CUS protocol is useful for eliciting depression-like symptoms in mice. These protocols can be modified to mild stressors, which is more useful in examining sensitivity to developing depression-like behaviours. The stressors presented here were selected because they do not induce pain or directly alter food intake or weight gain. The mild stress paradigm consisted of 7 different stressors over a period of 4 weeks (Figure 7). Mice were exposed to a different stressor each day in a randomized order that was consistent for all animals. The stressors included:

Constant light: mice were exposed to 36 hours of constant light, disrupting their typical dark cycle.

Novel object exposure: mice were exposed to an unfamiliar object (plastic beads) mixed in with their bedding during their dark cycle for 12 hours.

Saturated bedding: the cage bedding was saturated with 700mL of room temperature water for 12 hours during the dark cycle.

Slanted cage: the cage was tilted 45° for a duration of 12 hours during the dark cycle.
Novel environment: mice were introduced to a foreign environment with unfamiliar cage substrate (e.g. wood shavings, sand, 10 cm diameter plastic tubing) for 15 minutes during the dark cycle.

2.4 Plasma Sample Collection and Preparation

Plasma corticosterone in the CUS cohort was measured at three time points: baseline, after restraint stress, and after recovery from restraint stress (Figure 6). Restraint stress was delivered by restraining mice for 15 minutes using a 50 mL plastic cylinder with ample ventilation. Blood samples were collected from the saphenous vein of conscious, restrained mice within 30 seconds of removing the mouse from its home cage. This occurred between 17:00-19:00, a time period coinciding with the beginning of the light cycle (19:00). The blood was collected in heparinized capillary tubes and centrifuged at 1500 x g for 10 minutes in a refrigerated centrifuge. The plasma was removed and then stored at -80 degrees Celsius until analysis.

2.5 Fecal Sample Preparation

Fecal samples were collected from CUS male and female and control male mice between 17:00-19:00, a time period coinciding with the beginning of the light cycle (19:00). Samples were dried in an oven at 50°C until there was no weight change, indicating water loss had ceased and the samples were dry. Samples weighing 0.05-0.2 g were powdered and stored at -80 °C until analysis. Corticosterone was extracted from fecal samples by homogenization with 100% reagent alcohol (85% ethanol, 5% methanol, 5% isopropanol, 5% water) (0.1g feces/mL). The homogenate was centrifuged at 5000 x g for 15 minutes. The supernatant was retained and centrifuged at 7000 x g for 5 minutes. The supernatant was recovered and 1 mL was evaporated under nitrogen and then re-dissolved in 100 μL reagent alcohol.
2.6 Corticosterone Concentration Analysis

The plasma and fecal corticosterone concentrations were quantified by an enzyme-linked immunosorbent assay (ELISA) (Arbor Assays Inc., Ann Arbor, MI, USA) according to the manufacturer’s instructions. The kit has been verified with cross-reactivity equivalent to 12.3% desoxycorticosterone, 0.76% tetrahydrocorticosterone, 0.62% aldosterone, 0.38% cortisol, 0.24% progesterone, and 0.12% dexamethasone. Plasma samples were diluted 1:200 and analyzed. Fecal samples were diluted 1:9 and analyzed. The absorbencies were recorded at 450 nm using a SpectraMax iD3 plate reader (Molecular Devices, LLC, San Jose, CA, USA). Concentrations of corticosterone are expressed as ng/mL of plasma or ng/g of feces.

2.7 Estrous Cycle Evaluation

Vaginal swaps were collected from female mice as described previously (107) using a cotton tipped swab soaked in ambient temperature saline solution. The swab was inserted into the vagina of the mouse and gently rolled along the vaginal wall. Cells from the swab were transferred to a glass slide. After air drying, the slides were stained with Dip Quick stain (Jorgensen Laboratories, Loveland, CO, USA) according to manufacturer’s instructions. The slides were then rinsed with distilled water and viewed under magnified bright field illumination. The stage of the estrous cycle was determined by the relative abundance of nucleated epithelial cells, leukocytes, and cornified epithelial cells as described previously (107). Estrous cycle testing was performed within two hours of blood collection.

2.8 Adrenal Gland Dissection

18 month old male and female wild type and Aldh2<sup>−/−</sup> mice were subjected to 3 rounds of 4 week long mild CUS and several rounds of behavioural testing as described above. After sacrifice, the right and left adrenal glands were isolated under a light microscope and rinsed with phosphate buffered saline (PBS), allowed to dry, and the weights recorded. Relative weight was determined by averaging each animal’s left and right adrenal gland weight (mg) and dividing by its body weight (g).
2.9 Statistical Analysis

All statistical analyses were performed using GraphPad Prism Version 6.00 (GraphPad Software, San Diego, CA). Comparisons between two groups were performed using the unpaired Student’s \( t \) test where appropriate. The relationships between behavioural features were examined over time and between genotype, sex, and stress groups using one-, two-, and three-way analysis of variance (ANOVA). Bonferroni’s post-hoc test was applied, as appropriate. Data are expressed as the mean ± the standard deviation. Differences between means from the experimental groups were considered significant at \( p < 0.05 \).
Figure 5. Voltage-dependent lick measurer set up

Positive terminals are connected to water and sucrose water spouts. Negative terminals are connected to a metal sheet at the bottom of a mouse cage. Voltage produced when an animal stands on the metal plate and touches a spout is converted to a digital number representing the magnitude of the voltage at a given time point by the analog-to-digital converter.
<table>
<thead>
<tr>
<th>Baseline</th>
<th>CORT</th>
<th>15 min acute restraint stress</th>
<th>CORT</th>
<th>30 min recovery</th>
<th>CORT</th>
</tr>
</thead>
</table>

**Figure 6. Acute stress procedure and plasma corticosterone collection timeline**

Plasma collection for corticosterone (CORT) concentration analysis was done at baseline, after 15 minutes of acute stress, and after 30 minutes of recovery.
<table>
<thead>
<tr>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 Saturated bedding</td>
<td>30 Slanted cage</td>
<td>31 Tail suspension</td>
<td>1 Novel environment</td>
<td>2 Continuous light</td>
<td>3 No bedding</td>
<td>4 Novel environment</td>
</tr>
<tr>
<td>5 Tail suspension</td>
<td>6 Saturated bedding</td>
<td>7 No bedding</td>
<td>8 Novel environment</td>
<td>9 Tail suspension</td>
<td>10 Continuous light</td>
<td>11 Slanted cage</td>
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<tr>
<td>12 Slanted cage</td>
<td>13 Saturated bedding</td>
<td>14 No bedding</td>
<td>15 Tail suspension</td>
<td>16 Saturated bedding</td>
<td>17 Novel environment</td>
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<td>19 Slanted cage</td>
<td>20 Novel environment</td>
<td>21 Tail suspension</td>
<td>22 Saturated bedding</td>
<td>23 No bedding</td>
<td>24 Continuous light</td>
<td>25 Slanted cage</td>
</tr>
</tbody>
</table>

**Figure 7. Sample 4 week CUS protocol**

Chronic unpredictable stress (CUS) delivered by a randomized order of mild stressors over a 28 day period.
Chapter 3

Results

3.1.1 Mobility and Free Exploration Assessment

The open field behavioural task was used to assess ambulatory movement and revealed no difference between *Aldh2*−/− and wild type mice in total time moving or total distance travelled (Figure 8) at any time point. Similarly, exploratory behaviour as measured by time spent rearing revealed no difference between *Aldh2*−/− and wild type mice at any age measured (Figure 8). Time spent rearing decreased significantly between ages 3 and 7 months (*p*<0.0001, *p*<0.001), and 7 and 11 months (*p*<0.0001, *p*<0.0001) in both *Aldh2*−/− and wild type mice, respectively, and between ages 7 and 11 months in wild type mice (*p*<0.05).
Figure 8. Ambulation and exploration by Aldh2−/− and wild type mice in the open field test.

The pattern of movement shows no significant differences in ambulatory movement as assessed by total time moving (A) and total distance travelled (B) between Aldh2−/− and wild type mice at ages 3, 7, and 11 months. No differences in exploratory behaviour were observed as assessed by total time rearing (C) between Aldh2−/− and wild type mice at ages 3, 7, and 11 months. Time rearing decreased significantly between ages 3 and 7 months, and 3 and 11 months in both Aldh2−/− and wild type mice, and between ages 7 and 11 months in wild type mice. Data are presented as mean ± SD (wild type n=9-18, Aldh2−/− n=9-18) and were analyzed by two-way ANOVA and a Bonferroni post-hoc test. There were significant effects of age on time mobile, total distance, and time rearing ($F_{2,77}=5.1, p<0.01; F_{2,77}=9.5, p<0.001; F_{2,77}=48.2, p<0.0001$). Significant differences reported as indicated (* $p<0.05$, *** $p<0.001$).
3.1.2 Male and Female Performance in Anxiogenic Environments

Using the light/dark box (LDB), a classical test for anxiety as measured by aversion to the illuminated side of the arena, we found that Aldh2\textsuperscript{-/-} mice showed a preference for the dark side compared to wild type controls at ages 7 (31 ± 24 s, 74 ± 53 s; \( p=0.0029 \)) and 11 months (33 ± 17 s, 78 ± 40 s; \( p\leq0.001 \); Figure 9), but no difference between groups was observed at age 3 months. The Aldh2\textsuperscript{-/-} progressively spent less time in the light chamber as they aged, comparing 3 and 7 months, and 3 and 11 months of age (\( p<0.001; p<0.001 \)).

The elevated plus maze (EPM) was also used to assess anxiety behaviours measured by preference for exploring the open arms and hiding in the closed arms of the apparatus. The EMP revealed no differences in time spent in the open or closed arms between genotypes at age 3 months (Figure 10). The time spent in the closed arm (Figure 10A) was significantly different between Aldh2\textsuperscript{-/-} and wild type mice at 7 months (271 ± 14s, 232 ± 30s; \( p\leq0.001 \)), but not at 3 and 11 months, although comparison of Aldh2\textsuperscript{-/-} males revealed a trend toward significance (\( p=0.062 \)). The time spent in the open arm (Figure 10B) was significantly decreased in Aldh2\textsuperscript{-/-} males compared to wild type males at 7 (7 ± 5 s, 24 ± 14 s; \( p\leq0.01 \)) and 11 (1 ± 5 s, 35 ± 32 s; \( p\leq0.01 \)) months, but not in Aldh2\textsuperscript{-/-} females compared to wild type females. There was a trend toward a significant effect of sex on time in the open arm (\( F_{1,35}=4.0, \quad p=0.053 \)).
Figure 9. Time spent in illuminated chamber of the light/dark box by wild type and Aldh2−/− mice.

Male and female mice were subjected to the light/dark box test. Male and female data sets were not significantly different and were combined. Significant differences were observed between Aldh2−/− and wild type mice at age 7 months and 11 months, and between Aldh2−/− cohorts at ages 3 to 7 months and 3 to 11 months. The amount of time spent in the illuminated chamber was analyzed by 2-way ANOVA and a Bonferroni post-hoc test. Data are presented as mean ± SD (wild type n=17-20, Aldh2−/− n=14-20). There were significant effects of genotype and age on time in the illuminated chamber ($F_{1,102}=22.5, p<0.0001;$ $F_{1,102}=13.9, p<0.0001$). Significant differences reported as indicated (** $p<0.01$, *** $p<0.001$, **** $p<0.0001$).
Figure 10. Time spent by wild type and Aldh2\(^{-/-}\) mice exploring the closed (A) and open (B) arms of the elevated plus maze.

Male and female mice were allowed to explore the elevated plus maze freely for 6 minute trials at ages 3, 7, and 11 months. The amount of time spent in the open arm, closed arm, and middle were recorded. Significant differences were observed between Aldh2\(^{-/-}\) and wild type mice at age 7 months (A). Male and female cohorts were separated for analysis of time spent in the open arm (B). Significant differences were observed between Aldh2\(^{-/-}\) and wild type male mice at ages 7 and 11 months. Data are presented as mean ± SD (wild type male n=8-10, Aldh2\(^{-/-}\) male n=8-10; wild type female n=8-10, Aldh2\(^{-/-}\) female n=8-10). Data were analyzed by 2-way ANOVA and Bonferroni post-hoc test. There were significant effects of genotype on time in the closed arm, age on time in the closed arm, and age on time in the open arm (\(F_{1,103}=8.6, p<0.01\); \(F_{1,103}=5.2, p<0.01\); \(F_{2,95}=10.89, p<0.0001\)). Significant differences reported as indicated (** \(p<0.01\), *** \(p<0.001\)).
3.1.3 Male and Female Despair Behaviours in the Forced Swim and Tail Suspension Tests

The forced swim test (FST) and tail suspension test (TST) were used to assess depression-like behaviours as measured by time spent actively attempting escape from an aversive situation. The TST revealed no differences in time moving at any time point (Figure 11). The FST revealed no differences in time spent mobile at ages 3 and 11 months, but revealed Aldh2<sup>−/−</sup> animals spent less time moving than wild type controls at age 7 months (96 ± 18 s, 123 ± 23 s; \( p \leq 0.001 \); Figure 12).
Figure 11. Time spent actively attempting escape in the tail suspension test.

Male and female mice were subjected to the tail suspension test at ages 3, 7, and 11 months. The amount of time spent mobile and immobile were recorded. Data were analyzed by 2-way ANOVA. Significant differences were not observed between genotypes or sexes at any age. Data are presented as mean ± SD (wild type n=11-14, Aldh2+/– n=9-16).
Male and female mice were subjected to the forced swim test for 6 minute trials at ages 3, 7, and 11 months. The amount of time spent mobile and immobile were recorded. Data presented was analyzed as described. Significant differences were observed between *Aldh2*/*-*/ and wild type mice at age 7 months. Data were analyzed by 2-way ANOVA and a Bonferroni post-hoc test. Data are presented as mean ± SD (wild type *n*=15-20, *Aldh2*/*-*/ *n*=15-20). Significant differences reported as indicated (**p*<0.001).
3.1.4 Anxiety Behaviour in Light/Dark Box Following CUS

Twelve month old male and female mice were subjected to three intervals of four weeks of CUS at 3-4, 6-7, and 10-11 months of age (Figure 13). The LDB test was used to determine if stress could be a factor responsible for exacerbating anxiety behaviours. A two-way ANOVA revealed an interaction between sex and stress condition so the groups were not combined ($p=0.002$). The prior significant difference observed in the control group at 11 months (Figure 9) was abolished by the CUS protocol. No differences were observed between $Aldh2^{-/-}$ and wild type mice within the CUS groups.
Figure 13. Exploration of the illuminated chamber of the light/dark box by male and female mice subjected to CUS.

Male and female mice were subjected to the Light/Dark Box at age 11 months after three intervals of four weeks of CUS at ages 3-4, 6-7, and 10-11 months. The amount of time exploring the illuminated side of the chamber was recorded. Data were analyzed by three-way ANOVA and a Bonferroni post-hoc test. Significant differences were observed between control wild type males and control Aldh2+/− males, and control wild type females and control Aldh2+/− females. Data are presented as mean ± SD (male: wild type n=10-11, Aldh2+/− n=9-15; female: wild type n=7-9, Aldh2+/− n=8-10). There were significant effects of genotype × stress, and sex × stress on time in the illuminated chamber (F1,70=87.7, p<0.01; F1,70=6.0, p<0.05). Significant differences reported as indicated (* p<0.05, ** p<0.01).
3.1.5 Male and Female Anhedonia Behaviour in the Sucrose Preference Test

Male and female wild type and Aldh2<sup>−/−</sup> mice were subjected to the sucrose preference test (SPT) to assess anhedonia after CUS at ages 4, 7, and 11 months, and in control (unstressed) animals at ages 7 and 11 months. Preference for the sucrose solution was determined by the ratio of licking events (defined previously) with the sucrose spout relative to the total number of licking events. The weight of the bottles before and after the 24 hour test period was also recorded and analyzed. A 2-way ANOVA revealed significant differences between the two methods of analysis within in each group at age 7 months, but not at age 4 months. At age 4 months, no differences were observed between the CUS groups using either method of analysis (Figure 14). No differences were observed between female wild type and Aldh2<sup>−/−</sup> mice in the control group or the CUS group at age 7 months (Figure 15). Male wild type mice showed a significantly increased preference for the sucrose solution compared to male Aldh2<sup>−/−</sup> mice. The wild type males subjected to CUS showed a diminished preference for sucrose compared to the unstressed wild type males. There was no difference observed between the wild type and Aldh2<sup>−/−</sup> males subjected to CUS at 7 months. No differences were observed in males or females at age 11 months.

Percent preference was determined two ways: by dividing the number of sucrose spout interactions by the combined number of interactions with the sucrose and water spouts to give a relative score of licking events (defined previously); and by the difference in weight of the sucrose bottle after 24 hours of testing divided by the combined weight differences of the sucrose and water bottles. These two methods did not generate results that were significantly different ($F_{1,101}=3.722, p=0.057$). A trial was not considered in the analysis when there was evidence of a spill of more than 20mL. The use of events allowed for an inclusion of more data points in cases where the weight was unreliable due to spillage.
Figure 14. Sucrose solution preference by licking events (A) and solution mass (B) of mice subjected to CUS at age 4 months.

Male and female mice were subjected to the sucrose preference test at age 4 months. The number of interactions with the water and sucrose solution spouts and the weight of the bottles were recorded. Percent preference was determined by dividing the number of sucrose spout interactions by the combined number of interactions with the sucrose and water spouts (A), or by the amount of sucrose solution consumed divided by the total amount of liquid consumed (B). Data were analyzed by 2-way ANOVA. Data are presented as mean ± SD (male: wild type n=10, Aldh2^{-/-} n=12; female: wild type n=9, Aldh2^{-/-} n=7).
Figure 15. Sucrose solution preference of male (A, C) and female (B, D) mice at age 7 months.

Male and female mice were subjected to the sucrose preference test at age 7 months. The number of interactions with the water and sucrose solution spouts and the weight difference of the bottles were recorded. Percent preference was determined by dividing the number of sucrose spout interactions by the combined number of interactions with the sucrose and water spouts (A, B), or by the amount of sucrose solution consumed divided by the total amount of liquid consumed (C, D). Data were analyzed by 2-way ANOVA and a Bonferroni post-hoc test. Data are presented as mean ± SD (male: wild type n=9-11, Aldh2+/− n=8-12; female: wild type n=5-9, Aldh2+/− n=3-8). Significant difference reported as indicated (* p<0.05, ** p<0.01).
Figure 16. Sucrose solution preference of male (A) and female (B) mice at age 11 months.

Male and female mice were subjected to the sucrose preference test at age 11 months. The number of interactions with the water and sucrose solution spouts were recorded. Percent preference was determined by dividing the number of sucrose spout interactions by the combined number of interactions with the sucrose and water spouts. Data were analyzed by two-way ANOVA. Data are presented as mean ± SD (male: wild type n=2-4, Aldh2+/− n=3-7; female: wild type n=3-4, Aldh2+/− n=2-4).
Figure 17. Sucrose solution preference by weight of solution consumed (A) and solution consumption relative to body weight (B) in 11 month old mice.

Male and female mice were subjected to the sucrose preference test at age 11 months. Sucrose solution preference relative to body weight was determined by dividing the percent preference determined by bottle weight by each animal’s body mass and multiplied by 100. Data were analyzed by 2-way ANOVA. Data are presented as mean ± SD (male: wild type n=6, Aldh2<sup>−/−</sup> n=8; female: wild type n=5, Aldh2<sup>−/−</sup> n=2).
3.1.6 Fecal Corticosterone Response to Chronic Unpredictable Stress

Fecal samples were collected from male and female wild type and Aldh2−/− mice subjected to CUS and analyzed for corticosterone concentration (Figure 18). Samples were collected within 2 hours of the beginning of the light cycle (19:00hrs). In female mice subjected to CUS, there appears to be a trend toward higher corticosterone concentration that failed to reach significance. This result suggests that there is no clear difference between the baseline fecal corticosterone levels of wild type and Aldh2−/− mice.
Figure 18. Fecal corticosterone concentrations in male (A) and female (B) mice subjected to CUS.

Fecal samples were collected from CUS mice at age 16 months and control male mice at age 10-11 months. Data were analyzed by two-way ANOVA (A) or Student’s t-test for unpaired data (B). Significant differences were not observed between genotypes in female or male CUS groups, or in male control groups. Data are presented as mean ± SD (male: wild type n=6-7 Aldh2-/- n=6-7; female: wild type n=7, Aldh2-/- n=5).
3.1.7 Plasma Corticosterone Response to Acute Stress Post-Chronic Unpredictable Stress

Plasma samples were collected from male and female wild type and Aldh2−/− mice subjected to CUS and analyzed for corticosterone concentration (Figure 19). Samples were collected at three time points to gain an understanding of the pattern of the HPA axis response. The initial sample was taken within 30 seconds of removing the animal from its home cage in order to prevent the baseline sample from alteration by the stress of the procedure. The second sample was collected after 15 minutes of restraint stress, a novel stressor to this cohort. The final sample was collected after 30 minutes of recovery in their home cages, again within 30 seconds of removal from the cage. No significant differences were observed between the genotypes, however there appears to be a trend showing lower initial values in both sexes and genotypes, but this failed to reach significance.

3.1.8 Male and Female Adrenal Gland Weight Post-Chronic Unpredictable Stress

Male and female mice were sacrificed at age 18 months and left and right adrenal glands were dissected and weighed (Table 1). There were no significant differences between left and right (One-way ANOVA and Bonferroni post-hoc test) so the two values were averaged for each mouse. The body weights of each mouse were recorded immediately before sacrifice in order to determine the weight of the adrenal gland relative to the mouse’s weight (mg/g). No significant differences were observed in the average weight of the adrenal glands, the body weight, or the adrenal weight relative to body weight.
Figure 19. Plasma corticosterone concentration response to acute stress in male (A) or female (B) wild type and Aldh2−/− mice subjected to CUS at age 16 months.

Plasma samples were collected from male and female mice at three time points: within 30 seconds of removal from home cage, after 15 minutes of restraint stress, and after 30 minutes undisturbed in their home cages. Data were analyzed by one-way ANOVA. There were no differences observed between sex and genotype groups. Data are presented as mean ± SD (male: wild type n=6, Aldh2−/− n=6; female: wild type n=5, Aldh2−/− n=3.)
Table 1. Adrenal weights relative to body weights following CUS.

Left and right adrenal glands from 18 month old male and female mice were dissected and weighed immediately after sacrifice. Body weights were determined immediately before sacrifice. The adrenal gland weight relative to the body weight was used to determine a relative score. One-way ANOVA revealed no significant differences between genotypes within sexes. Data are presented as mean ± SD.

<table>
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<th>Genotype</th>
<th>Female Wild Type</th>
<th>Female Aldh2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Male Wild Type</th>
<th>Male Aldh2&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Significant difference</th>
</tr>
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<td>3</td>
<td>5</td>
<td>7</td>
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<tr>
<td>Body weight (g)</td>
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<td>50.8±9.7</td>
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</tr>
<tr>
<td>Average adrenal gland weight (mg)</td>
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<td>61.8±31.9</td>
<td>68.2±19.2</td>
<td>83.2±23.7</td>
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<tr>
<td>Relative weight (mg adrenal/g body weight)</td>
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<td>1.3±0.3</td>
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</tr>
</tbody>
</table>
3.1.9 Effect of a D-PUFA-enriched Diet on Ambulation and Exploratory Behaviour

After 10 weeks on a diet of either H-PUFA or D-PUFA, male and female Aldh2<sup>−/−</sup> mice were subjected to the OFT for 5 minute trials. This was done to determine if any exploratory behaviour differences existed before proceeding with further behavioural testing. No differences were observed in time moving (D-PUFA, 161 ± 29 s; H-PUFA, 163 ± 17 s, p > 0.05, Student's t-test for unpaired data) or total distance travelled (D-PUFA, 83 ± 20 m; H-PUFA, 83 ± 18 m, p > 0.05, Student's t-test for unpaired data; Figure 20). Time spent rearing was considered an indicator of exploratory behaviour and revealed no difference between the two groups (D-PUFA, 33 ± 16 s; H-PUFA, 29 ± 11 s, p > 0.05, Student's t-test for unpaired data). These data suggest that treatment with D-PUFA does not change exploratory or pattern of movement.

3.1.10 Effect of a D-PUFA-enriched Diet on Aversion to Illumination in the Light/Dark Box

Five month old male and female Aldh2<sup>−/−</sup> mice were fed either D-PUFA or H-PUFA diets for 10 weeks then subjected to the LDB test to determine if the anxiety behaviour previously observed was rescued following administration of the D-PUFA diet. A two-way ANOVA revealed no sex differences in the observed behaviour so the data were combined. A significant decrease in both number of crosses to the illuminated chamber and total time spent in the illuminated chamber were observed in the H-PUFA group compared to the D-PUFA group (Student’s t-test for unpaired data, p<0.05) (Figure 21).
Figure 20. Ambulation and exploration by Aldh2⁻/⁻ mice fed H-PUFA and D-PUFA diets in the open field test.

Male and female mice were subjected to the OFT test at age 5 months after 10 weeks on either a D-PUFA- or H-PUFA- enriched diet. The pattern of movement shows no significant differences in ambulatory movement as assessed by total time moving (A) and total distance travelled (B) nor exploratory behaviour measured by total time rearing (C) between Aldh2⁻/⁻ mice fed D-PUFA and H-PUFA diets for 10 weeks. Data are presented as mean ± SD (D-PUFA n=16, H-PUFA n=13) and were analyzed by Student's t-test for unpaired data.
Figure 21. Exploration of the illuminated chamber of the light/dark box by mice fed diets enriched in D-PUFAs or H-PUFAs.

Male and female mice were subjected to the light/dark box (LDB) test at age 5 months after 10 weeks on either a D-PUFA- or H-PUFA- enriched diet. The number of crosses to the illuminated side and total time spent in the illuminated side during a 5 minute trial were recorded. Data are presented as mean ± SD (D-PUFA n=16, H-PUFA n=13) and were analyzed by Student’s t-test for unpaired data. Significant differences reported as indicated (* p<0.05).
Chapter 4

Discussion

Previous studies conducted by our laboratory have demonstrated that $Aldh2^{-/-}$ mice exhibit a multitude of pathological features of AD. These include increases in HNE adduct formation at age 3 months, and at age 9 months significant increases in the classic AD markers $A\beta$ and hyper-phosphorylated tau, significant increases in markers of neurodegeneration including activated caspases 3 and 6, significant decreases in PSD-95 and CREB, and significant impairments in spatial and working memory. It was demonstrated that $Aldh2^{-/-}$ mice show memory deficits beginning at 3-4 months of age that reach a plateau at 7 months (4). In order to further classify the neurological impairments related to AD present in this model, the present study examined anxiety-like and depression-like behaviours, stress response, and the ability of D-PUFAs to reverse anxiety-like behaviour.

Both anxiety and depression are prevalent in patients in early phases of cognitive decline and in patients with dementia (52,58,108–110). Previous studies examining non-cognitive symptoms of AD-like pathology in mouse models have primarily focused on behavioural differences induced by the expression of aberrant human genes implicated in early-onset AD. Findings from these studies include characterizations from several different mouse models and show mixed results, spanning the spectrum from elevated anxiety- and depression-like behaviours to disinhibition behaviours resulting in reduced anxiety- and depression-like behaviours (111). There is also considerable evidence of HPA axis dysfunction during midlife as a risk factor in the development of AD and the presence of elevated GCs in MCI and AD (69,93,112).

The present study consisted of three phases: 1) determination of the presence of non-cognitive behavioural abnormalities in $Aldh2^{-/-}$ mice, 2) determination of the influence of chronic mild stress on the development of non-cognitive impairments and the stress response system, 3) determination of the therapeutic benefits of dietary D-PUFAs. The presence and temporal progression of anxiety- and
depression-like behaviours in a mouse model of AD-like cognitive impairment due to elevated oxidative stress (OS) had not yet been characterized. This study revealed progressively worsening anxiety-like behaviours in both males and females (Figures 9,10), significantly increased anhedonia in Aldh2−/− males (Figures 14, 15, 16), and no notable differences in susceptibility to anhedonia following CUS (Figures 14, 15, 16). There were no observed differences in corticosterone concentration following exposure to acute and chronic stressors (Figures 18, 19). No differences were observed in anxiety-like behaviours between groups at age 3-4 months, a time period before the onset of cognitive impairment (Figures 9,10) (4). This suggests that disease processes that may contribute to anhedonia and anxiety-like behaviours, which may exhibit a temporal progression similar to that of memory loss within this oxidative stress model. Male Aldh2−/− mice that were not stressed showed the greatest number of behavioural abnormalities in the LDB, EPM, and SPT compared to wild type males. Females showed anxiety-like behaviours in the LDB and EPM, but appear to be more resilient to the development of non-cognitive behavioural comorbidities. The findings here reveal a previously unknown behavioural change in which Aldh2−/− mice are more prone to anxiety-like behaviours and anhedonia than wild type mice. This difference appears to be more pronounced in male Aldh2−/− mice than female Aldh2−/− mice.

4.1 Movement Analysis

The open field test (OFT) was used as a mobility and exploratory behaviour screen. The Aldh2−/− mice showed no differences from wild type mice in terms of their mobility or exploratory behaviour at any time point evaluated (Figure 8), indicating differences observed in subsequent tests are not attributable to mobility or exploration differences. Both wild type and Aldh2−/− mice showed decreases in rearing behaviour as they aged (Figure 8). This is indicative of decreased exploratory behaviour, however there were no differences between sexes or genotypes suggesting this may simply be a feature of aging. Prior analyses of the strain also revealed no phenotypic differences related to mobility (4). The SHIRPA (SmithKline Beecham, Harwell, Imperial College, Royal London Hospital, phenotype assessment), a standardized battery used to assess motor and sensory phenotype of genetically modified mice, was
performed at ages 2-3 months and 5-6 months and revealed no differences in motor behaviour phenotype (113). This included analysis of reflexes, aggression, respiration rate, and other motor behaviours. It can be reasonably concluded that no motor differences exist within this model at any of the time points evaluated here, and any behavioural differences may be attributed to differences in cognition or motivation.

4.2 Neuropsychiatric Comorbidities

4.2.1 Anxiety-like Behaviours

The elevated plus maze (EPM) test for anxiety-like behaviours revealed Aldh2−/− males spend significantly less time exploring the open arm than wild type males at age 7 and 11 months, which is indicative of increased anxiety-like behaviour. This difference was not observed in females. Both male and female Aldh2−/− mice spent more time in the closed arm of the apparatus compared to wild type mice at age 7 months (Figure 10A). There was no difference observed between males and females at this time point. The time spent in the open arm is likely a more sensitive measure of anxiety-like behaviours because it requires a mouse to choose to actively explore an anxiogenic environment and better relates to the amount of time spent in the illuminated chamber of the light dark box (described below).

The light/dark box (LDB) revealed a significant difference in aversion to bright illumination and diminished preference for exploration in 7 and 11 month old Aldh2−/− mice (Figure 9). Given that the OFT showed no differences in exploratory behaviour, this behaviour can be attributed to aversion to the testing parameter. Both male and female Aldh2−/− mice showed this behaviour, suggesting ALDH2 dysfunction affects anxiety-like behaviours in both males and female mice similarly in this particular behavioural test of anxiety.

Anxiety-like behavioural tests can be broadly divided into unconditioned and conditioned responses. Unconditioned response tests require no training and are considered to have high ethological validity, whereas conditioned response tests require a high degree of training and are sensitive to
interference from motivation and memory differences (114). Only unconditioned response tests were used in this study. Because Aldh2/− mice exhibit memory deficits, conditioned response tests would not be appropriate. This is a potential limitation of anxiety testing in this model, however the LDB an unconditioned response test that is robust and relatively specific. The EPM is limited in that there are essentially three chambers, one open (anxiogenic), one closed (secure), and one in the middle that is ambiguous. The LDB has only two chambers, the illuminated (anxiogenic) and the dark (secure). It is also noteworthy that the EMP considers behaviour to be anxiety-like when animals show aversion to the open arms of the apparatus, however the size of the animal may confound the subjective perception of danger in this test. Because male mice tend to be heavier in body weight than female mice, it is possible that female mice experienced less instability on the open arms and have a platform that is larger relative to their body size. This may explain why female Aldh2/− mice did not display a significantly different aversion to the open arm of the EPM, but did display significantly increased aversion to the illuminated chamber of the LDB compared to female wild type mice.

4.2.2 Depression-like Behaviours

Depression is a complex, heterogeneous collection of disorders that may be manifested in several different behaviours. For this reason, it is important to consider behaviours reflective of several aspects of depression. This study considered behavioural despair, as quantified by time actively attempting escape in an aversive environment and anhedonia.

The FST revealed that 7 month old Aldh2/− mice spent less time mobile than wild type mice, which is representative of despair (Figure 12). This decrease in time spent actively attempting escape was not observed at other time points in the FST or in the TST (Figure 11). The absence of depression-like behaviours observed was somewhat surprising because cognitive impairment and depression overlap in several physiological attributes, including oxidative stress and reduction in hippocampal volume (115). It was established previously that Aldh2/− mice exhibit both elevated markers of oxidative stress and hippocampal atrophy (4).
The TST is limited in validity because it is meant to examine behaviour in an inescapable situation, however, several animals learnt to escape by climbing their tails. The animals that learnt this behaviour had to be excluded from the trials. Additionally, both the FST and TST are very stressful to the animals and can elicit anxiety-like responses and hyper-locomotion that may confound measurements of depressed behaviours (116). Further, aversive testing conditions (e.g., handling, lighting, etc.) can elicit anxiety-like behaviours. In an attempt to minimize such interference, the FST and TST were conducted in a dark room illuminated by red light, though this may not have been sufficient given the aversive nature of these tests. The use of tests that elicit depression-related behaviours in less aversive conditions, such as the SPT, are considered more sensitive (117) because there are fewer confounding motivations that may alter behaviour, such as those related to pain or escape strategies. Additionally, exposing mice to stressors early in life can enhance the sensitivity of depression behavioural testing in rodents (117). The mice were exposed to tests in an increasing order of severity in order to mitigate this effect, however it is notable that depression-like behaviour was observed in the 7 month cohort in the FST which was administered last. It is possible that the three cohorts exposed to these tests were subjected to different levels of environmental stress during the testing procedures which could explain the appearance of depression-like behaviour in the 7 month old cohort and not 11 month old cohort. However, the use of CUS did not appear to selectively alter depression-related behaviour in the Aldh2−/− mice, as described below.

4.2.3 Anhedonia Observed in the Sucrose Preference Test

The sucrose preference test (SPT) is a measure of anhedonia in rodents. Anhedonia is considered a component of depressive disorders in the DSM-V and is characterized by either a reduced ability to experience pleasure or diminished interest in pursuing pleasurable activities (49). Rodents have an innate interest in sweet foods or solutions. The DMS V includes anhedonia as a feature of major depressive disorder (MDD), which is represented in the SPT. Other features of MDD include depressed mood, change in appetite, change in sleep, fatigue, feelings of guilt, diminished concentration, and suicidality.
The SPT revealed that Aldh2−/− males have a reduced preference for sucrose water compared to wild type controls at 7 months (Figure 15A). This result is indicative of anhedonia, which suggests that a previously unreported behavioural difference in reward-seeking behaviour between male and female Aldh2−/− mice at this age. It is notable that this difference was no longer observed at age 11 months (Figure 16) and there was also a marked decline in rearing behaviour at this same time point (Figure 8). It is possible that this set up of the SPT is not sensitive enough to detect anhedonia in older mice because rearing is a necessary component of reaching for the different water bottles. Several features of MDD are difficult to characterize in rodents, therefore we consider anhedonia as a feature of depression rather than conclusive evidence of depression in this model.

It is well-established that sucrose solution preference is attenuated in rodent models of depression and in rodents subjected to chronic stressors (118). In the present study, anhedonia was considered evident when animals did not demonstrate a preference for the sucrose solution greater than 50%. A 2% sucrose solution was selected after preliminary testing revealed a preference consistently above 50% in male and female mice at age 5 months (data not shown). Prior to testing, all animals were housed with the water bottle set up described for this test for at least 48 hours and until there was evidence of volume change from both bottles indicating the animal had interacted with both spouts. As the mice aged, several individuals did not reach this 50% preference threshold. Because the naïve mice initially preferred the sucrose solution, it is possible that the older mice and mice subjected to CUS were no longer motivated to search for the sucrose water, or were not cognitively able to remember the location of the sucrose water.

Using the weight of the bottles to measure consumption of each liquid is limited in accuracy because of the frequency of liquid spillage. To correct for this variability, a voltage recorder was used to determine the number of interactions with each spout. Mouse drinking behaviour is highly rhythmic in that mice typically lick at a rate of 10 licks/second (119). To obtain a more representative measure of licking, rather than touching the spout, an interaction with the spout was counted when rapid, consecutive touches were recorded. Using this measure of licking behaviour allowed for the inclusion of more data
points than bottle weight alone, because several animals spilled considerable volumes of liquid, as observed by wet bedding. Measuring licking events is more sensitive to small changes and allowed for a more accurate representation of the variability of behaviour. Use of bottle weight alone resulted in muted variation (Figure 14 Figure 15) that was not representative of the true drinking behaviour and exploration of the bottles. This also allowed for the inclusion of more data points, as any trial in which an animal had completely spilled a bottle was not included.

No cognitive differences are known to exist between male and female Aldh2−/− mice and no differences were observed in the preference for the sucrose solution, except between the unstressed Aldh2−/− males and females at age 7 months (Figure 15). This suggests that the difference observed is due to anhedonia behaviour present in Aldh2−/− males and not due to cognitive differences between the groups.

4.2.4 Neuropsychiatric Comorbidity Conclusions

The OFT, LDB, EPM, FST, TST, and SPT are classical behavioural tests that are prevalent in literature and relatively standardized. Using a battery of tests for both anxiety and depression in mice is beneficial, as similar behavioural alterations may be elicited in response to different testing parameters (114). Because avoidance of aversive, anxiogenic situations was observed in both the LDB, which elicits fear responses via illumination, and the EPM, which elicits fear responses via height, it can be reasonably concluded that Aldh2−/− mice exhibit elevated anxiety-like behaviours compared to wild type control mice. Male Aldh2−/− mice displayed anxiety-like behaviours in the EPM and anhedonia in the SPT. Together with knowledge that Aldh2 loss of function mutations in humans selectively alter the risk of developing AD in males (87, 120), it appears that the loss of functioning Aldh2 differently effects males and females.

4.3 HPA Axis Dysfunction

Dysfunction of the HPA axis is one of many pathogenic features of AD in humans. The hippocampus plays an essential role in terminating glucocorticoid release from the adrenal glands during the stress response. The hyperactivity of the HPA axis appears to be a consequence of neurodegeneration.
In MCI, cortisol is not elevated in the CSF, however, it is significantly elevated in patients with AD (121,122). Furthermore, GR expression is decreased in AD patients compared to healthy individuals, however, this difference disappears when controlling for neurodegeneration (ratio of synaptophysin to PSD-95), suggesting HPA axis dysregulation is a consequence of the disease state (121). Conversely, HPA axis dysfunction may arise from stresses of daily living with anxiety and depression disorders and may in turn predispose an individual to neurodegeneration. Psychological stress increases the excessive production of toxic oxygen metabolites in human and animal studies (62,74,75,90). It is possible that such psychiatric disorders contribute to an individual’s risk of developing AD. Exposure to stressful stimuli can induce or exacerbate depression in individuals who are prone to developing a depression disorder, but does not do so in the majority of the population (123,124).

4.3.1 Chronic Unpredictable Stress

CUS is one of the most widely used animal models of depression and represents a more natural development of psychological distress in response to adverse life events. CUS involves exposing animals to mild stressors on an unpredictable schedule for several weeks. The use of CUS protocols is well-established in rat models of depression and stress-related disorders, however, a notable challenge exists in using mice. The C57BL/6 mouse is a commonly used background strain for genetically modified mice, however, this breed is more resilient to stress than other mouse breeds (125). An 8 week CUS protocol described by Strekalova et al. (106) appears to be more efficient in inducing consistent neuroendocrine, behavioural, and immune changes following CUS exposure when compared to a more commonly used 4 week protocol. In the present study, the goal was not to induce a consistent stress response. Rather, we aimed to establish whether there were differences in stress response between Aldh2−/− mice and wild type mice. For this reason, a 4 week protocol consisting of mild stressors was chosen. In this study, we did not include predatory stressors such as animal odours or sounds, or social defeat. No stressors were used that disturbed food or water consumption, thus eliminating any possible metabolic confounds. The stressors chosen included continuous light (36 hrs), no bedding, saturated bedding, exposure to a novel
environment, and slanted cage. Because of the duration and stressors used, we consider the protocol described here to be a mild CUS protocol. The CUS protocol used here was repeated three times (age 3 months, 6 months, and 10 months) because this study aims to understand progressive impairments related to aging. Repeated bouts of stress are more representative of what an individual will experience in an uncontrolled environment, and challenges the animal to cope with varying allostatic load over its lifetime. It is unclear if any observable effects of earlier stressors would still be evident in mice 3-7 months later. Furthermore, it would not be possible to observe progressive behavioural differences related to CUS if each time point varied in the amount of time since the CUS protocol had occurred. For these reasons, a mild CUS protocol was repeated 4 weeks before testing.

Typically, CUS induces anhedonia in a subset of animals (106) while others are resilient to the effects of the stressors, however, this did not appear to be the case with the wild type and Aldh2+/− mice in this study. This particular protocol did not induce anhedonia in females of either genotype, but did induce anhedonia in wild type males as measured by the SPT (Figure 15A). Following CUS, there was no observable consistent behavioural response, which suggests that this protocol was sufficiently mild to determine if specific vulnerabilities to CUS exist within the Aldh2+/− strain. The Aldh2+/− mouse does not appear to be more susceptible to anhedonia behaviour following CUS than wild type mice.

In addition to anhedonia, CUS can elicit elevated corticosterone concentrations (126). HPA axis hyperactivity is a common feature of AD, anxiety, and depression (69,112,127). Because anxiety, depression, and AD are frequently comorbid, it is possible that a common etiological factor is involved. In order to obtain an understanding of acute and chronic stress responses in the Aldh2+/− mouse, plasma and fecal samples were analyzed for corticosterone concentration.

4.3.2 Fecal Corticosterone Following CUS

Fecal samples were analyzed as an undisturbed measure of baseline corticosterone levels. Following three sessions of 4-weeklong CUS, no differences in fecal corticosterone levels were observed between wild type and Aldh2+/− animals (Figure 18). However, there was a trend towards an increase in
baseline corticosterone in CUS Aldh2−/− female and unstressed Aldh2−/− males, and significantly different variance in the CUS females. It is possible that, with further testing and larger sample sizes, a significant increase would emerge. Fecal corticosterone levels are typically reported relatively as there can be considerable variation in the extraction methods used. A possible confounding factor in this analysis could arise as a consequence of other phenotypic differences between wild type and Aldh2−/− mice that do not relate to the HPA axis. One potential explanation could arise from gastrointestinal differences that may exist between the strains. We anecdotally observed gastrointestinal abnormalities during the dissection of several Aldh2−/− mice at ages 12-16 months. The ALDH2 polymorphism is associated with elevated incidence of gastric (128) and colorectal cancer in humans (120). If Aldh2−/− mice have an increased susceptibility to gastrointestinal abnormalities, it is possible that the fecal composition and fecal output are also atypical, which may in turn alter the corticosterone concentration present in a given fecal sample.

A limitation of this protocol was the gap in time between the CUS protocol and the corticosterone analysis. The CUS protocol was completed at age 11 months and corticosterone analysis was not possible until age 16 months. It is well-established that the behavioural effects of CUS administered in adulthood are observable 30 days after the end of the CUS protocol (124), but it is not known if the effects of this CUS protocol diminished over 5 months.

4.3.3 Adrenal Gland Weight Following CUS

Because prolonged exposure to stress is known to alter the activity of the adrenal glands, we considered the weight of the adrenal glands in the CUS mice. No significant differences in adrenal weight as a raw value or relative to body size were observed between males or females subjected to CUS (Table 1). Adrenal gland weights observed here are within the typical range of C57BL/6 mice, though females typically have higher adrenal weights relative to body size which was not observed here (129). It is worth noting that the animals used here were considerably older (18 months vs. 3-4 months).
4.3.4 Plasma Corticosterone Following Acute Stress

Plasma samples were collected at baseline, after 15 minutes of a novel stressor (restraint stress), and after 30 minutes of undisturbed recovery. This was done in order to obtain an understanding of the response to an acute stressor throughout activation, adaptation, and recovery phases. No differences were observed between wild type and Aldh2−/− mice in neither males nor females (Figure 19). The plasma corticosterone levels observed here are consistent with those of similar stress experiments in C57BL/6 mice using various stressors such as capsaicin injection (130) and foot shock (125). Using 15 minutes of restraint stress produced plasma corticosterone levels comparable to those obtained using 40 minutes of restraint stress (131). This result is not indicative of HPA axis dysfunction in this model. Because no differences were observed in anhedonia post-CUS (Figure 14, Figure 15, Figure 16) or adrenal weights (Table 1), it is perhaps not surprising that there was no corticosterone dysregulation observed in fecal or plasma samples.

4.3.5 Sucrose Preference Test Following CUS

Female Aldh2−/− mice did not show a diminished preference for sucrose prior to or following CUS, whereas male Aldh2−/− mice showed a lower preference for sucrose prior to CUS that no longer existed following CUS (Figure 15). Male wild type mice were the most susceptible to anhedonia following CUS, possibly due to the higher initial preference observed. These data do not support the hypothesis that male Aldh2−/− mice are more susceptible to anhedonia following CUS, but rather suggests that male Aldh2−/− mice exhibit baseline anhedonia that does not appear to progressively worsen with CUS.

Because anxiety- and depression-like behaviours were observed in our mouse model, we hypothesized Aldh2−/− mice may be more sensitive to the effects of CUS. However, following three intermittent periods of four weeks of CUS no differences were observed in anhedonia behaviour, corticosterone levels, or adrenal weight.
4.3.6 Corticosterone and the Estrous Cycle

Plasma corticosterone levels in female mice fluctuate throughout the estrous cycle. Corticosterone concentration is significantly elevated at proestrous and estrous (132). To account for this, female mice were tested daily to determine estrous cycle and samples were collected during diestrous. In human females, postmenopausal females have greater HPA reactivity to acute stress (133) that is reduced after estrogen replacement therapy (134). Because female mice do not display similar reproductive changes in older adulthood, this model may not be appropriate to represent this feature of aging. Female humans typically have elevated incidences of anxiety, depression, and dementia (29,135–137), however, this study did not observe greater behavioural impairments in female Aldh2−/− mice.

4.4 D-PUFA Treatment

4.4.1 Anxiety-like Behaviour

Oral administration of deuterium-reinforced PUFAs was associated with reduced anxiety-like behaviour in Aldh2−/− mice in the light/dark box test (Figure 21). Both groups showed a preference for the dark chamber, however the group fed the D-PUFA diet for 10 weeks displayed a significant increase in the amount of time spent in the light side and number of crosses from the dark side to light side. Prior analysis of exploratory behaviour using the open field test revealed no significant differences (Figure 20), which suggests that this change in behaviour resulted from a change in anxiety-like behaviour. Wild type animals fed D-PUFA or H-PUFA diets were not used in the present study, however the performance of Aldh2−/− mice fed D-PUFA diet for 10 weeks did not differ significantly from Aldh2+/− mice fed LabDiet® 5015 Mouse diet (Figure 9).

4.4.2 D-PUFA Diet Reduction in Oxidative Stress and Anxiety-like Behaviours

The prevention of the development of anxiety-like behaviour by the D-PUFA supplemented diet was observed along with a significant reduction in non-enzymatic LPO products, and a reversal of impairments in both spatial reference and working memory, as assessed by improved performance in the
novel object recognition test, spontaneous alternation in the Y-maze task, and the Morris Water Maze task (4). This is consistent with literature demonstrating higher markers of LPO in individuals with anxiety disorders (138). The prevention of anxiety-like behaviours by treatment with D-PUFAs shown in the present study coincided with decreased cortex and hippocampal F₂ isoprostanes by 55% and PGF₂α isoprostane by 20-25% compared to mice fed the H-PUFA diet. This strengthens the relationship between oxidative stress-induced injury and the development of anxiety behaviours in this model.

4.5 Limitations of this Study

Modelling anxiety and depression in rodents is complicated by the fact that both these disorders are highly heterogeneous and have manifestations at the physiological, psychological, and behavioural levels, but only certain responses can be observed and measured experimentally. In the diagnosis of human patients, clinicians rely largely on self-reported data, a measure that has no correlate in rodents. The justification for rodent models of anxiety and depression stems largely from the hypothesis that anxiety and depression behaviours are highly conserved in evolution, and show common features across species (138-139). This includes behavioural responses (i.e. freezing, hiding, etc.) to fearful stimuli. Although this idea remains controversial, anxiety- and depression-like behaviours provide a valuable tool in research using rodent models when interpreted in the light of literature outlining typical patterns of behaviour in mice. It is important not to anthropomorphize the behaviours of mice and maintain the distinction between atypical behaviours from what is expected in a mouse strain and what is observed clinically in humans.

A significant limitation of the HPA dysfunction analysis in this study is an inability to ascertain the stress exposure of the control cohort. It is likely that the animal housing facility subjects the mice to very minor daily stressors, such as handling for health checks, construction noises, and the presence of other animals that could be considered threatening or predatory. Furthermore, this analysis was performed five months after the completion of the CUS protocol. It is possible that a more immediate test could have revealed a difference that diminished over time. The primary interest of this study is determining long-
term and progressive neurodegenerative pathways. It may be appropriate to conduct an analysis of neurodegenerative markers in hippocampal tissue from the $Aldh2^{-/-}$ mice subjected to CUS.

4.6 Sexual Dimorphisms Affecting the Study of AD in Rodents

Rodent studies are often criticized for their underrepresentation of females. It is clear that a sexual dimorphism exists in dementia, however it remains poorly understood. A notable limitation of modelling AD in rodents is the absence of a menopause equivalent in females. In mice, females will continue to have regular estrous cycles until very old age (~20 months or later) (141). Age-related differences in the estrous cycle exist. Notably, the luteinizing hormone surge responsible for triggering ovulation declines substantially after age 16 months in C57BL/6 mice (141). In humans, menopause occurs at mid-age and results in cessation of ovulation. It is important to consider the menstrual cycle in evaluations of human dementia because of the clear relationship between estrogen and memory in females. In humans, females may show marked cognitive decline in the years following menopause (142,143). Female gonadal estradiol can influence performance in learning and memory tasks in rodents and humans (142). Additionally, estrogen receptors are distributed throughout cortical and limbic areas, suggesting a modulatory role in learning and memory (137). Furthermore, estrogens have been shown to protect against neurodegeneration by Aβ, oxidative stress, and ischemic insult (144). Estrogen depletion in the CNS confers significant risk in the development of AD (143). In a PS-1, APP, and tau overexpressing transgenic mouse model of AD (3xTG), ovariectomy of adult female mice was associated with a significant increase in the accumulation of Aβ and significantly increased impairments in memory (29). It is currently theorized that the decrease in estrogens post-menopause is responsible for some of the increased risk of AD in human females. Similarly, the higher incidence of depression during the reproductive years of females is correlated with atypical gonadal hormone levels (142). A potential explanation of these sex differences in adult humans could arise from the neuroprotective effects of estrogen, as human females have an increased risk of both anxiety and cognitive impairment after menopause (145). Unlike humans, mice do not stop their estrous cycle in late adulthood, and continue to
cycle until age 16-20 months (146), which was beyond the age range evaluated in this study.

Alternatively, the existence of a sex effect in the Aldh2 gene is further supported by a longitudinal human study that show a significant association between males carrying the Glu504Lys loss of function mutation in ALDH2 and risk of AD development (88). An additional factor to consider is the fact that some males were housed individually due to aggressive behaviours with cage mates. Social isolation is considered a stressor in rodents that can alter behaviour (100). The number of males isolated was minimal and was similarly distributed in Aldh2−/− and wild type mice, so it is unlikely that this would offer a complete explanation for the sex differences observed. Furthermore, all CUS animals and animals that completed the SPT had to be housed individually regardless of sex. Although no sex differences were observed in spatial memory impairment in the Aldh2−/− strain (4), this suggests that there could be a sex-related factor influencing the progression or severity of non-cognitive behavioural features in this model. Given the sex differences observed here in Aldh2−/− mice, performing similar cognitive and non-cognitive behavioural tests with ovariectomized females may provide a better model of AD pathogenesis in females.

4.7 Future Directions

Characterizing the baseline behavioural performance and the temporal progression of behavioural deficits of Aldh2−/− mice could provide a valuable tool in future preclinical testing for therapeutic interventions targeting disease processes related to oxidative stress in AD, other dementias, and other neurological conditions associated with excessive OS (e.g., Parkinson’s and Huntington’s diseases). By better understanding the multitude of disease processes and symptoms throughout disease stages, we may be better able to diagnose AD earlier. This may afford more effective treatment options. Additionally, the interactions between stress, mental illness, and dementia suggest that treatment for Alzheimer’s disease may require a multi-faceted approach.

The data presented here suggest that anxiety-like behaviours and anhedonia may be attributed to elevated oxidative stress. In the preclinical study of AD, it may be beneficial to consider experimental
endpoints broader than degree of memory impairment. Including measures of non-cognitive behavioural features may provide greater insight into the effectiveness of a novel treatment.

A previously unknown sex difference was observed here, suggesting ALDH2 plays a role in the development of anxiety and depression behaviours in males more than females. Females displayed some increased anxiety, but generally appeared to be more resilient to the effects of ALDH2 on non-cognitive behavioural impairments. Loss of function of ALDH2 also preferentially effects the risk of developing AD in male humans. The Aldh2−/− mouse may also model features that contribute to this sex difference.

Currently, AD treatments are limited to NMDA receptor antagonists and cholinesterase inhibitors that are only effective for a short period. No disease modifying treatments currently exist to prevent or reverse neurodegeneration. Because of the multitude of disease processes at play in AD, exploring other avenues, such as OS, may yield novel therapeutic interventions. Although treating OS may not be curative, it could provide improvements in quality of life to patients and caregivers. D-PUFAs appear to be effective in reducing OS load and reversing cognitive impairment and anxiety-like behaviour in Aldh2−/− mice. This intervention warrants further analysis and understanding of how it may be applied to human patients.

4.8 Conclusions

Although no animal model can fully replicate a complex, multifactorial, human neurological disease such as AD, investigating elements of AD pathology in different mouse models can provide valuable insights into disease mechanisms and potential therapeutic interventions. Existing data show that the Aldh2−/− mouse exhibits cognitive impairments and molecular markers of neurodegeneration, however it was not known if this model replicates the often debilitating non-cognitive features of AD such as anxiety and depression. The findings presented here suggest that Aldh2−/− mice exhibit anxiety-related behavioural disturbances and anhedonia that appear in tandem with the onset of cognitive decline. This strengthens the use of Aldh2−/− mice as a model of AD-like cognitive impairment that represents progressive age-related onset.
The findings of this study add to the constellation of pathological features of this model. By better understanding the multitude of disease processes and symptoms throughout disease stages, and how they differentially affect males and females, we may be better able to predict or diagnose AD earlier. This may afford more effective treatment options, which target specific elements of AD. The Aldh2−/− mouse may be a useful tool in the preclinical testing of new pharmacological interventions alongside existing AD models. Additionally, the interactions between stress, mental illness, and dementia suggest that treatment for Alzheimer’s disease may require a multi-faceted approach that could vary, depending on the genetic predisposition and sex of an individual diagnosed with AD.
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